Transcriptional analysis of the role of $CD8^+$ T lymphocytes in acute neural herpes simplex virus infection

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Dedicated to the memory of

Elfriede Muetzelfeldt

(1914 - 1996)

1 John 5:4
This is the victory... even our faith.

Contents

Abstract

$CD8^+$ T cells have a crucial role in clearance of herpes simplex virus (HSV) from the peripheral nervous systems of infected mice, but the mechanism of this protection is not known. To approach this problem, comparative transcriptional analyses of sensory ganglia from (i) HSV infected immunocompetent mice and (ii) HSV infected mice depleted of $CD8^+$ cells were done. Two types of analysis were undertaken. The first was directed at mRNAs encoding molecules of known importance in immunity and the second utilized mRNA differential display, a non-directed method for identifying differentially expressed genes. The directed analyses examined the effect of $CD8^+$ cell depletion on IL-2, IL-4, IL-6, IL-10 and IFN-γ mRNA levels in ganglia of mice at the peak (day five) or the recovery phase (day seven) of HSV infection. Transcription of each cytokine tested was upregulated in sensory ganglia in response to HSV infection. IL-4 mRNA levels were increased by the depletion of $CD8^+$ cells both at day five and day seven, raising the possibility that overexpression of IL-4 may be detrimental to clearance of HSV. IL-2 and IL-6 mRNAs were more abundant in the $CD8^+$ cell depleted mice at day five and day seven, respectively. The remaining cytokine transcripts were not significantly affected by $CD8^+$ cell depletion. Notably, mRNA for IFN-γ, a candidate effector of $CD8^+$ T cell function, was not altered. An mRNA differential display based analysis was developed, with the aim of detecting transcripts whose abundance in HSV infected sensory ganglia is dependent on the presence (or absence) of $CD8^+$ T lymphocytes. Nine such mRNAs were found and partial cDNAs of five of these were cloned. Northern blotting confirmed that two of the differential display clones, designated CC28 and CT03, represented mRNAs that were more abundant in HSV infected ganglia of immunocompetent mice compared with $CD8^+$ cell depleted mice. Nucleic acid sequence analyses disclosed that CC28 contains an as yet unreported sequence and CT03 is likely to represent mRNA for the α subunit of G_{olf}, a heterotrimeric G protein, not found before in sensory nerve ganglia.

Declaration of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this thesis being made available for loan and photocopying.

David C. Tscharke

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Solo Deo Gratia

Abbreviations

~	approximately
>	greater than
<	less than
^{0}C	degrees Celsius
Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
AIDS	acquired immune deficiency syndrome
α/β IFN	α and β interferon
bp	base pair
CD	cluster of differentiation (eg. CD8)
cDNA	complementary DNA
Ci	curie
CMV	cytomegalovirus
CNS	central nervous system
CPM	counts per minute
CRE	cAMP responsive element
CTL	cytotoxic T lymphocyte
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
DD	mRNA differential display
dGTP	deoxyguanosine-5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide, either dATP, dCTP, dGTP or dTTP
DTH	delayed type hypersensitivity
DTT	dithiothreitol
dTTP	deoxythymidine-5'-triphosphate
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
G+C (%)	guanosine and cytosine content in a nucleic acid sequence
g/l	gram per litre
GAPDH	glyceradehyde-3-phosphate dehydrogenase

H-2	murine major histocompatibility complex
hr	hour
HSV	herpes simplex virus
HSV pol	HSV polymerase
HSV-1 (or -2)	herpes simplex virus type 1 (or type 2)
ICP	infected cell polypeptide (eg. ICP4)
ICTV	International Committee on Taxonomy of Viruses
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin (eg. IL-2 = interleukin-2)
kb	kilobase pair
LATs	latency associated transcripts
M	moles per litre (molar)
mA	milliamperes
mAb	monoclonal Ab
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
mm	millimetre
mM	millimole per litre (millimolar)
mmol	millimol
mol	moles
MOPS	3-[N-Morpholino] propanesulphonic acid
mRNA	messenger RNA
μCi	microcurie
μg	microgram
μl	microlitre
μM	micromole per litre (micromolar)
NCBI	National Centre for Biological Information (USA)
ng	nanogram
NK cells	natural killer cells

nm	nanometre
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pmol	picomole
®	registered trademark
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	reverse transcription
SDS	sodium dodecylsulphate
sec	second
TCR	T cell receptor
TEMED	N,N,N',N'-Tetramethylethylenediamine
Th cells	helper T cells
Th1	type 1 helper T cells
Th2	type 2 helper T cells
TNF	tumour necrosis factor
Tris	Tris(hydroxymethyl)Aminomethane
u	units
UV	ultraviolet
v	version
V	Volts
VP	virion polypeptide (eg. VP16)
W	Watts
www	world wide web
× g	times gravity

Manuscripts and Presentations Arising

Manuscripts in preparation:

Tscharke DC, Simmons A. A strategy for purifying and cloning differential display cDNAs.

Tscharke DC, Simmons A. Use of differential display to study anti-viral $CD8^+$ T lymphocyte function in sensory nerve ganglia.

Tscharke DC, Simmons A. $CD8^+$ cell depletion alters cytokine gene expression in the peripheral nervous systems of herpes simplex virus infected mice.

Conference presentations:

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D. Tscharke, A. Simmons. $CD8^+$ cell depletion alters cytokine gene expression in the peripheral nervous systems of herpes simplex virus infected mice. Poster presented at the First Congress of the Federation of Immunological Societies of Asia-Oceania (FIMSA), Adelaide, Australia, 1996.

D. Tscharke, R. Wilkinson and A. Simmons. mRNA differential display identifies a novel cellular transcript modulated by $CD8^+$ T cells in HSV infected sensory ganglia. Abstract submitted for presentation at the 22nd International Herpesvirus Workshop, San Diego, CA, USA, 1997.

1. INTRODUCTION AND LITERATURE REVIEW

1.1 *Herpesviridae*

Herpesviruses are a diverse family of viruses, whose members can be found associated with at least one species in all of the vertebrate classes. The first herpesvirus probably arose relatively early in evolutionary history as evidenced by the complex relationships between current herpesviruses and their natural hosts, indicative of long periods of co-evolution, and the wide distribution of this family of viruses amongst vertebrates.

1.1.1 General properties and characteristics

The current criteria for inclusion of a virus into the family *Herpesviridae*, as defined by the Sixth Report of the International Committee on Taxonomy of Viruses (ICTV), is largely based on virion morphology (Roizman *et al.*, 1995). Distinguishing features of herpesvirus virions include (i) a core containing a linear double stranded DNA genome of at least 120 kb (Furlong *et al.*, 1972); (ii) an icosahedral capsid surrounding the core comprised of 162 capsomers and approximately 100 nm in diameter (Wildy and Watson, 1963); (iii) a lipid envelope derived from the host cell and containing viral proteins (Stannard *et al.*, 1987); and (iv) an amorphous material composed of viral proteins existing between the capsid and the envelope termed the tegument (Morgan *et al.*, 1968).

In addition to the virion architecture, herpesviruses share a number of other biological properties. These include replication in the nucleus of an infected cell using a wide range

of virally encoded enzymes, a lytic infection cycle *in vitro* and the ability to establish and maintain a latent infection, which is characterized by highly restricted viral gene expression, in infected hosts (Roizman *et al.*, 1995).

The ICTV has further subdivided the family *Herpesviridae* into three subfamilies, *alphaherpesvirinae*, *betaherpesvirinae* and *gammaherpesvirinae*, based on biological properties. A formal binomial nomenclature for the herpesviridae is not used and the current convention is that herpesviruses are named according to their natural host, followed by the virus family (or in some cases sub-family) name and finally an arabic number assigned according to the chronology of isolation (Roizman, 1996). Despite this convention, where they exist, common names for herpesviruses are still widely in use (for example, human herpesvirus 1 is generally referred to as herpes simplex virus type 1 (HSV-1), ictalurid herpesvirus 1 is more commonly called channel catfish herpesvirus).

1.1.2 *Alphaherpesvirinae*

Alphaherpesviruses infect a broad range of hosts, have a rapid replication cycle and spread quickly in culture. In addition these agents are able to establish latent infections in sensory ganglia. There are as yet no genetic attributes that distinguish this sub-family (Roizman *et al.*, 1995). These viruses have a preference for growth in cells of ectodermal origin, which predominate in a variety of tissues including skin, pharyngeal and genital mucosa, nervous system, eyes and adrenal medulla, but may grow in many cell types under appropriate conditions (Wildy *et al.*, 1982).

There are two genera in the subfamily, *Simplexvirus* and *Varicellovirus*, each with a distinctive genome structure and different disease progressions. *Simplexviruses*, the type species of which is HSV-1, generally cause primary disease at the inoculated skin or mucosal surface, followed by spread to the peripheral nervous system where latency is established. Thereafter, latency may be interrupted by sporadic reactivation of productive infection. Human diseases caused by viruses in this genus include lesions commonly termed "cold sores" and genital herpes. *Varicelloviruses*, of which varicella-zoster virus (or human herpesvirus 3) is the type species, cause disseminated primary infections, but recurrences are restricted to a single dermatome. In human varicella-zoster virus infections, the primary and recurrent diseases are more commonly known as "chicken pox" and "shingles", respectively.

1.1.3 *Betaherpesvirinae*

In contrast to the alphaherpesviruses, members of the subfamily *betaherpesvirinae* have a highly restricted host range, a long replication cycle and their infections in culture progress slowly. This subfamily of viruses can persist in a latent state in lymphoreticular cells and sometimes in secretory glands and kidneys (Wright, 1973; Roizman *et al.*, 1995).

Three genera, *Cryptomegalovirus*, *Muromegalovirus* and *Roseolovirus*, have been defined on the basis of genome structure and in the case of the latter, an ability to be isolated from lymphocytes. According to the latest report of the ICTV, each genus has only a single member assigned to it. Species of *Cryptomegalovirus* and *Roseolovirus* are able to cause human diseases, these viruses are cytomegalovirus (CMV or human herpesvirus 5) and

human herpesvirus 6 respectively (Roizman *et al.*, 1995). A recently defined herpesvirus, human herpesvirus 7, which is very similar to human herpesvirus six, is unofficially considered to be a member of the *Roseolovirus* genus. Human infections with these virus are generally subclinical, however where the host is immunocompromised the consequences may be grave. Another clinical problem caused by a betaherpesvirus is congenital malformation of babies born to mothers following an episode of productive cytomegalovirus infection during pregnancy.

1.1.4 *Gammaherpesvirinae*

The one feature that all gammaherpesviruses have in common is the ability to replicate in lymphoblastoid cells *in vitro*. Generally either B or T lymphocytes can be infected by these viruses, but rarely both, and such lymphocytic infections may be non-productive. Gammaherpesviruses like betaherpesviruses, have a narrow host range, often restricted to the family or order of which the natural host is a member (Roizman *et al.*, 1995). Latency is frequently established in lymphoid tissue and may require limited viral gene expression to maintain the genome as a circular episome (Adams and Lindahl, 1975).

The subfamily *gammaherpesvirinae* has two genera, *Lymphocryptovirus* and *Rhadinovirus*. Each has a characteristic genome structure and the former can establish latent infections in B lymphocytes, but this property is not exclusive (Roizman *et al.*, 1995). The type species of the genus *Lymphocryptovirus* is human herpesvirus 4, also known as Epstein-Barr Virus (EBV), and is a common cause of infectious mononucleosis or "glandular fever" in humans (Henle *et al.*, 1968). EBV is also associated with two human cancers, Burkitt's lymphoma

and nasopharyngeal carcinoma and can immortalize cells in culture (Pattengale *et al.*, 1973), indicating a possible etiological link between EBV and neoplasia. The genus *Rhadinovirus* has a single member, ateline herpesvirus 2, the natural host of which is a species of New World monkey. A newly identified herpesvirus, human herpesvirus 8 (also known as Kaposi's sarcoma herpesvirus), is also considered to be a gammaherpesvirus.

1.1.5 Other comments on herpesvirus classification

In original classifications of herpesviruses, the definition of groups was dependent on clinical findings and these groupings were modified to incorporate the physicochemical properties of virions, after techniques such as electron microscopy became available. With the vast amount of work done to characterize the biological properties and ultimately the genomes of many herpesviruses, their taxonomy has become more refined. In the most recent report of the ICTV, the subdivision of herpesviruses is based both on biological properties and DNA sequence data, but in a few cases these sets of information do not agree. Arriving at workable decisions about how such ambiguities are best resolved is a challenge which faces all contemporary taxonomists. In addition to the many herpesviruses that have been definitely assigned into subfamilies and genera, there are many more, some of which are only tentatively assigned and others which remain unassigned at either the subfamily or genus levels. The number of these viruses is likely to remain high because, while new data about many viruses will allow their assignment, still more hitherto unknown herpesviruses will be discovered.

1.2 Herpes simplex virus (HSV)

Based originally on serology, two subtypes of herpes simplex virus, type 1 (HSV-1) and type 2 (HSV-2), have been defined (Schneweis, 1962). The formal designations of HSV-1 and 2 are human herpes virus 1 and 2 respectively, and they are in the *alphaherpesvirinae* subfamily and the *Simplexvirus* genus (Roizman *et al.*, 1995). The two types of HSV produce very similar infections *in vivo* and *in vitro* and their genomes have approximately 50% homology (Kieff *et al.*, 1972). Herpes simplex viruses are the only human herpesvirus to include the term "herpes" in their common name, and in 1970 when herpesviruses as a group were assigned the taxonomic rank of genus, HSV-1 was the type species for the entire group (Wildy, 1973).

1.2.1 History

"Herpes" has been known as a medical term for at least two and a half millennia, but over that time has been used to describe a variety of skin conditions (Beswick, 1962). The original form of the word was the Greek, ερπης, the meaning of which translates as "the creep", and was used to refer to spreading skin diseases. According to Wildy, the current usage of the term was probably established by Willan and Bateman in 1814 in a book entitled *A practical synopsis of cutaneous diseases according to the arrangement of Dr. Willan* (Wildy, 1973). The first experimental transmission of a herpes simplex virus was to humans by Vidal in 1873 and transfer to another species was first achieved by Grüter, using the cornea of rabbits, in 1911. Nearly 30 years was to elapse before Burnet and Williams published their seminal article in which they proposed the current paradigm of

HSV pathogenesis (Burnet and Williams, 1939). Stated briefly, the proposal was that, following primary infection, HSV remains quiescent in the body between sporadic recurrences of disease. In the same article, Burnet and Williams noted the paradox of recurrent herpetic disease occurring in the face of well developed anti-HSV immunity, still a relevant issue today.

1.2.2 Clinical aspects

HSV disease is endemic in every nation on earth and is highly prevalent in most, if not all communities. The large number of people affected by HSV makes it a highly significant pathogen. Primary infection commonly occurs in the first three years of life and is the result of close physical contact with another person, themselves undergoing an acute infection (primary or recurrent), which is not necessarily clinically apparent. HSV was isolated from nearly 1% of all pregnant women and nursery staff at a single time in one report (Hatherly *et al.*, 1980); and a recent study of over 100 women with known genital herpes reported that nearly 51% of this population had shed virus asymptomatically at some time in an approximately 100 day period (Wald *et al.*, 1995).

The most common diseases caused by HSV of either type are primary and recurrent orofacial and genital lesions. HSV-1 and HSV-2 tend to be associated with infections around the mouth and genitalia, respectively, but these associations are far from exclusive. HSV has the ability to cause a variety of other infections when the skin barrier is broken, examples of which include herpetic whitlow, an infection under the fingernail and herpes gladiatorum which presents as a rash on the trunk, temple, ear or forehead and is most

prevalent among wrestlers and rugby players (Becker, 1992). Although these conditions cause much morbidity, there are a number of much more severe infections caused by HSV. Herpetic keratitis may lead to blindness as a result of recurrent episodes of corneal scarring (Pavan-Langston, 1984) and is thought to have a significant immunopathological component (Missotten, 1994; Babu *et al.*, 1995). Neonates infected *in utero*, or more commonly in the birth canal, may develop disseminated HSV infections with a fatal outcome because their poorly developed immune systems fail to adequately fight the viral challenge (Stagno and Whitley, 1985). Perhaps the least understood complication of herpes simplex is herpes encephalitis, which strikes unpredictably during primary or recurrent infections and is rapidly fatal unless treated promptly (Whitley *et al.*, 1982). In addition to these syndromes, severe herpetic diseases in immunocompromised patients (Whittaker and Hardson, 1978; Whorton *et al.*, 1983) are clinical issues of growing importance, owing to the current prevalence of AIDS, and increasing numbers of individuals (eg. transplant recipients) undergoing treatments requiring immunosuppression.

A development which had a massive impact on the way HSV diseases are treated was the discovery of the antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine, also known as acyclovir (Elion *et al.*, 1977; Schaeffer *et al.*, 1978). Acyclovir is a nucleoside analogue that, when converted to its active form by a virally encoded enzyme (thymidine kinase), is able to terminate HSV DNA replication (Elion *et al.*, 1977). The use of this drug has saved countless lives from otherwise fatal herpes infections and reduced the suffering of many people with recurrent lesions. Unfortunately, acyclovir is not a panacea. In most cases it must be administered at the very onset of clinical symptoms to be of value and acyclovir resistant viral mutants have already been described (Coen and Schaffer, 1980; Crumpacker

et al., 1982; Coen, 1994; Hwang *et al.*, 1994). Further, patients rescued from herpes encephalitis with acyclovir may not escape permanent neural damage (Barthez Carpentier *et al.*, 1995).

1.2.3 Terminology associated with the genome, genes and proteins

The approximately 152 kb genome is divided into two segments, termed long and short (L and S), which are roughly 126 kb and 26 kb respectively. Both segments comprise unique regions of DNA, designated U_L and U_S, flanked by inverted repeats designated *ab* and *b'a'* for U_L, and *a'c'* and *ca* for U_S; the *"a"* sequences are reiterated at the junction of the L and S segments and at the terminus of L, the number of iterations being different in different HSV strains (Sheldrick and Berthelot, 1974; Wadsworth *et al.*, 1975). The orientations of the long and short segments invert with respect to each other during virus replication, giving rise to four isomers which are equally abundant in all virus preparations (Hayward *et al.*, 1975; Clements *et al.*, 1976; Delius and Clements, 1976). The entire DNA sequence for one strain (strain 17) is known (GenBank accession X14112, (McGeoch *et al.*, 1985; McGeoch *et al.*, 1986; McGeoch *et al.*, 1988; Perry and McGeoch, 1988; Dolan *et al.*, 1992)) and at least 80 genes or transcription units have identified (Roizman and Sears, 1996).

HSV open reading frames are numbered sequentially in the unique regions, and are referred to according to segment and number (eg. the gene encoding thymidine kinase is U_L23); genes in the repeats, being diploid, are generally named after their protein product (McGeoch and Schaffer, 1993; Roizman and Sears, 1996). Gene expression, at least in cell

culture, follows an ordered pattern leading to alternate classification systems, in which genes are divided into classes with similar expression kinetics. Three main classes are defined, α (immediate early, IE) which are mainly regulatory genes; β (early, E) whose expression is dependant on α gene expression and are largely involved viral DNA replication; and γ (late, L) whose expression is dependent on viral DNA replication and generally encode virion structural proteins (Honess and Roizman, 1974). Some genes, especially those in the repeat regions, are more commonly referred to by their kinetic class and a number (eg. α4). HSV proteins are often referred to with descriptive names related to function. Various nomenclatures have also been used and current convention is: non-glycosylated proteins present in the virion are designated VP (virion polypeptide) followed by a number; proteins found in infected cells are designated ICP (infected cell polypeptide) followed by a number; and glycoproteins by g and a capitalized letter (for example, VP16, ICP36 and gB respectively) (McGeoch and Schaffer, 1993; Roizman and Sears, 1996).

1.2.4 The herpes simplex virus virion

HSV has a characteristic virion structure shared by all herpesviruses, comprising a DNA core covered by a proteinaceous capsid that in turn is covered by an envelope; the space between the capsid and envelope is filled with proteins and is termed the tegument. At least 33 viral genes encode structural components of the virion, and some of these genes contribute more than one protein product to the virion (Haarr and Skulstad, 1994).

The core is composed mainly, if not entirely, of the DNA genome which has a G+C content of 68-69% and is predominantly linear and double stranded (Becker *et al.*, 1968; Kieff *et*

al., 1971; McGeoch *et al.*, 1988; Rixon, 1993). The exact conformation assumed by the genome in a mature virion is not known. One hypothesis is that the DNA is wound around a chemical plug (Furlong *et al.*, 1972), but recently Booy *et al* (1991) used computer enhanced cryoelectron microscopy to visualize the core and found no evidence for substances other than DNA.

Seven HSV proteins are thought to exist in natural capsids, but synthetic capsids can be assembled in a cell free system using only 5 proteins, VP5, VP19, VP23, VP22a, and the U_L26 protease (Rixon, 1993; Newcomb *et al.*, 1994). Studies using viral mutants and degradative analyses of virions, indicate that VP5 is the major constituent of HSV capsomers, the basic units from which capsids are built (Weller *et al.*, 1987; Newcomb and Brown, 1989; Desai *et al.*, 1993).

The tegument contains at least as many proteins as the capsid and provides a source of viral products that can function as soon as the virus penetrates a new host cell. Some tegument proteins have had functions assigned to them while others remain less well understood. Examples of tegument proteins include, VP16, a transactivator of α gene expression (Stern *et al.*, 1989; O'Hare, 1993; Wu *et al.*, 1994); the product of U_L41, known as the virion host shut off (vhs) factor, which destabilizes mRNAs, reducing host protein synthesis (Nishioka and Silverstein, 1977; Read and Frenkel, 1983; Kwong and Frenkel, 1987; Kwong *et al.*, 1988); and VP18.8, a protein kinase capable of self phosphorylation but of unknown function (Cunningham *et al.*, 1992).

HSV acquires the lipid component of its envelope from the host cell and at least seven viral

glycoproteins are known to be inserted into the HSV-1 membrane (Stannard *et al.*, 1987; Spear, 1993). Three of these, gB, gD and gH, are essential for virus growth in culture and the majority are required for full infectivity *in vitro* and *in vivo* (Spear, 1993; Dingwell *et al.*, 1995; Roizman and Sears, 1996). Viral glycoproteins have long been considered important targets for immune recognition, for example antibodies raised against gB, gC, gD, gE, gH and gI are all able to neutralize virus infectivity (Simmons and Nash, 1985; Gompels and Minson, 1986; Eis-Hübinger *et al.*, 1993; Sanchez Pescador *et al.*, 1993; Burioni *et al.*, 1994; Ghiasi *et al.*, 1994).

1.2.5 Productive replication cycle

HSV grows rapidly in culture and the processes involved have been studied using many tools including electron microscopy, viral and cellular mutants and chemicals that block specific cellular functions. It is generally assumed that the mechanisms revealed by analysis of HSV infected cultures are reflected in all HSV-cell interactions, but there is evidence that this may not be the case for neurons *in vivo* (Simmons and Tscharke, 1992). The replication of HSV can be broken into four main stages, namely (i) virus attatchment, penetration and translocation of nucleocapsids and tegument proteins to the nucleus, (ii) viral gene expression, (iii) replication of the genome, and (iv) virion assembly and egress.

1.2.5.1 Virus attatchment, penetration and translocation to the nucleus

Heparan sulphate moieties are the primary, but not exclusive, receptors for HSV and their almost ubiquitous distribution across cell types helps to explain the wide host range of the

virus (Wudunn and Spear, 1989; Shieh *et al.*, 1992; Gruenheid *et al.*, 1993). A variety of evidence implicates gC in the binding of HSV to cells via Heparan sulphate (Campadelli-Fiume *et al.*, 1990; Herold *et al.*, 1991). In spite of the apparent importance of gC for attachment of wild type HSV to cells, mutants devoid of gC retain infectivity, albeit at a much reduced efficiency. The mechanism of gC independent attachment of HSV is not known, but interactions between gD and Heparan sulphate residues (Herold *et al.*, 1994) or another receptor (Johnson and Ligas, 1988) have been suggested. Following attachment, fusion between viral and cellular membranes occurs, releasing the tegument coated nucleocapsid into the cell (Morgan *et al.*, 1968; Johnson *et al.*, 1984). Studies with neutralizing antibodies and viral mutants have disclosed that three viral glycoproteins, gB, gD and gH are required for penetration (Sarmiento *et al.*, 1979; Little *et al.*, 1981; Fuller and Spear, 1987; Highlander *et al.*, 1987; Cai *et al.*, 1988; Desai *et al.*, 1988; Ligas and Johnson, 1988; Fuller *et al.*, 1989; Forrester *et al.*, 1992). In addition to direct fusion, HSV can be endocytosed, but it is not known whether this mode of entry can lead to a productive infection (Holmes and Watson, 1961; Campadelli-Fiume *et al.*, 1988). Having entered a cell, the nucleocapsid and at least some of the tegument proteins are transported to the nucleus where HSV replicates. Transport across the cytoplasm is most likely mediated by cellular cytoskeletal components (Kristensson *et al.*, 1986) and access to the nucleus is via nuclear pores (Tognon *et al.*, 1981). In the nuclei of infected cells, the genome circularizes (Roizman and Sears, 1987) with the aid of host factors, a virion component, or a both (Poffenberger and Roizman, 1985).

1.2.5.2 Viral gene expression

Transcription and translation of viral genes is very similar to that of cellular genes. Viral mRNAs are transcribed by cellular RNA polymerase II and are capped, methylated and polyadenylated (Bachenheimer and Roizman, 1972). As mentioned, HSV expression occurs as a sequential cascade with three stages, α, β and γ.

Alpha genes have been studied intensively because they are the instigators of acute infection and because they may be involved in reactivation from latency (Leib *et al.*, 1989b). Five α proteins are known, α0, α4, α22, α27 and α47, two of which (α4 and α27) are essential for replication in culture (Preston, 1979b; Preston, 1979a; Sacks *et al.*, 1985; Sacks and Schaffer, 1987). The promoters for α genes contain elements not found in other viral genes and their transcription is dependent on a complex of viral and cellular transcription factors (Campbell *et al.*, 1984; Kristie and Roizman, 1987; O'Hare and Goding, 1988; O'Hare, 1993). Expression of α genes peaks two to four hours after infection, and is maintained at lower levels throughout infection (Honess and Roizman, 1974). The α proteins regulate expression of all classes of viral genes, including their own (Knipe, 1989; Hay and Ruyechan, 1992).

Beta gene expression is dependent on the action of α proteins and their synthesis is maximal between five and seven hours after infection (Honess and Roizman, 1974; Honess and Roizman, 1975). This class of genes can be further subdivided into two groups, namely β_1 genes, the expression of which overlaps that of α genes, but requires the presence of α4, and β_2 genes that are synthesised later (Roizman and Sears, 1996). Beta

gene products, many of which are responsible for viral DNA replication, include the viral polymerase, the origin-binding protein and the helicase-primase, all of which are essential for HSV growth in culture (Challberg, 1986). Other β proteins have roles in nucleoside metabolism and although not required for growth in culture, may be important *in vivo*: for example the viral thymidine kinase is needed for replication of HSV in mouse neurons (Tenser *et al.*, 1979).

Gamma genes, the synthesis of which begins nine hours after infection, are mainly virion components. Like β genes, γ genes are divided into two groups with slightly different expression kinetics. Expression of genes in the first group (γ_1) is not dependent on viral DNA replication and precedes that of genes in the second group (γ_2). Expression of γ_2 genes is dependent on viral DNA synthesis and for this reason they are sometimes referred to as "true late genes". Examples of γ genes are those encoding the capsid subunits, glycoproteins and tegument proteins.

1.2.5.3 Replication of the genome

HSV DNA replication begins concurrently with β gene expression but the mechanisms employed by the virus are not fully understood. Three origins of replication have been identified, one in U_L (Ori_L), and a diploid origin (Ori_S) in the repeats flanking U_S (Hammerschmidt and Sugden, 1990). Mutational analyses indicate that none of the three origins are uniquely required for replication (Longnecker and Roizman, 1986; Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993), and the reason why all three have been maintained, when any single origin will suffice is unclear. Circular genomes are present

early in the replication process (Poffenberger and Roizman, 1985) and at later times, there are large masses of high molecular weight intermediates of viral DNA replication, in the form of head-to-tail concatemers (Ben-Porat *et al.*, 1976; Ben-Porat *et al.*, 1977; Jacob and Roizman, 1977; Jacob *et al.*, 1979; Jongeneel and Bachenheimer, 1981). Taken together, these results have been used to propose a rolling circle mechanism of HSV DNA synthesis. Such a mechanism is predicted to utilize circular DNA templates and to produce large concatemers of the genome, which are subsequently cleaved to yield unit length molecules (Poffenberger and Roizman, 1985; Garber *et al.*, 1993).

Four isomeric forms of the genome (in which the long and short segments are inverted with respect to each other) are generated during replication. Two of the four isomers could be formed by cleavage, at alternate junctions of long and short segments, of a concatemer of HSV genomes generated by rolling circle replication (Jacob *et al.*, 1979; Vlazny *et al.*, 1982). The other two isomers are not easily explained by the rolling circle model, and a role for homologous recombination has been suggested (Mocarski *et al.*, 1980; Smiley *et al.*, 1981; Mocarski and Roizman, 1982; Bruckner *et al.*, 1992; Dutch *et al.*, 1992). Recently two groups showed that in concatemers produced by HSV replication, adjacent monomers have the long segment inverted (Zhang *et al.*, 1994; Martinez *et al.*, 1996). Cleavage of these concatemers, at alternate junctions of long and short segments, could account for all four isomeric forms of the genome.

There are two other observations which suggest that a simple rolling circle model is inadequate to explain HSV DNA replication. First, rolling circle replication predicts a linear increase in new DNA over time, each genome taking 20-40 minutes to be made; in

contrast, a several hundred fold amplification of DNA is observed in HSV infected cells over a few hours. Second, replicative intermediates found in HSV infected cells are extensively branched and it is difficult to conceive how a simple rolling circle could produce so many of these structures (Martinez *et al.*, 1996; Severini *et al.*, 1996). Two models are currently favoured to explain HSV genomic replication in the light of these findings. The first is that theta-type replication begins from a circular template and homologous recombination takes place between a replicated viral repeat sequence and its as yet unreplicated counterpart, resulting in chasing rather than opposing replication forks, analogous to the replicative mechanism postulated for the 2μm plasmid of yeast (Futcher, 1986). Such a process would generate multimeric circles with long segments inverted, consistent with the observed concatemers (Hammerschmidt and Mankertz, 1991; Zhang *et al.*, 1994). The second model proposes that after initial replication, new replication forks are produced by recombination between free termini (generated by cleavage) and a homologous internal sequence, as proposed for T4 bacteriophage (Mosig, 1987). This process produces masses of branched concatemers and has enough inherent recombination to account for concatemers in which 50% of monomers have inverted long regions (Martinez *et al.*, 1996; Severini *et al.*, 1996). Further work on the structure and accumulation of replicative intermediates and the roles of genes required for producing mature replicated HSV genomes is needed to resolve these issues.

1.2.5.4 Virion assembly and egress

Once synthesised, capsid proteins are transported to the nucleus where they are assembled. The mechanism of assembly is not known but capsids can be made in insect cells infected

with recombinant baculoviruses carrying the structural components (Thomsen *et al.*, 1994). Recently a similar result has been achieved in a cell free system, indicating that the capsid proteins are capable of self-assembly (Newcomb *et al.*, 1994), but the production of mature capsids containing DNA requires the products of at least another eight genes (Roizman and Sears, 1996). Capsids are preformed and DNA is packaged into them at the same time as it is cleaved from replication intermediates (Vlazny *et al.*, 1982). Cleavage and packaging are directed by recognition of signals located in the *"a"* sequences of the viral genome (Varmuza and Smiley, 1985; Deiss *et al.*, 1986; Deiss and Frenkel, 1986).

Late in infection, accumulation of viral proteins and glycoproteins around and in the nuclear membrane causes patches of thickening visible by electron microscopy (Nii *et al.*, 1968). Newly formed nucleocapsids are also associated with these patches and at least their initial envelopment, which is dependant on the presence of full length DNA in capsids (Vlazny *et al.*, 1982), occurs at this membrane. The original mechanism proposed for HSV envelopment and release from infected cells suggests that capsids are enveloped and de-enveloped as they pass through the nuclear membrane, and either re-enveloped by the endoplasmic reticulum and released by fusion of vesicles with the cell membrane, or acquire an envelope by budding through the cell membrane (Stackpole, 1969). Electronmicrographs of capsids associated with all of these membranes support this hypothesis, but because they are only snapshots, the fate of these capsids can not be divined (Nii *et al.*, 1968; Schwartz and Roizman, 1969; Rixon *et al.*, 1992). Indeed, in at least some studies, many of the capsids found to be adjacent to the plasma membrane are at least partially degraded (Whealy *et al.*, 1991). An alternative hypothesis postulates that nucleocapsids acquire their envelope as they bud through the nuclear membrane and are

then transported via the Golgi apparatus to the cell surface for release by exocytosis (Johnson and Spear, 1982). This second hypothesis is currently favoured but more evidence will be needed before a consensus is reached.

1.2.6 HSV Pathogenesis

1.2.6.1 Anatomy

HSV infections involve the skin or mucosal membranes and the peripheral (and occasionally central) nervous system. The cells responsible for collecting sensory information from the skin and communicating it to the central nervous system (CNS) are primary sensory neurons. Primary sensory neurons are large cells extending a single process that divides into two branches, one branch connecting with the CNS and the other extending to the skin (Ham, 1969b). Cell bodies of primary sensory neurons are collected together in structures known as ganglia. Two ganglia are involved in the sensory innervation of the face, these are the right and left trigeminal ganglia, lying beneath the brain. Sensory ganglia for the rest of the body reside inside the spinal column, two (right and left) at each vertebral level (Ham, 1969a), and are known as dorsal root ganglia. The area of skin innervated by a single ganglion is termed a dermatome. For example, in a human, the dermatome associated with the left ganglion inside the tenth thoracic vertebra is a band of skin at the same vertebral level, running around the left hand side of the trunk from anterior to posterior midline, and between five to ten centimetres wide. Almost every part of a primary sensory neuron is tended by glial cells; the cell body is surrounded by satellite cells and the neuronal fibres, or axons, are ensheathed by Schwann cells (Pannese

et al., 1972; Pannese, 1981). These two peripheral nervous system glial cell types are very similar to each other and closely related to CNS macroglia (Pannese, 1981). All the organs and cell types mentioned above play roles in natural HSV infections, and their properties and interactions define the environment in which the virus has evolved and is maintained.

1.2.6.2 Primary infection

HSV infection in a susceptible (ie. seronegative) human, begins with inoculation of broken skin or a mucosal surface by close contact with a source of virus. Epithelial cells are the primary site of viral replication, infected cells undergoing characteristic changes including condensation of chromatin, nuclear degeneration and finally death (Wagner and Roizman, 1969; Darlington and Granoff, 1973). Infected cells may fuse with neighbouring cells allowing the virus to be propagated without exposure to extracellular spaces, thus evading neutralization by antibodies (Simmons and Nash, 1987). The cell damage caused by infection leads to formation of vesicles full of infectious virus, cellular debris and inflammatory cells. As infiltration of leukocytes into the lesion proceeds, vesicles develop into pustules and finally, when the infection is controlled, scabs form. Symptomatic primary infection usually lasts for two to three weeks. Alternatively, primary HSV infections can be asymptomatic (Mertz, 1993).

During acute infection of the skin, viral particles gain access to sensory nerve endings and travel to neuronal cell bodies in ganglia. The hypothesis that HSV spreads to the nervous system via axonal flow was proposed in the 1920s (Goodpasture and Teague, 1923; Goodpasture, 1925) and further evidence has been collected over time, first in animal

models (Kristensson *et al.*, 1971; Bastian *et al.*, 1972; Baringer and Swoveland, 1973; Cook and Stevens, 1973) and more recently using *in vitro* culture systems (Ziegler and Herman, 1980; Lycke *et al.*, 1984). Schwann cells can be productively infected by HSV and sequential infection of these cells may be an alternative, albeit minor, pathway of nervous system invasion (Johnson, 1964a; Wildy *et al.*, 1982). Unenveloped nucleocapsids have been visualized in axons by electron microscopy, but it is not known whether these particles are infectious, or what direction they are travelling (Hill *et al.*, 1972; Kristensson *et al.*, 1974; Lycke *et al.*, 1984; Penfold *et al.*, 1994). Spread of virus between ganglionic neurons is thought to occur via the CNS as shown in infected trigeminal ganglia of mice (Tullo *et al.*, 1982), or by a so called "round trip" to the skin where fresh nerves, and hence neurons become involved (Klein, 1976). There is other evidence that this spread occurs outside ganglia. Isolated HSV antigen positive neurons are frequently visualized by immunohistochemical staining of infected ganglia, but in contrast, spreading foci of infection are rare (Wildy *et al.*, 1982). Additionally, satellite glia in ganglia appear to be resistant to HSV infection *in vivo* (Dillard *et al.*, 1972; Cook and Stevens, 1973), whereas at least some glial cells in the CNS are permissive, and also may be in contact with, and able to spread virus to many nerves (Townsend and Baringer, 1978). In immunocompetent mice, primary sensory neurons can survive HSV infection, at least to the stage of late gene expression, unless the animal is overwhelmed by too high an inoculum of virus (Simmons *et al.*, 1992b; Simmons and Tscharke, 1992). In humans, it is not known whether productive neuronal infection is a common outcome, however it can occur in severe cases (Mok, 1971; Music *et al.*, 1971), and is likely to be a prerequisite for herpes encephalitis. Both in human and experimental infections, latent infection is established in some primary sensory neurons as a result of HSV neuroinvasion (Stevens and Cook, 1971; Baringer and

Swoveland, 1973; Stevens *et al.*, 1987; Arthur *et al.*, 1993). Additionally, the severity of primary skin and nervous system infections affects the number of neurons accessed by virus, and hence the number of latent sites (Walz *et al.*, 1976).

1.2.6.3 Latency and reactivation

The ability of HSV to establish and maintain latent infections contributes significantly to its success as a parasite. Latent infection in primary sensory neurons is characterized by maintenance of the viral genome in a non replicating state with highly restricted viral gene expression. No infectious virus can be isolated from homogenates of latently infected ganglia. However, this result can be reversed by a period of explant culture (Stevens and Cook, 1971). The ability to be reactivated by explantation has become a working definition of latent HSV infection, but the biological relevance of reactivation by such extreme measures is not known (Roizman and Sears, 1987; Ho, 1992). There are also *in vivo* models of reactivation, including severing of nerves (Walz *et al.*, 1974; Ecob Prince *et al.*, 1993), use of a variety of physical stimuli (Schmidt and Rasmussen, 1960; Blyth *et al.*, 1976; Nesburn *et al.*, 1977; Hill *et al.*, 1978; Harbour *et al.*, 1983; Hill *et al.*, 1985; Bobrowski *et al.*, 1991; Sawtell and Thompson, 1992; Fawl and Roizman, 1993) and some animals latently infected with HSV undergo regular spontaneous reactivations (Nesburn *et al.*, 1967; Scriba, 1975; Stanberry *et al.*, 1982; Berman and Hill, 1985; Stanberry, 1992). These models, and many more have been exploited extensively in studies aimed at elucidating the molecular basis of latency and reactivation.

Structure of latent DNA

The structure of latent HSV DNA is not known precisely. Restriction endonuclease analysis and Southern blotting has shown latent HSV genomes in animals and humans lack free termini (Rock and Fraser, 1983; Efstathiou *et al.*, 1986). In further analyses, most of the terminal fragments were shown to be fused, forming sequences similar to those at the junction region between short and long segments of the genome, and not deleted or integrated with host DNA. These data indicate that the genome is either circular, or a large concatemer (possibly integrated) (Rock and Fraser, 1985). Most, but not all HSV DNA from latently infected mice can be separated from host chromosomes by density gradient centrifugation and therefore, although the bulk of viral DNA is almost certainly episomal, integration of a small number of genomes cannot be excluded (Mellerick and Fraser, 1987). Finally, latent HSV DNA is bound by histones in much the same way as the cellular genome (Deshmane and Fraser, 1989).

Latency associated transcripts

Viral activity during HSV latency is limited to transcription from a small region of the genome. The major (most abundant) latency associated transcripts (LATs) were detected first by *in situ* hybridization studies and originate from the repeat regions of the long segment being antisense in part to the message for α0 (Deatly *et al.*, 1987; Spivack and Fraser, 1987; Stevens *et al.*, 1987). RNA species related to LATs are made by both HSV types and at least two other alphaherpesviruses, bovine herpesvirus 1 (Rock *et al.*, 1987; Kutish *et al.*, 1990) and pseudorabies virus (Rock *et al.*, 1988; Cheung, 1989; Priola *et al.*, 1990) and therefore, are considered to play important roles in aspects of latency. This evolutionary argument for the significance of LATs seems to be at odds with the

demonstration that LAT deletion mutants are able to establish, maintain and reactivate from latency *in vivo*. However, reactivation is impaired in some models (Leib *et al.*, 1989a; Steiner *et al.*, 1989; Hill *et al.*, 1990; Trousdale *et al.*, 1991; Trowsdale *et al.*, 1991). An understanding of LATs has been considered a vital step in understanding latency itself and the vast amount of work on their properties, promoters and potential functions has been reviewed extensively (Roizman and Sears, 1987; Stevens, 1989; Latchman, 1990; Garcia-Blanco and Cullen, 1991; Fraser *et al.*, 1992; Ho, 1992). HSV-1 major LATs are a series of co-linear, non-polyadenylated RNAs for which, at least *in vivo*, no protein product has been detected. Larger, but less abundant RNAs (minor LATs) are transcribed during latency from the same region of the genome and it has been suggested that major LATs may be stable introns derived from minor LATs (Dobson *et al.*, 1989; Farrell *et al.*, 1991; Spivack *et al.*, 1991). The reasons why HSV might employ such a strategy, and the roles of the putative spliced product and a stable intron are not clear. Major LATs do not encode obvious protein products, however they do contain two open reading frames (ORFs) and antibodies raised against a bacterial fusion protein made with one of these ORFs detected a protein in an *in vitro* latency system (Doerig *et al.*, 1991). The product remains undetected during latency *in vivo*, but this protein may function briefly or in low copy number in the establishment or reactivation phases. Other potential ORFs have been identified within the region from which minor LATs arise and the expression of one such ORF (ORF P) has been shown, but only in viral mutants lacking active α4 genes (Lagunoff and Roizman, 1994; Lagunoff and Roizman, 1995). Roles for the product of ORF P during latency have yet to be defined. In the absence of a protein product, LATs may function as structural RNAs. For example, anti-sense regulation of α0 by LATs has been suggested (Stevens *et al.*, 1987). There is no evidence of α gene dysregulation in experiments with

LAT deficient viruses, but the large number of LAT copies, estimated at between 20 and 50,000 per cell (Wagner *et al.*, 1988a; Wagner *et al.*, 1988b), makes the structural RNA hypothesis attractive. In searching for possible functions, links have been made between LATs, cellular cAMP levels and reactivation. A number of physiological stimuli which induce reactivation also affect cellular cAMP levels and a cAMP responsive element (CRE) has been discovered in one of the LAT promoters (Leib *et al.*, 1991; Rader *et al.*, 1993). The association of cAMP with reactivation has been strengthened by experiments showing that inducers of high cAMP levels were able to accelerate reactivation in explanted ganglia (Foster *et al.*, 1989; Sainz de la Maza *et al.*, 1989; Leib *et al.*, 1991) and in an *in vitro* model of latency (Rodriguez *et al.*, 1991). However the *in vivo* significance of these observations is not known. There is no evidence to suggest that LAT copy number is related to reactivation and at least one other HSV gene has a CRE in its promoter (Deb *et al.*, 1994). In spite of huge amounts of work on LATs *in vitro* and *in vivo*, their function remains unknown.

Does HSV DNA persist in a single form?

Many models of HSV latency are based on the premise that viral DNA persists in a single form. Several lines of evidence suggest that this may not be the case. First, attempts to quantify the number of latent genomes have produced results in the order of tens to hundreds of copies of HSV per neuron (Efstathiou *et al.*, 1986; Simmons *et al.*, 1992a). These data are difficult to reconcile with the observation that latency can be established experimentally in the absence of HSV DNA replication (Clements and Stow, 1989; Coen *et al.*, 1989; Leib *et al.*, 1989a; Steiner *et al.*, 1990; Speck and Simmons, 1991; Speck and Simmons, 1992). If a single type of latency exists, either viral DNA can be replicated in

the absence of detectable viral gene expression or the number of latently infected sites has been grossly underestimated. Second, studies using *in situ* polymerase chain reactions (PCRs) to localize viral DNA indicate some HSV genomes are transcriptionally silent, while others produce LATs (Ramakrishnan *et al.*, 1994; Mehta *et al.*, 1995). Finally, in a rabbit model of HSV latency where two types of reactivation can be demonstrated (spontaneous and induced), some mutations in the LAT region affect one but not both types of reactivation (Hill *et al.*, 1990). These data indicate that there are two pathways of reactivation, possibly originating from two types of latency. Experiments to address the issue of genome copy number have shown that HSV DNA is not distributed evenly amongst all LAT positive neurons; roughly ten fold more genomes persist per cell at sites where acute infection precedes latency, compared with sites where productive infection was not detected (Simmons *et al.*, 1992a). These results show that there are indeed two patterns of HSV DNA persistence, one characterized by high and another by low copy number. The biological significance, transcriptional status and reactivation criteria of these forms of persistence remain unclear, but the path by which some neurons come to harbour hundreds of copies of the genome is of interest. Slobedman *et al* (1994) used a thymidine kinase deficient (therefore replication defective in neurons) mutant of HSV to demonstrate that the high genome copy number form of latency could only be established by viruses able to productively infect neurons. These results implicate productive neuronal infection in production of high genome copy number latency, leading to the hypothesis that some neurons undergo an aborted productive infection with HSV, and survive carrying many copies of the genome (Simmons and Tscharke, 1992). In support of this idea, it has been shown that many neurons can withstand acute viral infection at least to the stage of γ gene expression, but only in the presence of intact immunity (Simmons and Tscharke, 1992).

Other models cannot be ruled out, the assumption that all persistent HSV DNA is in neurons needs definitive proof, although the current understanding is supported by *in situ* PCR reports (Ramakrishnan *et al.*, 1994; Mehta *et al.*, 1995). Also the absolute number of latent sites needs to be defined and amplification of viral sequences by cellular enzymes remains a possibility.

Latency outside of sensory ganglia

Persistence of HSV DNA has been observed in a variety of sites other than sensory ganglia including autonomic ganglia (Price and Schmitz, 1978; Warren *et al.*, 1978), adrenal medulla (Cook and Stevens, 1976), eyes (Sabbaga *et al.*, 1988; Claoue *et al.*, 1990) and skin (Scriba, 1977; Hill *et al.*, 1980; Clements and Subak-Sharpe, 1988; Simmons *et al.*, 1996), but the significance of these observations, and in some cases the ability of virus to be reactivated is questionable. There is little doubt however, that neuronal latency in sensory ganglia plays a pivotal role in HSV pathogenesis and is an important reservoir of infection.

1.2.6.4 Recurrence

Recurrent HSV infection occurs when latent virus in sensory ganglia is reactivated, spreads via axons to the skin and initiates a new episode of cutaneous infection; if this infection becomes clinically apparent it is termed a recrudescence. Reactivation in sensory neurons may not necessarily lead to recurrence because the latter requires transport of infectious virus to the periphery and infection of the skin (Wildy *et al.*, 1982). Likewise recrudescence is not an inevitable consequence of recurrence, as indicated by studies

demonstrating asymptomatic shedding of HSV in various human populations (Rattray *et al.*, 1978; Mertz *et al.*, 1992; Mertz, 1993). The role played by the immune system in preventing the progression from reactivation to recurrence and recrudescence is not defined. Immunosuppression increases the frequency and severity of recrudescence (Bagdades *et al.*, 1992), yet recurrences occur in spite of apparently intact specific anti-herpetic immunity (Burnet and Williams, 1939). Also the administration of neutralizing antibody can prevent seeding of infection in the skin from nerves, but antibody levels several fold higher than normal are required for this protection (Simmons and Nash, 1985).

Recrudescent HSV lesions are usually confined to the same dermatome as the primary lesion, but are frequently at a different site. For example, primary orofacial lesions are often inside the mouth, while recrudescences following such an infection are frequently at the mucocutaneous junctions of the lips. Likewise primary genital lesions of women often involve the cervix, but recurrences are usually on the skin of the vulva (Corey *et al.*, 1983). Neither the reasons for, nor significance of the disparity between the locations of primary and recurrent lesions are understood. However, the fact that it is frequently observed indicates that spread of virus in the peripheral nervous system (possibly by productive neuronal infection) during the initial infection, may play a significant role in HSV pathogenesis.

1.3 Immunity to herpes simplex virus infections

The immunobiology of HSV infections has been studied extensively using *in vivo* models in animals, and *ex vivo* and *in vitro* systems based on human cells. Each of these systems

has strengths and limitations, and comparisons of data between them is difficult. Animal models have had great value in answering basic questions about the nature of immune responses to HSV and mouse models stand out as the most frequently used. Reasons for the popularity of mouse models include fidelity to natural disease in humans (depending on route of inoculation), availability of well characterized inbred strains and immunological reagents, cost and ease of handling. Experiments using human cells *in vitro* have been instrumental in demonstrating the existence of a variety of immune responses in natural HSV infections and, in general, responses defined originally in animal models have also been found to be active in humans.

Any breakdown of the immune system into discrete parts is artificial because of the integrated nature of the system itself. The way in which the immune response is generally divided is firstly into non-adaptive and adaptive immunity, and adaptive immunity is then divided into humoral and cell mediated immunity. This view is largely historically based and persists despite evidence that non-adaptive immunity and adaptive immunity share many of the same mediators, and humoral responses are as reliant on cells (and in most cases T cells) as the so called cell mediated responses. However the immense complexity of immune responses necessitates division of the whole system in smaller pieces and the divisions stated above have served immunology well. For these practical reasons, the divisions mentioned above form the basic structure of the following discussion of HSV immunity.

1.3.1 Non-adaptive defences

Non-adaptive defence mechanisms are the first obstruction to successful infection that is encountered by any pathogen. Experimental inoculation of the skin with HSV is dependent on the use of physical trauma in nearly all models, indicating the strength of unbroken skin as a barrier to infection. Once the skin has been breached, other defences are encountered, including macrophages, neutrophils, natural killer (NK) cells, complement, and α and β interferons (α/β IFN). Evidence has been found for the activity of each of these mediators in resistance to HSV infection.

1.3.1.1 Macrophages

Macrophages have long been considered an important component of the anti-HSV response. Suckling mice are very susceptible to intraperitoneal HSV infection when compared with adults and partial protection against lethal challenge can be adoptively transferred to young mice by adult macrophages (Johnson, 1964b; Hirsch *et al.*, 1970). In addition, injection of anti-macrophage serum or silica particles with an intraperitoneal inoculum of HSV markedly reduces survival rates in adult mice (Zisman *et al.*, 1970). Two studies have used ^{89}Sr treatment which reduces the levels of circulating bone marrow derived cells (including monocytes and NK cells) without affecting tissue macrophages. Both studies used intraperitoneal inoculation of virus, but different strains of virus and inbred mice were used and conflicting results were reported (Lopez *et al.*, 1980; Morahan *et al.*, 1986). These reports highlight the difficulty in drawing general conclusions about the mechanisms of resistance to infection from experiments done using a single animal or

viral genetic background. In humans, age related resistance to HSV has been reported but the extent to which macrophage maturation contributes to this phenomenon is uncertain. As with mice, monocytes and macrophages derived from neonatal humans are more susceptible to infection *in vitro* than those from adults (Wu and Morahan, 1992). In addition, the ability of some HSV strains to replicate in macrophages has been linked to pathogenicity in the mouse intraperitoneal model (Ben-Hur *et al.*, 1988). However, because the infection that follows intraperitoneal inoculation with HSV is not similar to natural human diseases, the relevance of these findings to HSV infections in humans is unknown. As well as their role in natural resistance, macrophages collaborate with cells of the adaptive immune system and these responses are discussed below, with other aspects of cell mediated immunity.

1.3.1.2 Neutrophils

Neutrophils infiltrate HSV infections of the cornea and are the predominant cell type in resulting lesions (Doymaz and Rouse, 1992a; Niemialtowski and Rouse, 1992). Further, neutrophils are able to adhere to HSV infected cells and phagocytose antibody coated virions *in vitro* (Van Strijp *et al.*, 1988; Van Strijp *et al.*, 1989; Van Strijp *et al.*, 1991). Recently it was shown that depletion of neutrophils *in vivo*, resulted in enhanced viral growth in a mouse model of HSV eye infection (Tumpey *et al.*, 1996). However, to date, there has been no work on the role of neutrophils in human HSV infections.

1.3.1.3 Natural killer cells

NK cells are a heterogeneous population comprising subgroups which differ in ^{89}Sr sensitivity, expression of surface markers including asialo-Gm_1 and their targets for lysis (Lust *et al.*, 1981; Minato *et al.*, 1981). This heterogeneity is also observed in populations able to cause natural lysis of HSV infected cells (Colmenares and Lopez, 1986). A number of experiments done to determine the role of NK cells in resistance of mice to HSV have been reported, but these have all used ^{89}Sr or anti-asialo-Gm_1 antibody (Ab) to deplete NK cells, measured lysis of a single infected cell type or a combination of these methods. The heterogeneity of NK cells makes these experiments hard to interpret and further complications include the non-specificity of depleting agents, use of single mouse genetic backgrounds and use of intraperitoneal infections. Not surprisingly, conflicting conclusions have been drawn from these studies, some investigators finding that NK cells are involved in resistance to HSV (Lopez *et al.*, 1980; Habu *et al.*, 1984; Rager-Zisman *et al.*, 1987) and others finding the opposite (Bukowski and Welsh, 1986; Morahan *et al.*, 1986). The role of NK cells in humans is similarly unclear. There are reports of patients with severe HSV infections and significantly lower NK function than the general population (Ching and Lopez, 1979; Lopez *et al.*, 1983; Biron *et al.*, 1989). T lymphocyte deficiency was generally ruled out by the authors as a cause of susceptibility to infection in these studies, but the patients may have had other undefined immunodeficiencies contributing to severe disease.

1.3.1.4 α/β Interferon

α/β IFN is released by cells infected by many viruses including HSV and is thought to help activate cells involved in non-adaptive immunity (Bukowski and Welsh, 1986; Domke-Opitz *et al.*, 1986; Straub *et al.*, 1986). α/β IFN limits HSV growth *in vitro* and all studies using HSV infected mice have concluded that depletion of α/β IFN results in a more exaggerated disease (Gresser *et al.*, 1976; Zawatsky *et al.*, 1982; Bukowski and Welsh, 1986). Again, these experiments were carried out using intraperitoneal inoculation of virus and the human α/β IFN response has yet to be characterized.

1.3.1.5 Genetics of natural resistance

A different approach to the investigation of host responses to infection is the identification of host genetic elements that confer resistance or susceptibility. The first work of this nature was done using the intraperitoneal model of HSV infection and disclosed that mouse strains fell into three groups: resistant, moderately susceptible and susceptible. Resistant mice could survive approximately six orders of magnitude more virus than susceptible mice, and the trait was dominantly inherited (Lopez, 1975). The ability of mice to survive in this model was subsequently found to be controlled by two autosomal loci, and the major histocompatibility complex (MHC), a large cluster of genes involved in the regulation of adaptive immunity, was not involved (Lopez, 1980). Using survival as the only measure of resistance to infection, especially after intraperitoneal inoculation of virus, may not provide information relevant to human disease, which is generally self limited. The distribution of susceptibility to HSV amongst inbred mouse strains is the same after

cutaneous inoculation as that described for intraperitoneal models, but the genetics of resistance differ (Simmons and La Vista, 1989). Following cutaneous infection, neuroinvasion is delayed in resistant C57BL/10 mice when compared with BALB/c mice, and this effect is under complex control with possibly four independent loci involved (Simmons and La Vista, 1989). In contrast, survival in this model was determined by a single locus, highlighting that the use of death as an endpoint may be misleading.

1.3.2 Humoral immunity

1.3.2.1 Role of antibody in HSV infections

The observation that mice born to immune mothers were themselves immune to a normally lethal dose of virus was the first evidence that immunity to HSV could be passively transferred (Berry and Slavin, 1943). In subsequent experiments, it was demonstrated that the natural mode of transfer was largely mammary, and the effect could be mimicked by the administration of serum from HSV infected rabbits (Berry and Slavin, 1943). By 1946 there were five reports in the literature relating to the ability of passively transferred anti-HSV Ab to reduce mortality after experimental inoculation of young mice (Evans *et al.*, 1946). These experiments were repeated and refined by various workers, and the use of specific antiserum in the treatment or prophylaxis of human neonatal HSV infections was suggested (Luyet *et al.*, 1975; Baron *et al.*, 1976). This work demonstrates that passively transferred Ab can provide some protection against a lethal viral challenge, but the role of natural Ab responses in fighting herpetic disease was not approached.

The significance of work which aims to show the importance of natural Ab responses using passive transfer experiments is questionable as unphysiologically high doses of serum (Cheever and Daikos, 1950; Notkins, 1974) or monoclonal Abs (mAbs) (Dix *et al.*, 1981; Balachandran *et al.*, 1982) have, in general, been administered at times earlier than Ab naturally appears. Where more physiological amounts of Ab are used, they fail to protect mice from infection *in vivo*, even when transferred early (Oakes, 1975). Administration of anti-HSV Ab to immunocompromised mice reduced disease, but ultimately failed to eliminate infection (Oakes and Rosemond-Hornbeak, 1978; Nagafuchi *et al.*, 1979; Openshaw *et al.*, 1979; Kapoor *et al.*, 1982b; Kino *et al.*, 1982), except in studies where intraperitoneal inoculation (Worthington *et al.*, 1980), or certain high titre mAbs (Eis-Hübinger *et al.*, 1993) were used. These findings indicate that although Ab is potentially useful therapeutically, it is probably not the primary agent of anti-HSV immunity.

B lymphocyte suppressed mice are a useful tool for examining the role of natural Ab responses (Lawton *et al.*, 1972). B cell suppressed mice have normal cellular immune responses but do not produce antiviral Ab and, in spite of this deficiency, they recover from HSV infection normally (Kapoor *et al.*, 1982a). Cutaneous HSV infection was not found to be greater in B cell suppressed mice when compared with immunocompetent mice, but there was evidence that acute and latent ganglionic infections were enhanced (Kapoor *et al.*, 1982a). Simmons and Nash (1987) confirmed these results and extended them, showing that following a primary infection, immunity to reinfection was not compromised by B lymphocyte suppression unless high doses of virus were used. The apparent inability of Ab to combat HSV infection in the skin, while providing some protection from neural infection, had also been noted in passive immunization experiments (Kapoor *et al.*, 1982b).

HSV can be transmitted by cell fusion *in vitro*, allowing viral spread in the presence of neutralizing antiserum (Stoker, 1958; Ennis, 1973a; Hooks *et al.*, 1976). In addition there is histological evidence that HSV spreads via cell fusion in the skin but not nervous system, providing an explanation of the differential efficacy of Ab at these two sites (Simmons and Nash, 1987). Virus is also likely to be exposed to neutralizing Ab when infecting nerve endings, and when cutaneous infection is seeded from nerves, as must occur during recurrence (Simmons and Nash, 1985). From the above it can be concluded that Ab plays a supporting role in anti-HSV immunity and most likely acts predominantly by neutralizing extracellular virus, reducing spread of infection to and within the nervous system.

1.3.2.2 Functions of non-neutralizing anti-HSV antibody

Neutralization is not the only mechanism by which anti-herpetic Ab may act. Activation of cytotoxic cells by antibody-antigen complexes leading to lysis of infected cells, known as antibody dependent cellular cytotoxicity (ADCC), has strong support from some workers (Kohl, 1991; Kohl, 1992). Interactions of antibodies with other mediators can be stopped by removing a portion of the Ab molecules by proteolytic digestion (forming fragments called $F(ab')_2$) without damaging the neutralizing potential of the serum. Anti-HSV $F(ab')_2$ fragments are not as effective in transferring protection against herpetic infection to mice as full Ab molecules, and two studies ruled out the action of complement components which can also act with Ab to lyse infected cells (McKendall *et al.*, 1979; Oakes and Lausch, 1981; Hayashida *et al.*, 1982). Further data interpreted to support ADCC is that passively transferred antibody is most efficacious in the presence of leukocytes, either endogenous (Oakes, 1975; Oakes and Rosemond-Hornbeak, 1978), or transferred with

serum into immunocompromised mice (Rager-Zisman and Allison, 1976; Kohl and Loo, 1982). All the evidence for ADCC (i) relies on passive transfer experiments, the pitfalls of which have already been stated, and (ii) proposes a role for antibody in virus clearance from cutaneous lesions which heal normally in the absence of Ab. Additionally, evidence for the importance of the various cell types thought to mediate ADCC (NK cells, neutrophils and macrophages) is far from conclusive. Therefore, although ADCC can be demonstrated experimentally, the contribution made by this response in natural infections is probably minor.

Another possible mechanism of Ab action is manifest only in the nervous system, where there is an established role for naturally produced anti-HSV antibody (Simmons *et al.*, 1992b and see above). Infection of cells of neuronal origin with a sinbis, rabies and measles viruses can be halted or even cleared by Ab in the absence of any other immune mediators (Levine *et al.*, 1991; Dietzschold *et al.*, 1992; Schneider Schaulies *et al.*, 1992 respectively). In some cases the antibodies are able to downregulate viral gene expression at the level of transcription and, so far, all mAbs found to be active against infected neurons *in vitro* are protective when administered *in vivo*, as reviewed by Dietzschold (1993). HSV infection can be down-regulated *in vitro* by non-neutralizing mAbs directed against gB and gE (Oakes and Lausch, 1984). The cause of viral down-regulation has yet to be defined but was dependent on the constant presence of mAbs. As demonstrated for other viruses, mAbs active against HSV *in vitro* were found to be protective *in vivo* (Rector *et al.*, 1982; Oakes and Lausch, 1984). More evidence is required before this response can be considered significant. It may be relevant in this context that infiltrating B cells can be found in ganglia as early as four days after cutaneous inoculation (Cook and Stevens, 1983). At this

time there are HSV infected neurons, but probably little extracellular virus amenable to neutralization (Tullo *et al.*, 1982; Simmons and Nash, 1987).

1.3.2.3 Antibody in human HSV infections

In contrast to the large body of evidence suggesting that exogenously supplied polyclonal or monoclonal Ab can ameliorate disease in experimentally infected mice, the efficacy of antibody therapy in human disease has not been shown convincingly (Kohl, 1992). Although most clinical evidence in this field is discouraging, recent work to identify new target epitopes (Fuller and Spear, 1987; Staats *et al.*, 1991; Eis-Hübinger *et al.*, 1993) and new technologies for producing mAbs, such as phage display recombinatorial antibody libraries (Burioni *et al.*, 1994; Cattani *et al.*, 1995; Sanna *et al.*, 1995) may provide ways forward. The natural role of antibody in fighting herpetic disease in humans is also not well defined. There are two reports of patients with Bruton's (or x-linked) agammaglobulinemia, a purely B cell defect, and unusually severe HSV infections, however each case was complicated with other viral infections and immune system anomalies (Kohl, 1992). Other evidence from the literature suggests that uncomplicated Bruton's does not affect the healing of cutaneous herpetic lesions (Merigan and Stevens, 1971). A number of studies have been done that attempted to link severity of neonatal disease with low levels of circulating Ab, but the results thus far are conflicting (Kohl, 1992). Passive transfer of Ab from immune mothers has been cited as a reason why babies exposed during birth to HSV from recurrent, rather than primary, infections are at less risk of neonatal herpes (Kohl, 1992). However this interpretation of the data is not conclusive because recurrent lesions are associated with less viral shedding than primary lesions

(Corey *et al.*, 1983). In view of the above considerations, it is likely that in humans, as in mice, antibody plays a supporting rather than primary role in anti-herpetic immunity.

1.3.3 T lymphocyte mediated immunity

There is little doubt that T lymphocytes contribute significantly to anti-HSV immunity in mice and humans. Evidence that cell mediated immunity is active against HSV started to accumulate approximately 25 years ago. Zisman *et al* (1970) showed that mice treated with anti-lymphocyte serum are compromised in their ability to survive intraperitoneal HSV infections. Independent groups demonstrated that HSV infections *in vitro* could be reduced by addition of immune cells to cultures and that this effect was not attributable to antibody (Ennis, 1973a; Lodmell *et al.*, 1973). Subsequently Ennis (1973b) used the cells shown to be effective *in vitro* to protect mice from an otherwise lethal challenge with HSV. However, these early experiments were neither able to accurately define the relevant cell types nor give mechanistic insights.

The availability of mice depleted of thymocytes, either through the use of anti-thymocyte serum or because of a congenital defect (nude mice), allowed more definitive experiments demonstrating that T lymphocytes are an essential component of cellular anti-HSV responses to be done (Oakes, 1975; Walz *et al.*, 1976). Significantly, resistance could be transferred using HSV-immune splenocytes to these susceptible mice (Oakes, 1975; Nagafuchi *et al.*, 1979; Kapoor *et al.*, 1982b) or mice subjected to general immunosuppressive regimes (eg. cyclophophamide treatment) (Rager-Zisman and Allison, 1976). However, the protection was abrogated if the spleen cells were depleted of

thymocytes (Oakes, 1975; Rager-Zisman and Allison, 1976; Nagafuchi *et al.*, 1979). More recently, studies of HSV infections in MHC congenic mice, which differ genetically only at a region known to control T cell responses, disclosed that some variations at this locus confer susceptibility, further implicating T cell mediated immunity in resistance to HSV (Simmons, 1989). Additionally, transgenic mice unable to make functional T lymphocytes are compromised in their ability to survive HSV infections (Manickan and Rouse, 1995).

Corey *et al* (1978) noted an inverse correlation between the peak of peripheral blood lymphocyte proliferation in response to HSV and the length of virus shedding in patients experiencing primary genital herpes simplex. Additionally, severe and life threatening HSV infections are largely restricted to patients with profound deficiencies in cellular immunity, providing strong evidence that T lymphocytes play a crucial role in human anti-herpetic immunity (Merigan and Stevens, 1971; Schmid and Rouse, 1992; Simmons *et al.*, 1992b). Uncontrolled recurrence of latent infection is not uncommon following kidney transplantation and the associated immunosuppressive therapy (Naraqi *et al.*, 1977; Pass *et al.*, 1979). Similarly, infection with the human immunodeficiency virus is associated with increased severity of HSV disease (Siegal *et al.*, 1981; Quinnan *et al.*, 1984).

1.3.3.1 Major histocompatibility complex: T cell subsets and their regulation

The MHC is a large cluster of genes involved in the regulation of mammalian adaptive immunity and the murine and human MHCs are known as H-2 and HLA, respectively. In mice and humans, the MHC is naturally highly polymorphic. Inbred mouse strains provide well characterized populations of animals with identical MHC genes, each designated a H-2

haplotype. MHC gene products are cell surface glycoproteins and, based on structure and function, they can be divided into two major classes, class I and class II. Class I products involved in adaptive immunity are encoded mainly in the H-2K, D and L loci, and HLA-A, B and C loci of mice and humans respectively. Relevant class II regions include H-2I in mice and HLA-DP, DQ and DR in humans. The significance of MHC products in adaptive immunity is that they are required to display antigenic epitopes at the cell surface for recognition by T lymphocytes. As determined by x-ray crystallography, the gross structures of both classes of MHC products are similar. The distal portion of an MHC molecule contains a groove (of which the sides are defined by two α-helices and the floor by a series of anti-parallel β-sheets) that binds an antigenic peptide such that it is available to interact with receptors on T cells (Bjorkman *et al.*, 1987; Madden *et al.*, 1991; Fremont *et al.*, 1992; Brown *et al.*, 1993; Fremont *et al.*, 1996). Antigen receptors on T cells (TCRs) are thought to recognize both the peptide and parts of the presenting MHC molecule (Davis and Bjorkman, 1988; Bentley *et al.*, 1995; Fields *et al.*, 1995). A cognate interaction occurring between an MHC-peptide complex and a TCR is essential but not sufficient for T cell activation; a number of additional molecular interactions between the antigen presenting cell and the T cell are required (Dustin and Springer, 1991). One of these interactions is the binding of either CD4 or CD8 (expressed on the T cell) to an MHC product at a site independent to that which contacts the TCR (Littman, 1987). CD8 and CD4 are not co-expressed on mature T cells and interact exclusively with class I and class II MHC products respectively (Swain, 1981; Marrack *et al.*, 1983; Swain, 1983; Littman, 1987). As a result, T cells can be divided into two populations, $CD8^+$ and $CD4^+$, which recognize epitopes presented in the context of class I and class II MHC products respectively (Kappler and Marrack, 1976; Marrack and Kappler, 1987). Thus the

population of T cells responding to a given epitope is restricted according to the MHC product with which the epitope is associated (Swain, 1983; Townsend and Bodmer, 1989). Classically, $CD4^+$ T lymphocytes were considered to be involved in regulating and enhancing all facets of adaptive immunity and were termed helper T (Th) cells. $CD8^+$ T lymphocytes were thought to kill infected, or otherwise aberrant, cells and were termed cytotoxic T lymphocytes (CTLs). It is now clear that this association between phenotype and function is not absolute and either subset of T cell can have multiple activities even at the level of individual cells (Yasukawa and Zarling, 1984b; Johnson *et al.*, 1990). Another dogma that has been breaking down recently, is the concept that class I and class II MHC products present only endogenously and exogenously derived antigens (with respect to the antigen presenting cell), respectively. Investigations into the pathways of antigen processing and MHC assembly led to this model (Brodsky and Guagliardi, 1991), but it now seems that the processing pathways are less discrete than originally thought (Weiss and Bogen, 1991; Malnati *et al.*, 1992; Pfeifer *et al.*, 1993; Kovacsovics Bankowski and Rock, 1995) and viral proteins are included in the list of antigens which contradict the dogma (Bohm *et al.*, 1995; Oxenius *et al.*, 1995). During development of the immune system, T cells that recognize only non-self peptides complexed to self MHC products survive (Davis and Bjorkman, 1988). A consequence of this process is that mature T cells recognize antigens presented only by MHC molecules from a syngeneic background (Shevach and Rosenthal, 1973; Zinkernagel and Doherty, 1974), hence the usefulness of inbred animals when studying T lymphocyte mediated immunity.

1.3.3.2 Relative roles of HSV-specific $CD4^+$ and $CD8^+$ T lymphocytes

The relative importance of $CD4^+$ and $CD8^+$ T cells in murine anti-HSV immunity has been a contentious issue and, while primacy of each subset been proposed (Nagafuchi *et al.*, 1982; Larsen *et al.*, 1983; Sethi *et al.*, 1983; Bonneau and Jennings, 1989; Manickan and Rouse, 1995; Manickan *et al.*, 1995b), other workers have favoured complementary roles for $CD4^+$ and $CD8^+$ cells (Howes *et al.*, 1979; Larsen *et al.*, 1984; Nash *et al.*, 1987; Smith *et al.*, 1994). The majority of inquiries into the relative roles of $CD4^+$ and $CD8^+$ cells have used adoptive transfers and demonstrate only that the T cell subset in question can be used therapeutically or prophylactically against HSV infection (Simmons *et al.*, 1992b). In addition, results gained with adoptive transfers are affected by the way in which donor animals were immunized and the degree of *in vitro* manipulation or culturing of cells before transfer (Larsen *et al.*, 1984). Information has also been gained using immunization protocols that preferentially prime one subset of T cells (Nash *et al.*, 1981a; Martin *et al.*, 1987; Mishkin *et al.*, 1992; Mercadal *et al.*, 1993; Manickan *et al.*, 1995a; Manickan and Rouse, 1995; Manickan *et al.*, 1995b). These experiments disclose more about the value of the particular immunization than the relative value of T cell subsets in a natural infection. Finally, the effects of depleting mice, *in vivo*, of lymphocyte subsets, by injection of subset-specific mAbs, have been studied. This work is most likely to provide useful information about natural immunity to HSV, with the caveat that compensatory immune mechanisms are likely to be developed in T cell depleted animals. In two reports, each on a different genetic background, the effect of $CD4^+$ and $CD8^+$ cell depletions on HSV infection was compared, and each subset was shown to contribute to the elimination of infectious virus (Nash *et al.*, 1987; Smith *et al.*, 1994). In contrast, results of a series of

experiments with transgenic mice lacking either $CD4^+$ or $CD8^+$ cells, were interpreted as suggesting that $CD8^+$ T lymphocytes are dispensable in immunity to HSV (Manickan and Rouse, 1995). However, zosteriform lesion formation, which is a crude clinical marker, was used to judge the level of infection in these experiments. Additionally, mice of the haplotype used do not adequately generate HSV-specific $CD8^+$ T cells in the absence of $CD4^+$ cells (Jennings *et al.*, 1991), complicating the interpretation of data gained with $CD4^+$ cell deficient mice. $CD4^+$ and $CD8^+$ T cells are present in HSV infected tissues of mice (Chan *et al.*, 1989; Cantin *et al.*, 1995; Shimeld *et al.*, 1995; Liu *et al.*, 1996) and humans (Cunningham *et al.*, 1985; Sobel *et al.*, 1986). In human skin lesions, $CD4^+$ cells are present in higher numbers than $CD8^+$ cells, particularly early in infection, but the antigen specificity and activation stage of these lymphocytes is unknown (Cunningham *et al.*, 1985). Viewing the literature as a whole, it seems likely that $CD4^+$ and $CD8^+$ T lymphocytes contribute to anti-HSV immunity.

1.3.3.3 Analysis of HSV-specific T lymphocytes by function

Historically, HSV-specific T lymphocytes have been subdivided and characterized primarily according to measurable effector functions, rather than surface phenotype. There are two easily measurable anti-HSV cell mediated effector mechanisms, namely delayed type hypersensitivity (DTH) and lysis of infected cells by CTLs (Simmons *et al.*, 1992b). Both mechanisms have been useful as functional markers for different subsets of T cells but, as will be discussed, they are clearly not the only anti-viral responses, and their *in vivo* relevance has yet to be adequately demonstrated.

Delayed type hypersensitivity

DTH is an assay used to measure the recruitment and activation of macrophages, or other non-specific effector cells, by antigen activated T cells via the secretion of cytokines. *In vivo*, DTH can be observed as local inflammation at a site of secondary antigen challenge (Nash *et al.*, 1980a). Secretion of anti-viral cytokines, for example interferon-γ (IFN-γ), in addition to activation of macrophages may contribute to clearance of virus (Blanden, 1971). However it must be noted that cytokine expression is not limited to responses measurable as DTH and DTH can be mounted, albeit less efficiently, in the absence of interferon-γ (Bouley *et al.*, 1995).

DTH responses to HSV have been shown in humans (Anderson and Kilbourne, 1961) and experimentally infected mice (Nash *et al.*, 1980a) and guinea pigs (Lausch *et al.*, 1966). If a mouse ear pinna is used as the challenge site, the amount of inflammation, and hence strength of a DTH response, can be assessed by measuring ear thickness using a screw gauge micrometer (Nash *et al.*, 1980a). DTH to HSV can be transferred with draining lymph node cells from cutaneously infected mice to naive mice, provided that donor and recipient are H-2 matched at the class II locus, implicating $CD4^+$ cells in this response (Nash *et al.*, 1981b). In agreement with this finding, cell populations enriched for $CD4^+$, but not $CD8^+$, T cells are able to transfer DTH (Nash and Gell, 1983; Larsen *et al.*, 1984) and depletion *in vivo* of $CD4^+$ but not $CD8^+$ cells abrogates DTH in mice (Nash *et al.*, 1987). In some models, adoptive transfer of cells that mediate DTH has been correlated with protection, as measured by reduction of virus titres in skin, after HSV challenge (Nash *et al.*, 1981b; Nash and Gell, 1983). Further, in mice immunized with certain HSV glycoproteins, protection against disease has been equated with induction of DTH (Schrier

et al., 1983). However, there are data to suggest that the ability of mice to mount a DTH response to HSV may not be a good measure of protective anti-viral immunity, or even $CD4^+$ T cell mediated immunity. First, tolerance to DTH can be selectively induced by intravenous inoculation of virus, without compromising virus clearance (Nash *et al.*, 1981a). Second, $CD4^+$ splenocytes taken from mice immunized by the intraperitoneal route and transferred directly to naive syngeneic mice are very poor producers of DTH, but are protective against an HSV challenge (Larsen *et al.*, 1984). Finally, mice immunized with HSV α4 (ICP4) generated DTH responses to whole virus, but not protective immunity (Martin *et al.*, 1990). There is also some indication that DTH can cause tissue damage in HSV infections in the CNS (Altmann and Blyth, 1985; Chan *et al.*, 1989) and eye (Doymaz and Rouse, 1992a; Doymaz and Rouse, 1992b), and in one report, a vaccination protocol that induced tolerance to DTH reduced this immunopathology (Altmann and Blyth, 1985).

Cytotoxic T lymphocytes

CTLs are T lymphocytes that can kill virally infected cells, or other cells presenting non-self antigens, in an MHC restricted manner (Zinkernagel and Doherty, 1974). Such killing is observed when antigen-primed CTLs are mixed *in vitro* with MHC-matched target cells presenting appropriate antigens. In these assays, cytotoxicity is generally measured by quantifying release of $Na_2{}^{51}CrO_4$, with which the targets were previously labelled (Shinohara, 1992). Killing of virally infected cells by CTLs before infectious progeny are released is considered an important mechanism in anti-viral immunity (Pfizenmaier *et al.*, 1977b; Zinkernagel and Althage, 1977; Martz and Gamble, 1992).

HSV-specific CTL precursors can be isolated from local draining lymph nodes

(Pfizenmaier *et al.*, 1977a; Pfizenmaier *et al.*, 1977b) and spleens (Lawman *et al.*, 1980b; Nash *et al.*, 1980b) of mice infected with the virus, or peripheral blood of seropositive humans (Sethi *et al.*, 1980; Yasukawa *et al.*, 1983; Schmid, 1988; Torpey III *et al.*, 1989). The cells are referred to as CTL precursors because they require a period of culture *in vitro* before they have activity in cytotoxicity assays (Pfizenmaier *et al.*, 1977b; Sethi *et al.*, 1980). It has been suggested from murine studies that immune suppressor factors exist in lymphoid tissue and removal of these factors, by culture, allows proliferation and differentiation of CTL precursors to an active phenotype (Pfizenmaier *et al.*, 1977a; Lawman *et al.*, 1980a). The detection of a splenocyte derived factor that can suppress the activity of CTLs *in vitro* has been cited as further evidence for this hypothesis (Horohov *et al.*, 1986). Recently, active CTLs have been isolated directly from herpetic lesions in the brains of mice, indicating that CTL precursors like those found in lymphoid tissue, may be activated locally, at a site of infection (Hudson and Streilein, 1994). The *in vivo* priming requirements of murine HSV specific CTLs varies amongst different inbred mouse strains. $CD8^+$ CTLs can be generated in the absence of $CD4^+$ T lymphocytes in $H\text{-}2^k$ mice (Nash *et al.*, 1987; Mercadal *et al.*, 1991), but not $H\text{-}2^b$ or $H\text{-}2^d$ mice (Jennings *et al.*, 1991; Mercadal *et al.*, 1991 respectively). These differences highlight (i) the extent to which genetic factors, presumably within the MHC, shape the immune response to pathogens and (ii) the dangers of extrapolating results gained using a single inbred mouse strain to other strains or outbred populations.

In mice, the majority of HSV-specific CTLs have been shown to be MHC class I restricted (Pfizenmaier *et al.*, 1977a; Sethi *et al.*, 1983), but the restricting class I region varies amongst inbred mouse strains (Jennings *et al.*, 1984; Bonneau and Jennings, 1990). In

agreement with these results, most murine CTLs bear the CD8 surface marker (Rouse *et al.*, 1983), although $CD4^+$ CTLs are generated at a lower frequency (Kolaitis *et al.*, 1990; Doymaz *et al.*, 1991; Niemialtowski *et al.*, 1994). There is some controversy over the predominant phenotype of human HSV-specific CTLs. Cytotoxicity mediated by human cells was originally shown to be class I restricted, but the possibility of class II restricted CTLs was not tested (Sethi *et al.*, 1980; Yasukawa *et al.*, 1983). The existence of $CD4^+$, class II restricted CTLs was first reported in clonal analyses of T cells derived from the blood of HSV seropositive patients (Yasukawa and Zarling, 1984a; Yasukawa and Zarling, 1984b). In these studies, the majority of cytolytic clones were $CD4^+$. A subsequent experiment with short term bulk cultured human CTLs, which were also largely $CD4^+$, seemed to confirm these results (Schmid, 1988). HSV infected keratinocytes that have been shown to express class II MHC have been proposed to be the natural targets for $CD4^+$ CTLs *in vivo* (Cunningham and Noble, 1989). In addition, $CD4^+$ CTL clones have now been derived directly from recurrent genital lesions (Koelle *et al.*, 1994a). The main criticism of these data is that blood derived lymphocytes must be stimulated with antigen *in vitro*, which presumably amplifies a rare subset of cells, before they demonstrate CTL activity and the form of antigen used can determine the phenotype of resultant CTLs (Yasukawa *et al.*, 1989). HSV antigens that preferentially associate with class II MHC products, such as heat inactivated virus, tend to stimulate $CD4^+$ cells whereas HSV infected fibroblasts, which are more likely to present antigens in association with class I products, stimulate $CD8^+$ cells (Yasukawa *et al.*, 1989; Posavad *et al.*, 1996). Accordingly, more recent studies using virally infected cells as stimulators have shown that $CD8^+$ CTL precursors occur at frequencies as high as those reported for $CD4^+$ CTL (Posavad *et al.*, 1996). Therefore it seems that $CD4^+$ and $CD8^+$ CTL precursors are present in HSV-

seropositive individuals, but it is less clear whether these precursors mature to an active phenotype and function as cytotoxic cells *in vivo*.

The antigen specificity of the CTL response to HSV has been investigated extensively, with the aim of defining the range of HSV proteins which are presented to T lymphocytes and identifying so called immunodominant antigens. However, a single MHC product can only present a limited range of antigenic peptides (Falk *et al.*, 1990; Romero *et al.*, 1991; Arnaiz-Villena, 1993) and therefore, owing to MHC polymorphism, animals of different MHC type present different epitopes to T cells. This consideration leads to the conclusion that different outbred hosts might recognize different viral products, especially if the virus is large and, while this concept is not upheld with all viruses, probably for co-evolutionary reasons, it appears to be valid in the case of HSV. Most work of this nature has been carried out in inbred mice of MHC types H-2^b, H-2^d and H-2^k and CTL recognition of six HSV proteins, gB, gC, ICP0, ICP4, ICP27 and ICP47 has been examined in all of these haplotypes. H-2 matched $CD8^+$ CTLs derived from the lymph nodes or spleens of HSV infected mice can recognize (and kill) cells of H-2^b haplotype expressing gB (Rosenthal *et al.*, 1987; Witmer *et al.*, 1990; Bonneau *et al.*, 1993; Nugent *et al.*, 1994), gC (Glorioso *et al.*, 1985; Martin *et al.*, 1993) or ICP27 (Nugent *et al.*, 1995) but not ICP4 (Martin *et al.*, 1990). In contrast, similarly derived H-2^d restricted CTLs recognize cells expressing gB (Witmer *et al.*, 1990; Hanke *et al.*, 1991) and ICP27 (Banks *et al.*, 1991; Banks *et al.*, 1993; Rouse *et al.*, 1994), but not gC or ICP4 (Martin *et al.*, 1990; Martin *et al.*, 1993). A third, overlapping set of HSV proteins can be recognized by *ex vivo* H-2^k CTLs which includes gC (Rosenthal *et al.*, 1987; Martin *et al.*, 1993) and ICP4 (Martin *et al.*, 1990) but not gB or ICP27 (Rosenthal *et al.*, 1987; Martin *et al.*, 1989; Martin *et al.*, 1990; Banks *et al.*,

1991; Banks *et al.*, 1994). Cells expressing ICP0 or ICP47 could not be lysed by anti-HSV CTLs irrespective of MHC type (Martin *et al.*, 1990; Banks *et al.*, 1994). Further considerations arising from the above literature and related work include: (i) None of the HSV proteins examined could be recognized by T cells from all haplotypes, and therefore not all individuals in an outbred population would be expected to respond to a single antigen. These results imply that vaccines comprising a single HSV protein (or even a few) may not be useful. (ii) The context in which proteins are delivered to mice may determine whether or not specific T lymphocytes are generated. For example H-2^k mice produce ICP4 specific CTLs in response to HSV infection, but not in response to immunization with a recombinant vaccinia virus expressing ICP4 (Martin *et al.*, 1990; Manickan *et al.*, 1995a). With gC, the reverse situation with a vaccinia recombinant and HSV infection has been reported in the same mouse haplotype (Martin *et al.*, 1993). Significantly, vaccinia virus recombinants expressing viral proteins known to be recognized by at least one component of a natural anti-HSV response do not necessarily confer protection when used to immunize mice (Martin *et al.*, 1990). (iii) Infection with different types, and therefore by extrapolation different strains, of virus may elicit T cell responses directed at different ranges of proteins (Salvucci *et al.*, 1995). (iv) The route of inoculation may affect the range of proteins to which the immune system is directed. For example gC specific CTL are generated in H-2^k mice after footpad (Rosenthal *et al.*, 1987), but not intraperitoneal (Martin *et al.*, 1993), inoculation of HSV. (v) Different protocols for *in vitro* manipulation or expansion of T lymphocytes before they are tested for target recognition may change the range of targets recognized (Nugent *et al.*, 1995). (vi) Although the investigations in this area have been done almost exclusively with $CD8^+$ (MHC class I restricted) T cells, the lessons learned are likely to be equally applicable to $CD4^+$ T cells, because antigens are

presented physically by MHC class I and II products in similar ways. (vii) Human studies indicate that in natural HSV infections, $CD4^+$ and $CD8^+$ T lymphocytes that recognize multiple viral proteins, rather than a single immunodominant epitope, are generated (Tigges *et al.*, 1992; Koelle *et al.*, 1994b). This result would be anticipated from an overall consideration of the murine studies.

The importance of cytotoxicity as an *in vivo* mechanism for the clearance of viral infection has best been demonstrated using transgenic mice that have $CD8^+$ T cells but are unable to generate CTLs because of a targeted deletion in the perforin gene (Kägi *et al.*, 1994). In these studies, a clear role for cytotoxicity in immunity against lymphocytic choriomeningitis virus was demonstrated (Kägi *et al.*, 1994). No similar experiments have been done to establish a role for anti-HSV CTLs and the literature in this area is not definitive. Mice immunized with vaccinia recombinants expressing herpes glycoproteins induce a DTH response and antibody capable of neutralizing herpes simplex but do not generate anti-herpes CTLs (Martin *et al.*, 1987; Martin *et al.*, 1989). The ability of these vaccinia virus immunized mice to survive a challenge with a high dose of HSV is poor compared with mice vaccinated with a low dose of HSV. It has been suggested that this discrepancy is the result of poor CTL activation by the recombinant vaccinia virus immunized mice (Martin *et al.*, 1989). As with investigations of DTH, adoptive transfers were done, but in these experiments, protection against HSV was shown to be transferred with highly lytic T cell populations (Sethi *et al.*, 1983; Bonneau and Jennings, 1989). These experiments, like all adoptive transfers, cannot be used to infer a definite role for the transferred cells in a natural infection. Further, it is possible that adoptively transferred cells do not act cytolytically *in vivo*, so these reports do not give mechanistic insights.

There are examples of protection being transferred or developed in the absence of CTLs (Martin *et al.*, 1987; Nash *et al.*, 1987; Smith *et al.*, 1994; Manickan *et al.*, 1995b), and of immunizations which generate CTLs, but not protection (Banks *et al.*, 1991). Finally, H-2^k mice generate a moderate CTL response, as judged by precursor frequencies (Rouse *et al.*, 1983; Niemialtowski *et al.*, 1994), yet this haplotype has been associated with extremely poor immune control of HSV infections (Simmons, 1989). Therefore although there is likely to be a role for MHC restricted cytotoxicity in anti-HSV immunity, its significance remains undefined.

Other functions of HSV-specific T lymphocytes.

If the activity of T lymphocytes in immunity to HSV infections cannot be adequately explained in terms of DTH and CTLs, what then are the other functions of these cells? Other defined mechanisms of anti-viral T cell activity rely on the effects of soluble anti-viral mediators such as cytokines, which may be produced in the absence of detectable DTH. Cytokines may be produced by $CD4^+$ and $CD8^+$ lymphocytes (Mosmann and Coffman, 1989; Kelso, 1995) and some have established anti-HSV activity *in vitro* (Balish *et al.*, 1992; Chen *et al.*, 1993). To further define mechanisms of immunity against herpes simplex, the direct analysis of cytokines, and other known immunomodulatory molecules, is likely to be more useful than analysis of broader and less well defined phenomena, such as DTH. In addition, there may be protective mechanisms that are beyond our present knowledge, and the best hope for identifying these lies in the application new strategies to well defined models of HSV pathogenesis and immunity.

1.3.3.4 The role of cytokines and helper T cell subsets

Cytokines are a large family of intercellular messenger molecules that are released by a variety of cells to alter the responses of their neighbours. In immune reactions, cytokines can regulate host responses to antigens and in some cases they have potent anti-viral effects (Paul and Seder, 1994). The regulatory roles of cytokines are yet to be fully elucidated, but it has become apparent that helper T cells can be considered to fall on a gradient between two extremes, according to cytokine profiles (Kelso, 1995). The two extremes, designated Th1 and Th2, were defined originally by observations of T cell clones (Mosmann *et al.*, 1986; Bottomly, 1989; Mosmann and Coffman, 1989) and these observations are supported by evidence that protective responses to some pathogens *in vivo* are also weighted towards production of either Th1 or Th2 cytokines (Murray *et al.*, 1989; Heinzel *et al.*, 1991; Clerici and Shearer, 1993; Wesselingh *et al.*, 1994; Finke *et al.*, 1995; Huber *et al.*, 1996). The predominant cytokines in Th1 skewed responses are interleukin-2 (IL-2), IFN-γ and tumour necrosis factor-β (TNF-β), and they support macrophage activation, DTH, CTLs and production of antibody of IgG2a subtype by B cells. The cytokines characteristic of a Th2 weighted response are interleukins 4, 5, 6, 10 and 13 (IL-4, 5, 6, 10 and 13). They strongly support B cell activation and humoral immunity in general and, in particular, the production of IgG1 and IgE isotypes. A third type of T cell clone, designated Th0, has been defined on the basis of IL-2 production and also the production of cytokines typical of both the other types of Th clones. Th0 cells may be the precursors for Th1 and Th2 cells (Kamogawa *et al.*, 1993) or Th cells that fall somewhere toward the middle of the gradient between the Th1 and Th2 extremes (Kelso, 1995) or a mixed population of both. Cytokines themselves are amongst the most important factors shaping the characteristics of a Th

response. For example, naive T cells primed in the presence of IFN-γ and IL-4 tend to mature into Th1 and Th2 cells respectively (Seder *et al.*, 1992; Paul and Seder, 1994; Liblau *et al.*, 1995). The working hypothesis that Th responses can be roughly divided into Th1 and Th2 and that expression of cytokines not only reflects, but maintains the balance between Th1 and Th2 responses can be referred to as the Th1/Th2 paradigm.

Murine HSV infections are thought to induce a predominantly Th1 response as determined by the pattern of cytokines expressed in infected tissue (Hendricks *et al.*, 1992; Niemialtowski and Rouse, 1992; Babu *et al.*, 1995) and the production of IgG2a antibody (Nguyen *et al.*, 1994). In addition, *in vivo* depletion of IFN-γ, a key Th1 cytokine, delays clearance of HSV from infected mice, and transgenic mice deficient in either IFN-γ or the IFN-γ-receptor, are similarly unusually susceptible to HSV infections (Hendricks *et al.*, 1992; Smith *et al.*, 1994; Bouley *et al.*, 1995; Cantin *et al.*, 1995). Although these studies demonstrate that IFN-γ (and by extrapolation, presumably a Th1 weighted response) is needed for normal immune control of HSV infection, the effect of IFN-γ depletion on infection is not as profound as total T cell depletion (Smith *et al.*, 1994), indicating that IFN-γ-independent T cell mediated immune mechanisms also exist. In humans, a correlation has been made between peak IFN-γ production by peripheral blood mononuclear cells during a HSV recurrence and the time to next recurrence (Cunningham and Merigan, 1983). Therefore IFN-γ is considered to be a an important cytokine in anti-HSV immunity, but it remains to be seen whether its role is primarily regulatory or in mediating direct anti-viral effects. Two other molecules that have direct anti-viral activity *in vitro*, namely tumour necrosis factor-α (TNF-α) and CD40 ligand, both come from the TNF family and are not dependent on the type of Th response (Feduchi *et al.*, 1989;

Lidbury *et al.*, 1995; Ruby *et al.*, 1995). These molecules are discussed further below, in the context of ganglionic HSV infection. The role of Th2 cytokines in HSV infections has not received as much attention as Th1 cytokines, although two independent groups have found that treatment with IL-4, or transfer of IL-4 producing T cells, can exacerbate HSV mediated disease in mice (Jayaraman *et al.*, 1993; Ikemoto *et al.*, 1995a; Ikemoto *et al.*, 1995b). In the context of the Th1/Th2 paradigm, the action of IL-4 in these instances might be to bias immune responses away from protective IFN-γ production, thus hindering normal virus clearance.

1.3.3.6 T lymphocytes and ganglionic HSV infections

There is evidence that processes of immunity to HSV in the nervous system may differ from those in the skin. First, primary infection is initiated in the skin when the immune system is naive whereas, by the time productive infection is established in ganglia, there has already been peripheral priming of some lymphocytes (Wildy *et al.*, 1982). Second, depletion *in vivo* of $CD8^+$ cells affects clearance of virus from sensory ganglia in all H-2 haplotypes, but skin clearance is not altered in $H\text{-}2^k$ mice (Simmons, 1989; Simmons *et al.*, 1992b). Third, in humans infiltrates in cutaneous but not CNS HSV lesions contain significantly more $CD4^+$ cells than $CD8^+$ cells (Cunningham *et al.*, 1985; Sobel *et al.*, 1986). Lastly, in mouse hepatitis virus infections T cells with a unique antigen specificity were found in nervous system tissues (Stohlman *et al.*, 1993), indicating that different epitopes may be presented to anti-viral T cells at different sites of infection.

It is noteworthy that two of the lines of evidence stated above relate specifically to $CD8^+$

T cells. The findings that depletion *in vivo* of $CD8^+$ cells affects clearance of ganglionic HSV in all H-2 haplotypes tested and that the ratio of $CD8^+$ to $CD4^+$ cells is higher in neural lesions when compared with skin, could be taken to imply a role for $CD8^+$ T lymphocytes in the control of neural HSV infections. Further, one of the earliest experiments to map the MHC restriction of the anti-HSV response *in vivo* demonstrated that mice lacking T cells could be rescued from rapid death with neurological symptoms by transfer of immune cells compatible at class I loci alone (Howes *et al.*, 1979). As mentioned, H-2^d and H-2^b mice fail to mount an adequate $CD8^+$T cell response in the absence of $CD4^+$ cells and therefore, it is difficult to assess the roles of these cell types independently (Smith *et al.*, 1994). However in H-2^k mice, $CD4^+$ cells can be depleted without affecting CTL generation and DTH and humoral responses are maintained in the absence of $CD8^+$ cells. Experiments comparing the effects of $CD4^+$ and $CD8^+$ cell depletions on HSV infections in H-2^k mice have indicated that $CD4^+$T cells are responsible for controlling skin infections, while the $CD8^+$ T cells are involved in limiting viral replication in ganglia (Nash *et al.*, 1987). H-2^d mice treated *in vivo* with anti-CD8 mAb cells have intact humoral and DTH responses, yet suffer more widespread peripheral nervous system infection after cutaneous inoculation of HSV (Simmons *et al.*, 1992b; Simmons and Tscharke, 1992). Additionally, it was shown that in immunocompetent HSV infected mice, the majority of HSV infected ganglionic neurons survive the infection but, in stark contrast, there was high neuronal loss (>35% of total neurons) in HSV infected $CD8^+$ T cell depleted mice (Simmons and Tscharke, 1992). These data have two main implications with respect to the role of $CD8^+$ T cells. First, $CD8^+$ T cells are important in the control of neuronal HSV infection (at least in H-2^d mice) and second, the mechanism of $CD8^+$ cell-mediated protection is non-lytic (Simmons and Tscharke, 1992).

It might be envisaged then, that HSV infected neurons are rescued by local production of anti-viral cytokines or other soluble mediators (known or unknown) by $CD8^+$ T lymphocytes or via another cell as a result of $CD8^+$ lymphocyte activity (Simmons *et al.*, 1992b; Simmons and Tscharke, 1992).

A candidate mediator of $CD8^+$ T cell activity, which has been linked to the control of HSV *in vivo*, is IFN-γ (Smith *et al.*, 1994; Cantin *et al.*, 1995). However, IFN-γ can be made by $CD4^+$ and $CD8^+$ T cells and a definitive link between $CD8^+$ T lymphocytes and IFN-γ is lacking. TNF-α has also been proposed as an important effector in immunity to HSV because administration of TNF-α protected mice against a lethal HSV challenge (Rossol Voth *et al.*, 1991). Supporting a role for TNF-α, a recent immunohistochemical analysis of HSV infected ganglia found that levels of TNF-α expression correlated with $CD8^+$ T cell infiltration (Liu *et al.*, 1996). However, other possible sources of TNF-α in sensory ganglia need to be taken into account when interpreting this data (Murphy *et al.*, 1995). Another molecule with direct anti-HSV activity *in vitro* is the ligand for CD40 but, as yet, these observations have not been extended to studies *in vivo* (Ruby *et al.*, 1995). Finally, there may be many unknown anti-viral molecules in the armamentarium of the immune system and it is possible that one, or a combination, of these is the final effector through which $CD8^+$ T lymphocytes control neuronal infections with HSV.

1.4 Aims

The final mediators of immunity to HSV in the nervous system have not been defined at a molecular level and, while there are some candidates for this role, it is reasonable to suggest that as yet uncharacterised immune effectors may be involved. In addition, there is a well characterized model of ganglionic HSV infection, in which virus clearance is dependent on $CD8^+$ T cells. Therefore the broad aim of this thesis is to analyse the molecular events associated with $CD8^+$ T lymphocyte activity in HSV infected sensory ganglia. Two complementary approaches to this aim were taken. First, the role of $CD8^+$ T cells in cytokine responses to ganglionic HSV infection was investigated, with particular reference to the Th1/Th2 paradigm and a known anti-viral mediator, IFN-γ. Second, a non-directed method of mRNA analysis, namely mRNA differential display, was applied to HSV infected ganglia with the specific aim of identifying transcripts (and hence polypeptides) that may be associated with $CD8^+$ T cell activity in the nervous system.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Mice

Female BALB/c mice (H-2^d) were obtained from Animal Resource Centre (ARC), a specific pathogen free facility. Mice were used at greater than eight weeks of age in all experiments and housed in the IMVS basement animal house.

2.1.2 Virus

A low passage strain of an oral isolate of HSV-1, designated SC16 (Field *et al.*, 1979) was used in all experiments. In mice, SC16 is neurovirulent when inoculated cutaneously, producing a transient productive infection both in the skin and nervous system (Harbour *et al.*, 1981; Simmons and Nash, 1984; Simmons and La Vista, 1989; Simmons *et al.*, 1992b). Working stocks of virus were produced by infecting roller bottle cultures of African green monkey kidney (vero) cells with submaster stocks, retrieved from liquid nitrogen storage. Cell monolayers were inoculated at 0.1 plaque forming units (pfu) per cell, harvested after approximately 40 hr and disrupted by sonication to liberate intracellular virus. Working stocks with titres in excess of 1×10^{10} ml, determined by a standard plaque assay (Russell, 1962), were stored at -70^{0}C in 0.2 ml lots. Virus for animal inoculation was thawed rapidly immediately before use, diluted in phosphate buffered saline (PBS) and kept on ice for up to 1 hour.

2.1.3 Anti-CD8 monoclonal antibody

A well characterized anti-CD8 monoclonal Ab, known to be effective in depleting mice of HSV-specific $CD8^+$ cells, was used for all experiments requiring anti-CD8 treatment (Cobbold *et al.*, 1984; Cobbold *et al.*, 1986; Nash *et al.*, 1987; Ghobrial *et al.*, 1989; Simmons *et al.*, 1992b; Simmons and Tscharke, 1992). This rat IgG2b antibody, specific for the Lyt-2.1 chain of murine CD8, is secreted by hybridoma YTS 169.4. To produce stocks of anti-CD8, six (LOU/M × DA)F_1 rats (ARC) were injected intraperitoneally with pristane (2,6,10,14-Tetramethylpentadecane), rested for 10 days and then injected intraperitoneally with 4.3×10^7 hybridoma cells in a volume of 1 ml. Rats were killed by CO_2 asphyxiation 10 or 11 days after injection of cells. An average of 27 ml of ascitic fluid was collected from each rat, allowed sit at 4^0C overnight and the clot removed by centrifugation. Ascites from all six rats was pooled and stored in 1 ml lots at -70^0C. The pooled ascites had a total protein concentration of 42 mg/ml and could be used at a 1×10^5 dilution to detect CD8 immunohistochemically in frozen sections of mouse spleen using the methods of Simmons and Tscharke (1992).

2.1.4 Oligonucleotides

Unless otherwise stated, oligonucleotides were designed using nucleic acid sequences obtained from GenBank (using the world wide web (www) Entrez service at the National Centre for Biological Information (NCBI)) with the aid of Primer Designer v 2.0 for DOS (Scientific and Educational Software) and produced by Molecular Pathology, IMVS. They were synthesised at 40 nm scale and provided in 1 ml of concentrated ammonia solution.

Before use, oligonucleotides were split into two lots, each of which was precipitated with two sequential butanol extractions. The precipitates were washed with ethanol, dried at room temperature and resuspended in 100 µl sterile deionized water (Commonwealth Serum Laboratories, CSL, referred to as CSL water). The oligonucleotide solutions were quantified by UV spectroscopy at 260 nm and had concentrations in the order of 1 µg/µl (approximately 100 - 200 µM for a 20mer). Working stocks were made at 100 ng/µl (~12-15 pmol/µl for 20 - 22mers) in CSL water and stored at -20^{0}C.

2.1.5 Solutions, gel mixes and miscellaneous reagents

Unless stated otherwise, all solutions were made using deionized water purified by reverse osmosis (RO water) provided by the Media Production Unit, Infectious Diseases Laboratories, IMVS and most chemicals were purchased from Ajax, BDH and Sigma. Concentrations given as percentages indicate % weight per volume for dissolved solids and % volume per volume for liquids.

2TY broth: 16 g/l tryptone, 10 g/l yeast extract, 85 mM NaCl (yeast and tryptone Difco).

2TY agar: 1.5% technical grade agar in 2TY broth.

Acid Wash: 100 ml concentrated sulphuric acid in 340 mM potassium dichromate.

Bacteria: All plasmids were grown in *Escherichia coli* strain DH5α (Hanahan, 1983; Sambrook *et al.*, 1989)

Competent bacteria (*E coli* strain DH5α): a 200 ml culture of log phase DH5α (OD_{550} approximately 0.3), grown in 2TY broth was cooled on ice for 15 minutes (min) before removing the broth by centrifugation and replacing it with 64 ml cold transforming buffer 1 (100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate pH 7.5, 10 mM $CaCl_2$, 15% glycerol, final pH 5.8). Bacteria were incubated in transforming buffer 1 for 15 min on ice, then this solution was replaced with 32 ml cold transforming buffer 2 (10 mM MOPS (3-[N-Morpholino] propanesulphonic acid) free acid, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol). 1 ml lots of competent cells in transforming buffer 2 were frozen using liquid nitrogen and stored at -70^{0}C.

CSL water: Cell culture grade sterile deionized water (Commonwealth Serum Laboratories).

Denaturing DNA gel loading buffer: 98% deionized formamide, 10 mM disodium EDTA (Ethylenediaminetetraacetic acid), 0.1% bromophenol blue, 0.1% xylene cyanol.

Denhardts solution (100×): 2 mg/ml polyvinyl pyrrolidone, 2 mg/ml bovine serum albumin, 2 mg/ml ficoll 400.

DNA gel loading buffer: 50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol.

DNase buffer (1×): 50 mM Tris(hydroxymethyl)Aminomethane (Tris), 7 mM $MgCl_2$

Elution Buffer (for extracting DNA from polyacrylamide gels): 0.1% sodium dodecylsulphate (SDS), 10 mM magnesium acetate, 0.5 M ammonium acetate, 1 mM disodium EDTA.

Hybridization solutions for DNA detections (including colony, electro and Southern blots probed with random primed and oligonucleotide probes):

Prehybridization: 2× Denhardts solution, 6× SSC, 0.5% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4.

Hybridization: 3× Denhardts solution, 4× SSC, 0.5% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 10% dextran sulphate.

Hybridization solutions for northern blotting:

Prehybridization: 5× Denhardts solution, 5× SSC, 0.1% SDS, 50% deionized formamide, 200 µg/ml sheared and denatured salmon sperm DNA, 50 mM Na_2HPO_4, 50 mM NaH_2PO_4.

Hybridization: 1× Denhardts solution, 5× SSC, 0.1% SDS, 50% deionized formamide, 100 µg/ml sheared and denatured salmon sperm DNA, 10% dextran sulphate.

Ligation buffer (1×): 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT).

MOPS buffer (1×): 20 mM MOPS free acid, 5 mM sodium acetate, 1 mM disodium EDTA, pH 7.0.

PBS: 140 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4.

Polyacrylamide gels:

Denaturing gels (DNA sequencing gels): 6% acrylamide (acrylamide:bis-acrylamide 19:1), 1× TBE, 8 M urea. Gels polymerised by adding ammonium persulphate to 0.05% and TEMED (N,N,N',N'-Tetramethylethylenediamine) to 0.2%.

Non-denaturing gels for differential displays: 6% acrylamide (acrylamide:bis-acrylamide 29:1), 2× TBE, 10% glycerol. Gels polymerised by adding ammonium persulphate to 0.1% and TEMED to 0.075%.

Polyacrylamide minigels: 6% acrylamide (acrylamide:bis-acrylamide 29:1), 1× TBE. Gels polymerised by adding ammonium persulphate to 0.1% and TEMED to 0.075%.

Radiolabelled DNA size markers: 5 µg of *Hpa* II cut pUC-19 marker (Bresatec) was incubated for 90 min at 37^{0}C in a 30 µl reaction containing 3 mM DTT, 1× 1-phor-all buffer (pharmacia), 30 µCi [γ-^{32}P]ATP (4000 Ci/mmol, Bresatec) and 10 u (units) T4 polynucleotide kinase (Pharmacia). Labelled markers were passed through sephadex G25 columns to remove unincorporated label before use.

RNA gel loading buffer: 50% glycerol, 1× MOPS buffer, 0.25% bromophenol blue.

Solution D for RNA extractions: 4 M guanidine thiocyanate, 25 mM sodium citrate pH 7, 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol (Chomczynski and Sacci, 1987).

SSC (1×): 150 mM NaCl, 15 mM trisodium citrate, pH 7.0.

T-tailed Bluescript (SK) vector: 10 µg "Bluescript (SK) +" vector (Stratagene), linearized with EcoRV, was placed in a reaction containing 1 mM dTTP, 1.5 mM $MgCl_2$, 1× PCR buffer II and 5 u AmpliTaq Polymerase (Buffer and Taq, Perkin-Elmer) and incubated for 2 hours (hr) at 75^{0}C. T-tailed vector was extracted from the reaction mix, resuspended in 50 µl of CSL water and its concentration estimated as described in Ausubel *et al* (1988).

TBE (1×): 90 mM Tris, 90 mM Boric Acid and 2.4 mM disodium EDTA

TE, 10:1: 10 mM Tris pH 8.0, 1 mM disodium EDTA pH 8.0.

2.2 Methods

2.2.1 Inoculation of mice

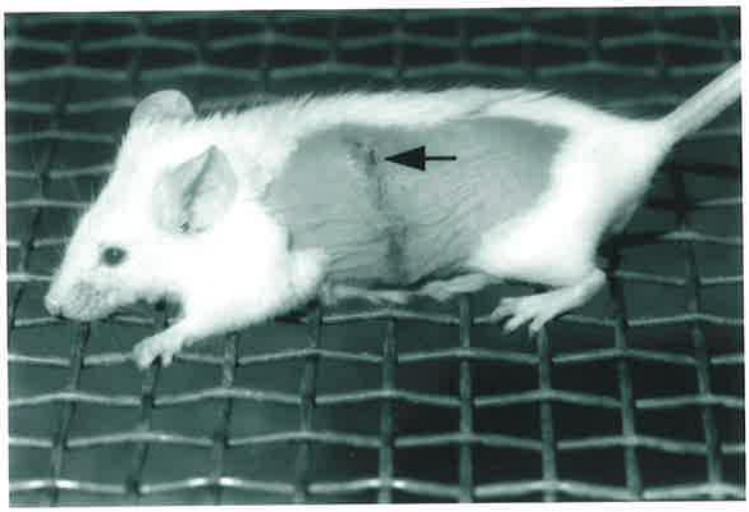
2.2.1.1 Zosteriform model

A well characterized zosteriform model of HSV infection (Simmons and Nash, 1984) was used for the majority of experiments and groups of between ten and twenty mice were infected concurrently. Fur from shoulder to thigh on the left side was shaved using an electric clipper (Golden A5, Oster). Nair depilatory cream (Carter Wallace) was applied to the shaved area for five minutes, then wiped off, along with remaining fur, with a warm damp cloth. After being rested for roughly an hour, mice were anaesthetised with diethyl ether before inoculation. The left tenth thoracic dermatome of each mouse was located by visualizing the tip of the spleen through the skin and marked with a 10 µl drop of virus suspension at 1.5×10^7 pfu/ml (1.5×10^5 pfu total). 20 scratches were made through the drop of virus with a 27 gauge needle (Terumo) covering a 5 mm^2 area of skin.

In this model, acute ganglionic infection is confined to neurons and has been shown to peak on the fifth day, resolving by the ninth day after inoculation (Simmons and Nash, 1984; Simmons *et al.*, 1992b). In all groups of mice infected, by the seventh day after inoculation 90% all mice (regardless of immune status) developed zosteriform lesions that are a characteristic of this model (figure 2.1). Zosteriform lesions are a marker of productive ganglionic infection. Over the duration of experiments, the proportion of mice that had to be culled or died from acute infection never rose above 20% in any group of mice.

Figure 2.1

Characteristic zosteriform lesion on the left flank of a BALB/c mouse six days after inoculation with HSV-1 strain SC16 by scarification at the site indicated by the arrow.



2.2.1.2 Ear model

The mouse ear model of Hill *et al* (1975) was used where experiments required HSV infected skin, or lymph nodes draining an infected site. Groups of five mice were anaesthetised with ether and the left ear pinna injected intradermally with 20 µl of HSV-1 at 5×10^6 pfu/ml using a 27 gauge needle (Terumo). No mice infected in this way died or were culled for humane reasons.

2.2.2 *In vivo* depletion of $CD8^+$ cells by anti-CD8 treatment

To deplete mice of $CD8^+$ cells, anti-CD8 monoclonal Ab was injected intraperitoneally at intervals of four days commencing four days before inoculation with virus (Simmons and Tscharke, 1992). Using immunohistochemistry, this regime has been shown previously to remove $CD8^+$ cells from the spleens of HSV infected mice (Simmons and Tscharke, 1992). Removal of $CD8^+$ T cells by the batch of anti-CD8 used for experiments in this thesis was confirmed by the same methods (not shown). Depletion of cells transcribing mRNA encoding CD8 was demonstrated by experiments documented in section 3.4. Control mice were given phosphate buffered saline. Where paired groups of HSV infected immunocompetent and CD8 depleted mice were required, infection of the groups was done concurrently.

2.2.3 Removal of tissue samples from mice

Mice were killed by intraperitoneal injection of 9 mg sodium pentobarbitone (Nembutal®, Boehringer Ingelheim). All tissues were instantly frozen in 1.5 ml tubes placed in a liquid nitrogen bath and stored at -70^{0}C until RNA extractions were done.

2.2.3.1 Sensory ganglia

Viscera were removed through an anterior midline incision, exposing the anterior surface of the spinal column. The thirteenth thoracic vertebra was identified by its articulation with the lowest rib and was used as a reference for other levels. Fine forceps were used to tease apart the vertebrae at the intervertebral disc, exposing the dorsal root ganglion which was removed. In all experiments where HSV infected ganglia were required, left ganglia were collected from the eighth to twelfth thoracic levels.

2.2.3.2 Draining lymph nodes

An incision was made to remove skin from the anterior surface of the neck and using fine forceps, two to three cervical lymph nodes were taken from the left side.

2.2.3.3 Skin

HSV infected ear pinnae were cut off with small dissecting scissors, taking care to avoid fur.

2.2.4 Extraction of RNA

RNA was extracted from tissues according to the method of Chomczynski and Sacci (1987). In the preparation of solutions and extraction and handling of RNA, all glassware used was treated with acid wash overnight. Where possible, sterile, machine packed plasticware was used and nuclease free filtered pipette tips (ART, Molecular *Bio*-Products) were used for all liquid transfers. Where paired RNA samples were required from tissues of immunocompetent and CD8 depleted mice, the extractions were done in parallel.

Frozen tissue was homogenised in "Solution D" (see 2.1.5) at room temperature using at least 50 passes in a 1 ml glass homogeniser (Wheaton). The volume of Solution D depended on the tissue: between 0.5 ml and 1 ml was used for pools of 50 to 100 ganglia, 0.6 ml was used for five ears and 0.6 ml for the lymph nodes taken from five mice. To each homogenate was added a tenth of a volume of 2 M sodium acetate, pH 4, 1 volume of water saturated phenol and a fifth of a volume of chloroform-isoamyl alcohol (49:1) in that sequence and with thorough mixing between additions. This mixture was shaken vigorously for 10 seconds (sec) then incubated on ice for 15-20 min before centrifugation at 10,000 × g for 20 min at 4^0C. The aqueous (upper) phase was recovered, mixed with 1 volume of isopropanol and placed at -20^0C for at least 1 hr (generally 2 hr - overnight). The resultant precipitate was recovered by centrifugation, dissolved in 50 µl of Solution D, and re-precipitated by the addition of 50 µl of isopropanol and cooling at -20^0C for at least 1 hour. The precipitate was again recovered by centrifugation (10,000 × g, 20 min, 4^0C), and washed with 75% ethanol in CSL water, dried at room temperature and dissolved in 50 - 100 µl of CSL water with the aid of a 10 min incubation at 65^0C. The quality and

quantity of these RNA samples was determined by UV spectroscopy. RNA obtained in this way was used directly for northern blotting, or treated with DNase for use in reverse transcription (RT)-PCR based methods.

2.2.5 DNase treatment of RNA samples

RNA samples were incubated with 10 u of DNase (Boehringer), 40 u of RNasin (Promega) in 5 mM DTT and 1× DNase buffer at 37^0C for 30 - 40 min. Following this treatment, the RNA was purified using one phenol/chloroform extraction, two chloroform extractions and precipitation with a tenth of a volume of 3 M sodium acetate and 3 volumes of ethanol. Finally, RNA precipitates were dissolved in CSL water and the quantity and quality of the resulting samples determined by UV spectroscopy.

2.2.6 RT-PCR to detect known mRNAs

RTs were done in accordance with the instructions provided by the enzyme manufacturer (Gibco BRL). Generally more than one reaction was assembled at a time and in that case, a master mix of the above reagents, excluding RNA, was made and RNA was added to samples of this mix. To avoid contamination of reagents, the RNA was added last and in a laboratory separate from that in which the rest of the reaction was prepared. 20 µl reaction mixtures contained:

100 ng	oligo-$dT_{(12\text{-}18)}$ (Pharmacia)
0.5 mM	each deoxynucleotide (dNTP) (Pharmacia)
20 u	RNasin (Promega)
10 mM	DTT (Gibco BRL)
1×	First Strand Buffer (Gibco BRL)
200 u	MMLV reverse transcriptase (Gibco BRL)
100 ng	DNase treated RNA

Reactions were incubated at 37^0C for 60 min before being stopped by heating to 95^0C for 5 min. Completed RTs were diluted to a total volume of 50 µl and stored at -20^0C if not required immediately.

PCRs were done using the primers given in table 3.1, and the same reagent concentrations were suitable for all primer pairs. PCR reactions, excluding input cDNA were assembled in a laminar flow hood using a set of pipettes reserved exclusively for that purpose. Where possible, master mixes of the reagents were made and then divided into single reaction lots. Reagents were assembled to give the following final concentrations in a total of 50 µl,

100 ng	3' primer
100 ng	5' primer
200 µM	each dNTP (Pharmacia)
1 mM	$MgCl_2$ (Perkin-Elmer)
1×	PCR buffer II (Perkin-Elmer)
1 u	AmpliTaq polymerase (Perkin-Elmer)

The mix was divided into 45 µl lots that were overlayed with 2 drops of paraffin oil (Paraffin liquid BP, Faulding) and then removed to another laboratory where 5 µl of a

diluted RT product was added (ie. one tenth of the product of an RT, effectively the reverse transcribed products of 10 ng of input RNA). Care was taken to ensure that RT products were mixed well before samples were transferred into PCR reactions, especially after they had been frozen and they were transferred into PCRs using a pipette with disposable positive displacement tips (Bresatec). The tubes were spun at 12,000 × g for 10 sec and placed in a thermal cycler (PTC-100, MJ Research). A single cycling profile was used to amplify cDNAs corresponding to IL-2, IL-6, IL-10, IFN-γ, Lyt 2 and HSV pol transcripts and was: 25 cycles, each comprising 94^{0}C for 1 min, 60^{0}C for 1 min and 72^{0}C for 45 sec Detection of GAPDH mRNA required only 15 cycles of the above profile and IL-4 required 30 cycles and an annealing temperature of 50^{0}C (rather than 60^{0}C). Products of these reactions were collected from under the layer of oil and electrophoresed on polyacrylamide minigels or dot blotted using a vacuum manifold.

2.2.7 Polyacrylamide minigel electrophoresis and electroblotting

DNA samples were loaded onto non-denaturing polyacrylamide minigels in 1× DNA gel loading buffer and electrophoresed at 120 V. Gels were stained with ethidium bromide and DNA visualized by UV illumination, or transferred to nylon membranes (Hybond N+, Amersham).

To transfer DNA from polyacrylamide gels to nylon, the gel was first soaked in 0.3× TBE for 5-10 min before being placed in a stack comprising, from top to bottom, 2 sheets of Whatman 3MM chromatography paper (Whatman), the gel, a piece of nylon membrane and 2 further sheets 3MM. All the components in the stack were pre-cut to match the size of

the gel and pre-wet with 0.3× TBE. The stack was placed between the electrodes of a TransBlot® SD semi-dry transfer cell (Bio-Rad) and a current of 100 mA was passed for 90 min. After disassembling the stack, DNA was fixed to the nylon by placing the membrane on a sheet of 3MM soaked with 0.4 M NaOH for 20 min. Filters were rinsed in 2× SSC before storing at 4^0C or immediate use in hybridizations. The same transfer method was used to blot differential display gels.

2.2.8 Dot blotting

Samples of PCR products were denatured in 96 well microtitre trays at 4^0C for 10 min with 0.4 M NaOH and 20 mM disodium EDTA in a final volume of 60 µl. A piece of nylon membrane was cut to fit a Hybri•Dot® 96 well filtration manifold (Gibco BRL) exactly, including holes punched to accommodate the screws. Filters were moistened with TE (10:1) before being fixed into the apparatus. 50 µl of a 0.4 M NaOH and 20 mM disodium EDTA solution of was placed into each well of the apparatus and a vacuum was applied. With the suction still on, 50 µl of the denatured samples were added to the appropriate wells using an 8 channel multitrack pipette and the wells were then washed with a further 100 µl of the NaOH/EDTA solution. The vacuum was released to allow removal of the filter, which was placed on a piece of 3MM soaked in 0.4 M NaOH for 20 min. Finally the filter was rinsed with 2× SSC.

2.2.9 Generation of radio-labelled DNA probes

2.2.9.1 Oligonucleotide probes

Oligonucleotides designed for use as probes were labelled using T4 polynucleotide kinase to transfer the terminal (γ) phosphate from [γ-^{32}P]ATP to their 5'-hydroxyl termini (Chaconas and van de Sande, 1980; Ausubel *et al.*, 1988). 50 µl reactions contained:

100 ng	oligonucleotide (~15 pmole for a 22mer)
2 mM	DTT
1×	1-phor-all (Pharmacia)
5 µCi	[γ-^{32}P]ATP (4000 µCi/mmol, Bresatec)
10 u	T4 polynucleotide kinase (Pharmacia)

and were incubated at 37^{0}C for 40 min. Oligonucleotide probes were purified by passing them through a sephadex G25 column to remove unincorporated radioactivity. To determine the activity of the probe, a 1 µl sample was spotted onto filter paper (Whatman S42), placed in liquid scintillation fluid (OptiPhase HiSafe 3, LKB Scintillation Products) and radioactive counts measured with a Beckman LS6800 scintillation counter. The total number of counts per minute (CPM) in the original sample was calculated and divided by the number of µg of oligonucleotide, to estimate the specific activity for the probe (assuming that all the unincorporated radioactivity was removed). Specific activities of oligonucleotide probes were between 1×10^8 and 1×10^9 CPM/µg and usually $>5\times10^8$ CPM/µg. Probes were usually used within a few hours of being made. When enough probe for 2 hybridizations was generated, probes were stored at -20^{0}C for up to a week before use. 2.5×10^6 CPM of these probes were used per ml of hybridization mixture.

2.2.9.2 Random primed probes

Where DNA probes of longer than 100 bp were required, probes were generated by random primed labelling (Feinburg and Vogelstein, 1983). When the DNA templates were derived from plasmids, the inserts containing the desired sequences were excised with restriction endonucleases and separated from vector sequences on agarose gels. DNA was extracted from agarose gel fragments using a BRESAclean® kit (Bresatec), the basis of which is differential binding of DNA to silica beads in high and low salt solutions. The size and quantity of recovered insert DNA were estimated by comparison with DNA size markers of known quantity on ethidium bromide stained agarose gels. 20-25 ng of these inserts were used as the templates for random priming. In some situations, uncloned cDNAs, extracted from polyacrylamide gels by shaking the gel slice with elution buffer overnight at 37^0C, were used as the template to generate probes. All reagents in random primed reactions, except the [α-^{32}P]dATP (Bresatec), were from a kit (Random Primed DNA Labelling Kit, Boehringer). 20 µl reactions comprised:

20-25 ng	denatured DNA template (where possible)
25 µM	each of dCTP, dGTP and dTTP
50 µCi	[α-^{32}P]dATP (3000 Ci/mmol)
1×	random hexanucleotide primer reaction mix
2 u	Klenow enzyme

and proceeded at 37^0C for 60 min. Newly made probes were diluted to a final volume of 120 µl and unincorporated nucleotides were removed with a sephadex G25 spun column. Two 1 µl samples of a probe, one taken before and the second after purification were counted using the method outlined above for oligonucleotide probes. The percentage of

total available radioactivity incorporated into a probe was estimated from the quotient of CPM remaining after purification and CPM present before purification. Where optimal amounts of template were available, incorporations ranged from 50-85% and the total number of counts emitted by probes was generally in the order of 1×10^8 CPM. Occasionally, probes were generated using less than 1 ng of template DNA. Such probes were useful for colony blot hybridization even when only 4% of available radioactivity was incorporated and total emitted counts were 5×10^6 CPM. Probes were usually used within a few hours of being made, but for some DNA hybridizations where the amount of target was relatively high, probes kept for up to a week at -20^{0}C were occasionally used. 2.5×10^6 CPM of these probes were used per ml of hybridization mixture.

2.2.10 DNA hybridizations with oligonucleotide probes

Nylon membranes to which target DNA had been transferred and fixed were rinsed in 6× SSC before being placed in siliconized (Coatasil, Ajax Chemicals) hybridization tubes (Robbins Scientific) containing 10-20 ml (depending on the size of the filter) of prehybridization solution (for DNA detections). The tubes were incubated at 55^{0}C for at least 4 hr in a Robbins Scientific, Model 310 rolling hybridization incubator. Prehybridization solution was then replaced with 10-20 ml hybridization solution (for DNA detections) containing 1×10^6-2.5×10^6 CPM/ml of oligonucleotide probe and the tubes incubated at 55^{0}C for 90 min. After hybridization, the probes were decanted and filters washed with approximately 100 ml of pre-warmed 5× SSC, 0.1% SDS solution, once at room temperature for approximately 1 min and then 3 times at 65^{0}C for 20 min. All oligonucleotide probes were designed to require this level of stringency. Filters were

removed from the hybridization tubes, blotted on 3MM paper and wrapped in plastic wrap (GLAD Wrap, Glad Products) before exposure to PhosphorImage storage screens (Molecular Dynamics) or X-ray film. Exposure to both media was routinely overnight, however results could often be obtained from PhosphorImage screens within 2 hr.

2.2.11 DNA hybridizations with random primed probes

Hybridization of DNA on nylon filters with probes generated by random priming was done using the same basic protocol and hybridization solutions as described above for oligonucleotide probes. However, most temperatures, times and wash solutions were different. Prehybridization and hybridization were at 65^0C for at least 4 hr and overnight (14-18 hr), respectively. Washes were as follows, one 1 min wash at room temperature and one 30 min wash at 65^0C with a solution of 2× SSC and 0.1% SDS, followed by one 1 min wash and two 30 min washes at 65^0C in a pre-warmed solution of 0.1× SSC and 0.1% SDS. Where the G+C content of the probe was known and according to the formula for the calculation of Tm_{50} (the temperature at which 50% of nucleic acid hybrids will dissociate):

$$Tm_{50}(DNA/DNA) = 16.6\log[Na^+] + 0.41(\%G+C) + 81.5$$

the above washing conditions provided a stringency equivalent to Tm_{50}-15^0C or higher (Meinkoth and Wahl, 1984). After washing, wet filters were wrapped in plastic to facilitate stripping of probes if required and exposed to X-ray film or PhosphorImage screens for various times.

2.2.12 Northern blot hybridization

2.2.12.1 Electrophoresis of RNA and northern blotting

RNA samples (5-10 μg) were denatured by incubation at 70^{0}C for 5 min in 50% formamide, 2 M filtered formaldehyde and 1× MOPS buffer. Samples were placed briefly on ice and $^1/_{10}$ of a volume of 10× RNA loading buffer was added before they were loaded onto gels comprising 1% agarose, 2.2 M formaldehyde and 1× MOPS buffer. Electrophoresis was done in 2.2 M formaldehyde, 1× MOPS buffer solutions for 3-4 hr at 90 V. A sample of RNA marker (0.28-6.58 kb, Gibco BRL), or an extra RNA sample (typically mouse spleen RNA) was run in one of the end lanes of all gels. Marker lanes were cut from gels, stained with ethidium bromide, placed on a UV transilluminator and photographed next to a ruler. These photographs were later aligned with autoradiographs obtained after northern blot hybridizations. The major portion of the gels was rinsed three times for 5 min in RO water and then soaked in 20× SSC for 45 min before the RNA was transferred to nitrocellulose filters (Schleicher and Schuell) by standard northern blotting (Sambrook *et al.*, 1989). After blotting, nitrocellulose filters were baked for 60 min at 80^{0}C under vacuum and rinsed with 2× SSC before storing at -20^{0}C or being used immediately in hybridizations.

2.2.12.2 Hybridization of northern blot filters

Probes for northern blot hybridizations were generated by random priming of cloned templates and used immediately after they were made. The β-actin clone used to reprobe

northern blots, allowing for correction of loading errors, was pAL41 (Alonso *et al.*, 1986) and the β-actin containing insert was cut from the vector using *Pst*I. Where the %G+C contents of probes were known, the Tm_{50} of probe/RNA hybrids in 0.1× SSC, 0.1% SDS (the stringent wash solution) were calculated using the equations of Meinkoth and Wahl (1984):

$$Tm_{50}(RNA/DNA) = \frac{Tm_{50}(DNA/DNA) + Tm_{50}(RNA/RNA)}{2}$$

where Tm_{50}(DNA/DNA) is given by the formula in section 2.2.11 and

$$Tm_{50}(RNA/RNA) = 79.8 + 18.5\log[Na^+] - 0.584(\%G+C) + 11.8(\%G+C)^2$$

Hybridization of northern blots was done using the same apparatus as the other hybridizations reported above. Prehybridization and hybridization using northern blot hybridization solutions, which include 50% formamide, were at 65^{0}C for at least 6 hr and overnight (14-18 hr) respectively. Washes were: one 1 min wash at room temperature and one 30 min wash at 65^{0}C with a solution of 2× SSC and 0.1% SDS, followed by one 1 min wash and two 30 min washes at 65^{0}C in a pre-warmed solution of 0.1× SSC and 0.1% SDS. For probes with G+C contents lower than 45%, the hybridization and washing temperatures were reduced to 60^{0}C to maintain a stringency of between Tm_{50}-15 and Tm_{50}-10. After washing, wet filters were wrapped in plastic to facilitate subsequent stripping of probes and exposed to PhosphorImage screens overnight or X-ray film for up to 5 days.

2.2.13 Autoradiography and digital imaging

Filters and gels were exposed to X-ray films (XOMAT-AR or XK, Kodak) in cassettes lined with intensifying screens (Cronex® Hi-Plus, Dupont) and unless stated, exposures longer than 2 hr were done at -70^{0}C. The orientation of autoradiographs was marked by cutting off the top left corner of filters after nucleic acid transfer and films when the exposure was set up.

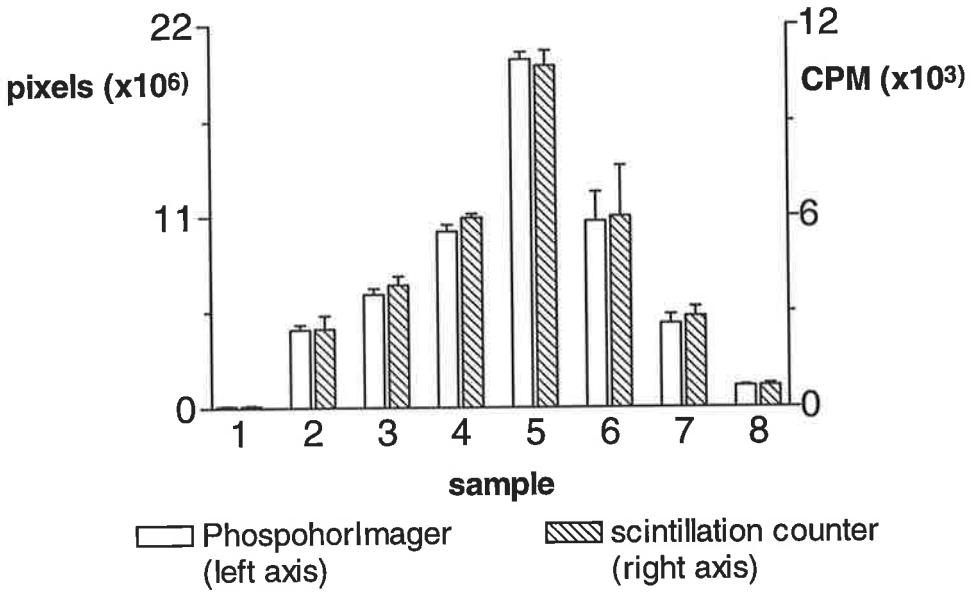
Many probed filters were exposed to PhoshorImage storage screens and scanned with a 400 series PhosporImager, allowing the images to be analysed digitally using ImageQuant software v 3.0 (all PhosphorImage hardware and software, Molecular Dynamics). Where quantification was required, the above digital system (using the "Volume Integration" function of ImageQuant) was always used. The validity of these quantifications was demonstrated in a dot blot experiment by comparing results obtained using the PhosphorImage system with those gained by cutting the filter according to the pattern of dots and using a scintillation counter to measure the radioactive emissions of each dot (figure 2.2). In terms of relative quantification of samples, the two methods of analysis yielded the same result.

In figures presented in the following chapters, all radiographic images are referred to as autoradiographs, irrespective of whether they were produced by traditional autoradiography with X-ray film, or using digital technology starting with exposure of materials to PhosporImage screens.

Figure 2.2

Comparison of data obtained by PhosphorImager and scintillation counter analysis of a dot blot hybridization experiment. Samples were dot blotted in duplicate and bars represent the mean counts and standard error for each sample. PhosphorImage data (open bar, left axis) was generated by exposure of the hybridized filter to a Phosphor Storage screen, scanning the screen with a PhosphorImager and using the "Volume Integration" function of ImageQuant software. Scintillation counter data (hashed bar, right axis) were generated by cutting the filter into pieces corresponding to the dots and counting the radiation emitted from each piece using a scintillation counter.

Analysis of a dot blot by two methods



2.2.14 mRNA differential display (DD)

The procedure used for DD was based on published methods (Liang and Pardee, 1992; Bauer *et al.*, 1993). DD uses RT-PCRs to reverse transcribe, amplify and radiolabel random samples of RNA populations. The resultant cDNAs are then separated on large polyacrylamide gels and visualized by autoradiography providing a partial RNA population fingerprint. If two similar, but non-identical RNA samples are processed in tandem, differences in transcription can be visualized (figures 4.1 and 5.1). Furthermore, cDNAs that are differentially displayed in this manner can be eluted from the polyacrylamide gel, re-amplified and cloned, allowing them to be characterized. Further discussions on the basis of the method, design of RT-PCR primers and the development of protocols appear in chapter 4.

2.2.14.1 DD RT-PCRs and display gels

The majority of DD RT-PCRs were done in large batches and the logistics and reaction conditions of these experiments is described below. Where batch sizes varied from those described, the same logistical considerations were taken into account, but reaction master mixes and numbers were scaled appropriately. During development of methods, the constituents of master mixes varied (section 4.3 and table 4.1). In optimization experiments where reagent concentrations were varied, master mixes were still used and the reagents being titrated were added after the mix was dispensed into smaller volumes. The precautions taken to avoid contamination of reactions with extraneous nucleic acids that are noted in section 2.2.6, were strictly adhered to in all DD RT-PCRs.

Usually, when two RNA sources were being displayed, three RTs were done for each RNA source and the products of each RT were pooled according to source. RT reaction mixes were made to give the following final concentrations in a 20 µl reaction:

2.5 µM	$dT_{11}VN$ primer (where V is A, G or C and N is A, G, C or T)
100 µM	each dNTP
1×	first strand buffer (Gibco BRL)
1 mM	DTT
300 u	MMLV-RT (Gibco BRL)

Master mixes were divided into 15 µl lots, to which was added 100 ng of input RNA in 5 µl. Denaturation of the RNA template prior to RT was found to make no difference to the final DD fingerprints and thereafter was not done. RTs were incubated at 35^0C for 60 min, after which reactions were stopped by heating to 95^0C for 5 min.

Products of each RT were diluted to a final volume of 50 µl with CSL water and after appropriate pooling were stored at -20^0C if not required immediately. 5 µl of these diluted RT products ($=^1/_{10}$ of an original RT) was used as the template in DD PCRs. All reactants were present in excess in the RT reactions and therefore they were considered to be carried over into the PCRs at $^1/_{10}$ of their original concentration in the RT. For most reagents, this carry over is negligible and was ignored, with one important exception, namely dNTPs. The concentration of dNTPs in DD PCRs was $^1/_{10}$ of their concentration in the RT and therefore the total requirement for dNTPs in the PCRs was supplied by carry over with RT products. A practical ramification of this situation was that during the development of DD, modulation of dNTP concentrations in PCRs was achieved by changing dNTP concentrations in the RT. Experiments were designed to ensure that the quality of RT was

not compromised by the changes and these experiments disclosed that the lowest dNTP concentration that allowed normal RT was 50 µM.

PCR reagents were also assembled in master mixes (usually for 50 reactions) that were made to give the following final concentrations in a 20 µl PCR,

1.5 mM	$MgCl_2$ (Perkin-Elmer)
1×	PCR buffer II (Perkin-Elmer)
2.5 µM	$dT_{11}VN$ primer
5 µCi	[α-^{33}P]dATP (1500 Ci/mmol, Bresatec)
1.5 u	AmpliTaq (Perkin-Elmer)

13 µl of PCR master mix was added to 0.5 ml tubes already containing 2 µl of a decamer primer (at 5 µM, to give 0.5 µM in 20 µl). Typically 48 tubes, two for each of the 24 decamers (table 2.1), were prepared, allowing the two RNA sources to be displayed by one $dT_{11}VN$ primer in combination with each of the 24 decamers in a single experiment. Reactions were covered with a drop of paraffin oil and removed to another laboratory where 5 µl of a diluted RT product was added. Care was taken to ensure that RT products were mixed well before samples were transferred into PCR reactions, especially after they had been frozen. Tubes were centrifuged for 10 sec at 12,000 × g and placed in a thermocycler (MJ Research). The thermal cycling profile used was: 30 cycles, each comprising 94^{0}C for 30 sec, 40^0 C for 2 min and 72 C for 30 sec, followed by a final extension at 72^{0}C for 5 min.

6 µl of DD PCR products were mixed with 1 µl of standard 10× gel loading buffer before loading onto 400 mm×300 mm×0.4 mm polyacrylamide gels. To facilitate comparisons

Table 2.1 Decamer primer sequences used in DD

name	sequence (5' -> 3')	source
OPA-01	caggcccttc	Operon*
OPA-02	tgccgagctg	"
OPA-03	agtcagccac	"
OPA-04	aatcgggctg	"
OPA-05	aggggtcttg	"
OPA-07	gaaacgggtg	"
OPA-08	gtgacgtagg	"
OPA-09	gggtaacgcc	"
OPA-10	gtgatcgcag	"
OPA-11	caatcgccgt	"
OPA-12	tcggcgatag	"
OPA-13	cagcacccac	"
OPA-14	tctgtgctgg	"
OPA-15	ttccgaaccc	"
OPA-16	agccagcgaa	"
OPA-18	aggtgaccgt	"
OPA-19	caaacgtcgg	"
OPA-20	gttgcgatcc	"
DT-21	gtactcaacc	Bresatec†
DT-28	tagtcaccga	"
DT-30 (10lyt2)	cgatgagtcc	"
DT-31	gtcagacagg	"
DT-32	gtggctaagg	"
DT-33	cgccaagcat	"

*Primers were from 10-mer Kit A, Operon Technologies. Two primers, namely OPA-6 and OPA-17, than were included in this kit were not used in DD, because DD PCRs primed with them and any $dT_{11}VN$ primer amplified <10 cDNAs.

†Primers were designed arbitrarily (taking care to avoid self complementarity and maintaining 50-60% G+C), or were chosen to bind exactly to a known mRNA (for example 10lyt2, see section 4.5) and were synthesised by Bresatec.

between fingerprints, pairs of RT-PCR products produced from the two different input RNA populations, but using the same primers, were loaded in adjacent lanes. Non-denaturing gels containing 6% polyacrylamide, 10% glycerol 2×TBE (sections 2.1.5 and 4.2) were used routinely and were electrophoresed at constant power (65 W) for five hours in 2× TBE buffer. Denaturing gels containing 6% polyacrylamide, 8 M urea and 1× TBE (section 2.1.5) were used occasionally during DD development and these were electrophoresed at 60 W for 2 hr in 1× TBE buffer. Irrespective of gel composition, the same Model S2 Sequencing Gel Electrophoresis Apparatus (Gibco BRL) was used. After electrophoresis, the gel plates were prised apart and the gel picked up on a sheet of 3MM chromatography paper before being dried for 2 hr at 80^0C under vacuum (Model 583 Gel Dryer, Bio-Rad). Dried gels were exposed to X-ray film at room temperature overnight (12-15 hr). Two holes were punched through the gel and film after they were put together to facilitate realignment after the film was developed. Attempts were made to use PhosphorImage technology and ImageQuant software to analyse DD gels, but the automated detection of bands by this system (using area integration and peak detection functions) was not able to accurately identify differences between DD fingerprints.

2.2.14.2 Cloning DD cDNAs

As described in section 4.4, various procedures for cloning re-amplified cDNAs were tried. The basic methods used in all of the approaches were the same, but different strategies were employed to purify cDNAs before cloning and screen resultant clones. Details of the successful strategy and methods common to all approaches are presented here; the specifics of the two unsuccessful strategies are documented in sections 4.4.1 and 4.4.2.

Reproducible differentially displayed cDNA tags were located on gels using the autoradiograph and cut out, leaving behind as much of the backing paper as possible. Polyacrylamide gel slices were rehydrated and macerated in CSL water and placed directly into re-amplification PCRs with appropriate primers. Re-amplification reactions were identical in all respects to those used to produce the original displays. 5 µl of a given first round re-amplification product was used to seed a second identical round of re-amplification. 10 µl of first and second round re-amplification products were purified by separation on differential display gels, appropriate products being identified by overnight autoradiography and eluted from gel slices by shaking overnight at 37^0C in elution buffer.

Half of each second round product was ligated into T-tailed pBS SK + overnight at room temperature or 14^0C in 20 µl reactions comprising:

8 µl	second round product
60 ng	T-tailed pBS SK + (section 2.1.5)
1×	ligase buffer
2 mM	ATP (adenosine-5'-phosphate)
2.5 u	T4 DNA ligase (Pharmacia)

Controls containing only vector DNA were always done. This strategy is known as A-T cloning (Ausubel *et al.*, 1988) and is based on the fact that Taq polymerase adds a number of extra adenosine residues to the 3' ends of PCR products (Clark, 1988). Cloning PCR products into T-tailed vectors eliminates the need to blunt end PCR products before cloning and the 1-2 bp A-T overlap between vector and insert facilitates ligation. Half of each ligation was mixed with 150 µl of competent bacteria (section 2.5.1) and cooled on ice for 40 min with occasional gentle shaking before being heat shocked at 42^0C for 90 sec.

Transformation mixtures were plated on 2TY agar containing 100 µg/ml amoxycillin (Moxacin, CSL), 40 µg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyronoside (X-Gal) and Isopropyl β-D-Thiogalactopyronoside (IPTG) which were incubated overnight at 37^0C. White colonies were assumed to harbour plasmids containing inserts. When required, inserts were excised by restriction endonuclease digestion with *Hind*III and *Pst*I.

2.2.14.3 Screening of DD clones by colony blotting

White colonies were re-plated onto fresh medium, and onto medium overlayed with nylon membranes. Colonies grown on nylon were lysed and fixed by placing filters sequentially onto sheets of 3MM paper moistened with the following solutions: 0.5 M NaOH for 3 min, 3 lots of 1 M Tris pH 7 for 1 min and 1.5 M NaCl in 1 M Tris pH 7 for 3 min. Colony blots were hybridized with probes generated by random priming of first round re-amplification products eluted from the purification gel, using protocols documented in sections 2.2.9.2 and 2.2.11. Plasmid preparations were made using the alkaline lysis method (Sambrook *et al.*, 1989) from colonies selected on the basis of hybridization to probes in these colony blots. These plasmids were then tested by hybridization (after generation of random primed probes) to electroblotted differential displays and northern blots.

2.2.15 DNA sequence analysis

DNA sequence analysis was done to characterize a number of cDNA clones derived from DD throughout the project. Initially all DNA sequencing was done using the manual method but later, when automated sequencing became available, this second method was

occasionally used. Most sequences presented in this thesis were compiled from data generated by both systems. Both systems are based on termination of enzymatic DNA chain extension by dideoxynucleotides (Sanger *et al.*, 1977).

2.2.15.1 Manual sequencing

The manual sequencing protocol used asymmetric PCR with ^{32}P-labelled primers to generate sequence ladders. All enzymes and buffers were supplied by a kit (Taq Track® Sequencing Systems, Deaza, Promega) but a modified protocol was used. Primers were labelled in 10 µl reactions incubated for 10 min at 37^{0}C and comprising:

10 pmol	sequencing primer (USP-17mer and RSP-25mer, Bresatec)
1×	T4 Polynucleotide Kinase buffer
5 u	T4 Polynucleotide Kinase (Pharmacia)
30 µCi	[γ-^{32}P]ATP (4000 Ci/mmol, Bresatec)

Labelled primers were used as soon as possible, but kept at -20^{0}C if there was any delay. The kit supplied four deoxynucleotide/dideoxynucleotide solutions (d/ddATP, d/ddCTP, d/ddGTP and d/ddTTP) and 1 µl of each was dispensed into one 0.5 µl tube (on ice) for each clone being sequenced. The following 26 µl mix was assembled for each clone:

100 ng (~1.6 pmol)	plasmid DNA (alkaline lysis plasmid preparations)
1×	Taq buffer
2 µl (2 pmol)	^{32}P-labelled primer (from the above reaction)
5 u	Taq polymerase (sequencing grade)

6 µl of these mixes were added to appropriate tubes already containing d/ddNTP solutions.

A drop of paraffin oil was added the tubes which were then thermocycled: 20 cycles, each comprising 95^0C for 30 sec, 45^0C for 1 sec and 70^0C for 30 sec.

Each reaction was diluted with 10 µl CSL water and a 5 µl sample added to 0.5 µl of 10× denaturing gel loading buffer and heated to 70^0C before loading on a denaturing polyacrylamide gel (6% polyacrylamide, 8 M urea and 1× TBE, section 2.1.5) which had been pre-electrophoresed for 30 min at 70 W. Samples were loaded in the standard order for sequencing. The gel was electrophoresed for 2½ hr at 70 W after which time, a second 5 µl sample of each reaction was loaded in unused lanes before a 2 further hours of electrophoresis at 70 W. Sequencing gels were dried onto 3MM paper for 2 hr at 80^0C and autoradiographed overnight. The double loading of each sample enabled longer runs of sequence to be read; up to 300 bp of DNA sequence could be determined in a single experiment using the above method.

2.2.15.2 Automated sequencing

Automated sequencing was done with an ABI Prism (ABI) automated sequencer, utilizing the supplied dye terminator cycle sequencing reaction kit and adhering to all protocols therein. The same primers that were used in manual sequencing were used with the automated system (USP-17mer and RSP-25mer, Bresatec). Up to 350 bp of sequence data were provided by automated runs and no attempt was made to optimize the system further.

2.2.16.3 DNA sequence construction and database searches

All the data available for a given clone were compiled with the aid of Sequence Navigator and Dnasis software (v 1.0.1 for Apple Macintosh, ABI Prism and v 7.00 for MS-DOS, Hitachi software engineering respectively). During this process, original autoradiographs of manual sequence reactions and chromatographs produced by automated sequencing were examined to help resolve any ambiguities. Nucleic acid database comparisons were done at NCBI via their www site. The blastn program was used to search the non-redundant (nr) database. Blastn utilizes the Basic Local Alignment Search Tool algorithm of Altschul *et al* (1990) and the nr database contains all the sequences held in GenBank, EMBL and the DNA DataBank of Japan. Submission of sequences to GenBank was also done via a www page maintained at NCBI, using BankIt.

2.3 List of suppliers

Ajax Chemicals, Auburn NSW, Australia

Amersham, Amersham International plc, Little Chalfont, Buckinghamshire, UK

Animal Resource Centre (ARC), WA, Australia

ABI, Applied Biotechnology Inc., Division of Perkin-Elmer Corporation

BDH, BDH Chemicals Australia Pty Ltd, Kilsyth Vic, Australia

Beckman, Beckman Instruments Inc., Fullerton CA, USA

Bio-Rad, Bio-Rad Laboratories, Hercules CA, USA

Boehringer Ingelheim, Ingelheim, Germany

Boehringer, Boehringer Mannheim, Mannheim, Germany

Bresatec, Bresatec Pty Ltd, Adelaide SA, Australia

Carter Wallace, Carter Wallace Pty Ltd, Frenchs Forest NSW, Australia

CSL, Commonwealth Serum Laboratories, Melbourne Vic, Australia

Difco, Difco Laboratories, Detroit Michigan, USA

Faulding, F. H. Faulding Pty Ltd, Thebarton SA, Australia

Gibco BRL, Life Technologies, Gaithersburg MD, USA

Glad Products of Australia, Rhodes NSW, Australia

Kodak, Eastman Kodak, Rochester NY, USA

LKB Scintillation Products, Pharmacia LKB Biotechnology, Bromma, Sweden

MJ Research, products sold and serviced by Bresatec Pty Ltd

Molecular *Bio*-Products, San Diego CA, USA

Molecular Dynamics, Inc., Sunnyvale CA, USA

Molecular Pathology, IMVS, Adelaide SA, Australia

Operon Technologies, Inc., Alameda CA, USA

Oster, Division of Sunbeam Corporation, Milwaukee WI, USA

Perkin-Elmer Corporation, Roche Molecular Systems, Inc., Branchberg NJ, USA

Pharmacia, Pharmacia Biotech, Uppsala, Sweden

Promega, Promega Corporation, Madison WI, USA

Robbins Scientific, Robbins Scientific Corporation, Sunnyvale CA, USA

Schleicher and Schuell, Dassel, Germany

Sigma, Sigma Chemical Company, Sigma-Aldrich Pty Ltd, St. Louis MO, USA

Stratagene, Stratagene Cloning Systems, La Jolla CA, USA

Terumo, Tokyo, Japan

Whatman, Whatman International Ltd, Maidstone, UK

Wheaton, Millville NJ, USA

3. EFFECT OF DEPLETING CD8$^+$ CELLS ON CYTOKINE mRNA LEVELS IN HSV INFECTED GANGLIA

The ganglionic infection that follows cutaneous inoculation of the flanks of mice with HSV provides a well characterized model of neural HSV infection (Simmons and Nash, 1984). In this model, known as the zosteriform model, immune control of ganglionic HSV infection is dependent on the presence of CD8$^+$ cells (Simmons and Tscharke, 1992), but it is not known how these cells act. Cytokines are involved in all facets of anti-viral immunity from initiation and regulation of responses, to the mechanisms of virus clearance. In addition, many cytokines and their functions are well characterized at the molecular level in mice and humans. With respect to regulation of immune responses, the role of CD4$^+$ T lymphocytes in cytokine secretion has been studied extensively. However, the role of CD8$^+$ T lymphocytes in promoting appropriate cytokine responses has received much less attention, despite the fact that CD8$^+$ and CD4$^+$ cells secrete the same range of cytokines (Kelso, 1995). Further, it has been suggested that CD8$^+$ T cells, mediate anti-HSV activity in the nervous system via IFN-γ (Cantin *et al.*, 1995). Therefore cytokines are obvious targets for a molecular investigation into the activity of HSV-specific CD8$^+$ T cells in the nervous system.

The CD8$^+$ T cell depleted mouse is a potentially useful tool for identifying cytokines associated with CD8$^+$ T lymphocyte mediated immunity; mice lacking CD8$^+$T cells should also lack a CD8$^+$ T cell dependent cytokine expression profile, if such a profile exists. Cytokines usually act in the local environment in which they are produced and therefore measurement of cytokine production in infected tissue is an appropriate way to gauge

cytokine responses. In this study, sensory ganglia removed from groups of HSV infected immunocompetent and anti-CD8 treated mice were compared with respect to various cytokine mRNA levels.

3.1 Generation of experimental material

Four groups of 20 BALB/c mice, of which two were treated with anti-CD8 and the other two were immunocompetent controls, were infected with HSV according to the zosteriform model. One immunocompetent group and one anti-CD8 treated group were killed five days after inoculation and the remaining mice were killed seven days after inoculation. These times were chosen because they correlate with the peak and recovery phases of acute ganglionic infection in the zosteriform model (Simmons *et al.*, 1992b). At the time of killing, left dorsal root ganglia from the eighth to tenth thoracic levels were pooled from each group of mice and snap frozen. Dorsal root ganglia were also removed from five uninfected mice. All experiments in this section were done using material extracted from these five groups of ganglia.

3.2 Development of a semi-quantitative RT-PCR method

An approach based on quantitative RT-PCRs to detect levels of cytokine transcription was chosen for the measurement of cytokine expression in HSV infected ganglia. The RT-PCR method compared levels of each mRNA amongst samples but did not determine the absolute number of transcripts in each sample. Therefore the method is referred to as "semi-quantitative RT-PCR".

PCR primers were designed to amplify transcripts for IL-2, IL-4, IL-6, IL-10, IFN-γ, Lyt 2 (CD8), HSV polymerase (HSV pol), and glyceradehyde-3-phosphate dehydrogenase (GAPDH, a constitutively expressed eukaryotic "house keeping" gene). In addition, an oligonucleotide probe for a region internal to the PCR primers was designed to allow detection of PCR products by hybridization. All oligonucleotides, the GenBank accessions of the nucleic acid sequences from which they were designed and the expected sizes of PCR products are listed in table 3.1. All RTs were primed with oligo $dT_{(12-18)}$. RT-PCR conditions were optimized for each primer pair so that a single product of the anticipated size was amplified from HSV infected ganglionic RNA, as judged by hybridization analyses of electroblotted polyacrylamide minigels (figure 3.1). In each case, the number of PCR cycles was set at the lowest number that reliably yielded amounts of product detectable by electroblot hybridization. To show that RNA and not DNA was the original nucleic acid template for RT-PCRs, experiments in which reverse transcriptase was omitted from the RT reaction were done. All such experiments gave no detectable products. Also, in those cases where PCR primer pairs spanned one or more introns, RT-PCR product sizes were always consistent with the template being a spliced mRNA and not the gene. Controls for DNA contamination, in which input RNA was substituted with water, were also included.

To ensure that RT-PCRs could determine the relative quantities of an mRNA in different samples, control samples (referred to as quantity controls) were prepared that comprised a day seven HSV infected ganglionic RNA sample diluted in uninfected ganglionic RNA. Several quantity controls, each with a different ratio of infected to uninfected ganglionic RNA, were included in each experiment. Amplified product was quantified by

Table 3.1 PCR primers, oligo probes and expected PCR product sizes for semi-quantitative PCR analyses.

mRNA*	5' primer	3' primer	oligonucleotide probe	product size
IL-2 (X01772)	gcagctgttgatggacctac	tccaccacagttgctgactc	gcagaggtccaagttcatcttc	306 bp
IL-4 (x05064)†	agctagttgtcatcctgctc	agtgatgtggacttggactc	atatgcgaagcaccttggaagc	355 bp
IL-6 (j03783)	gataccactcccaacagacc	tctctctgaaggactctggc	ggtagctatggtactccagaag	323 bp
IL-10 (m37897)	caataactgcacccacttcc	aatcactcttcacctgctcc	tttctgggccatgcttctctgc	356 bp
IFN-γ (k00083)	ctcatggctgtttctggctg	tttccgcttcctgaggctgg	tgttgctgatggcctgattgtc	414 bp
Lyt 2 (y00157)	cgccaggaagctacaactac	gctctggtgttacagtctgc	ggtgtgatggggacagttcctt	218 bp
HSV pol‡	atcaacttcgactggcccttc	ccgtacatgtcgatgttcacc	cgcgtgtgggacattggccagagccactt	174 bp
GAPDH (m32599)	atggccttccgtgttcctac	ttgctcagtgtccttgctgg	ggctggcattgctctcaatgac	358 bp

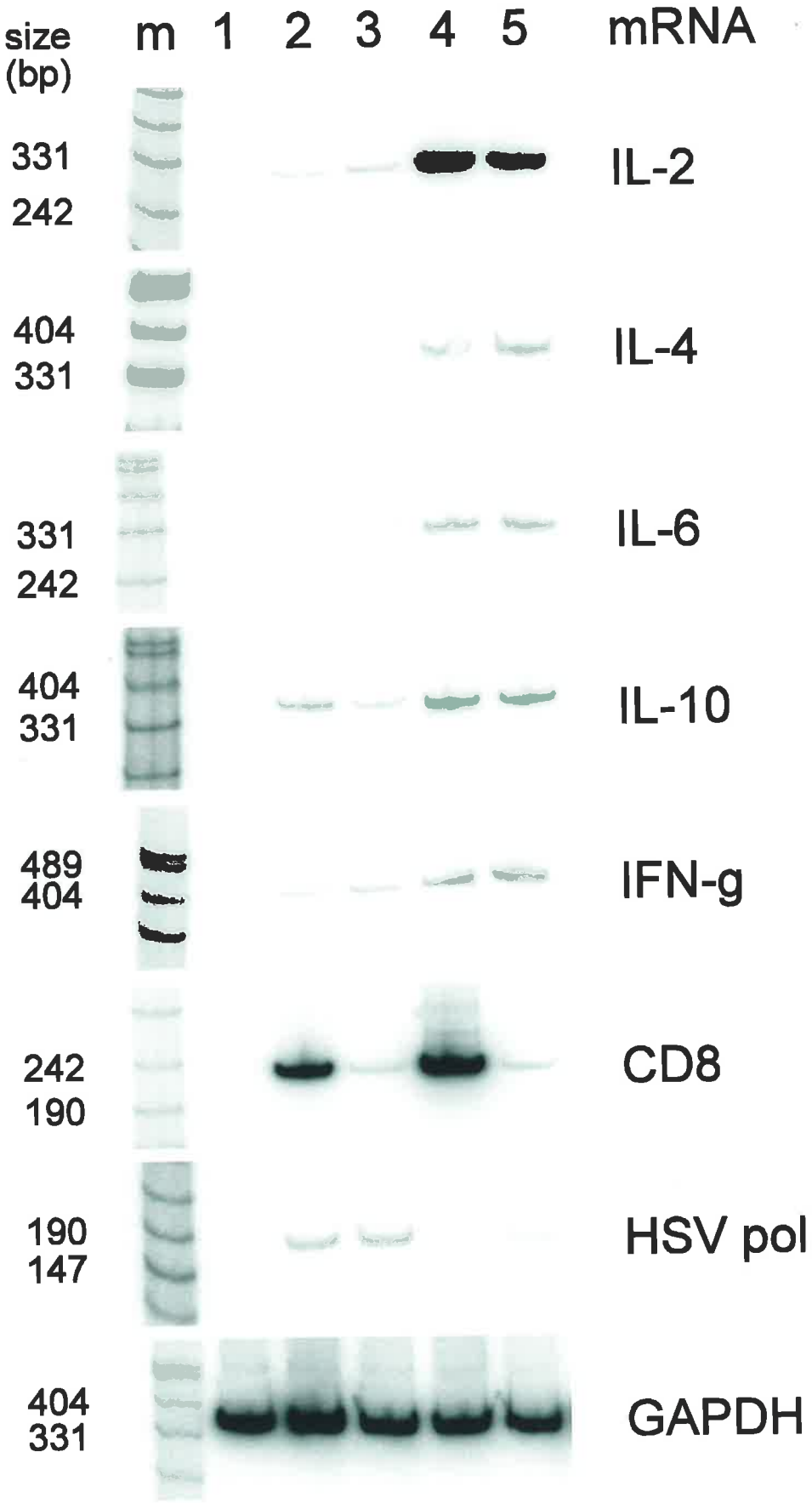
*GenBank accessions of nucleic acid sequences from which oligonucleotides were designed are bracketed

†accessions X05252 and X05253 also used

‡HSV pol oligonucleotides from Lakeman *et al* (1995)

Figure 3.1

Autoradiographs of electroblotted polyacrylamide gels loaded with RT-PCR products generated with primers specific for the transcripts indicated on the right and hybridized with appropriate radio-labelled oligonucleotide probes. RT-PCRs were seeded with ganglionic RNA from uninfected (1) and HSV infected (2-5) mice. Radiolabelled *Hpa*II digested pUC-19 plasmid DNA was included on each gel as a size marker (m) and sizes of relevant marker fragments are indicated on the left. (Primer and probe sequences can be found in table 3.1, as can the expected size of each RT-PCR product.)


size (bp)
m
1
2
3
4
5
mRNA
331
242
IL-2
404
331
IL-4
331
242
IL-6
404
331
IL-10
489
404
IFN-g
242
190
CD8
190
147
HSV pol
404
331
GAPDH

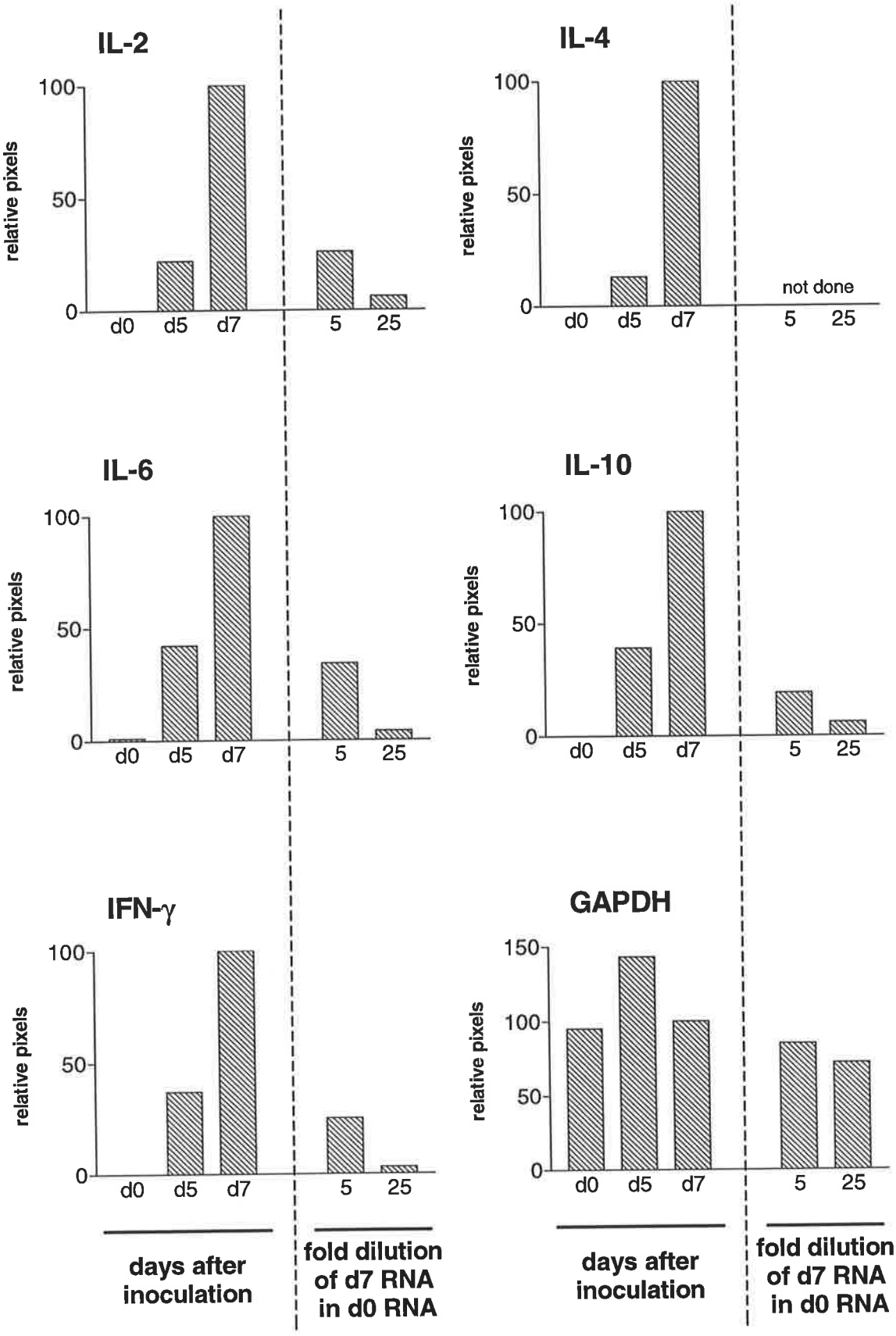
PhosphorImage analysis of hybridized blots. To demonstrate the reproducibility of semi-quantitative RT-PCR, up to eight replicate RT-PCRs were done on each RNA sample. The products of each set of replicate reactions were dot blotted onto a single membrane for hybridization with an appropriate oligonucleotide probe. After hybridization, the statistical mean and standard error of PhosphorImage pixel counts from each set of replicates were calculated. The standard errors were construed as a measure of the reproducibility of RT-PCRs.

3.3 Cytokine expression in HSV infected ganglia

The previous section documented the development of semi-quantitative RT-PCR to detect transcription of five cytokines, namely IL-2, IL-4, IL-6, IL-10 and IFN-γ. To further establish the utility of semi-quantitative RT-PCR for detection of cytokine transcription, a preliminary analysis of cytokine expression in HSV infected ganglia was done. Transcription of all five cytokines was examined in uninfected ganglia, and ganglia taken from mice five and seven days after inoculation. Semi-quantitative RT-PCR was used and quantity controls were included in these experiments, but replicate RT-PCRs were not done. The results of these experiments are presented as graphs in figure 3.2. The only cytokine mRNA detected in uninfected ganglia was for IL-6, but the levels of this transcript were extremely low and did not appear to disturb the quantity controls in this experiment. Transcripts for all five cytokines were detected in ganglia five days after inoculation of mice with HSV and the abundance of these mRNAs was increased further on day seven. These data agree with results of immunohistochemical experiments published for IL-4, IL-10 and IFN-γ (Cantin *et al.*, 1995; Liu *et al.*, 1996) and extend current knowledge of

Figure 3.2

Semi-quantitative PCR analysis of cytokine mRNA expression in ganglia of uninfected mice (d0) and infected mice, five (d5) and seven (d7) days after inoculation with HSV. The cytokines examined are indicated above each graph and the bars represent ImageQuant pixel counts from dot blotted RT-PCRs, made relative to day seven values set arbitrarily at 100. To the right of the dashed line in both columns of graphs are quantity controls, 5 and 25 fold dilutions of d7 RNA in d0 RNA. Transcripts for GAPDH (bottom right) were quantified in each sample to control for RNA handling errors.


IL-2
IL-4
IL-6
IL-10
IFN-γ
GAPDH
relative pixels
100
50
0
150
d0
d5
d7
5
25
not done
days after inoculation
fold dilution of d7 RNA in d0 RNA

cytokine expression in HSV infected ganglia to include IL-2 and IL-6. The correlation of semi-quantitative RT-PCR data with previously published results demonstrates the utility of this method in studies of cytokine expression.

3.4 Verification of $CD8^+$ cell depletion

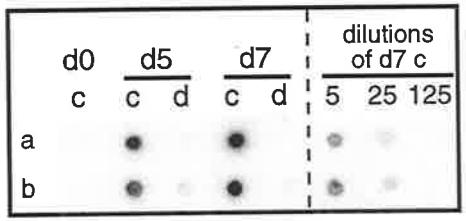
The efficacy of anti-CD8 treatment has been measured previously by fluorescent flow cytometric analysis (Cobbold *et al.*, 1984; Nash *et al.*, 1987; Smith *et al.*, 1994), immunohistochemistry (Simmons and Tscharke, 1992) and functional assays such as cytotoxicity (Cobbold *et al.*, 1984; Nash *et al.*, 1987). These methods have shown that anti-CD8 treatment can deplete cells bearing functional surface CD8 molecules from lymphoid tissue. To date, the efficacy of anti-CD8 treatment has not been assessed at the transcriptional level or at a site of infection.

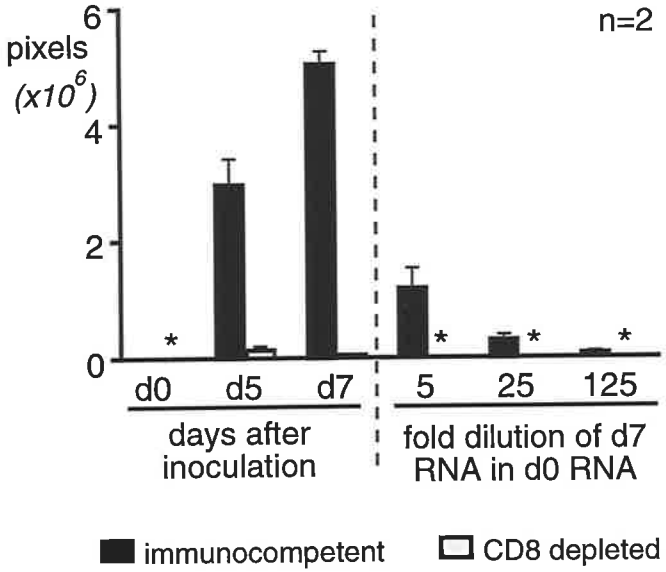
Lyt 2 is a component of murine CD8 and the level of Lyt 2 mRNA was used as a measure of CD8 gene expression. To compare relative levels of Lyt 2 mRNA in ganglia from the uninfected group of mice and ganglia taken from the immunocompetent and anti-CD8 treated groups of mice, total ganglionic RNA samples were analysed by semi-quantitative RT-PCR (figure 3.3A). In concordance with the prior observation that $CD8^+$ T cells could not be detected by immunohistochemistry in uninfected sensory ganglia (Cantin *et al.*, 1995), Lyt 2 transcripts were not detected in RNA from uninfected mice. Conversely, in ganglionic RNA from immunocompetent mice, Lyt 2 transcripts were detected easily five and seven days after HSV infection. These data are consistent with accumulation of $CD8^+$ T cells in HSV infected ganglia during the course of infection, shown previously by

Figure 3.3

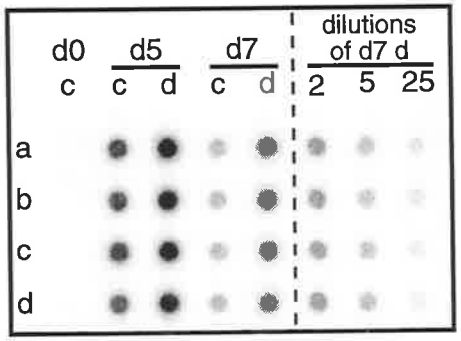
Semi-quantitative RT-PCR analyses of Lyt 2 (**A**) and HSV pol (**B**) mRNA in ganglia of uninfected mice (d0) and infected mice, five (d5) and seven (d7) days after inoculation with HSV. Mice were either immunocompetent (c, black bar) or $CD8^+$ cell depleted (d, grey bar). Above each graph is the dot blot on which quantifications were based, note that each RT-PCR was done in duplicate (n=2, Lyt 2) or quadruplicate (n=4, HSV pol). Bars on graphs represent means and standard errors of ImageQuant pixel counts from replicate RT-PCRs. To the right of the dashed line both on dot blots and graphs are quantity controls, the stated dilutions of d7 RNA in d0 RNA. *not done.

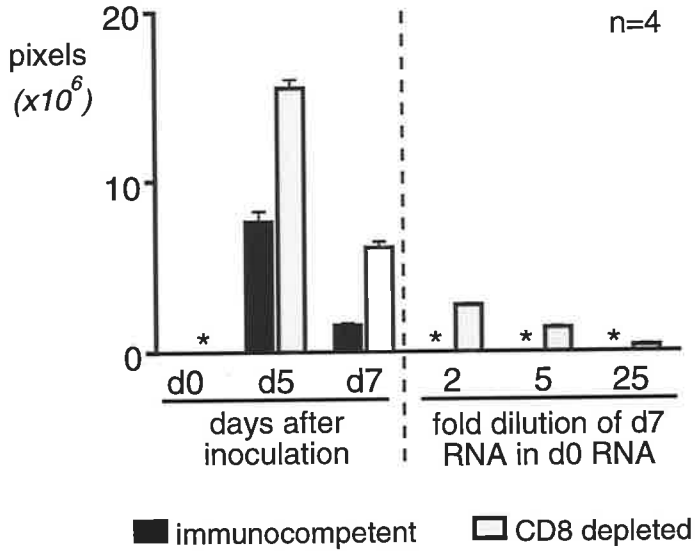
A


d0
d5
d7
dilutions of d7 c
c
c d
c d
5 25 125
a
b


pixels (x10^6)
6
4
2
0
n=2
*
d0 d5 d7
5 25 125
days after inoculation
fold dilution of d7 RNA in d0 RNA
immunocompetent
CD8 depleted

B


d0
d5
d7
dilutions of d7 d
c
c d
c d
2 5 25
a
b
c
d


pixels (x10^6)
20
10
0
n=4
*
d0 d5 d7
2 5 25
days after inoculation
fold dilution of d7 RNA in d0 RNA
immunocompetent
CD8 depleted

immunohistochemistry (Cantin *et al.*, 1995; Liu *et al.*, 1996). Significantly, levels of Lyt 2 transcripts in RNA samples from anti-CD8 treated mice were at least 100 fold less than those found in immunocompetent mice. GAPDH mRNA levels, as measured by semi-quantitative RT-PCR, varied less than 1.7 fold amongst all samples analysed (not shown). Therefore, it was concluded that the apparent differences in Lyt 2 mRNA levels amongst samples were not a result of RNA handling errors. The profound deficit in Lyt 2 transcripts in ganglia from anti-CD8 treated mice is consistent with the belief that anti-CD8 treatment depletes mice of the vast majority of cells that transcribe mRNA for CD8. Furthermore, these data indicate that depletion of CD8 expressing cells can be maintained at a site of viral infection, despite strong, sustained antigenic stimulation.

3.5 $CD8^+$ cell depletion increases HSV replication

The expression of a HSV gene was used to gauge the effect of anti-CD8 treatment on ganglionic HSV replication. U_L30, a β gene that encodes the HSV DNA polymerase, was chosen for this purpose (Lakeman *et al.*, 1995). Semi-quantitative RT-PCR was used to detect the relative levels of HSV pol transcription in ganglia from uninfected mice and ganglia taken from immunocompetent and anti-CD8 treated mice five and seven days after inoculation (figure 3.3B). HSV pol transcripts were not detected in ganglia from uninfected mice, reflecting the specificity of the oligonucleotides used as PCR primers and the probe. Both in immunocompetent and anti-CD8 treated mice, the amount of HSV pol mRNA in ganglia was higher five compared with seven days after inoculation. At both times, higher levels of mRNA for HSV pol were detected in ganglia from $CD8^+$ cell depleted mice compared with ganglia from immunocompetent mice, with the difference

being most marked seven days after infection. GAPDH mRNA levels showed that both day five samples were slightly overloaded (figure 3.4, bottom right), but nonetheless, it was apparent that anti-CD8 treatment increased ganglionic HSV replication.

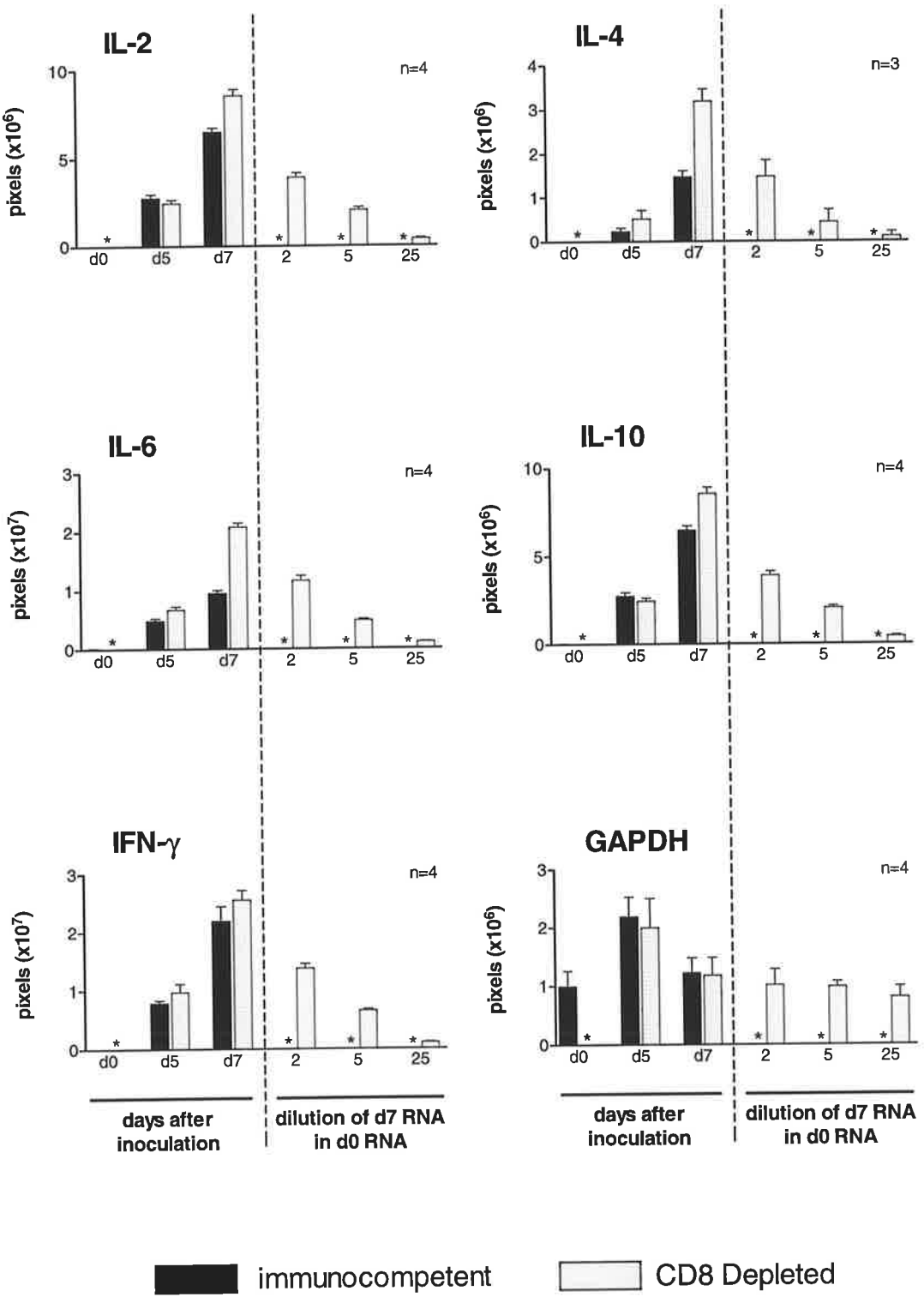
3.6 $CD8^+$ cell depletion alters cytokine mRNA levels in HSV infected ganglia

Experiments were designed to address the hypothesis that depletion of $CD8^+$ T lymphocytes *in vivo* alters the cytokine response to HSV in infected ganglia.

Five cytokines were selected for this study, namely IL-2 and IFN-γ, which are considered to be Th1 cytokines and IL-4, IL-6 and IL-10, which are Th2 cytokines. GAPDH mRNA levels were used to control for RNA handling errors. Semi-quantitative RT-PCR was used to compare the mRNA levels for each of these molecules in ganglia from uninfected mice and ganglia taken from immunocompetent and anti-CD8 treated mice five and seven days after inoculation (figure 3.4). RT-PCRs for each cytokine transcript were done at least in triplicate, and the majority in quadruplicate. The upregulation of cytokine transcription in ganglia during HSV infection described in section 3.3 was confirmed by these experiments and further, it was shown that the increase in the level of each cytokine was not dependent on $CD8^+$ T cells. The comparative amounts of GAPDH mRNA detected showed that both day five samples were overloaded with respect to the uninfected and day seven samples. Therefore the differences in cytokine mRNA levels between days five and seven are, at face value, underestimated. In examining the data for differences between anti-CD8 treated and immunocompetent mice, each day after infection was considered separately. On the fifth

Figure 3.4

Semi-quantitative RT-PCR analyses of cytokine mRNA in ganglia of uninfected mice (d0) and infected mice, five (d5) and seven (d7) days after inoculation with HSV. Mice were either immunocompetent (black bar) or $CD8^+$ cell depleted (grey bar). The cytokines examined are indicated above each graph and bars represent the mean and standard errors of ImageQuant pixel counts from dot blotted RT-PCRs done in triplicate (n=3) or in most cases quadruplicate (n=4). To the right of the dashed line in both columns of graphs are quantity controls, 2, 5 and 25 fold dilutions of d7 RNA in d0 RNA. Transcripts for GAPDH (bottom right) were quantified in each sample to control for RNA handling errors. *not done.


IL-2
n=4
pixels (x10⁶)
10
5
0
d0
d5
d7
2
5
25
IL-4
n=3
pixels (x10⁶)
4
3
2
1
0
IL-6
n=4
pixels (x10⁷)
3
2
1
0
IL-10
n=4
pixels (x10⁶)
10
5
0
IFN-γ
n=4
pixels (x10⁷)
3
2
1
0
GAPDH
n=4
pixels (x10⁶)
3
2
1
0
days after inoculation
dilution of d7 RNA in d0 RNA
days after inoculation
dilution of d7 RNA in d0 RNA
immunocompetent
CD8 Depleted

day of infection, transcripts for at least IL-2 and IL-4 appeared to be more abundant in ganglia from anti-CD8 treated mice than immunocompetent mice. However, the quantity controls did not adequately cover the appropriate range and the standard errors were large in comparison with the amounts of transcripts detected. For these reasons, the significance of the observed differences is not certain. Seven days after inoculation, with reference to the quantity controls, the amounts of IL-4 and IL-6 transcripts were two fold higher in ganglia of $CD8^+$ cell depleted when compared with immunocompetent mice. Smaller differences between immunocompetent and anti-CD8 treated mice were shown for IL-2 and IL-10, but the significance of these differences was considered to be questionable. IFN-γ mRNA levels were not significantly affected by anti-CD8 treatment.

Three conclusions about the effect of $CD8^+$ cell depletion on ganglionic cytokine mRNA levels in HSV infected ganglia were drawn from this set of experiments. First, the levels of two cytokines, namely IL-4 and IL-6, were increased by anti-CD8 treatment. Second, anti-CD8 treatment did not affect IFN-γ expression and therefore third, the pattern of cytokines affected by CD8 depletion cannot be easily explained in terms of the Th1/Th2 paradigm.

3.7 Cytokine expression five days after infection

The experiments presented in the previous section were designed to investigate ganglionic cytokine mRNA levels five and seven days after inoculation of mice, but the day five results were inconclusive. To examine cytokine transcription more closely five days after inoculation, which corresponds to the time when clearance of infectious HSV from ganglia

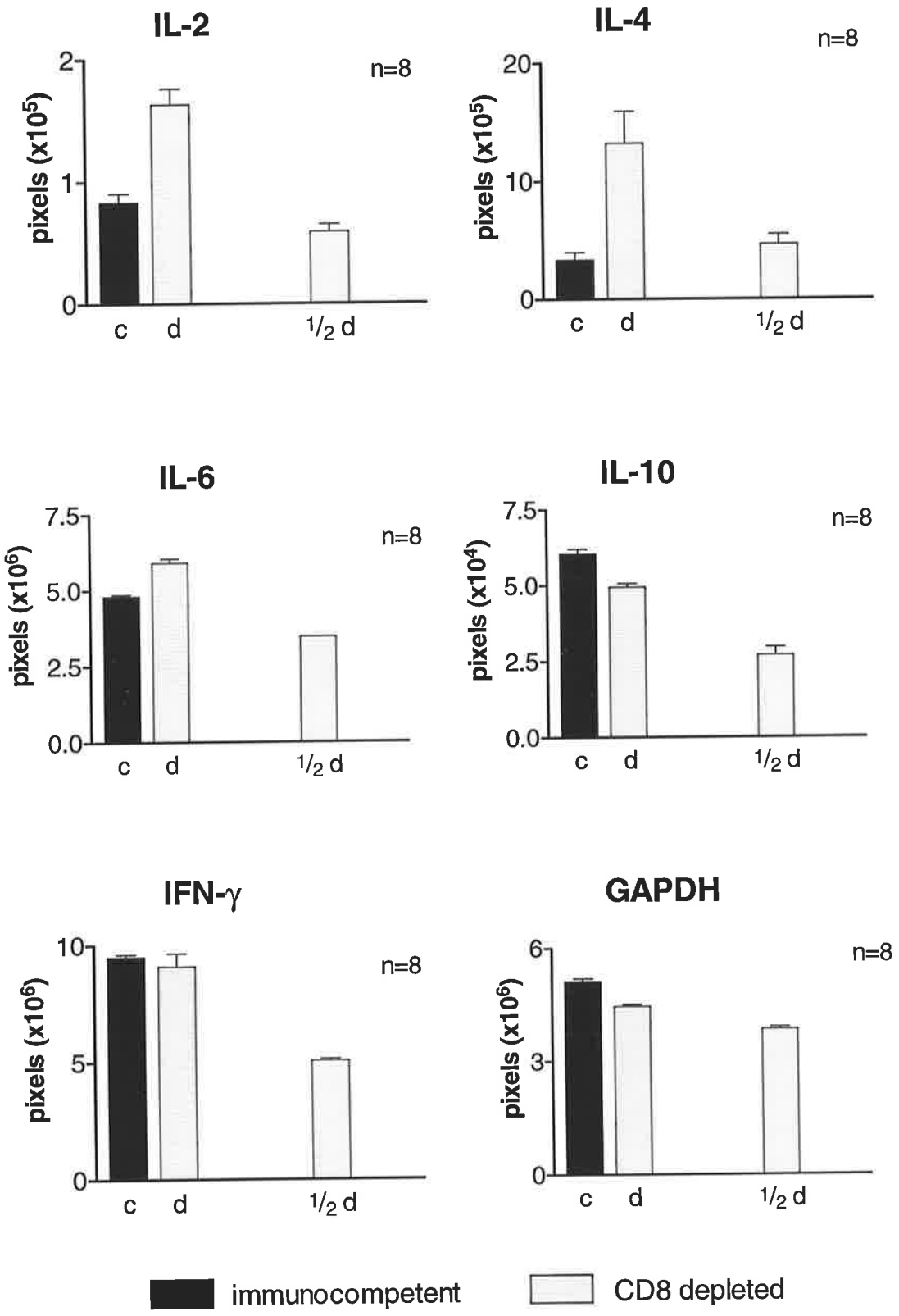
is initiated, the semi-quantitative RT-PCR analysis of the day five RNA samples was repeated using an appropriate quantity control. In addition, RT-PCRs for each cytokine mRNA were done in octuplicate, to improve confidence in the results. The data from these experiments are presented as graphs in figure 3.5 and are consistent with the trends documented in the last section. That is, in anti-CD8 treated mice, ganglionic IL-2 and IL-4 mRNA levels were increased significantly compared with immunocompetent mice, five days after inoculation with HSV. The other cytokines tested (IL-6, IL-10 and IFN-γ) were not considered to be significantly affected by $CD8^+$ cell depletion at this time.

3.8 Discussion

Mice depleted of $CD8^+$ T lymphocytes have intact HSV-specific DTH and humoral responses and the only documented defect of anti-HSV immune function in these mice, is the failure to generate MHC class I restricted CTLs (Nash *et al.*, 1987). A specific aspect of immune system regulation is the role played by cytokines in shaping helper T cell responses. The Th1/Th2 paradigm predicts that changes in Th1 cytokine expression should induce a reciprocal change in Th2 cytokine expression and vice versa (Heinzel *et al.*, 1993; Sharma *et al.*, 1996). This model is not supported by the data presented above pertaining to changes in ganglionic cytokine mRNA levels caused by anti-CD8 treatment of HSV infected mice. In ganglia, the abundance of mRNA for only one cytokine, namely IL-4, was altered five and seven days after HSV inoculation by anti-CD8 treatment. Transcripts for other Th2 cytokines were not similarly affected and the level of mRNA for IFN-γ, a Th1 cytokine, was also unchanged. Furthermore, on the fifth day of infection, $CD8^+$ cell depleted mice had raised levels of transcripts for IL-2 and IL-4, which are considered to be

Figure 3.5

Semi-quantitative RT-PCR analyses of cytokine mRNA in ganglia taken from mice five days after inoculation with HSV. Mice were either immunocompetent controls (c, black bar) or $CD8^+$ cell depleted (d, grey bar). The cytokines examined are indicated above each graph and bars represent the mean and standard errors of ImageQuant pixel counts from dot blotted RT-PCRs done in octuplicate (n=8). A quantity control (½d) consisting of d RNA diluted two fold in uninfected ganglionic RNA was included. Transcripts for GAPDH (bottom right) were quantified in each sample to control for RNA handling errors.


IL-2
n=8
pixels (x10^5)
2
1
0
c
d
1/2 d
IL-4
n=8
pixels (x10^5)
20
10
0
c
d
1/2 d
IL-6
n=8
pixels (x10^6)
7.5
5.0
2.5
0.0
c
d
1/2 d
IL-10
n=8
pixels (x10^4)
7.5
5.0
2.5
0.0
c
d
1/2 d
IFN-γ
n=8
pixels (x10^6)
10
5
0
c
d
1/2 d
GAPDH
n=8
pixels (x10^6)
6
3
0
c
d
1/2 d
immunocompetent
CD8 depleted

Th1 and Th2 cytokines respectively. Ashman *et al* (1995) reported that differences in cytokine expression between two strains of mice with different susceptibilities to severe central nervous system lesions caused by *Candida albicans* yeast, do not fit the Th1/Th2 paradigm. Thus, there is a precedent for a breakdown of the Th1/Th2 paradigm in a nervous system infection. However, direct application of the Th1/Th2 paradigm to anti-HSV immunity in ganglia may be too simplistic. For instance, the biasing of individual Th cells towards Th1 or Th2 cytokine expression is thought to occur at the time of priming, presumably in lymphoid tissue (Seder *et al.*, 1992) and the increased IL-4 transcription observed in HSV infected ganglia may not be occurring in the vicinity of T cell priming. Also, there is evidence for the expression of cytokines, including IL-4, IL-6 and IL-10 by resident cells of the nervous system (Benveniste, 1992; Umehara *et al.*, 1994; Wesselingh *et al.*, 1994; Murphy *et al.*, 1995) and therefore, the source of increased levels of cytokine mRNAs might not be lymphocytes. These issues could be partly resolved by expansion of the current line of investigation to include lymphoid tissue, absolute quantification of cytokines in lymphoid and nervous system tissue, and *in situ* studies to determine the cellular origin of the various cytokine transcripts detected in HSV infected ganglia.

There may be implications of changes in cytokine transcription induced by anti-CD8 treatment that lie outside the confines of immune regulation and Th responses. In experiments presented in this chapter, anti-CD8 treatment of mice resulted both in increased IL-4 transcription and impaired ability to clear HSV from infected ganglia. These data are of particular interest because mice given recombinant IL-4 and HSV intranasally suffer more severe encephalitis than mice given HSV alone (Ikemoto *et al.*, 1995b). Additionally, therapy with anti-IL-4 mAbs reduced mortality after an intranasal

challenge with HSV. It might be envisaged that the effect of recombinant IL-4 on HSV infection is mediated via a reciprocal decrease in IFN-γ expression, but in the light of data present here and the above discussion, this cannot be assumed. Although a causal link between increased IL-4 expression and inefficient virus clearance cannot be made on the basis of the current data, the possibility that IL-4 is detrimental to resolution of HSV infection merits further investigation.

IL-2 mRNA was more abundant in anti-CD8 treated mice, compared with immunocompetent mice, at five days after infection. There is little literature which either supports, or sheds light on the reasons for the apparent IL-2 upregulation. According to one report, there may be a population of $CD8^+$ suppressor cells, the removal of which allows the generation of more IL-2 producing cells (Pyrmowicz *et al.*, 1985). However, if anti-CD8 treatment releases $CD4^+$ T cells from suppression, an equivalent increase in transcription of other cytokines might be expected. One might speculate that an increase in IL-2 producing cells reflects an early compensation for the lack of $CD8^+$ T cells.

Unlike the increase in IL-2 transcription at five days after inoculation, there is a likely explanation for increased IL-6 transcription in ganglia of $CD8^+$ cell depleted mice seven days after HSV inoculation. Bolin *et al* (1995) and Murphy *et al* (1995) have each documented IL-6 production by resident cells of the peripheral nervous system in response to neuronal or axonal damage and further, primary sensory neurons have been identified as a source of IL-6 (Murphy *et al.*, 1995). Anti-CD8 treatment exacerbates HSV infection and it is feasible that increased neuronal damage, caused by HSV in the absence of $CD8^+$ T cells, results in higher levels of IL-6 transcription. This hypothesis could be confirmed

by *in situ* studies to determine the source of IL-6 production in HSV infected ganglia. The role of IL-6 in situations of neural damage is not completely understood. Increased expression of IL-6 has been noticed in areas of the brain resistant to ischemia (Maeda *et al.*, 1994) and IL-6 can promote the survival of several types of neurons in culture (Hama *et al.*, 1991; Kushima *et al.*, 1992; Kushima and Hatanaka, 1992; Yamada and Hatanaka, 1994). In addition, IL-6 has been shown to cause cultured astrocytes to secrete nerve growth factor, an important neurotrophic factor (Williams *et al.*, 1986), providing a possible mechanism of IL-6 mediated protection of neurons (Frei *et al.*, 1989). IL-6 production has been noted in many viral infections in the central nervous system, including those caused by Borna disease virus (Shankar *et al.*, 1992), lymphocytic choriomeningitis virus (Frei *et al.*, 1989; Moskophidis *et al.*, 1991), vesicular stomatitis virus (Frei *et al.*, 1989), murine hepatitis virus (Sun *et al.*, 1995), Theiler's murine encephalomyelits virus (Rubio and Sierra, 1993) and enterovirus (Gillespie *et al.*, 1993). It has been suggested that the role of IL-6 in these infections is to protect nervous system tissue from viral damage (Frei *et al.*, 1989; Rodriguez *et al.*, 1994) and this may be the case in ganglionic HSV infections. However, putative rescue of HSV infected neurons by IL-6 production is not a unique property of $CD8^+$ T cells, because IL-6 mRNA was more abundant in ganglia of anti-CD8 treated mice.

IFN-γ is an established mediator of anti-HSV immunity and was of particular interest in this investigation. Production of IFN-γ has been proposed as an important effector mechanism by which $CD4^+$ and $CD8^+$ T lymphocytes terminate HSV infection in the skin (Smith *et al.*, 1994). In HSV infected ganglia, where $CD8^+$ T cells are crucial for adequate immunity, a more modest role for IFN-γ was shown by Cantin *et al* (1995). The current

semi-quantitative RT-PCR analysis of IFN-γ transcription in anti-CD8 treated and immunocompetent mice demonstrates that $CD8^+$ T lymphocytes are not required for IFN-γ production in HSV infected ganglia. This means either that $CD8^+$ T lymphocytes do not contribute significantly to IFN-γ production or that if IFN-γ is made by $CD8^+$ T cells, other cells compensate in $CD8^+$ cell depleted mice. Either way, the anti-viral activity unique to HSV-specific $CD8^+$ T lymphocytes is independent of IFN-γ.

In summary, the experiments reported in this chapter raise new issues about the nature of cytokine regulation in sensory ganglia during the immune response to HSV infections and the role of IL-4 in HSV pathogenesis. However, none of the changes in cytokine transcription caused by anti-CD8 treatment adequately explain why $CD8^+$ T cells appear to be essential for clearance of HSV from murine ganglia. Therefore the mechanisms of $CD8^+$ T lymphocyte action in the nervous system remain unknown. Furthermore, it remains to be shown whether the effects of anti-CD8 treatment documented in this chapter can be reproduced in other models of infection and on other viral and mouse genetic backgrounds.

4. DEVELOPMENT AND VERIFICATION OF mRNA DIFFERENTIAL DISPLAY

New approaches, which are not confined by the boundaries of present knowledge of immune mechanisms, may increase our understanding of the function of HSV-specific $CD8^+$ T lymphocytes. Comparison of HSV infections in immunocompetent and $CD8^+$ cell depleted mice identified previously a role for $CD8^+$ T cells and this defined model can be exploited further to gain molecular insights. A complete catalogue of the molecular differences between HSV infected ganglia of immunocompetent and $CD8^+$ cell depleted mice must include the effectors by which $CD8^+$ T cells mediate their anti-viral effect. Investigation based on such a catalogue of differences is not limited to, but may be aided by, present knowledge and constitutes a new approach to elucidating $CD8^+$ T cell functions.

Several methods have been developed for comparing molecular repertoires. Some methods make comparisons at the polypeptide level (eg. protein fingerprinting using two-dimensional PAGE), but techniques based on mRNA analysis are more sensitive, facilitate further analysis of molecules of interest and hence are more popular. There are three basic strategies for identifying differentially expressed mRNAs: differential screening of cDNA libraries, subtractive hybridization and mRNA population fingerprinting. With a few recently published exceptions (Coche *et al.*, 1994; Hubank and Schatz, 1994; Zeng *et al.*, 1994), most successful implementations of the first two strategies have used large amounts of RNA (ie. more than 50 μg), making them unattractive options for analysis of murine dorsal root ganglia. In contrast, mRNA population fingerprinting methods, which are PCR based, require small amounts of input RNA and are at least as sensitive as the other

techniques. The experiments in this chapter describe the development and validation of mRNA differential display (DD), an mRNA population fingerprinting method, for use in the identification mRNAs that are expressed at different levels in HSV infected ganglia from immunocompetent and $CD8^+$ cell depleted mice.

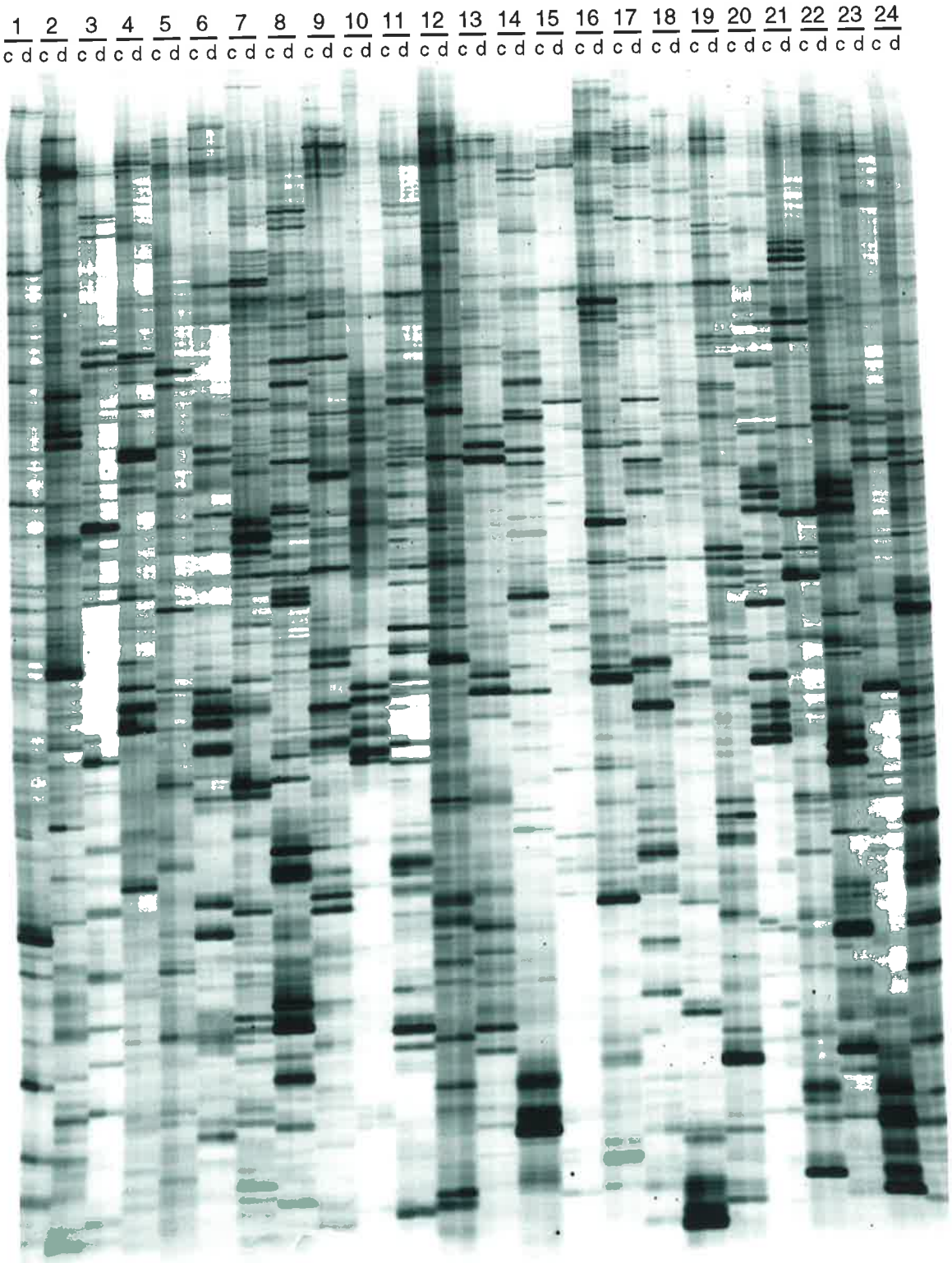
4.1 Basis of DD and considerations on the choice of primers

Mammalian cells are estimated to contain over 20,000 different species of mRNA and therefore displaying the full diversity of mRNAs from a group of cells at once is impossible. DD, introduced by Liang and Pardee (1992), uses degenerately primed RT-PCRs to divide the total pool of mRNAs into sets of cDNA samples, each comprising between 50 and 150 different species, that can be displayed on large polyacrylamide gels (eg. figure 4.1). Displaying RNA populations concurrently from two or more sources allows differences in transcription between the sources to be visualized. After a difference is identified on a display gel, this partial cDNA can be excised from the gel, amplified by PCR and then cloned, allowing further analysis. In the five years since DD was first published, there have been over 200 publications based on the method, including several recent reviews (Liang and Pardee, 1995; McClelland *et al.*, 1995; Sunday, 1995). The technology is currently available in kit form from a number of suppliers.

The key to DD is the design of primers used in RT-PCRs. According to the original protocol, a series of 12 RTs are done, each primed with one of 12 possible oligonucleotides of the form TTTTTTTTTTVN, where V is A, G or C and N is A, G, C or T (ie. $dT_{11}AT$, $dT_{11}AG$, $dT_{11}AC$, etc). RTs using primers of this form initiate transcription at the

Figure 4.1

Autoradiograph of a differential display of RNA from HSV infected ganglia of immunocompetent (c) and $CD8^+$ cell depleted (d) mice using $T_{11}AC$ with 24 different decamers (1-24).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d c d

polyadenylated 3' end of mRNAs and, owing to the specificity conferred by the last two bases of the primer, one twelfth of the input mRNAs should theoretically be transcribed. However, it has been shown that the penultimate base does not contribute greatly to the specificity of priming (Liang *et al.*, 1993) and therefore, primers with a single non-dT base at the 3' end ($dT_{11}V$) have been recommended (Liang *et al.*, 1994). Irrespective of the primer system chosen, the net effect is that a series of cDNA pools are generated, each of which represents a subset of the original population of mRNAs. Proponents of $dT_{11}V$ primers argue that cDNA pools generated by $dT_{11}VN$ primers are largely overlapping (Liang *et al.*, 1992; Liang *et al.*, 1993; Linskens *et al.*, 1995) and it has been shown that $dT_{11}V$ primers reduce this redundancy (Liang *et al.*, 1994), but this gain may come at the expense of diversity. There is little doubt, however, that the newer system, which apparently entails one quarter of the work required by the original protocol for the same result, is more marketable and most DD kits provide primers of the newer ($dT_{11}V$) form.

Following the RT reactions, a subset of each cDNA pool is PCR amplified in the presence of a radioactive nucleotide using an arbitrary decamer in combination with the RT primer. The radiolabelled products of such reactions are then separated on large polyacrylamide gels and visualized by autoradiography. It has been estimated that a series of 25 decamers are required to sample adequately all the cDNAs in a pool generated using a $dT_{11}VN$ primer (Bauer *et al.*, 1993). However, this statistical estimate ignores two important empirical findings regarding DD: (i) DD is greatly biased towards the display of abundant mRNAs and rare transcripts are under-represented (Bertoli *et al.*, 1995) and (ii) abundant mRNAs are often represented on DD gels many times (Liang *et al.*, 1992; Linskens *et al.*, 1995). Based on these empirical findings, Bertoli (1995) suggested that fewer than 25

decamers are required to sample the most abundant cDNAs, which comprise the vast majority of products visualized on a display gel, but far more primers are required to render effectively even a fraction of the rare cDNAs. No estimations of this kind based on the use of $dT_{11}V$ primers have been published. The question: How many decamer primers is enough? remains unanswered, but in most applications this consideration is of limited practical value because transcriptional differences are often identified by DD with fewer than a dozen primer combinations. Exceptions to this practical rule arise when DD is applied to situations where there are few differences, and when differentially expressed mRNAs are of low abundance in their original samples. The abundance of mRNAs of interest is a potentially important limitation when using DD to identify transcripts associated with $CD8^+$ T cells in HSV infected ganglia, because the cell types of greatest interest, $CD8^+$ cells and infected neurons, are greatly outnumbered (at least 100:1) by other cells.

In this project, a combination of twelve $dT_{11}VN$ primers and 24 decamer primers was used. Figure 4.1 shows an example of a DD gel on which paired ganglionic RNA samples were displayed using all 24 decamers in combination with a single $dT_{11}VN$ primer. Unless otherwise stated, RNA extracted from vero (African green monkey kidney) cells was used in all developmental experiments reported in this chapter.

4.2 Choice of gels for use in DD

The original description of DD and nearly all subsequent publications have used denaturing polyacrylamide gels, identical to those used for DNA sequencing, to separate DD products,

despite an early publication suggesting that non-denaturing (or native) gels are more suitable (Bauer *et al.*, 1993). The main point stated in favour of non-denaturing gels was that they display, at a single position, variants of a PCR product that differ only in the number of additional adenosine residues at their 3' end (generated by Taq polymerase (Clark, 1988)) whereas denaturing gels display each variant at a unique position, leading to unwanted complexity (Bauer *et al.*, 1993). In addition, unlike sequencing gels that resolve DNA molecules strictly according to size, non-denaturing gels resolve DNA according both to size and base composition and therefore may separate two DNA species of identical size. For these reasons, non-denaturing gels were tested for their utility in resolving DD RT-PCR products.

Figure 4.2A shows an autoradiograph of three pairs of differential displays on a standard sequencing gel (denaturing) and a non-denaturing polyacrylamide gel. The compositions and electrophoretic conditions of these gels were as per sections 2.2.15.1 and 2.2.14.1 respectively. In concordance with the findings of Bauer *et al* (1993), multiple bands on the denaturing gel (especially seen below approximately 150 bp), produced by polyadenylated variants of the same PCR product, were resolved as single bands under non-denaturing conditions. In addition, although there is no known matrix that separates products from 500 bp to 100 bp linearly (a property that would be ideal for DD), the native gel came closest to this goal. In contrast, products between 100 and 200 bp occupied over half the length of the denaturing gel. From this result and other similar experiments where different electrophoresis conditions were tested for both types of gel, it was concluded that non-denaturing gels are more useful than denaturing gels for separating of DD RT-PCR products.

Figure 4.2

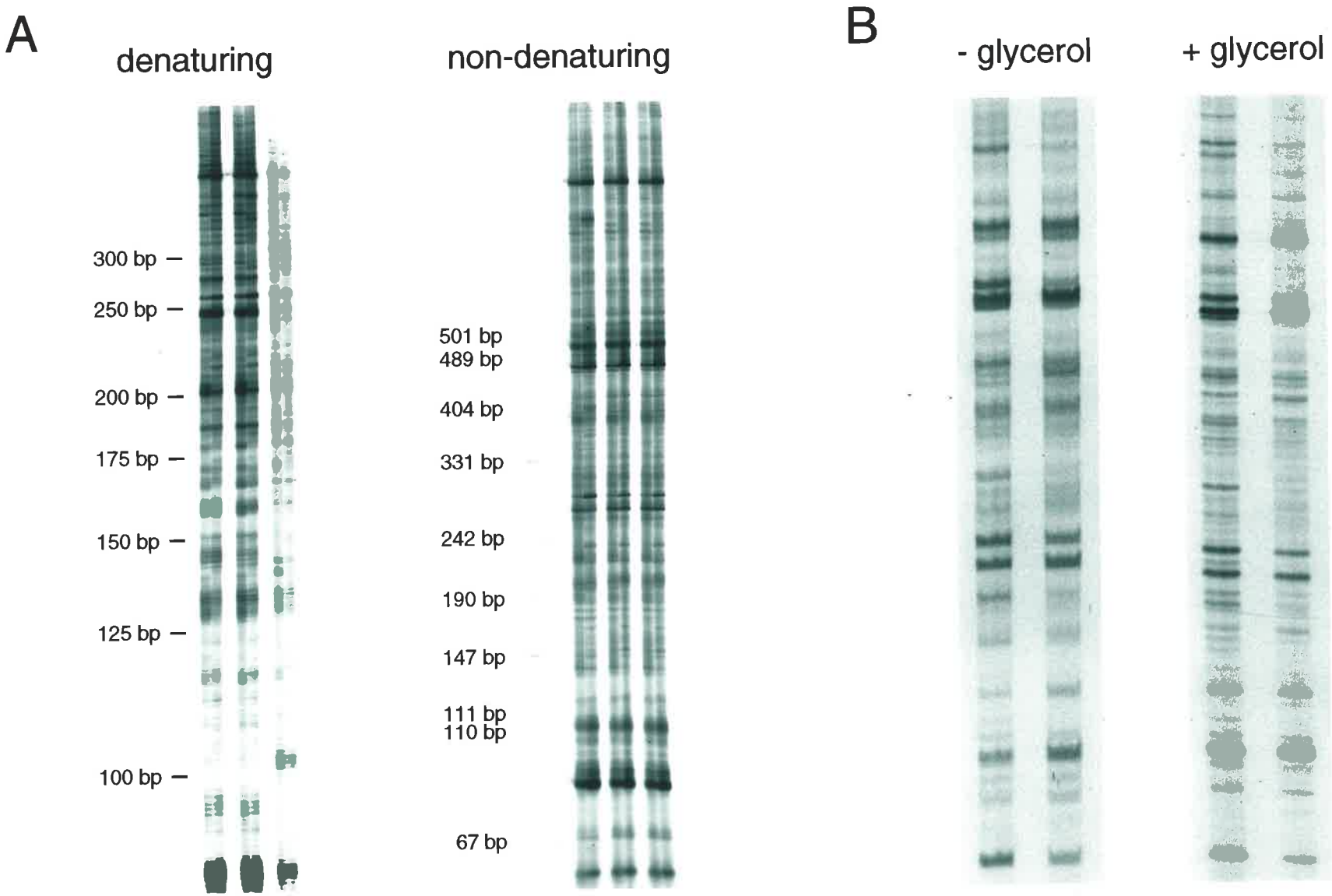
Autoradiographs of various 6% polyacrylamide gel types tested for use in DD.

A

Products of three pairs of DD RT-PCRs fractionated on denaturing and non-denaturing gels. The DNA sizes indicated on the left of each gel were generated using a DNA sequence ladder (not shown) on the denaturing gel and radio-labelled *Hpa*II digested pUC-19 plasmid DNA (to the left of DD products) on the non-denaturing gel. Note the different DNA fragment size separating ranges of the two gel types and the "laddering" effect on the denaturing gel especially below approximately 150 bp.

B

Products of two DD RT-PCRs separated on non-denaturing polyacrylamide gels prepared without glycerol (-) and with 20% glycerol (+). Note the difference in resolution between the two gels.


A
denaturing
non-denaturing
300 bp
250 bp
200 bp
175 bp
150 bp
125 bp
100 bp
501 bp
489 bp
404 bp
331 bp
242 bp
190 bp
147 bp
111 bp
110 bp
67 bp
B
- glycerol
+ glycerol

A disadvantage of non-denaturing gels was that, despite resolving a wider range of DNA fragment sizes, the resolution of fragments on these gels was inferior to that of denaturing gels. Changing the concentrations of TBE buffer, ratios of acrylamide:bis-acrylamide and the electrophoretic conditions produced no significant improvement (data not shown). Glycerol is sometimes added to non-denaturing gels to prevent cracking during the drying process, a problem that reduces resolution. Although cracking did not seem to be a source of poor resolution on DD gels (ie. the gels appeared to be intact after drying and the appearance of subsequent autoradiographs was not indicative of cracking), the addition of glycerol was tested because none of the other attempts to increase resolution had been fruitful. Figure 4.2B shows sections of non-denaturing gels that have been prepared with or without 20% glycerol. In this experiment, resolution was enhanced by the addition of glycerol to the gel matrix, to the extent that it approached that of denaturing gels. Further experiments tested lower concentrations of glycerol because gels prepared with 20% glycerol remain tacky after drying, causing problems with autoradiography and storage. Gels with 10% glycerol provided the best compromise between good resolution and ease of handling and were chosen for use in DD.

The gel composition and electrophoretic conditions established by the above set of experiments were: 6% polyacrylamide (acrylamide:bis-acrylamide 29:1, polymerised with 0.1% ammonium persulphate and 0.075% TEMED), 10% glycerol in 2× TBE buffer and electrophoresed at 60 W for 5-6 hours in 2× TBE buffer. These parameters were used in all subsequent DD based experiments.

4.2 Optimization of RT-PCR conditions for DD

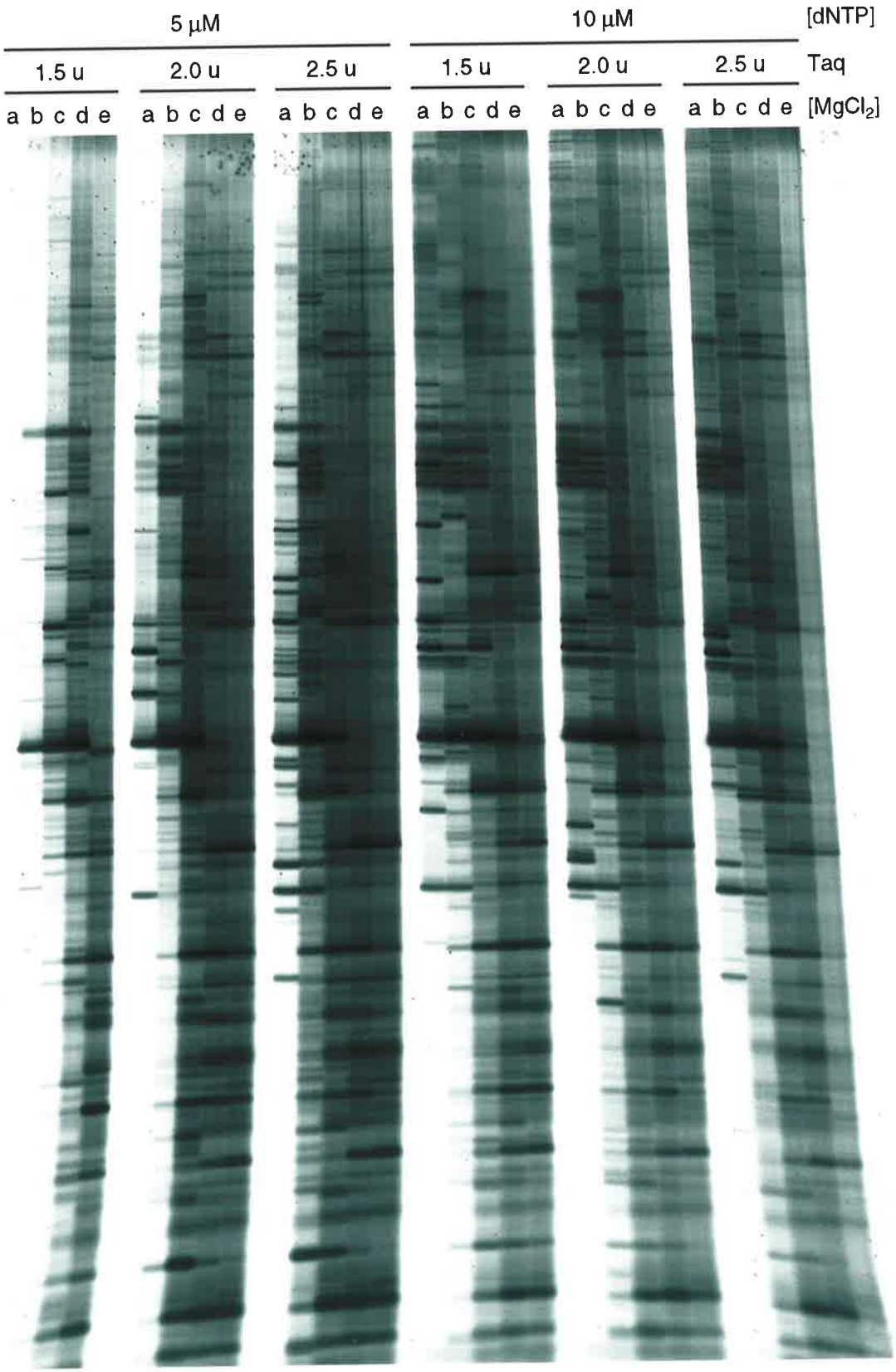
No special modifications were required to adapt standard RT protocols for use in DD because the aim of RT reactions in DD is the same as that for most other applications, ie. to fully transcribe into single stranded cDNA each RNA molecule that can bind the chosen primer. All PCR reactions require optimization for maximum performance in a given situation. PCRs are most frequently used to amplify single, defined sequences of nucleic acid and optimal reaction conditions can be very specific. The optimization of such a reaction is easily monitored by measuring increases in the amount of desired product after each successive refinement. In contrast, the aim of DD PCRs are the production of approximately 100 undefined products of varying size, but most preferably under 1 kb, by each of a number of different primers pairs and therefore, the essence of PCR optimization for DD is compromise rather than specificity.

A DD PCR reaction comprises template cDNA, a $dT_{11}VN$ primer, an arbitrary decamer primer, each of the four dNTPs, radiolabelled dATP, $MgCl_2$, a buffer (Tris-HCl and KCl, defined by the manufacturer of Taq) and Taq polymerase. The amounts of each constituent can be altered to change the number of discrete products and the background seen between the products when visualized on a gel. Two parameters of PCR thermocycling are critical for DD, (i) the temperature and length of annealing steps, which partially determines the level of degenerate priming, and (ii) the number of cycles, which affects the amount of each product and background on DD gels. A change in one of the above reagent amounts or thermocycler settings generally affects the remaining parameters and therefore, simultaneous titration of all reagents and thermocycling profiles is, theoretically, the most

efficient approach to optimization. However, simultaneous titration of ten parameters is not feasible and therefore, some conditions were not modified and others were fixed according to independent criteria (for example, the need to use as little RNA as possible), reducing the number of permutations. Primers were used at the concentrations originally specified by Liang and Pardee (1992) (2.5 M and 0.5 M for $dT_{11}VN$ and decamer primers respectively) because these concentrations are constant in most of the DD literature. The buffer was not changed from the manufacturer's recommendation. Another three parameters were fixed for economic or practical reasons. First, only small quantities of RNA were available and therefore the lowest amount of input cDNA that reliably produced approximately 100 PCR products when amplified with the majority of primer pairs was used (equivalent of 10 ng of mRNA per reaction, assuming 100% efficiency in the RT). Second, to reduce expense and exposure to radioactivity, the smallest amount of [α-^{33}P] dATP that enabled visualization of products after overnight autoradiography of gels was used (5 μCi per reaction). Third, the number of iterations in the thermocycling profile was reduced from the originally recommended 40 cycles (Liang and Pardee, 1992) to 30 cycles, to save time and because early optimization experiments indicated that this reduction in cycles lowered background on DD gels without a significant loss of discrete products over a wide range of other parameter settings. The remaining four parameters, namely concentrations of dNTPs, $MgCl_2$ and Taq and the annealing temperature, interact to determine the stringency of priming and polymerase stability, which in turn influence the final number of PCR products visualized on a DD gel. These parameters were titrated in groups of three over a series of experiments, an example of which is represented by the autoradiograph in figure 4.3. This particular experiment tested all possible combinations of five $MgCl_2$ concentrations, three dNTP concentrations and two amounts of Taq

Figure 4.3

An example of an experiment in which three DD PCR conditions were titrated simultaneously, dNTP concentration ([dNTP]), amount of Taq polymerase used (Taq) and concentration of $MgCl_2$ ([$MgCl_2$]: a = 1 mM; b = 1.5 mM; c = 2 mM; d = 2.5 mM and e = 3 mM). The autoradiograph depicted is of a DD gel on which RNA extracted from vero cells was displayed with $dT_{11}AA$ and decamer DT-21 using the PCR conditions indicated above each lane.


5 µM
10 µM
[dNTP]
1.5 u
2.0 u
2.5 u
1.5 u
2.0 u
2.5 u
Taq
a b c d e
a b c d e
a b c d e
a b c d e
a b c d e
a b c d e
[MgCl2]

polymerase. Of particular note is the observation that some products are amplified best at the highest and not at all at the lowest $MgCl_2$ concentration, whereas other products have the opposite $MgCl_2$ requirement, emphasising the need for compromise conditions in DD PCRs. The interactions amongst the three parameters is also demonstrated by this experiment, in which raising dNTP concentration, the amount of Taq, or both, reduces the $MgCl_2$ concentration needed to support amplification of the same number of products. A set of conditions for the four parameters was chosen after a series of titration experiments using one pair of primers. The choice was then checked, and modified if necessary, in further experiments using more than 12 different primer pairs. The final conditions chosen after this process of optimization were: dNTP and $MgCl_2$ concentrations of 10 μM and 1.5 mM respectively, 1.5 units of Taq per reaction and an annealing temperature of 40^0C for 2 minutes. The conditions chosen for all parameters, along with the variations tested for each, are summarized in table 4.1.

4.3 Reliability of DD

Two important reliability issues relating to differential displays are: (i) ensuring that the products of DD PCRs are amplified cDNA, not chromosomal DNA and (ii) that the range of products produced by RNA from one source with a given pair of primers is reproducible. Experiments to test these concerns were done throughout the development of DD and figure 4.4 shows exemplary autoradiographs.

To demonstrate that DD PCR products were not derived from contaminating genomic or other DNA, duplicate RNA samples were subjected to the same RT-PCR procedure, except

Table 4.1 Parameter conditions used in development of DD PCRs

Parameter	Conditions tested	Final conditions
input cDNA	equivalent to 200, 20, 10 and 2 ng of RNA	≡ 10 ng of RNA
[dT_{11}VN primer]	2.5 μM	2.5 μM
[decamer primer]	0.5 μM	0.5 μM
[dNTP]	2, 5, 10, 15 and 20 μM	10 μM
[$MgCl_2$]	0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 mM	1.5 mM
[α-^{33}]dATP*	3, 4, 5, 7.5, 10 μCi/reaction	5 μCi/reaction
Taq†	1, 1.5, 2, 2.5 u/reaction	1.5 u/reaction
[PCR buffer]	1×	1×
annealing	37, 38, 40, 42°C for 1 or 2 min	40°C for 2 min
cycle number	30, 35 and 40 cycles	30

*[α-^{35}S]dATP at 7 and 14 μCi/reaction and [α-^{32}P]dATP at 0.5 μCi/reaction also tested.

†Taq from a second supplier (not Perkin-Elmer) was tested at 0.5, 1 and 2 u/reaction, but could not be used after being withdrawn from the market in early 1994.

the reverse transcriptase enzyme was omitted from one of the RTs reactions. Any DNA fragments amplified by PCR from the products of a sham RT were considered to be contaminants. If DNA contamination was found, the cleanliness of reagents and equipment was checked using reactions in which input RNA was replaced with water. As reported by other investigators (Liang *et al.*, 1993), occasional PCR products, made in the absence of reverse transcription, were not found in the range of products made by the corresponding full RT-PCR (see arrow in figure 4.4A). Therefore, such products were not considered to be a significant problem. The only situation in which DNA contamination was found to affect DD RT-PCRs was when RNA samples were not DNase treated (section 2.2.5). For this reason, all RNA was routinely DNase treated before use in DD.

It has been reported that more than 95% of products produced by duplicate DD RT-PCRs seeded with the same input RNA are identical (Liang *et al.*, 1993). To test the this aspect of DD reliability under various reaction conditions, multiple DD RT-PCRs using vero cell RNA were done and the resultant displays compared. Figure 4.4B shows an autoradiograph from one such experiment in which five independent reactions were done. A total of 69 discrete products were present in all five displays and a maximum of three differences can be found between any two displays. In this experiment, it was concluded that >95% of the cDNAs displayed were identical and these data are in agreement with the majority of other similar reliability tests. However, it is noteworthy that, if thousands of cDNAs are to be displayed, an inherent variability of 5% (or even 1%) can be expected to produce a significant number of putative differences that are spurious (often referred to as "false positives"). Inherent variability in DD was documented as a problem within a year of the first DD publication (Liang *et al.*, 1993). An accepted solution is to repeat, with

Figure 4.4

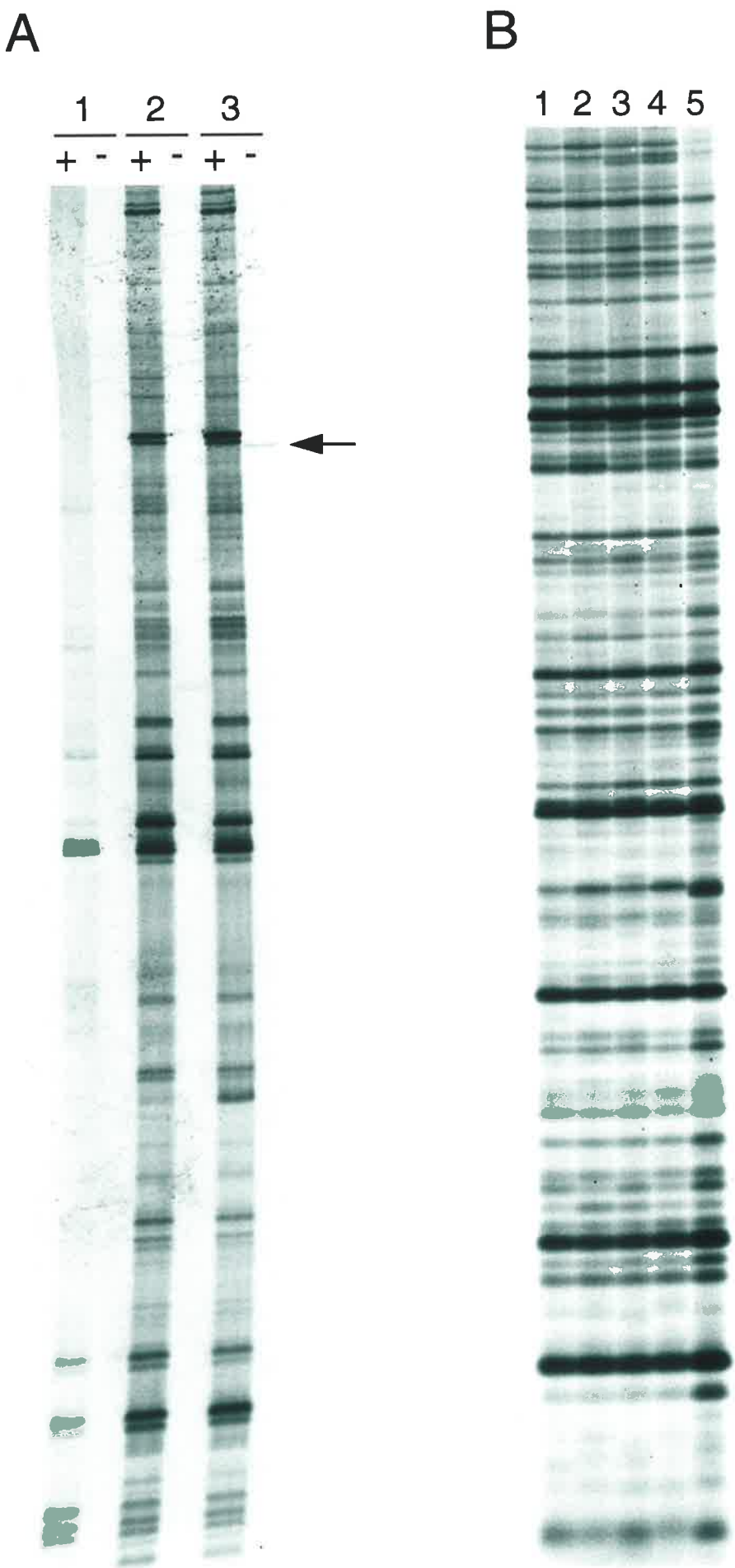
Two aspects of the reliability of DD are demonstrated, namely dependence on reverse transcription (**A**) and reproducibility of products (**B**). Both autoradiographs show vero cell RNA displayed with $dT_{11}AA$ and decamer DT-21.

A

Three different PCR conditions (1-3) were used and reverse transcriptase was included (+) or omitted (-) during the RT step. The arrow shows a DD fragment produced despite the omission of reverse transcriptase, note however, that this product cannot be seen in normal displays.

B

A single RNA sample was displayed five times (1-5) using identical RT-PCR conditions. Note that although there is variation in abundance, nearly all of the products can be identified in all lanes.


A
1 2 3
+ - + - + -
B
1 2 3 4 5

independent RNA samples, any DD RT-PCRs that generate putative differences, discarding differences that cannot be visualized on both occasions (Liang *et al.*, 1993; Sompayrac *et al.*, 1995).

4.4 Cloning differentially displayed cDNAs

An important feature of DD is that it enables cloning of differentially displayed cDNAs facilitating (i) verification that the cDNAs in question truly represent differentially expressed transcripts and if so, (ii) further analysis of such transcripts. However, cloning of the partial cDNAs produced by DD can be problematic. Most cloning problems in DD arise because cDNAs must be cut from display gels and re-amplified using the same short primers with which they were originally amplified. As published by Callard *et al* (1994) and shown in experiments below, DNA extracted from DD gels is heterogeneous, comprising the product of interest and contaminants. This problem is compounded when this mixture of cDNAs is re-amplified prior to cloning. The following three sections document the application of three different strategies for cloning DD cDNAs, the third of which was successful.

4.4.1 The "shot gun" approach

In the original publications by Liang and Pardee (1992) and Liang *et al* (1992), cDNAs of interest were simply cut from display gels, re-amplified and cloned. The sizes of re-amplified cDNAs were checked by conventional agarose gel electrophoresis prior to cloning, but no other tests of authenticity were done. Northern blotting showed that at least

some of the clones obtained in this way contained cDNA representing genuinely differentially expressed mRNA. The first attempts to clone a differentially displayed cDNA derived from HSV infected ganglia used this simple approach.

In a pilot experiment, DD was used to compare ganglionic RNA from $CD8^+$ cell depleted and immunocompetent mice, five days after inoculation with HSV-1. A differentially displayed cDNA, designated GT12, was identified (figure 4.5A) and subsequently reproduced using independent ganglionic RNA samples. GT12, which was found in displays of ganglionic RNA from HSV infected immunocompetent mice and not HSV infected $CD8^+$ cell depleted mice, was cut from a display gel and placed directly into a re-amplification PCR identical to the reaction used to produce the original display, except [α-^{33}P]dATP was omitted. Products of the re-amplification (5 µl) were used to seed a second identical reaction, the products of which were fractionated on a 2% agarose gel. Unlike the results of similar experiments published by Liang *et al* (1992), several DNA species were re-amplified (figure 4.5B). The most prominent re-amplification product, which was approximately the anticipated size, was cloned using the A-T cloning method (section 2.2.14.2). A second attempt to re-amplify and clone GT12 was made and again, products of re-amplification were found to be heterogeneous. DNA sequence analysis was done to test the heterogeneity of clones derived from these two experiments. Of nine clones containing appropriately sized inserts, only two were the same with respect to DNA sequence. It was concluded that the heterogeneity amongst clones was derived from heterogeneity originally present in the DNA extracted from the DD gel.

Three of the unique clones were used to probe northern blots of the original ganglionic

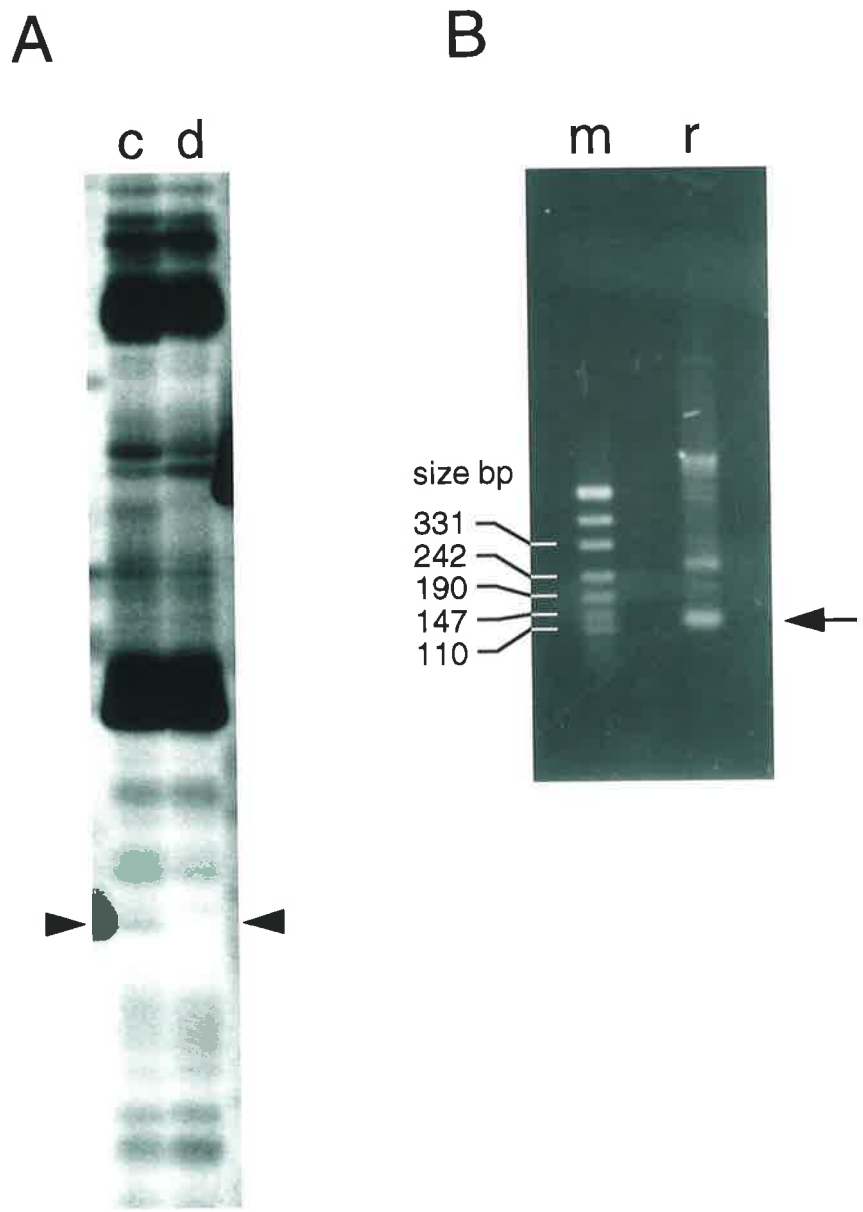
Figure 4.5

A

A section of an mRNA differential display of ganglionic RNA from HSV infected immunocompetent control (c) and $CD8^+$ cell depleted (d) mice. The primers used were $T_{11}GT$ and OPA-12 and a differentially displayed cDNA, designated GT12, is indicated by the arrow heads.

B

An ethidium bromide stained agarose gel on which the final products of two sequential PCR re-amplifications of GT12 cDNA (r), extracted from a DD gel, were separated. The size marker (m) was *Hpa*II digested pUC-19 plasmid DNA and the arrow indicates the product thought to be GT12.


A
c d
B
m r
size bp
331
242
190
147
110

RNA samples. No hybridization was observed with any of the probes suggesting that the clones tested were not derived from the original RNA, or were derived from transcripts present at levels below the sensitivity of the northern blots. In either case, it could not be shown whether or not the clones represented differentially expressed mRNAs. Further screening of the eight unique clones (and others not yet sequenced) by hybridization was abandoned because it was apparent that production of DD clones using the "shot gun" approach was very inefficient.

4.4.2 Screening of DD clones by differential hybridization

A second approach was tried in which clones were produced as before, but were then screened as follows. Duplicate filters containing dot blotted clones or fixed colonies were probed with radiolabelled cDNA from one or other of the two original RNA samples, based on the premise that clones containing differentially expressed cDNAs should bind only one of the probes. The probes were prepared by the standard RT-PCR protocol used for DD, but products were labelled by adding 50 μCi of [α-^{32}P]dATP to PCRs instead of the usual 5 μCi of [α-^{33}P]dATP. The screening strategy was applied to colonies obtained in the attempt to clone GT12 and despite high background hybridization, two candidate clones were identified (figure 4.6A).

To conserve RNA, rather than testing the clones by northern blotting, they were used to probe electroblotted DD gels. The display gels were loaded with samples from the original DD reactions and products of new reactions in which the ^{33}P label had been omitted. The labelled (original) DD products acted as size markers on these blots; positive clones were

Figure 4.6

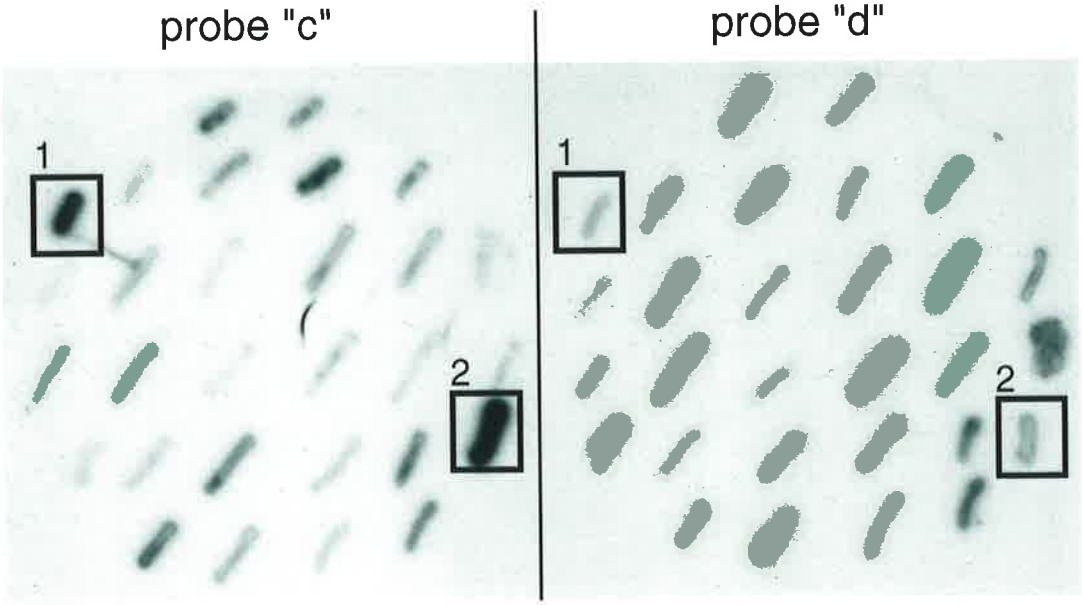
A

Autoradiographs of duplicate colony blots hybridized with ^{32}P-labelled DD RT-PCR products generated by using primers $T_{11}GT$ and OPA-12 with ganglionic RNA from HSV infected immunocompetent control mice (probe "c") or $CD8^+$ cell depleted mice (probe "d"). The colonies are transformants from an attempt to clone GT12, a DD cDNA. Boxes 1 and 2 indicate colonies hybridizing much more strongly to probe "c" than probe "d", the anticipated result for a clone containing GT12.

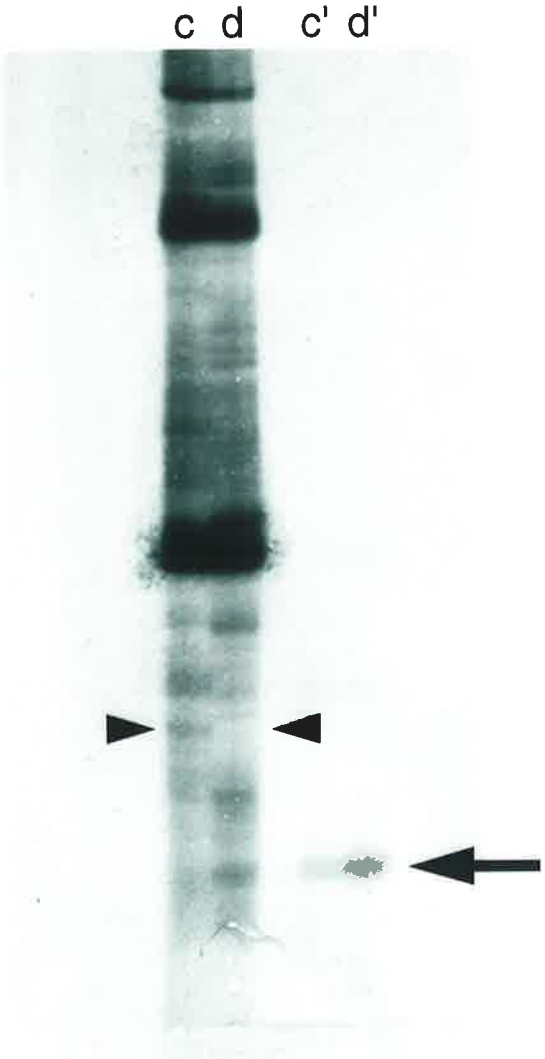
B

Autoradiograph of a DD gel electroblotted onto nylon and hybridized with a probe generated from the putative GT12 clone in box 2 in **A** above. The displays were of ganglionic RNA from HSV infected immunocompetent control mice (c and c') and $CD8^+$ cell depleted mice (d and d') and were radiolabelled (c and d) or unlabelled (c' and d'). The differentially displayed cDNA, GT12, is marked by two arrow heads and the large arrow indicates a cDNA (not differentially displayed) detected by the clone.

A



B



expected to hybridize differentially between displays from the different RNA sources and to products of the correct size. Figure 4.6B depicts an autoradiograph from one of the electroblots, probed with a clone, designated p64-1, chosen on the basis of the colony blot in figure 4.6A. This clone hybridized neither differentially nor to a product of the correct size. Screening of further clones by differential hybridization identified three more clones of potential interest. Before the three new clones were used to probe differential display electroblots, the colony blots from which they were identified were stripped and reprobed with clone p64-1. The only colonies which bound p64-1, were the three colonies thought to contain the correct insert (not shown). All colonies on these blots bore plasmids, as judged by antibiotic resistance (and in some cases β-galactosidase activity) and therefore it was concluded that the three new clones contained the same insert as p64-1. To confirm this conclusion, a combined probe made from all three new clones was used to reprobe the differential display electroblot probed originally with p64-1 (and shown in figure 4.6B). As anticipated, the combined probe hybridized to the same products as p64-1 (not shown), consistent with the conclusion that all four clones represented the same cDNA.

The above results led to the conclusion that differential hybridization is not a satisfactory strategy for screening putative DD clones in the current context. However, it is noted that screening strategies based on differential hybridization have, on occasions, been successful (Mou *et al.*, 1994; Vögeli Lange *et al.*, 1996; Zhang *et al.*, 1996). A possible reason why differential hybridization was found not to be useful in experiments presented here is that GT12 was not abundant in the original display. Two of the three successful applications of differential hybridization used cDNA directly labelled by RT rather than labelled DD RT-PCR products to probe colony blots (Mou *et al.*, 1994; Zhang *et al.*, 1996). The

success of this approach may be related to the fact that the abundance of a product in a differential display does not necessarily predict the abundance of the mRNA it represents in the original RNA population (Liang *et al.*, 1993). However this methodology requires significant amounts (up to 50 µg) of each RNA sample to make probes for the differential hybridizations (Zhang *et al.*, 1996) and therefore, was not used to screen DD clones derived from ganglionic RNA.

4.4.3 An integrated strategy for purifying and cloning DD cDNAs

The problem of screening clones would be greatly reduced if re-amplified cDNAs could be highly purified prior to ligation with a vector. To this end, a protocol was developed in which differentially displayed cDNAs were purified on full length DD gels before cloning. Re-amplified cDNAs were radiolabelled and samples from the original DD RT-PCRs were loaded as a size marker on purification gels, allowing empirical identification of the correct re-amplified cDNA by autoradiography. Irrespective of the method used to purify cDNA tags before cloning, it is still possible that not all resultant clones will contain the desired insert. Callard *et al* (1994) published a method for screening DD clones that is not based on differential hybridization. Differential displays containing differences were repeated using ^{32}P in the reactions; after recovering the correct cDNA, half was re-amplified and cloned and the other half was used to screen the clones by slot blot hybridization. A modification of Callard's screening strategy, integrated with purification of re-amplified differential cDNAs on DD gels, was developed and used to clone GT12 (summarized in figure 4.7).

Figure 4.7

An outline of the gel purification and cloning strategy described in section 4.4.3. Includes four autoradiographs from the successful cloning of cDNA GT12 as an example.

A

A differential display of two ganglionic RNA samples, "c" and "d". A differentially displayed cDNA, designated GT12, is boxed.

B

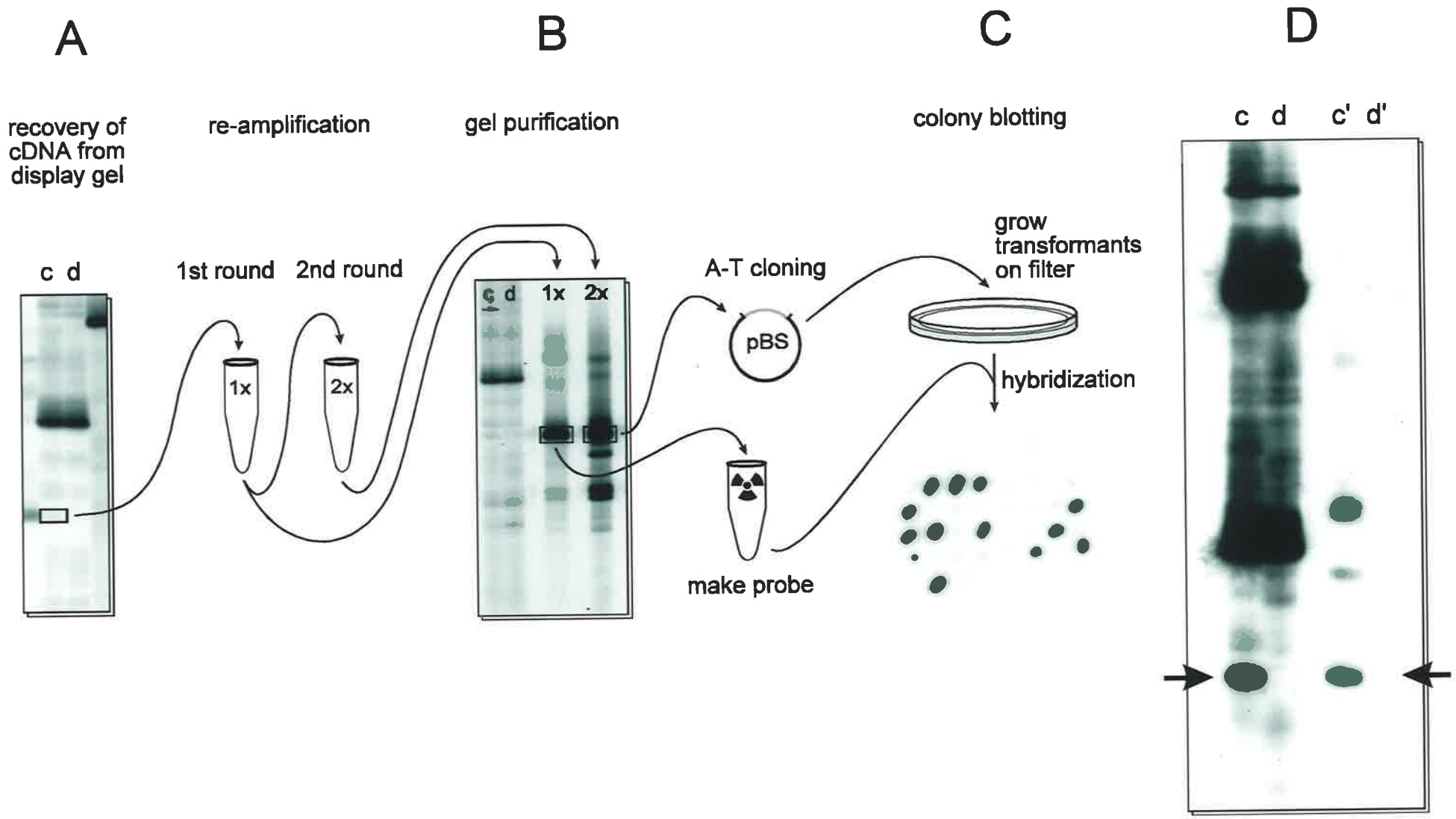
A purification gel with samples of the original displays (c and d) and products of the first and second rounds of re-amplification (1× and 2× respectively).

C

A colony blot of clones generated by ligation of 2× re-amplified products into a vector, hybridized with radiolabelled 1× re-amplified products.

D

An electroblot of labelled (c and d) and unlabelled (c' and d') differential displays of ganglionic RNA samples hybridized with a putative GT12 clone selected on the basis of a positive colony blot result. Arrows indicate detection of the differentially displayed cDNA by the clone. Other products were detected by the probe (see lane c'), indicating that the mRNA from which GT12 was derived is represented by DD several times.


A
B
C
D
recovery of cDNA from display gel
re-amplification
gel purification
colony blotting
c d
1st round
2nd round
1x
2x
c d 1x 2x
A-T cloning
pBS
grow transformants on filter
hybridization
make probe
c d c' d'

Re-amplification of GT12 was done as stated in section 4.4.1 with modifications: (i) [α-^{33}P] was included in each reaction and (ii), first and second round re-amplification products (10 µl of each) were fractionated on a DD gel adjacent to samples of the original display PCRs (figure 4.7B). PhosphorImage analysis of the purification gel, shown in figure 4.6B, disclosed that GT12 constituted 80% of the total DNA after the first round of re-amplification, and 60% after the second round of re-amplification. However during the course of this project, the desired cDNA was not always found to be the most abundant species after re-amplification. Once recovered from the gel, half of the second round re-amplified GT12 was cloned using the A-T method. Transformants were grown overnight on two nutrient agar plates, one of which was overlayed with a nylon membrane. Colonies grown on nylon were lysed and fixed, before hybridization with the gel purified, ^{32}P labelled, first round re-amplified GT12 (Fig 4.7C). Colonies containing only vector sequences were included as negative controls, and none of these bound the probe.

The differential display electroblot described in section 4.4.2 (and shown in figure 4.6B) was stripped and reprobed with a clone selected using the new screening method (figure 4.7D). In this experiment, the probe hybridized to a product of the correct size in displays of ganglionic RNA from HSV infected immunocompetent mice but not HSV infected, anti-CD8 treated mice. It was concluded that this clone contained GT12, a differentially displayed cDNA. Also demonstrated in figure 4.7D, is the redundancy inherent in differential displays: in addition to the expected product, the probe bound three other cDNAs, indicating that the mRNA represented by GT12 was represented at least four times in the same display. DNA sequence analysis disclosed that the clone confirmed to be GT12 was identical to two other colony blot positive clones, providing evidence for the reliability

of the cloning methodology. When compared with the eight DNA sequences obtained from clones described in section 4.4.1, GT12 was found to be unique, ie. none of the previously obtained clones contained GT12. The sequence of GT12 (figure 4.8) was also compared with data in the non-redundant combined DNA database at NCBI and no significant similarities were identified.

Electroblots of DD products can be used to demonstrate that the insert of a clone is derived from a particular differentially displayed cDNA, but they do not confirm independently that the mRNA represented by that cDNA is differentially expressed. Northern blotting is considered to be the "gold standard" for verifying the authenticity of putative differentially expressed mRNAs (Liang and Pardee, 1995). The clone of GT12 was used to probe three northern blots with amounts of ganglionic RNA ranging from 6 - 10 μg but no hybridization was detected. It has been suggested that DD cDNAs smaller than 200 bp are not useful because the short probes generated from these templates are not efficient in hybridizations (Liang *et al.*, 1993). GT12 was only 120 bp, including the PCR primers at each end. Reducing the stringency of hybridization, in case the GT12 probe was acting more like an oligonucleotide, did not result in detectable hybridization. The small size and the low G+C content of GT12 also prohibited the design of efficient primers for semi-quantitative RT-PCR. Therefore the status of the mRNA represented by GT12 with respect to differential expression in HSV infected ganglia of immunocompent and $CD8^+$ cell depleted mice remains unknown. Nonetheless, the cloning of GT12 led to the development of a cloning strategy robust enough to clone poorly displayed cDNAs.

Figure 4.8

Nucleic acid sequence of the DD clone designated GT12. Sequences determined by the primers used in DD to generate this clone are indicated by bold typeface.

GT12

GenBank	Name: MMU73907, Accession: U73907
Sequence	108 bp, linear RNA
base count	33 a 24 c 25 g 26 t

1 **tcggcgatag** gaggtagcgg gtaactgtac ttcacagaga tctggtttaa
51 ggctcgatag tcaatacacg ggcgcagacc tccttccttt tcttc**acaaa**
101 **aaaaaaaa**

4.5 Validation of DD in an *in vivo* setting

Having developed conditions for all aspects of DD, from production of displays to the cloning of differentially displayed cDNAs, the techniques were validated using a transcript for CD8 as a model. Lyt 2, one of the chains of murine CD8, is expressed differentially in normal and anti-CD8 treated mice (section 3.4). To facilitate detection of Lyt 2 cDNA on differential displays, a decamer primer (10lyt2) matching the published genomic DNA sequence for Lyt 2 (Nakauchi *et al.*, 1987, GenBank accession Y00157) was designed. For this experiment, draining lymph nodes from immunocompetent and $CD8^+$ cell depleted mice were used as sources of CD8 positive and negative tissue respectively.

RNA populations from draining lymph nodes of HSV infected immunocompetent mice and HSV infected anti-CD8 treated mice were displayed using a variety of $dT_{11}VN$ and decamer primers. Based on nucleic acid sequence data, it was predicted that a 755 bp cDNA, corresponding to Lyt 2 mRNA, should be visible only in mRNA displays of RNA from a source of $CD8^+$ cells primed with $dT_{11}GT$ and 10lyt2. However, displays of lymph node RNA from normal and $CD8^+$ cell depleted mice using these primers contained no differences. There were three possible interpretations of this result, (i) the display method was not sensitive enough to detect Lyt 2 mRNA, (ii) DD was sensitive enough to detect Lyt 2 mRNA produced by a few remaining $CD8^+$ cells in anti-CD8 treated mice (iii), the primers, which were designed using DNA sequence from B10.A mice, could not bind BALB/c Lyt 2 mRNA. The last of these interpretations was investigated because although the decamer, 10lyt2, was designed from a coding region of Lyt 2, and was hence likely to

be conserved amongst mouse strains, the other primer was chosen on the basis of two base pairs at the predicted 3' end of the transcript. In case $dT_{11}GT$ was an inappropriate 3' primer, other $dT_{11}VN$ primers were used in combination with 10lyt2 to display the two lymph node RNA samples. A differentially displayed cDNA (designated GAlyt) was found in displays primed with $dT_{11}GA$ and 10lyt2. Display reactions primed with 10lyt2 and $dT_{11}GA$ were repeated using the original RNA sources and RNA from lymph nodes of uninfected mice, a second independent source of Lyt 2 mRNA. This three-way differential display (figure 4.9A) demonstrated that GAlyt, the putative Lyt 2 cDNA, was present only in displays of RNA from lymph nodes expected to contain $CD8^+$ cells. Although the cDNA was not the predicted length, it was cloned, as verified by hybridization to an electroblotted display (figure 4.9B), and the DNA sequence was determined. The sequence was compared with that previously published for Lyt 2. Excluding terminal regions that are determined by the PCR primers, the two sequences were found to be identical with the exception of two single base insertions in the newly cloned cDNA (figure 4.10). Both insertions are in the 3' untranslated region and may represent sequencing errors or genuine variation between the two mouse strains. Sequences at the ends of the cloned cDNA indicated that it was amplified by a combination of two 10lyt2 primers, rather than a 10lyt2 primer and a $dT_{11}GA$ primer. DD cDNAs containing a decamer at both ends have been reported previously (Guimarães *et al.*, 1995) and, in this instance, explains the smaller than expected size of the cDNA. Although 10lyt2 was used exclusively by the PCR, primer $dT_{11}GT$ provided specificity in the RT step. It was considered likely that DNA sequence variation between mouse strains, or problems in predicting the exact 3' end of the transcript were the reason why $dT_{11}GA$ and not dT_{11} GT (the originally chosen 3' primer) was needed to display a Lyt 2 cDNA. These results and conclusions highlight the difficulty of accurately

Figure 4.9

A

A three way differential display of draining lymph node RNA from HSV infected immunocompetent mice (c), HSV infected $CD8^+$ cell depleted mice (d) and uninfected mice (u). The arrow indicates the differentially displayed cDNA designated GAlyt.

B

An autoradiograph of an electroblot of labelled (c and d) and unlabelled (c' and d') differential displays of "c" and "d" lymph node RNA samples hybridized with a clone selected on the basis of a positive colony blot result. Arrows indicate detection of the differentially displayed GAlyt cDNA by the clone.

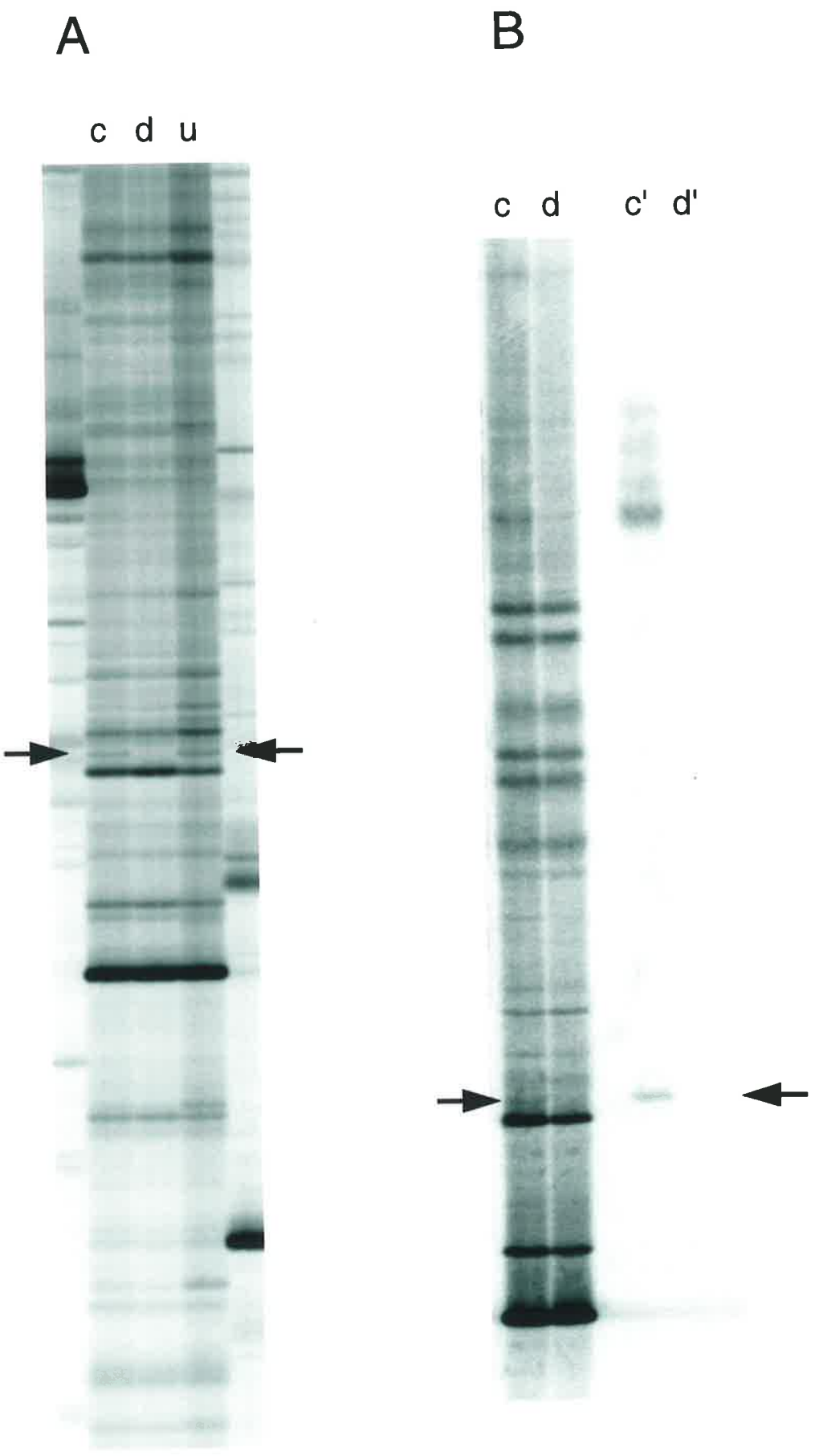

A
c d u
B
c d c' d'

Figure 4.10

Nucleic acid sequence alignment of cloned differential display cDNA GAlyt (GALYT) with genomic DNA encoding Lyt 2.2, accession Y00157 (MLYT2). The primers used in DD RT-PCRs are indicated in bold typeface. In this case the decamer (10lyt2) was found on both ends of the cDNA tag.

```
                10        20        30        40        50
GALYT   gtcagacaggaaggcaagcccagaccttcagagaaaattgtgtaaaatgg
        ::::::::::::::::::::::::::::::::::::::::::::::::::
MLYT2   gtcagacaggaaggcaagcccagaccttcagagaaaattgtgtaaaatgg
        3740      3750      3760      3770      3780
                60        70        80        90       100
GALYT   caccgccaggaagctacaactactacatgacttcagagatctcttcttgc
        ::::::::::::::::::::::::::::::::::::::::::::::::::
MLYT2   caccgccaggaagctacaactactacatgacttcagagatctcttcttgc
        3790      3800      3810      3820      3830
               110       120       130       140       150
GALYT   aagaggccaggccctccttttttcaagtttcctgctgtcttatgtattgcc
        :::: ::::::::::::::::::::::::::::::::::::::::::::::
MLYT2   aaga-gccaggccctccttttttcaagtttcctgctgtcttatgtattgcc
        3840      3850      3860      3870      3880
               160       170       180       190       200
GALYT   ctctgtattgttttagtaggggtgtgatggggacagttccttttttcttta
        ::::::::::::::::::::::::::::::::::::::::::::::::::
MLYT2   ctctgtattgttttagtaggggtgtgatggggacagttccttttttcttta
         3890      3900      3910      3920      3930
               210       220       230       240       250
GALYT   tgaattctctttgacacaaagcatacttgtatgcatacaatgggagtaat
        ::::::::::::::::::::::::::::::::::::::::::::::::::
MLYT2   tgaattctctttgacacaaagcatacttgtatgcatacaatgggagtaat
          3940      3950      3960      3970      3980
               260       270       280       290       300
GALYT   gagcagactgtaacaccagagctagttccagtttcggggtccatgtcgct
        ::::::::::::::::::::::::::::::::::::::::::::::::::
MLYT2   gagcagactgtaacaccagagctagttccagtttcggggtccatgtcgct
         3990      4000      4010      4020      4030
               310       320       330       340
GALYT   ggtggcctcagcacccaccttgatataaatctcctgtctgac
        :::::::::::::::: :::::::::::::::::::::: :
MLYT2   ggtggcctcagcaccca-cttgatataaatctcctgtctgcc
         4040      4050      4060      4070
```

99.1% identity in 342 bp overlap

predicting from nucleic acid sequence data, the existence or size of a given product in an mRNA display.

4.6 Summary

The experimental work documented in this chapter aimed to develop and verify the utility of DD for use in identifying mRNAs that are expressed at different levels in ganglia from HSV infected immunocompetent mice and ganglia from HSV infected $CD8^+$ cell depleted mice. DD was developed and used to clone a cDNA from ganglionic RNA. Also, a cDNA representing Lyt 2 was cloned by DD (documented in section 4.5) verifying that DD can be successfully combined with *in vivo* depletion of $CD8^+$ T cells to obtain cDNA clones of transcripts associated with $CD8^+$ T cells. Therefore it was concluded that DD is a suitable approach for the molecular analysis of $CD8^+$ T cell activity in HSV infected sensory ganglia.

5. AN mRNA DIFFERENTIAL DISPLAY BASED ANALYSIS OF THE ROLE OF CD8$^+$ T CELLS IN HSV INFECTED SENSORY GANGLIA

This chapter describes the application of DD to the study of anti-HSV CD8$^+$ cell activity in spinal ganglia and initial characterization of several mRNAs that were differentially displayed in HSV infected ganglia of immunocompetent, versus anti-CD8 treated mice. The combination of *in vivo* T cell subset depletion and DD as a strategy for studying viral immunity and pathogenesis is discussed, as is the potential significance of findings.

5.1 Differential display of RNA from HSV infected ganglia of immunocompetent and CD8$^+$ cell depleted mice

To detect mRNAs whose expression in sensory nerve ganglia is altered by depletion of CD8$^+$ cells *in vivo*, DD was used to compare ganglionic RNA from groups of 10 anti-CD8 treated and 10 immunocompetent mice, seven days after inoculation with HSV-1. The presence of CD8$^+$ cells in HSV infected ganglia of untreated mice, and their absence in anti-CD8 treated mice was confirmed using semi-quantitative RT-PCR to detect Lyt 2 mRNA as established in chapter 3. The protocols used in this application of DD were those developed and validated in chapter 4.

Approximately 20,000 partial cDNAs were visualized, 52 of which were differentially displayed. To identify reproducible differences, RNA from a duplicate mouse experiment

was used in a second round of differential displays. Nine of the original 52 differences were confirmed, eight of which were associated exclusively with RNA from untreated mice and one with RNA from anti-CD8 treated mice (figure 5.1 and table 5.1). Five of the confirmed differentially displayed cDNAs (designated CC28, CT03, CG17, AG16 and AG18) were cloned readily and the remaining four await cloning to enable further analysis. The clones were named according to their inserted cDNAs.

5.2 Northern blot analyses based on DD clones

5.2.1 Ganglionic RNA

Northern blotting was used to test that the five cloned differentially displayed cDNAs represent differentially expressed mRNAs and to investigate the differential expression of these transcripts in ganglia at various times after HSV infection. The northern blots were prepared with RNA samples generated independently from those used for DD.

To confirm that the five cDNAs cloned using DD represent RNA transcripts whose abundance is influenced by the presence of $CD8^+$ cells, each of the clones was used to probe northern blots of ganglionic RNA extracted seven days after infection from immunocompetent and anti-CD8 treated mice. Two of the clones, CC28 and CT03, detected transcripts (hereafter referred to as CC28 mRNA and CT03 mRNA) that were more abundant in ganglia from immunocompetent compared with anti-CD8 treated mice (eg. figure 5.2, lanes 4 and 5). Of the remaining clones, CG17 detected a transcript whose abundance was too low for an accurate comparison between samples to be made. AG16

Figure 5.1

Sections of nine differential displays of ganglionic RNA from HSV infected immunocompetent mice (c) and HSV infected $CD8^+$ cell depleted mice (d). Differentially displayed cDNAs are marked by paired arrow heads and the designation of each is given at the top of the displays. Note that although differentially displayed cDNAs other than those indicated can be seen, only the marked ones were able to be reproduced with independent RNA samples.

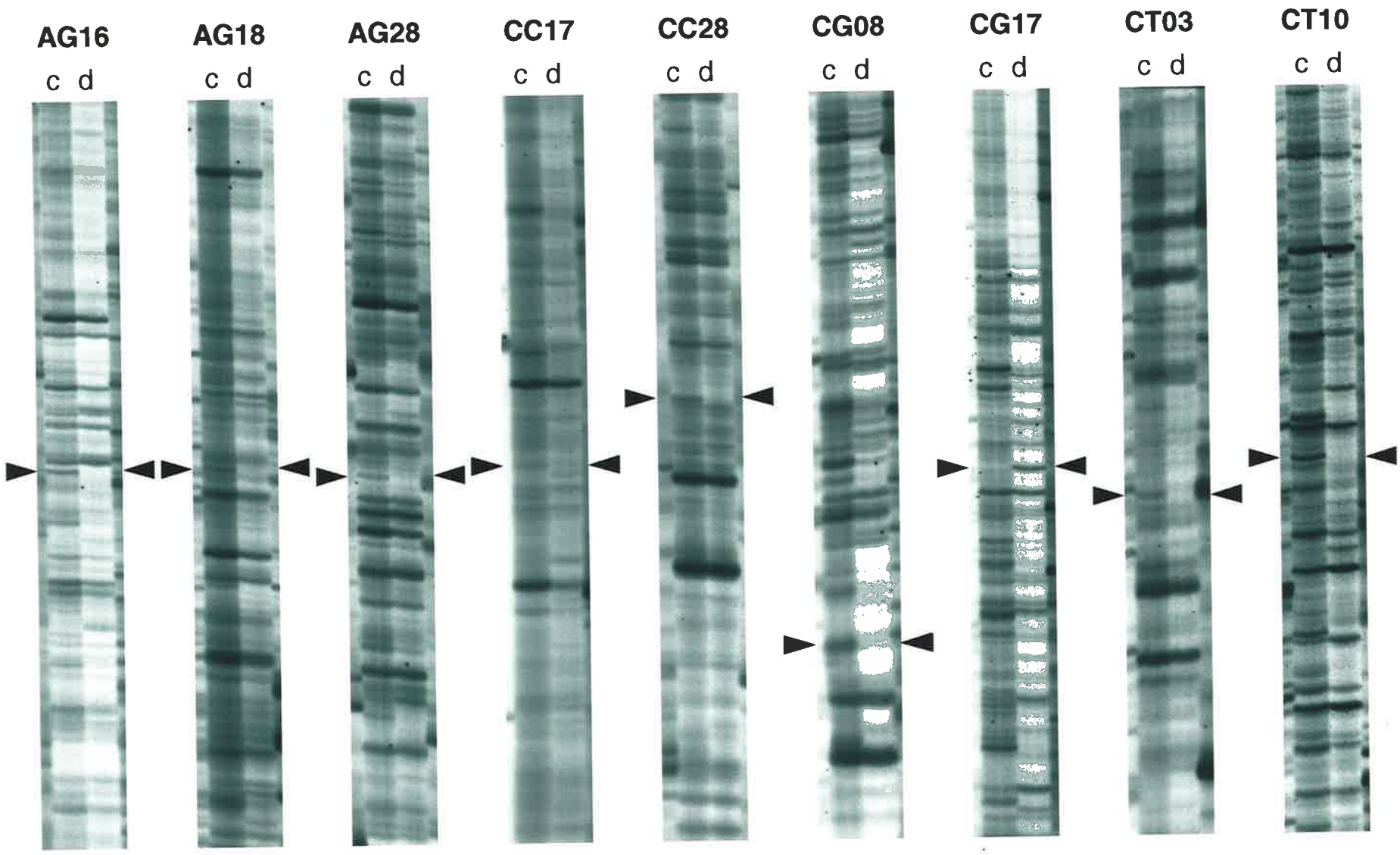

AG16
c d
AG18
c d
AG28
c d
CC17
c d
CC28
c d
CG08
c d
CG17
c d
CT03
c d
CT10
c d

Table 5.1 Summary of findings based on DD cDNAs

	Differential Display		Northern Blot Analysis			Sequence Analysis	
cDNA	Source	*Size	DD confirmed?	mRNA Sizes	tissue distribution§	accession¶	Homologies
AG16	immunocompetent	144 bp	no	many	G, L, S		mouse 28s rRNA (X00525)
AG18	immunocompetent	450bp	nd†		nd	U73908	human cDNA (T78507)
AG28	immunocompetent	200 bp					
CC17	immunocompetent	340 bp					
CC28	immunocompetent	375 bp	yes	6.6 kb	G, L, S	U73905	none
CG08	immunocompetent	100 bp					
CG17	CD8 depleted	600 bp	nq‡	3.5 kb	G, L	U73906	human DNA (Z22371)
CT03	immunocompetent	303 bp	yes	5.8, 3.2 kb	G	U73904	Rat $G_{olf}\alpha$ (M26718)
CT10	immunocompetent	480 bp					

*With the exception of CT03, CC28 and AG16 sizes are estimates only
†nd, not detected
‡nq, not quantified
§G, ganglia; L, lymph node; S, skin
¶Accession numbers of DD clone sequences submitted to GenBank

hybridized strongly with RNA co-migrating with rRNA and several other transcripts, none of which were differentially expressed. Finally, no messages corresponding to AG18 could be detected by northern blotting. It was concluded that at least two of the DD clones, CC28 and CT03, represent RNA molecules whose abundance in HSV infected ganglia is reduced by anti-CD8 treatment.

To explore further the association between the presence of $CD8^+$ cells during HSV infection and the levels of mRNA detected by the DD clones, the clones were used to probe northern blots of RNA from uninfected ganglia and ganglia removed five or seven days after inoculation of immunocompetent and anti-CD8 treated mice (figures 5.2 and 5.3). RNA from anti-CD8 treated, uninfected mice was not included, because there is no evidence for the presence of $CD8^+$ cells in uninfected ganglia (see section 3.4) and therefore, anti-CD8 treatment is unlikely to affect this tissue. Of most interest were the results of blots probed with clones detecting the two differentially expressed transcripts, CC28 mRNA and CT03 mRNA. CC28 mRNA was detected in sensory ganglia from all groups of mice. Its abundance was altered by HSV infection and, in infected ganglia, by prior anti-CD8 treatment (figure 5.3A). In immunocompetent mice, a significant increase in the abundance of this transcript was observed five days after HSV inoculation. In contrast, in anti-CD8 treated mice, the increase in CC28 mRNA levels over the same time interval was, at best, modest. Seven days after HSV inoculation, CC28 mRNA levels were reduced both in untreated and anti-CD8 treated mice when compared with the fifth day of infection. The reduction was much greater in $CD8^+$ cell depleted mice, widening the difference apparent at day five, between CC28 mRNA levels in anti-CD8 treatment and immunocompetent mice. Three conclusions can be drawn from these data. First, CC28

Figure 5.2

Northern blots of total RNA (10 μg) from uninfected ganglia (1), ganglia taken five days after HSV inoculation from immunocompetent or $CD8^+$ cell depleted mice (2 and 3 respectively) and ganglia taken seven days after HSV inoculation from immunocompetent or $CD8^+$ cell depleted mice (4 and 5 respectively). Probes used are indicated on the left. The size marker (M) was stained with ethidium bromide and the photograph re-aligned with autoradiographs.

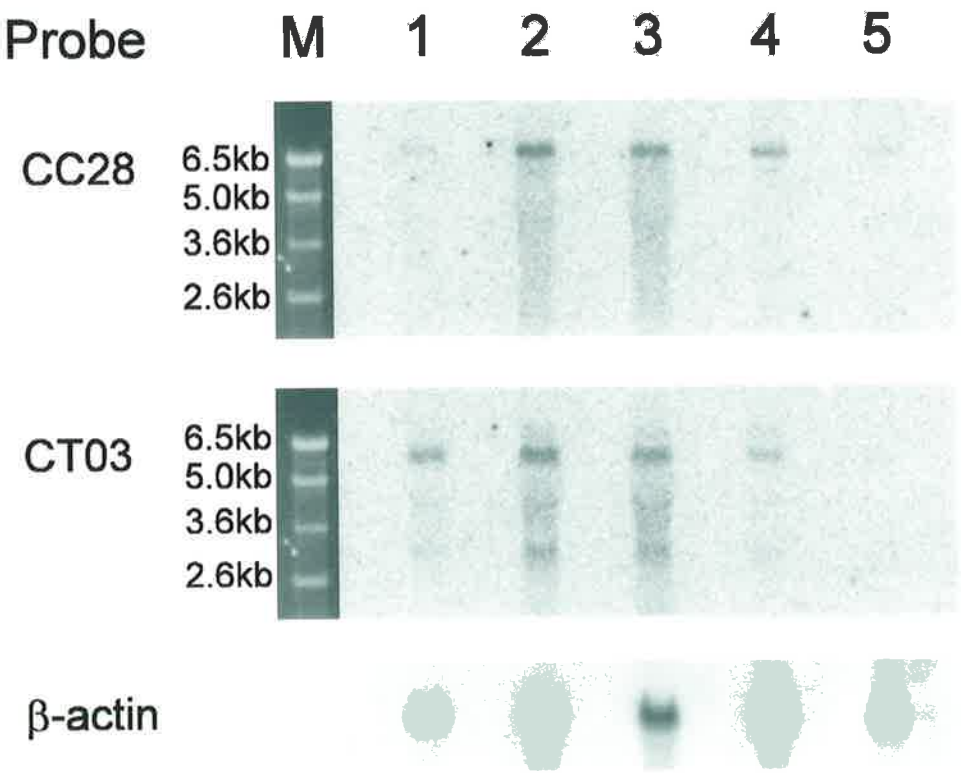
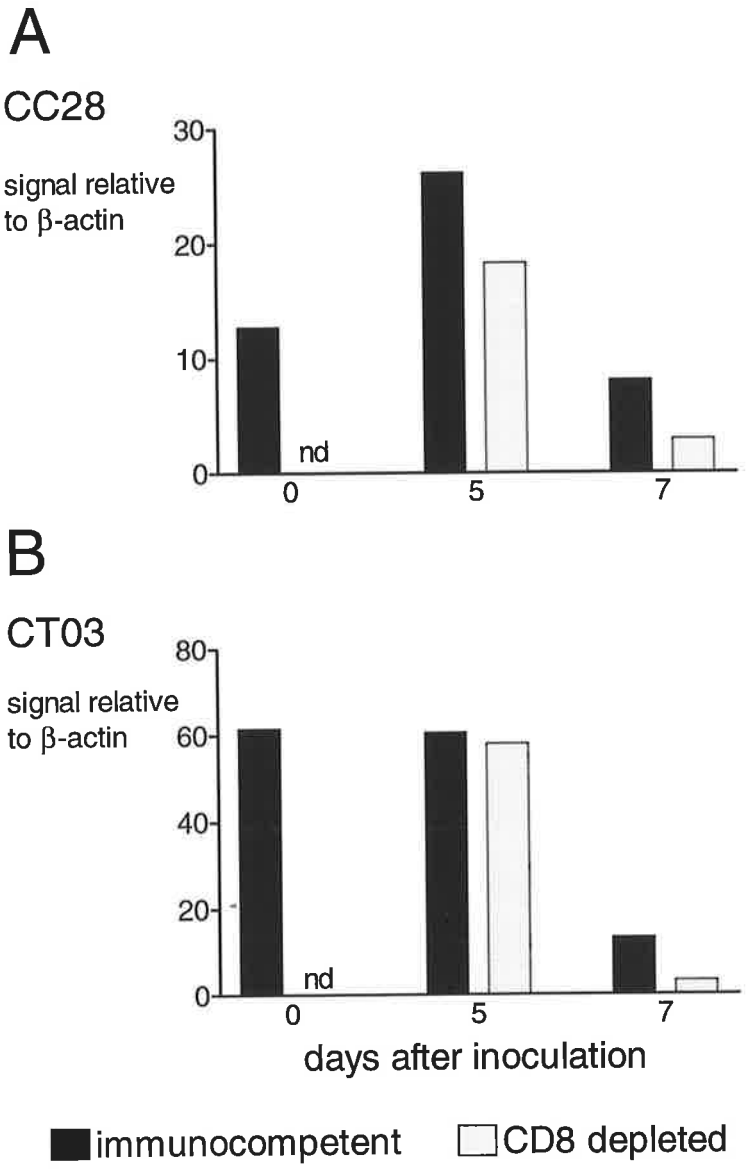

Probe
M
1
2
3
4
5
CC28
6.5kb
5.0kb
3.6kb
2.6kb
CT03
6.5kb
5.0kb
3.6kb
2.6kb
β-actin

Figure 5.3

Expression levels of the mRNAs represented by clones CC28 (**A**) and CT03 (**B**) in sensory ganglia of immunocompetent (solid bars) and $CD8^+$ cell depleted (grey bars) mice at various times after inoculation with HSV. The data were provided by PhosphorImage analysis of northern blots and were normalized for loading errors after re-probing with β-actin (see text and methods). nd, not done.


A
CC28
signal relative
to β-actin
30
20
10
0
nd
0
5
7
B
CT03
signal relative
to β-actin
80
60
40
20
0
nd
0
5
7
days after inoculation
immunocompetent
CD8 depleted

mRNA is expressed in the sensory nervous systems of normal mice. Second, in immunocompetent mice, the increase and decline in CC28 mRNA levels coincide with the previously described increase and decline in HSV gene expression associated with productive ganglionic infection (Speck and Simmons, 1991; Simmons and Tscharke, 1992). Third, anti-CD8 treatment reduces the abundance of CC28 mRNA in HSV infected sensory nerve ganglia.

CT03 mRNA was also detected in uninfected ganglia but unlike CC28 mRNA, its abundance was unchanged five days after inoculation irrespective of anti-CD8 treatment (figure 5.3B). However, seven days after inoculation, a reduction in CT03 mRNA abundance was observed and as with CC28 mRNA, the reduction of CT03 mRNA levels between day five and seven was much greater in anti-CD8 treated mice. The main implications of these data are, (i) CT03 mRNA is present in normal dorsal root ganglia, (ii) CT03 mRNA is down regulated in ganglia as a result of HSV infection and (iii) anti-CD8 treatment enhances the HSV infection-associated reduction in CT03 mRNA.

Clone CG17 detected a transcript in ganglia regardless of infection or immune status but as in the previous experiment, its levels were too low to allow accurate comparative quantification. Finally, clone AG16 showed strong hybridization to RNA co-migrating with rRNA and numerous other transcripts in all samples and, as found previously, no transcripts were detected by clone AG18. The only conclusion from these data was that clone AG16 appeared to be closely related to a structural RNA.

5.2.2 RNA from draining lymph nodes and HSV infected skin

As a step towards characterizing the mRNAs represented by DD clones, the expression of these transcripts in draining lymph nodes and skin of HSV infected mice was investigated. Five mice were inoculated in their left ear pinnae and, seven days later, RNA was extracted from infected ears and ipsilateral cervical lymph nodes for analysis by northern blotting (summarized in table 5.1).

CC28 mRNA was detected in skin and lymph nodes but, in contrast, CT03 failed to detect transcripts in either of the non-neural tissues. CG17 hybridized only with RNA from lymph nodes and, as with the northern blot of ganglionic RNA, the hybridization was weak. Clone AG16 showed the same pattern of hybridization in skin and lymphoid tissue as it did in ganglia and the message corresponding to AG18 was undetectable. The most significant conclusions from these experiments are that, amongst the tissues examined, CT03 mRNA expression is restricted to the nervous system, whereas CC28 mRNA is also present in skin and lymphoid tissue.

5.3 DNA sequence analyses of DD clones

One feature of DD that makes it particularly powerful, is that clones of differentially displayed cDNAs are produced, allowing nucleic acid sequence data to be obtained easily. If a differentially expressed mRNA can be aligned with known sequences, existing knowledge relating to that sequence may be applied to the biological problem at hand, saving much time and energy. A brief review of the DD literature was done to estimate the

approximately proportion of DD cDNA sequences that have been identified by nucleic acid database searches. In six publications, chosen because each presented at least six DD cDNAs, a total of 136 cDNAs were examined and 56 (41%) of these were found to be identical to sequences already in databases or to be homologues of a gene described in another species (Aiello *et al.*, 1994; Utans *et al.*, 1994; Graf and Torok Storb, 1995; Kozian and Augustin, 1995; Linskens *et al.*, 1995; Traianedes *et al.*, 1995). The rapid rate at which nucleic acid sequence data bases are growing means that this percentage will only rise over time.

All five clones obtained in the application of DD to HSV infected ganglia were either fully or partially sequenced and the data compared with sequences in the non-redundant combined DNA database at the NCBI. These sequences appear in figure 5.4 and accession codes for sequences submitted to GenBank and the results of homology searches are summarized in table 5.1. CT03 was found to have >88% homology over the full length of the cDNA tag (303 bp) with the 3' untranslated region of the mRNA encoding rat $G_{olf}\alpha$ (figure 5.5). $G_{olf}\alpha$ is the α-subunit of a heterotrimeric G protein originally found in olfactory cilia (Jones and Reed, 1989). CC28 had no significant homology to any known sequences and may constitute an as yet uncharacterised transcript. 203 bp of the partial sequence of CG17 had 95% homology with an unpublished DNA sequence produced by shot-gun sequencing of the human genome (EMBL accession Z22371). The full insert of AG16 had 100% homology with sequences from murine 28s rRNA. The final clone, AG18, had 77% homology over 208 bp of partial sequence with an unpublished cDNA derived from a human fetal liver and spleen library (GenBank accession T78507). In conclusion, of the five clones derived from HSV infected ganglia, only one had no

Figure 5.4

Nucleic acid sequences of five cloned differentially displayed cDNAs. Names and accession numbers are supplied for sequences submitted to GenBank.

AG16

GenBank not submitted
Sequence 144 bp, linear RNA
base count 44 a 37 c 40 g 23 t

```
  1 agccagcgaa accacagcca agggaacggg cttggcggaa tcagcgggga aagaagaccc
 61 tgttgagctt gactctcgcc cgtcacgttg aacgcacgtt cgtgtggaac ctggcgctaa
121 accattcgta gctaaaaaaa aaaa
```

AG18

GenBank Name: MMU73908, Accession: U73908
Sequence 249 bp, linear RNA
base count 72 a 61 c 67 g 49 t

```
  1 aggtgaccgt gggtgaatat cacactcgtc aaagaaacca aggagaatgg ggaaacagaa
 61 gtggatgcct gtaccccagc agagccaggt tggaaggcag tgttgaccat cctgttggcc
121 cacaagcgtt ctaaccagcc agctgagaca gactccatga gtctctctga aaaatcaacc
181 aaagtgttcc gaatattccg gcagtgggag tcttcatcct catcgtgaag atgctccgga
241 cagactgag
```

CC28

GenBank Name: MMU73905, Accession: U73905
Sequence 375 bp, linear RNA
base count 139 a 93 c 100 g 43 t

```
  1 tagtcaccga tgtcaaaaag caaagggaaa ggaaagtctc tccaacaacg taaggccaag
 61 cctccgaaga agcaggagga gaaggagaag agggggaagg ggaagccgca ggaggatgag
121 ctgaaggacg ctctggccga cgacgacagt tcctccacca ccacagagac ctccaacccc
181 gacacagagc cactcctcag ggaggataca gagaagcaca aggggagacc agccgtgcct
241 gaaaaacagg aaagtgaact gtctcaaggg aagcccaaga gcaaaaagct cttaaacgct
301 aagaaagaaa tcccaacaga tgtgaaaggc agttcctttg aactaccgta cactccctca
361 ctggaaaaaa aaaaa
```

CG17

GenBank Name: MMU73906, Accession: U73906
Sequence 252 bp, linear RNA
base count 68 a 76 c 37 g 71 t

```
  1 tgagtgagta ctccccacgc tctcctctcc ccgtgttagg tcctgctgtc ccctgacagt
 61 ttgccttcta ccttcagatc gcatacacac cgtggtttca tgtctctcta caaagctcag
121 gagctacaaa taagagcaca catatgctgt ttgtcctccc tacaccagct aacccccttaa
181 taggaccatc tctggataca tccataaaat cactctttat aaaagtcatt tctctttgcc
241 gaaaaaaaaa aa
```

CT03

GenBank Name: MMU73904, Accession: U73904
Sequence 303 bp, linear RNA
base count 94 a 63 c 70 g 76 t

```
  1 tagtcagcca ccaaggcaca gaactctaca aggaggattc ttgagtagcc atagaaaaga
 61 gtattacatc ttggggtctc tacagtctgg ttaaagcaac aacagaaaga agataggaat
121 gactatgcta ttgtaaaggt gtagaggacc ccttgtttat aaacaggaca ccatttgcag
181 tagtgaatgt ggcaggtttt ctgcccaagt gtaaaaggct cccagaagct ctctccaaat
241 gcccaaggct tacagccatt gcaactgtgg ctgaaaaagt tcctcttatt ctgtggctga
301 cta
```

Figure 5.5

Nucleic acid sequence alignment of clone CT03 (CT03) with mRNA for rat $G_{olf}\alpha$, GenBank accession M26718 (GOLF). The primers used in DD RT-PCRs are indicated in bold typeface, in this case the decamer was found on both ends of the cDNA tag.

```
                10        20        30        40        50
CT03    tagtcagccacagaataagaggaacttttcagccacagttgcaatggct
          x::::: :::::: : ::::: :::::::::::::::::::::::::
GOLF    acgtcagcaacagaagacgaggacctttttcagccacagttgcaatggct
         1740      1750      1760      1770      1780
                60        70        80        90
CT03    gtaagccttgggcattt-ggagagag--cttctgggagcctttacactt
         ::::::::::::::: ::  :::: ::::::::::::::::::::::
GOLF    ataagccttgggcatttgggttagagcacttctgggagcctttacactt
         1790      1800      1810      1820      1830
        100       110       120       130       140
CT03    gggcagaaaacctgccacattcactactgcaaatggtgtcctgtttataa
        ::::::::: :: :::::::::::::::   :::::::::::: :::::
GOLF    gggcagaaagccggccacattcactact---aatggtgtcctgtctataa
         1840      1850      1860        1870      1880
        150       160       170          180       190
CT03    acaaggggtcctctacacctttacaata-----gcatagtcattcctatc
        ::::::::::::::::::::    :::     :::::::: ::::: ::
GOLF    acaaggggtcctctacaccttcctgatagctgggcatagtccttcctgtc
           1890      1900      1910      1920      1930
             200       210       220       230       240
CT03    ttctttctgttgttgctttaaccagactgtagagaccccaagatgtaata
        :::::::::::::::::: :::::::::: ::::: ::::::::: ::
GOLF    ttctttctgttgttgcttt-accagactgtacagaccacaagatgtacta
           1940      1950       1960      1970      1980
             250       260       270       280       290
CT03    ctcttttctatggctactcaagaatcctccttgtagagttctgtgccttg
        ::::::::: ::::::::: :::: ::::::::::::::: ::::::::
GOLF    ctcttttctgtggctactccagaagcctccttgtagagttccgtgccttg
            1990      2000      2010      2020      2030
             300
CT03    gtggctgacta
        ::::: :              88.2% identity in 305 bp overlap
GOLF    gtggcggctat
            2040
```

similarity with known sequences and three were almost certainly homologues of sequences in the databases.

5.4 Discussion

DD has been applied to a variety of biological problems both *in vivo* and *in vitro*, but it has not been used before to investigate aspects of pathogenesis or immunity. The application of DD reported in this chapter produced nine consistently differentially displayed mRNAs, five were cloned and at least two are genuinely differentially expressed. In spite of the complex nature of the *in vivo* setting used and the fact that the cells of greatest interest, $CD8^+$ cells and infected neurons, are greatly out numbered by other cell types in HSV infected sensory ganglia, DD produced clones worthy of further study. This finding demonstrates the utility of DD in this context, despite the recent report suggesting that it is biased towards relatively abundant messages (Bertoli *et al.*, 1995).

The primary aim of using DD to catalogue transcriptional differences between HSV infected ganglia of immunocompetent versus anti-CD8 treated mice was to stimulate new lines of research into the *in vivo* action of HSV-specific $CD8^+$ T lymphocytes. The production of clone CC28 demonstrates that this aim was feasible. CC28 mRNA levels rise in dorsal root ganglia in response to HSV infection and this upregulation is reduced in mice depleted of $CD8^+$ T lymphocytes. Therefore upregulation of this transcript may be linked to the presence or even action of $CD8^+$ T cells. With respect to HSV pathogenesis, the transcript is of interest because to our knowledge, CC28 mRNA is the first cellular gene to be identified with expression kinetics that mimic those of viral genes in infected sensory

nerve ganglia. In addition, CC28 has no similarity with any sequences in the nucleic acid sequence databases and may represent a novel transcript. Further work to investigate the possible link between CC28 mRNA and anti-HSV $CD8^+$ T cells should include studies *in situ* to determine the cell types expressing this transcript at sites of HSV infection, in the presence and absence of $CD8^+$ cells. Also, because CC28 mRNA was found in HSV infected skin, the relationship between CC28 transcription, HSV and $CD8^+$ T cells at this site merits investigation. These experiments need to include mouse strains other than BALB/c to confirm that any findings are not mouse strain-specific. Finally, obtaining and characterizing a full length cDNA clone of CC28 mRNA is an important future goal.

Applying DD to an *in vivo* model of viral disease in normal and immunocompromised mice has the potential to detect transcriptional changes associated with different levels of infection and clone CT03 may represent such a transcript. The level of CT03 mRNA was reduced in ganglia during HSV infection and this reduction was enhanced by anti-CD8 treatment, possibly as a consequence of the increased severity of neural infection known to be associated with $CD8^+$ T cell depletion (Simmons and Tscharke, 1992). If levels of CT03 mRNA are modulated by infection, the transcript is of significant interest in HSV pathogenesis, particularly because CT03 mRNA it is almost certainly a murine homologue of the message for $G_{olf}\alpha$, the α subunit of a heterotrimeric G protein. CT03 has high homology over its whole length with the 3' untranslated region of rat $G_{olf}\alpha$ mRNA. On northern blots, the products detected by CT03 are identical to those on blots published previously for murine $G_{olf}\alpha$ messages (Strathmann *et al.*, 1989) and CT03 mRNA was detected in neural tissue but not skin or lymph nodes, an expected result if the clone contains cDNA of the $G_{olf}\alpha$ message. G_{olf} expression was originally thought to be olfactory

specific (Jones and Reed, 1989), but has since been found in brain, testis, spleen, lung and pancreatic islets (Zigman *et al.*, 1993). Of these tissues, however, only in the olfactory epithelium, brain and testis has expression of $G_{olf}\alpha$ mRNA been detected by northern blotting (Zigman *et al.*, 1993). The current work is the first evidence for $G_{olf}\alpha$ expression in dorsal root ganglia. Previously, expression of G_{olf} in the nervous system has been associated exclusively with neurons (Jones and Reed, 1989; Herve *et al.*, 1993) making primary sensory neurons the most likely source of the $G_{olf}\alpha$ transcripts detected in ganglia.

Assuming that ganglionic $G_{olf}\alpha$ was derived only from neurons, there are two possible explanations for the observed reduction of $G_{olf}\alpha$ mRNA levels in ganglia infected by HSV. The first is a dilution effect, caused by an increase in the ratio between non-neuronal and neuronal cells as a result of HSV induced ganglionitis. While this possibility was not formally excluded, it is considered unlikely, because neither the timing nor the extent of the decrease in $G_{olf}\alpha$ levels coincide with the increase in non-neuronal cell numbers in HSV infected murine sensory ganglia (Cook and Stevens, 1973; Simmons and Tscharke, 1992; Cantin *et al.*, 1995 and unpublished observations; Liu *et al.*, 1996). The other explanation is that $G_{olf}\alpha$ mRNA abundance is altered as a result of HSV infection and if this is the case, it is the first indication that viral infection may modulate heterotrimeric G protein synthesis. Heterotrimeric G proteins couple a wide array of surface receptors to generators of intracellular signals and therefore they are an essential part of many signal transduction pathways (Neer, 1995). The ramifications of viral interference with these signal transduction pathways are substantial because a vast range of receptors is coupled to G proteins. G protein coupled receptors include those for androgens, serotonins, histamine, dopamine, ordorant molecules, ecosanoids and β-chemokines (Simon *et al.*, 1991; Ahuja

et al., 1994). Many of these ligands directly affect viral pathogenesis and the β-chemokines illustrate this point well. β-chemokines have been shown to be of importance in immunity to viruses (Cook *et al.*, 1995) and at least two herpesviruses carry homologues for β-chemokine receptors which are thought to subvert chemokine signalling (Ahuja *et al.*, 1994). It can be envisaged that down regulation of specific G proteins coupled to these receptors is another mechanism by which viruses may interfere with chemokine signal transduction.

Specific consideration of why HSV might regulate $G_{olf}\alpha$ in sensory ganglia is also warranted. In other neural tissues where G_{olf} is found, it is associated with a G_{olf}-specific adenylyl cyclase and thus plays a role in regulation of cellular cAMP levels (Menco *et al.*, 1992; Herve *et al.*, 1993; Menco *et al.*, 1994). Two HSV transcription units, namely LAT and U_L9, have cAMP responsive elements (CREs) in their promoter regions (Leib *et al.*, 1991; Rader *et al.*, 1993; Deb *et al.*, 1994). LATs are thought to be involved in aspects of latency and the U_L9 product, the origin-binding protein, is essential for productive HSV infection (Challberg, 1986). In addition, experimental links have been made between levels of cAMP and the stability of herpes simplex latency (Foster *et al.*, 1989; Sainz de la Maza *et al.*, 1989; Leib *et al.*, 1991; Rodriguez *et al.*, 1991). The possibility that there is a functional link between G_{olf} levels, cAMP and HSV pathogenesis warrants further investigation.

Studies *in situ* are required to define the cellular source of $G_{olf}\alpha$ mRNA in sensory ganglia and to determine whether it is down regulated in areas of HSV infection. In addition, quantification of $G_{olf}\alpha$ mRNA or G_{olf} protein throughout the course of HSV infection,

including the productive, latent and reactivation stages may further define the role of HSV infection in regulation of G_{olf} levels. From a neurobiological point of view, technology exists to identify the cell surface receptor and the generators of intracellular signals linked to G_{olf}, steps that will aid the assignment of function to this G protein.

Four cDNA tags were identified which have not yet been cloned, and two clones await the application of more sensitive methods to confirm their putative association with the presence of $CD8^+$ T lymphocytes in HSV infected ganglia. Identifying the transcripts represented by these tags is a focus for further work. Two clones, CG17 and AG18, have homology with anonymous sequences in the nucleic acid databases. Detection of these transcripts in murine sensory ganglia, and possible association with immune processes adds to the pool of knowledge concerning them and may be useful in assignment of function. One clone produced, AG16, is probably an artifact of DD because it appears to be derived entirely of sequences from 28s rRNA. Why 28s rRNA was differentially displayed in our experiments is difficult to explain, but its abundance in total cellular RNA preparations probably accounts for the ease with which it was amplified and cloned. The use of poly A selected RNA would prevent the detection of structural RNAs, but is not practical when only small amounts of RNA are available.

In summary, the identification of two clones associated with aspects of HSV pathogenesis and immunology and the detection of six more transcripts of interest demonstrates the ability of DD to stimulate novel lines of investigation in a complex setting. Specifically, the combination of DD and *in vivo* depletion of lymphocyte subsets may be a useful new approach to immunological problems.

6. CONCLUDING DISCUSSION

The first part of this discussion focuses on potential applications of DD and three broad fields of research are considered: (i) HSV pathogenesis, (ii) virology in general and (iii) immunology.

(i) The molecular basis of some aspects of HSV pathogenesis may be approached using DD if appropriate models are found and exploited. DD has been used successfully to monitor changes in gene expression over time *in vivo*, by concurrent display of RNA samples taken at various times after organ transplantation (Utans *et al.*, 1994) and balloon angioplasty (Autieri *et al.*, 1995). A similar approach could be used to catalogue changes in gene expression occurring during experimental HSV infections. The modulation of unknown transcripts visualized on DD gels could be compared with the expression kinetics of various viral genes (for example LATs) and markers of the host response (for example markers of lymphocyte populations). By exploiting different *in vivo* models of HSV infection, various aspects of HSV infection may be studied in isolation. For instance, there are *in vivo* models in which latency is established in the absence of a productive infection (Clements and Stow, 1989; Leib *et al.*, 1989b; Speck and Simmons, 1991; Speck and Simmons, 1992). In these models, transcriptional events associated with the establishment of latency may be examined in the absence of a complicating productive infection. Mice with long term latent infections (longer than six months) are another example of an animal model which may be useful in this context. In the ganglia of these mice, productive infection, the immune response to productive infection and presumably the establishment phase of latency have all dissipated (Pereira *et al.*, 1994; Cantin *et al.*, 1995; Liu *et al.*, 1996). If HSV latency is

a dynamic, rather than static process, comparison of transcription in such instances of stable latency with transcription in uninfected ganglia may reveal an ongoing host response associated with the maintenance of latency. Models in which reactivation of HSV from latency can be induced are also available (see section 1.2.6.3). DD could be used in conjunction with these models to produce a list of mRNAs whose abundance is altered following a reactivation stimulus. Finally, the assignment of phenotypes to some HSV mutants may be aided by a DD based analysis of transcription in tissue infected by mutant and wild type HSVs.

(ii) The use of DD to study the effects of mutation on viral infections is not restricted to HSV; DD may be used to study viral genetic variation or alternatively, host genetic variation in many virus-host interactions. For example, the transcriptional differences amongst cell lines that are permissive, semi-permissive and non-permissive for a given viral infection may be catalogued by DD. There are also *in vivo* applications of this approach, because the pathogenesis of a virus frequently differs across the range of different inbred mouse strains. This idea can be expanded to include analyses utilizing transgenic animals and DD might be useful in disclosing downstream effects of targeted gene disruptions, or over-expressions.

(iii) There are many possible applications of DD in the field of immunology. In work presented in this thesis, DD was used to investigate the effects of *in vivo* depletion of $CD8^+$ T cells on HSV infections. This approach could be applied to other lymphocyte subsets and other models of pathogenesis. Another possible *in vivo* application of DD is the comparison of transcription in lymph nodes of infected and uninfected animals to identify

new lymphocyte activation markers. DD might also be of use in clonal analyses of T lymphocytes. Cloning of T cells to allow investigation of immune function at the level of individual cells is commonplace. However, characterization of the differences amongst clones has thus far not proceeded beyond molecules of known immunological significance, for example cytokines. DD may enable a more extensive catalogue of differences between lymphocyte clones to be compiled. A problem with some T cell clones is that they do not behave like freshly derived *ex vivo* lymphocytes when re-introduced to animals. In particular, the ability of cloned T cells to home to correct tissues is often impaired (Leung and Nash, 1984). DD may be a suitable tool to define differences between cloned T cells and T cells that have been derived freshly from animals. Indeed, DD could be used to directly examine the effects of *in vitro* culture, under various conditions, on lymphocytes.

The preceding paragraphs constitute a list of possible applications of DD to problems in the fields virology and immunology. However, DD is not suggested as a sole approach in these instances. There are technical difficulties associated with DD and, although these are being overcome as the method is constantly refined, these problems need to be considered before embarking on a DD based strategy. The most important aspect of any application of DD is the choice of starting material. In DD experiments documented in this thesis, although the primary aim was to isolate mRNAs associated with anti-HSV $CD8^+$ T cells, one of the cDNAs produced, namely CT03 (encoding mRNA for $G_{olf}\alpha$), is more likely to associated with different levels of HSV infection. This example illustrates the need to use carefully defined models, especially for *in vivo* applications of DD. At the same time, this example highlights the flexibility of DD and demonstrates the technique's potential to produce unexpected findings of significant interest. DD gels on which multiple RNA samples are

displayed disclose more information about the expression of transcripts than gels displaying only two RNA populations. Therefore multiple displays may allow greater discrimination to be exercised when selecting differentially displayed cDNAs for further analysis. For example, the concurrent display of RNA from lymph nodes of uninfected mice, HSV infected mice and anti-CD8 treated, HSV infected mice facilitated the selection of a putative Lyt 2 cDNA (section 4.5, figure 4.9A). The selection of differentially displayed cDNAs of interest from displays of ganglionic RNA, as described in section 5.1, may have been aided by a similar strategy. RNA from immunocompetent and anti-CD8 treated mice at several times after HSV infection could be displayed concurrently. Such displays may facilitate the selection of cDNAs that are associated with $CD8^+$ cells, because the kinetics of $CD8^+$ cell infiltration and virus production, in ganglia of HSV infected mice, are known.

There are few biological problems where molecular analyses have come to a complete halt through lack of genes of interest. DD may be useful in these instances, but is not a replacement for investigations and careful characterizations based on molecules of known importance. In the case of immune control of ganglionic HSV infections, cytokine expression and the role of $CD8^+$ T cells in the regulation of cytokine expression are obvious targets for investigation. The data gained through the characterization of cytokine expression in HSV infected ganglia presented in chapter 3 provide several insights. First, anti-CD8 treatment affects the abundance of some cytokine transcripts in HSV infected ganglia. Second, the assumption that the Th1/Th2 paradigm is broadly applicable to all immune responses has been challenged by evidence that in the case of ganglionic HSV infections, reciprocal regulation of IFN-γ and IL-4 does not occur. Third, attention has been drawn to the possibility that increased IL-4 expression may be detrimental to recovery

of mice from ganglionic HSV infections. Finally, IFN-γ mRNA levels were not affected by anti-CD8 treatment, leading to the conclusion that the failure of CD8 depleted mice to clear productive ganglionic HSV infection is not linked to inadequate IFN-γ production.

Like the results obtained by the application of DD to the search for anti-HSV $CD8^+$ T cell function, data from the cytokine experiments provide new avenues of investigation. Unlike DD, the directed characterization of cytokine expression was able to narrow the range of molecules of interest in this context, by demonstrating that IFN-γ mRNA levels are not affected by removal of $CD8^+$ cells. In addition, this finding is unequivocal, whereas all DD based findings remain possibilities to be explored. Therefore, the use of a flexible, but unfocussed tool such as DD in parallel with an analysis focussed on established knowledge provides stronger approach than either strategy alone.

In summary, the mechanism by which $CD8^+$ T lymphocytes clear HSV from infected sensory ganglia of mice remains unknown. There are however, powerful approaches to this problem. Investigations based on cytokines and other host proteins with established roles in immunity may reveal mediators of $CD8^+$ T cell activity. At the same time, the application of new molecular techniques, such as DD, to well defined *in vivo* models of HSV disease has the potential to extend the list of candidate effector molecules.

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