T LYMPHOCYTE RESPONSES TO GLYCOPROTEIN B OF HERPES SIMPLEX VIRUS TYPE 1.

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Thesis submitted in accordance with the requirements of the

UNIVERSITY OF LONDON

for the degree of

DOCTOR OF PHILOSOPHY.

December 1989.

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ABSTRACT.

Glycoprotein B (gB) of herpes simplex virus type 1 (HSV-1) is expressed on the virus envelope and on the surface of infected cells. It is an important viral protein, both functionally and as a target for the immune response of the infected host. Although gB of HSV-1 (gB-1) does not induce viral neutralising antibody, subcutaneous priming with immunopurified gB-1 can protect mice against a lethal infection with HSV-1. This protective effect could be adoptively transferred by specific T helper cells.

This project further investigated the role of T cells specific for HSV-1 and gB-1 in the response to the virus. This was carried out in three areas:-

1. A comparison of the T cell response to, and induced by, gB-1 and its unglycosylated precursor form, pgB-1, to determine if glycosylation of gB had any effect on the T cell response. T cell proliferation assays showed that HSV primed cells could proliferate equally well to gB or pgB *in vitro*. Priming with pgB *in vivo* induced virus and glycoprotein specific T cells as effectively as gB, and could protect mice against a lethal challenge with HSV-1. Therefore, glycosylation is not important in the recognition of gB by T helper cells, either *in vitro* or *in vivo.*

2. The production of lymphokines (IL-1, IL-2, IL-3 and IFN-γ) *in vivo*, post infection, in mice primed with whole virus, gB-1 or pgB-1 was examined. Again, pgB was as

effective as gD in inducing lymphokine production. Priming appeared to induce earlier, elevated lymphokine levels compared to that seen in the control mice. However, control mice which survived beyond 10 days post infection developed lymphokine levels comparable to those detected in the primed animals. Thus, the early production of lymphokines in the primed animals may contribute to their protection against infection.

3. A panel of T cell clones were developed which recognized HSV-1 and, in some cases, gB-1. These were $CD4^+$ and $I\text{-}A^d$ restricted. In response to antigen restimulation *in vitro*, all the clones produced IL-2, IL-3 and IFN-γ. Therefore, the clones could be tentatively classed as Th cells.

ACKNOWLEDGEMENTS.

I would like to thank my supervisors, Dr W.L. Chan and Dr F.Y. Liew, for their help, guidance and discussions throughout my work and for giving me an insight into a life in science.

Thanks also to Professor C.A. Mims, Dept. of Microbiology, Guy's Hospital Medical School for his interest and encouragement during my stay in his department.

I would also like to thank Dr J. Tite, Dr R. Lelchuk and Mrs E. Abdulla, Wellcome Biotech Ltd. for the gifts of cell lines and reagents and the Dept. of Photography, Wellcome Research Laboratories, for their excellent graphic work.

My thanks to all those in the Dept. of Experimental Immunobiology, Wellcome Biotech Ltd., especially Ms Val Swain, and in the Dept. of Microbiology, Guy's Hospital Medical School, especially Dr Penny Hatfield and Mr Mark Tizzard, for their support, friendship and encouragement.

Thanks also to the Science and Engineering Research Council and to the Friend's of Guy's Trustees Fund for financial support.

Finally, I must thank my husband, Neil, for his continual support, encouragement and understanding throughout my work and especially while writing my thesis.

DEDICATION.

I would like to dedicate my thesis to the memory of my Mum and Dad. Without their support in the early years, I would not have progressed as far as I have. Thank you.

ABBREVIATIONS.

AIDS	Acquired immune deficiency syndrome.
APC	Antigen presenting cell.
BHK	Baby hamster kidney.
CMC	Carboxymethylcellulose.
CNS	Central nervous system.
Con A	Concanavalin A.
cpm	Counts per minute.
CTL(s)	Cytotoxic T cell(s).
DLN(s)	Draining lymph node(s).
DNA	Deoxyribonucleic acid.
DTH	Delayed type hypersensitivity.
EBV	Epstein Barr virus.
Endo H	Endo-β-N-acetylglucominidase H.
FCA	Freund's complete adjuvant.
FCS	Foetal calf serum.
gA	Glycoprotein A.
gB	Glycoprotein B.
gB-EBV	Glycoprotein B of Epstein Barr virus.
gC	Glycoprotein C.
G-CSF	Granulocyte colony stimulating factor.
gD	Glycoprotein D.
gE	Glycoprotein E.
gG	Glycoprotein G.
gH	Glycoprotein H.
gI	Glycoprotein I.

GM-CSF	Granulocyte-macrophage colony stimulating factor.
gP	Glycoprotein.
HI	Heat inactivated.
HSV-1	Herpes simplex virus type 1.
HSV-2	Herpes simplex virus type 2.
HCMV	Human cytomegalovirus.
HHV-6	Human herpes virus-6.
IFN(s)	Interferon(s).
IL(s)	Interleukin(s).
i.p.	Intraperitoneal.
kDa	Kilodalton.
LD	Lethal dose.
MAb(s)	Monoclonal antibody(ies).
mar	Monoclonal antibody resistant.
MCMV	Murine cytomegalovirus.
MHC	Major histocompatibility complex.
M.O.I.	Multiplicity of infection.
mRNA	Messenger ribonucleic acid.
NK	Natural killer.
NMS	Normal mouse serum.
N.T.	Not tested.
PAS	Protein A-Sepharose.
PBSA	Phosphate buffered saline.
PFU	Plaque forming units.
pgB	Precursor glycoprotein B.
pgP	Precursor glycoprotein.

p.i.	Post infection.
PPD	Purified protein derivative.
r	Recombinant.
RIA	Radioimmunoassay.
RNA	Ribonucleic acid.
rpm	Revs per minute.
s.c.	Subcutaneous.
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
SEM	Standard error of the mean.
S.I.	Stimulation index.
SK	Stromal keratitis.
syn	Syncytia-forming.
TCGF	T cell growth factor.
T-DH	T-delayed hypersensitivity.
Th	T helper.
TM	Tunicamycin.
TNF	Tumour necrosis factor.
ts	Temperature sensitive.
Ts	T suppressor.
U_L	Unique long.
U_S	Unique short.
UV-HSV-1	Ultraviolet light inactivated herpes simplex virus type 1.
VZV	Varicella zoster virus.

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CHAPTER 1.

GENERAL INTRODUCTION.

1.1 THE FAMILY HERPESVIRIDAE.

1.1.1. General properties.

The herpesviruses are a large family of enveloped, DNA viruses which can infect many species, both vertebrate and invertebrate. All the viruses share certain structural features. Their nucleic acid is double stranded DNA, enclosed within an icosahedral capsid consisting of 162 capsomeres. The capsid is, in turn, surrounded by a tegument of globular material. Finally, a bilayered membrane, i.e. the envelope, containing surface projections, surrounds the virion, (reviewed by Roizman, 1982).

The replication of herpesviruses occurs in 3 stages:-

1. Entry - The viral envelope adsorbs to receptors on the host cell surface, fusing with the plasma membrane. The capsid is released into cell cytoplasm and a DNA-protein complex is transported to the nucleus.
2. Replication - Transcription of viral DNA occurs in the nucleus and mRNA generated from the DNA is translated in the cytoplasm. Viral DNA formed in the nucleus is also used in the nucleocapsid of new virus particles.
3. Maturation and egress - The capsids become enveloped by budding through cellular membranes, including the inner lamella of the nuclear membrane. Virus particles accumulate in the nuclear membrane and the endoplasmic reticulum, before being transported to the cell surface and released.

1.1.2. Human herpesviruses.

There are 5 well characterized members of the human herpesvirus family:-

1. Herpes simplex virus type 1 (HSV-1).
2. Herpes simplex virus type 2 (HSV-2).
3. Epstein-Barr virus (EBV).
4. Human cytomegalovirus (HCMV).
5. Varicella zoster virus (VZV).

Recently, a sixth member has been described, human herpesvirus-6 (HHV-6; Ablashi et al., 1987). This virus was isolated from patients with lymphoproliferative disorders or AIDS, and was originally called human B-lymphotrophic virus because it infects human B cells (Salahuddin et al., 1986). However, HHV-6 has since been shown to infect a number of cell types, including megakaryocytes, glioblastoma cells and T cells, particularly immature T cells (Ablashi et al., 1987; Lusso et al., 1987).

The herpesviruses exhibit certain properties:-

1. Each herpesvirus preferentially infects a certain cell type, i.e. they exhibit trophism. See Table 1.1.1.
2. After an initial infection, the viruses can become latent and may not be reactivated for many years. In the case of herpes simplex virus, latency occurs in the ganglia closest to the site of the original infection (see Section 1.2.3.).
3. Some herpesviruses, e.g. EBV, CMV and HSV, can transform

cells *in vitro.* This transforming ability has led to great interest in the possible role of herpesviruses in cancer, particularly as EBV is associated with Burkitt's lymphoma and nasopharyngeal carcinoma. (For reviews, see Rapp & Westmoreland, 1976; Ito, 1986; Lenoir, 1986).

The herpesviruses have been divided into three subfamilies according to their biological properties. These include their host range, the duration of their reproductive cycle, cytopathology and latent infection characteristics (Roizman, 1982). The subfamilies are designated the α, β, and γ herpesviruses (see Table 1.1.1.).

Table 1.1.1. General characteristics of the human herpesviruses.

Virus	Trophism	Latent infection site	Subtype
HSV-1	Nerve cells	Trigeminal ganglion	α
HSV-2	Nerve cells	Sacral ganglion	α
EBV	B cells	Lymphoid tissues	γ
VZV	Nerve cells	Neural ganglia	α
HCMV	Various; includes epithelial and interstitial cells	Various; includes secretory glands, kidneys and lymphoreticular cells	β
HHV-6	Various; includes B and T cells	Lymphoid tissues	–

As the work in this thesis involved herpes simplex virus type 1, (HSV-1), the rest of this chapter will deal mainly with this herpesvirus.

1.2. HERPES SIMPLEX VIRUS.

1.2.1. Herpes simplex virus types 1 and 2.

Herpes simplex viruses are divided into 2 serological subtypes, which can be differentiated by neutralization assays. These are termed HSV type 1 (HSV-1) and HSV type 2 (HSV-2; Pauls & Dowdle, 1967). HSV-1 and HSV-2 share approximately 50% DNA sequence homology (Kieff et al., 1972), and many HSV-1-encoded proteins are structurally and functionally equivalent to those of HSV-2 (Spear, 1984).

1.2.2. Clinical symptoms of infection by HSV-1 or HSV-2.

Infection with HSV-1 or HSV-2 can be symptomatic or asymptomatic, particularly in healthy individuals, when infection is rarely serious. However, in neonates and in immunosuppressed individuals, with incomplete or suppressed immune responses, infection with HSV can be very serious and lead to death.

HSV-1 and HSV-2 infect and replicate in mucosal and submucosal tissues at the site of viral entry. This is generally around the mouth, for HSV-1, and the genitals, for HSV-2 (Dowdle et al., 1967; Plummer et al., 1970). However, this division is not absolute and HSV-2 can be isolated from oral lesions and vice versa, although these infections can be less severe (Reeves et al., 1981). HSV infection can also lead to encephalitis and, in the eye, HSV infection can lead to stromal keratitis (reviewed by Rouse (1985)).

1.2.3. HSV and latency.

In common with the other herpesviruses, HSV-1 and 2 can become latent after a primary infection. The virus enters the peripheral nerve endings during infection, travels along the sensory nerves to the ganglion and establishes latency in the neurones of the local ganglion. With HSV-1 this occurs in the trigeminal ganglion and with HSV-2 in the sacral ganglion. During latency, no virus particles can be detected in the ganglion. The virus can remain latent for many years before reactivation, usually due to stress or immunosuppression, when it travels back along the sensory nerves and re-establishes a mucosal infection. The subject of HSV latency is fully reviewed by Hill (1985), Roizman & Sears, (1987) and Baichwal & Sugden (1988).

1.2.4. The HSV genome.

HSV-1 and HSV-2 each have linear, double stranded DNA consisting of 2 covalently linked segments, i.e. the unique long (U_L) and unique short (U_S) sequences. These are flanked by a pair of inverted repeat sequences (Roizman, 1979). The U_L and U_S sequences comprise 82 and 18% of viral DNA respectively. The HSV-1 genome has a total length of 152,260 residues and has recently been completely sequenced (summarized by McGeoch *et al.*, 1988). It is presently estimated that there are 72 protein-coding genes in the HSV-1 genome, although with 2 genes represented twice, this means a total of 70 distinct polypeptides can be encoded by the genome. These polypeptides form 3 groups, the α , β and γ polypeptides, and their synthesis is coordinately regulated and sequentially ordered (Honess & Roizman, 1974). The genes encoding the α polypeptides appear to map partly within the repeat sequences of the L and S regions, whereas the β and γ polypeptide genes are located mainly in the unique sequences of the L and S regions (Roizman, 1979).

1.2.5. The HSV glycoproteins.

Both HSV-1 and HSV-2 express glycoproteins, which are exposed on the virion envelope (Stannard et al., 1987) and on the surface of infected cells (La Thangue et al., 1984). These are involved in many functions including viral entry into cells, viral replication, cell fusion and are major targets of the infected host's immune response. They are usually designated by a letter followed by the serotype of the virus they are located on, e.g. glycoprotein B of HSV-1 is gB-1. Due to their importance, they have been the subject of many reviews, (Norrild, 1980; Spear, 1980, 1984, 1985).

HSV-1 is now known to encode at least 7 glycoproteins, glycoproteins B, C, D, E, G, H and I (McGeogh et al., 1988). The exact number encoded by HSV-2 is unclear, however it is known to be at least 5, i.e. glycoproteins B, C, D, E and G (Spear, 1985). Over recent years, a number of groups have located and sequenced many of the glycoproteins and this is summarized in Table 1.2.1. The genes encoding the HSV-2 glycoproteins are presumed to be located on the HSV-2 genome in positions co-linear with the HSV-1 glycoprotein genes, as each HSV-1 glycoprotein appears to be structurally and antigenically related to its HSV-2 counterpart. In addition, analysis using HSV-1 x HSV-2 recombinants has shown that the genomes are predominantly, if not completely, co-linear (Preston et al., 1978).

It should be noted that earlier confusion over the naming of some of the glycoproteins has now been resolved. The HSV-2 glycoprotein now termed gC-2 (Zweig et al., 1983) was originally called gF (Balachandran et al., 1982a). Also, gG-2 (Roizman et al., 1984) was originally gC (Ruyechan et al., 1979). Finally, gA (Spear, 1976) is now known to be a form of gB (Eberle & Courtney, 1980; Pereira et al., 1981, 1982; Balachandran et al., 1982b).

Table 1.2.1. Nucleotide sequences of the glycoproteins of HSV-1 and HSV-2 - References.

Glycoprotein	Reference
gB-1	Bzik *et al.* (1984a) Pellet *et al.* (1985a)
gC-1	Frink *et al.* (1983)
gD-1	Watson *et al.* (1982)
gE-1	McGeogh *et al.* (1985)
gG-1	McGeogh *et al.* (1985)
gH-1	Gompels & Minson (1986)
gI-1	McGeogh *et al.* (1985)
gB-2	Bzik *et al.* (1986) Stuve *et al.* (1987)
gC-2	Swain *et al.* (1985)
gD-2	Watson (1983)

1.2.6. Glycoprotein functions.

The glycoproteins have been implicated in various functions, including viral adsorption and penetration into cells, fusion of cells and receptors for complement and immunoglobulin. gB is involved in a number of these functions and will be discussed in greater detail in Section 1.4. However, the role of the other glycoproteins is summarized in Table 1.2.2.

Table 1.2.2. Functions of the HSV glycoproteins - References.

Glycoprotein	Function	Reference
gB	Viral penetration	DeLuca *et al.* (1982)
	Cell fusion	Manservigi *et al.* (1977)
	Viral infectivity	Sarmiento *et al.* (1979)
		Cai *et al.* (1988)
gC	Viral adsorption	Fuller & Spear (1985)
	C3b receptor	Friedman *et al.* (1984)
gD	Viral adsorption	Fuller & Spear (1985)
	Viral penetration	Highlander *et al.* (1987)
		Ligas & Johnson (1988)
	Cell fusion	Noble *et al.* (1983)
gE	Viral adsorption	Fuller & Spear (1985)
	Fc receptor	Para *et al.* (1982)
gH	Viral penetration	Fuller *et al.* (1989)
	Viral infectivity	Desai *et al.* (1988)
gI	Fc receptor	Johnson & Feenstra (1987)

Therefore, glycoproteins B, D and H are essential for viral entry into cells and hence essential for viral replication. However, glycoproteins C, E, G and I are not essential for viral replication (Longnecker & Roizman, 1987; Zezulak & Spear, 1984a). As yet, no role has been found for gG.

The glycoproteins are also targets for the host immune response, as they are expressed on the surface of infected cells (La Thangue *et al.*, 1984). Infection with HSV leads to a strong humoral response against the glycoproteins

(Norrild, 1980). The passive transfer of monoclonal antibodies raised against gB, gC, gD and gE of HSV-2 were all found to protect mice against a lethal challenge with virus (Balachandran et al., 1982a). HSV glycoproteins have also been implicated as targets for cell mediated immunity (Carter et al., 1981). In particular, immunization with gD has been found to induce both an antibody response and a delayed type hypersensitivity (DTH) response (Blacklaws et al., 1987) and to protect mice against viral challenge (Blacklaws et al., 1987; Rooney et al., 1988; Krishna et al., 1989). Some glycoproteins were also shown to be targets for cytotoxic T cells, including gB (Blacklaws et al., 1987) and gC (Rosenthal et al., 1987). Glycoprotein B is a major target for both antibody and cell-mediated responses, and this will be discussed fully in Section 1.6.

1.3. GLYCOPROTEIN B.

1.3.1. The genome location of glycoprotein B.

The area of the genome containing the gene for gB was first located using the temperature sensitive mutants (ts) tsB5 (Manservigi et al., 1977) and tsJ12 (Little et al., 1981). At the non-permissive temperature, these mutants did not synthesize mature gB. The mutation in tsJ12 mapped to the DNA fragment 0.345 to 0.399 map units (Little et al., 1981) while the mutation in tsB5 mapped between 0.360 and 0.368 map units (DeLuca et al., 1982; Holland et al., 1983a). tsB5 also contained a syncytia-inducing (syn) mutation (Manservigi et al., 1977). The syn mutation was found to be close to, but separate from, the ts mutation (Honess et al., 1980) and mapped between 0.345 and 0.355 map units (DeLuca et al., 1982). A monoclonal antibody, which recognized an antigenic variant of gB (marB1.1), was also used and the mutation in marB1.1 mapped between 0.350 and 0.361 map units (Holland et al., 1983a).

Thus, the combined data using DNA fragments from ts, syn and monoclonal antibody resistant (mar) mutants showed that the gene for gB lay between 0.345 and 0.399 map units. Rafield & Knipe (1984) isolated a 3.4kb RNA homologous to the DNA sequence between 0.343 and 0.386 map units and showed that the translation products were recognized by a gB-specific antiserum. Finally, Bzik et al., (1984a) presented the nucleotide sequence for gB of HSV-1 strain KOS, (gB-1 (KOS)). The gB gene was located between 0.368 and 0.348 map units, transcribed from right to left (Bzik et al., 1984a, 1984b) and yielded an RNA species of 3.1-kb, containing approximately 3050 nucleotides. This was translated to a 100.3-kilodalton polypeptide of 903 amino acids. (For details on the structure of gB, see Section 1.3.2.).

After gB-1 (KOS) was sequenced, the nucleotide sequences of gB-1 strain F and gB-1 strain Patton were reported (Pellet et al., 1985a; Stuve et al., 1987). When these strains were compared, there were only 29 amino acid differences amongst them. These were followed by the sequences for gB-2 strain HG52 (Bzik et al., 1986) and gB-2 strain 333 (Stuve et al., 1987; Zwaagstra & Leung, 1987). gB-2 was found to have approximately 85% homology with the nucleotide and amino acid sequences of gB-1 (Bzik et al., 1986; Stuve et al., 1987).

1.3.2. The structure of glycoprotein B-1.

The gB gene encodes a 100 kilodalton polypeptide, 903 amino acids in length (Bzik et al., 1984a; Pellet et al., 1985a). The polypeptide consists of a number of domains and has the structural characteristics of a glycoprotein:-

1. An N-terminal signal sequence of 29 (Pellet et al., 1985a) or 41 (Bzik et al., 1984a) amino acids in length, although why the estimated size is different between the groups is unclear. This sequence is cleaved during post-translational processing, resulting in a 95.7 kDa, 862 amino acid polypeptide (Bzik et al., 1984a) or 97kDa, 874 amino acid polypeptide (Pellet et al., 1985a). The size of the unglycosylated gB polypeptide was previously estimated to be between 92 and 98 kDa (Norrild & Pedersen, 1982; Kousoulas et al., 1983; Wenske & Courtney, 1983).
2. A hydrophilic surface domain of approximately 700 amino acids. gB contains a number of N-linked glycosylation sites, (9 sites on gB-1 (KOS); 6 sites on gB-1 (F)). These sites are marked by the sequence asn-X-ser/thr and are all located

on this extracellular portion of the glycoprotein.

3. A hydrophobic membrane-spanning domain. Again, Bzik and Pellet differ on the number of amino acids contained in this sequence, and on the number of times it spans the cell membrane. Bzik et al., (1984a) estimated the sequence to be 44 amino acids in length and to span the membrane twice, while Pellet et al., (1985a) claim it to be 69 amino acids long and to cross the membrane three times. However, recent work on the membrane insertion of gB-1 supports the theory that gB crosses the cell membrane three times (Claesson-Welsh & Spear, 1987).

4. A highly charged cytoplasmic domain of 109 amino acids, which is the anchor sequence.

The addition of N-linked (Wenske et al., 1982) and O-linked (Oloffson et al, 1981) oligosaccharides to non-glycosylated gB is responsible for increasing its molecular weight to that of the mature glycoprotein, i.e. approximately 120kDa (see Section 1.3.3.). Also, in common with the other glycoproteins, gB contains inorganic sulphate (Hope et al., 1982).

1.3.3. Glycosylation of gB-1.

N- and O-linked oligosaccharides are added to the non-glycosylated polypeptide during post-translational processing events (reviewed by Campadelli-Fiume & Serafini-Cessi, 1984; Spear, 1984). Early work demonstrated that the glycoproteins were formed by the stepwise addition of oligosaccharide chains to the polypeptide backbone of the glycoprotein (Honess & Roizman, 1975). However the type of side chains involved, i.e. N- or O-linked, were not elucidated.

Evidence for the presence of N-linked oligosaccharides on all the glycoproteins, including gB, has been obtained by treating HSV-infected cells with tunicamycin, which blocks glycoprotein synthesis. After tunicamycin treatment, lower molecular weight non-glycosylated molecules were obtained which were structurally and antigenically related to the mature glycoproteins (Pizer et al., 1980; Norrild & Pedersen, 1982; Peake et al., 1982; Kousoulas et al., 1983). Endo- β -N-acetylglucominidase H (Endo H) was also used to remove high-mannose oligosaccharides from immature glycoprotein forms (Wenske et al., 1982; Johnson & Spear, 1983). The mature gB was partially sensitive to Endo H treatment, hence it contained both complex- and high-mannose-type oligosaccharides. However, its precursor form, then termed gA, was completely sensitive to Endo H, thus it contained only the high-mannose-type oligosaccharides.

The HSV glycoproteins, including gB, also contain O-linked oligosaccharides (Oloffson et al., 1981). The addition of the O-linked glycans occurs in the Golgi apparatus at the same time as the high-mannose-type N-linked glycans are processed to complex-type glycans (Johnson & Spear, 1983).

While the mature glycoproteins, including gB, are expressed on the infected cell surface (Spear, 1976), the precursor forms, containing the high-mannose type chains, are located in the nuclear fraction of the infected cells (Compton & Courtney, 1984). It appears that the precursor forms are acquired by the virion at the nuclear membrane. It then passes through the rough endoplasmic reticulum and Golgi apparatus, where the envelope glycoproteins are further processed to fully mature species.

Recent work using a truncated gB protein synthesized in mammalian cells has shown that its secretion is dependent on glycosylation, implying that transport to the cell surface is also dependent on glycosylation (Ali et al., 1987).

1.3.4. Relationship of gA and gB.

Spear (1976) demonstrated that the major HSV-1 glycoproteins were derived from 4 antigenically distinct polypeptides, designated gA, gB, gC and gD, and these were separable on the basis of their electrophoretic mobilities. However, it was later found that anti-gA and anti-gB antiserum could recognise both glycoproteins (Eberle & Courtney, 1980). The structural identity of gA and gB was also confirmed in HSV-2 (Balachandran et al., 1982b). Thus, gA was found to be a lower molecular weight precursor of gB, due to differences in glycosylation. Also, the in vitro infection of different cell types led to variation in the molecular weight of gB and its precursors (Pereira et al., 1981, 1982).

1.3.5. Homologues of gB.

Extensive nucleotide and amino acid homology has been demonstrated between gB of HSV-1 and HSV-2 (Bzik et al., 1986) and with the equivalent gB-like molecule in other herpesviruses. This was first reported by Pellet et al., (1985b) who demonstrated homology between gB and the protein encoded by the EBV DNA open reading frame BALF 4. This was confirmed by Balachandran et al., (1987), Gong et al., (1987) and Chan (1989).

A homologous "gB-like" protein has now been found in many of the herpesviruses, including cytomegalovirus (Cranage et al., 1986; Balachandran et al., 1987; Stannard et al., 1989), varicella zoster virus (Edson et al., 1985; Keller et al., 1986; Kitamura et al., 1986), equine herpes virus type-1 (Snowden et al., 1985; Allen & Yeargan, 1987; Meredith et al., 1989; Whalley et al., 1989), equine herpes virus type-4 (Meredith et al., 1989), bovine herpes virus-1 (Lawrence et al., 1986; Whitbeck et al., 1988) and bovine herpes virus type-2 (Snowden et al., 1985).

The extensive homology of gB amongst so many herpesviruses implies that it is a conserved protein. Therefore, it must have an important and necessary function (or functions) to the virus.

1.4. FUNCTIONS OF gB.

The temperature sensitive (ts), syncytial-forming (syn) and monoclonal antibody resistant (mar) mutants which were used in locating the gene for gB, (see Section 1.3.1.), have also been used to establish the functions of gB.

gB appears to be multifunctional, i.e. it is required for both virus penetration and replication and is also involved in cell fusion.

The use of ts mutants (tsB5 and tsJ12) indicated a role for gB in viral penetration and infectivity. These strains contain mutations in the gB gene (Little et al., 1981; DeLuca et al., 1982; Bzik et al., 1984a, 1984b) which prevent the normal processing of gB at the non-permissive temperature (Manservigi et al., 1977; Sarmiento et al., 1979; Little et al., 1981), leading to the production of non-infectious virions. This block in infectivity was at the level of penetration because the virions were able to bind to the cells and polyethylene glycol treatment, which promotes membrane fusion, led to an increase in infectivity (Sarmiento et al., 1979; Little et al., 1981). Viral penetration was also prevented by monoclonal antibodies which bind to gB (Highlander et al., 1988) and gB-free virions failed to infect cells until polyethylene glycol was used (Cai et al., 1988). It should be noted that in all the above examples, although the absence of gB affected viral penetration and infectivity, the virions were still able to bind to the cell surface. However, in a system using liposomes expressing viral glycoproteins, the liposomes bound the cells more efficiently when gB was present than they did when it was absent. This was taken to indicate a role for gB in viral adsorption as well (Johnson et al., 1984). The reason for this discrepancy is unclear, but may reflect differences in the experimental systems used.

gB is also essential in HSV-induced cell fusion. Again, use of the ts mutant tsB5 demonstrated that the lack of gB expression at the non-permissive temperature correlated with the absence of cell fusion (Manservigi et al., 1977). This syncytial-inducing mutation was also mapped to the gB gene ((DeLuca et al., 1982; Bzik et al., 1984a, 1984b). Later work using gB expressed in mammalian cells (Ali et al., 1987) or gB-free virions (Cai et al., 1988) have confirmed the role of gB in cell fusion.

As well as its important role in viral infectivity and replication, or perhaps because of it, gB is also an important target in the host immune response to HSV infection (see Section 1.6.).

1.5. IMMUNE RESPONSES IN HSV INFECTION.

The immune mechanisms involved in the response to HSV infection are many and varied, and involve both specific and non-specific defence mechanisms. The glycoproteins are major targets of the immune response, inducing both the production of antibody (Norrild, 1980, 1985) and cell-mediated responses (Carter et al., 1981; Nash et al., 1985). The following sections will review some of the mechanisms induced by whole virus and the glycoproteins.

1.5.1. Antibody responses.

Antibody is not effective in preventing the cell-to-cell spread of HSV in infected tissue culture monolayers (Hooks et al., 1976). In vivo, neutralizing antibody production has been demonstrated in mice (Nash et al., 1980a) and humans (Mann & Hilty, 1982; Zweerink & Corey, 1982) in response to a primary HSV infection, although detectable levels were not seen until 1-2 weeks after infection, by which time virus, at least in mice, had been cleared (Nash et al., 1980a). However, neutralizing antibody may be more important in restricting viral spread to the central nervous system. The passive transfer of neutralizing polyclonal anti-HSV antiserum or neutralizing monoclonal anti-gD antiserum greatly reduced the amount of virus present in the ganglia and spinal cord of athymic nude mice, but not in the pinna (Kapoor et al., 1982a). Neutralizing antibody may also be involved in recurrent HSV infection, when latent virus is reactivated and recrudesence occurs. B cell suppressed mice recovered from a primary infection with HSV , but had a higher incidence of latent infection. In addition, primary infection of the peripheral and central nervous system was more florid (Kapoor et al., 1982b). As virus reaches the ganglia within 48 hours post infection (McKendall et al., 1979; Openshaw et al., 1979) , and antibody does not appear until 1-2 weeks post infection (p.i.), it may be ineffective in a primary response. However, when virus is reactivated and passes from the nerve endings to the epidermal cells, antibody could neutralise it before it enters the cell (Simmons & Nash, 1985).

The passive transfer of monoclonal antibodies specific for HSV glycoproteins can protect mice against a lethal challenge with HSV (Balachandran et al., 1982a; Chan et al., 1985). Balachandran and his co-workers (1982a) demonstrated that administration of monoclonal antibodies recognising gB, gC, gD, gE and gG of HSV-2 could protect mice against HSV-2 infection. However, Chan et al., (1985) found that their anti-gB-1 MAb could not passively protect infected mice, although anti-gD-1 and anti-gH-1 MAbs could. This discrepancy is probably due to differences between the monoclonal antibodies.

Therefore, neutralizing antibody appears to be less important in clearing primary infections from epidermal sites, e.g. the pinna, but is important in restricting viral spread to the CNS and in preventing recrudesence in the form of a zosteriform rash.

1.5.2. T cell responses.

Evidence for the involvement of T lymphocytes in response to HSV infection has come from a number of sources.

Neonatally thymectomized mice were found to be much more susceptible to HSV infection, whether or not they had been immunized against the virus (Mori et al., 1967). Infection of athymic nude mice, i.e. with a congenital defect of the thymus, led to a progressive infection of the peripheral and central nervous systems which was not seen in their normal immunocompetent littermates (Kapoor etal., 1982b). Although the passive transfer of neutralizing anti-HSV antiserum reduced the amount of virus in the ganglia and spinal cord, only the transfer of HSV immune T cells led to virus elimination and the survival of the mice.

Adult mice immunosuppressed by X-irradiation (Oakes, 1975) or cyclophosphamide (Rager-Zisman & Allison, 1976) were extremely susceptible to HSV infection, but were protected by the transfer of immune spleen cells. However, such protectivity was abolished if the cells were treated with anti-Thy-1 antiserum and complement before transfer. Therefore, T cells are clearly important in protection against HSV infection.

It has been demonstrated that protection can be mediated by immune cells possessing either I region or K-D region MHC compatibility with immunosupressed recipients (Howes et al., 1979), but long-term protection, i.e. up to 60 days post infection, was dependent on I region compatibility. This implied that both helper T cells (Th) and cytotoxic T cells (CTL) were involved in protection, but that Th cells had the greater long-term capacity for protection.

The various T cell populations involved in the immune response to HSV infection will now be discussed individually.

1.5.3. T helper cells.

As mentioned above, long term protection in immunosuppressed mice required the transfer of immune T cells expressing I-region compatibility with the recipient (Howes et al., 1979). The cell involved was presumed to be a Th cell, although the mechanism of help was unclear. It could have involved help for CTL, for B cells or for the production of lymphokines.

Lyt-1^+23^- cells were shown to mediate viral clearance in mice infected subcutaneously or intraperitoneally (Larsen et al., 1984). Th cells could also be induced by immunization with gB and the adoptive transfer of these cells protected mice against infection (Chan et al., 1985). Protection correlated with the induction of neutralizing antibody in the serum of the recipients, and this help for antibody production was postulated to be involved in the protective mechanism. An HSV-specific Th cell clone was also found to protect mice and induce B cells to produce anti-HSV antibody after adoptive transfer (Leung et al., 1984). This clone produced interleukin 3 and interferon-γ in vitro and enhanced the virocidal activity of macrophages (Seid et al., 1986). So, it could be exerting its effect by inducing antibody production or by releasing lymphokines which could act on other cells, either to activate them or to recruit them into the infected area.

Th cells could also provide help for the induction of HSV-specific CTL responses. The activation of these helper cells required priming with infectious virus and reexposure to virus in vitro (Schmid & Rouse, 1983). This was supported by the finding that Th cell precursors required priming with infectious virus and reexposure to viral antigen in vitro before detectable expansion of Th precursors occurred (Prymowicz et al., 1985). In both cases, priming with inactivated virus did not lead to activation of Th cells.

Recent work in mice depleted of individual T cell subsets before infection has revealed that L3T4-deficient mice did not produce DTH responses or anti-HSV antibodies, but CTL responses were induced (Nash et al., 1987). However, these mice cleared virus from the pinna more slowly than the untreated, infected mice, suggesting that L3T4 cells were the principal cell involved in viral clearance from the skin (Nash et al., 1987). These mice also had a more florid infection of the nervous system, reflecting the situation in B cell suppressed mice (Kapoor et al., 1982b), thus showing the involvement of L3T4 Th cells in providing help for antibody production.

1.5.4. T cells mediating delayed type hypersensitivity.

HSV-specific T cells mediating DTH (T-DH) are induced after the injection of either infectious or UV-inactivated virus using the intra-dermal (Nash *et al.*, 1980b) or subcutaneous (Schrier *et al.*, 1982) routes. DTH was observed 3-5 days post infection and could be induced up to 18 months later in sensitized mice. DTH was also adoptively transferred to naive recipients using T cells from the draining lymph nodes (Schrier *et al.*, 1982), but this was only possible using cells from donors infected 6-10 days previously (Nash *et al.*, 1980b). As well as transferring DTH, these cells could also protect the mice by clearing infectious virus (Nash *et al.*, 1980b; Larsen *et al.*, 1984; Schrier *et al.*, 1985). The adoptive transfer of DTH was restricted by MHC class-II antigens, although the clearance of virus required both class I and class II restricted T cells (Nash *et al.*, 1981a). The transfer of DTH also required an Lyt-1^+23^- cell (Nash & Gell, 1983; Larsen *et al.*, 1984), but not an Lyt-1^-23^+ cell. Individual glycoproteins could also prime for and elict DTH to HSV, including gD, gC (Schrier *et al.*, 1983a) and gB (Chan *et al.*, 1985).

Following the intravenous injection of virus, DTH was not induced. This was probably due to the development of suppressor T cells (Nash *et al.*, 1981b; Schrier *et al.*, 1983b), and is discussed in Section 1.5.5. Cells from these tolerized mice failed to transfer DTH and could not clear infectious virus (Nash & Ashford, 1982). Therefore, DTH may be an important early defence mechanism, active before the development of neutralizing antibody and CTL responses, and mediating protection by the clearance of virus.

As well as being a possible mechanism of protection *in vivo*, DTH can also mediate immune pathology if it is uncontrolled, e.g. in HSV-induced stromal keratitis (Newell *et al.*, 1989). By selectively depleting mice of $CD4^+$ or $CD8^+$ T cells, the role of each subset in the pathogenesis of herpetic stromal keratitis (SK) may be analysed. In $CD4^+$-depleted mice, which showed normal CTL responses but no anti-viral or DTH response, SK was significantly reduced. However in $CD8^+$-depleted mice, which had reduced CTL responses but normal antibody and DTH responses, SK developed more rapidly and was more severe. Also, the ability of athymic mice to generate SK could be reconstituted by the transfer of T cells mediating DTH. Therefore, a DTH response appears to be one mechanism involved in the immunopathology of SK (Rouse, 1985).

1.5.5. T suppressor cells.

There have been a number of reports on the involvement of suppressor T cells (Ts) in the regulation of HSV-specific T cell responses.

The demonstration of HSV-specific CTL activity *in vitro* requires either pretreatment of the mice with cyclophosphamide or in vitro cultivation for a few days (Pfizenmaier et al., 1977a, 1977b; Lawman et al., 1980a). Therefore it was suggested that CTL induction was regulated by suppressor cells which could be inactivated by these treatments. It was then demonstrated that the in vitro restimulation of HSV-immune spleen cells with virus resulted in the production of supernatant factors which could suppress the induction of CTL (Horohov et al., 1985a). Most of the factors obtained were antigen non-specific, however one factor, SF-200, was shown to be virus specific and genetically restricted in it's action. Further characterization of SF-200 showed it to be I-J$^+$, and it was postulated to be involved in a suppressor cell cascade (Horohov et al., 1986). Suppressor T cells were also implicated in the regulation of HSV-specific lymphoproliferation (Horohov et al., 1985b). These cells were Lyt-1$^-$23$^+$ and postulated to interact with Lyt-1$^+$23$^-$ "suppressor inducer" T cells and I-J$^+$ antigen presenting cells, as proposed in models of suppressor cell cascades (Greene et al., 1983; Dorf & Benacerraf, 1984).

The role of Ts cells in the regulation of DTH has been better characterized. The intravenous injection of infectious HSV, inactivated HSV or HSV-infected spleen cells all led to the virus-specific suppression of DTH (Nash et al., 1981b; Schrier et al., 1983b). Two distinct Ts populations could be induced, depending on the type of tolerogen used. The injection of whole virus preparations led to Ts cells which acted on the induction of DTH, i.e. T-afferent suppression, while the injection of virus-infected syngeneic spleen cell preparations led to Ts cells which acted on the expression of DTH, i.e. T-efferent suppression (Nash et al., 1981b, 1981c; Schrier et al., 1983b). Further work showed that the Ts-aff cells were induced by adsorbed HSV antigens on the spleen cells, whereas Ts-eff were induced by nascent HSV antigens expressed on the spleen cells (Ishioka et al., 1985). Seven days after injection of virus Lyt-$1^{+}23^{-}$, I-J^{+} cells were detected which could transfer suppression. Later, at 28 days p.i., 2 populations were detected which could transfer suppression, one Lyt-$1^{+}23^{-}$, I-J^{+} and the other Lyt-$1^{-}23^{+}$, I-J^{+} (Nash & Gell, 1983). Again, these cells were postulated to be part of a suppressor cell cascade.

The suppression observed in these systems appeared to be specific for DTH responses, i.e. CTL and antibody responses were unaffected, so a form of split-tolerance existed (Nash & Ashford,1982). However, cells from suppressed mice could not transfer DTH to syngeneic recipients, nor could these cells transfer protection or eliminate infectious virus, implying that the T-DH cell is an important protective T cell in vivo. (See Section 1.5.4.). In contrast, mice in which DTH tolerance was induced were protected from herpes-induced encephalitis and from CNS demyelination, and this protection could be transferred by their remaining T cells (Altmann & Blyth, 1985). Thus, in this situation, Ts cells may protect mice from DTH-mediated immunopathology in the CNS.

1.5.6. Cytotoxic T cells.

Cytotoxic T cells (CTL) were detected in the draining lymph nodes of infected mice 4 days post infection (p.i.) and this activity peaked between 6-9 days p.i., then diminished (Pfizenmaier *et al.*, 1977a, 1977b; Lawman *et al.*, 1980a; Nash *et al.*, 1980a). However, generation of detectable CTL activity required either pretreatment of the mice with cyclophosphamide (Pfizenmaier *et al.*, 1977b; Lawman *et al.*, 1980a) or 3 days *in vitro* culture with HSV (Nash *et al.*, 1980a). These findings are thought to imply the presence of a Ts cell population regulating the induction of CTL activity (see Section 1.5.5.).

The induction of CTL required priming with infectious virus, although the *in vitro* restimulation could be carried out using ultraviolet light- or glutaraldehyde-inactivated virus preparations, but not heat-inactivated (HI) virus or detergent-extracted antigens (Lawman *et al.*, 1980a). The inability of HI virus to induce CTL cells appears to be due to its inability to activate the Th cells required in this reaction (Schmid & Rouse, 1983) as strong *in vitro* CTL responses were generated if the cultures were supplemented with non-specific helper factors (Rouse & Lawman, 1980).

After priming with infectious virus, the frequency of CTL precursors increased from 1/250,000 to between 1/3,500 and 1/15,670 and expressed the Lyt-1^{+}23^{+} phenotype (Rouse *et al.*, 1983).

The adoptive transfer of spleen cells from HSV infected mice, restimulated *in vitro*, were shown to protect lethally infected syngeneic recipients (Larsen *et al.*, 1983). However, treatment of these cells with anti-Lyt-2 antiserum plus complement before transfer abrogated this protective response. A CTL clone was also able to protect against lethal infection (Sethi *et al.*, 1983). Lyt-1^{-}23^{+} T cells from mice infected 6 weeks previously could clear virus from the pinna (Larsen *et al.*, 1984). This contrasted with the protective T cell population from recently infected mice, i.e. infected less than 6 weeks, which was Lyt-1^{+}23^{-}. A study of Lyt-2-deficient, infected mice revealed that they had normal DTH and serum antibody responses, but no MHC class-I restricted CTL responses (Nash *et al.*, 1987). These mice were able to clear infectious virus in the pinna, but developed a severe infection of the nervous system, implying that the major role of Lyt-1^{-}23^{+} CTL cells was in protecting the nervous system. However, this result was obtained from acutely infected mice, i.e. no more than 9 days p.i., and the cell type responsible for viral clearance is known to differ between the acute, primed and memory cell populations (Larsen *et al.*, 1984). Also, both Lyt-1^{+}23^{-} and Lyt-1^{-}23^{+}

cells are involved in the elimination of acute HSV infections (Howes et al., 1979; Nash et al., 1981a).

The target recognised by HSV-specific CTL cells is unclear and there may, in fact, be more than one. The HSV glycoproteins were originally shown to be targets for virus-specific CTL response (Carter et al., 1981), and in particular the VP123 region glycoproteins (Lawman et al., 1980b) which contained the glycoproteins gA/gB and gC (Spear 1976). Later work showed that gB could induce and be recognised by CTL cells in mice (Blacklaws et al., 1987) and in humans (Zarling et al., 1986). gD could also induce CTL cells in humans (Zarling et al., 1986), but not in mice (Blacklaws et al., 1987). However, others have demonstrated virus-specific CTLs which recognised only gC and none of the other surface glycoproteins (Rosenthal et al., 1987). Recently, it has been shown that a significant proportion of HSV-specific CTLs recognise the non-structural immediate-early gene products, which cannot be recognised serologically (Martin et al., 1988). This is in line with other viruses, where CTLs can also recognise antigens not detectable on the cell surface by serological techniques. These include the influenza A viruses where CTL cells recognise the internal nucleoprotein (Townsend et al., 1984) and matrix protein (Gotch et al., 1987a) and also the RNA polymerases (Gotch et al., 1987b). Within the herpesviruses, CTL cells have been found to recognise the latent membrane protein/p63 of EBV (Thorley-Lawson & Israelson, 1987) and the immediate-early antigens of murine CMV (Reddehase et al., 1986; Reddehase et al., 1987a).

Effector CTL cells were shown to be MHC class-I restricted (Sethi et al., 1983; Nash et al., 1987). However, MHC class-II restricted CTLs have also been demonstrated in humans (Yasukawa & Zarling, 1984a; Schmid, 1988), as has been shown in the influenza virus system (Lukacher et al., 1985). Their in vivo function is not yet clear, however they have been found to inhibit the replication of HSV in vitro (Yasukawa & Kobayashi, 1985) and to help B cells with antibody production (Yasukawa et al., 1988). They also exhibit helper activity by proliferating and producing IL-2 when restimulated by HSV in vitro (Yasukawa & Zarling, 1984b) and can recognise, and respond to, viral glycoproteins, including gB, gC, gD and gE (Yasukawa & Zarling, 1985). Recently, class-II restricted CTL cells have been generated which can lyse HSV-infected B cell lines (Schmid 1988), HSV-infected peripheral blood monocytes and in vitro cultured, infected macrophages (Torpey et al., 1989). In the former system, no class-I restricted CTLs were detected. However, in the latter system, class-I restricted CTLs were also detected.

Thus, CTL cells appear to have an important role in the protection against and clearance of virus, at least in the later stages of infection. They may also have an important role to play in the protection of the CNS against viral infection.

1.5.7. Natural killer cells.

Natural killer (NK) cells are probably important in the initial response to HSV infection, before the specific immune responses of T cells and antibody have developed, and may be one factor in determining the intrinsic resistance of certain mouse strains to HSV infection (Lopez, 1975; Lopez, 1984).

It was demonstrated that human NK cells isolated from peripheral blood could lyse HSV-infected fibroblasts and epithelial cells *in vitro* (Ching & Lopez, 1979; Fitzgerald *et al.*, 1982; Bishop *et al.*, 1983a; Yasukawa & Zarling, 1983). Recently, NK cells have also been shown to kill HSV-infected B cells (Schmid, 1988), and NK cell clones were able to inhibit HSV-1 and HSV-2 replication *in vitro* in both syngeneic and allogeneic cells (Yasukawa & Kobayashi, 1985). This MHC unrestricted pattern of NK cell lysis, and their lack of specificity in recognising HSV subtypes, had already been demonstrated *in vitro* (Yasukawa & Zarling, 1983; Schmid, 1988). However the target antigen recognized by the NK cells is unclear.

Susceptibility of the infected target cells to NK-mediated lysis was found to be related to the expression of HSV glycoproteins on the target cell surface (Bishop *et al.*, 1983a). On further investigation, Bishop *et al.* (1984, 1986) implicated the glycoproteins gB, gC and gD as possible targets for NK cell recognition. Glycosylation may be necessary for NK cell susceptibility, as treatment of the infected cells with 2-deoxy-D-glucose or with tunicamycin led to a reduction in the susceptibility of the cells to NK-mediated lysis (Bishop *et al.*, 1983a; Lopez-Guerrero *et al.*, 1988). However, recent work has shown that these glycoproteins alone may not be sufficient for recognition by NK cells and it was suggested that the host cell transferrin receptor may also be involved (Lopez-Guerrero *et al.*, 1988).

NK cells may also be involved in the *in vivo* protection of mice. Euthymic mice, depleted of NK cells before infection with HSV-1, were shown to have increased virus titres in the brain, liver and spleen and an increased rate of mortality compared with undepleted controls (Habu *et al.*, 1984). To ensure that T cells were not involved, athymic nude mice were also depleted of NK cells before infection and these mice died more rapidly than the undepleted control mice. However, NK cell depletion had to occur early in infection for any effect to be observed, indicating that NK cells *in vivo* are effective during the early stages of HSV infection. The transfer of NK cells to infected, immunosuppressed mice also induces protection and can reduce viral titres in the liver and brain of the recipients (Rager-Zisman *et al.*, 1987).

Thus, NK cells appear to be an early defence mechanism against HSV infection, allowing time for the specific defence mechanisms to develop.

1.5.8. Lymphokines.

As the number of well characterized lymphokines continues to increase, the realization of their importance *in vivo*, in response to viral infections has grown, and herpetic infections are no exception.

Interferon (IFN) production was detected from HSV immune peritoneal exudate cells (Lodmell & Notkins, 1974) and from HSV immune spleen cells (Fujibayashi *et al.*, 1975) after *in vitro* incubation with HSV. The interferon detected in the former system was IFN-α or β, while in the latter system it was IFN-γ. Treatment of mice with anti-interferon antiserum before infection resulted in a decreased latency period and a great increase in mortality (Gresser *et al.*, 1976). A correlation was also detected between interferon production and the resistance of certain mouse strains to HSV infection (Zawatzky *et al.*, 1981), as spleen cells from the resistant strain produced much greater levels of interferon than the susceptible strain. Interferon was also detected in the serum of these mice 8 hours after infection, however this required very high infectious doses of virus. Domke *et al.* (1985) demonstrated that interferon-α, β and γ were able to inhibit virus replication in murine macrophages *in vitro* at an early stage, either prior to or during the synthesis of the early proteins, with IFN-γ acting synergistically with IFN-α or IFN-β . IFN-α and β may also be involved in the activation of NK cells, as infection i.p. with HSV led to an increase in NK activity as well as high titres of interferon (Engler et al., 1981). However, other groups have found that NK cell activity can be separated from the induction of IFN and that the role of IFN may be to augment, rather than induce, NK cell activity (Fitzgerald et al., 1982; Bishop et al., 1983b).

IFN production can also be used to predict the time to recurrence of HSV infection. IFN was detected in both the serum (Cunningham & Merigan, 1983) and herpetic vesicles (Torseth & Merigan, 1986) of patients with recurrent herpes labialis and the titre of this IFN correlated directly with the time to the next occurrence of herpetic lesions.

IL-2 has also been investigated, although this was mainly concerned with the use of recombinant IL-2 (rIL-2) to augment immunity to HSV-2 infection. In guinea pigs, the administration of rIL-2 protected the animals against both acute (Weinberg et al., 1986a) and recurrent infection (Weinberg et al., 1987). Those animals which escaped infection after rIL-2 treatment appeared to lack immunologic memory for HSV, which was taken to imply the importance of early non-specific defence mechanisms in the response to HSV. Elimination of NK cells in these animals also eliminated the rIL-2 mediated protection, so the protective mechanism involved here was probably NK-mediated killing (Weinberg et al., 1986b).

rIL-2 has also been successfully used as an adjuvant. When administered in conjunction with viral extract or with recombinant gD, enhanced protection against HSV-2 infection was observed (Weinberg & Merigan, 1988). This appeared to be mediated by virus-specific CTL and was enhanced by the rIL-2. Purified IL-2 can also increase the effectiveness of viral clearance mediated by the adoptive transfer of immune T cells, particularly of the Lyt-1^-23^+ subset (Rouse et al., 1985). Thus, IL-2 appears to be useful as a means of augmenting the immune response in vivo and so increasing protection. This has also been demonstrated with MCMV infection, where the administration of IL-2 in vivo significantly improved the control of virus multiplication by enhancing the antiviral effect of CTL cells (Reddehase et al., 1987b).

It has been reported that freshly isolated T cells can develop permissiveness for HSV replication after treatment with IL-2 (Braun & Kirchner, 1986). However, this was restricted to a 5% subset of the total T cell population and it is difficult to ascertain what role these cells would play in a viral infection in vivo.

There is little information on the involvement of any other lymphokines in HSV infection so far. Recently, Chan et al. (submitted for publication) have shown that IL-3 may have an important role in affecting the susceptibility of cells to HSV-1 infection. Mice recovering from HSV infection produced high levels of IL-3 in vivo, whereas the injection of anti-IL-3 monoclonal antibody into infected animals resulted in disease exacerbation. The treatment of primary mouse embryonic brain cells with IL-3 rendered them resistant to HSV replication. It was suggested that IL-3 affects cell susceptibility to HSV infection by affecting cell growth and differentiation. The administration of IFN-γ, G-CSF, GM-CSF and IL-1β have also been reported to enhance host resistance to HSV infection in mice (Iida et al., 1989).

Therefore, as lymphokines become better characterized, their role in the prevention, or exacerbation, of HSV infection will become clearer.

1.6. IMMUNE RESPONSES TO gB.

gB is expressed on the surface of virions (Stannard et al., 1987) and infected cells (La Thangue et al., 1984) and can be extracted from these sources as detergent-stable, heat-dissociable oligomers, principally dimers (Sarmiento & Spear, 1979; Claesson-Welsh & Spear, 1986). Therefore, gB is an important target in the host immune response and is able to induce a range of responses, both humoral and T cell-mediated.

1.6.1. Antibody responses.

In vitro, gB can induce virus neutralizing antibodies (Glorioso *et al.*, 1984; Snowden *et al.*, 1985) and antibodies which mediate the immunocytolysis of virally infected cells (Norrild *et al.*, 1979). The role of anti-gB virus neutralizing antibodies in protection against HSV infection was examined (Glorioso *et al.*, 1984). Neutralizing antibody contributed to protection against intra-cranial infection, but not against intraperitoneal infection. A reduction in antibody titre was associated with an increased susceptibility to viral challenge, but antibody was not solely responsible for protection. However, anti-HSV neutralizing antibody is generally important in infections of the CNS, as it can restrict the movement of virus within the CNS (Kapoor *et al.*, 1982a, 1982b).

The passive transfer of gB-specific monoclonal antibody (MAb) has led to mixed results with regard to protection. Balachandran *et al.* (1982a) found that an anti-gB-2 MAb could protect mice against infection with HSV-2. Although the antibody did not neutralize virus, it did mediate antibody-dependent cell-mediated cytolysis. However, an anti-gB-1 MAb was unable to neutralize virus or to protect mice against HSV-1 infection (Chan *et al.*, 1985). It is likely that these results were due to intrinsic differences in the monoclonal antibody preparations used and that the two antibodies act in different ways on separate areas of the immune response.

Evidence is accumulating for a causal role of T cells in the protection and recovery from HSV infections, and gB is an important target for T cells.

1.6.2. T cell responses.

Priming with immunopurified gB, by the intraperitoneal, subcutaneous or intracerebral routes, induced protection in mice challenged with live virus (Chan et al., 1985; Roberts et al., 1985). Vaccinia virus-gB recombinants and mouse L cells expressing gB have also been used to protect mice against HSV infection (Cantin et al., 1987; Willey et al., 1988; Blacklaws et al.,1987). However, a recent report has shown that immunization of mice with a gB-vaccinia recombinant did not completely protect mice on HSV challenge, nor could the animals eliminate an epithelial infection (Martin et al., 1989). Although Lyt-1^{+}23^{-} T cells were induced, Lyt-1^{-}23^{+} cells were not and this may have contributed to the failure to develop complete protection.

Immunization with gB can induce both Th cells and CTL cells. Chan et al., (1985), demonstrated that immunization with gB induced strong proliferative T cell responses to HSV. Protection was adoptively transferred by L3T4^{+} T cells and could enhance the level of neutralizing antibody to HSV in the recipients. Therefore, gB appeared to be capable of activating Th cells which provided help for HSV-specific neutralizing antibody production. gB could also induce a strong DTH response to HSV in vivo (Chan et al., 1985; Blacklaws et al., 1987), and this could be an important protective mechanism (see Section 1.5.4.).

CTL cells play a major role in the elimination of infectious virus, particularly in epithelial sites of infection (see Section 1.5.6.). Immunization with gB can also induce CTL cells (Zarling et al., 1986; Blacklaws et al., 1987; McLaughlin-Taylor et al., 1988), although one group found that gB did not induce CTL cells (Rosenthal et al., 1987). However, high numbers of effector cells are required per target cell, so there may be other more important CTL targets than the glycoproteins (see Section 1.5.6.).

Therefore, gB appears to be an important target antigen in the host immune response against HSV infection, inducing both humoral and cell-mediated immunity. The importance of gB in viral functions and as a conserved protein have been discussed already (Sections 1.4. and 1.3.5. respectively). Some confirmation of this was recently provided by the finding that a MAb against gB of HSV-1 could be used to immunopurify the gB homologue from EBV infected cells (gB-EBV). gB-EBV stimulated HSV-1 immune T cells to proliferate and could protect mice against HSV-1 infection in vivo (Chan, 1989).

1.7. AIMS.

The aim of the present study was to examine T cell responses to gB of HSV-1. This was approached in 3 ways:-

1. By examining the role of glycosylation of the gB molecule in determining T cell responses. The proliferation of immune T cells to gB, and to its non-glycosylated precursor pgB, were examined to ascertain if glycosylation was involved in the T cell recognition of, and response to, gB. The protective ability of pgB compared with gB and whole virus was also examined.
2. By examining the *in vivo* production of lymphokines induced by HSV infection, and if prior priming with virus, gB or pgB would affect the levels of lymphokine production achieved. Also, to ascertain if the production of lymphokines *in vivo* could correlate with protection.
3. By developing a panel of T cell clones, specific for gB and whole virus. These clones would be characterized according to their antigen specificity, cell surface phenotype and MHC restriction. Each clone would also be analysed for the production of lymphokines.

CHAPTER 2.

MATERIALS AND METHODS.

2.1. ANIMALS.

2.1.1. Mice.

BALB/c mice aged 8-14 weeks, and of either sex, and male CBA mice, aged 5-7 weeks, were kindly gifted by Dr. F.Y. Liew, Wellcome Biotech Ltd., Beckenham, Kent. Male C57BL/6 mice, aged 8 weeks, were obtained from Harlan Olac Ltd., Oxon.

Within each experiment, all mice were age- and sex-matched.

2.1.2. Rats.

Young adult male Sprague-Dawley rats were obtained from Harlan Olac Ltd., Oxon. These were used in the production of T cell growth factor (see Section 2.15.).

2.2 CELL LINES.

2.2.1. BHK cell line.

BHK cells (baby hamster kidney cells) were grown as monolayer cultures in Dulbecco's Modified Eagles medium (DMEM; Gibco, Paisley, Scotland) supplemented with penicillin, streptomycin, 2 mM L-glutamine and 10% heat-inactivated (HI) foetal calf serum (FCS; Gibco). During routine culture, cells were split twice a week into fresh medium.

FCS was inactivated by heating to 56° C for 40-45 minutes in a water bath, then stored at -20° C until required.

2.2.2. Vero cell line.

Vero cells (African green monkey kidney cells) were grown as monolayer cultures in RPMI-1640 medium (Gibco) supplemented with penicillin, streptomycin, 2mM L-glutamine and 10% HI FCS. These cells were sub-cultured once a week into fresh medium.

2.2.3. MLA-144 cell line.

MLA-144 cells are an IL-2 producing T cell line established from a gibbon lymphosarcoma (Rabin *et al.*, 1981) and were the kind gift of Dr R. Lelchuk, Wellcome Biotech Ltd., Beckenham, Kent. These were maintained in RPMI-1640 medium plus penicillin, streptomycin, 2mM L-glutamine, 2×10^{-5} M beta-mercaptoethanol and 10% HI FCS. During routine culture, cells were split 1/10 twice a week.

MLA cell-free supernatant was used to support the growth of the CTL-L cell line (see 2.2.5.). To obtain cell-free supernatant, the MLA cells were grown in suspension until 100-300ml were obtained. The cells were then washed, resuspended in serum-free medium at 5×10^6 cells per ml and incubated for 24 hours at 37° C in 5% CO_2. The supernatant was then removed, spun at 3500rpm for 15 minutes and millipored through a 0.22µm filter (Flow Labs., England). The supernatant was aliquoted and stored at -20° C until used.

2.2.4. WEHI-3 cell line.

The WEHI-3 cell line is an IL-3 producing BALB/c leukaemic line, possibly of lymphocytic origin (Lee et al., 1982), also obtained from Dr R. Lelchuk. The cells were maintained in RPMI-1640 medium, supplemented with penicillin, streptomycin, 2mM L-glutamine, 2×10^{-5}M beta-mercaptoethanol and 10% HI FCS. During routine culture, cells were split twice a week into fresh medium.

WEHI-3 cell-free supernatant was used to maintain growth of the 32-D cell line (see Section 2.2.6.). The generation of supernatant was based on the method described by Curtsinger and Fan, (1984). Essentially, WEHI-3 cells were grown in bulk until 300-500ml were obtained. The cells were washed, resuspended in serum-free medium at 1×10^{6} cells/ml and incubated at 37° C for 48 hours. The supernatant was collected, spun at 3500rpm for 15 minutes, millipored through a 0.22µm filter (Flow Labs., England) and stored at -20° C until used.

2.2.5. CTL-L cell line.

The CTL-L cell line, derived from C57BL/6 tumour specific cytotoxic T cells, is an IL-2 dependent cell line first described by Gillis and Smith, (1977) and kindly supplied by Mr G. Wallace, University College, London. The cells were grown in RPMI-1640 medium plus penicillin, streptomycin, 2mM L-glutamine, 2×10^{-5}M beta-mercaptoethanol, 10% HI FCS and 25% MLA-144 cell-free supernatant. The cells were normally split 1/10, three times a week, into fresh medium. However, for use in IL-1 and IL-2 detection assays, the cells were split 1/5 on the previous day.

2.2.6. 32-D cell line.

The 32-D cell line is an IL-3 dependent murine cell line, derived from C3H/HeJ bone marrow cultures, (Ihle et al., 1982.) and supplied by Dr R. Lelchuk. 32-D cells were maintained in RPMI-1640 medium containing penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M 2-mercaptoethanol, 10% HI FCS and 10% WEHI-3 cell-free supernatant. The cells were split 1/10, twice a week, into fresh medium. For use in IL-3 detection assays, cells were split 1/5 on the previous day.

2.2.7. EL-4 NOB-1 cell line.

The EL-4 NOB-1 cell line, a subclone of the murine EL-4 thymoma line, produces high levels of IL-2 in response to IL-1 (Gearing et al., 1987) and was kindly supplied by Mrs. E. Abdulla, Wellcome Biotech Ltd., Beckenham, Kent. The cells were grown in RPMI-1640 medium supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M 2-mercaptoethanol and 10% HI FCS. The cell line was split 1/5 twice a week.

2.2.8. WEHI-279 cell line.

The WEHI-279 cell line, a murine B cell lymphoma (Sibley et al., 1980), is used in the detection of IFN-γ and was the gift of Dr J.Tite, Wellcome Biotech Ltd., Beckenham, Kent. The line was grown in RPMI-1640 plus penicillin, streptomycin, 2mM L-glutamine, 2×10^{-5}M 2-mercaptoethanol and 10% HI FCS. The cells were split 1/10 twice a week into fresh medium.

2.3. VIRUS STOCKS.

2.3.1. Virus strains.

The following herpes simplex virus (HSV) type 1 and 2 strains were used:-

HSV type 1 strain Krueger [HSV-1(Krueger)].

HSV type 1 strain KOS [HSV-1(KOS)].

HSV type 2 strain 333 [HSV-2(333)].

All HSV strains used were prepared and stored in the same way. Unless stated otherwise, either in the Materials and Methods or the Figure Legends, all stocks of HSV-1, gB-1 and pgB-1 used were derived from HSV-1(Krueger).

2.3.2. Virus preparation.

Virus stocks were prepared from BHK cell monolayers infected at a multiplicity of infection (M.O.I.) of 0.1 plaque-forming units (PFU) of virus/cell. The virus was allowed to absorb to the cells for 1 hour at 37°C, the cells washed once and fresh DMEM + 10% HI FCS added. Incubation was continued for 48-72 hours, until clear cytopathic effects were seen, i.e. cells rounding up and becoming non-adherent. The cells were then resuspended, pelleted by centrifugation, washed in serum-free medium and disrupted by ultrasonication on ice, in a small volume of serum-free medium, for 1 minute. The resulting lysate was pelleted by centrifugation at 3500rpm for 15 minutes, resuspended in 1-2ml of serum-free DMEM and 200µl aliquots stored at -70° C until required.

2.3.3. Determination of viral titre.

Virus stocks were titrated on newly confluent monolayers of Vero cells grown in 24-well flat bottomed plates (Corning, England). Virus stocks were serially diluted 10-fold until a dilution factor of 10^{-5} was reached, and then 5-fold dilutions were used. Most of the medium covering the Vero cells was removed and 100µl of each viral dilution was added, in triplicate, to the cells. The virus was allowed to absorb for 2 hours at 37° C, then removed and the cells covered with RPMI + 10% HI FCS + 0.6% carboxymethylcellulose (CMC; BDH Chemicals Ltd., England.). After 72 hours incubation at 37° C in 5% CO_2, the RPMI + 10% HI FCS + 0.6% CMC was removed and the cells fixed and stained using crystal violet stain dissolved in methanol (10% w/v), formaldehyde and distilled water. After 30 minutes, the stain was washed off and the plates air dried.

Viral plaques are seen as clear holes against a purple background. The plaques found in the lowest viral dilution are counted and the number of plaque forming units (PFU) per ml are calculated. The virus titre was usually 1×10^{8} - 4×10^{9} PFU/ml, depending on the virus strain used.

2.3.4. Heat-inactivation of virus.

Virus stocks were, when required, inactivated by heating in a water bath at 56° C for 1 hour. Such stocks are designated HI HSV. After heat inactivation, the virus was stored in small aliquots at -20° C.

2.3.5. UV-inactivation of virus.

HSV-1(Krueger) stocks were also inactivated using a UV-light source. Viral stocks were diluted in Click's medium (Irvine Scientific, California, USA) at 1×10^{8} PFU/ml in a 45mm petri dish. The diluted virus was placed 7.2cm from the UV source and irradiated for 12 minutes. 500μl aliquots were stored at -70° C until required. Such stocks are designated UV-HSV-1.

UV-inactivation reduced the infectious virus titre by approximately 10^{5} PFU/ml.

2.4. PREPARATION OF A GLYCOPROTEIN B-SPECIFIC IMMUNOAFFINITY COLUMN.

2.4.1. HSV-1 glycoprotein B-specific monoclonal antibody.

The HSV-1 glycoprotein B (gB-1) specific monoclonal antibody (MAb) TI57, an IgG2b MAb, has already been characterized (La Thangue & Chan, 1984; Chan *et al.*, 1985).

2.4.2. Purification of TI57 immunoglobulin.

TI57 immunoglobulin was purified using a staphylococcal protein A-Sepharose column (PAS; Pharmacia Ltd., England) according to the method of Ey *et al.* (1978). The immunoglobulin was adjusted to pH 8.25 using 0.1M Na_2HPO_4, loaded onto the PAS column and allowed to sit at room temperature for 1 hour to let the antibody bind to the column, then washed extensively with phosphate-buffered saline (PBSA). The immunoglobulin was eluted at pH 3.5 using citrate buffer, and immediately neutralized by dripping into 1M Tris HCl, pH 9.6. The immunoglobulin was then dialyzed overnight at 4° C against 0.1M carbonate buffer, pH 9.5.

2.4.3. Coupling of TI57 to Sepharose CL4B.

The purified TI57 was coupled to Sepharose CL4B (Pharmacia Ltd., England) using sodium metaperiodate activation, based on the method of Wilson and Nakane (1976). Essentially, Sepharose CL4B was added to a solution of sodium metaperiodate (100mg per ml of distilled water) at a concentration of 2ml of solution per gram of Sepharose and stirred for 4 hours. The gel was washed with saline, then 10% aqueous ethanediol (ethylene glycol; BDH Chemicals Ltd., England) was slowly run through it. Finally, the gel was washed with 0.1M sodium carbonate buffer, pH 9.5.

The activated Sepharose was added to the TI57 immunoglobulin solution and mixed for 18 hours at 4° C. The Sepharose was then washed, dried, transferred into an aqueous solution of sodium borohydride (5mg/ml; BDH Chemicals Ltd., England) and mixed for 2 hours. Finally, the gel was thoroughly washed with PBSA and packed into a 10-ml column. Before use, the column was extensively washed with PBSA.

The protein concentration of the MAb was measured before and after coupling to determine the amount of antibody bound to the column.

2.5. GLYCOPROTEIN STOCKS.

2.5.1. Preparation of glycoprotein stocks.

Glycoprotein stocks of HSV-1 and HSV-2 (gP-1 and gP-2) were each prepared in the same way. BHK cell monolayers were infected with virus at an M.O.I. of 2.0 PFU/cell. The virus was allowed to absorb for 1 hour, the cells washed once and fresh DMEM + 10% HI FCS added to each flask. The infected cells were incubated at 37° C in 5% CO_2 for 48 hours until clear cytopathic effects were seen, i.e. cells rounding up and becoming non-adherent. The cells were harvested, washed to remove the medium, and disrupted by ultrasonication for 1-1.5 minutes in extraction buffer at 2ml per flask, i.e. 1% Nonidet P-40 in 150mM sodium chloride, 25 mM Tris HCl buffer, pH 8.0, containing 1% aprotinin, 1mM tosyl-lysyl-chloromethylketone (TLCK), 1mM *p*-hydroxymercuribenzoate, 1mM phenylmethylsulphonylfluoride (PMSF), 10mM EDTA and 2mM EGTA. It is important to add the above protease inhibitors to minimise proteolytic cleavage during extraction (Pereira et al., 1982; Zezulak & Spear, 1984b). The cell lysate was centrifuged at 100,000g, 4° C for 1 hour, the supernatant collected and aliquots stored at -20° C until required.

2.5.2. Preparation of precursor glycoprotein stocks.

Precursor glycoprotein stocks of HSV-1 and HSV-2 (pgP-1 and pgP-2) were grown and prepared using the same method as given in Section 2.5.1. However, 1 hour after infection, the antibiotic tunicamycin (Sigma, U.K.), which blocks glycoprotein synthesis, was added to the infected cells at a concentration of 0.5µg/ml of medium (Carter et al., 1981).

2.5.3. Immunopurification of glycoprotein B and precursor glycoprotein B.

Purification of HSV-1 glycoprotein B (gB-1) and its precursor form (pgB-1) was performed using the method described by Chan (1983); Chan et al., (1985).

Infected cell extracts were applied to the Sepharose CL4B-TI57 monoclonal antibody column (see Section 2.4.3.). The extract was allowed to absorb to the column for 2-4 hours at 4° C, then the column was sequentially washed with:-

(1) PBSA overnight - approximately 10 column volumes.

(2) 25mM Tris HCl, pH 8.0, 50mM sodium chloride, 0.5% Nonidet P-40 and 0.5% sodium deoxycholate - 4-5 column volumes.

(3) 50mM Tris HCl, pH 6.8 - 4-5 column volumes.

(4) 9M ethanediol, 0.3M sodium chloride and 0.0075M sodium phosphate, pH 7.4 - 3-4 column volumes.

The gB or pgB was eluted at pH 3.0 with 9M ethanediol, 0.3M sodium chloride and 0.1M citric acid - 8-10 column volumes. To prevent protein degradation, the eluted protein was returned to pH 7.0 by dripping into 1M Tris HCl buffer, pH 9.6.

The eluted proteins were dialyzed overnight against 0.02M sodium phosphate buffer, pH 7.4 and then extensively against 0.002M sodium phosphate buffer. The proteins were lyophilized, resuspended in distilled water and the protein concentration determined by optical density at 280nm. Aliquots were stored at -20° C until required.

2.6. IODINATION OF RABBIT ANTI-MOUSE IMMUNOGLOBULIN.

Iodination of rabbit anti-mouse immunoglobulin (^{125}I-RαMIg) was carried out using the Chloramine-T method. Free iodine-125 was obtained from Amersham, Bucks. The iodination was kindly done for me by Dr W.L. Chan, Guy's Hospital Medical School. ^{125}I incorporation into the protein was determined by TCA precipitation. Briefly, 10µl of iodinated protein was diluted in 0.5ml PBSA + 10% normal calf serum. 0.5ml cold trichloroacetic acid (TCA) was added, the sample left on ice for 30 minutes and then microfuged for 5 minutes. The supernatant and the pellet were separated and each was counted in a gamma-counter.

% incorporation = c.p.m. of pellet/total c.p.m. x 100%.

2.7. SDS-PAGE AND WESTERN BLOTTING.

2.7.1. Preparation of gP-1 and pgP-1 samples.

HSV-1 glycoprotein (gP-1) and precursor glycoprotein (pgP-1) samples were prepared from BHK-infected cells as previously described (see Sections 2.5.1. and 2.5.2.). However, concentrated samples were prepared for electrophoresis. Briefly, gP-1 and pgP-1 were each prepared from one infected flask and the cells were harvested into 1ml of extraction buffer before sonication. The cell lysates were then spun in an Eppendorf centrifuge for 30 minutes at 4° C, the supernatant removed and aliquots stored at -20° C until required.

2.7.2. SDS-PAGE and Western blotting.

gP-1 and pgP-1 samples were reduced by boiling for 3 minutes in sample buffer containing beta-mercaptoethanol. The samples, along with pre-stained molecular weight markers (BRL, England), were electrophoresed in a 7% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) at 55mA for 2-3 hours.

After electrophoresis, the gel was transblotted overnight onto a nitrocellulose membrane at a current of 45mA, increased to 240mA for 1 hour the next morning. The blot was removed and washed extensively in 5% Marvel, 0.01% sodium azide in distilled water. Further washes used 0.5% Tween 20 in 150mM NaCl, 50mM Tris HCl, pH 7.5 (TSB buffer). The antibodies were diluted in 1% Marvel, 0.01% sodium azide in TSB. The gB-specific monoclonal antibody, TI57, was used in the first layer and ^{125}I-rabbit anti-mouse immunoglobulin in the second layer. Both antibodies were incubated overnight at 4° C. The blot was dried, exposed to Fuji RX X-ray film and left at -70° C for 6 days before developing.

2.8. PREPARATION OF NORMAL MOUSE SERUM.

Normal BALB/c mice were killed by cervical dislocation and bled by cardiac puncture. The blood was left at room temperature until it clotted, spun in an Eppendorf centrifuge for 4 minutes and the serum collected. The serum was heat-inactivated at 56° C for 30 minutes, aliquoted and stored at -20° C until required.

2.9. T CELL PROLIFERATION ASSAYS.

2.9.1. Priming.

In all T cell proliferation experiments, the mice were immunized subcutaneously (s.c.) in the hind footpads or in the hind footpads and the base of the tail. The mice were primed with HI HSV-1 at 5×10^6 PFU/mouse; ovalbumin and the glycoproteins were used at 30μg/mouse. In early experiments, the antigens were emulsified with an equal volume of Freund's complete adjuvant (FCA). However, in later experiments, this was changed to aluminium hydroxide (alum; 2mg/ml). Equal volumes of alum and antigen were mixed and left on ice for several hours before injection.

In some experiments, the mice were boosted 14 days after priming with antigen but no adjuvant was used. The proliferation assays were set up 5 days after boosting.

2.9.2. Proliferation assays.

The draining lymph nodes (DLN) were removed from young adult BALB/c mice at various times after priming or 5 days after boosting. The DLN removed depended on the injection sites used:-

(i) Hind footpads only - Popliteal lymph nodes.

(ii) Hind footpads and base of tail - Popliteal, para-aortic and inguinal lymph nodes.

Lymph node suspensions were plated out at $4x10^5$ cells/well in 96-well flat-bottomed tissue culture plates in 100µl Click's EHAA medium (Click's Extra High Amino Acids; Irvine Scientific, USA) supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 0.5% normal mouse serum (NMS). Antigens, (as indicated in the Figure Legends), or medium alone were incubated, in triplicate, in 100µl of medium per well for 96 hours at 37°C in 5% CO_2, pulsed with 1µCi/well [^{3}H]-thymidine (Amersham, Bucks.) for 18 hours and harvested with a cell harvester onto glass fibre paper. The glass fibre discs were dispensed into vials, 0.5ml scintillation fluid added (Optiscint "Hi-Safe"; LKB, England) and counted in a beta-counter.

Cell proliferation is expressed as S.I. (stimulation index = [c.p.m. with antigen]-[c.p.m. without antigen]).

2.10. RADIOIMMUNOASSAY FOR VIRAL-SPECIFIC ANTIBODIES.

The levels of HSV-1-specific antibody in the serum of mice immunized with various antigens (see Figure Legend 3.2.7.) were assayed in a solid-phase radioimmunoassay (RIA). U-bottomed flexiplates (Becton Dickinson, U.K.) were coated with HI HSV-1 (8.0×10^{7} PFU per ml; 50µl/well) and left at room temperature (RT) overnight. After washing with PBSA, the wells were blocked with 1% bovine serum albumin (BSA) in PBSA at RT for 2-3 hours. The samples were serially diluted in 10% BSA in PBSA and, after washing the plates with PBSA, were plated out, in triplicate, at 50µl per well and incubated at RT for 1 hour. The plates were washed with PBSA + 0.05% Tween 20, followed by PBSA alone, then the ^{125}I-rabbit anti-mouse immunoglobulin was added (1×10^{8} c.p.m. per ml; 50µl per well), diluted in 10% BSA in PBSA, and incubated at 4°C overnight. After extensive washing, each well was counted in a gamma-counter.

Results are expressed as c.p.m. ± SEM $\times10^{3}$.

2.11. DETERMINATION OF THE LD_{50} IN CBA MICE.

7 week old male CBA mice were used to determine the LD_{50} of HSV-1, i.e. the dose necessary to kill 50% of the mice. The virus was serially diluted 1/5 and the mice infected subcutaneously in the hind footpads (50µl per mouse; 5 mice per dilution). The mice were observed for visible signs of infection and for mortality over the following 3 weeks. All deaths, except one, occurred within the first 11 days.

The LD_{50} was calculated to be 3.2×10^5 PFU/mouse.

In the subsequent protection experiments, it was essential to use the same batch of HSV-1 and to infect the CBA mice at exactly 7 weeks of age, as the susceptibility of the mice to infection was extremely sensitive to factors such as age and ambient temperature (Chan et al.,1985).

2.12. ACTIVE PROTECTION AGAINST HSV-1 INFECTION.

5 week old male CBA mice were immunized subcutaneously (s.c.) in 4 sites in the flanks with HI HSV-1 at 5×10^6 PFU/mouse; ovalbumin, gB-1 or pgB-1 at 30µg/mouse. The antigens were mixed with an equal volume of aluminium hydroxide (alum; concentration = 2mg/ml) and incubated on ice for 2-3 hours before injection. 10 days later, all the groups were boosted with the same amount of antigen, but without the use of alum. At 7 weeks old, the mice were infected s.c. in the hind footpads with a dose of HSV-1 10x

LD_{50}, i.e. 3.2×10^6 PFU/mouse, in 50µl PBSA. The mice were observed daily for visible signs of infection and for mortality until 60 days after infection.

2.13. PRODUCTION OF LYMPHOKINE SUPERNATANTS.

2.13.1. Lymphokine production after sublethal infection.

Adult BALB/C mice were immunized subcutaneously (s.c.) in 4 sites in the flanks with HI HSV-1 at 5×10^6 PFU/mouse; ovalbumin or gB-1 at 30µg/mouse. The antigens were emulsified in an equal volume of Freund's complete adjuvant (FCA). Two weeks after priming, the mice were boosted with the same amount of antigen, but without using FCA. Five days later, the mice were infected s.c. in the hind footpads with 1.5×10^6 PFU HSV-1 per mouse. Due to the age and strain of mice involved, (Sprecher & Becker, 1987), this was a sublethal dose of virus.

On days 0, 2, and 6 post infection (p.i.), the spleen and the draining lymph nodes (DLN; popliteal, para-aortic and inguinal lymph nodes) were removed from 2 or 3 mice per group. For the culture system, see section 2.13.3.

2.13.2. Lymphokine production after lethal infection.

Male CBA mice were immunized and infected in a similar manner to that used for the protection experiments (see Section 2.12.). At 5 weeks old, the mice were immunized subcutaneously (s.c.) in 4 sites in the flank with HI HSV-1 at $5x10^6$ PFU per mouse; ovalbumin, gB-1 or pgB-1 at 30μg/mouse. These antigens were mixed with an equal volume of aluminium hydroxide (alum; concentration = 2mg/ml). 10 days later, all groups were boosted with the same amount of antigen, but without the use of alum. At 7 weeks of age, the mice were infected s.c. in the hind footpads with a dose of HSV-1 5x LD_{50}, i.e. $1.6x10^6$ PFU/mouse.

On days 0-16 post infection, at 4 day intervals, the spleens were removed from 2 or 3 mice per group. For the culture system, see Section 2.13.3.

2.13.3. Culture system for the production of lymphokines.

The pooled spleens and the pooled DLNs from sublethally infected BALB/c mice, or the spleens from individual lethally infected CBA mice were homogenized, washed, and resuspended at $5x10^6$ cells/ml in RPMI-1640 medium supplemented with penicillin, streptomycin, 2mM L-glutamine and $2x10^{-5}$M beta-mercaptoethanol. The cells were plated out in 24-well flat-bottomed tissue culture plates (Corning, U.K.) at 1ml/well, i.e. $5x10^6$ cells/well. The wells were restimulated with $6x10^6$ PFU HI HSV-1 per well in 50μl of medium or with 50μl of medium alone.

The cultures were incubated at 37°C in 5% CO_2 for either 24 or 48 hours, depending on which lymphokine the supernatant was to be tested for, i.e.

(i) IL-2 and IL-3 - 24 hours in vitro culture.

(ii) IL-1 and IFN-γ - 48 hours in vitro culture.

Cell-free supernatants, 800µl/well, were collected and aliquots stored at -70°C until assayed.

2.14. LYMPHOKINE ASSAYS.

2.14.1. Assay to detect IL-2.

Cell-free supernatants, generated as described in Section 2.13.3., were assayed on the IL-2 dependent cell line CTL-L (see Section 2.2.5.). All assays were set up in 96-well flat-bottomed tissue culture plates. CTL-L cells were plated out at $1x10^4$ cells per well in 100µl of RPMI-1640 medium supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 10% HI FCS. Supernatant samples, collected 24 hours after in vitro culture, and controls were added, in triplicate, at 100µl per well. Cells cultured with medium only were used as the negative control, with serial dilutions of MLA cell supernatant or mouse recombinant IL-2 (rIL-2; Genzyme) used as a positive control. Cultures were incubated at 37°C in 5% CO_2 for 24 hours, pulsed with 1µCi/well [^{3}H]-thymidine (Amersham, Bucks.) for 18 hours then harvested onto glass fibre paper. The glass fibre discs were dispensed into vials, 0.5ml of scintillation fluid (Optiscint "Hi-Safe"; LKB, England) added to each and counted in a beta-counter.

Results are expressed either as c.p.m. ± SEM $x10^3$ or as units of IL-2 per ml (U/ml) which were calculated from a rIL-2 standard curve.

2.14.2. Assay to detect IL-3.

The cell-free supernatants were assayed on 32-D cells, an IL-3 dependent cell line (see Section 2.2.6.). All assays were set up in 96-well flat-bottomed plates. The 32-D cells were plated out at $2x10^4$ cells/well in 100µl of RPMI-1640 medium supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 10% HI FCS. Supernatant samples, from 24 hour in vitro cultures, and controls were added, in triplicate, at 100µl per well, with medium only as the negative control and serial dilutions of either WEHI-3 cell supernatant or mouse recombinant IL-3 (rIL-3; Genzyme) as the positive control. Cultures were incubated for 24 hours at 37°C in 5% CO_2, pulsed with 1µCi/well [^{3}H]-thymidine for 18 hours, harvested onto glass fibre paper and dispensed into vials. 0.5ml scintillation fluid was added to each to each vial and then counted in a beta-counter.

Results are expressed as either c.p.m. ± SEM $x10^3$ or as units of IL-3 per ml, based on a standard curve using rIL-3.

2.14.3. Assay to detect IL-1.

To detect IL-1, the supernatant samples were assayed on the IL-1 responsive cell line EL-4 NOB-1 (see Section 2.2.7.) and the IL-2 dependent line CTL-L, based on the method of Gearing et al., (1987). Briefly, the addition of IL-1 to these cultures induces the EL-4 NOB-1 cells to produce IL-2, which stimulates the proliferation of the

CTL-L cells. However, as the EL-4 NOB-1 cells are pretreated with mitomycin C, they do not proliferate, so any cell proliferation measured is due only to the CTL-L cells.

EL-4 NOB-1 cells were treated with mitomycin C (Sigma, U.K.) at a concentration of 33μg/5x10^6 cells for 30 minutes at 37°C. After extensive washing, the EL-4 NOB-1 cells were plated out at 2x10^5 cells/well in 100μl of medium along with the CTL-L cells at 4x10^3 cells/well in 50μl of medium in 96-well flat-bottomed plates. The medium used throughout these assays was RPMI-1640 plus penicillin, streptomycin, 2mM L-glutamine, 2x10^{-5}M beta-mercaptoethanol and 5% HI FCS. (The amount of FCS used was reduced as this lowered the background number of counts.) Supernatant samples, collected after 48 hours in vitro culture, and the controls were added, in triplicate, at 50μl per well. The negative control was medium only and the positive control was serial dilutions of mouse recombinant IL-1 (rIL-1). Cultures were incubated for 24 hours at 37°C in 5% CO_2, pulsed with 1μCi/well [^{3}H]-thymidine for 18 hours and harvested onto glass fibre paper. The glass fibre discs were dispensed into vials, 0.5ml scintillation fluid added to each and the vials counted in a beta-counter.

Results are expressed as c.p.m. ± SEM x10^3 or as units of rIL-1 per ml (U/ml) calculated from an IL-1 standard curve.

2.14.4. Assay to detect IFN-γ .

To detect IFN-γ , the cell-free supernatants were assayed on the WEHI-279 cell line (see Section 2.2.8.), based on the method of Reynolds et al. (1987). In the presence of IFN-γ , proliferation of this cell line is inhibited.

The cells were plated out in 96-well flat bottomed plates at $2x10^3$ cells/well in 100μl of RPMI-1640 medium plus penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 10% HI FCS. The supernatant samples, collected after 48 hours in vitro culture, and the controls were plated out, in triplicate, at 100μl/well. Medium only was used as the negative control and serial dilutions of mouse recombinant IFN-γ (rIFN-γ) as the positive control. Cultures were incubated at 37°C in 5% CO_2 for 72 hours, pulsed with $[^3H]$-thymidine for 18 hours and harvested onto glass fibre paper. The discs were put into vials, 0.5ml scintillant added to each and counted in a beta-counter.

Results are expressed as c.p.m. ± SEM $x10^3$.

2.14.5. Titration of lymphokine-containing supernatants.

In some experiments, the cell-free supernatants were titrated to ensure that the effect seen was lymphokine-specific and not due to any non-specific factors.

For each lymphokine tested, the culture system was as already described (Sections 2.14.1-4.) and all assays were set up in triplicate. For each sample to be tested, the supernatant was diluted by serial 2-fold dilutions in medium and then plated onto the cells at 100μl per well. Pulsing and harvesting was as described above.

Results are expressed as c.p.m. ± SEM $x10^3$.

2.15. PRODUCTION OF T CELL GROWTH FACTOR.

The methods of preparing and testing the T cell growth factor are based on those derived by Gillis *et al.*, (1978).

2.15.1. Preparation of T cell growth factor.

TCGF was prepared from Concanavalin A (Con A) stimulated rat spleen cells as follows.

Ten young adult male Sprague-Dawley rats were killed by CO_2 inhalation and cervical dislocation. The spleens were removed, homogenized, washed, and resuspended at $5x10^6$ cells/ml in RPMI supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 5% HI FCS. Con A (Sigma; U.K.) was added to the spleen cells at 2.5µg/ml and cultured at 37°C in 5% CO_2 for 48 hours. The supernatant was then pooled and spun in a Sorvall ultracentrifuge at 8000 rpm for 30 minutes to remove any remaining cells. Methyl α-D-mannopyranoside (Sigma, U.K.) was added at 1g per 50ml of supernatant, to neutralize the effects of any residual Con A. The pooled supernatant was then aliquoted into 100ml bottles and stored at -20°C. Before use, each bottle of TCGF was millipored through a 0.22µm filter (Flow Labs., England) and further aliquoted into 20ml universals.

2.15.2. Testing the TCGF.

Before use, the actvity of the TCGF was tested on CTL-L cells. The CTL-L cells were plated out in a 96-well flat-bottomed tissue culture plate at $1x10^4$ cells/well in 100µl RPMI plus penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 10% HI FCS. The TCGF was serially diluted 1/2 in medium and plated out, in triplicate, at 100µl per well. Medium alone was used as the negative control and MLA-144 cell supernatant, also serially diluted 1/2 in medium, was used as the positive control. The cultures were incubated at 37°C in 5% CO_2 for 24 hours, pulsed with 1µCi/well [^{3}H]-thymidine for 18 hours and harvested with a cell harvester onto glass fibre paper. The harvested discs were placed in vials, 0.5ml scintillant added and the vials counted in a beta-counter.

The level of proliferation obtained with the TCGF was greatest at a dilution of 1/4 (data not shown). The proliferation achieved then reached a plateau until the much lower dilution of 1/32. Therefore it was decided to use the TCGF at a concentration of 1/10, i.e. 10%.

2.16. THE DEVELOPMENT AND MAINTAINANCE OF T CELL LINES AND CLONES.

2.16.1. Immunization and setting-up of T cell flasks.

BALB/c mice were immunized subcutaneously in the hind footpads and base of tail with gB-1 at 30µg/mouse. The gB-1 was emulsified in an equal volume of Freund's complete adjuvant (FCA). 14 days after priming, the mice were each boosted with 30μg of gB-1, but FCA was not used. 5 days later, the mice were killed by cervical dislocation and the draining lymph nodes, i.e. the popliteal, para-aortic and inguinal lymph nodes, were removed. The lymph nodes were homogenized and a single cell suspension prepared of $1x10^7$ cells/ml in Click's EHAA medium (Irvine Scientific, USA) supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 0.5% NMS. The cells were set up as 5ml upright cultures in $25cm^2$ flasks (Sterilin, England) and restimulated with UV-inactivated HSV-1 (UV-HSV-1) at $5x10^7$ PFU per flask. The flasks were then fed regularly with medium for 10 days before being restimulated.

To restimulate the flasks, the spleens were removed from 2 normal BALB/c mice, homogenized and the erythrocytes removed by hypo-osmotic shock, i.e. the cells were pelleted, 900μl of sterile, distilled water was added to the cells followed, a few seconds later, by 100μl of 10-times PBSA. The remaining cells were incubated with UV-HSV-1 at an M.O.I. of 0.5 per $1x10^8$ spleen cells, i.e. $5x10^7$ PFU of virus per 10^8 cells, in a final volume of 500μl for 1 hour at 37°C. The cells were then treated with mitomycin C (Sigma, U.K.) at 20μg per 10^7 cells in a final volume of 1ml PBSA per 10^7 cells. The splenocytes were incubated with the mitomycin C at 37°C for 30-45 minutes, then extensively washed with PBSA to remove all traces of mitomycin C and resuspended at 10^8 cells per ml in Click's + 0.5% NMS. These antigen presenting cells (APC) were added to the T cell flasks at $2x10^7$ APC per flask.

The flasks were restimulated weekly with APC and fed with Click's + 0.5% NMS in between restimulation for the first 2 weeks to reduce the number of non-specific T cells produced. The flasks were then tested and found to be viral specific (data not shown). The medium was then altered to Click's supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol, 10% HI FCS and 4% TCGF.

2.16.2. Setting up and maintaining T cell lines.

After the specificity of the T cell flasks was ascertained and the lines established for 6-8 weeks, the cells were cloned out at 30 cells/well. This does not lead to pure T cell clones, but does give rise to T cell lines.

Cloning was set up in 96-well U-bottomed tissue culture plates (Flow Labs., England) 5-6 days after the cells were restimulated. Antigen presenting cells (APC) were prepared in the same manner as described above (Section 2.16.1.). Briefly, a spleen cell suspension was prepared from the spleens of normal BALB/c mice. The spleens were homogenized, erythrocytes removed by hypo-osmotic shock and the cells infected with UV-HSV-1, at an M.O.I. of 0.5 PFU per APC, in a volume of 500µl for 1 hour at 37°C. The cells were then treated with mitomycin C at a concentration of 20µg per 10^7 cells, in a total volume of 1ml PBSA per 10^7 cells, at 37°C for 30-45 minutes. The cells were washed to remove the mitomycin C and resuspended at 1×10^8 cells/ml in Click's medium supplemented with penicillin, streptomycin, 2mM L-glutamine, 2×10^{-5}M beta-mercaptoethanol, 10% HI FCS and 4% TCGF. The APC were plated out at 2×10^5 cells per well in 100µl of medium.

T cells from the flasks were washed and resuspended to give 30 T cells per well, i.e. 30 cells per 100µl = 3×10^3 T cells per 10ml. The T cells were plated onto the APC and fed regularly with Click's + 10% HI FCS + 4% TCGF. After 10 days, the cells were restimulated with APC and UV-HSV-1, and restimulated every week after that. After about 4 weeks, clumps of larger, refractory, dividing cells could be seen in a large number of wells. These wells were restimulated, fed and, eventually, some were expanded into 24-well plates.

2.16.3. Expansion of T cell lines into 24-well plates.

APC were prepared as previously described, Sections 2.16.1 & 2, and plated out in 24-well tissue culture plates (Corning, U.K.) at 5×10^6 cells/well in 500µl Click's + 10% HI FCS and 4% TCGF. The T cells were resuspended in the 96-well plates and 200-400µl of cells transferred to a well in the 24-well plate. The wells were fed regularly with medium and restimulated every 7-12 days with APC and UV-HSV-1 at 2×10^6 APC/well. The amount of TCGF used was increased to 10%

when the T cell growth was found to be slow. The T cells were expanded into 5 or 6 wells, tested for their specificity and then expanded into flasks.

2.16.4. Expansion of T cell lines into flasks.

The T cell lines were finally expanded into 25cm^2 flasks (Sterilin, U.K.). The APC and UV-HSV-1 were prepared as before and used at 1x10^7 cells per flask in Click's plus 10% HI FCS and 10% TCGF. The T cells in the 24-well plates were resuspended and 500-800µl of T cells transferred to each flask. The total volume of each flask was 2-3ml. The flasks were fed regularly with medium and restimulated with APC and UV-HSV-1, at 10^7 APC per flask, every 7-12 days.

2.16.5. Development of T cell clones.

T cell cloning was carried out from 3 T cell lines. The method used was essentially the same as previously described for the T cell lines (see Section 2.16.2.). The BALB/c antigen presenting cells (APC) with UV-HSV-1 were prepared as already described and plated out in 96-well U-bottomed tissue culture plates at 2x10^5 cells per well in 100µl of Click's medium supplemented with penicillin, streptomycin, 2mM L-glutamine, 2x10^{-5}M beta-mercaptoethanol, 10% HI FCS and 10% TCGF. T cells were taken from the 24-well plates or flasks, washed and resuspended at 1 T cell per well, i.e. 100 T cells per 10ml, and plated out in 100µl of medium per well, onto the APC and UV-HSV-1. The cells were fed regularly with medium for 10 days and then restimulated with APC and UV-HSV-1. The wells were restimulated every 7-12 days and screened for the development of T cell clones.

As the T cell clones grew, the cells were transferred to 24-well plates and then to 25cm^2 flasks using the methods already described. (See Sections 2.16.3.& 4.)

2.17. TESTING THE SPECIFICITY OF T CELL LINES AND CLONES.

The method used was the same for both the T cell lines and the T cell clones. Spleens from normal BALB/c mice were homogenized, the erythrocytes removed and the cells incubated at 37°C with mitomycin C (Sigma, U.K.) at 20µg per 10^7 cells for 30-45 minutes. After extensive washing to remove the mitomycin C, the cells were plated out in 96-well flat-bottomed tissue culture plates at $2x10^5$ cells/well in 50µl of Click's medium supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 0.5% NMS. Antigen, as described in the Figure Legends, or medium alone, was added to the wells, in triplicate, in 50µl of medium and incubated at 37°C for 1 hour.

The T cell lines or clones were washed to remove FCS and TCGF, resuspended in medium and plated onto the APC and antigen at $1x10^4$ T cells per well in 100µl of medium. The cultures were incubated for 48 hours at 37°C in 5% CO_2, pulsed with 1µCi/well [^{3}H]-thymidine (Amersham, Bucks.) for 18 hours and harvested with a cell harvester onto glass fibre paper. The glass fibre discs were distributed into vials, 0.5ml scintillant (Optiscint "Hi-Safe", LKB, England) added per vial and counted in a beta-counter.

The results are expressed as c.p.m. ± SEM and as a stimulation index (S.I. = [c.p.m. with antigen]/[c.p.m. without antigen]).

2.18. T CELL CLONE RESPONSES TO ANTIGEN PRESENTED ON APC OF DIFFERENT HAPLOTYPES.

Spleens from normal BALB/c, CBA and C57BL/6 mice were removed, homogenized, erythrocytes lysed by hypo-osmotic shock and the cells treated with mitomycin C (Sigma, U.K.) at 20μg per 10^7 antigen presenting cells (APC) for 30-45 minutes at 37°C. After extensive washing, the APC were plated out in 96-well U-bottomed plates at $2x10^5$ APC per well in 50μl of Click's supplemented with penicillin, streptomycin, 2mM L-glutamine, $2x10^{-5}$M beta-mercaptoethanol and 0.5% NMS. UV-HSV-1, at an M.O.I. of 5.0 PFU per APC in 50μl of medium was added to the APC in triplicate and incubated at 37°C for 1 hour.

The T cells were washed, resuspended in medium and plated onto the APC and UV-HSV-1 at $1x10^4$ cells/well in 100μl of medium. Cultures were incubated for 48 hours at 37°C in 5% CO_2, pulsed with 1μCi/well [^{3}H]-thymidine for 18 hours and harvested as previously described (see Section 2.17.).

Results are expressed as c.p.m. ± SEM and as a stimulation index (S.I. = [c.p.m. with antigen]/[c.p.m. without antigen]).

2.19. PRODUCTION OF LYMPHOKINES BY THE T CELL CLONES.

2.19.1. Culture system for the production of lymphokines.

The in vitro culture system used to produce lymphokine-containing supernatants was based on the assay system set up by Ertl & Finberg (1984). Essentially, BALB/c spleen cell suspensions were prepared as before and split into 2 groups. One group was incubated with UV-HSV-1 at an M.O.I. of 0.5 PFU per APC in 500µl of Click's medium. The second group was incubated with 500µl of medium only. After 1 hour at 37°C, both groups were treated with mitomycin C at 20µg per 10^7 APC, in a total volume of 1 ml PBSA per 10^7 cells, for 30-45 minutes at 37°C. The cells were then extensively washed, resuspended and plated out in 24-well plates at 5×10^6 APC/well in 500µl of Click's medium supplemented with penicillin, streptomycin, 2mM L-glutamine, 2×10^{-5}M beta-mercaptoethanol and 2% FCS.

The T cell clones were washed to remove FCS and TCGF, resuspended in medium and plated out at 1.5×10^5 T cells per well in 500µl. The cultures were incubated at 37°C in 5% CO_2 for 24 or 48 hours, depending on the lymphokine to be tested:-

IL-2, IL-3 - 24 hours in vitro culture.

IFN-γ - 48 hours in vitro culture.

800µl of cell-free supernatant per well was collected and aliquots stored at -70° C until assayed.

2.19.2. Assays to detect lymphokines.

Cell-free supernatants from the T cell clones were analyzed for the presence of IL-2, IL-3 and IFN-γ. The assays used have already been described in Sections 2.14.1, 2 & 4.

2.20. $CD4^+/CD8^+$ PHENOTYPING OF THE T CELL CLONES.

2.20.1. Monoclonal antibodies.

YTS 191.1 is a rat IgG2b, anti-CD4 monoclonal antibody (MAb). YTS 169.4 is a rat IgG2b, anti-CD8 MAb. Both have been well characterized by Cobbold et al. (1984).

GK1.5 is a rat IgG2b, anti-CD4 MAb (Dialynas et al., 1983).

These MAbs were the kind gift of Dr F.Y. Liew, Wellcome Biotech Ltd., Beckenham, Kent.

2.20.2. Phenotyping of the T cell clones.

The T cell clones were washed to remove FCS and TCGF, resuspended and plated out in 96-well U-bottomed plates at 10^4 cells/well in 50µl of Click's plus 0.5% NMS. The MAb were plated in triplicate, in 50µl of medium, onto the T cells and incubated at 37° C for 45 minutes. Varying concentrations of antibody were used, depending on the experiment (see Figure Legends for details).

BALB/c spleen cell suspensions were treated with mitomycin C (20µg/10^7 cells) at 37°C for 30-45 minutes. After washing, the cells were incubated at 37°C for 1 hour with UV-HSV-1 at an M.O.I. of 0.5 PFU per APC in 500µl of medium. Then, the volume was adjusted to give $2x10^5$ APC per well in 100µl of medium and the APC plus UV-HSV-1 were plated onto the T cells plus MAb. The cultures were incubated at 37°C in 5% CO_2 for 48 hours, pulsed with $[^3H]$-thymidine for 18 hours, harvested as before and counted in a beta-counter.

Results are expressed as c.p.m. ± SEM $X10^3$.

In one experiment, this protocol was reversed. The APC and virus were plated out first and the MAb-treated T cell clones plated on top. See the Figure Legend to Fig. 5.2.2. for details.

2.21. MHC CLASS I/II PHENOTYPING OF THE T CELL CLONES.

2.21.1. Monoclonal antibodies.

MK-D6 is a murine IgG2a, anti-I-A^d MAb (Kappler et al., 1981; Kappler et al., 1982).

Y-3P is a murine anti-I-A MAb which recognises the I-A subregion of various haplotypes, including I-A^b, I-A^p and I-A^s, but does not recognise I-A^d (Janeway et al., 1984). For ease of definition, Y-3P is defined as an anti-I-A^s MAb.

These were also gifted by Dr F.Y.Liew.

2.21.2. Phenotyping of the T cell clones.

BALB/c spleen cells were treated with mitomycin C, 20µg per 10^7 cells, at 37° C for 30-45 minutes. After extensive washing, the APC were resuspended and plated out in 96-well U-bottomed plates at $2x10^5$ APC/well in 50µl Click's plus 0.5% NMS. The MAb MK-D6 or Y-3P were plated onto the APC in 50µl of medium, and incubated at 37° C for 45 minutes. The MAbs were used at varying concentrations, depending on the experiment (see Figure Legends for details). UV-HSV-1, at an M.O.I. of 0.5 PFU per cell in 50µl of medium, was then plated onto the APC and MAb and incubated at 37° C for a further 45 minutes. The T cell clones were washed, resuspended in medium, then dispensed into the wells at 10^4 T cells per well in 50µl of medium. The cultures were incubated at 37° C in 5% CO_2 for 48 hours, pulsed with 1µCi/well [^{3}H]-thymidine, harvested and counted as before.

The results are expressed as c.p.m. ± SEM $X10^3$.

2.22. STATISTICAL ANALYSIS.

The significance of the results were analysed by Student's *t*-test; $p < 0.05$ is considered significant.

CHAPTER 3.

COMPARISON OF T CELL RESPONSES TO GLYCOPROTEIN B AND ITS NON-GLYCOSYLATED PRECURSOR FORM, pgB.

3.1. INTRODUCTION.

The immune response to HSV-1 involves both antibody and cell-mediated immune mechanisms, and the HSV-1 glycoproteins, expressed on the virion and on infected cell membranes, are the major targets of these responses. (For reviews, see Norrild, 1980; Nash, Leung & Wildy, 1985; Spear, 1984, 1985). Glycoprotein B (gB) has been found to be an important target in the host immune response. In mice, antibodies to gB can neutralize viral infectivity in vitro (Glorioso et al., 1984; Snowden et al., 1985) or cause immunocytolysis of virally infected cells (Norrild et al., 1979). gB can also induce helper T cells which are protective against a lethal challenge with HSV-1 (Chan et al., 1985), delayed-type hypersensitivity (Chan et al.,1985; Blacklaws et al., 1987) and cytotoxic T cells (Lawman et al., 1980b; Blacklaws et al., 1987). In man, cytotoxic T cells (Zarling et al.,1986) and specific natural killer cells (Bishop et al., 1986) have been found which recognize gB.

In studying the role of gB in eliciting viral immune responses, several systems have been used including immunopurified gB (Chan et al., 1985) and viral mutants unable to express gB on the infected cell surface (Glorioso et al., 1984). Recently, use has been made of vaccinia virus recombinants (Cantin et al., 1987; McLaughlin-Taylor et al., 1988) and eukaryotic cell lines (Blacklaws et al., 1987;

Rosenthal et al., 1987) which express only gB. In these latter systems, a less glycosylated or unglycosylated precursor of gB has also been expressed. However, it is not clear if the carbohydrate side chains on the mature, fully glycosylated gB are important in the T cell recognition of gB.

The HSV glycoproteins, including gB-1, are known to contain both N-linked and O-linked oligosaccharide side chains, added during post-translational modifications, (reviewed by Campadelli-Fiume & Serafini-Cessi, 1984; Spear, 1984). The presence of N-linked glycans was demonstrated by Wenske et al. (1982) using endo-β-N-acetylglucosaminidase (Endo H), which cleaves N-linked glycans from immature forms of the HSV glycoproteins. Treatment of gB with Endo H resulted in a decrease in molecular weight, as seen by SDS-PAGE, indicative of the cleavage of N-linked carbohydrate chains. The treatment of HSV-infected cells with tunicamycin (TM) was also used to examine N-linked glycosylation on the glycoproteins (Pizer et al., 1980; Norrild & Pedersen, 1982; Peake et al., 1982; Kousoulas et al., 1983). TM blocks the synthesis of glycoproteins, including gB-1, by inhibiting the attachment of N-acetylglucosamine to dolichol phosphate which in turn prevents the addition of the oligosaccharide chains to the protein backbone. TM inhibited both the production of infectious virus particles and the glycosylation of viral glycoproteins. Lower molecular-weight polypeptides, antigenically and structurally related to the fully glycosylated proteins, were detected in cell extracts but these were more susceptible to proteolytic degradation, perhaps due to the lack of carbohydrate side chains (Pizer et al., 1980). Other groups also found that TM treatment resulted in a loss of glycoprotein expression on the virions and on the infected cells (Norrild & Pedersen, 1982; Peake et al., 1982), and that the immunological specificity was mainly in the protein backbone as gB-reactive antibodies could still recognize the unglycosylated protein (Norrild & Pedersen, 1982). After TM treatment, gB was no longer functional as a target for antibody-dependent, cell-mediated cytotoxicity (Norrild & Pedersen, 1982). In T cell-mediated lysis of infected cells, treatment with TM left the infected target cells more resistant to lysis (Carter et al., 1981), although the role of individual glycoproteins was not examined. However, a possible reason for this loss in target function could be due to TM inhibiting glycoprotein expression on the cell surface.

Evidence for O-linked glycosylation was first obtained by Oloffson et al., (1981). Treatment of gB with N-acetylgalactosamine (GalNAc) oligosaccharidase, which cleaves O-linked glycans, resulted in a decrease in its molecular weight, confirming the presence of O-linked carbohydrate chains (Johnson & Spear, 1983).

To investigate the possible role of N-linked oligosaccharide side chains in T cell recognition of the glycoproteins, immunopurified gB and its unglycosylated precursor gB (pgB) were prepared and used both as immunogens _in vivo_ and as restimulating antigens _in vitro_.

The aims of this chapter are:-

1. To examine the specificity of the HSV-1 T cell response and its kinetics _in vitro_.
2. To examine T cell responses using gB for priming and for _in vitro_ restimulation.
3. To use pgB for priming and for _in vitro_ restimulation and compare the T cell responses with those induced by gB.
4. To examine the capacity of pgB to protect mice against a lethal challenge with HSV-1.

3.2. RESULTS.

3.2.1. In vitro proliferative responses by heat-inactivated (HI) HSV-1 or saline primed lymph node cells against HI HSV-1, HI HSV-2 or purified protein derivative.

BALB/c mice were immunized subcutaneously (s.c.) using HI HSV-1 or saline. T cell proliferation assays were set up, using the popliteal lymph nodes, on days 7, 9 and 11 after priming to test the specificity of the primed cells for HSV.

The saline primed cells gave no significant response against either HI HSV-1 or HI HSV-2 on any of the days tested (Figure 3.2.1.). There was a slightly higher response to purified protein derivative (PPD) on days 7 and 9, but this was only seen at the highest concentration of 10µg per well and was probably due to non-specific mitogenic effects. The HSV-1 primed cells did not respond significantly to PPD on any day tested. However, the cells responded well to both HI HSV-1 and HI HSV-2. On day 7 after priming, the cells responded equally well to both HSV-1 and HSV-2 with a stimulation index (S.I.) of approximately 30. By day 9, the response to HSV-1 had almost doubled, however the response to HSV-2 had fallen slightly. There was little change on day 11, with the responses to HSV-1 falling only slightly and the response to HSV-2 remaining consistent.

Thus, a strong, virus-specific response to HI HSV-1 was seen 7 days after priming and this increased rapidly. A good cross-reactive response to HI HSV-2 was also detected, but

Figure 3.2.1. In vitro proliferative responses by heat-inactivated (HI) HSV-1 or saline primed lymph node cells against HI HSV-1, HI HSV-2 or purified protein derivative.

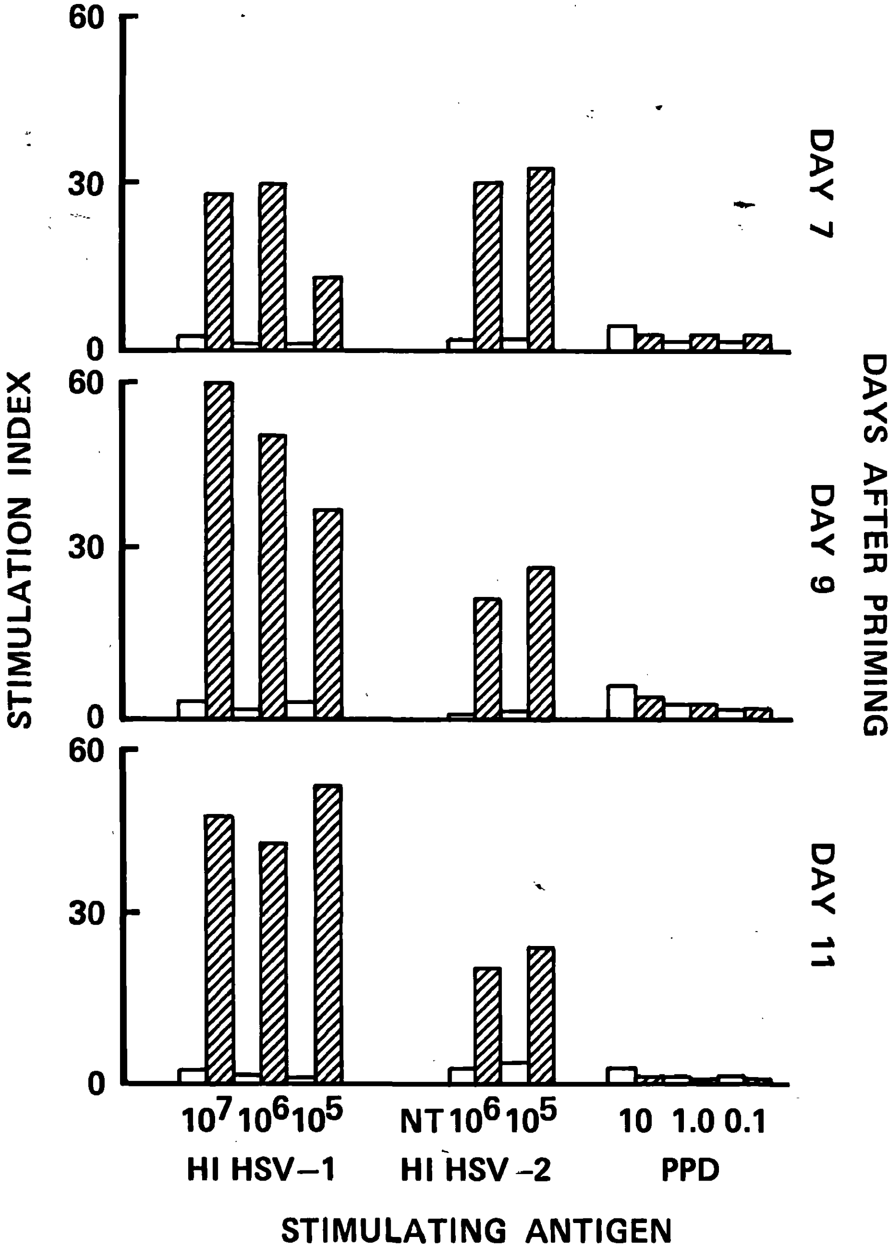
BALB/c mice were immunized subcutaneously in the hind footpads with HI HSV-1(Krueger) at 5x10^6 PFU/mouse or with saline, both emulsified with FCA. On days 7, 9 and 11 after priming, assays were set up using the popliteal lymph nodes from 3 mice per group. Stimulating antigens used were HI HSV-1(Krueger) or HI HSV-2(333) at 10^7-10^5 PFU/well or purified protein derivative (PPD) at 10-0.1μg/well. All samples were set up in triplicate.

Cell proliferation is expressed as a stimulation index (S.I. = [c.p.m. with antigen]/[c.p.m. without antigen]).

☐ Saline primed.

▨ HI HSV-1 primed.

N.T.- Not tested


60
30
0
STIMULATION INDEX
DAY 7
DAY 9
DAY 11
DAYS AFTER PRIMING
10^7 10^6 10^5
HI HSV–1
NT 10^6 10^5
HI HSV–2
10 1.0 0.1
PPD
STIMULATING ANTIGEN

this fell slightly by day 9. The response titrated out in some cases, e.g. day 9, HI HSV-1 group. However 10^5 PFU of virus per well was as efficient at stimulating a T cell response as 10^7 PFU per well, indicating that a plateau was reached by 10^5 PFU of virus per well.

3.2.2. In vitro proliferative responses of HI HSV-1, HI HSV-2 or saline primed lymph node cells against HI HSV-1-kinetics of the response.

Saline primed cells showed no response to HI HSV-1 on any day tested (see Figure 3.2.2.). HI HSV-1 primed cells gave a marked response on day 7, which increased steadily and peaked on day 11. There was then a sharp decrease by day 13 (S.I. of 50.0 falling to an S.I. of 12.0) and the response continued to decrease, reaching background levels by day 17. HI HSV-2 primed cells responded best to HSV-1 on day 7, then the response steadily decreased, also reaching background levels by day 17.

The data shown in Figure 3.2.2. was obtained using 10^6 PFU of HI HSV-1 per well. In experiments using 10^7 and 10^5 PFU per well, similar results were obtained (data not shown).

Figure 3.2.2. In vitro proliferative responses of HI HSV-1, HI HSV-2 or saline primed lymph node cells against HI HSV-1-kinetics of the response.

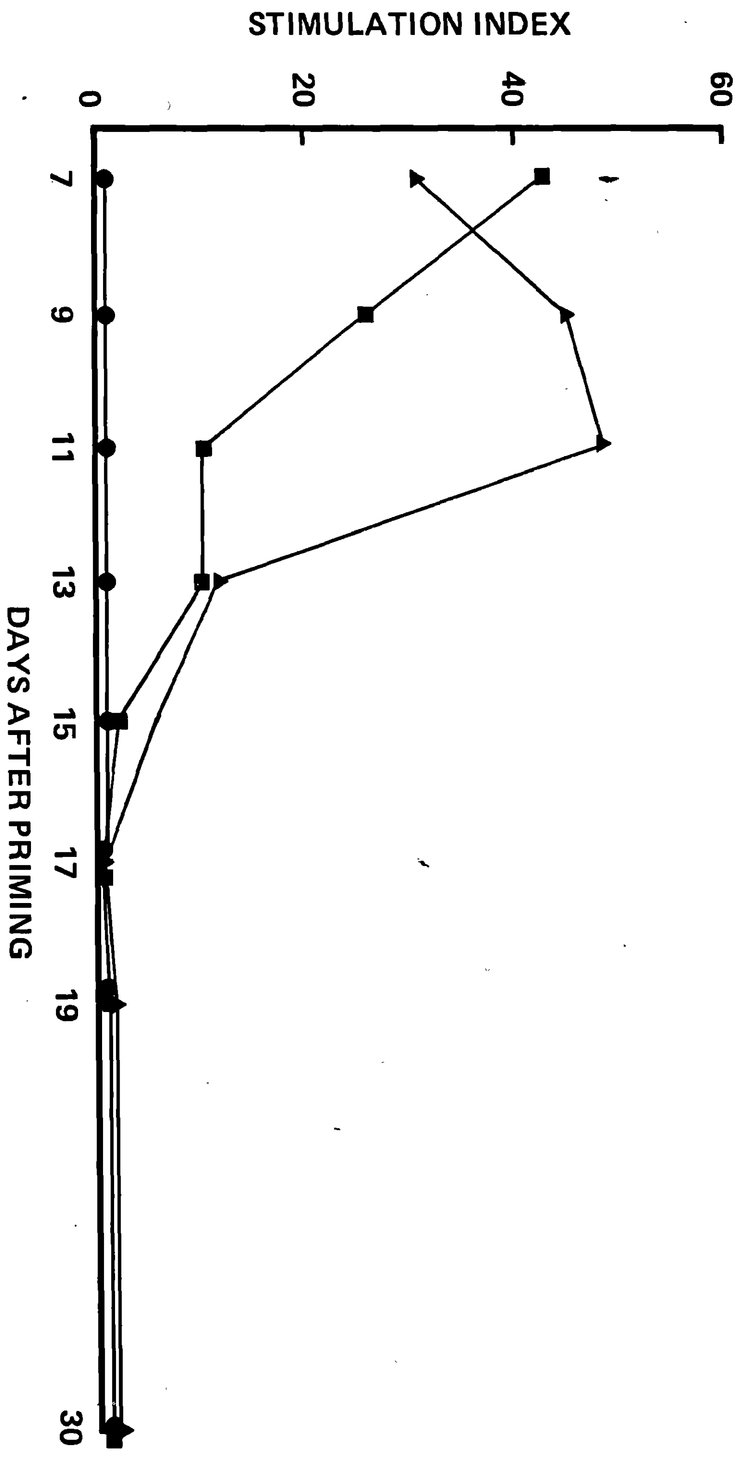
BALB/c mice were immunized subcutaneously in the hind footpads with HI HSV-1(Krueger) or HI HSV-2(333) at $5x10^6$ PFU/mouse or with saline, each emulsified with FCA. Assays were set up on days 7-19, at 2 day intervals, and on day 30 after priming, using the popliteal lymph nodes from 3 mice/group. Primed cells were restimulated in triplicate with HI HSV-1(Krueger) at 10^6 PFU per well.

Cell proliferation is expressed as a stimulation index (S.I. = [c.p.m. with antigen]/[c.p.m. without antigen]).

● Saline primed.

▲ HI HSV-1 primed.

■ HI HSV-2 primed.


STIMULATION INDEX
0
20
40
60
7
9
11
13
15
17
19
30
DAYS AFTER PRIMING

3.2.3. In vitro proliferative responses of HI HSV-1, HI HSV-2 or saline primed lymph node cells against HI HSV-2 - kinetics of the response.

Again, the saline primed cells did not respond to HI HSV-2 (Fig. 3.2.3.). However, HSV-1 primed cells gave a high response to HSV-2 on days 7 and 9 after priming, which then steadily decreased and reached background levels by day 17. HSV-2 primed cells also responded best on day 7, then the response decreased slowly, reaching background levels by day 17.

The results shown in Fig. 3.2.3. were obtained after restimulation with 10^6 PFU of HI HSV-2 per well. In experiments using 10^7 and 10^5 PFU of HSV-2 per well, similar results were obtained (data not shown).

In all subsequent proliferation assays, mice were boosted 14 days after priming, and T cell proliferation assays set up 5 days after boosting.

Figure 3.2.3. In vitro proliferative responses of HI HSV-1, HI HSV-2 or saline primed lymph node cells against HI HSV-2- kinetics of the response.

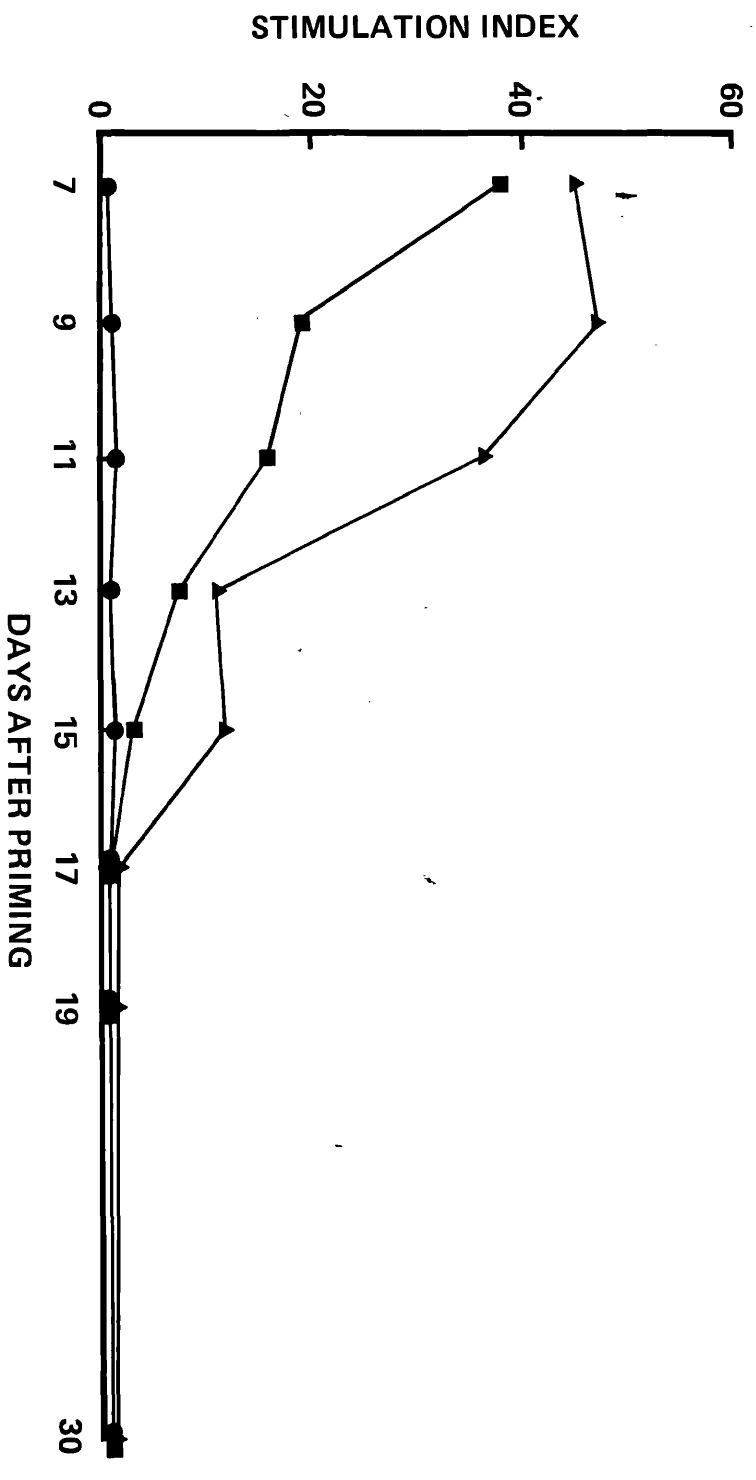
BALB/c mice were immunized as previously described and cell cultures set up on days 7-19 and day 30 after priming as before (see Fig. 3.2.2). Cells were restimulated in triplicate with HI HSV-2(333) at 10^6 PFU/well.

Cell proliferation is expressed as a stimulation index (S.I. = [c.p.m. with antigen]/[c.p.m. without antigen]).

● Saline primed.

▲ HI HSV-1 primed.

■ HI HSV-2 primed.


STIMULATION INDEX
0
20
40
60
7
9
11
13
15
17
19
30
DAYS AFTER PRIMING

3.2.4. In vitro proliferative responses of ovalbumin, HI HSV-1 or gB-1 primed lymph node cells against HI HSV-1, HI HSV-2 or gB-1 of different viral strains.

Ovalbumin primed cells gave little or no response to all the antigens tested, with an S.I. of approximately 2.0 in each case, (Figure 3.2.4.). This showed that the glycoprotein samples used were relatively pure and did not have mitogenic contaminants causing non-specific proliferation.

The HI HSV-1 primed cells responded almost equally well to HI HSV-2 and to both of the HSV-1 strains used in vitro. They also responded to restimulation with gB-1 of different HSV-1 strains, where there was a slightly better response to gB-1 of the strain used to immunise the mice.

The gB-1 primed cells also responded to the 3 virus strains used, although the response was slightly lower to HI HSV-2 than to HI HSV-1. The response was also significantly lower than that seen with the virus primed cells. When restimulated with gB-1 in vitro, the cells responded equally well to both strains and gave a much stronger response to gB-1 than the virally primed cells did.

Therefore, priming with HSV-1 induced T cells capable of recognising both HSV-1 and HSV-2 and which also responded well to gB-1. Priming with gB-1 elicted gB reactive T cells which also recognised whole virus of either type. The different strains used did not affect the response.

Figure 3.2.4. In vitro proliferative responses of ovalbumin, HI HSV-1 or gB-1 primed lymph node cells against HI HSV-1, HI HSV-2 or gB-1 of different viral strains.

BALB/c mice were immunized subcutaneously in the hind footpads with HI HSV-1(KOS) at $5x10^6$ PFU/mouse or with ovalbumin, gB-1(KOS) or gB-1(Krueger) at 30µg/mouse, each emulsified in FCA. After 14 days, the mice were boosted with antigen, but no FCA. Five days after boosting, the assay was set up using the popliteal lymph nodes from 2 mice per group. Stimulating antigens used were HI HSV-1(KOS), HI HSV-1(Krueger) and HI HSV-2(333) at 10^5 PFU/well, gB-1(KOS) and gB-1(Krueger) at 10µg/well.

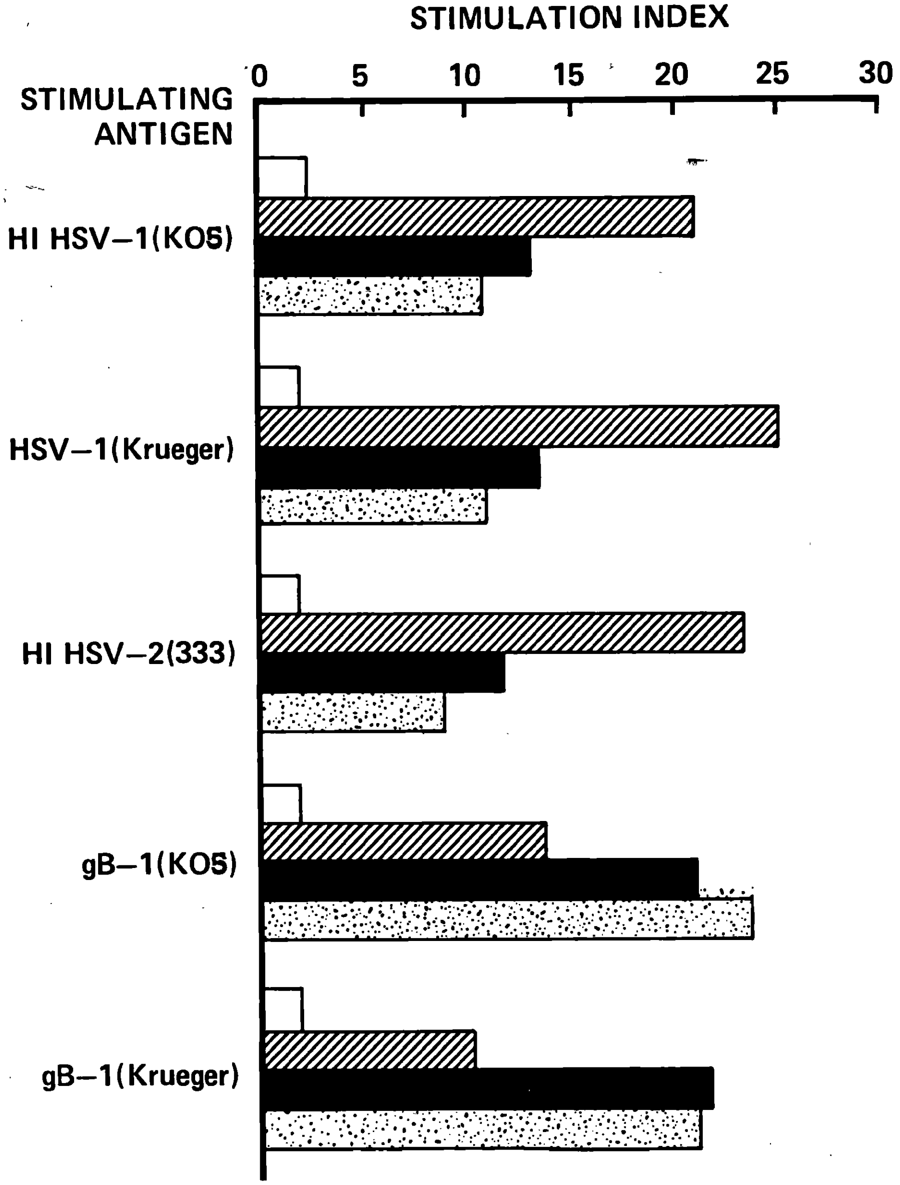
Cell proliferation is expressed as a stimulation index (S.I. = [c.p.m. with antigen]/[c.p.m. without antigen]).

□ Ovalbumin primed.

▧ HI HSV-1(KOS) primed.

□ gB-1(KOS) primed.

▒ gB-1(Krueger) primed.


STIMULATION INDEX
0
5
10
15
20
25
30
STIMULATING ANTIGEN
HI HSV–1(KOS)
HI HSV–1(Krueger)
HI HSV–2(333)
gB–1(KOS)
gB–1(Krueger)

3.2.5. Western blot analysis of HSV-1 infected BHK cell extracts, treated with or without tunicamycin.

To ensure that tunicamycin (TM) treatment did produce non-glycosylated precursor glycoprotein (pgP-1), treated and untreated glycoprotein samples were electrophoresed on SDS-PAGE and Western blotted using a gB-1 specific monoclonal antibody.

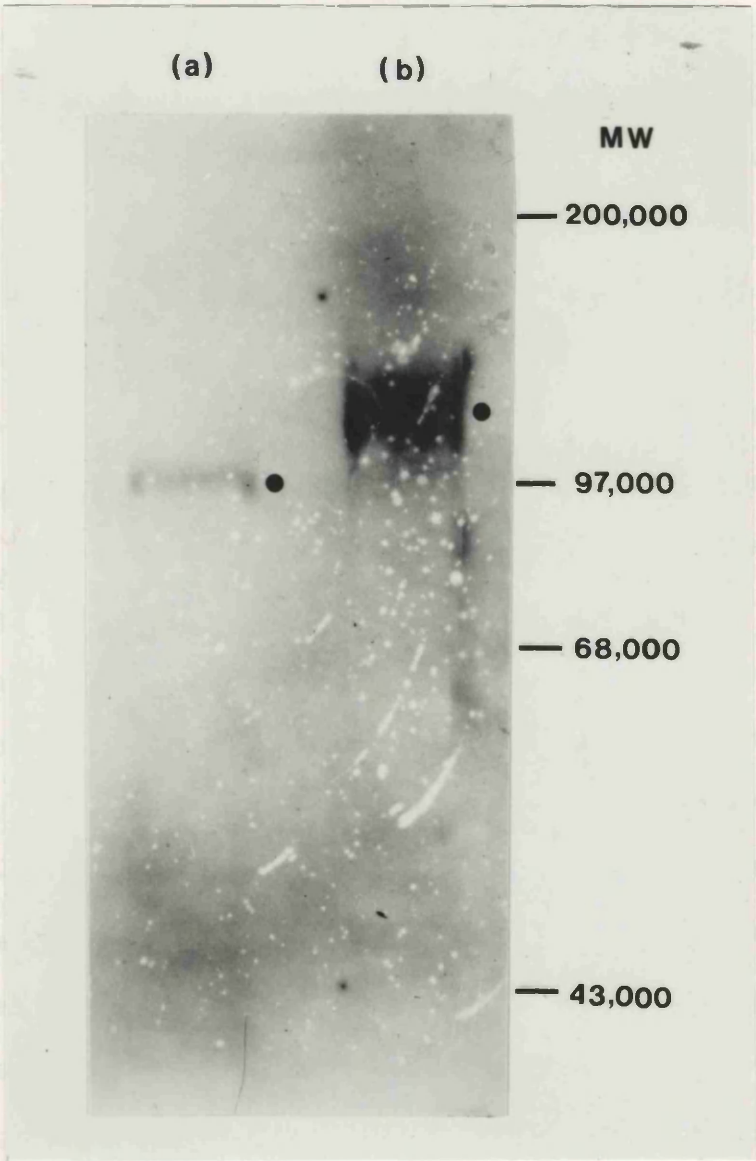
Untreated glycoprotein (gP-1) was detected as a large discrete band of 110,000 - 115,000 MW (see Fig. 3.2.5., Lane b). TM treated glycoprotein (pgP-1) was detected at approximately 97,000 MW (Lane a). No other bands were seen in either preparation. Therefore, the tunicamycin concentration used was sufficient to prevent glycosylation taking place. As no smaller fragments were detected, the protease inhibitors used had prevented proteolytic degradation of gB occurring during extraction.

Figure 3.2.5. Western blot analysis of HSV-1 infected BHK cell extracts, treated with or without tunicamycin.

Lane (a)- HSV-1 infected BHK cell extract, with tunicamycin treatment, i.e. pgP-1.

Lane (b)- HSV-1 infected BHK cell extract, without tunicamycin treatment, i.e. gP-1.

gP-1 and pgP-1 samples were electrophoresed in a 7% sodium dodecyl sulphate-polyacrylamide gel, under reducing conditions, and blotted onto a nitrocellulose membrane. The blot was washed extensively and incubated overnight at 4° C with the gB-1 specific monoclonal antibody TI57. After further washing, the blot was then incubated overnight at 4° C with ^{125}I-rabbit anti-mouse immunoglobulin. Finally, after further washing, the blot was dried and exposed to film at -70° C for 6 days before developing.


(a)
(b)
MW
200,000
97,000
68,000
43,000

3.2.6. In vitro proliferative responses of primed lymph node cells against ovalbumin, HI HSV-1, gB-1 or pgB-1.

To examine if the lack of glycosylation on pgB-1 would affect its recognition by T cells, proliferation assays involving priming and in vitro restimulation using pgB-1 were set up and the results obtained compared with those using whole heat-inactivated virus, live virus and gB-1.

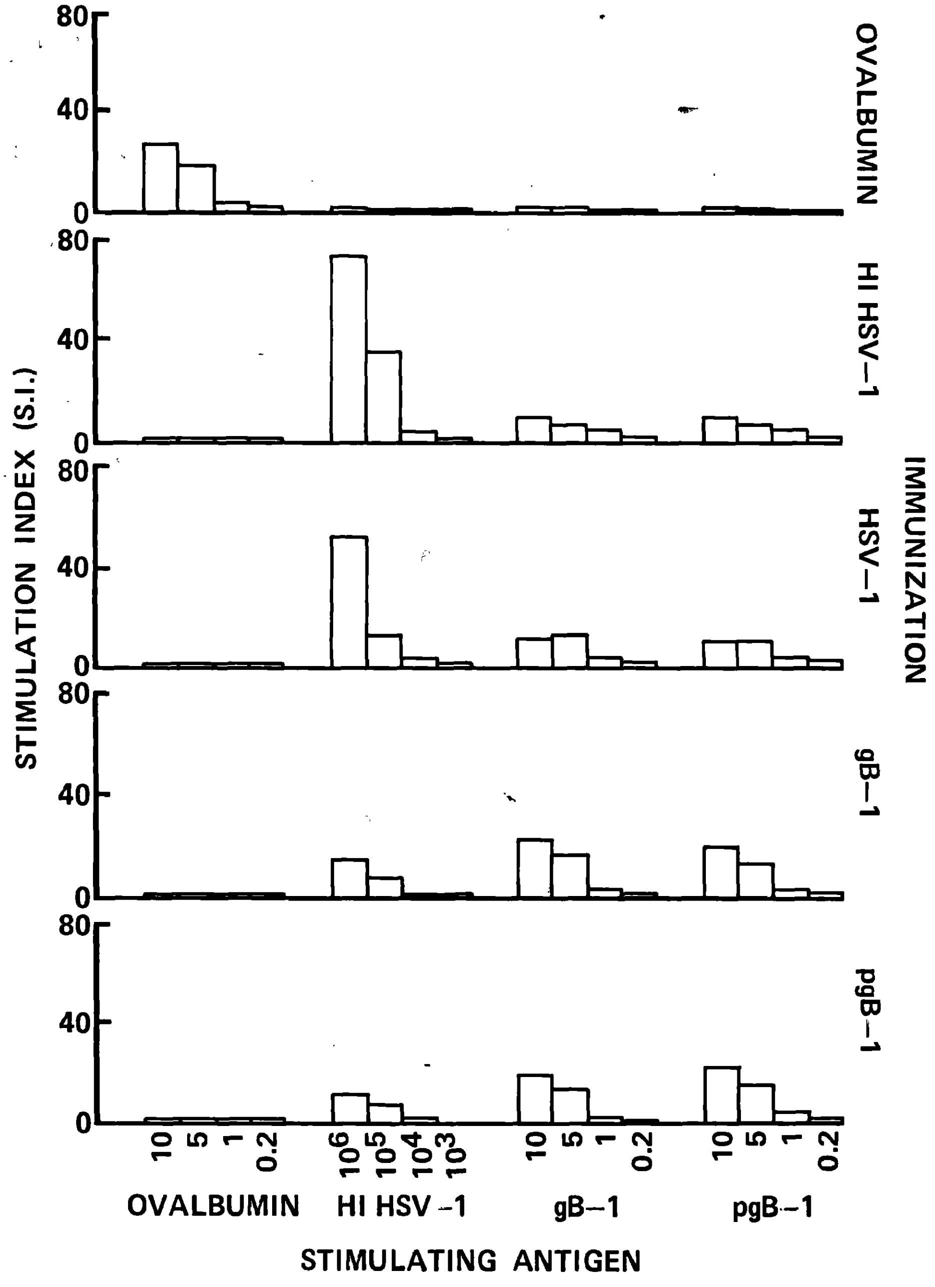
The ovalbumin primed cells responded only to in vitro restimulation with ovalbumin (see Figure 3.2.6.). There was no response to whole virus or to either of the glycoproteins. No other group of primed cells responded to ovalbumin in vitro. Cells primed with a low dose of HSV-1 or with HI HSV-1 responded well to restimulation with HI HSV-1 in vitro. Both also responded to gB-1 and pgB-1 with similar levels of restimulation in each case, but this was significantly lower than the response to whole virus. gB-1 primed cells responded equally well to restimulation with both gB-1 and pgB-1. These cells also proliferated in response to HI HSV-1, but the level of proliferation was less than that to the glycoproteins. The cells primed with pgB-1 gave results very similar to those obtained with gB-1, i.e. a good proliferative response to gB-1 and pgB-1 and a lower response to whole virus.

Thus, priming with gB-1 or pgB-1 induces T cells which can respond in vitro to whole virus as well as to the glycoproteins. As the pattern of response is so similar

Figure 3.2.6. In vitro proliferative responses of primed lymph node cells against ovalbumin, HI HSV-1, gB-1 or pgB-1.

BALB/c mice were immunized subcutaneously in the base of the tail and hind footpads with HI HSV-1(Krueger) at $5x10^6$ PFU/mouse; HSV-1(Krueger) at 10^5 PFU/mouse; ovalbumin, gB-1(Krueger) or pgB-1(Krueger) at 30µg/mouse, each mixed with alum. 14 days later, all the groups were boosted as before, but without using alum. 5 days later, the assay was set up using the draining lymph nodes, i.e. popliteal, para-aortic and inguinal nodes, from 3 mice per group. The stimulating antigens used were HI HSV-1 at 10^6-10^3 PFU/well; ovalbumin, gB-1 or pgB-1 at 10-0.2µg/well. All antigens were plated out in triplicate.

Cell proliferation is expressed as a stimulation index (S.I. = [c.p.m. with antigen]/[c.p.m. without antigen]).



STIMULATION INDEX (S.I.)
80
40
0
IMMUNIZATION
OVALBUMIN
HI HSV–1
HSV–1
gB–1
pgB–1
10
5
1
0.2
10^6
10^5
10^4
10^3
OVALBUMIN
HI HSV–1
gB–1
pgB–1
STIMULATING ANTIGEN

between gB and pgB, it appears that the lack of glycosylation does not affect T cell recognition of pgB nor its ability to prime for T cells.

3.2.7. Detection of anti-HSV-1 antibodies in the sera of mice primed with ovalbumin, HI HSV-1, live HSV-1, gB-1 or pgB-1.

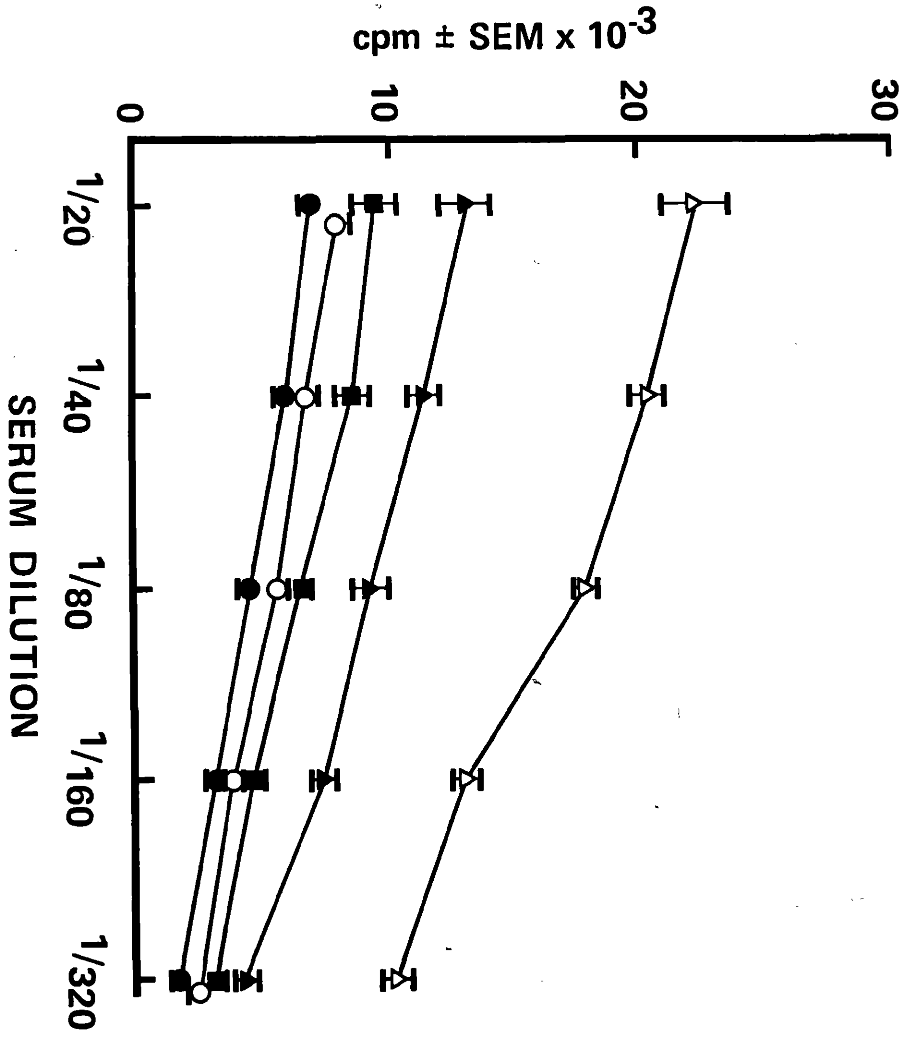
The counts obtained with the serum from the ovalbumin primed mice were used as the background control. The highest levels of anti-HSV antibody were detected in the pooled sera of mice primed with live HSV-1 and even at a dilution of 1/320, significantly higher amounts of antibody were detected than in the control serum (Fig. 3.2.7.). Priming with HI HSV-1 also led to detectable amounts of antibody, although not as high as with live HSV-1, and by the lowest dilution of 1/320 there was not a great difference. However, the counts obtained with the gB and pgB sera were not significantly different from those obtained with the ovalbumin serum. Therefore, it appears that no anti-HSV-1 antibody was present in these samples.

Figure 3.2.7. Detection of anti-HSV-1 antibodies in the sera of mice primed with ovalbumin, HI HSV-1, live HSV-1, gB-1 or pgB-1.

BALB/c mice were primed and boosted as previously described (see Fig. 3.2.6). Serum dilutions were plated, in triplicate, in 96-well flexiplates coated with HI HSV-1 and incubated at room temperature for 1 hour. The plates were washed and a second layer of ^{125}I-rabbit anti-mouse immunoglobulin was added and incubated overnight at 4° C. After extensive washing, the plates were dried and counted in a gamma-counter.

Results are expressed as c.p.m. ± SEM $\times 10^3$.

● Ovalbumin primed.
▲ HI HSV-1 primed.
△ HSV-1 primed.
■ gB-1 primed.
○ pgB-1 primed.


cpm ± SEM x 10-3
0
10
20
30
1/20
1/40
1/80
1/160
1/320
SERUM DILUTION

3.2.8. Active protection of mice primed with ovalbumin, HI HSV-1, gB-1 or pgB-1 against a lethal challenge with HSV-1.

It has already been shown that gB-1 can protect mice against a lethal challenge with HSV-1 (Chan *et al.*, 1985). However, the protective value of pgB-1 was not known.

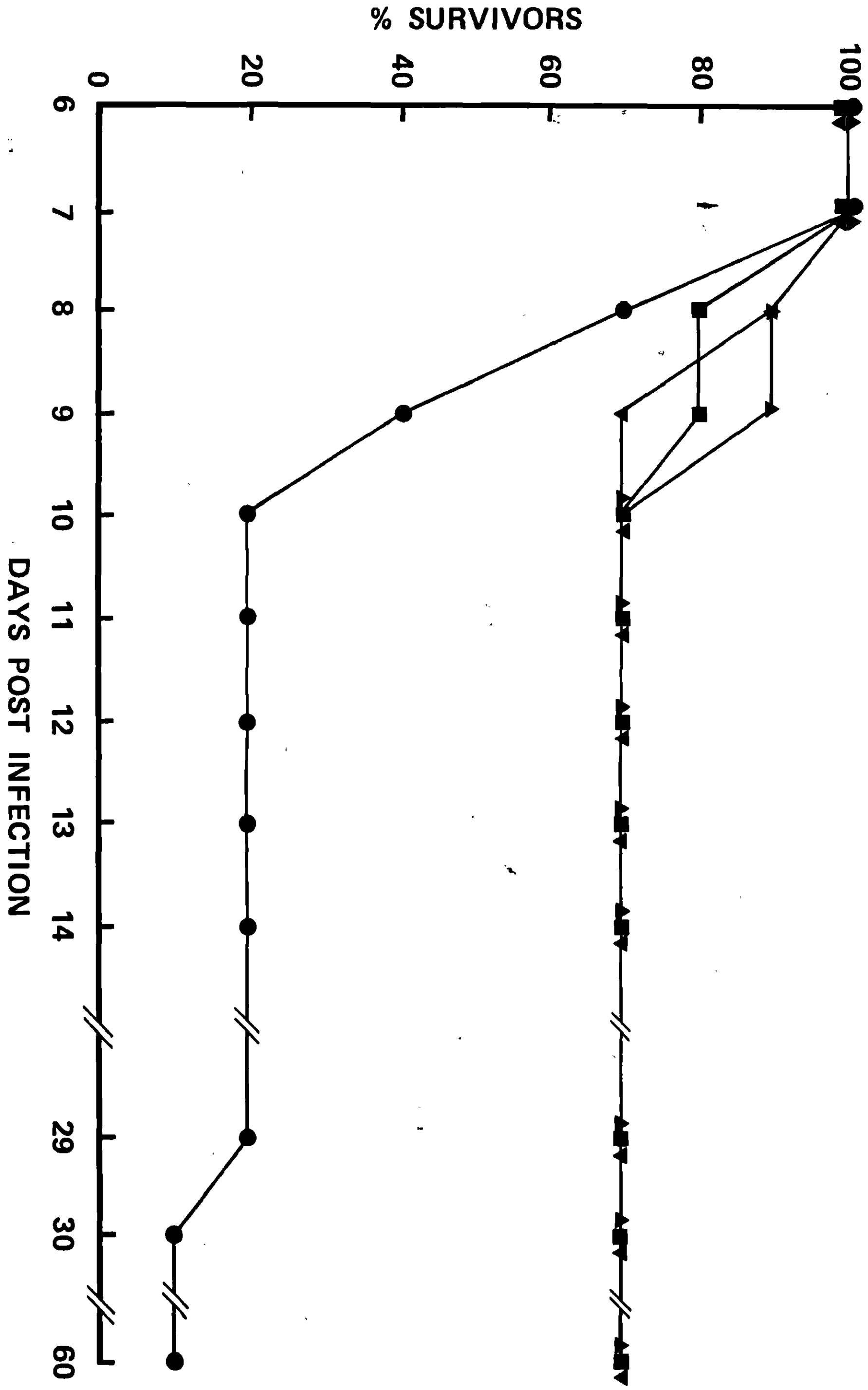
Mice were immunized and boosted with HI HSV-1, gB-1, pgB-1 or ovalbumin (10 mice per group). Five days after boosting, the mice were lethally challenged in the hind footpads with a 10x LD_{50} dose of HSV-1 and monitored for visible signs of infection and mortality. As expected, priming with ovalbumin gave no protection against HSV-1 infection and by day 10 post infection (p.i.) only 2 mice had survived (Fig.3.2.8.). HI HSV-1 gave good protection against infection, as did gB-1, with only 3 mice in each group dying. pgB-1 protected as well as the whole virus and gB-1, again with only 3 mice dying.

Therefore, pgB-1 could protect against a lethal infection with HSV-1 as well as HI HSV-1 or gB-1.

Figure 3.2.8. Active protection of mice primed with ovalbumin, HI HSV-1, gB-1 or pgB-1 against a lethal challenge with HSV-1.

5 week old CBA mice were injected subcutaneously (s.c.) in four sites in the flank with HI HSV-1(Krueger) at $5x10^6$ PFU per mouse; ovalbumin, gB-1(Krueger) or pgB-1(Krueger) at 30µg per mouse, each mixed with alum. 10 days later, all the groups were boosted as above, but without using alum. At 7 weeks old, the mice were infected s.c. in the hind footpads with 10x LD_{50} of HSV-1. The mice were observed for visible signs of infection and mortality until 60 days after infection.

● Ovalbumin primed.	▲ HI HSV-1 primed.
■ gB-1 primed.	▼ pgB-1 primed.


% SURVIVORS
100
80
60
40
20
0
6
7
8
9
10
11
12
13
14
29
30
60
DAYS POST INFECTION

3.3. DISCUSSION.

The aims of this chapter were to demonstrate the specificity and kinetics of the T cell response to herpes simplex virus, to examine the T cell response involving gB-1 and to compare these responses to those obtained with the unglycosylated precursor form, pgB-1.

As expected, priming mice with whole virus induced a virus-specific T cell response. This response was cross-reactive, i.e. priming with HI HSV-1 induced T cells reactive to both HSV-1 and HSV-2, and vice-versa. It has been shown that HSV-1 and HSV-2 share approximately 50% DNA homology (Kieff et al., 1972). Also, antigenic determinants have been detected on the glycoproteins that are specific for each serotype (type-specific) as well as determinants that are shared between the serotypes (type-common; Honess et al., 1974; Glorioso et al., 1978). Priming with HI HSV-1 also induced T cells capable of recognising gB-1 in vitro, confirming the findings from others (Chan, 1989; Martin et al., 1989). Priming with gB-1 induced T cells which recognised both HSV-1 and HSV-2, as well as gB-1. It has been shown that gB-1 has a homologue in HSV-2 (Bzik et al., 1986; Stuve et al., 1987; Zwaagstra & Leung, 1987) and gB-1 specific antisera and monoclonal antibodies have been found to cross-react with gB of HSV-2 (Snowden et al., 1985; Balachandran et al., 1987). Therefore, gB-1 can also prime for cross-reactive T cells which can recognise and proliferate to HSV-2 in vitro. The levels of proliferation obtained using different strains of gB-1 were very similar. However, as there are very few differences in the amino acid sequence of gB-1 between strains (Stuve et al., 1987), the T cell response should not be greatly affected.

Having shown that gB-1 could prime for and restimulate T cells, it was decided to examine the T cell responses involving the non-glycosylated precursor form, pgB-1, to determine if the lack of carbohydrate side chains would affect the T cell response to this protein.

Carbohydrate side chains have been found to be involved in determining the epitopes for antibody recognition in some viral systems (Kaluza et al., 1980; Elder et al.,1986; Hongo et al., 1986), although this is probably by influencing the conformation of the epitope, rather than involving direct recognition of the carbohydrate by the B cell. In contrast, Gitelman et al. (1981), studying the haemagglutinin of influenza A virus, found the carbohydrate portion was not involved in antibody recognition of the haemagglutinin. With HSV-1, a panel of gC-1 specific monoclonal antibodies were used to demonstrate B cell epitopes dependent on carbohydrate for their antigenic conformation (Sjoblom et al., 1987). Norrild & Pedersen (1982) showed that after tunicamycin (TM) treatment, gB ceased to be a target for antibody-dependent cell-mediated cytotoxicity. TM treatment also lead to a decrease in cytotoxic T cell-mediated lysis of HSV infected target cells, although the role of individual glycoproteins was not examined (Carter et al., 1981). This finding agrees with a study by Pimlott & Miller (1986) who showed that the treatment of tumour cell targets with TM led to a marked decrease in their susceptibility to cytotoxic T cell-mediated lysis. It was postulated that CTL recognition of target cells involved an interaction with N-linked glycans on the target cell surface, but as TM is known to inhibit glycoprotein expression on the cell surface, this may explain the lack of CTL recognition. A gB-1-specific hyperimmune antiserum was shown to recognise unglycosylated gB (Norrild & Pedersen, 1982), therefore the immunological reactivity was not dependent on the oligosaccharide side chains.

In this work, non-glycosylated pgB was obtained by tunicamycin treatment. It is thought that TM treatment results in the loss of both N- and O-linked carbohydrate chains in most of the glycoproteins, including gB-1. The tunicamycin concentration chosen (0.5µg/ml) was known to inhibit glycoprotein synthesis and to inhibit the production of infectious virus (Carter et al., 1981). When the gB-1 monoclonal antibody TI57 was used in Western blots, it detected a large band of 110,000-115,000 MW from an HSV-1 infected BHK cell extract. This corresponds to gB-1 (Chan et al., 1985). It also detected a smaller band of approximately 97,000 MW from an HSV-1 infected, TM treated

BHK cell extract. This appears to correspond to pgB-1,as other groups have reported pgB-1 to be 92,000-98,000 MW (Norrild & Pedersen, 1982; Kousoulas _et al._, 1983; Wenske & Courtney, 1983) or 105,000 MW (Peake _et al._, 1982). The difference in the molecular weight of pgB-1 found by these groups is probably due to the use of different cell lines to grow the virus. It has been reported that the electrophoretic mobilities of the glycoproteins can vary, depending on the cell line they are cultured in (Pereira _et al._, 1981). The amount of pgB detected was much less than the amount of gB detected, probably due to TM inhibiting the production of virus particles, so less glycoprotein is produced anyway.

The T cell response induced by priming with pgB or by _in vitro_ restimulation using pgB was also examined and found to be very similar to the response generated by gB. Priming with gB induced T cells which recognised and proliferated to whole virus, gB and pgB _in vitro_, with the response to the glycoproteins being very similar. Priming with pgB also elicted T cells reactive to virus, gB and pgB, with the levels of proliferation achieved very similar to those with gB. Both gB and pgB could also be used to stimulate HSV-1 primed cells _in vitro_, again with very similar results. Therefore, the results obtained from _in vitro_ T cell proliferation experiments showed that the lack of glycosylation in pgB did not seem to affect the T cell epitopes on the protein. Cells primed with pgB could recognise the restimulating antigens as well as gB primed cells and pgB, as a restimulating antigen in vitro, was as efficient as gB. Using a low dose of live HSV-1 for priming was no better than using HI HSV-1 in inducing a response to the glycoproteins in vitro.

Attempts to measure the level of anti-HSV-1 antibodies in the sera of mice primed with gB-1 and pgB-1 were not so successful. The amount of antibody detected was not significantly above the background level obtained from the ovalbumin primed mice, although the mice primed with virus, particularly a low dose of live virus, gave a good antibody response. It is likely that the time scale chosen, which was primarily for the T cell proliferation assay, was not optimal for the production of antibody. Also, the T cells which primarily help the antibody response may be distinct from the IL-2 producing cells which are probably involved in the proliferative response to antigen (Mosmann & Coffman, 1987; Powers & Miller, 1987). However, even with the low level detected, there was very little difference between gB and pgB.

The ability of pgB to protect mice against a lethal challenge with HSV-1 was also investigated. It has already been found that gB-1 can protect mice against a lethal infection (Chan et al., 1985). Immunopurified pgB-1 was found to be as effective in protecting mice against a lethal challenge with HSV-1 as gB-1 and whole virus. The major immune mechanism involved in the protection to and clearance of HSV-1 is mediated by T cells (for a review see Nash et al., 1985). Therefore, the T cells must recognise determinants on the protein backbone of pgB, and not on the carbohydrate side chains, for pgB to be as effective as gB in protection.

The results presented here, along with some later results presented in Chapter 4, confirm that the carbohydrate side chains present on glycoprotein B are not recognised by gB- or HSV-specific T cells as the non-glycosylated pgB is as efficient in T cell priming, T cell proliferation and protection as the fully glycosylated gB. Therefore, if unglycosylated gB is incorporated into vaccinia virus recombinants or into eukaryotic cell lines, the T cell responses induced by these preparations should be unaffected by the lack of carbohydrate side chains.

CHAPTER 4.

THE IN VIVO PRODUCTION OF LYMPHOKINES IN MICE INFECTED WITH HSV-1.

4.1. INTRODUCTION.

When the immune system encounters foreign antigens, either expressed by viruses or on the surface of infected cells, it mounts a specific response in an attempt to eradicate the infection. This response involves B lymphocytes, which produce antibody, and T lymphocytes, which regulate antibody production and mediate their own effector mechanisms, e.g. direct killing by cytotoxic T cells, delayed type hypersensitivity by helper T cells. However, in recent years, another important effector mechanism involved in the control of the immune response has been uncovered. This is the production of lymphokines, or interleukins, which are soluble protein factors produced by the T cells and antigen presenting cells involved in the immune response, although other cell types can also secrete them.

Cells producing these factors include T cells, monocytes, macrophages, natural killer cells, B-cell lines, fibroblasts, astrocytes and keratinocytes, depending on the lymphokine examined. Each lymphokine is also pleiotropic in its effects, i.e. it can act on different cell types in many ways. (For a fuller review, see O'Garra *et al.* 1988).

The results in this chapter deal with the lymphokines IL-1, IL-2, IL-3 and IFN-γ, so a brief description of each will be given here. However, it is very likely that other lymphokines are also involved in the *in vivo* response to viral infections.

IL-2 was originally called T cell growth factor (TCGF) and was described by Gillis et al., (1978). T cells release IL-2 when activated by specific antigen or by mitogens, Thowever IL-1 is also required. This induces the production of IL-2 (Smith et al., 1980; Gillis & Mizel, 1981) and the expression of IL-2 receptors (Kaye et al., 1984), which in turn leads to the proliferation of other T cells (reviewed by Smith, 1988). IL-2 has multiple biological activities. As well as acting as a growth factor for thymocytes and T cells (Gillis & Smith, 1977), IL-2 can mediate the production of gamma-interferon (Farrar et al., 1981; Kasahara et al., 1983; Kawase et al., 1983) and, via this mechanism, induce the maturation of cytotoxic T cells (Farrar et al., 1981; Maraskovsky et al., 1989). Other activities of IL-2 include the stimulation of natural killer cell activity (Handa et al., 1983), the induction of B cell differentiation and maturation into antibody-secreting cells (Parker, 1982; Leibson et al., 1984) and, in humans, augmenting monocyte cytotoxicity (Malkovsky et al., 1987). As well as up-regulation of the immune response, recent evidence has suggested that IL-2 may down-regulate the activation of T helper cells involved in the regulation of antibody responses (Kennedy et al., 1987) and may also down-regulate the cytotoxic activity of some MHC class II-restricted cytotoxic T lymphocytes (Shih & Truitt, 1987).

IL-3 was first described by Ihle et al., (1981). It is produced by activated T cells (Schrader, 1986) and constitutively by the WEHI-3 cell line (Lee et al., 1982). IL-3 acts on many cell types, including mast cells, multipotential stem cells and progenitor cells, hence it's other description as a panspecific haemopoietin (reviewed by Schrader, 1986). Its functions include supporting the growth of mast cells (Yokota et al., 1984), supporting the growth and differentiation of multipotential stem cells and committed progenitor cells (Nicola & Vadas, 1984; Rennick et al., 1985) and supporting the growth of pre-B cells (Palacios et al., 1984). It has also been reported that IL-3 can augment the response of cytotoxic T cells to allogeneic tumour cells in vitro (Curtsinger & Fan, 1984). Astrocytes have also been found to produce IL-3 (Frei et al., 1985) and this acts as a growth factor for microglia cells and for peritoneal macrophages (Frei et al., 1986).

IL-1 is produced by a wide variety of cell types, principally monocytes and macrophages (Gery & Waksman, 1972), but also by dendritic cells, natural killer cells, B cells, epithelial cells, fibroblasts, astrocytes and keratinocytes, among others, (for fuller reviews, see Dinarello, 1984; Durum et al., 1985). IL-1 is probably the most pleiotropic of the lymphokines, with a wide variety of biological activities. For example, IL-1 is involved in fever, tissue catabolism, prostaglandin E_2 release and the synthesis of the acute phase proteins, (for greater detail, see the reviews listed above). Within the immune system, IL-1 acts as a cofactor in T cell growth (Farrar et al., 1980; Oppenheim et al., 1986), induces lymphokine release from activated T cells (Smith et al., 1980; Gillis & Mizel, 1981; Kasahara et al., 1985) and increases NK cell activity (Durum et al., 1985; Oppenheim et al., 1986). IL-1 is also required for antibody synthesis via a direct effect on B cell activation and proliferation (Wood, 1979). However, the effect of IL-1 on the immune system is often indirect, by enhancing the activity of IL-2 and IFN-γ .

Recently, it has become clear that some of the activities attributed solely to IL-1 are affected by IL-6 as IL-1 is a potent inducer of this lymphokine and their activities are known to overlap (Wong & Clark, 1988). Therefore, it will be necessary to reassess many of the above biological activities.

Gamma- interferon (IFN-γ) or immune interferon is produced by antigen-specific T cells (Farrar et al., 1981; Morris et al., 1982) and by natural killer cells (Handa et al., 1983). IFN-γ is distinct from IFN-α (leukocyte IFN) and IFN-β (fibroblast IFN) and has important immunoregulatory effects. It induces increased Ia expression on macrophages (Warren & Vogel, 1985) and increases their phagocytic activity (Pace et al., 1983). IFN-γ also enhances NK cell activity (Trinchieri & Perussia, 1985), is involved in the induction of cytotoxic T cells (CTLs) by IL-2 (Farrar *et al.*, 1981; Maraskovsky *et al.*, 1989), and can indirectly support B cell proliferation and differentiation by synergizing with other lymphokines, e.g. IL-1 and IL-2 (O'Garra *et al.*, 1988). Another important role for IFN-γ, and also for IFN-α and β, is their ability to inhibit viral replication (Friedman & Vogel, 1983).

Although many of the individual effects of the lymphokines have been characterized, their induction and possible relationship *in vivo* in response to a viral infection is not well understood. Sheridan *et al.* (1982) showed that in humans the development of recurrent HSV-2 lesions correlated with depressed levels of the lymphokine leukocyte migration-inhibition factor, which returned to normal when the lesion healed. The level of IFN-γ secreted by T cells after a recurrence of herpes labialis could be used to predict the time to the next recurrence (Cunningham & Merigan, 1983; Torseth & Merigan, 1986). Injection with HSV-1 resulted in the production of IFN-α, β and γ by peritoneal exudate cells and this in turn led to the activation of NK cells (Engler *et al.*, 1981). Infection with malignant rabbit fibroma virus, an oncogenic and immunosuppresive virus, did not affect the ability of rabbit adherent cells to produce or respond to IL-1, but did render them unable to produce or respond to IL-2 (Strayer *et al.*, 1986). Spleen cells from mice infected with mouse hepatitis virus type 4 spontaneously produced IL-2 and IL-3 *in vitro* (Kyuwa *et al.*, 1988), while the spleen cells of mice infected with LCMV transcribed IL-2 *in vivo* during the course of the infection (Kasaian & Biron, 1989). The time course of the transcription and production of IL-2 correlated with CTL proliferation.

The aims of this chapter are :-

1. To examine the *in vivo* production of IL-1, IL-2, IL-3 and IFN-γ in immunized mice, after a sub-lethal infection with HSV-1, and to determine if immunization results in earlier or increased lymphokine production.
2. To determine if there is any correlation between lymphokine production and the protective effects of immunization with HI HSV-1, glycoprotein B of HSV-1 (gB-1) and its non-glycosylated precursor form pgB-1

4.2. RESULTS.

4.2.1. IL-2 production by ovalbumin, heat-inactivated (HI) HSV-1 or gB-1 primed spleen cells before and after HSV-1 infection.

No IL-2 was detected at days 0 and 2 post infection (p.i.) in the ovalbumin or gB-1 primed groups (see Figure 4.2.1.). However, by day 6 p.i., low levels were detected in the ovalbumin primed group. IL-2 levels in the gB primed group had risen significantly by day 6 p.i., but were still much lower than in the HSV-1 group. With the HSV-1 primed cells, IL-2 was detected even on day 0. This remained steady at day 2 p.i., but had increased sharply by day 6.

No IL-2 was detected in any group in the absence of *in vitro* restimulation using HI HSV-1.

4.2.2. IL-2 production by ovalbumin, HI HSV-1 or gB-1 primed draining lymph node cells before and after HSV-1 infection.

As with the spleen cell populations, almost no IL-2 was detected without HI HSV-1 restimulation. Restimulation of ovalbumin primed cells resulted in significant levels of IL-2 only on day 6 (Fig. 4.2.2.). Low levels of IL-2 were detected on days 0 and 2 in the gB-1 primed group. By day 6, this had sharply increased and more IL-2 was detected in this group than in the virus primed group. The amount of IL-2 from HI HSV-1 primed mice was of a consistently high

Figure 4.2.1. IL-2 production by ovalbumin, heat-inactivated (HI) HSV-1 or gB-1 primed spleen cells before and after HSV-1 infection.

BALB/c mice were immunized with HI HSV-1(Krueger) at 5x10^6 PFU/mouse; ovalbumin or gB-1(Krueger) at 30µg/mouse. Infection in the hind footpads was with 1.5x10^6 PFU HSV-1(Krueger) per mouse. On days 0, 2 and 6 post infection (p.i.), the spleens were removed from 2 or 3 mice per group and cell cultures to obtain lymphokine-containing supernatants were set up as described in Materials and Methods Section 2.13.3.

To detect IL-2, supernatant samples collected after 24 hours *in vitro* culture were assayed on the CTL-L cell line. Cells were plated out in 96-well flat bottomed plates at 1x10^4 cells/well with either supernatant samples, MLA-144 cell supernatant or medium alone. The samples and controls were assayed in triplicate. The cultures were incubated for 24 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

Results are expressed as c.p.m. ± SEM x10^3.

● Ovalbumin primed.

▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.

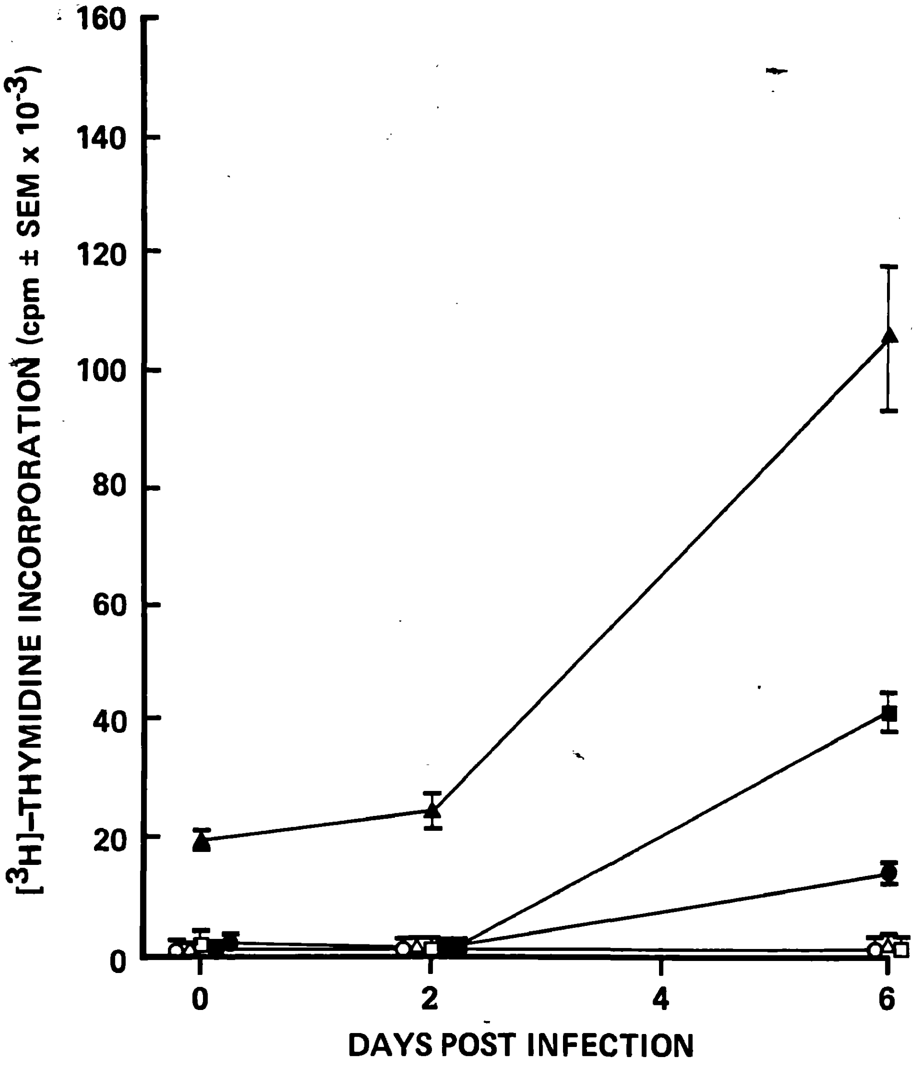

[^{3}H]-THYMIDINE INCORPORATION (cpm ± SEM x 10^{-3})
160
140
120
100
80
60
40
20
0
0
2
4
6
DAYS POST INFECTION

Figure 4.2.2. IL-2 production by ovalbumin, HI HSV-1 or gB-1 primed draining lymph node cells before and after HSV-1 infection.

For the protocol, see Figure 4.2.1. On days 0, 2 and 6 p.i., the draining lymph nodes, i.e. the inguinal, para-aortic and popliteal lymph nodes, were removed and cell cultures set up.

The presence of IL-2 was detected by assaying the supernatants on CTL-L cells as described in Figure 4.2.1.

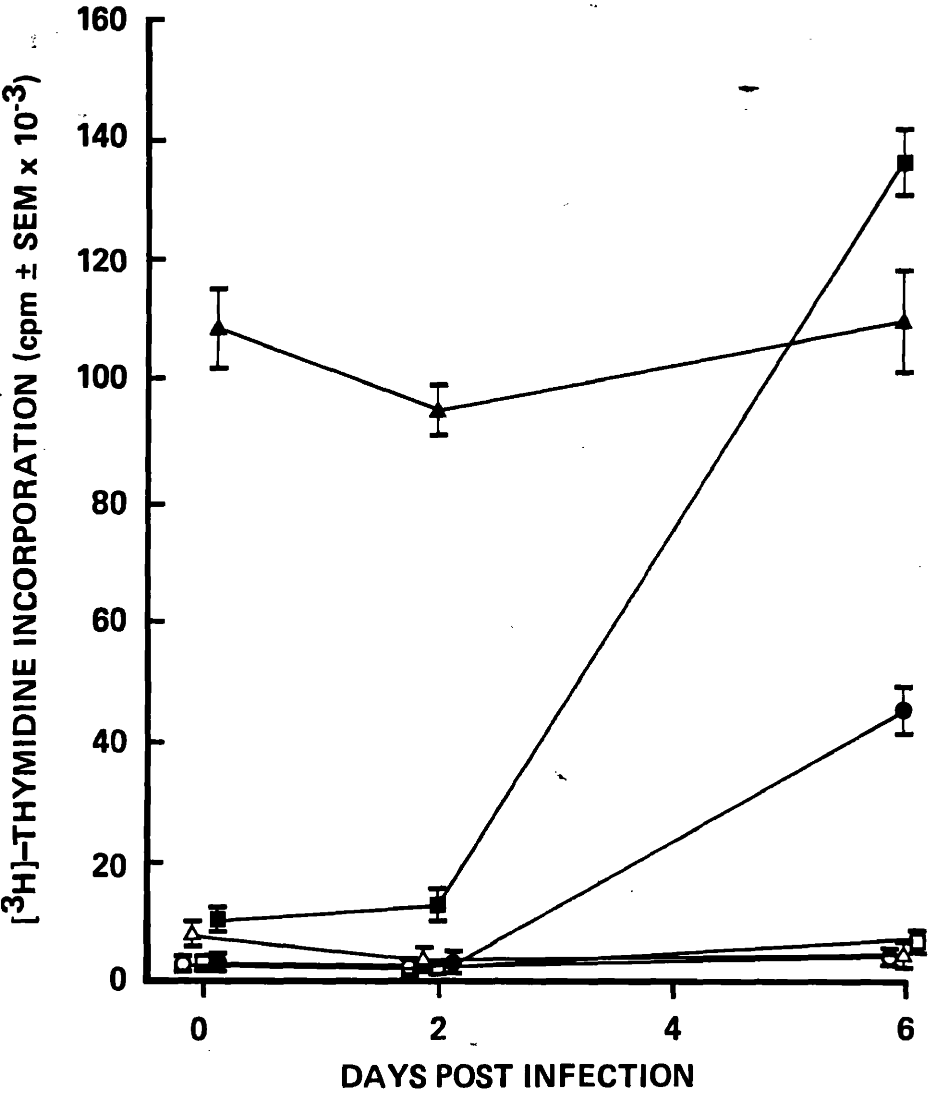
Results are expressed as c.p.m. ± SEM x10^3.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.


[^{3}H]-THYMIDINE INCORPORATION (cpm ± SEM x 10^{-3})
160
140
120
100
80
60
40
20
0
0
2
4
6
DAYS POST INFECTION

level on all the days tested.

4.2.3. Titration of IL-2 containing supernatants.

Supernatants from cultures set up on day 6 p.i. were titrated on CTL-L cells. As expected, the group not restimulated with virus in vitro did not produce any IL-2, even when undiluted supernatant was used (Fig. 4.2.3.). Supernatants from the restimulated groups did contain IL-2, with the highest levels present in the undiluted samples. As the samples were diluted, the IL-2-dependent proliferation of the CTL-L cells decreased linearly. This suggests that the observed proliferation was due to the effects of IL-2. In all samples tested, except the unstimulated group, the DLN cells gave a greater response than the equivalent spleen cell group.

4.2.4. IL-3 production by ovalbumin, HI HSV-1 or gB-1 primed spleen cells before and after HSV-1 infection.

Significant IL-3 production was only detected after in vitro restimulation with HI HSV-1. In the culture supernatant of ovalbumin and gB-1 primed cells restimulated with virus, no IL-3 was detected on days 0 and 2 p.i. (Fig. 4.2.4.). By day 6 p.i., significant levels of IL-3 were detected in both groups. However, the amount of IL-3 was much greater in the gB group than in the ovalbumin group. At day 0, there was a low but detectable level of IL-3 in the culture supernatant from the virus primed group. This rose

Figure 4.2.3. Titration of IL-2 containing supernatants.

Supernatant samples from primed spleen and draining lymph node (DLN) cell cultures, set up on day 6 post infection, were serially diluted in medium. The dilutions were plated, in triplicate, onto CTL-L cells in a 96-well flat bottomed plate. Cultures were incubated for 24 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

Results are expressed as c.p.m. ± SEM x10^3.

(a) Supernatants from primed spleen cells.

(b) Supernatants from primed DLN cells.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.

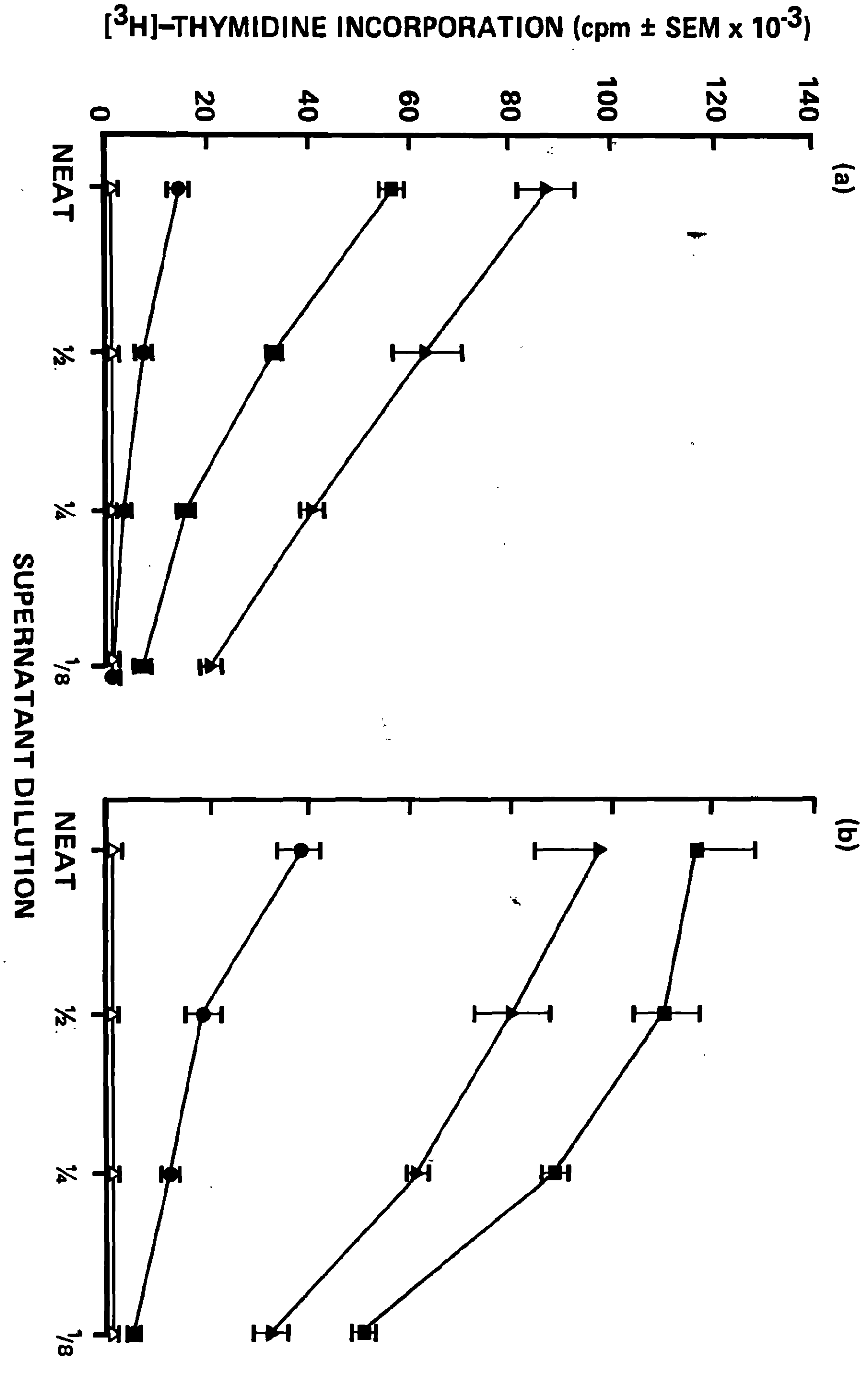

[^{3}H]-THYMIDINE INCORPORATION (cpm ± SEM x 10^{-3})
0
20
40
60
80
100
120
140
(a)
(b)
NEAT
½
¼
⅛
NEAT
½
¼
⅛
SUPERNATANT DILUTION

Figure 4.2.4. IL-3 production by ovalbumin, HI HSV-1 or gB-1 primed spleen cells before and after HSV-1 infection.

Cell-free supernatant samples were collected from primed spleen cells after *in vitro* culture for 24 hours and assayed for IL-3 - see Figure 4.2.1 for details.

To detect IL-3, supernatant samples obtained after 24 hours *in vitro* culture were assayed on the 32-D cell line. Cells were plated out in 96-well flat bottomed plates at 2×10^4 cells/well with either supernatant samples, WEHI-3 cell supernatant or medium alone added in triplicate. Cultures were incubated for 24 hours, pulsed with $[^3H]$-thymidine for 18 hours then harvested.

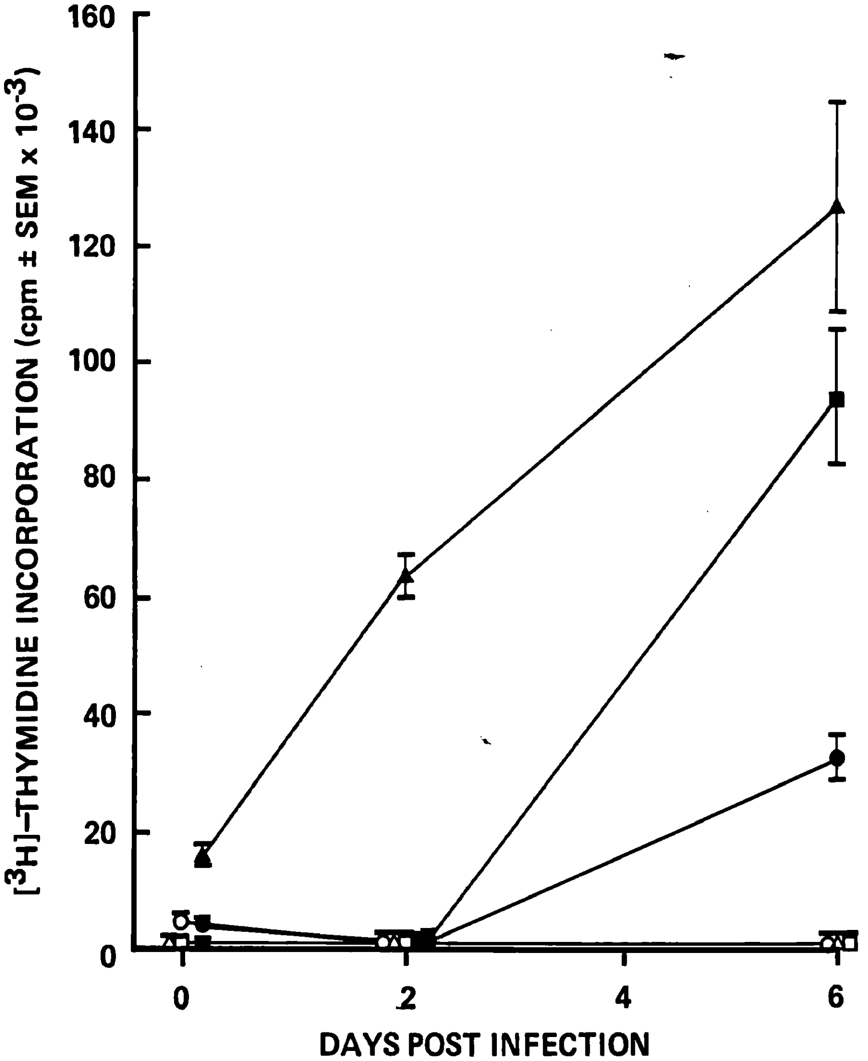
Results are expressed as c.p.m. ± SEM $\times10^3$.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.



160
140
120
100
80
60
40
20
0
[^{3}H]-THYMIDINE INCORPORATION (cpm ± SEM x 10^{-3})
0
2
4
6
DAYS POST INFECTION

steadily and was significantly higher than the ovalbumin group on both days 2 and 6 p.i.

4.2.5. IL-3 production by ovalbumin, HI HSV-1 or gB-1 primed DLN cells before and after HSV-1 infection.

IL-3 levels obtained from the DLN cells were similar to those from spleen cells. No IL-3 was seen in the ovalbumin or gB-1 primed groups on days 0 and 2 p.i., however significant levels were found on day 6 (Fig. 4.2.5.). Significantly greater amounts of IL-3 were detected in the virus primed cell supernatant on day 0 p.i. and this rose steadily on days 2 and 6. No IL-3 was seen without in vitro restimulation.

4.2.6. Titration of IL-3 containing supernatants.

Supernatants from day 6 p.i. cell cultures were titrated on the 32-D cell line. IL-3 was detected in all groups restimulated with whole virus in vitro but not in the unstimulated group (Fig. 4.2.6.). The level of 32-D proliferation decreased linearly as the supernatant samples were diluted. The amounts of IL-3 detected in the spleen and DLN cell cultures were similar.

Figure 4.2.5. IL-3 production by ovalbumin, HI HSV-1 or gB-1 primed draining lymph nodes before and after HSV-1 infection.

On days 0, 2 and 6 post infection, the draining lymph nodes, i.e. the inguinal, para-aortic and popliteal lymph nodes, were removed and set up as in Figure 4.2.1.

The presence of IL-3 was detected by assaying the supernatant samples on 32-D cells as described in Figure 4.2.4.

Results are expressed as c.p.m. ± SEM x10^3.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.

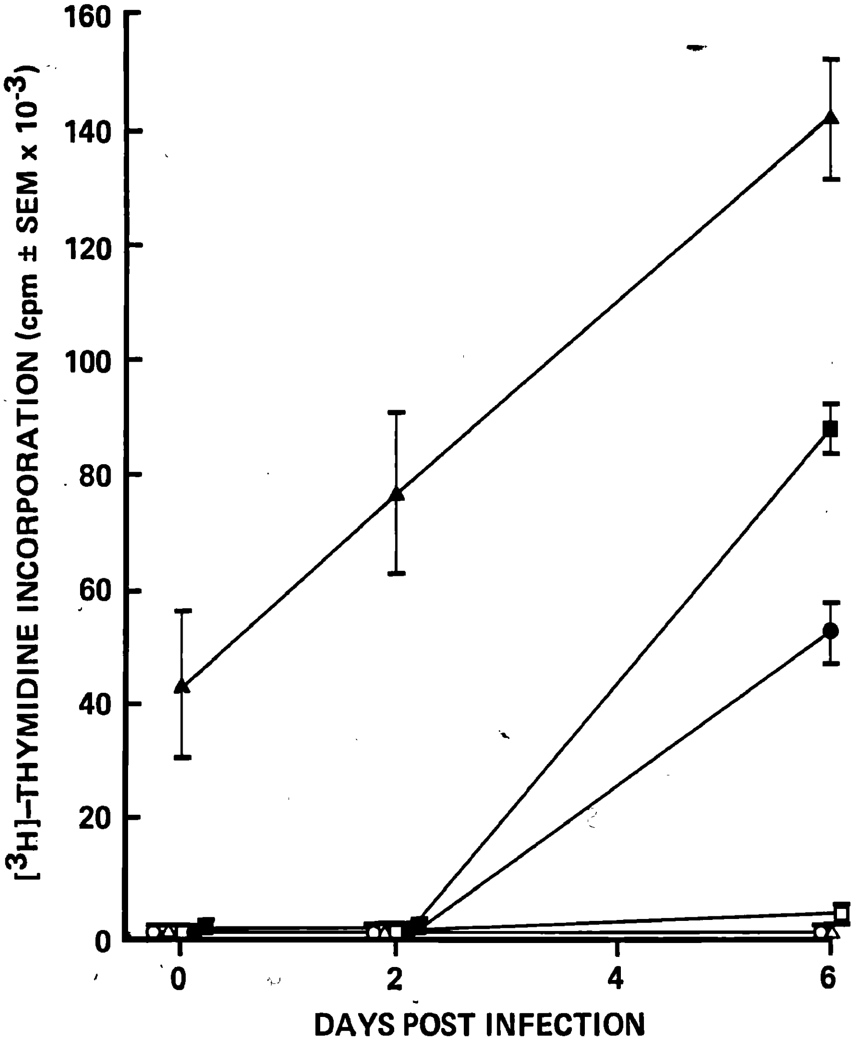

160
140
120
100
80
60
40
20
0
[3H]-THYMIDINE INCORPORATION (cpm ± SEM x 10-3)
0
2
4
6
DAYS POST INFECTION

Figure 4.2.6. Titration of IL-3 containing supernatants.

Supernatant samples from primed spleen and draining lymph node (DLN) cell cultures set up on day 6 post infection were serially diluted in medium. The dilutions were plated in triplicate onto 32-D cells in a 96-well flat bottomed plate. The cultures were incubated for 24 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

Results are expressed as c.p.m. ± SEM x10^3.

(a) Supernatants from primed spleen cells.

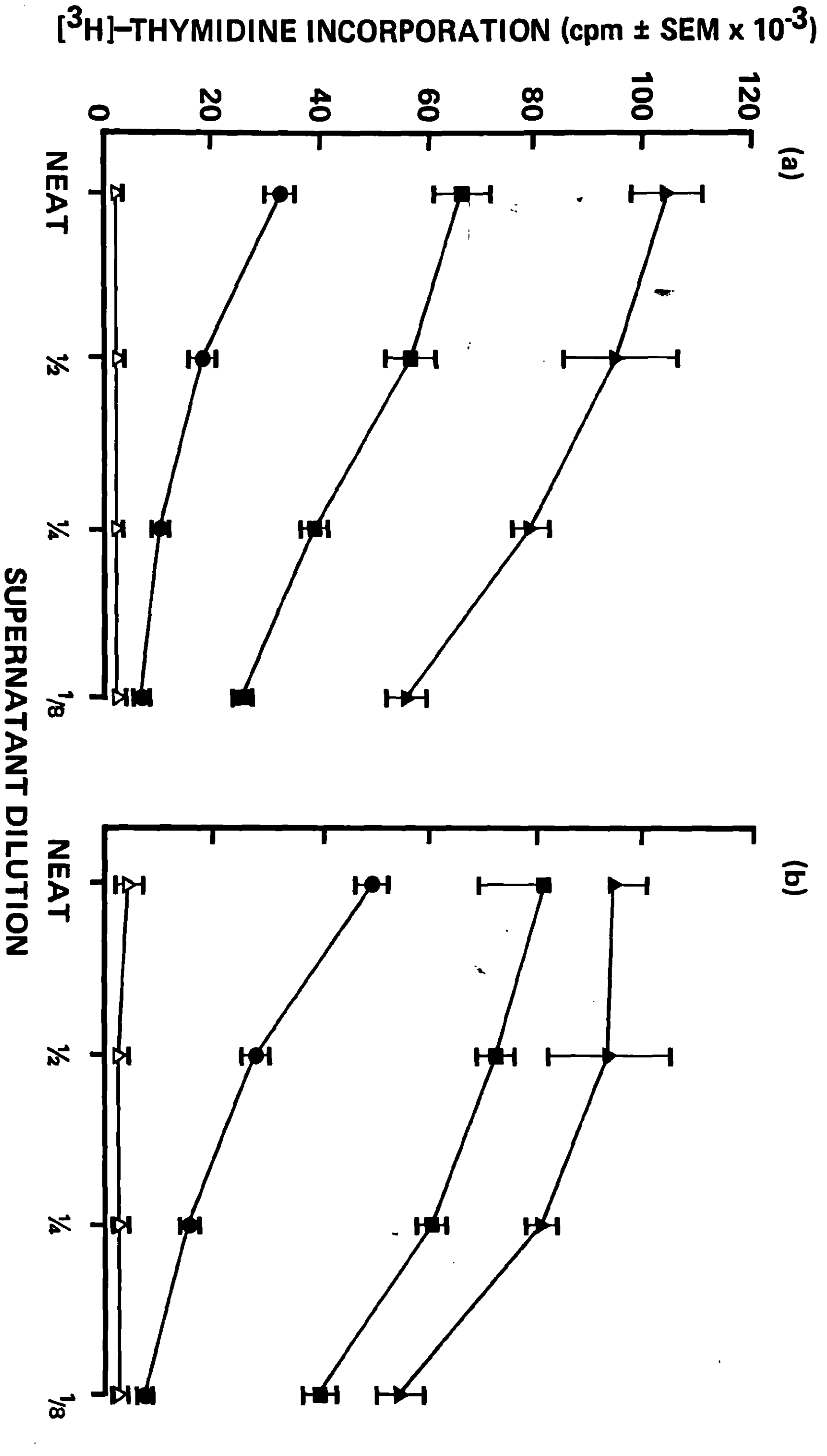
(b) Supernatants from primed DLN cells.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No in vitro restimulation.

Closed symbols - In vitro restimulation using HI HSV-1.


[3H]-THYMIDINE INCORPORATION (cpm ± SEM x 10-3)
0
20
40
60
80
100
120
(a)
(b)
NEAT
½
¼
⅛
NEAT
½
¼
⅛
SUPERNATANT DILUTION

4.2.7. IL-1 production by ovalbumin, HI HSV-1 or gB-1 primed spleen cells before and after HSV-1 infection.

Significant levels of IL-1 were detected in the supernatants collected from both stimulated and unstimulated cells. The level of IL-1 seen in the unstimulated groups was lower on days 0 and 2 p.i. than in the equivalent restimulated group (Fig. 4.2.7.). By day 6, these levels had risen and were similar to the groups restimulated with HI HSV-1. The amount of IL-1 in the restimulated cell supernatants remained consistent throughout the assay period. The amount of IL-1 detected in the HSV-1 and gB-1 groups rose slightly, but not significantly, on day 6.

4.2.8. IL-1 production by ovalbumin, HI HSV-1 or gB-1 primed DLN cells before and after HSV-1 infection.

Again, IL-1 was detected in both the stimulated and unstimulated groups. Only the ovalbumin primed groups were tested on day 0 p.i. The unstimulated cell supernatant had slightly lower levels of IL-1 than the restimulated cells, however by day 2 the two groups were very similar (Fig. 4.2.8.). By day 6 p.i., the amount of IL-1 in the unstimulated group remained consistent but, in the restimulated group, had become significantly higher. IL-1 levels in the HSV-1 and gB-1 primed groups, restimulated *in vitro*, were significantly higher than in the equivalent unstimulated groups. The IL-1 detected in the restimulated gB-1 group showed a marked increase from days 2 to

Figure 4.2.7. IL-1 production by ovalbumin, HI HSV-1 or gB-1 primed spleen cells before and after HSV-1 infection.

Cell-free supernatants were collected from the primed cells as described in Figure 4.2.1. However, the cells were cultured for 48 hours *in vitro* before the supernatants were collected.

To detect IL-1, supernatant samples were assayed on the EL-4 NOB-1 and CTL-L cell lines. Briefly, EL-4 NOB-1 cells were treated with mitomycin C ($33\mu g/5x10^6$ cells) at 37° C for 30 minutes. After extensive washing, the EL-4 NOB-1 cells were plated out at $2x10^5$ cells/well along with the CTL-L cells at $4x10^3$ cells/well in 96-well flat bottomed plates. Supernatant samples, rIL-1 or medium alone were added, in triplicate, to the cells. Cultures were incubated for 24 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

Results are expressed as c.p.m. ± SEM $x10^3$.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.

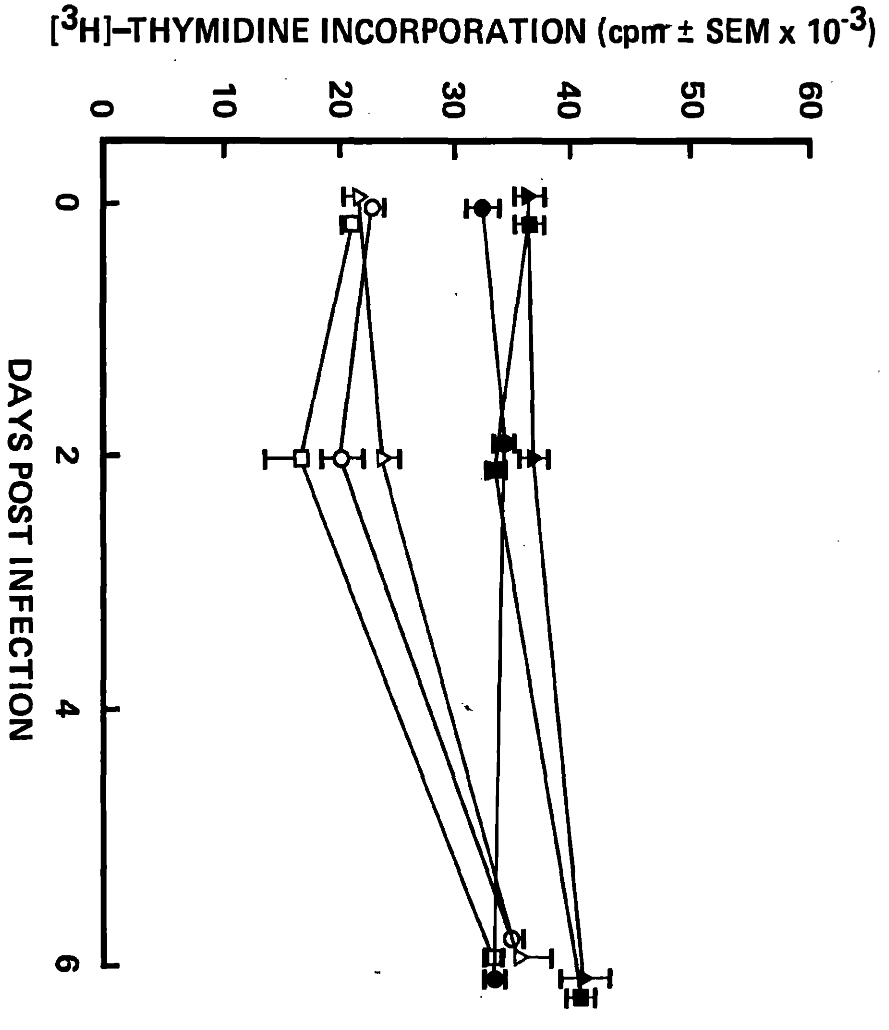

$[^3H]$–THYMIDINE INCORPORATION (cpm ± SEM x 10^{-3})
0
10
20
30
40
50
60
0
2
4
6
DAYS POST INFECTION

Figure 4.2.8. IL-1 production by ovalbumin, HI HSV-1 or gB-1 primed draining lymph node cells before and after HSV-1 infection.

On days 0, 2 and 6 post infection, the draining lymph nodes, i.e. the inguinal, para-aortic and popliteal lymph nodes, were removed and assayed as in Figure 4.2.1.

The presence of IL-1 was detected by assaying supernatant samples on EL-4 NOB-1 and CTL-L cells as described in Figure 4.2.7.

Results are expressed as c.p.m. ± SEM $x10^3$.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.

6, whereas the restimulated HSV-1 group remained steady.

4.2.9. Titration of IL-1 containing supernatants.

The supernatants from draining lymph node cell cultures set up on day 6 p.i. were titrated. IL-1 was detected in all the samples, however the unstimulated sample did have the lowest levels of IL-1 (Fig. 4.2.9.). The IL-1-dependent cell proliferation decreased linearly in every group as the samples were titrated.

4.2.10. IFN-γ production by ovalbumin, HI HSV-1 or gB-1 primed spleen cells before and after HSV-1 infection.

The supernatant samples were assayed on the WEHI-279 cell line. In the presence of IFN-γ , proliferation of this cell line is inhibited. IFN-γ was detected in all the supernatant samples as the counts obtained were lower than the counts from the cells alone (data not shown). Less IFN-γ was present in the unstimulated cell samples on days 0 and 2 p.i. (see Fig. 4.2.10.). By day 6 IFN-γ levels had increased in each group, but particularly in the gB-1 primed cells, where there was a sharp increase in the level of IFN-γ . The amount of IFN-γ detected in the cell supernatants restimulated with HI HSV-1 *in vitro* was significantly greater than in the equivalent unstimulated group. The HSV-1 primed cells had high levels of IFN-γ on all the days tested. High levels of IFN-γ were detected in

Figure 4.2.9. Titration of IL-1 containing supernatants.

Supernatant samples from the primed draining lymph node cultures set up on day 6 post infection were serially diluted in medium. The dilutions were plated in triplicate onto EL-4 NOB-1 and CTL-L cells in a 96-well flat bottomed plate. Cultures were incubated for 24 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

Results are expressed as c.p.m. ± SEM x10^3.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.

Figure 4.2.10. IFN-γ production by ovalbumin, HI HSV-1 or gB-1 primed spleen cells before and after HSV-1 infection.

Cell-free supernatants were collected from the primed cells as described in Figure 4.2.1. However, the cells were cultured *in vitro* for 48 hours before the supernatant was collected.

To detect IFN-γ, the supernatant samples were assayed on the WEHI-279 cell line. Cells were plated out at 2×10^3 cells/well in 96-well flat bottomed plates with either supernatant samples, rIFN-γ or medium alone. The samples and controls were added in triplicate. The cultures were incubated for 72 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

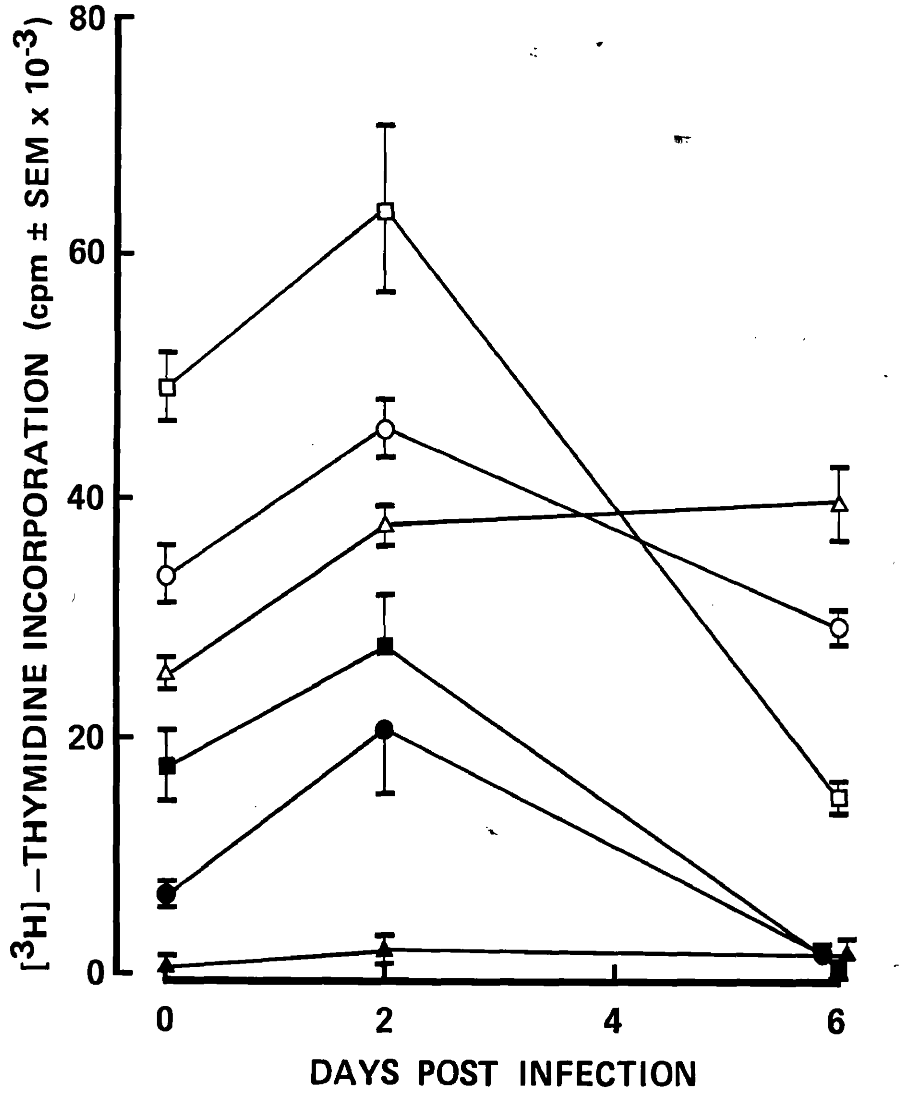
Results are expressed as c.p.m. ± SEM $\times 10^3$. The presence of IFN-γ is detected by an inhibition in the proliferation of the WEHI-279 cell line.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.


80
60
40
20
0
[^{3}H]–THYMIDINE INCORPORATION (cpm ± SEM x 10^{-3})
0
2
4
6
DAYS POST INFECTION

the ovalbumin and gB primed groups on day 0. These levels dropped slightly on day 2 and then increased significantly by day 6 p.i. More IFN-γ was found in the ovalbumin group than in the gB group on days 0 and 2 p.i., reflecting the situation with the unstimulated supernatants. However, by day 6, the levels were the same.

4.2.11. IFN-γ production by ovalbumin, HI HSV-1 or gB-1 primed DLN cells before and after HSV-1 infection.

IFN-γ was again detected in both stimulated and unstimulated cell supernatants. Only the ovalbumin primed supernatants were tested on day 0 p.i. IFN-γ was found in the unstimulated ovalbumin primed supernatant on day 0 and the levels increased steadily over the assay period (Fig. 4.2.11.). IFN-γ was also detected in the HSV-1 and gB-1 primed groups on day 2, at a similar level to the ovalbumin group, and had increased by day 6. Ovalbumin primed cells, restimulated *in vitro* with HI HSV-1, had the same level of IFN-γ as the unstimulated cells on day 0. However, the level sharply increased and was significantly higher on days 2 and 6. High levels of IFN-γ were also found in the HSV and gB groups restimulated *in vitro*. The amount detected from the gB primed cells was high on day 2 p.i. and increased sharply by day 6. IFN-γ levels from the HSV-1 primed cells were consistently high on days 2 and 6 p.i.

Figure 4.2.11. IFN-γ production by ovalbumin, HI HSV-1 or gB-1 primed draining lymph node cells before and after HSV-1 infection.

On days 0, 2 and 6 post infection, the draining lymph nodes, i.e. inguinal, para-aortic and popliteal lymph nodes, were removed and cultured as described in Figure 4.2.1.

The presence of IFN-γ was detected by assaying the supernatant samples on WEHI-279 cells as described in Figure 4.2.10.

Results are expressed as c.p.m. ± SEM $\times 10^3$.

● Ovalbumin primed.　　▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.

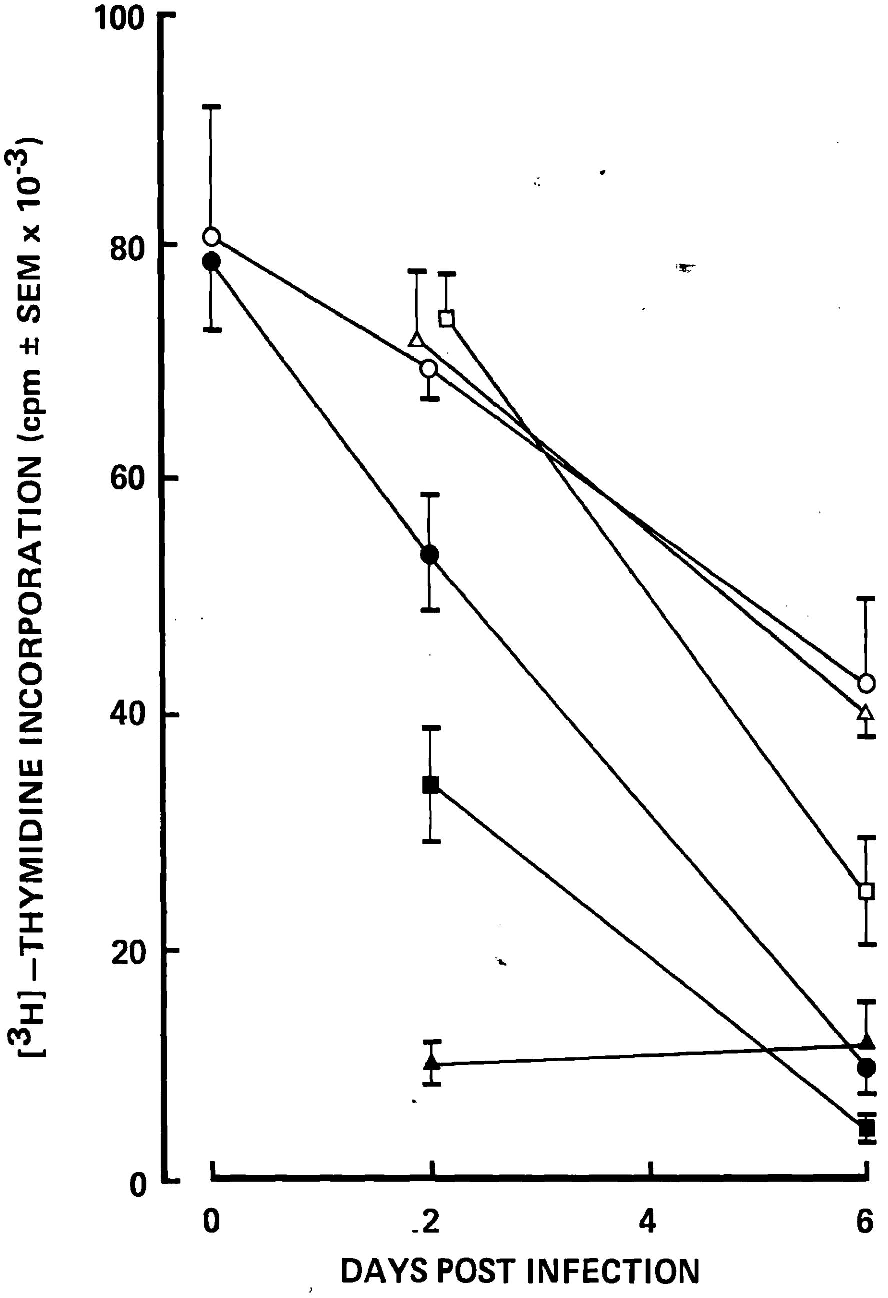

[3H]–THYMIDINE INCORPORATION (cpm ± SEM x 10-3)
100
80
60
40
20
0
0
2
4
6
DAYS POST INFECTION

Figure 4.2.12. Titration of IFN-γ containing supernatants.

Supernatant samples from primed spleen and draining lymph node (DLN) cell cultures set up on day 6 post infection were serially diluted in medium. The dilutions were plated in triplicate onto WEHI-279 cells in a 96-well flat bottomed plate. Cultures were incubated for 72 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

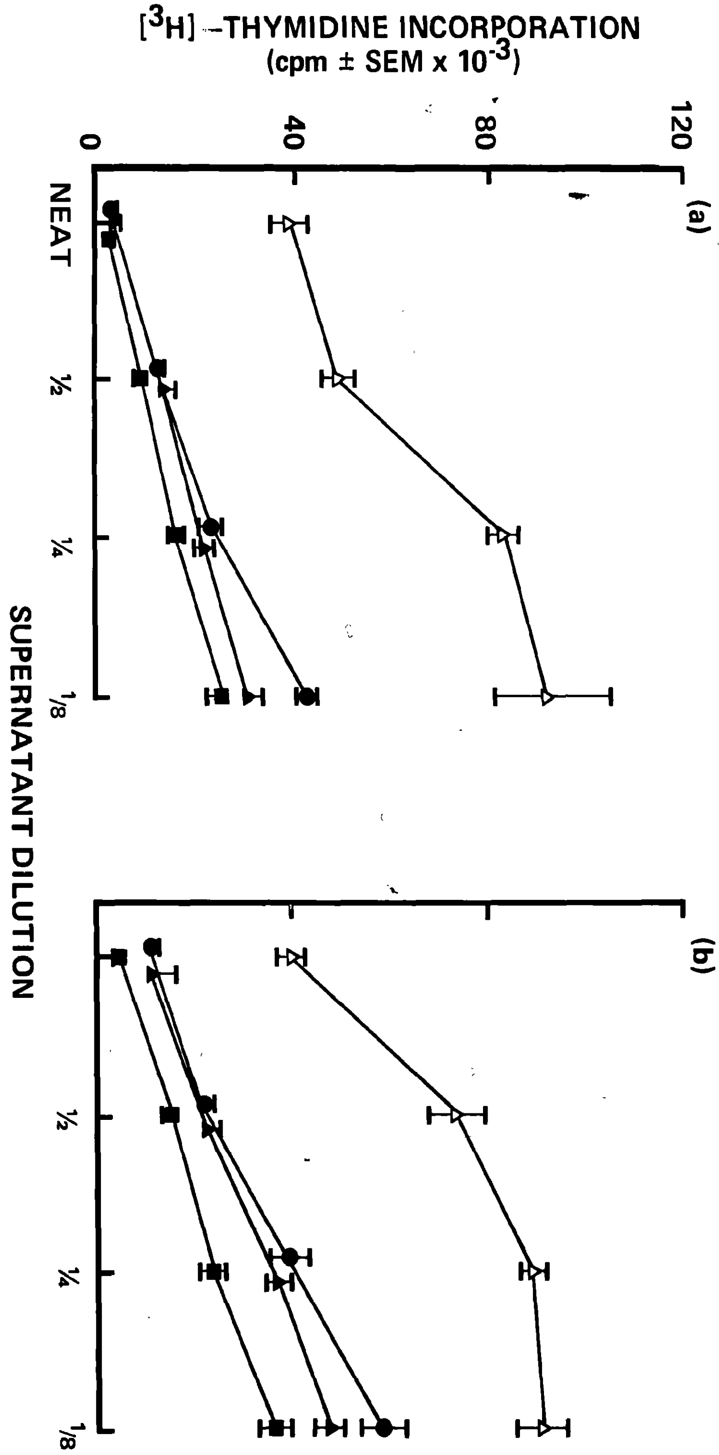
Results are expressed as c.p.m. ± SEM x10^3.

● Ovalbumin primed. ▲ HI HSV-1 primed.

■ gB-1 primed.

Open symbols - No *in vitro* restimulation.

Closed symbols - *In vitro* restimulation using HI HSV-1.


[3H] –THYMIDINE INCORPORATION
(cpm ± SEM x 10-3)
0
40
80
120
(a)
(b)
NEAT
½
¼
⅛
½
¼
⅛
SUPERNATANT DILUTION

4.2.13. IL-2 production by ovalbumin, HI HSV-1, gB-1 or pgB-1 primed spleen cells from individual mice before and after HSV-1 infection.

In an attempt to correlate the protective effects of whole virus and the glycoproteins (see Section 3.2.8) with lymphokine production in individual mice, 5 week old CBA mice were immunized with ovalbumin, HI HSV-1, gB-1 or pgB-1. At 7 weeks, the mice were infected with a high dose of HSV-1. The spleen cells from individual mice were cultured for lymphokine production on days 0-16 post infection, at 4 day intervals.

Most of the mice showed no visible signs of infection (Fig. 4.2.13.). Of those which did, 2 were seriously ill and dying, i.e. day 8, ovalbumin (a); day 10, ovalbumin (a). One mouse, i.e. day 8, ovalbumin (b), was fairly ill, but may have recovered if it had not been sacrificed.

There was no significant IL-2 production without in vitro restimulation with HI HSV-1. Supernatant from the ovalbumin primed mice did not contain significant levels of IL-2 until day 8 post infection (see Fig. 4.2.13.). The levels then slowly increased and, by day 16 p.i., IL-2 production equalled the other primed groups. No IL-2 was detected in the supernatant obtained from the spleen of the dying mouse on day 10. Significant levels of IL-2 were found in the HSV-1 primed samples on day 0. The levels increased rapidly by day 4 p.i. and remained high for the rest of the assay

Figure 4.2.13. IL-2 production by ovalbumin, HI HSV-1, gB-1 or pgB-1 primed spleen cells from individual mice before and after HSV-1 infection.

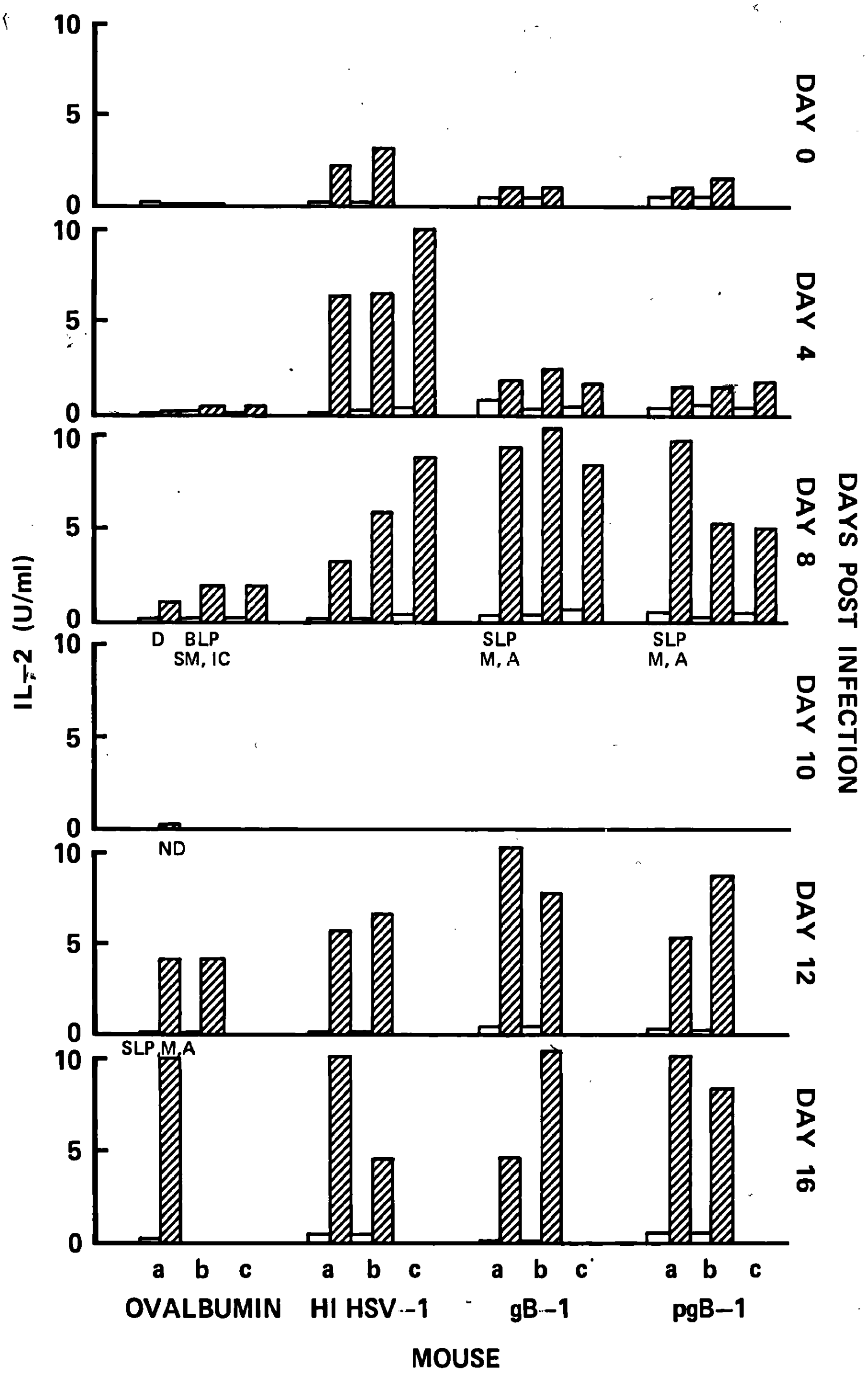
5 week old CBA mice were immunized with HI HSV-1(Krueger) at 5x10^6 PFU/mouse; ovalbumin, gB-1(Krueger) or pgB-1(Krueger) at 30μg/mouse. At 7 weeks, the mice were infected with HSV-1(Krueger) using a dose 5x LD$_{50}$. (See Materials and Methods Section 2.13.2. for fuller details.) On days 0-16 post infection, at 4 day intervals, the spleens were removed from individual mice and cell-free supernatants prepared as described in Materials and Methods 2.13.3.

To detect IL-2, supernatant samples collected after 24 hours *in vitro* culture, were assayed on the CTL-L cell line. Cells were plated out at 1x10^4 cells/well in 96-well flat bottomed plates with either supernatant samples, rIL-2 or medium alone. The samples and controls were added in triplicate. Cultures were incubated for 24 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

Results are expressed as units per ml (U/ml) of IL-2.

☐ No *in vitro* restimulation.

▨ *In vitro* restimulation using HI HSV-1.



ND - Near death.
BLP - Both hind limbs paralysed.
IC - Incontinent.
M - Mobile.

D - Dying.
SLP - Single hind limb paralysed.
SM - Semi-mobile.
A - Alert.

period, although individual variation was observed. Detectable levels of IL-2 were found in the day 0 samples of the gB and pgB primed groups. This increased slightly by day 4, then rapidly by day 8 p.i. and remained high. The amount of IL-2 detected from day 8 onwards was similar to that observed in the virus primed supernatants. Throughout the assay period, the amount of IL-2 detected from the gB or pgB primed supernatants was similar.

4.2.14. IL-3 production by ovalbumin, HI HSV-1, gB-1 or pgB-1 primed spleen cells from individual mice before and after HSV-1 infection.

IL-3 was not detected without in vitro restimulation. No IL-3 was detected in the ovalbumin primed supernatants until day 12, with the exception of one mouse on day 8 which produced low levels of IL-3 (FIg. 4.2.14.). IL-3 production was then the same on days 12 and 16. With HI HSV primed supernatants, IL-3 was first observed on day 4 p.i. and, by day 8, it had increased sharply in 2 of the mice tested. The levels fell by day 12, but were still reasonably high, however by day 16 they were less than 10 units per ml. With the gB and pgB samples, IL-3 was first seen on day 4. The levels then remained fairly consistent, with some individual variation, until day 12. By day 16, IL-3 levels were quite low. Except on day 4, the amount of IL-3 from the glycoprotein primed mice was generally lower than from the virally primed mice. However, the levels were similar

Figure 4.2.14. IL-3 production by ovalbumin, HI HSV-1, gB-1 or pgB-1 primed spleen cells from individual mice before and after HSV-1 infection.

Cell-free supernatants were prepared from the primed spleen cells as described in Figure 4.2.13. The cells were cultured for 24 hours before the supernatant was collected.

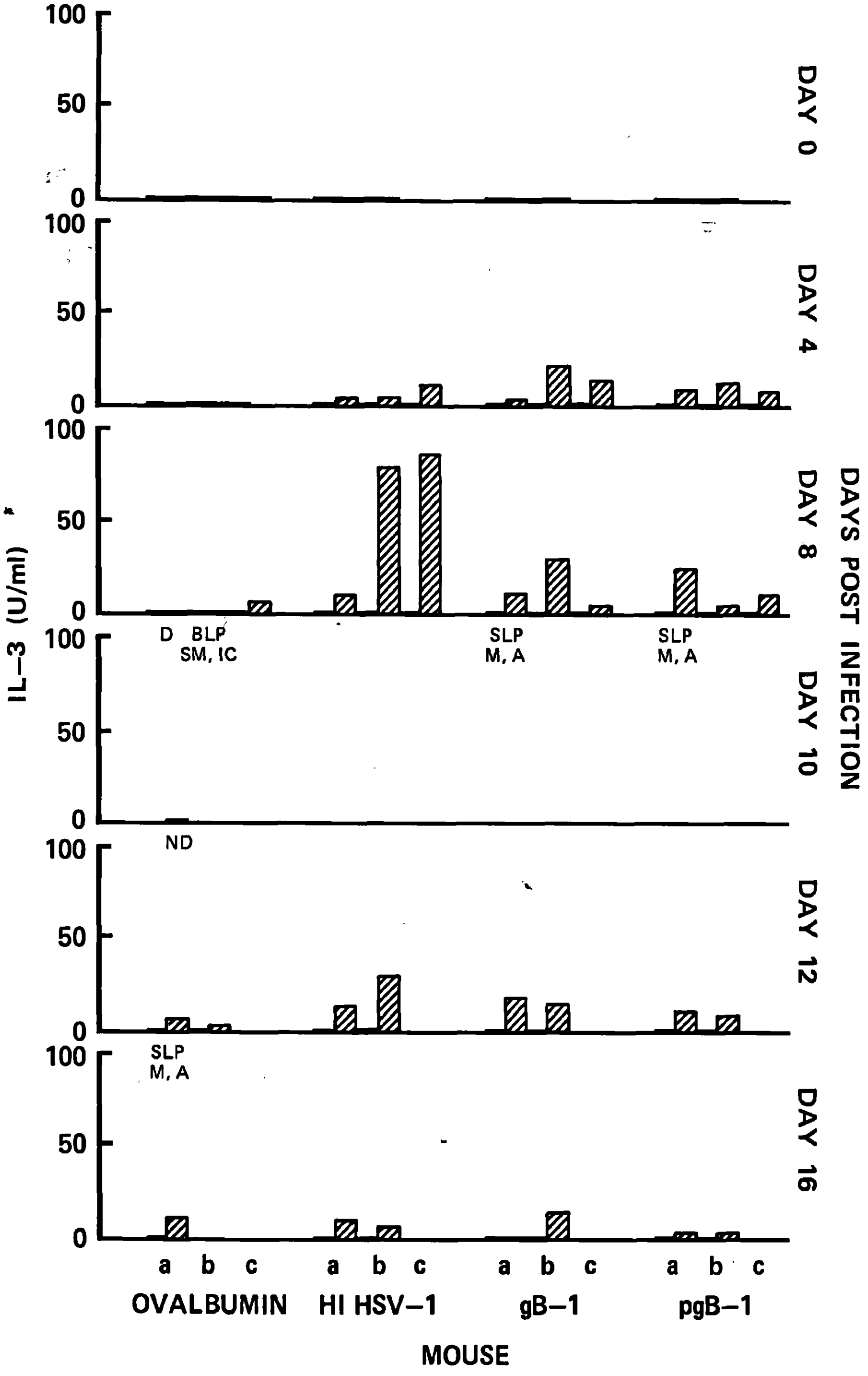
To detect IL-3, the samples were assayed on the 32-D cell line. Cells were plated out in 96-well flat bottomed plates at $2\text{x}10^4$ cells/well with either supernatant samples, rIL-3 or medium alone added in triplicate. The cultures were incubated for 24 hours, pulsed with $[^3H]$-thymidine for 18 hours then harvested.

Results are expressed as units per ml (U/ml) of IL-3.

□ No *in vitro* restimulation.

▨ *In vitro* restimulation using HI HSV-1.

Most mice showed no visible signs of infection, however those which did are indicated. For the key, see Figure 4.2.13.


IL–3 (U/ml)
100
50
0
DAY 0
DAY 4
DAY 8
DAY 10
DAY 12
DAY 16
DAYS POST INFECTION
D BLP
SM, IC
SLP
M, A
SLP
M, A
ND
SLP
M, A
a b c
a b c
a b c
a b c
OVALBUMIN
HI HSV–1
gB–1
pgB–1
MOUSE

between the gB and pgB primed groups.

4.2.15. IL-1 production by ovalbumin, HI HSV-1, gB-1 or pgB-1 primed spleen cells from individual mice before and after HSV-1 infection.

Some IL-1 was detected without *in vitro* restimulation and the amount produced increased towards the end of the assay. Ovalbumin primed supernatants produced low levels of IL-1 during the first 8 days p.i., but this increased by day 12 and remained high (see Fig. 4.2.15.). Supernatant from the dying mouse on day 10 contained no IL-1. IL-1 was found in the supernatants of the HI HSV-1 group on day 0, with one mouse producing a significantly high amount. The level remained steady throughout the assay period, with the background increasing slightly towards the end. There appeared to be an increase in production on day 16, with one mouse producing slightly more and the other producing a significantly greater amount of IL-1. The gB and pgB primed cell supernatants produced similar amounts of IL-1. The amount observed on day 0 was low and there was little difference between the stimulated and unstimulated samples. An increase in IL-1 was seen by day 8 and the background production had fallen. The amount of IL-1 then remained steady until the end of the assay period, although the amount of IL-1 detected from the unstimulated cells did increase on day 12. From day 8 onwards, the amount of IL-1 in the glycoprotein supernatants was similar to that from the virus primed supernatants.

Figure 4.2.15. IL-1 production by ovalbumin, HI HSV-1, gB-1 or pgB-1 primed spleen cells from individual mice before and after HSV-1 infection.

Cell-free supernatant cultures were prepared from the primed cells as described in Figure 4.2.13. However, the cells were cultured for 48 hours *in vitro* before the supernatant was collected.

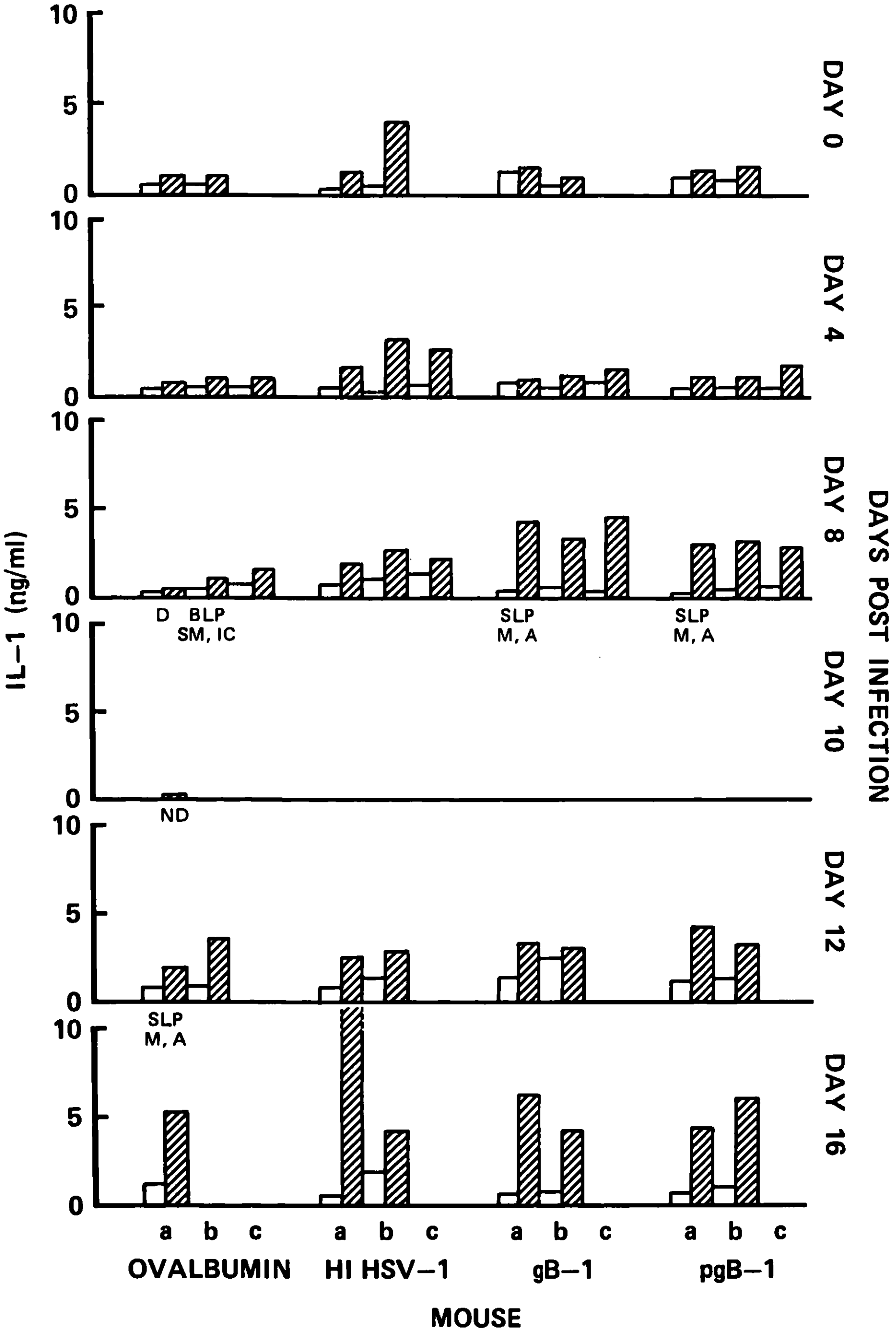
To detect IL-1, the samples were assayed on the EL-4 NOB-1 and CTL-L cell lines. Mitomycin C treated EL-4 NOB-1 cells at $2x10^5$/well plus CTL-L cells at $4x10^3$/well were plated out in 96-well flat bottomed plates. Supernatant samples, rIL-1 or medium alone were added in triplicate. Cultures were incubated for 24 hours, pulsed with [^{3}H]-thymidine for 18 hours then harvested.

Results are expressed as ng per ml of IL-1.

☐ No *in vitro* restimulation.

▨ *In vitro* restimulation using HI HSV-1.

Most mice showed no visible signs of infection, however those which did are indicated. For the key, see Figure 4.2.13.


IL–1 (ng/ml)
10
5
0
DAY 0
DAY 4
DAY 8
DAY 10
DAY 12
DAY 16
DAYS POST INFECTION
D BLP
SM, IC
SLP
M, A
SLP
M, A
ND
SLP
M, A
a b c
OVALBUMIN
HI HSV–1
gB–1
pgB–1
MOUSE

4.3. DISCUSSION.

The aims of this chapter were to examine the in vivo production of lymphokines after a sublethal infection with HSV-1 and to attempt to correlate the protective effects of immunization using HI HSV-1, gB-1 and pgB-1 with lymphokine production in vivo.

On examining the lymphokine production from primed spleen and draining lymph node (DLN) cells, it was generally found that the DLN cultures had slightly higher levels of lymphokine production, particularly with IL-2. However, as the general trends were similar, this discussion will not always differentiate between the two cell populations.

IL-2 and IL-3 could only be detected after in vitro restimulation using HI HSV-1. However, both IL-1 and IFN-γ could be detected without restimulation. The reasons for this are unclear. IL-1 can be induced by various stimulants, including peptidoglycans and other components of the bacterial cell wall (Dinarello, 1984). In earlier experiments, the mice were immunized with Freund's complete adjuvant (FCA), which contains a bacterial suspension. This may have contributed to a non-specific release of IL-1, as adjuvants, including FCA and aluminium hydroxide, are potent inducers of IL-1 (Oppenheim & Gery, 1982). Serum components can also induce IL-1 production and lowering the amount of serum used to 5% did decrease the non-specific background achieved. T cells produce IFN-γ (Farrar et al.,1981;

Morris et al., 1982) and this involves IL-2 (Farrar et al., 1981; Kasahara et al., 1983). IL-2 production is, in turn, dependent on the production of IL-1 (Smith et al., 1980; Kasahara et al., 1985; Kaye et al., 1984). Therefore, if IL-1 was induced by the immunization, this could have led to low levels of IL-2 and IFN-γ being produced in vivo, and the WEHI-279 cells may have been more sensitive to low levels of IFN than the CTL-L cells were to IL-2. NK cells also produce IFN-γ (Handa et al., 1983), so some of the detected IFN may have originated from NK cells as they are known to have a role in protection against HSV (Habu et al., 1984; Rager-Zisman et al., 1987).

Priming with HI HSV-1 produced detectable levels of IL-2 and IL-3 on all the days assayed. However, priming with ovalbumin or gB-1 did not generally lead to detectable levels of these lymphokines until day 6 post infection (p.i.), with levels of IL-2 and IL-3 significantly greater in the gB samples than in the ovalbumin samples. When IL-2 production from the DLNs was examined, the gB primed cells produced low levels of IL-2 even on days 0 and 2 and the virus primed cells produced consistently high levels throughout the assay period (see Figure 4.2.2.). In contrast, IL-1 was found in the supernatant of all three groups of primed spleen cells throughout the assay period at a consistent level. Production of IL-1 from ovalbumin and gB primed DLN cells did increase by day 6, but was constant in the HSV primed cells. IFN-γ production was also detected from each group throughout the assay period. However, with the spleen cells, the level decreased slightly on day 2 before sharply increasing by day 6.

It appeared from these experiments that priming with whole virus and gB-1 led to an early and marked increase in lymphokine production, particularly of IL-2 and IL-3. CTLs have an important role in protection against HSV infection by killing infected target cells (Pfizenmaier et al., 1977a; Nash et al., 1980a) and this activity is at it's peak 6-7 days after infection (Nash et al., 1985). IL-2 (Farrar et al., 1981; Gromo et al., 1987; Kasaian & Biron, 1989; Maraskovsky et al., 1989) and IL-3 (Curtsinger & Fan, 1984) have been shown to be involved in the regulation and augmentation of CTL responses, as has IFN-γ (Farrar et al., 1981; Gromo et al., 1987; Maraskovsky et al., 1989). IL-1 is also involved in the generation of CTL responses (Farrar et al., 1980; Mizuochi et al., 1988). As IL-2 and IL-3 levels were shown to be increasing at day 6 p.i., particularly in the HSV and gB primed groups, it is possible that they were involved in an early, protective immune response against the HSV-1 infection by inducing CTLs. However, these experiments were carried out using pooled cell populations. HSV-1 infection has been shown to affect an individual animal's ability to produce IL-3 (Chan et al., submitted for publication), so it was decided to examine lymphokine production by individual mice rather than using pooled populations. To obtain enough cells, the spleens were used instead of the DLN, even though the DLN often yielded slightly higher responses. In order to correlate lymphokine production with protection, the murine strain used was changed to the CBA strain and the immunization protocol adapted to that used in the protection experiments (see Materials and Methods Section 2.12. & 2.13.2.). Finally, lymphokine production induced by immunization with pgB-1 was also assessed. It had been shown that priming with pgB protected mice against lethal infection as well as priming with whole virus or gB and that the lack of glycosylation in pgB did not affect T cell responses (see Chapter 3). However, it was not known if the induction of lymphokines by pgB would be different.

Few of the mice showed visible signs of infection. Of those which did, only two were seriously ill. These were on day 8 post infection, ovalbumin (a) which was dying and day 10 post infection, ovalbumin (a) which was almost dead when sacrificed. One other mouse, day 8 p.i.,ovalbumin (b), was markedly ill when sacrificed, but it is possible that it would have made a good recovery. The other mice which showed visible signs of infection were not seriously ill.

Again, detectable production of IL-2 and IL-3 required in vitro restimulation with HI HSV-1, while IL-1 was detectable without this restimulation. Priming with whole virus, gB-1 or pgB-1 resulted in the marked early production of IL-2 and IL-3. In the ovalbumin primed mice, these lymphokines were not detected until at least 8 days post infection. In the HSV and glycoprotein primed mice, the lymphokine levels then remained high or decreased slowly from days 8 to 16 p.i. However, in the ovalbumin primed mice, they increased slowly from day 8 onwards and by the end of the assay period were similar to those found in the other groups. IL-1 was detected in all groups from day 0. However, from day 0 to 8 p.i., the levels increased more quickly in the HSV and glycoprotein primed groups. After this, IL-1 levels in all the groups were similar. It should also be noted that the lymphokine levels induced by gB and pgB priming were very similar. Thus, the lack of glycosylation on pgB does not affect either T cell responses (see Chapter 3) or the induction of lymphokines.

Except in the seriously ill animals, particularly those dying, the signs of visible infection did not correlate with a decrease in lymphokine levels. However, mice showing only single limb paralysis often do not get any worse and, in many cases, recover. The levels detected in these mice appeared to be the same as in the other, unaffected mice in their group and indicates that these mice had their infection under control.

It would appear from this work that lymphokine production is important in two ways:-

1. An early and marked production of lymphokines, particularly from day 0 up to day 8 is important in the protective response against infection. This response is seen in the virus and glycoprotein primed groups, where lymphokine production is detected earlier and increases more quickly than in the ovalbumin primed group.
2. As the time after infection increases, those mice which survive infection, particularly in the ovalbumin group, develop or sustain lymphokine production. This lymphokine production is probably in direct response to the viral infection.

So, although the early, increased production of lymphokines appears to be important in the protection of mice against HSV-1 infection, the mechanism or mechanisms involved are unclear. CTLs are thought to have an important role in the clearance of virus, particularly when the infection involves high doses of virus (Nash _et al._, 1985). Various lymphokines, including IL-1, IL-2, IL-3 and IFN-γ (references cited above) have been implicated in the induction, differentiation and augmentation of the CTL response, either directly or indirectly. The early induction of lymphokines could also stimulate the development of Th cells. These are involved in helping a number of immune responses, including antibody production and DTH (Leung _et al._, 1984) and the induction of CTL responses (Schmid & Rouse, 1983). However, Th cells may be more relevant to the secondary immune responses generated in response to a recurrent infection.

A role for DTH as an early defence mechanism has also been postulated with a strong correlation between T-DH cells and viral clearance shown (Nash & Ashford, 1982). Recently, IFN-γ has been shown to play a major role in recruiting lymphocytes into cutaneous DTH reactions (Issekutz et al., 1988).

IFN-γ also has potent anti-viral activity. Leucocytes from mice infected with HSV-1 were found to produce IFN-γ within a few hours of in vitro culture with inactivated HSV-1 (Lodmell & Notkins, 1974; Fujibayashi et al., 1975). Mice immunized with anti-interferon globulin were much more susceptible to infection with HSV-1 (Gresser et al., 1976). In a study of murine strains resistant or susceptible to HSV infection, no difference was found in their humoral or cellular immunity, but there was a difference in HSV-induced interferon levels in the serum with a correlation between high IFN-γ production and in vivo resistance (Zawatzky et al., 1981). IFN was detected 8 hours after i.p. injection of high doses of HSV, however no IFN was detected if low doses of HSV were used.

Another defence mechanism which may play an important early role in protection are the NK cells. Transfer of NK cells into infected recipients led to a significant reduction in virus titres in the brain and liver of these recipients (Habu et al., 1984; Rager-Zisman et al., 1987). Both IL-2 and IFN-γ can augment NK cell activity (Handa et al., 1983; Kawase et al., 1983), and some groups have shown that IFN-γ can augment NK-mediated lysis of HSV-infected target cells (Engler et al., 1981; Fitzgerald et al., 1982). However, the induction of NK activity can be separated from the induction of IFN-γ (Bishop et al., 1983b).

IL-3 may also have a more important role in clearance of HSV infections. Curtsinger & Fan (1984) showed that IL-3 was able to augment CTL responses. However, recent work (Chan et al., submitted for publication) has shown a more direct role for IL-3 in HSV infections. Treatment of primary mouse embryonic brain cells with IL-3 before infection with HSV-1 led to a significant decrease in virus titre compared with the untreated controls. The addition of anti-IL-3 antibody abrogated this resistance. Also, the injection of anti-IL-3 monoclonal antibody into infected mice led to an exacerbation of disease. HSV-1 is a neurotropic virus and infection with a lethal dose leads to pathology of the CNS, resulting in paraplegia and encephalitis. IL-3, or an IL-3-like factor, is known to be synthesised by astrocytes of the brain (Frei et al., 1985) and this functions as a growth factor for microglia (Frei et al., 1986), So, IL-3 may be an important defense mechanism against HSV infection in the CNS, by affecting cell growth and differentiation and inducing the production of other lymphokines, e.g. the interferons.

Therefore, the early and increased production of lymphokines induced by immunization with whole virus or the glycoproteins appears to be an important defence mechanism. These lymphokines may function directly against the infected target cells or indirectly by inducing or augmenting both the specific (e.g. CTL, DTH, antibody) and the non-specific (e.g. NK cells) defence mechanisms. If, however, unimmunized or unprotected mice survive the early infection, their own lymphokine production will increase in response to the infection and match that of the protected mice in the later stages of infection and clearance.

CHAPTER 5.

ISOLATION AND CHARACTERIZATION OF A PANEL OF HSV-1 AND gB-1 SPECIFIC T LYMPHOCYTE CLONES.

5.1. INTRODUCTION.

The role of T cells in the response to large and complex antigens, like herpes simplex virus, are many and varied. However, until the development of clonal populations of T cells, it was not possible to examine the contribution of discrete T cell subsets to the overall immune response.

It was shown that antigen-specific T cells could be propagated and enriched for specificity *in vitro* by repeated exposure to the antigen (Ben-Sasson *et al.*, 1975). Then, using soft agar culture techniques, it became possible to clone mitogen responsive T cells (Rozenszajn *et al.*, 1975; Sredni *et al.*, 1976). However, it was the discovery of T cell growth factor or interleukin-2 (IL-2) which made it possible to grow and maintain T cells *in vitro* for long periods of time (Gillis & Smith, 1977; Ruscetti *et al.*, 1977; Lotze *et al.*, 1980.) This led to the development of cloned populations of cytolytic T cells (Gillis & Smith, 1977; Schreier *et al*., 1980; Glasebrook *et al.*, 1981), and helper T cells (Watson, 1979; Schreier *et al.*, 1980; Glasebrook *et al.*, 1981).

However, there are potential problems inherent in the development of T cell clones including:-

(1) Selection of T cells which are responsive to media components or to TCGF, but not to the specific antigen.

(2) Selection of T cells with a skewed set of specificities for the antigen, therefore these cells would not be

representative of an in vivo population of T cells.

(3) Loss of specificity of the T cell clones during prolonged in vitro culture.

The methods used to avoid some of these problems will be discussed during this chapter.

Murine and human T cell clones have been used in a number of viral systems in an attempt to understand the role of different T cell subsets and their interactions. Cytotoxic T cell clones have been developed to various viruses, including herpes simplex virus (Sethi *et al.*, 1983; Yasukawa & Zarling, 1984a), influenza virus (Lin & Askonas, 1980; Lukacher *et al.*, 1985; Taylor *et al.*, 1985), murine cytomegalovirus (Reddehase *et al.*, 1986) and respiratory syncytial virus (Cannon & Bangham, 1989). Helper T cell clones were developed which also recognised herpes simplex virus (Leung *et al*., 1984) and influenza virus (Lamb & Green, 1983; Mills *et al.*, 1986a) as well as rabies virus (Celis *et al.*, 1986) and Sendai virus (Ertl & Finberg, 1984).

Many of these clones have been characterized both phenotypically and functionally, particularly those recognising influenza virus, with some interesting results. Braciale and co-workers have found their influenza virus-specific CTL clones recognize antigen in the context of the MHC class-II antigens I-A and I-E and express a helper cell phenotype, i.e. Lyt-1^+23^-, L3T4^+ (Lukacher *et al.*, 1985).

Yasukawa and Zarling (1984a) also found MHC class-II restricted human CTL clones with the helper cell phenotype directed against HSV-infected target cells. However, although the CD4 (L3T4) surface marker was originally thought to correlate with helper T cells and CD8 (Lyt-23) to correlate with cytotoxic T cells (Reinherz et al., 1979), it has now become clear that the expression of CD4 and CD8 markers on the surface of the T cells is more closely correlated with the MHC specificity of the T cell, i.e. CD4 with class-II restriction and CD8 with class-I restriction, than with it's function (reviewed by Swain (1983)). Of course, exceptions can be found, e.g. a $CD8^+$, class-II restricted influenza virus-specific CTL clone (Morrison et al., 1985) and a similar panel of alloreactive CTL clones (Shinohara et al., 1988).

T cell clones can also be analyzed for their lymphokine production, as this can distinguish two subsets of T helper cells (for reviews, see Mosmann & Coffman, 1987, 1989). One subset, Th1, secrete IL-2, IFN-γ, lymphotoxin, IL-3 and GM-CSF whereas the other subset, Th2, secrete IL-4 and IL-5 as well as IL-3 and GM-CSF (Cherwinski et al., 1987). (Th2 cells probably also secrete IL-6 (Mosmann & Coffman, 1989)). Also, Th1 cells can help polyclonal Ig secretion, mediate DTH and kill appropriate targets cells, while Th2 cells can provide help for both polyclonal and specific antibody responses. This has led to the suggestion that Th1 cells be termed inflammatory T cells and Th2 cells be termed helper T cells (Bottomly, 1988). The Th2/helper cells, with their ability to help B cell responses could play a major role in the defence against extracellular pathogens, while the Th1/inflammatory T cells could be involved against intracellular pathogens. Some murine Lyt23$^+$ CTL clones have been shown to have a lymphokine secretion pattern closely resembling that of the Th1 cells (Prystowsky *et al.*, 1982; Kelso & Glasebrook, 1984) and thus could also be expected to play an important role in the defence against intracellular pathogens. Certainly, CTLs are a major defence mechanism against HSV infection (Nash *et al.*, 1985). Recently, a third set of Th clones have been described and termed Th0 cells (Firestein *et al.*, 1989). These clones secrete a broad spectrum of lymphokines, including IL-2, IL-3, IL-4 and IFN-γ. It has been suggested that the Th0 cells may be a precursor population which can give rise to either Th1 or Th2 cells (Firestein *et al.*, 1989; Mossmann & Coffman, 1989).

So far, the T cell clones developed against HSV have been raised against whole virus (Sethi *et al.*, 1983; Leung *et al.*, 1984; Yasukawa & Zarling, 1984a). Some human HSV-specific CTL clones were found to recognise glycoproteins B and D of HSV-1 (Zarling *et al.*, 1986), however, no T cell clones have been raised using glycoprotein B as the initial antigen.