HERPES SIMPLEX VIRUS TYPE 1 INFECTION

OF DENDRITIC LEUCOCYTES

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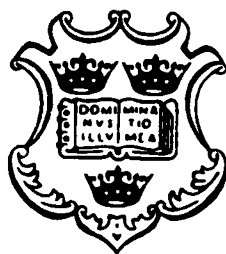

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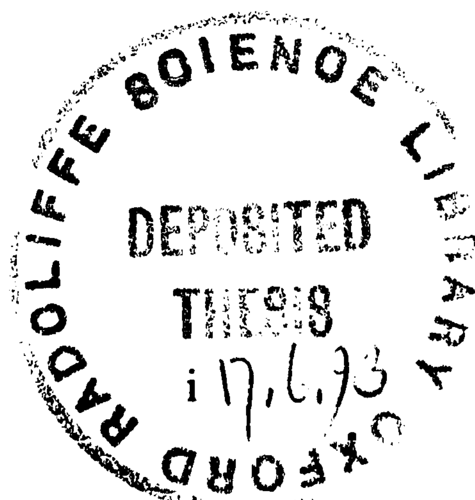

DEDICATION

To my parents,

Mary and George

"my supporters in the wings......

who provided moral and material support in the wise

belief that education was an opportunity to be seized."

Sir William Kerr-Fraser,
Principal, University of Glasgow.
12th July 1989

"Now to him who is able to do immeasurably more than
all we ask or imagine, according to his power that
is at work within us, to him be the glory in Christ Jesus,
for ever and ever."

Ephesians Ch.3 v.20,21

ABSTRACT

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Herpes Simplex Virus Type 1 Infection
of Dendritic Leucocytes

Dendritic leucocytes, or dendritic cells (DC), are a family of antigen presenting cells uniquely adapted for their role in the initiation of immune responses. Immature cells in peripheral tissues, such as Langerhans cells (LC) in the skin are capable of internalising and processing foreign antigens, such as viruses, and are available at all sites of virus entry. Migration to the lymphoid organs is accompanied by a phenotypic and functional maturation such that they develop into potent immunostimulatory cells which present antigenic peptides bound to MHC Class I or II molecules, and deliver the appropriate activation signals to resting T cells. LC are intimately involved in immunity to herpes simplex virus type 1 (HSV1) where they appear to function as the critical antigen presenting cell. However, the direct interaction of HSV1 with LC, or any DC, is poorly understood.

LC, in the form of bulk epidermal cell (EC) populations, were isolated from mouse skin and exposed to HSV1 in vitro. By UV microscopy and two-colour flow cytometry it was found that (i) HSV1 reduced the number of detectable LC, either by down regulation of MHC Class II or by killing the cells, (ii) in many instances, MHC Class II expression was reduced on the remaining infected LC, and (iii) >65% of the remaining infected LC expressed virus glycoproteins. Culturing of LC in vitro had no profound or consistent effect on the susceptibility of LC to infection by HSV1. In contrast to LC, DC isolated from mouse spleen (sDC) appeared to be relatively resistant to infection.

Experiments were set up to test whether HSV1 affected the immunostimulatory capacity of LC in the allogeneic mixed leucocyte reaction (MLR) and oxidative mitogenesis. These T cell proliferative assays are exquisitely sensitive to the presence of DC. A loss of function was observed using HSV1 infected EC but this was partly due to the release of virus which infected responding T cells. The problem of virus infection of responding T cells was over come using (i) a neutralising polyclonal antibody for HSV1, and (ii) an HSV1 mutant which produces non-infectious progeny. The subsequent loss of proliferation in the MLR and oxidative mitogenesis was found to be associated with an HSV1-induced loss of MHC Class II-positive leucocytes, LC, rather than a functional impairment of these cells.

The characterisation of a system which assesses the ability of DC, pre-pulsed in vitro, to initiate virus specific immune responses in vivo, was begun. Initial studies utilised splenic DC (sDC), pulsed in vitro with UV-inactivated HSV1. These cells were able to prime naive recipients for an HSV1-specific T cell proliferative response when administered subcutaneously, but not intravenously. No HSV1-specific antibody was detected in these two groups of animals. Further studies will be needed to extend this work which has shown the potential of DC to function as natural adjuvants. It is hoped that this will be used as a basis for future studies on the ability of LC pulsed in vitro to initiate an HSV1 specific response.

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I owe so much thanks to my family who have encouraged me and stood by me. I am especially indebted to my parents, Mary and George, who made me what I am ("warts and all!"), who have supported me in every way that mattered and who instilled in me the desire to succeed.

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these past months much more bearable and very special. I really could not have made it this far, without her.

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ABBREVIATIONS

ACV	acyclovir
ADCC	antibody dependent cellular cytotoxicity
MLR	allogeneic mixed leucocyte reaction
αMEM	minimal essential medium
αMEM+	αMEM complete tissue culture medium
APC	antigen presenting cell(s)
BFA	brefeldin A
Bfa	buffered formal acetone
BHK	baby hamster kidney
BSA	bovine serum albumin
CFA	complete Freunds' adjuvant
CMC	carboxy methyl cellulose
cpm	counts per minute
Cs	caesium
CTL	cytotoxic T lymphocytes
DC	dendritic cell(s)
E	early
EC	epidermal cell(s)
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
Expt.	experiment
FACS	fluorescent-activated cell sorter
FcR	Fc receptor
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
fp	footpad
FSC	forward scatter
g (prefix)	glycoprotein

GMCSF	granulocyte-macrophage colony stimulating factor
H-2	murine histocompatibility complex
HEL	hen-egg lysozyme
HGG	human γ-globulin
HRPO	horseradish peroxidase
HSPG	heparan sulphate proteoglycans
HSV	herpes simplex virus
ICA	infectious centre assay
ICP	infected cell protein
IE	immediate early
IFN	interferon
iv	intravenous
K	killer
KLH	keyhole limpet haemocyanin
L	late
LC	Langerhans' cells
LCA	leucocyte common antigen
LN	lymph node
LODAC	low density adherent spleen cells
MAb	monoclonal antibody
MCF	mean channel fluorescence
mϕ	macrophage(s)
MHC	major histocompatibility complex
MOI	multiplicity of infection = pfu/cell
NK	natural killer
NMS	normal mouse serum
OD	optical density
Ova	ovalbumin

PBS	phosphate buffered saline
pfu	plaque forming units
PMN	polymorphonuclear leucocytes
R10	RPMI complete tissue culture medium
sDC	splenic dendritic cell(s)
SFV	Semliki Forest virus
S.I.	stimulation index
SRBC	sheep red blood cells
SRBC-Ab	antibody-coated sheep red blood cells
SSC	side scatter
TB	trypan blue
TBAC	tris-buffered ammonium chloride
T_{DTH}	delayed-type hypersensitivity T cell
T_h	T helper cell
tk	thymidine kinase
TMV	tobacco mosaic virus
VP	virion polypeptide
WS	washing solution
wt	wild-type

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1.6 Summary

1.1 Herpes Simplex Virus Type 1 - An Overview

Herpesviruses are ubiquitous being found in animals of all classes of the animal kingdom, vertebrates as well as invertebrates. Herpes simplex virus type 1 (HSV1) is distributed worldwide in both developed and underdeveloped countries, and man remains the sole reservoir for transmission to other humans. Primary infections are usually acquired in infancy via the oropharynx or the eyes. Less than 1% of primary infections are clinically overt in immunologically competent individuals and, in general, infections are mostly apathogenic and only exceptionally do they become life threatening. Latency can follow primary infection by transmission of the virus or its genome via the sensory nerve pathways to the trigeminal ganglia where the virus resides in a non-replicating form. Virus can be reactivated and travel down the neural routes to an epithelial site where recurrent infections, recrudescences, can occur. The nature and extent of the recurrent episode is affected by cell mediated immune mechanisms and the number and competence of Langerhans cells (LC), the immune surveillance cells of the skin. Patients under immunosuppressive regimes to prevent rejection of kidney transplants frequently suffer HSV reactivation episodes which can easily disseminate. Reactivation in this case can result in progressive disease to the respiratory tract, oesophagus, and gastro-intestinal tract or, in severe cases, to the brain, usually resulting in fatal encephalitis if not controlled. Immune prevention strategies must not only limit and effectively eliminate epithelial infections, reducing the concentrations of virus able to establish latency, they must also prevent the entry of virus into the CNS, the site of latency and source of recurring virus.

1.2 Herpes Simplex Virus Type 1 Virology

1.2.1 General

Membership of the family Herpesviridae is based on similarities in virion architecture. They are large viruses, >100nm in diameter, and are composed of a core, containing linear dsDNA (MW 1-1.5 $\times 10^8$), an icosahedral capsid consisting of 162 capsomers, globular material surrounding the capsid, known as the tegument, and finally, the membrane or envelope. Virions of herpesviruses cannot be differentiated by electron microscopy but differ in biological properties, immunological specificity, size, base composition and genome arrangement. So far,

>90 herpesviruses have been identified, 7 of which are pathogenic for man. Herpes simplex virus type 1 (HSV1) is an alphaherpes virus, as is HSV2.

1.2.2 Viral Genome Expression and Regulation

In summary, three classes of viral proteins are expressed in the course of an HSV1 infection. Immediate early (IE), or α proteins, are expressed first, and in the absence of prior viral protein synthesis. Their expression peaks at 2-4 hours post-infection, but some continue to accumulate until late in infection. IE products, at least three of which act by transactivation and/or transrepression of viral genes, function mainly in the regulation of the remaining classes of virus proteins. The next class, early (E) or ß polypeptides, are divided into two groups, ß1 and ß2, on the basis of kinetics of expression. Their expression requires the presence of functional α proteins and peaks at 5-7 hours post-infection. Most of the ß gene products are involved exclusively in viral nucleic acid metabolism and their appearance signals the initiation of viral DNA synthesis. The final class comprises the late (L), or γ polypeptides, γ1 and γ2, separated on the basis of a requirement for viral DNA expression. γ2, but not γ1, products require viral DNA replication for their synthesis. γ gene products are mainly structural and include the viral glycoproteins and capsid components. Overall, the replication cycle of HSV1 takes about 18 hours from entry to exit of viral progeny, and invariably results in the destruction of infected cells. The generation of viral progeny after infection of a permissive cell typically involves four main processes: attachment and penetration, capsid entry, viral DNA replication and gene transcription, and envelopment and egress. These we will now consider in greater detail.

1.2.3 Attachment and Penetration

Initial attachment of HSV1 is thought to occur via cell surface proteoglycans, especially heparan sulphate proteoglycans (HSPG) [1]. The ubiquitous nature of these molecules on many cell types corresponds to the wide and variable host range of the virus. Heparin has long been known to inhibit HSV1 by binding to the virus and preventing normal attachment

Table 1.1 HSV1 Gene Products -A Summary

Kinetic Class	*Designation*	*Primary Function(s)*
Immediate Early	IE/α gene products	Transactivation/transrepression of later gene products
Early	E/β1 and β2	Viral nucleic acid metabolism
Late	L/γ1 and γ2	Structural components of virion

to cells, being identified as an inhibitory agent in leucocyte cultures [2] Recent studies have attempted to determine the specific interactions of virus molecules with the cell surface.

The exact structure of the cellular receptor is still unknown but a heparin moiety seems important. WuDunn et al [1] have shown that heparin reduced the adsorption and plaquing ability of both HSV1 and HSV2 on HEp-2 cells (human epithelioma cells), but only if present during the time of adsorption. Enzymatic removal of cell surface heparan sulphate, using heparinase (cleaves glycosidic linkages in heparin and heparan sulphate) or heparitinase (cleaves linkages in heparin sulphate) reduced plaque number and virus adsorption of HSV. Binding studies also showed that unlabelled HSV1 and HSV2 could competitively inhibit the binding of radio labelled HSV1, indicating that both types may compete for the same receptor.

The basic fibroblast growth factor receptor (bFGFR) has also been proposed as a "portal of entry" for HSV1 by Kaner *et al* [3] who observed that smooth muscle cells could, in part, be protected from infection with HSV1 by pre-incubation of cells with bFGF. They proposed that binding of HSV1 to cell surface proteoglycans enabled subsequent binding to the bFGFR. The exact or potential role of bFGF and its cellular receptor however seems confusing for the following reasons: (i) bFGF is known to bind directly to heparan sulphate, albeit with a lower affinity than to its own receptor, so the possibility remains that bFGF may act simply by blocking virus attachment to heparan sulphate, as proposed by WuDunn *et al* [1] (ii) Kaner *et al* [3] further demonstrated that a bFGF polypeptide, 103-120, which is known to inhibit the

binding of bFGF to its receptor, will inhibit HSV1 uptake. However, Baird *et al* [4] show that peptide 103-120 will bind heparin in a nitrocellulose binding assay. (iii) Kaner *et al* [3] provided further evidence by using chinese hamster ovary cells (CHO; which do not normally express bFGFR or take up HSV1) transfected with the bFGFR gene. They observed a >10-fold increase in uptake of radio labelled HSV1 in the transfected cells. In contrast to this, Shieh *et al* [5] showed that bFGFR transfectants *did not* show increased HSV1 binding. (iv) If HSV1 binds the bFGF receptor then its entry into the cell must diverge from that of bFGF, since bFGF normally enters by endocytosis [6], a process which may result in degradation of the virus [7]. (v) Entry of HSV1 by the bFGFR may be by direct binding of the virus or bFGF may act as a "bridge" for HSV1. Baird *et al* [8] have identified an immunoreactive bFGF-like protein associated with HSV1, and since no bFGF sequence can be found in the HSV1 genome it was suggested that the virus associates with host-cell derived bFGF and uses this to attach to the bFGFR. The assertion that HSV1 acquires bFGF from the host cell is hard to justify since in that same study bFGF is barely detectable in lysates of mock-infected cells.

In a more extensive study by Muggeridge *et al* [9] using a wider range of cell types and two strains of HSV1 and one of HSV2, they showed, like Kaner *et al* [3] that human recombinant bFGF will inhibit plaque formation on cells of vascular origin (arterial smooth muscle cells and umbilical vein endothelial cells) but little plaque reduction was seen on other cell types of a "non-vascular" origin, such as fibroblasts. A monoclonal antibody, bFM1, which blocks the biological activity of bFGF had no effect on HSV1 plaque formation.

Virus glycoproteins B (gB), gD,gH, gK and gL are essential for viral replication in vitro, but are known to act mainly in post-adsorption events [10-12]. The remaining glycoproteins gC, gE, gI, gJ, gG are all dispensable in vitro. To date, no HSV glycoproteins which are indispensable for virus attachment have been identified. The glycoprotein which has been most studied for its role in mediating virus attachment is gC. gC is a $\gamma 2$ polypeptide and is dispensable in vitro. Herold *et al* [13] attempted to define which virus gene product showed affinity for immobilised heparin. gC and gB both bound heparin sepharose. The binding of

gC-negative mutants to cells was also significantly impaired, whereas the gB-negative mutants bound normally. The small percentage of gC-negative virions which did attach to the cell were tightly bound and still sensitive to inhibition by heparin, indicating that the gC-negative mutants still interact via a second receptor with heparan sulphate. gB may therefore substitute for gC in adsorption to cells. However, in wild type HSV1 it is unlikely that gC and gB act co-operatively. Kuhn *et al* [14] studied the interaction of biotinylated cell surface molecules with radio labelled viral lysates. When co-incubated, the majority of viral proteins were confined to the detergent soluble (cell surface molecule) fraction. Using a variety of methods they showed that no single glycoprotein was responsible for attachment, but that gC, gB and gD were the only virus products associated with cell surface molecules from all the cell lines and virus isolates tested. No association was seen with gH, a glycoprotein known to be important in fusion of the virus with the host cell, indicating that this study reflects the events involved in adsorption, not penetration of the virus. Mutants of, and antibodies to gC, gB and gD only decreased binding of the individual glycoproteins. For example, a gC-negative mutant lysate did not show decreased binding of gD and gB, and a monospecific antibody to gB did not affect binding of gC and gD. Therefore, while gC, gB and gD all appear to be potentially involved in HSV1 binding to heparin, their action is not co-operative. This contrasts with gII, the Pseudorabies virus (PRV; an alphaherpesvirus), homologue of gB, which does not bind well to heparin when gIII (PRV gC) is absent [15]

Table 1.2 *Glycoprotein Function*

Name	*Known/suspected function*	*Reference*
gC	Initial adsorption of virion to cell surface HSPG	[1]
gB	May act in adsorption by binding HSPG Possible role in penetration of virion	[13] [16]
gD	Fusion of virion envelope with cell membrane	[17]
gH	Fusion; complexes with gL	[18]
gL	Fusion; complexes with gH	[19]
gK	Negative regulator of fusion	[20]
gE	HSV FcR; alone binds IgG complexes or associated with gI binds monomeric IgG	[21]
gI	HSV FcR component; associates with gE	[22]

Evidence points to the involvement of multiple receptor-ligand interactions in the attachment of HSV1, and indicates that different mechanisms have varying importance depending on cell type, virus strain, cellular environment and location. Sears *et al* [23] demonstrated, by infection of polarised, epithelial Madin-Darby canine kidney (MDCK) cells, the asymmetrical distribution of different cellular receptors for HSV1, one dependent on gC, the other not. R6012, an HSV1 mutant lacking the coding domain of gC, was able to infect continuous cell lines such as HEp-2, and that binding was more or less equivalent to the wild type. By exposing HSV1 wild type and R6012 to MDCK cells, which sort and secrete sets of proteins to apical and basal surfaces (heparan sulphate proteoglycans, for example, are known to be secreted primarily on the basal surface of epithelial cells) Sears *et al* [23] found that the mutant was less able to infect via the apical surface, as determined by plaque formation and expression of viral gene products, but that infection via the basal route was indistinguishable from wild type. Entry of HSV1 via the apical surface seems to require gC whereas basal entry utilises a second receptor. It could be that the second receptor is gB, which is known to bind heparin, and/or gD, which interacts with cell surface, possibly via heparin-like molecules, and would function by interaction with the much increased concentration of HSPG on the basal surface. So in this case what is determining entry is an abundance of HSPG on the basal, and a lack on the apical, surfaces. If the second receptor is dependent on HSPG then entry is much reduced due to the absence/reduced expression of HSPG's on the apical surface. This is coupled with the observation by Herold *et al* [13] that the putative second receptor is heparin sensitive.

Penetration of HSV1 involves the virus glycoproteins gB, gD and the gH/gL complex [19] which are essential for virus replication in culture. Early EM studies indicated that HSV1 could enter the cell by endocytosis, or viropexis [24]. However this observation is not completely supported by recent evidence. All enveloped viruses appear to enter cells by fusion of the virion envelope with the cell membrane. They can however be differentiated on the basis of where this fusion takes place, at the cell membrane or, after endocytosis, at the endosomal membrane . Viruses, such as Semliki Forest virus and influenza virus, enter by fusion *after* endocytosis, and are inhibitable by agents which raise lysosomal and endosomal pH, for

example, chloroquine and ammonium chloride [25;26]. In contrast, HSV1 penetration seems to have no requirement for low pH, and is therefore not affected by these agents [27;28]. Further evidence against endocytosis as the mechanism of entry which results in productive infection has been presented, but this seems by no means conclusive. Photosensitised HSV1 could be protected from light inactivation immediately after warming cells to 37°C, suggesting rapid penetration by fusion [29]. Nevertheless, it has been shown that such a temperature shift from 4°C to 37°C will initiate a high rate of endocytosis in cells [30]. Fc receptors characteristic of the virion envelope can be identified shortly after infection in the absence of de novo virus protein synthesis [31] but it is possible that this was due to membrane recycling after endocytosis. Campadielli-Fiume *et al* [7] demonstrated that HSV1 attached normally to the BJ cell line and was taken up in coated pit endocytic vesicles (This cell line constitutively expresses gD of HSV1 and does not support productive infection by HSV1 and 2). Most internalised virions were shown to be in a state of degradation and viral DNA reaching the nucleus was much reduced in copy number and decreased rapidly with time compared to the parental cell line, BHK tk-.

Binding of HSV1 to cells via gC may be a necessary prerequisite for binding to second receptors involved in penetration, for example via gD. In BJ cells the constitutive expression of gD is thought to sequester the cellular receptor for gD and results in penetration of the virus by a mechanism, endocytosis, which results in degradation. gB and gD are both known to interact with molecules on the cell surface [14] and gB was the first glycoprotein implicated in penetration [16]. Certain antibodies to these glycoproteins will inhibit penetration, but not binding of HSV1 virions. Highlander *et al* [32] have generated antibodies which identify five antigenic sites (I-V) on gB. Monoclonal antibodies (MAb) to sites I, III and IV, but not II, interfered with virion penetration. Sites I, III and IV were subsequently shown by immunoprecipitation to be located centrally in the external domain of gB, and site II was near the point of membrane insertion. Fuller *et al* [33] compared attachment and penetration of infectious virions with those that had been pre-incubated with anti-gD MAb, and found that both bound normally but, by EM, nucleocapsids could be seen in the cytoplasm near or just below

the plasma membrane of cells exposed to infectious virus. Cells exposed to neutralised virus gave no such pattern unless treated with low concentrations of polyethylene glycol (PEG), a fusogenic agent, indicating that anti-gD MAb block fusion. MAb to gH have been shown to act in a similar way [34] in that virions pre-treated with anti-gH MAb can bind but not fuse, as demonstrated by a lack of shutdown of host cell metabolism which is normally associated with productive infection, and lack of induction of viral protein synthesis. gH was initially identified as a 110K product immunoprecipitated by LP11, a type-specific MAb which neutralises HSV1 [35]. Evidence exists to suggest that gH is expressed in association with other virus gene product(s). Studies of mammalian cells transfected with gH [18;36] or infected with virus-gH recombinants [37], indicated that gH expressed alone was antigenically different and was not glycosylated or transported to the cell surface, and this may reflect a defect in transport out of the rER to the Golgi [38]. These cells, when co-infected with HSV1, however expressed gH indistinguishable from native gH. Only one known report has found gH when expressed alone to be similar to native gH [39]. Most recently, gL has been identified and was shown to associate with gH and to allow the full post-translational processing and cell surface expression of both products [19]. It is proposed that gH and gL form a hetero-oligomeric complex on the virion and cell surface which appears essential for virus replication, in that its absence renders the virions unable to penetrate the host cell.

Mutants which lack gB and gD cannot penetrate cells [40] but as yet no conditional lethal mutants have been found for gH. The relative importance of each of these components in penetration of HSV1 is unclear. gD-expressing cells, as discussed above, resist infection but gB expressing cells do not [7] and MAb to gD, but not to gB, inhibit syncytial formation [41]. Soluble gD from HSV1 and HSV2 inhibits plaque formation in vitro and binds to cell receptors in a saturable way, suggesting that gD binds to a limited cell receptor. This may explain why gD expressing cell lines are so resistant to infection, in that they need only sequester a receptor which exists in low concentrations. Recent evidence by Martin *et al* [42] has shown that soluble gD protected rats against ocular infection with HSV1 and reduced mortality. The receptor for gB, however, is not saturable suggesting a more abundant cell receptor, perhaps

heparan sulfate, to which gB is known to bind [13] and indicates why gB expressing cells do not resist infection. gK is thought to play an essential role in fusion [20]. Syncytial mutants of HSV1 which give rise to large multinucleated cells often have the defect mapped to the UL53 gene in the HSV1 genome [43]. Using anti-peptide antisera (generated by coupling four peptides spanning the predicted hydrophilic regions of UL53 to bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH)) Hutchinson *et al* [20] were able to immunoprecipitate a glycoprotein, gK, from purified virions. It appears that gK acts by negatively regulating the fusion process and that mutations in this region fail to regulate syncytial formation.

In summary, gB, gD, gH/L complex and gK are required for the penetration of HSV1 into cells. Entry is thought to be mediated by these molecules, all of which regulate, and are important in, fusion of the viral envelope with the host cell membrane. An alternative or accessory role for endocytosis in virion entry cannot be entirely discounted on the basis of current evidence.

1.2.4 Capsid Entry

On entering the cell, there is a release of tegument components. These are involved (i) in replication of the virus, for example VP16 (Vmw 65) is a trans-activator of α genes, and VP13 and VP14 which modulate VP16 function; and (ii) in early shut-off of host macromolecular synthesis. Early evidence indicated that HSV1 infection of different cell types resulted in a reduction in cell DNA, RNA, and protein synthesis with concomitant translation of viral mRNA [44;45]. This inhibition was seen to occur in two phases, one at 1-3 hours and another >6 hours post-infection, and was associated with a breakdown in host cell polyribosomes [46]. Since then a wealth of information has been produced concerning the virally-induced reduction in host cell metabolism. Studies of HSV1 infection of dimethyl sulfoxide-treated Friend erythroleukaemia cells showed the virus can also act to degrade cellular mRNA [47]. Studies with HSV2 demonstrated an inhibition mechanism which (i) occurs without de novo expression

of viral genes, in that HSV2 inactivated by low doses of UV irradiation also inhibits protein synthesis [47], whereas heat treated HSV does not, and (ii) affects synthesis of Sendai virus proteins, but not those specified by HSV1 [48]. The findings of Roizman et al [45] suggested a multi-step process of inhibition for which Nishioka et al [47], amongst others, provided direct evidence: initial breakdown of polyribosomes by a product in the infecting virion was followed by a degradation of cellular globin mRNA which required expression of viral gene products. Mutants have since been isolated which were defective in their ability to shut off host macromolecular synthesis, and were designated virion-associated host shut off (vhs) mutants [49]. These mutants adhere and penetrate normally and are only slightly impaired in their ability to form plaques in Vero or HEp-2 cells, therefore vhs may not be essential for virus growth. The vhs gene encodes a product which is a structural component of the virion and acts on pre-existing cellular mRNA's inducing rapid turnover of viral mRNA's [50;51].

1.2.5 Viral DNA and gene transcription

On entry, the nucleocapsids then move to the nuclear pores, a process thought to be mediated by the cell cytoskeleton [52]. The capsid uncoats and viral DNA enters the nucleus, circularising as it does so. HSV1 and HSV2 have a GC rich (≈70%) genome which consists of two unique (U) sequences, one long (UL) and one short (US), covalently linked and flanked by inverted repeats. The virus may become latent - a property which is possessed by all known herpesviruses. Viral DNA is transcribed in the nucleus by host cell RNA polymerase II throughout the replication cycle [53] but is under the influence of viral factors, such as VP16 (Vmw65), at all stages. α genes are the first viral genes to be expressed and this occurs in the absence of viral protein synthesis, that is, they are the only viral RNA's to be transcribed and accumulate in the presence of protein synthesis inhibitors. Transcription occurs in the nucleus but protein synthesis takes place in the cytoplasm of an infected cell. Once synthesised, the α gene products re-enter the nucleus where they exert their effects as trans-acting regulators of gene expression.

α gene expression

There are five α gene products. They are located near the termini of UL and US, the unique sequences, and form two clusters in the circularised genome, (i) α27, 0, 4 and 22, and (ii) α47,4 and 0. Each α gene has its own promoter regulatory region and transcription initiation and termination sites and may exist in more than one copy in the genome. Control of expression is, in part regulated by a conserved DNA sequence (minimum consensus TAATGARATTC; R=purine) and is required for trans-activation by the tegument component, VP16. However, IE promoter activity can be detected in the absence of VP16. The α gene products, infected cell polypeptides (ICP), are necessary for the subsequent expression of later classes. ICP4 (IE3, Vmw175) accumulates in the nucleus where it interacts with viral DNA [54] and appears to be involved directly or indirectly in the autoregulation of IE gene transcription. ICP22 (IE4, Vmw 68) may be important for neurovirulence but is not essential for virus growth in culture [55] or for the establishment of latency. ICP27 (IE2, Vmw 63) is an essential regulatory protein. ICP0 (IE1, Vmw 110) is probably not essential, except at low multiplicities of infection (MOI = pfu/cell). ICP47 (IE5,Vmw 12) is not essential since it is possible to get viable deletion mutants which lack the whole of this gene. ICP4, 27, 22, (and ICP0) are all found in the nucleus whereas ICP47 is located in the cytoplasm.

β Gene Expression

HSV1 encodes several proteins involved in synthesising viral DNA, and these are almost entirely restricted to the products of the β gene family. Viral DNA synthesis is detectable at 3 hours post-infection, continuing for a further 9-12 hours.

The first category of β gene products are seven proteins essential for amplification of DNA. These include products of genes UL5, UL8 and UL 52 which form a complex and function as a DNA primase-helicase [56]. These three proteins become associated in the cytoplasm and in so doing they expose or generate a site for localisation to the nucleus, their site of action [57]. UL8 protein is important [58] but UL5 and UL52 alone can function as the enzymatic unit [59]. Other essential genes include UL30 and UL 42, the products of which form the catalytic

subunits of the viral DNA polymerase [60], and the UL29 protein, a single stranded DNA binding protein [61].

The second category of β gene products are those, mostly non-essential, products involved in nucleic acid metabolism. HSV1 thymidine kinase (tk) is probably one of the most extensively studied β gene products. It is essential for normal virus multiplication in experimental infections, but not in cell culture [62], and its substrate range is far greater than that of its host counterpart. It is this latter characteristic which forms the basis for therapy using nucleoside analogues, such as acyclovir (ACV), in the treatment of HSV infections. The viral ribonucleotide reductase is a complex of proteins, both being required for activity, and it functions to create a pool of substrates for DNA synthesis. HSV1 also encodes a DNAse and an alkaline nuclease which localise to the nucleus.

Since viral DNA replication takes place in the nucleus, the β gene proteins that are expressed in the cytoplasm and which are directly involved in this process, must localise there. This is thought to occur by rapid, selective binding of protein at the nuclear membrane and a slower step of translocation across the membrane. Viral DNA synthesis occurs by a rolling circle mechanism to form long head to tail concatamers. Replication of DNA activates γ gene expression. At this time changes occur in the cell nucleus, specifically, swelling of the nucleolus [63] and chromatin condensation, with the chromosomes probably being altered by α and β gene products. As DNA synthesis proceeds, globular structures form in the nucleus which are the sites of viral DNA replication proteins [64].

γGene Expression, Capsid Formation and DNA Packaging

The γ gene products are mainly structural proteins of which the glycoproteins, gB, gC, gD, gE, gG, gH, gI, gJ, gK and gL are the major group found in the virion envelope. Non-envelope proteins are either associated with the nucleocapsid or the tegument [65] and these include VP5, the major capsid protein [66], and VP22, a major tegument protein [67]. Empty capsids form in the nucleus as viral DNA synthesis proceeds. Newly-synthesised DNA is cleaved to unit-

lengths (when an as yet unidentified capsid component is thought to complex with the DNA-protein complex) and is packaged into the preformed capsids. Viral DNA, in the form of a toroid, is packaged tightly into the core of all mature herpes virions. Recent evidence suggests that the DNA alone forms the core [68]. Nucleocapsids may be modified at this stage to enable binding to the nuclear membrane.

1.2.6 Envelopment and Egress

Exit of viral progeny may occur by various mechanisms but the one considered most likely has been termed "reverse phagocytosis" [69], conveying the direction rather than the mechanism of egress. Other studies have indicated (i) the involvement of a microtubular network [63], or (ii) envelopment at the inner lamella of the nuclear membrane, then de-envelopment at the outer lamella, followed by re-envelopment at the cytoplasmic membrane [70]. "Reverse phagocytosis" is thought to occur in the following manner. Non-glycosylated protein precursors are produced by the polyribosomes on the rough endoplasmic reticulum (rER) and are translocated into the lumen of the rER. Glycosylation is O-linked and N-linked, processing being mainly carried out by host cell machinery. Viral glycoproteins and tegument proteins accumulate at the inner nuclear lamellae where the DNA containing capsids then attach. Budding into the perinuclear space produces immature, enveloped virions which transit to the Golgi, where further modification of the glycoproteins takes place.

Mature, fully processed glycoproteins are known to be important in the egress of the virus. As previously mentioned, gH is essential in the fusion of the virion envelope with the host cell prior to capsid entry. Using a temperature sensitive mutant for gH, Desai *et al* [11] found infected cells accumulated infectious virus particles expressing an immature form of gH, which, when excreted, lacked gH and were non infectious. This and other evidence [71] suggests that the presence, but not complete processing of glycoproteins is sufficient for virion infectivity.

The Na+/K+ ionophore, monensin [72], which affects glycoprotein transport to the cell surface and membrane vesicle transport from the Golgi to the cell surface, induced the accumulation of

infectious HSV1 virions and an increased number of defective particles localised in large, intracytoplasmic vacuoles. Post-translational processing was also affected as evidenced by an increased number of immature forms of the envelope glycoproteins [71]. Brefeldin A (BFA) greatly reduced the release of infectious virions into the extracellular space by inducing the accumulation of enveloped nucleocapsids in the perinuclear space of HSV1 infected cells [73].

1.2.7 Modification of the Host Cell Surface by γ Gene Products

HSV1-infected cells have been shown to express receptors for the Fc region of IgG, derived from the virion envelope, after the infecting virion enters the host cell by fusion/endocytosis and prior to de novo protein synthesis [31]. Early studies [74] showed that infected cell monolayers acquired the ability to bind antibody-coated erythrocytes. This binding was prevented by pre-incubation of infected cell monolayers with anti-HSV1 antisera, indicating the involvement of viral products in rendering the cell monolayers 'sticky'. The presence of Fc receptors (FcR) has been confirmed on many cell types infected with HSV1 [75;76], HSV2 [77] and CMV [78]. The involvement of viral products in the formation of the FcR is apparent in a number of studies. Para *et al* [31] demonstrated that gE from viral extracts co-sedimented with IgG, and that this was the only virus glycoprotein to bind specifically to immobilised IgG. gE, however, can also be associated with gI to form an FcR [22]. gI was initially identified as a glycoprotein (g70) which could be co-precipitated with gE using rabbit or human immune serum or anti-gE MAb on infected cell extracts [21]. In an attempt to assess its physiological role, many studies have been made of the binding affinities and biochemical characteristics of the virally-encoded Fc receptor. The virally-encoded FcR is trypsin-sensitive [75]. Binding of IgG by the HSV FcR expressed on cell surfaces results in capping and is energy dependent, suggesting a possible interaction with the cytoskeleton, the bound ligand then being internalised [75].

The HSV FcR binds IgG in the region close to where Staphylococcal Protein A binds (Cγ2 and Cγ3 domain of IgG) [79], and is Ig subclass-specific, [78] in that it will bind all human subclasses except IgG3. It may also be species-specific and bind IgG from other primates,

swine and sheep, but not that from rodents, cats, dogs, horses and chickens [80]. This latter study however was only carried out using one concentration of IgG so its implications might be limited.

Perhaps most interesting of all is the suggestion that HSV1 encodes two functionally distinct FcR which differ in their binding characteristics [81]. The binding of IgG complexes to cells expressing gE alone was not enhanced by co-transfection of gI, and monomeric IgG could only bind to cells expressing both gE and gI. Indirect evidence for this phenomenon comes from other studies. Mouse cells transfected with gE do not bind monomeric IgG [21]. While sequential immunoprecipitation of viral extracts with an anti-gE MAb removed gE/I complexes, much of the gE remained uncomplexed, suggesting two separate receptors [22]. The role of the virally-encoded FcR is not at all clear. It may bind IgG and reducing virus production in the infected cell [77]. This may prevent abrupt cell lysis towards the end of the replicative cycle prior to the complete maturation of all progeny virions [82]. The HSV FcR could help establish or maintain latency in preventing the immune destruction of infected cells [76]. Basically, the HSV FcR binds the Fc portion of IgG rendering it inaccessible to complement components or FcR bearing effector cells. HSV1 infected cells also express a virally encoded C3b receptor induced by expression of HSV1 gC on endothelial cells [83].

In summary, HSV1 replicates within the host cell and as it does so its effects on the cell are profound. Attachment to the cell involves multiple receptor-ligand interactions primarily via gC and cell surface HSPG. Penetration of the nucleocapsid involves gD, gB, gK and the gH/L complex which control fusion of the virion envelope, although endocytosis cannot be ruled out as a means of entry. On entering the cell the virus immediately begins the inexorable process of cell shut-down, and this is in part mediated by the *vhs* components of the virion tegument. There follows a cascade of virus gene product expression, the functions of which are varied. Virion envelopment is intranuclear and progeny virions egress from the cell via the ER. Prior to this, virus glycoproteins are inserted into the cell membrane. These include gE and gI which form the HSV FcR. This may play a significant role in protecting the cell and therefore

the virus from the effector arm of the HSV1-specific immune response, the subject of the next section.

1.3 Effector Mechanisms in the Immune Response to HSV1

The following section is intended as a concise overview of the salient aspects of the humoral and cell-mediated mechanisms thought to be involved in immunity to HSV1. The immune response to HSV1 is potentially required to deal with the virus at two levels. Firstly, in the acute infection it must limit and/or abolish viral replication in the epithelial tissues. In the normal immunocompetent host this is apparently completely effective. Secondly, it must prevent access to the nervous system thereby eliminating the possibility of re-eruption and dissemination from the sensory ganglia where the virus is latent but rarely replicates. In this the host is apparently much less efficient and the virus appears to be able to establish latency quite easily after the acute primary infection. Many studies have attempted to establish the pre-eminent immune mechanism(s) which are involved, but it is apparent from the wealth of literature on this issue and the conclusions they support, at times apparently contradictory, that the protective mechanism is a complex interplay of innate and adaptive immune responses. The relative importance of the many components of the humoral and cell-mediated immune mechanisms is dependent on a great number of variables which can reflect the differing state of the host and often the experimental conditions under which the study is carried out.

1.3.1 HSV1 Infections - An Overview

Primary infections in man occur in epithelial cells. The route of infection by intradermal (subcutaneous) injection in the mouse most closely reflects the normal mucocutaneous route of infection in man and has been used extensively to study the factors acting locally at the site of infection which limit viral replication. The model system which has been most valuable in this respect is the ear clearance model in which varying infectious doses of HSV1 are introduced subcutaneously into the ear flap of mice of varying ages under different experimental conditions. Various parameters are then measured, for example, clearance of virus, antibody levels, in vitro proliferative and cytotoxic responses, and in vivo delayed type hypersensitivity

(DTH) reactions. Subcutaneous immunisation in the footpad resembles the ear model but has been less extensively studied. Peripheral inoculation by abrasion of the exposed abdominal skin, the "zosteriform model", is employed to assess immune prevention of viral spread within the peripheral nervous system. In this model, virus spreads to the local sensory ganglia then migrates to regions of the epithelia remote from the inoculation site. Intraperitoneal challenge by direct inoculation into the peritoneal cavity appears to be heavily influenced by innate mechanisms of immunity and bears little resemblance to a normal infection in man.

A typical scenario following infection with HSV1 involves an initial acute episode of replication at the site of infection and at other sites in the same neurodermatome. Virus titre in the periphery is highest at 2-6 days and is undetectable by day 10, the reduction in infectious load being mainly attributable to cell-mediated mechanisms. The dominant role of T cells is suggested by the apparent absence of complete protection afforded by antibody [84] and the ability of B cell deficient mice to clear virus in the epithelia [85]. However, passively transferred antibody can, if given at the appropriate time and in a high enough dose, prevent dissemination of the virus to the sensory ganglia and central nervous system [85]. These antibody sensitive periods determined by passive immunisation all occur prior to the appearance of neutralising antibody in the model systems, therefore antibody may not moderate the primary infection but rather limit recurrence. In contrast, athymic mice support virus replication in the pinna which rapidly spreads to the ganglia, brain and spinal cord, when challenged subcutaneously. This is almost entirely reversed by transfer of syngeneic lymphoid cells from normal infected donors [86].

1.3.2 The Role of Antibody

Neutralising antibody is first detectable at around 6 days post-infection and appears to act in several ways as a potential protective mechanism against HSV1. It can act by direct neutralisation of extracellular virus [87]. It can also act by binding to virus proteins on infected cells and allowing complement-mediated lysis [88] or attachment of FcR-positive effector cells,

such as macrophages (mϕ), natural killer (NK) cells and neutrophils. These release cytotoxic subtances [89] and mediate antibody-dependent cell-mediated cytotoxicity (ADCC).

Neutralisation

Direct neutralisation of virus can only occur prior to entry of the infecting virions into, or after exit of viral progeny out of, the cell. In man, recurrent infection occurs despite the presence of circulating neutralising antibody, i.e. individuals with frequent lesions showed no detectable difference in antibody levels compared to those with infrequent lesions [90]. Antibody may restrict virus access to the nervous system. Neutralising serum may decrease, not eliminate, infectious virus found in the sensory ganglia in vitro and in vivo, but may not affect the ability of the virus to re-activate [91;92]. Neutralising antibody may also act to preferentially protect certain areas of the CNS [93].

Complement-mediated Lysis

Antibody plus complement has long been known to mediate the lysis of HSV1-infected cells [94]. Even if the binding of antibody plus complement itself does not result in cell lysis there is the generation of chemotactically active mediators, for example C5a, which can recruit effector cells such as macrophages to the site of inflammation [95].

Antibody Dependent Cellular Cytotoxicity (ADCC)

ADCC appears around 3 days post-infection in mice [96] and is known to be an important defence mechanism [97]. It is important in limiting cell-to-cell spread [89] and most importantly in this respect it can act on cells which have only recently been infected [98]. thereby reducing the possibility of releasing infectious progeny. ADCC is demonstrable in mice but is more potent in humans [99]. In humans the effector cell appears to be the killer (K) or natural killer (NK) cell, whereas in mouse it is the mϕ in both spleen and peritoneal populations [100]. In both, the antibody involved is IgG [101]. C' is not a requirement [102] although it can actually enhance ADCC [103]. The importance of ADCC is its

effectiveness at low levels of antibody and that it can act on the cell at an early stage in infection [104].

A lack of competent ADCC has been associated with decreased resistance to HSV1. Neonatal and infant mice which are known to be highly susceptible to HSV1 have been shown to have a poor capacity for ADCC [105]. Using an innovative approach which assessed the activity of human ADCC this defect was reversed by transferring mononuclear cells from adult humans (neonatal mononuclear cells were ineffective), interferon and HSV-specific antibody to neonatal mice [106]. The impairment in the neonate therefore appeared to be an intrinsic defect in the effector cell, actually reversible by γ-interferon [107], rather than a difference in cell number between neonate and adult, and is progressively less apparent by 4-6 weeks of age in the mouse [105]. Recent studies emphasise the potential importance of ADCC in determining intraperitoneal susceptibility to HSV1. Intraperitoneal administration of recombinant cytokines such as macrophage colony stimulating factor (M-CSF-1) and IL-1 to neonatal C57/Bl/6 mice has been shown to reduce their susceptibility to HSV1 [108]. Cytokine-induced maturation of macrophages, the principal effector cell in murine ADCC, may be an important mechanism in this increased resistance.

Studies of mice passively immunised with HSV1-specific monoclonal antibodies have often shown that protection is not associated with neutralisation [109], but with an ability to activate ADCC in vitro [110]. All HSV glycoproteins are considered potential targets for ADCC and protection from lethal challenge by glycoprotein-specific MAb is well documented. Much depends on the route of challenge as to which antibodies are most effective. Antibodies to gC and gD are effective in intradermal challenge [86;111]. In zosteriform spread however, a range of antibodies to gC and gD, but only one to gB, were protective and this protection was not associated with a predominance of a single in vitro function such as neutralisation [112].

Antibody Binding by Infected Cells

Cells may modulate virus infection simply by binding IgG or Fc fragments via their FcR. Despite the fact that one antibody was inactive at neutralisation, C'-mediated lysis and ADCC it was found to protect from infection [110]. In a key study [113] a pool of non-neutralising monoclonal antibodies was employed in the absence of complement and lymphoid cells. The pool was shown to be highly effective at suppressing virus growth in ganglia removed from mice infected in the cornea and cultured in the presence of antibody in vitro. Neuroblastoma cells were also tested, ruling out the effects of any resident ADCC effector cells in the ganglia, and shown to be as susceptible to the effects of antibody. This effect was not due to cell lysis since removal of antibody was followed by a rapid rise in virus production. In this respect binding of the Fc portion of the antibodies via the virally encoded FcR may influence virus production. Whole IgG and Fc fragments alone have been shown to inhibit the cytopathic effect in HSV2 infected cultures of Vero and retinoblastoma cells [77].

Antibody in Immune Evasion

A mechanism of self preservation which is open to the virus is the simultaneous binding of HSV1-specific antibody via its Fc and antigen-binding Fab portions, so called antibody bipolar binding or bridging. It was previously known that the HSV FcR could protect cells from complement-mediated attack [114] and specifically that the HSV1 FcR bound anti-HSV1 IgG [115]. More recent evidence however has detailed a clear role for the virally-encoded FcR in protecting the infected cell. Studies of target cells infected with HSV1 wild type and an FcR-negative mutant (those which resist neutralisation with anti-gE antibodies) indicated that the expression of a functional HSV FcR and binding of anti-gD IgG partially protected the cells from ADCC [116]. In a similar investigation using wild type and FcR-ve strains of HSV1, a functional FcR reduced the availability of 'free' Fc ends as determined by a decrease in the binding of Protein A-FITC, and it also hindered the attachment of 51-Cr labelled polymorphonuclear leucocytes to infected cells [79]. In this latter study it was shown that low concentrations of antibody were most effective (0.1%). Higher concentrations of IgG bound

by the Fab domain may exceed the number of FcR expressed on the cell surface and therefore are still available for ADCC.

The role of antibody in immunity to HSV1 may involve the processes of neutralisation, ADCC and Fc-dependent functions, all of which may have a potential role in restricting virus infection. No single mechanism predominates but antibody may act, in the presence of an intact T cell response, to slow virus spread and delay the rate of replication, giving more time for specific cell-mediated recognition [117], the subject of the next section.

1.3.3 The Role of T Cells

T cells in general are known to function in various ways, such as, secretion of cytokines by T helper (T_h) cells, mediate DTH (T_{DTH}) and direct lysis of cells by T cytotoxic (CTL) cells. As was stated earlier, studies have only served to emphasise the complexity and inter relatedness of the HSV1-specific, in this case T cell, response. The relative role of the T cell subsets seems to be heavily dependent on mouse strain and virus dose.

T_h cells are divided into two groups differentiated in vitro by the spectrum of cytokines they produce [118]. T_h1 cells produce IL-2, IL-3 and γ-IFN and promote DTH reactions [119], whereas T_h2 cells secrete IL-3, IL-4, IL-5 and IL-10, the cytokines important in B-cell differentiation [120]. Both subtypes can be important in B cell help (although T_h1 are less effective, if at all [121]) and in the generation of CTL. In the context of HSV1, MHC Class II restricted $CD4^+$ $CD8^-$ T_h clones are important in low dose virus infections and are known to stimulate HSV1-primed B cells when injected with live or uv-inactivated HSV1 into irradiated mice. The resulting antibody response is type-common, and directed against a number of glycoproteins [122]. Recent evidence suggests that HSV1-specific T_h cells in humans exhibit considerable functional heterogeneity [123].

T_{DTH} Cells

T_{DTH} cells are an MHC Class II-restricted functional subset of T_h cells, and many studies have centred around their role in mediating recovery from low dose HSV1 infections. In the context of T_h1 vs. T_h2 subsets, it is thought that the classical tuberculin-type DTH (lymphocyte and mononuclear phagocyte cellular infiltrate) is a consequence of T_h1 and T_h2 cooperation [124]. Their main mode of action is the release of lymphokines which recruit nonspecific effector cells to control cutaneous HSV1 infections. The classical DTH response to HSV1 is measurable in man [125]. In mice the DTH response to HSV is strong and rapidly induced (3-4 days post-immunisation) and detectable 24h after challenge in the ear [126]. This reaction is transferable [126;127], with a fall of virus titre in the periphery due to a mononuclear cell infiltrate under T cell control, but only if the cells to be transferred are removed from the donor mice and transferred before a critical time, around day 12-13 [127]. There may be a requirement for H-2K and H-2 I-A compatibility for rapid clearance of virus from the inoculation site [128]. This apparent requirement for H-2 I-A compatibility, unlike other virus infections such as LCMV [129], may reflect the route of immunisation, the nature of the antigen involved, or a T_h cell-induced augmentation of a CTL clearing mechanism [130]. Other workers have suggested that DTH does not always correlate with immunity and that the ability of adoptively transferred T cells to mediate virus clearance may be a consequence of the nature of priming and restimulation of these cells [131]. Infectious or uv-inactivated HSV1 elicit DTH [128] and the resulting response can be type-common or type-specific [132]. T_{DTH} cells can also mediate immunopathology but only if their action is prolonged, or if it occurs in certain critical areas. Herpetic stromal keratitis is the leading nontraumatic cause of blindness in humans and is thought to be due to immune response-induced corneal scarring.

Cytotoxic T Cells (CTL)

The destruction of HSV1 infected cells by CTL is a well documented phenomenon [133], as is recovery from HSV1 infection mediated by CTL in the mouse [134]. CTL are detectable in the draining lymph nodes of mice 4 days after subcutaneous immunisation, they are maximal by 6-9 days, but by day 14 are undetectable [127]. One salient feature of the human and murine

response to HSV1 is the occurrence of $CD4^+$ CTL [135;136]. The likelihood that $CD4^+$ CTL are simply an experimental artefact due to an exogenous mode of antigen presentation is reduced given that high frequencies of these cells are induced even by the endogenous antigen-processing pathway [137]. Also these cells are found in herpetic lesions well before $CD8^+$ CTL which appear around the time that these lesions have already begun to resolve [138]. $CD4^+$ CTL can also aid HSV1-specific antibody production [139] similar to classical $CD4^+$ T_h cells.

It is interesting to note that freshly isolated cells from HSV1 infected animals show little or no cytotoxic capacity, but on culture for 48-72 hours in the presence *or* absence of HSV1 they can develop into effective CTL [140;141]. It seems likely that CTL in the freshly isolated population are restrained, perhaps by T suppressor (T_s) cells, the influence of the latter being diminished by culture in vitro. Cyclophosphamide which is thought to act on T_s cells can permit the detection of CTL in a freshly isolated population of draining lymph node cells [142]. CTL require only a short time of exposure to the infected cell to deliver the cytotoxic signal [141] approximately 30-60 mins.

The target antigens for CTL have also been the subject of extensive study. HSV1 glycoproteins have been suggested as the principal CTL targets [143]. Any impairment in glycoprotein expression using inhibitors of glycosylation, such as tunicamycin, or HSV1 mutants which are defective in glycoprotein synthesis, reduce killing by CTL [144;145]. Mouse cell lines which constitutively express gB can effectively generate and be recognised by CTL [146].

A more recent report however has suggested that HSV1-specific CTL recognise nonstructural proteins of HSV1 [147]. By interrupting HSV1 replication with cycloheximide, infected cells only express HSV1 immediate early (IE) proteins. These cells have been shown to be effectively recognised by CTL even though no IE proteins could be detected serologically. Indirect evidence supporting the recognition of HSV1 gene products produced early in infection

is the observation that cells exposed to infectious HSV1 for 30 mins at 37°C, then to HSV1-specific CTL for 60 mins were effectively killed. This did not occur if cells were exposed to the virus at 4°C suggesting that input virus glycoproteins are not the recognition elements [142]. In summary, it is possible that both glycoproteins and nonstructural components of HSV1 are recognised by CTL.

T_{DTH}/CTL Cooperation

There is a considerable body of evidence which strongly suggests that CTL cooperate with T_{DTH} in order to optimise the immune response against HSV1. As detailed earlier, optimal clearance of virus from the peripheral tissues requires compatibility at H-2K and H-2 I-A loci [128], the restricting elements for $CD8^+$ CTL and $CD4^+$ DTH cells. Despite the observation that in primed mice DTH was detectable up to around two years, it was only transferable maximally at day 6-9 but not after day 12-13. Coupled with the observation that transfer of CTL was impossible after day 13 it seems likely that transfer of DTH requires competent CTL *and* T_{DTH} cells [127]. It is reasonable to suggest that T_{DTH} cells arm and recruit macrophages to the site of inflammation and CTL lyse virally infected cells, both acting in concert.

Immunogenicity of Virus antigens

Many studies have concentrated on the ability of glycoproteins in the form of immunopurified proteins, peptides or expressed in the form of a vector, to act as immunogens. Responses to gB and gC include the whole gamut of HSV1-specific immune responses : non neutralising antibodies, CTL, in vitro T cell proliferation, transferable protection, reduction in ganglionic colonisation, enhanced clearance and T_{DTH} [112;148;149]. Responses to gD are similar [150] except that they can affect the immune response to both HSV1 and HSV2 even if the immunogen is HSV1 in origin [151;152]. In summary, while vaccinia virus constructs are effective, but not always wholly protective, there is essentially little difference between purified protein or that expressed on cells as vaccinia virus constructs or transfectants in the immune responses they appear to generate.

1.3.4 Innate Mechanisms of Resistance

Macrophages (mϕ) form a principal and formidable defence against HSV1. They can exert their effects in two ways. Firstly, by extrinsic resistance which involves (i) the inactivation of extracellular virus by activated mϕ [153], (ii) the destruction of infected cells [154] and (iii) the inhibition of viral replication in permissive cells for which the mϕ must contact the cell [155]. Secondly, by intrinsic resistance the mϕ can internalise [156] and digest viral particles and genomes. The exact role of polymorphonuclear leucocytes (PMN) and NK cells in innate resistance to HSV1 remains to be established and will not be discussed here.

Interferons have a protective role but it has been shown that their effects are varied. Interferon (IFN) α and β (α-IFN and β-IFN) can inhibit HSV1 replication in T cells but γ-IFN will not [157]. Replication of HSV1 in mouse macrophages is inhibited at the transcriptional level by α-IFN and β-IFN [158], but γ-IFN acts at the translational level [159]. In summary, the exact mechanisms of the action of IFN are not known but exogenous IFN or IFN induction maintains intrinsic resistance of monocytes to HSV1 [160] and perhaps by this mechanism optimise immunity to HSV1 by the intraperitoneal route.

Immunity to HSV1 involves different components of the effector arm of the immune response and for an optimal response this may require extensive co-operation between these components. The extent to which each component contributes towards the immune process may in part be dependent on the nature and route of antigen presentation.

1.4 Antigen Presentation - A Summary

Antigen presentation in the context of this discussion refers to the process whereby foreign antigens are presented to lymphocytes in a form which they can recognise and to which they can respond. Antigen presentation is the process which enables the stimulation and clonal expansion of T cells. B cells recognise native antigen via membrane-bound immunoglobulin whereas T cells can only recognise processed antigen in the form of peptides bound to molecules of the major histocompatability complex (MHC). Antigen is processed into

fragments by antigen presenting cells (APC) and the resulting peptides associate with MHC Class I or Class II molecules in specialised compartments within that cell. The genes encoding MHC Class I and II molecules are found in the H-2 region in mouse and the HLA region in humans. Technically, any cell which expresses MHC Class I or II can act as an APC and present antigen to primed T cells, but only those specialised cells which can deliver the necessary and appropriate stimulatory signals can initiate a primary immune response. The chief, if not only, group of cells which can present antigen and deliver the necessary additional signals are dendritic cells (DC). Before we consider the specialised nature of DC it is appropriate to detail the current views on the nature and mechanisms of antigen presentation by MHC Class I and II molecules.

MHC Class I

Classical MHC Class I molecules are encoded by the H-2D and -K (mouse) or HLA-A, -B and -C (human) loci. They are expressed or induced as integral membrane proteins on the surface of all nucleated cells and consist of a large α chain which traverses the plasma membrane and has three extracellular domains (α1-3). A smaller, entirely extracellular domain, β_2-microglobulin (β_2m), is encoded outside the MHC and is associated with the α chain via the membrane proximal α3 domain. β_2m functions to enable transport to the cell membrane and stabilise the expression of MHC Class I there. The crystal structure of HLA-A2 has been elucidated [161;162]. The putative antigen-binding cleft of MHC Class I is formed from the α1 and α2 domains and consists of a floor of β-pleated sheets topped by α helices.

MHC Class II

MHC Class II molecules are encoded by H-2A and H-2E (I-A and I-E) (mouse) or HLA-DP, DQ and DR (human) loci. They exist as integral membrane molecules but unlike MHC Class I they are constitutively expressed on a limited number of cells, including B cells, T cells (humans), mϕ (not constitutive) and DC. Consisting of two noncovalently linked polypeptides, α and β chains, both encoded by the MHC, they resemble MHC Class I in that membrane distal domains, α1 and β1, form the peptide binding site. This model of peptide binding to MHC

Class II has been proposed based on a comparison of conserved and polymorphic residues in MHC Class I and II but as yet no crystal structures are available. A third component, the invariant chain, is associated with MHC Class II intracellularly. In the absence of MHC Class II it is trimeric [163] but when complexed consists of three α, three β subunits and three invariant chains [164].

Antigen and the MHC

Despite functional similarities, in that both MHC Class I and II bind peptides and present them to T cells, there exist fundamental differences in the pathways by which these peptides are generated. Generally, MHC Class I molecules present peptides generated within the cytosol [165], such as viral antigens, although degradation of protein in the ER may supply some of the peptides [166]. Cytosolic degradation is thought to be mediated by proteasomes (multi-subunit degradative enzymes) [167] Peptide association with Class I is necessary for assembly with β_2m [168;169] and is thought to occur in the endoplasmic reticulum [165;170]. Peptide entry into the ER may be mediated by the TAP 1 and 2 (Transporter associated with Antigen Presentation) molecules which are encoded in the MHC and are proposed to act as carrier molecules from the cytosol into the ER. The resulting MHC Class I-peptide complex (Class I binds peptides of about 9 amino acids in length [171;172] is then transported to the Golgi. The transfer to the Golgi is possibly mediated by a recently reported 88kd protein which is retained in the ER and is associated with the α chain/β2 complex until the mid Golgi [173]. Brefeldin A (BFA) which inhibits MHC Class I presentation by inhibiting glycoprotein exit from the ER has been used to show the requirement for newly synthesised Class I molecules in antigen presentation [174;175].

In contrast, MHC Class II molecules encounter peptides as exogenous antigens much later en route to the cell surface. The α/β chain complex associates with the invariant chain soon after the insertion of these proteins in the ER and is thereby enabled to transport to the Golgi [163]. After leaving the Golgi, MHC Class II and the invariant chain must enter the endocytic pathway where they colocalise with endocytosed antigen [176]. MHC Class II and the invariant chain

are then dissociated. Inhibitors of MHC Class II presentation, such as, chloroquine, interfere with this dissociation and subsequent transport to the cell surface [177] (The primary action of chloroquine is to prevent acidification of lysosomes thereby inhibiting the action of the enzymes they contain). The invariant chain appears to be required for processing of an intact protein antigen. MHC Class II expressing fibroblasts (expressing low levels of endogenous invariant chain) will present peptide fragments but not intact protein, unless the cells are cotransfected with the invariant chain genes [178;179]. The requirement for invariant chain can be overcome by high antigen doses [Stockinger, B., Basel Institute for Immunology, Switzerland, unpublished] or by virus antigens. Measles-specific class II-restricted cytotoxic T cells will kill infected invariant chain-negative fibroblasts in a chloroquine insensitive manner [180]. Therefore, virus antigen can associate with MHC Class II, in the absence of invariant chain, in a nonendosomal compartment, possibly within the ER. This has also been observed for influenza [181;182] and was inhibited by BFA, an inhibitor of MHC Class I presentation. In the context of HSV1 it is tempting to speculate that the predominance in humans and occurrence in mice of class II restricted CTL is as a consequence of this "alternative" mode of presentation of endogenously derived peptides via MHC Class II molecules. It is also interesting to note that this invariant chain-independent non-endosomal pathway of MHC Class II presentation has only been described for viral antigens. It is conceivable that ongoing viral replication supplies an excess of antigen making the invariant chain dispensable. In conclusion, invariant chain is not an absolute requirement for presentation of exogenous antigens nor does it preclude the presentation of endogenous material. DC, which constitutively express MHC Class II molecules, are pivotal in the initiation of the primary immune response. It is our contention that, for the generation of an effective anti-viral immune response, viruses must interact with, and be processed and presented by, cells of this lineage.

1.5 Dendritic Cells (DC) - A Lineage of Specialised APC

Dendritic cells (DC) are a family of bone marrow-derived antigen-presenting cells widely distributed in most tissues of the body. They constitutively express high levels of MHC Class

I and II and several adhesion molecules, such as ICAM-1 (CD54) and LFA-3 (CD58). In non-lymphoid organs they act as "sentinels" to capture and process foreign antigens. These antigen-bearing DC migrate via the lymphatics or blood to the T cell areas of the lymph nodes or spleen. In lymphoid organs they may initially interact with T cells in an antigen independent manner. Subsequent recognition by the T cell-receptor (TcR) leads to the activation of those T cells which are specific for the peptide-MHC complex that the DC is expressing. It is the ability of DC to deliver the signal(s) necessary for the activation of naive T cells, so called immunostimulation, which establishes their unique position in the antigen presenting cell niche.

1.5.1 The Dendritic Cell System

DC were initially characterised as a novel cell type isolated from lymphoid organs which differed in structure and function from other leucocytes [183-185] In situ, DC exist as a network of stellate cells and in vitro, suspended in cell culture, they continually extend and retract sheet-like processes. Langerhans cell (LC) freshly isolated from mouse and human skin can efficiently process and present antigens to antigen-specific primed T cells. The process of maturation is thought to reflect the situation which is proposed to exist in vivo. LC, or for that matter any non-lymphoid DC, acquire antigen in the periphery and under the influence of certain stimuli migrate to the draining lymphoid organ. During the process of migration the capacity to process antigen is reduced but their immunostimulatory function is increased. In the lymphoid organs they present antigenic peptide in association with MHC Class I and II, and deliver the appropriate secondary signals, to activate naive antigen-specific T cells.

1.5.2 DC in Lymphoid Organs

DC can be identified in the thymus [186] spleen [187] lymph nodes, Peyer's patches and tonsils [188] by immunohistochemistry using antibodies to MHC Class II or DC-specific monoclonal antibodies. They are restricted to the T cell areas [189] and if administered into the blood or footpad will home to these regions [190]. Isolation of DC typically involves a period of culture in vitro and while these isolated populations share many features with the cells in situ it is possible that the resident population is more heterogeneous

[191;192]. On the basis of their expression of the integrin CD11c/CD18, identified by the antibody N418 DC, freshly isolated from mouse spleen (sDC), contain a small population of interdigitating cells which express NLDC145 [192] and a much larger population expressing 33D1 which normally lie in the marginal zone [193]. After a period of incubation in vitro, the NLDC145-positive population is much less apparent, if at all. Recent evidence has indicated that this phenotypic change may be cytokine-mediated, and freshly isolated N418-positive sDC can subsequently mature in culture as manifested by a loss or significant decrease in macrophage-specific markers such as F4/80 and FcR and a reduced ability to take up and present protein antigens [194]. The phenotype and function of sDC may therefore be seen to change in culture.

1.5.3 Non-lymphoid DC

The best characterised of these is the LC of the skin. LC constitute about 1% of bulk epidermal cell populations as determined by constitutive expression of MHC Class II and morphological characterisation [195]. Human LC can be identified by antibodies to CD1 (a non-MHC encoded but MHC Class I-like molecule [196]), whereas murine LC label specifically with NLDC145 [187]. LC are found in the dermis [197] and throughout the epidermis [187], where they are ideally situated to encounter foreign antigens, such as viruses. When freshly isolated, LC are poor immunostimulatory cells as judged by their ability to stimulate the mixed leucocyte reaction (MLR). This in vitro proliferative response is highly sensitive to the presence of DC and is used as a means of characterising cells as mature or maturing DC. In comparison, B cells and mϕ are weakly stimulatory for the MLR. For LC, this faculty is acquired after 24-72 hours maturation in culture and is primarily under the influence of GM-CSF [198;199]. Maturation is also associated with changes in phenotype and structure in that cultured LC express higher levels of MHC Class II and adhesion molecules and become enlarged. DC have been identified in other non-lymphoid organs such as heart [200], lung [201], lamina propria [202] . and liver [203].

1.5.4 DC in Blood and Lymph

DC in the form of veiled cells have been isolated from the afferent lymph of mouse , human, sheep, rat, rabbit and pig [204-209]. The lymph node is the terminal point since no DC have been detected in the efferent lymph. Intradermally administered antigen can be detected on the veiled cells of the draining lymphatics by their ability to stimulate antigen-specific T cell lines [206]. Veiled cells may be LC which have migrated from the skin and blood DC originate from the peripheral tissues. DC in the blood exist in two functionally distinct forms. As DC precursors en route from the bone marrow to non-lymphoid tissues [210;211] they exist as MHC Class II-negative cells which when isolated and cultured in vitro in the presence of GM-CSF will develop into classical veiled MHC Class II-positive immunostimulatory cells [210]. A migratory form of DC (non-lymphoid DC migrating from blood to the spleen) has been isolated from human peripheral blood after extensive purification [212]. They were characterised as large motile cells which expressed high levels of MHC Class I and II and adhesion molecules and could potently stimulate the MLR.

1.5.5 Dendritic Cell Surface Markers

Monoclonal antibodies to differentiation antigens which specifically identify DC have proved difficult to generate. In the mouse, N418 (anti-CD11c/CD18) reacts strongly with interdigitating cells (IDC) and marginal zone DC (MZDC) [193]. N418, in contrast to antibodies specific for MHC Class II, does not interfere with the ability of splenic DC to stimulate the MLR [192]. NLDC145 reacts with LC in the skin and interdigitating cells in the lymphoid organs [187]. 33D1 identifies most MZDC and Peyer's patch DC, but not those in skin [213]. These antibodies do not react with monocytes or macrophages suggesting that DC originate from a separate lineage of cells. A recently characterised antibody, ER-BMDM1 identifies macrophages and the maturing forms of DC - LC, veiled cells and interdigitating cells in the mouse [214]. While the authors contend that this is evidence for a common lineage, the expression of the ER-BMDM1 ligand is

consistent with the transient similarity between the maturing subpopulations of DC and cells of the monocyte/macrophage system.

The cell surface expression of certain other molecules on DC is entirely consistent with their role as highly specialised antigen presenting cells. Constitutive expression of MHC Class II which is quantitatively higher than that seen on B cells and macrophages exemplifies this [212]. It has been suggested that qualitative differences in MHC Class II between APC, in terms of proportion of sialic acid residues, may in part explain the potency of DC [215]. It is argued that the lower levels of glycoprotein sialylation on DC, compared to mϕ and B cells, enables them to more easily associate with T cells. Recent evidence, however, does not support this [216;217]. No cytokines have been identified which up regulate MHC products on DC. Even the observed increase in MHC Class II on culture of LC is apparently independent of GM-CSF, TNF-α, IL-4 and γ-IFN [218].

IL-2 receptors have been identified on cultured LC but their function is as yet unknown [219]. Freshly isolated DC (LC and a small percentage of splenic DC) express a functional FcγR (CD32; FcγRII) [192;220] which is profoundly down regulated on culture [192;221]. The FcR for IgE (CD23; FcεRII) has also been identified on human LC and is induced by γ-IFN and IL-4 [222]. Receptors for the complement component C3bi (CD11b) are also readily detectable on LC [223].

Adhesion molecules on the surface of DC have been studied in greater detail in recent years as more have been identified and reagents become available. ICAM-1(CD54; ligand=LFA-1) [224;225] and LFA-3 (CD58; ligand=CD2) have been shown to be up-regulated on human LC during in vitro culture. They are also expressed on human blood DC along with LFA-1 (CD11a/CD18) and Pgp-1 (CD44) [212;226]. These receptor-ligand interactions are known to be important in the interaction of blood DC and T cells leading to the activation of resting T cells. Antibodies to LFA-1 and ICAM-1 will strongly inhibit T cell-DC binding, anti-LFA-3 has no effect while antibodies to Pgp-1 actually enhanced the interaction [226].

Immature DC, such as LC, express Fc and C3bi receptors which may facilitate the internalisation of foreign antigens. As maturation proceeds, MHC Class II expression is up-regulated, as are the molecules important in clustering with (ICAM-1, LFA-3), and activation of (B7BB1), T cells.

1.5.6 Migration and Maturation of DC in vivo

The processes of DC migration and maturation are held to be concomitant and most information on the course of these two processes refers to LC. Various non-specific stimuli, such as abrasion, tape stripping and injection of hypertonic saline or steroids and are known to reduce the number of LC in the epidermis [227-229] but little is known of the cytokines that mediate these processes. Intradermal injection of TNFα has been shown to cause a concentration- and time-dependent increase in the number of lymph node DC, while GM-CSF had no effect [230]. TNFα is produced by keratinocytes [231] and maintains viability of LC [218]. GM-CSF has been shown to mediate the migration of LC precursors from the blood into the dermis [232]. A tumour-derived protein of MW>12,000 has been identified which induces LC migration into squamous-derived tumours [233]. Given its squamous origin, the authors have suggested that this protein may attract LC precursors into the epidermis.

UV-B irradiation has been extensively studied for its role in modulating the migration and function of LC. LC and DC are known to be effective at presenting HSV1 and HSV2 antigen to HSV-primed T cells and B cells [234-236]. The action of UV-irradiation reduces LC density. UV irradiation has been shown to induce a reversible loss and decrease in function of splenic DC [237;238]. It will suppress HSV1-specific DTH in vivo [239] by the generation of two phenotypically distinct HSV1-specific T-suppressor cells [240;241]. The process of migration appears to be mediated in part by cis-urocanic acid, the deamination product of histidine [241]. This acts to induce the local release of TNFα [242] which may directly affect LC. Recent evidence has shown that UV-B irradiation will affect LC function in terms of their ability to induce anti-CD3 mediated T cell proliferation [243] and subvert LC from cells which

generate DTH to those which induce unresponsiveness in the T_h1 T helper cell subset [244]. UV-B however has no effect if LC are cultured prior to irradiation [245].

In terms of function, freshly isolated LC, but not cultured LC, can efficiently present exogenous protein antigens to antigen-specific T cells [246]. Alternatively, peptides are readily presented by both cultured LC and mature DC [246]. This indicates that LC exist in the skin as highly efficient antigen processing cells, but this capacity is down regulated as the cells mature. This antigen processing capacity may be related to the level of MHC Class II biosynthesis [247] and may differ depending on the strain of mice and cell population tested [248-250].

GM-CSF is pivotal in the maturation of LC [198]. IL-1 may augment the effects of GM-CSF [199] and increase the ability of DC to cluster with T cells [251]. LC can, in fact, produce their own IL-1β and this increases on culture [252]. IL-10 produced by keratinocytes has been suggested to act on LC [253]. However, other evidence has shown IL-10 can inhibit mitogen-induced T cell proliferation mediated by mϕ but not by mature splenic DC [254]. The possibility still remains that IL-10 may act on LC which in their immature state more closely resemble mϕ than splenic DC.

Once in the lymph node, DC cluster with T cells in an antigen independent manner. IL-1, while not produced by mature DC [255;256], is produced by freshly isolated LC [257] and has been shown to increase the ability of DC to cluster with T cells [251]. This process of clustering is no doubt aided by the adhesion molecules detailed earlier and these may be further modulated by the cytokines which are produced during an ongoing response. The DC-specific molecules which activate resting T cells have not yet been identified. B7/BB1 (ligand=CD28) is considered to be important in the activation of resting T cells [258;259] and has been identified on cultured, but not freshly isolated, murine LC [260] and also on mature human blood DC [261].

1.5.7 DC as Initiators of the Primary Immune Response

In an elegant and impressive study, splenic DC pulsed in vivo and in vitro with protein antigens were shown to potently induce an MHC-restricted protein-specific proliferative response in vivo [262]. The use of DC pulsed in vitro demonstrated the need to expose these cells to protein at the time of isolation when they were capable of endocytic activity (demonstrated by the internalisation of rhodamine modified ovalbumin), rather than 24 h later. When introduced into naive animals these cells primed for a highly antigen-specific proliferative response which, at 5 days, was restricted to the lymph node draining the immunised footpad. Bulk spleen cells were much less efficient and peritoneal cells were completely ineffective thus emphasising the specialised nature of DC. More recent work has confirmed and extended these findings. Keyhole limpet haeomcyanin (KLH) pulsed DC were transferred into mice by the intravenous and subcutaneous routes. Subcutaneously immunised KLH-DC remained in the skin or localised to the local lymph nodes and generated a detectable DTH on ear challenge with KLH. KLH-DC administered intravenously migrated to the spleen and while increasing the levels of anti-KLH antibodies they did not generate DTH. In mice immunised with KLH in complete Freund's adjuvant (CFA), the simultaneous, intravenous transfer of KLH-DC resulted in an H-2 restricted suppression of DTH [263]. The authors suggested that the immune response was determined by the route of antigen presentation which in turn may be determined by the ratio of T_h1 to T_h2 cells in lymph nodes vs. spleen. T_h1 and T_h2 cells differ in ratio depending on their anatomical location [264] and differential stimulation may determine the immune response - induction of T_h1 leads to DTH and T_h2 to antibody production [119;265]. A recent study [266] has shown that DC pulsed with protein antigens in vitro and administered intravenously (followed by an antigen boost 5 days later) induced a "primary" humoral response. It is difficult to conceive how antigen delivered in the context of DC can prime B cells which recognise native, not processed, antigen. Internalised, undegraded antigen regurgitated by DC in vivo and thereby acting to prime B cells seems unlikely. The authors contend that DC induce a primary response, despite the fact that 5 d after the administration of DC 100µg of antigen is delivered to these animals prior to the detection of specific antibody. It seems more likely that antigen-pulsed DC prime antigen-specific Th cells, a well documented phenomenon, which are

then ready and waiting to amplify the B cell response generated when native antigen was administered later. This would generate a so called "primary" response but does not represent the priming of specific B cells directly by DC.

1.5.8 DC as Initiators of the Primary Antiviral Immune Response

The availability of DC at all potential sites of virus entry allows them to act as the first, and in many instances the most important, line of defence. Their ability to internalise and process antigens and then to subsequently mature into immunostimulatory cells on migration to draining lymph nodes or spleen renders them ideal for the initiation and maintenance of anti-viral immunity. Functionally competent LC occurring at critical levels in the epidermis appear necessary for the generation of an effective HSV1-specific immune response. Following infection with a pathogenic, but not a non-pathogenic, strain of HSV1 the number of LC in the epidermis increased [267]. These were presumably recruited from the circulation or the dermis to control infection. Keratinocytes produce IL-1α and this is down-regulated upon infection with HSV1 [268].

It has also been shown that depleting LC in the footpad using the non-specific stimuli detailed above (hypertonic saline followed by abrasion or steroid administration) the virulence of both pathogenic and non-pathogenic strains of HSV1 was significantly increased [228;229;267]. HSV1-bearing interdigitating cells can be detected in the draining lymph nodes; the lymph node T cells responded specifically to HSV1 antigen in vitro. Depletion of LC however abolished this in vitro HSV-1-specific proliferative response [229]. Conversely, increasing the density of LC by intradermal injection of the immunomodulator OK-432 led to an increased migration of DC to the lymph nodes and a decrease in HSV1 pathogenicity [229].

The central criticism which could be levelled against these studies is the unknown effects of the so called non-specific stimuli, other than the obvious decrease in LC number, which may also affect HSV1 pathogenicity. However, when mice were challenged with HSV1 in sites which normally possess lower numbers of LC, such as the ear pinna, the virulence of certain strains of

HSV1 was increased [228]. Additionally, LC density in the epidermis is decreased in old mice and this affected the ability of EC isolated from these animals to induce in vitro secondary responses [269]. Resolution of HSV1 infections of the cornea is associated with a recruitment of LC to the site of the lesion and is linked with the generation of $CD4^+$ T cells [270].

LC density has also been implicated in controlling vaccinia virus infections in the skin [271] . Human papilloma virus lesions are thought to be limited by the activation, and increased number, of LC in the vicinity of the wart [272;273] Foot and mouth disease virus (FMDV) antigen has been detected in the LC population after inoculation of guinea pigs with the virus [274].

There is as yet no evidence which demonstrates that LC can initiate a primary anti-viral response in vitro. HSV1-specific T cells could be generated by co-culturing naive T cells with spleen cells and HSV1 [275]. No such response was detected when EC, enriched for LC, were used as APC [228;275]. One report has suggested that EC could initiate an HSV1-specific primary response in naive T cells from individuals who tested negative serologically for HSV1 [276]. However, antibodies may not identify T cell epitopes so the above study may merely represent secondary proliferation of previously primed T cells. In the context of secondary restimulation LC act as efficient presenting cells for HSV1 antigen and therefore may also fulfil a role in the maintenance of the virus specific response in the peripheral tissues. LC are known to be associated with T cells in an HSV1-specific DTH lesion. In fact, infected keratinocytes produce cytokines, such as interferons [277] which may mediate migration of T cells to the lesion [278]. When isolated in vitro, EC can presented HSV antigen to primed T cells in murine [235] and human [234] systems, and this presentation was ablated when $CD1^+$ cells were depleted or when EC were pre-incubated with anti-MHC Class II antibodies [234]. LC also induce HSV1-specific antibody in an in vitro antibody induction system [236]. Keratinocytes which can be induced to express MHC Class II by β-IFN have been shown to act as HSV1-antigen presenting cells and indeed were susceptible to CTL-mediated lysis [138].

The evidence presented above which assessed the competence of LC in presenting HSV in primary and secondary immune responses is perhaps not so remarkable given the functional differences between freshly isolated and cultured LC. Freshly isolated LC cannot deliver the signals necessary for activation of naive T cells and therefore would not be expected to initiate a primary immune response. It is also possible that HSV1 may infect LC and thereby kill them or ablate their capacity to stimulate T cells. LC can however efficiently process antigen and present it to primed T cells, the requirement of which for further activation signals is much less stringent. To our knowledge no studies have addressed whether or not LC (cultured, to allow maturation) in the presence of HSV1 antigen can initiate an HSV1 response in naive T cells. However, at least some DC isolated from lymphoid organs appear to internalise and process antigen. These cells if incubated overnight in the presence of antigen not only express antigen-MHC complexes but are also capable of delivering the appropriate secondary signals to naive T cells. This may explain why HSV1-specific primary responses could be detected with bulk spleen cells but not with freshly isolated LC [275].

Splenic DC (sDC) pre-incubated with influenza virus AX/31 and added to syngeneic lymphocytes have been shown to initiate potent influenza-specific primary proliferative and cytotoxic responses in vitro. Peritoneal mϕ were ineffective [279]. DC (and mϕ) in this system were incubated overnight prior to exposure to the virus, but since influenza virus enters the cell by fusion with the cell membrane entry is presumably not limited by the inability of mature sDC to endocytose. The T cells that were primed recognised the same nucleoprotein (NP) epitope as CTL which are generated after priming mice with infectious virus [280] emphasising the ability of sDC to naturally select immunologically relevant epitopes. Influenza-treated DC were also highly efficient inducers of $CD8^+$ CTL and MHC Class II restricted $CD4^+$ [281]. DC pulsed with the MHC Class I restricted peptide NP 147-155 did not efficiently induce CTL in the absence of exogenously added helper factors [281]. This indicates the need for the expression of relevant peptides on MHC Class I, and $CD4^+$ T_h cells via MHC Class II, in the production of influenza specific CTL. DC treated with vaccinia virus recombinants expressing various respiratory syncytial virus (RSV) components efficiently

generated RSV-specific CTL from normal human adult peripheral blood mononuclear cells [282].

Earlier work demonstrated the ability of sDC to induce a virus-specific primary antibody response when administered in vivo to syngeneic mice [283]. Splenic DC (and mϕ) were pulsed in vitro with tobacco mosaic virus (TMV). DC and mϕ were administered intravenously and subsequent analyses demonstrated the highly efficient induction of TMV-specific antibody by DC but not by mϕ.

1.5.8 Virus Induced Impairment of DC Function

The interactions of DC with viruses must be viewed as a two-edged sword. Certain viruses in the course of replication within the cell may impair that cell's ability to function normally. Therefore DC may themselves be adversely affected by the interaction with infectious virus such that they are impaired in their ability to generate an effective immune response. Viruses may employ this strategy to subvert the immune system and thus prolong or alter the course of infection. Indeed, vaccinia and influenza A viruses have recently been shown to block the processing and presentation of an exogenous antigen, but not peptide, by B cell lines [284]. This suggests a virally-induced effect on intracellular metabolism. Additionally, lymphocytic choriomeningitis virus (LCMV) mediates the $CD8^+$ T-cell dependent killing of mϕ and destruction of follicles in lymphoid organs [285].

Several reports have centred around the ability of human immunodeficiency virus-1 (HIV) to functionally impair DC. It has been proposed that LC may act as a reservoir for HIV in the skin. Clinical-stage related reductions in LC number have been observed during HIV infection, where LC number ranges from 10-77% of that of normal controls [231;286;287]. Additionally, HIV-like particles and antigens have been detected in a small percentage of skin biopsies [288]. In one patient gross morphological changes in LC, such as cytoplasmic vacuoling and loss of dendritic morphology, and virus budding were observed in 2-5% of LC [288;289]. From this extremely limited and ambiguous evidence, and the fact that epidermal

sheets biopsied from HIV patients and epidermal cell cultures appear to support replication of HIV [290;291], the authors contend that "LC are an actual site of HIV-1 replication". This is not supported by other lines of evidence which show no decrease in the number of mucosal LC [292] and that certain antibodies to HIV will bind non-specifically to skin from normal individuals thereby generating false positives [292;293]. Against this backdrop a study was undertaken to clarify these issues [294]. A variety of methods including immunohistochemistry, in situ hybridisation and direct virus isolation were applied to detect HIV in 28 HIV-infected patients and 15 HIV-seronegative individuals. The authors failed to detect any decrease in LC number or change in LC morphology. Viral antigen and particles were not detected in any epidermal LC and infectious virus could not be recovered from blister fluid or epidermal samples. HIV could only be detected in the dermis, where a variety of cell types reside, and, in conclusion, the authors assert that their evidence does not support the notion that epidermis harbours HIV to any considerable extent.

HIV infection of peripheral blood DC has been studied in detail. Evidence has been presented which suggests that DC isolated from HIV positive individuals (HIV-DC) are productively infected and preferentially reduced in number [295]. In this and other studies assessments were made on cell populations which contained only 30% DC, with a further 70% being monocytes [296]. The authors claim that when 1000 autologous DC were titrated into peripheral blood T cells, from normal and HIV-infected individuals, and co-cultured with Con A, there was an apparent impairment in the ability of DC to stimulate this response [295]. However, from the data presented it appears that the responding cells used come from the same source as the DC, that is, normal DC stimulate normal T cells and HIV-DC stimulate T cells from HIV-positive patients. Therefore, any impairment detected could simply represent a T cell not DC defect. In the mixed leucocyte reaction this problem may be partly addressed by using normal DC and HIV-DC to stimulate normal T cells and an HIV related defect is still apparent. As the authors have stated however "equivalent numbers of DC from patients and normals were added as stimulants". Since DC decrease in number in HIV positive individuals the total number of cells which need be added (the majority of which are mϕ) is therefore increased. It

is known that mϕ from HIV infected patients suppress the generation of T cells clones [297] and produce IL-1 suppressors [298], so the apparent functional defect observed with DC from HIV infected individuals [295] may merely reflect the presence and increased concentrations of these inhibitory factors produced by an increased number of mϕ. It is unlikely that secondary infection of responding T cells (<2% T cells were found to be positive for HIV [299]) could explain this apparent defect since studies utilising dideoxy-adenosine (ddA) prevented replication of HIV in T cells but still showed an apparent DC defect [299]. In a manner analogous to that described for influenza [279], DC treated with HIV or HIV peptides have been reported to be effective at generating HIV-specific CTL in vitro [300;301].

The ability of HIV to productively infect and impair the function of peripheral blood DC, and prevent the initiation of an effective immune response is by no means clearly established. Purified populations of DC were infected with HIV and shown to be productively infected by in situ hybridisation [302]. Others [303] have reported that in individuals at different stages of HIV infection there is no preferential loss of DC, little, if any, functional impairment and no productive infection. They suggest that earlier reports by Knight *et al.* may be affected by contaminating mϕ and T cells. An alternative means of optimising HIV infection may be open to the virus other than by preventing the initiation of the primary immune response. In an elegant study, it has been suggested that DC, while not being productively infected with HIV, can efficiently transmit the virus to the T cells with which it is clustering and activating [304].

1.6 Summary

The replication cycle of HSV1 is complex and has wide ranging effects on the host cell. The immune response which has evolved to deal with HSV1 infection is equally complex and, as the experimental evidence indicates, involves a wealth of effector mechanisms which limit the effects of the virus. Humoral and cell-mediated immunity to HSV is comparatively easy to detect in humans and experimental models, such as the mouse, but it remains to be seen how these mechanisms interrelate to control HSV1 replication in the immunologically competent host. The mechanisms of immunity may in part be determined by the route of antigen

presentation. Dendritic cells (DC) are considered to be pre-eminently important in the initiation of any primary immune response and are strongly implicated in immunity to viruses such as HIV and influenza virus. LC, the immature form of DC in the skin, have been shown to affect the initiation of the primary response to HSV1. Treatment of LC in vitro and in vivo with various mediators, such as cytokines, and specific stimuli, such as UV-B irradiation have been shown to modulate or subvert the resulting immune response. While studies have concentrated on the role of LC in immunity to HSV1, little is known about the direct interaction of the virus with LC, or other members of the DC lineage, in vitro and in vivo. In this thesis we have attempted to assess the capacity of HSV1 to infect cells of the DC lineage, and the ability of HSV1 to impair the function of LC (and sDC) in T cell proliferative responses in vitro. In view of the well documented capacity for DC to act as effective adjuvants we have also begun to develop an immunisation protocol which uniquely assesses the capacity of HSV1 antigen pulsed DC to initiate primary anti-HSV1 cell-mediated and humoral immune responses.

Chapter 2

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2.1 Introduction

The following chapter does not provide an exhaustive list of all the materials and methods used, but presents those which are sufficiently common to all the experimental chapters that follow. Where necessary, particular methods which have been applied in each chapter are documented in detail at the end of the chapter in the section "Specialised Materials and Methods".

2.2 Media and Solutions

2.2.1 RPMI-1640

RPMI-1640 was prepared and tested by Mr. G. Plant (Nuffield Department of Surgery) from concentrated stocks obtained from Imperial Laboratories Ltd. (Cat.No. 2-540-14). Alternatively, pre-diluted, sterile stocks were obtained from the same source (GIBCO, Cat.No.041-01870-M). RPMI-1640 was the basic medium used in the culture of all primary cell populations.

2.2.2 αMEM

αMEM was obtained in concentrated form from Imperial Laboratories Ltd. (10X Minimal Essential Medium, Eagle with Earle's Salts, Cat.No.2-300-07) and diluted 1:10 with sterile double deionised water (ddH_2O).

2.2.3 Complete Tissue Culture Media

RPMI-1640 was supplemented with 10% Foetal Calf Serum (FCS, Imperial Laboratories Ltd., Cat.No.6-000-07), the complement components of which had been heat inactivated at 56°C for 30 mins prior to use. 2-mercaptoethanol (12.8M stock, Bio-Rad, Cat.No.161-0710) and glutamine were added at final concentrations of 2.5×10^{-5}M and 2mM respectively. Antibiotics were added, i.e. penicillin at 45μg/ml (GIBCO, Cat.No. 066-01830H), streptomycin at 45 μg/ml (GIBCO, Cat.No. 066-01860H) and kanamycin at 90 μg/ml (GIBCO, Cat.No. 860-1815IL). The resulting medium, R10, was used for the culture of all primary cell populations. αMEM was supplemented with 10% heat-inactivated newborn calf serum or FCS. Glutamine and tryptose phosphate broth (Oxoid, Cat.No. CM283) were added at a final concentration of

2mM and 3% respectively. Antibiotics were used, namely penicillin and streptomycin as detailed above. The final solution, αMEM+, was used for the short and long term culture of all the continuous cell lines detailed in this study.

2.2.4 *Phosphate Buffered Saline (PBS)*

Dulbecco A tablets (Oxoid, Cat.No.BR14a) were dissolved in distilled water (dH_2O), to a final volume of 100ml per tablet. The resulting solution was free of Ca^{2+} and Mg^{2+} and was sterilised by autoclaving at 10psi for 10 mins., when necessary.

2.2.5 *Washing solution (WS)*

WS used was PBS supplemented with 1% FCS. NaN_3 (Sigma Cat.No. S-2002) was added at a final concentration of 2mM to prevent contamination due to long term storage at 4°C and, on the few occasions where live cells were stained for analysis by flow cytometry, to prevent capping and internalisation of antigen-antibody complexes on the cell surface.

2.2.6 *Buffered Formol Acetone (Bfa)*

200mg Na_2HPO4 and 1g KH_2PO4 were dissolved in 400ml ddH20. The pH was adjusted to 6.8 with 1MNaOH and the volume made up to 450ml with ddH20. The final buffer was made by adding 460µl of the salt solution to 92µl formaldehyde and 450µl acetone and mixing. The cells to be permeabilised were adjusted to 10^7 cells/pellet and 250µl of Bfa added for 2 seconds followed by vortexing. The cells were washed immediately with 2 washes with WS.

2.2.7 *Bovine Serum Albumin (dense BSA)*

Bovine Serum Albumin fraction V was obtained in powder form (Sigma Cat.No. A7906) and prepared for use in density gradients. BSA powder (50g) was reconstituted in 32.5ml ddH_20 and 93ml PBS for 12-18 h at 4°C. After allowing the solution to come to room temperature the pH was adjusted to 7.4 with a recorded volume of 1M NaOH. This recorded volume was subtracted from 14.5 ml and the difference added to the solution as ddH_20. The solution, with

a resulting density of $\rho = 1.080$, was filter sterilised using 0.2μm pore size filter units (Nalgene Cat.No. 245-0045) and stored in 40-50 ml volumes at 4°C prior to use.

2.2.8 Heparin

Porcine Heparin was obtained from Sigma (H5640) and dissolved in PBS to a final concentration of 1-10mg/ml. The solution was forced through 0.22μm pore size filter units (ICN Biomedicals, Flowpore, Cat.No.64-001-04) and was stored at 4°C prior to use. When required, the stock solution was diluted appropriately in R10 or αMEM+ depending on the cell type to be treated.

2.2.9 Tris Buffered Ammonium Chloride (TBAC)

TBAC was prepared by mixing 0.16M NH_4Cl (Sigma Cat.No. A4514) and 0.17M Tris base (Sigma Cat.No. T1503) in the ratio of 9:1. The resulting solution was adjusted to pH 7.2 using 1M HCl and sterilised by autoclaving at 10 psi for 10mins. Red blood cell lysis was carried out by resuspending a pellet of cells in 1-3 ml of TBAC and incubating at room temperature for 3-5 mins. TBAC was subsequently removed by two washes with RPMI-1640.

2.2.10 Trypan Blue (TB)

TB at 0.4% was obtained from Sigma (Cat.No. T8154). TB was added, typically to a final concentration of 0.2-0.04%, to appropriate dilutions of cell samples. Viability was assessed on the basis of TB exclusion and live cells were counted using a Neubauer haemocytometer.

2.2.11 Turk's Solution

10 mg of Gentian Violet (BDH, Cat.No. 34033) was dissolved in 100 ml of 3% (vol/vol) acetic acid/dH_20. The resulting solution lysed red blood cells allowing a count of total nucleated cells to be made using a Neubauer haemocytometer, when diluted 10-40X in Turk's solution.

2.3 Plastics and Glassware

2.3.1 Glassware

All glassware was washed thoroughly in dilute detergent (Lipsol, LIP Ltd.) and rinsed in ddH$_2$0. Sterilisation, when necessary, was carried out by autoclaving at 20 psi for 20 mins.

2.3.2 Plastics

All plastics used for tissue culture procedures were supplied by Falcon (Becton Dickinson Ltd.) (Table 2.1).

2.4 Sources and Maintenance of Experimental Animals

Six to eight week old male and female Balb/c (H-2^d), C57/Bl/6 (H-2^b) and C3H (H-2^k) mice were supplied by Harlan Olac Ltd. (Bicester, Oxon.). The animals were maintained under standard conditions either by Biomedical Services Ltd., John Radcliffe Hospital or where appropriate in the Immunology division animal facility, Dept. of Pathology, University of Cambridge.

2.5 Virus Strains and Isolates

2.5.1 Virus Stocks

All virus stocks were generously supplied by various sources (Table 2.2). In all instances, virus stocks were stored at -70°C in the form of infected cell sonicates.

2.5.2 Infection of Cells

Unless otherwise stated, primary cells and continuous cell lines were incubated in the presence of appropriate dilutions of HSV1 or Vaccinia virus stocks for 1 h at 37°C. The amount of virus added to each cell population was, unless indicated, calculated on the basis of the number of cells to be infected. The mode of infection differed for certain populations of cells (Table 2.3). The final plaque forming unit (pfu) to cell ratio, usually in the range of 0.1 to 1.0 pfu/cell, was expressed as a multiplicity of infection (MOI) of the relevant virus stock. Inhibitors such as heparin and B114 were incubated with cells *during* the time of exposure to

virus. After 1 h any excess, unbound virus (and inhibitors) were removed by three washes in tissue culture media. Cells were incubated in tissue culture dishes or flasks in tissue culture media for 0-24 h post-infection, as indicated in the text.

Table 2.1 *Disposable Plastics*

Items	*Cat.No.*
Pipettes	
1ml, 5ml, 10ml and 25ml	7521, 7543, 7551, and 7525 resp.
Tubes	
50ml polypropylene conical	2070
15ml polystyrene conical	2095
15ml polypropylene conical	2096
4ml polypropylene	2063
Tissue culture dishes	
60mm and 100mm	3002 and 3003 resp.
Petri dishes	
60mm and 100mm	1007 and 1029 resp.
Tissue culture flasks	
25cm2, 75cm2 and 175cm2	3013, 3084 and 3028 resp.
Tissue culture plates	
6 well	3046
96 well flat bottom microtest plates	3072
Cell strainer	2350

Table 2.2 *Virus Stocks* §

Strain	Designation	Titre (pfu/ml)	Source
HSV1/34	Lab passaged clinical isolate	6.8×10^8	C.Drummond*
HSV1SC16	Wild type	5×10^9	A.A. Nash$
HSV1 Kos	Wild type	5×10^9	A.A. Nash
HSV1 in1411	VP16/Wmw65 mutant	4.4×10^7	A.A. Nash
HSV1 gH	gH-ve mutant of SC16	1×10^9	A. Minson+
HSV1 MDK	thymidine kinase mutant	2×10^9	A.A. Nash

§ All virus stocks were grown in confluent monolayers of BHK cells and cell free supernatant was sonicated and stored at -70°C in 100-500µl aliquots. The number of infectious units (pfu) was determined by conventional plaque assay (See section 4.12.11).

* Supplied by C. Drummond and Dr. R.P Eglin, Dept. of Virology, John Radcliffe Hospital, Oxford.

$ Supplied by Dr. A.A. Nash, Dept. of Pathology, Univ. of Cambridge.

+ Supplied by Prof. A.Minson, Dept. of Virology, Univ. of Cambridge.

Table 2.3 *Infection of different cell types*

Cell Type	Mode of Infection
EC	Pellet of known cell number resuspended in 100µl R10 containing xµl of the virus stock to give infection on a pfu/cell basis.
BHK	Pellet of known cell number resuspended in 100µl R10 containing xµl of the virus stock to give infection on a pfu/cell basis <u>or</u> infected as a confluent cell monolayer at $1.5\text{-}2 \times 10^7$ cells/ $75cm^2$ flask in a volume of 1-2 ml.
sDC	Pellet of low density pellicle cells of known cell number resuspended in 100-300µl R10, containing xµl of the virus stock, to give infection on a pfu/cell basis <u>or</u> infected as a tissue culture adherent population in a small volume of 2-3 ml.

2.6 Cell Isolation

2.6.1 Epidermal Cells (EC)

EC suspensions were prepared, as the source of Langerhans cells (LC), from ear skin by a method adapted from Witmer-Pack et al [198]. Both pinnae were removed from 15-20 normal Balb/c or C57/Bl/6 mice near the base of the ear flap and were sterilised by rinsing thoroughly in 70% ethanol. Excess ethanol was removed in a sterile flow cabinet and the ear flaps allowed to dry by spreading on open petri dishes. Once dry the ear flaps were separated into dorsal and ventral halves and incubated separately in petri dishes containing 6.5 ml of Hanks buffered salt solution (HBSS, GIBCO., Cat.No.041-04025-M), 2.5ml of Trypsin (GIBCO 10X, Cat.No.043-05090-H) and 1 ml of 0.2% EDTA. The dorsal halves were incubated for 20 mins. and the ventral halves for 40 mins, both at 37°C in covered petri dishes. After the appropriate incubation times, epidermal sheets were removed from the digested dorsal and ventral halves into a sieve in a petri dish containing 35-40 ml HBSS + 1% FCS. The sieve was then shaken to remove cells and the resulting cell suspension pipetted vigorously with a sterile pasteur pipette to break up cell aggregates and then passed through a cell strainer to remove debris and hair (typical cell yields $3x10^6$ bulk EC/mouse). These EC were either used at this point as freshly isolated EC or incubated in vitro for 24-96 h and used as cultured EC.

2.6.2 Splenic Dendritic Cells (sDC)

Isolation of sDC from the spleens of normal Balb/c and C57/Bl/6 mice was done on the basis of their low buoyant density and transient adherence to tissue culture plastic. The spleens of 15-45 mice were removed aseptically. Single cell suspensions of bulk spleen cells were prepared by teasing with forceps and by being passed through a mesh sieve, to disperse and remove any tissue debris. A sample of cells was removed for a total cell count using Turk's solution and the remaining cell suspension was pelleted. The spleen cell pellet was resuspended in dense BSA to approximately $2\ x10^8$ cells/ml and 4 ml samples were transferred into 15 ml conical tubes and overlaid with 2 ml R10 per tube. The resulting density gradients were spun in an MSE Coolspin centrifuge at 3000g for 20 mins. at 4°C. Cells with a density $r > 1.080$ were

pelleted while cells with a low buoyant density accumulated at the BSA/R10 interface as a discrete pellicle.

Pellicle cells were removed using a pasteur pipette and washed in RPMI-1640 prior to counting in Turk's solution. Cells were resuspended in R10 at a concentration of 10^7 cells/ml and plated in 10 ml volumes onto 100mm tissue culture plates. The plates were incubated for approximately 2 h at 37°C after which time the non adherent cell were removed by gently pipetting medium over the surface of the plate with a pasteur pipette. 10 ml of fresh R10 were added back to each plate and the adherent cells incubated overnight at 37°C. During this time sDC become non adherent while macrophages (mϕ) remain strongly attached. The resulting non adherent population of cells, referred to as LODAC (Low density adherent cells), were commonly found to consist of 70-75% sDC as determined by FACScan scatter profiles and MHC Class II expression. When necessary this LODAC population was further purified by removing contaminating B cells and mϕ (using the Fc rosetting method detailed below) to give a population of 90-95% sDC. Typical yields were 5×10^6 purified sDC/20mice.

2.6.3 Bulk Spleen Stimulator Cells

Spleens were removed aseptically from normal Balb/c and C57/Bl/6 mice and teased extensively, as for sDC above. The resulting cell suspension was pelleted and red blood cells lysed using TBAC. The number of viable cells was assessed by trypan blue exclusion and the cell pellet was irradiated for 4.4 mins at 1000 rads from a Caesium (Cs) source prior to use as a stimulator population in functional assays.

2.6.4 T cells

Lymph node or spleen populations enriched for T cells were prepared by passage over nylon wool. Nylon wool (Travenol Labs Ltd., Cat.No. 4C2906) was boiled extensively in two to three changes of ddH_20 and dried on aluminium foil in a warm oven for 1-2 days. Nylon wool columns were prepared by extensively teasing 3g of nylon wool and placing it in the barrel of a 30 ml syringe, and autoclaved at 20 psi for 20 mins before use. Columns were equilibrated

with RPMI-1640 then R10 by running volumes of media through the column, a butterfly clip (Venisystems™, Cat.No. 4492) was attached to allow controlled elution. The columns were incubated at 37°C for 45 mins to 1 h prior to use.

Bulk spleen or lymph node cell suspensions were prepared by teasing the organs extensively. These were loaded onto the nylon wool column at a concentration of 10^8 cells/ml in R10, at a maximum of 5ml per 3 g column, and incubated at 37°C for 1 h. Non adherent cells were eluted with 35ml of warmed R10, pelleted and depleted of red blood cells using TBAC. Viability (typically >90-95%) of the eluted cells was determined on the basis of trypan blue exclusion and the cells were used as per protocol with no further purification.

2.7 Cell Enrichment

2.7.1 Enrichment of sDC by Fc rosetting

Stock sheep red blood cells (SRBC) labelled with rabbit anti-sheep erythrocyte antibody were prepared from SRBC supplied in Alsevers solution (Becton Dickinson, Cat.No.210102) that were <2wks old before use. 5ml of SRBC was removed and washed twice in PBS, the pellet of SRBC having a final volume of approximately 1ml. The cell pellet was resuspended in RPMI-1640 + 2% FCS (R2) and 0.4ml was removed into a clean 15ml conical tube. 1.6ml R2 and 5µl of rabbit anti-sheep erythrocyte antibody was added and mixed well. The mixture was incubated for 15mins in a 37°C water bath before being checked for aggregation. At this point a 10µl sample was removed and counted and equalled approximately 4 x 10^8 cells/ml. After a further 15mins the antibody (Ab) coated SRBC were washed twice in PBS and the pellet resuspended in 2ml R2. This acts as the SRBC-Ab stock.

LODAC from overnight incubation (section 2.6.2) were removed by gentle pipetting of medium from the tissue culture dishes and pooled, where appropriate, into 15ml opaque conical tubes. After centrifugation, the cell pellet was resuspended in 2ml R2 and counted with a 1:2 dilution of trypan blue. Stock SRBC-Ab were mixed with LODAC in a ratio of 50:1 (50 parts SRBC-Ab: 1part Lodacs), where the SRBC-Ab stock was approximately 4 x 10^8 SRBC/ml. After

resuspension, the LODAC/SRBC-Ab mixture was centrifuged at 1000rpm for 5 mins and incubated on ice for 30 mins. 4ml of dense BSA were added to a 15ml conical tube and after 30 mins the SRBC-Ab-LODAC pellet was resuspended and overlain on the BSA so as to produce a sharp interface. The gradients were centrifuged at 3000g for 20 mins at 4°C, then the pellicle of cells at the interface were harvested removing as little dense BSA as possible. The cells were centrifuged and the red blood cells lysed by resuspending the cell pellet in 0.5-1ml TBAC. The remaining cells, typically 90-95% pure sDC, were counted using a 1:2 dilution of trypan blue.

2.7.2 Partial Purification of LC

EC cell suspensions were prepared and cultured (section 2.6.1) for a minimum of 18 h at 1-2 x 10^7 cells/ml on 100mm tissue culture dishes. After this time the cells, containing 2-5% LC, as defined by staining for MHC Class II, were removed and pelleted. 4ml aliquots of dense BSA were dispensed into 15ml conical tubes and the EC pellets resuspended in 2ml R10 and used to overlay the BSA. Typically, cells from a single 100mm tissue culture dish were added onto a single 4ml dense BSA aliquot. The gradients were spun at 3000g for 20mins at 4°C. Low density LC would collect at the R10/BSA interface and were removed carefully with a pasteur pipette. This population contained from 15-25% LC, and few (<0.5%), if any, LC were detected in the high density pellet fractions.

2.8 Continuous Cell Lines

2.8.1 Baby Hamster Kidney (BHK) Cells and Balb/c 3T3 Fibroblasts.

BHK cells (provided by C.Drummond and Dr. R.P. Eglin, Dept. of Virology, John Radcliffe Hospital) and Balb/c 3T3 Clone A31 mouse embryo fibroblasts (ECACC, Cat.No.86110401) were resuscitated from frozen stocks stored in liquid N_2. Aliquots of 2 x 10^7 cells were quickly thawed in a 37°C water bath and the cell suspension immediately transferred to 10-15ml αMEM+ and spun down. The pellet was resuspended in 10ml αMEM+, transferred to a 75cm^2 tissue culture flask and incubated at 37°C/5%CO_2 to confluency (2-3 days). Once confluent, all the medium was removed and the cell monolayer was washed once with sterile

PBS. Cells were detached by overlaying the monolayer with 1% trypsin for 2-3mins followed by gentle agitation and then transferred into 10ml tissue culture medium to stop the action of trypsin. Excess trypsinisation caused the cells to clump irreversibly. After centrifugation, the cell pellet from each $75cm^2$ tissue culture flasks was split into three fresh $75cm^2$ tissue culture flasks and incubated at $37^oC/5\%CO_2$ for a further 2-3 days until confluent. The number of cells in a confluent flask was in the range of 1.5-2 x 10^7cells/flask.

2.9 Primary Antibodies

2.9.1 Monoclonal Antibodies (MAb) to Cell Markers

Unless otherwise indicated, MAbs to cell markers were used in the form of tissue culture supernatant and stored at 4^oC until required. The main exception to this was the use of biotinylated B21-2 purified hybridoma supernatant (prepared and kindly supplied by J.Roake in this lab) in some two-colour flow cytometry experiments. Table 2.4 details the particular MAbs used in this study.

2.9.2 Antibodies to Virus Antigens

Antibodies to HSV1 were in the form of both MAb's and polyclonal antibodies (PAb's) and supplied from commercial and academic sources. Antibody to Vaccinia virus was in the form of PAb only. Table 2.5 overleaf summarises the relevant details of all the antibodies employed in this study.

2.10 Immunofluorescent Staining and UV Photomicroscopy

2.10.1 Preparation of Cytospins

Cells were resuspended in tissue culture medium to a concentration of 1-2 x 10^6/ml and 100µl was removed and pipetted into the each of twelve channels in a cytocentrifuge (Shandon Cytospin 2). Cells were spun down onto slides at 800rpm for 8 mins, all excess liquid being absorbed by the surrounding filter. After centrifugation, the slides were carefully removed

Table 2.4 *Monoclonal Antibodies*

	Cell Marker	*Species*	*Cell Specificity*	*Source*
B21-2	MHC Class II (Ia b,d)	Rat IgG2b	All DC	ATCC
N418	CD11c/CD18	Hamster	DC restricted:IDC and mzDC	ATCC
NLDC145		Rat IgG2a	LC, IDC, sDC subsets and thymic DC	Ref. 187
TIB122	LCA	Rat IgG2a	All bone marrow derived cells	ATCC
TIB105	CD8 (Ly-2)	Rat IgG2b	CD8+ T cells	ATCC
GK1.5	CD4 (L3T4)	Rat IgG2b	CD4+ T cells	ATCC
KT3	CD3	Rat IgG	Pan T cell	Ref. 318

Table 2.5 *Antibodies to Virus Antigens*

Specificity	Source	Catalogue No.	Working dilution
HSV1 major glycoproteins and 1 core protein	Dako	B114	1:30
HSV1 major glycoproteins and 1 core protein (B114-FITC conjugate)	Dako	F318	1:30
HSV1 internal phospho-protein	Serotec	MCA 406	1:50
HSV1(2?) gH	A.Minson*	LP11	1:200-1:500
HSV1 VP16	A.Minson	LP1	1:200-1:500
HSV1 gD	A.Minson	AP7	1:200-1:500
Vaccinia virus	G.Smith+	-	1:25-1:100

* kindly supplied by Prof. A. Minson, Division of Virology, University of Cambridge.

+ kindly supplied by Dr. Geoff Smith, Sir William Dunn School of Pathology, University of Oxford.

labelled and allowed to air dry overnight at room temperature. Individual cytospin slides were then used immediately or tightly wrapped in aluminium foil and stored at -20°C until required.

2.10.2 Single Colour Immunofluorescent Staining

Cytospin slides were thawed from frozen and allowed to come to room temperature before removing the aluminium foil to prevent condensation developing on the slide and cell sample. Cells were pre-fixed in acetone (BDH, Cat.No.100034Q) for 10 mins then air dried for a further 10 mins. Once dry the cell samples were re-hydrated in WS for 2-3 mins. All further procedures were carried out with the slides at room temperature in a moist box. The samples were pre-blocked by incubation in 5% normal rodent (rat, mouse or rabbit) serum for 15-20 mins then washed 3X with WS. Primary antibodies were added at the appropriate dilutions and the samples incubated for 45 mins to 1 h, after which time they were washed 3X with WS. If the primary antibodies were directly conjugated to the fluorochrome, fluorescein isothiocyanate (FITC), such as the anti-HSV1 PAb, F318, the slides were immediately mounted in Aquamount (BDH, Cat.No.36086) using No.1 coverslips. Alternatively, the relevant secondary antibodies were applied at the appropriate dilution for a further 45 mins to 1 h, then washed 3X with WS and mounted as above, apart from the phycoerythrin (PE) fluorochrome which were mounted in WS because PE is rapidly destroyed by glycerol based mountants, such as Aquamount.

2.10.3 Two Colour Immunofluorescent Staining

This basic procedure was carried out in a manner essentially the same as that described for single colour staining. Cytospin slides were fixed and prepared as described above and the primary antibody, MCA406 (Serotec, anti-HSV1 internal phosphorylated protein MAb) was applied for 45 mins to 1 h, washed and subsequently identified by a secondary antibody conjugated to FITC incubated for the same period of time. After washing, the second primary antibody, B21-2, was applied. Before the application of a biotinylated donkey anti-rat secondary (in the case of biotinylated B21-2 this step was not required), the cells were pre-incubated in 5% normal donkey serum to block non-specific binding of the secondary antibody.

Cells were then incubated with streptavidin-phycoerythrin (PE) for 45 mins to 1 h and washed thoroughly. Slides were mounted in WS only and analysed immediately.

Control, single colour slides were prepared in the same manner except for the omission of the relevant primary antibody in each case.

2.10.4 UV Photomicroscopy

Photographs of single and two colour stained cytospins were taken using a Wild-Leitz MPS 46/52 Photoautomat attached to a Wild-Leitz Ortholux II transmitted light microscope both in the Nuffield Dept. of Surgery and in the Immunology division, Dept. of Pathology, University of Cambridge. Film used was 400 ASA Kodacolor print film.

2.11 Flow Cytometry

2.11.1 Single Colour Fluorescence

Suspensions of cells pre-fixed for 15 mins in 4% formaldehyde (Sigma, 37% Formalin, Cat.No.F1635) were washed extensively and stained immediately or stored at 4°C for 12-36 h prior to staining. Individual samples of cells spun down in 1.5 ml Eppendorf tubes were resuspended in MAb or PAb for 45 mins to 1 h on ice. Samples were washed once in ice-cold WS and spun down. Supernatant was carefully aspirated off and when necessary, that is, when the primary antibody was not directly conjugated to a fluorochrome, an appropriate dilution of the relevant secondary antibody was used to resuspend the cell sample for a further 45 mins to 1 h. Unbound antibody was removed by washing with WS and the cells were then fixed by resuspending in 1% formaldehyde.

Fluorescence profiles were generated using the Becton Dickinson FACScan interfaced with the Hewlett Packard 310 computer. For each cell sample, cells were gated according to forward (FSC) and side scatter (SSC) profiles and wherever possible regularly analysed cell populations which consistently gave similar profiles, such as EC suspensions, were gated using the same FSC and SSC parameters, in an attempt to minimise inter-experimental differences. 10,000

FSC and SSC parameters, in an attempt to minimise inter-experimental differences. 10,000 events were acquired in each case. In all but one cell population the percentage of positively stained cells was assessed by setting a marker according to the negative control profiles, i.e. samples which had not received the relevant primary antibody. Except in rare occurrences, as detailed in the experimental text, the marker was set at the point above which only 0-1% of the control events appeared.

The singular exception to the above rule of the negative marker being set on the negative control was LC in an EC suspension. It has been shown in this lab and in some studies detailed in the experimental chapters of this study (see Chapter 4, section 4.9) that B21-2, an anti-MHC Class II MAb, can potentially define two populations in an EC suspension. Two-colour fluorescent staining with NLDC145, an LC specific MAb, and B21-2 has shown that a population of B21-2 positive cells does not express NLDC145, that is, were not LC. These cells however were noted for their weak expression of MHC Class II (MHC Class II ^{weak}). NLDC145 positive cells however were all brightly labelled with B21-2 (MHC Class II ^{bright}) (C.Reis e Sousa, personal communication). Because of this, LC in the EC suspensions are gated on the basis of the single positive control population stained for MHC Class II to exclude the MHC Class II ^{weak} cells, which are not LC (NLDC145 negative), and acquire only the MHC Class II ^{bright} cells which are LC (NLDC145 positive).

It is also possible that HSV1 infection of EC resulted in an up-regulation of MHC Class II on non-leucocytes and presented a similar problem, to that described above, when it came to gating and acquiring LC in an EC suspension on the basis of labelling for MHC Class II. However, (cf. Chapter 4, section 4.9) EC which were infected with HSV1 did not appear to have a significant population of MHC Class II-positive non-leucocytes, as defined by two colour staining for MHC Class II and leucocyte common antigen by 18 h post-infection, the normal time point for analysis. The majority of MHC Class II-positive cells were therefore LC and gating and acquisition was again made on the basis of a clear peak of MHC Class II ^{bright} cells.

2.11.2 Two Colour Fluorescence

Cell samples were pre-fixed in 4% formaldehyde and separated into 1.5ml eppendorfs according to the following protocol. Four categories of cell populations were needed for each analysis; namely a double negative sample, two single positive samples for each of the fluorochromes and the double positive sample stained for both markers. Staining of the double negative and appropriate single positive samples varied depending on the cells to be stained. In, for example, a cell population to be stained for two cell phenotypic markers no primary antibody, or only one relevant primary antibody was applied. In the case of staining HSV1 infected and control uninfected cells, the primary antibody, anti-HSV1 PAb F318 (Dako, Cat.No. F318) is directly conjugated to FITC so F318 was applied to all cell samples at a dilution of 1:30. No virus antigen would be expressed on the surface of uninfected cells therefore any binding of F318 to this population would be non-specific and used validly as the double negative (no second primary) or single positive (if stained with second primary). Populations were defined using uninfected cells stained with F318/second primary omitted/secondary antibody-fluorochrome complex (double negative) and with F318/second primary included/secondary antibody-fluorochrome complex (uninfected single positive for cell phenotype marker (PE)). HSV1 infected cells were stained with F318/second primary omitted/ (single positive for virus antigen (FITC)) or F318/second primary included/secondary antibody-fluorochrome complex (double positive for infected cell phenotype marker <u>and</u> virus antigen). Table 2.6 summarises the protocol for double labelling HSV1 infected cells described above.

In summary, for all two colour analyses saturating concentrations of the primary antibody were applied to the relevant samples and where necessary binding of the primary was then identified by using a species-specific FITC-conjugated secondary. Cell pellets were washed once and resuspended in the second primary antibody for 45 mins, washed once and followed by pre-blocking with an appropriate normal serum at 5%. Except in the case of biotinylated B21-2, a biotinylated-secondary specific for the second primary was used to resuspend the washed cell pellet. The final stage was a dilution of PE applied to the pellet of cells once all unbound secondary had been removed. Table 2.7 summarises the basic protocol.

Table 2.6

Cell Type	*Ab to Cell ?*	*Ab to Virus Ag ?*	*Nature of sample*
Control	No	Yes	**Double negative.**
Control	Yes	Yes	**Single positive** for cell marker.
HSV1 infected	No	Yes	**Single positive** for virus Ag.
HSV1 infected	Yes	Yes	**Double positive** or cell marker and virus Ag.

Table 2.7 *Basic Two-Colour Flow Cytometry Protocol*

	Double Negative	Single +ve marker 1.	Single +ve marker 2.	Double Positive
Primary antibody (FITC conjugate ?)	No	Yes	No	Yes
FITC secondary to 1st primary	Yes	Yes	Yes	Yes
Second primary	No	No	Yes	Yes
Pre-block	Yes	Yes	Yes	Yes
Biotin secondary to 2nd primary	Yes	Yes	Yes	Yes
PE	Yes	Yes	Yes	Yes

For single colour profiles, cells were gated on the basis of FSC and SSC, 10,000 events being acquired. However, because of the overlapping spectra of FITC and PE the FACscan had to be set to be compensated. This was achieved using the double negative and single positive cell samples in the following manner. Samples negative for both markers were allowed to run until an appreciable population (1000 events) was apparent as a discrete "ball" in the bottom left hand corner of an FL1 (FITC) vs. FL2 (PE) scatter profile. The PE single positive sample was run through and allowed to accumulate being visible up the left axis of the same scatter plot. FL2-FL1 compensation was adjusted where necessary to move this cell population as close to the y axis so that it did not go beyond the right hand margin of the negative control but was not pushed up against the y axis (overcompensated). This procedure was repeated with the FITC single positive sample and the FL2-FL1 compensation adjusted to move the population appropriately close to the x axis. The final result of this was an upper right hand "quadrant" of the FL1 vs. FL2 scatter plot as free as possible from spuriously double positive cells.

Cell populations were analysed using Consort 30 or Lysis II software and results were presented mainly as histogram plots or occasionally for Lysis II as FL1 vs. FL2 scatter plots. Markers were set as for single colour fluorescence using the double negative and reciprocal single positive cell samples.

2.11.3 Secondary Antisera

A complete list of all secondary antisera used in UV microscopy and FACS analyses is presented in Table 2.8

Table 2.8 Secondary Antisera used in UV Microscopy and Flow Cytometry

Specificity	Source	Catalogue No.	Working dilution
FITC Conjugates			
Goat anti-mouse	Jackson	115-095-100	1:50-1:100
Rabbit anti-rat	Jackson	312-095-003	1:50-1:100
Rabbit anti-hamster	Jackson	307-015-003	1:50-1:100
Goat anti-rabbit F(ab')$_2$	Jackson	111-096-006	1:50-1:100
Goat anti-rabbit	Sigma	F6005	1:25-1:30
Rabbit anti-rat	Sigma	F7631	1:25-1:30
Goat anti-rat	Sigma	F6258	1:25-1:30
Biotin Conjugates			
Donkey anti-rat	Jackson	712-065-138	1:50-1:100
Goat anti-hamster	Jackson	107-065-142	1:50-1:100
Goat anti-hamster F(ab')$_2$	Jackson	107-066-142	1:50-1:100
Sheep anti-mouse	Sigma	B8899	1:25-1:30
Goat anti-rat	Sigma	B7139	1:25-1:30
Streptavidin PE	Southern Biotech.	7100-09	1:50-1:100

Chapter 3

HSV1 Infection of Dendritic Cells

3.1 Introduction

3.2 Infection of LC determined by UV Microscopy

3.3 Infection of LC determined by FACS Analysis

3.3.1 Initial Observations

3.3.2 Expression of HSV1 Antigens by Infected LC

3.3.3 Time course of HSV1 Antigen Expression

3.3.4 Mouse and Virus Strain Dependency

3.3.5 MHC Class II Expression on Infected LC

3.4 Maturation of LC in culture, and the resultant effect on infection by HSV1

3.5 Infection of Splenic DC (sDC)

3.5.1 Method 1: Infection of sDC as low density pellicle cells

3.5.2 Method 2: Infection of sDC as low density adherent cells

3.5.3 Method 3: Parallel infection of sDC and LC

3.5.4 Method 4: Infection of 18 h cultured FcR-negative-sDC and LC

3.6 Chapter Summary

3.7 Specialised Materials and Methods: Infection of splenic DC

3.7.1 Method 1

3.7.2 Method 2

3.7.3 Method 3

3.7.4 Method 4

3.1 Introduction

Langerhans cells (LC) are intimately involved in the initiation of immunity to HSV1, where they appear to act as the critical antigen presenting cell [267;305]. Depletion of the skin LC population, prior to subcutaneous infection with HSV1, impairs the HSV1-specific T cell response [229]. It is conceivable that the virus itself may productively infect and destroy LC and thereby diminish the protective immune response. Some reports [229;306] have suggested that LC can transport virus or virus antigen from the skin to the draining lymph nodes, but are not productively infected. However, no previous studies have directly assessed the susceptibility of cells of the DC family to infection with HSV1. The studies detailed in the following chapter addressed this issue by investigating the interaction of LC and splenic DC (sDC) with HSV1 in vitro.

3.2 Infection of LC determined by UV Microscopy

The problem was initially approached by attempting to detect viral antigens in cells exposed to HSV1 in vitro. The intention was to develop a protocol whereby virus antigen could be detected on the surface of HSV1-infected dendritic cells, either LC or sDC. LC were chosen for the initial analyses and were infected in vitro, as a bulk epidermal cell (EC) population, with HSV1 (see Materials and Methods, section 2.5.2). Cytospins of infected or control (uninfected) cells were prepared and labelled with primary antibodies to MHC Class II (B2-12; anti-Ia^{b,d}) to identify LC, or HSV1 specific antibodies, such as MCA 406, (anti-HSV1 Internal Phosphorylated Protein (IPP)). Binding of primary antibody was, in the first instance, detected by incubating the slides with specific FITC-conjugated secondary antibodies and observed by UV microscopy.

Control staining for B21-2 expression alone showed that LC in an uninfected population could be easily identified using B21-2/FITC as brightly positive cells which, in most instances, had characteristic veiled morphology (Figure 3.1 (a)). This was especially evident at higher magnification (Figure 3.1 (b)). EC infected with HSV1/34, a clinical isolate of HSV1, were stained for B21-2 and compared with control (uninfected) cells.

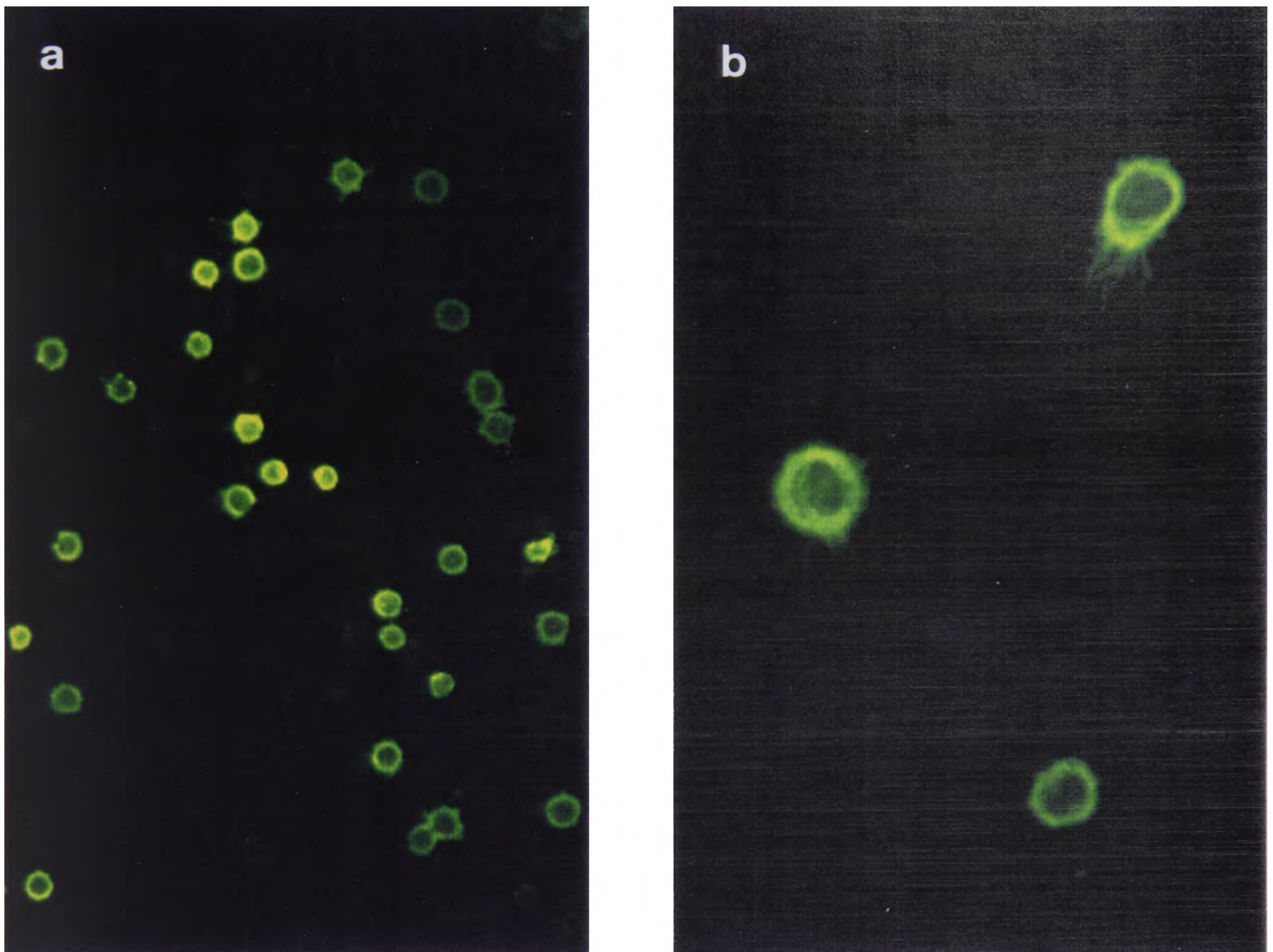


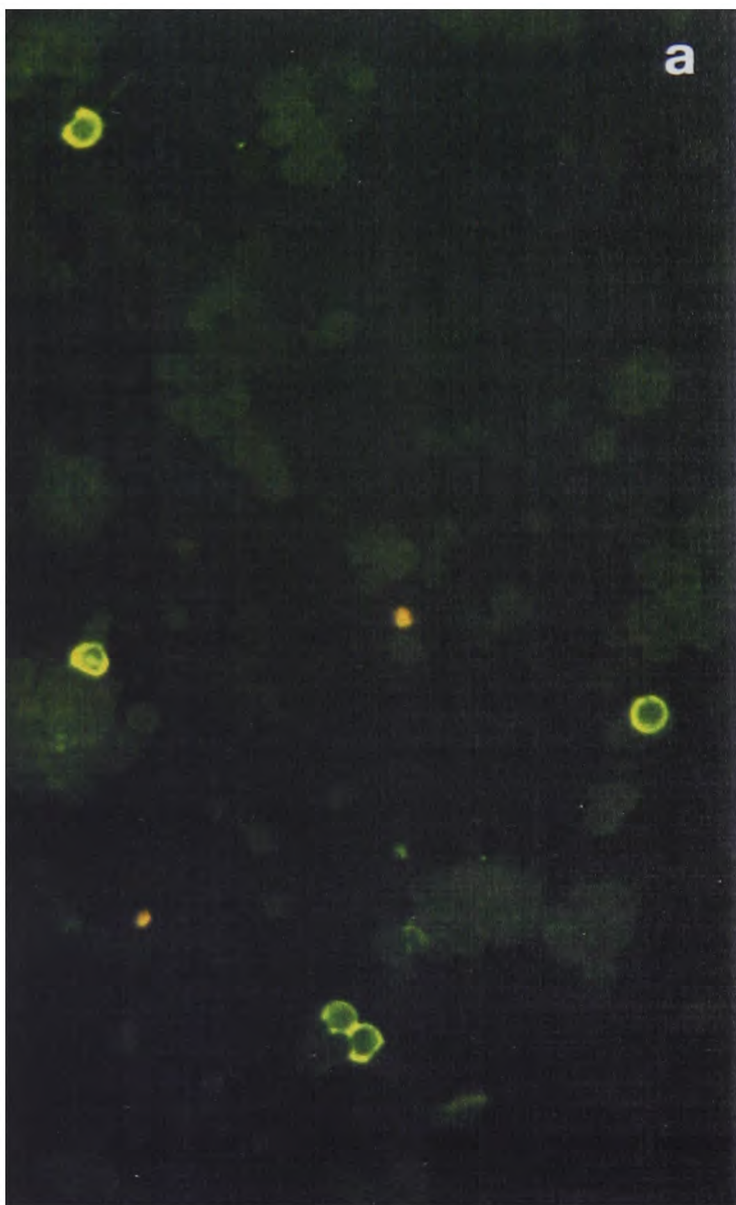
Figure 3.1 *MHC Class II expression on LC in an uninfected EC population. LC could be easily identified as brightly positive cells which, in many instances, had characteristic veiled morphology (a) (x200). This was especially evident at higher magnification (b) (x400).*

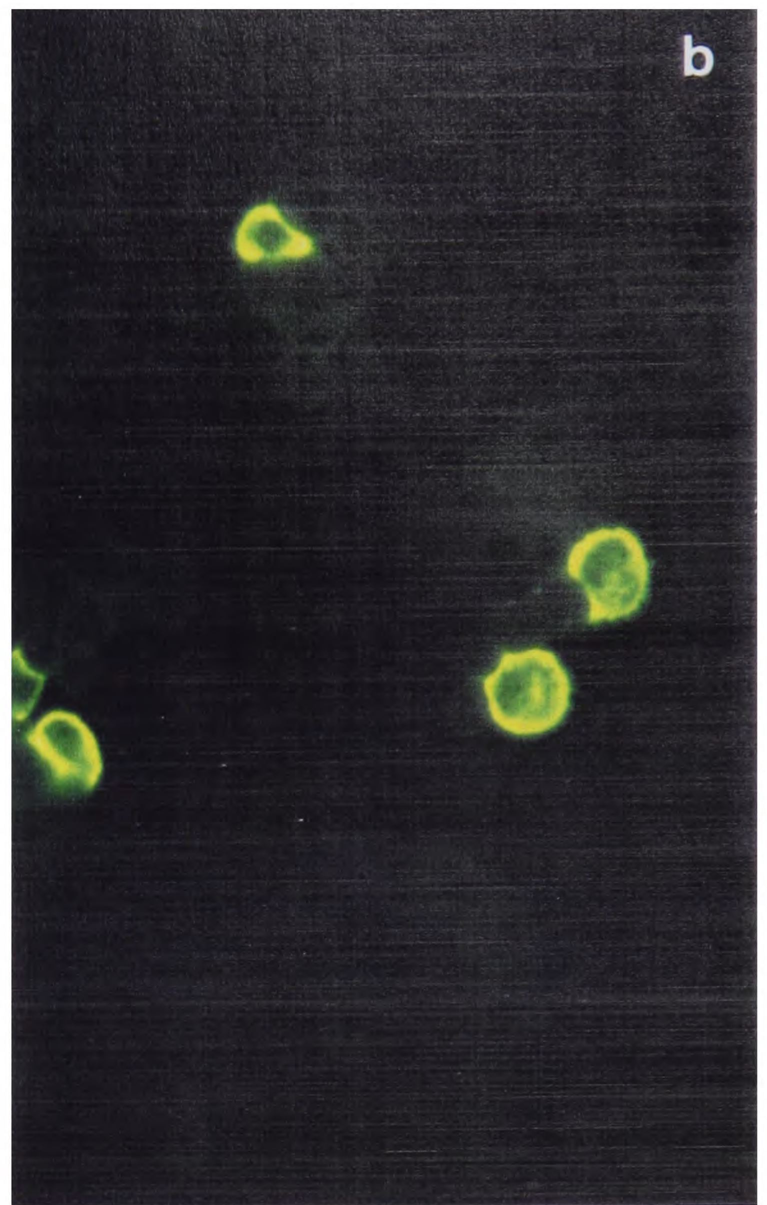
The control cells (Figure 3.2 (a) and (b)) closely resemble those seen above (cf. Figure 3.1 (a) and (b)). Upon infection the cells were reduced in number, were slightly enlarged and the brightness of individual LC was reduced (Figure 3.2 (c)). This again was more apparent at higher magnification (Figure 3.2 (d) cf. Figure 3.2(b)). This apparent decrease in intensity of MHC Class II on some, but not all, HSV1-infected LC was a consistent observation in a number of EC preparations.

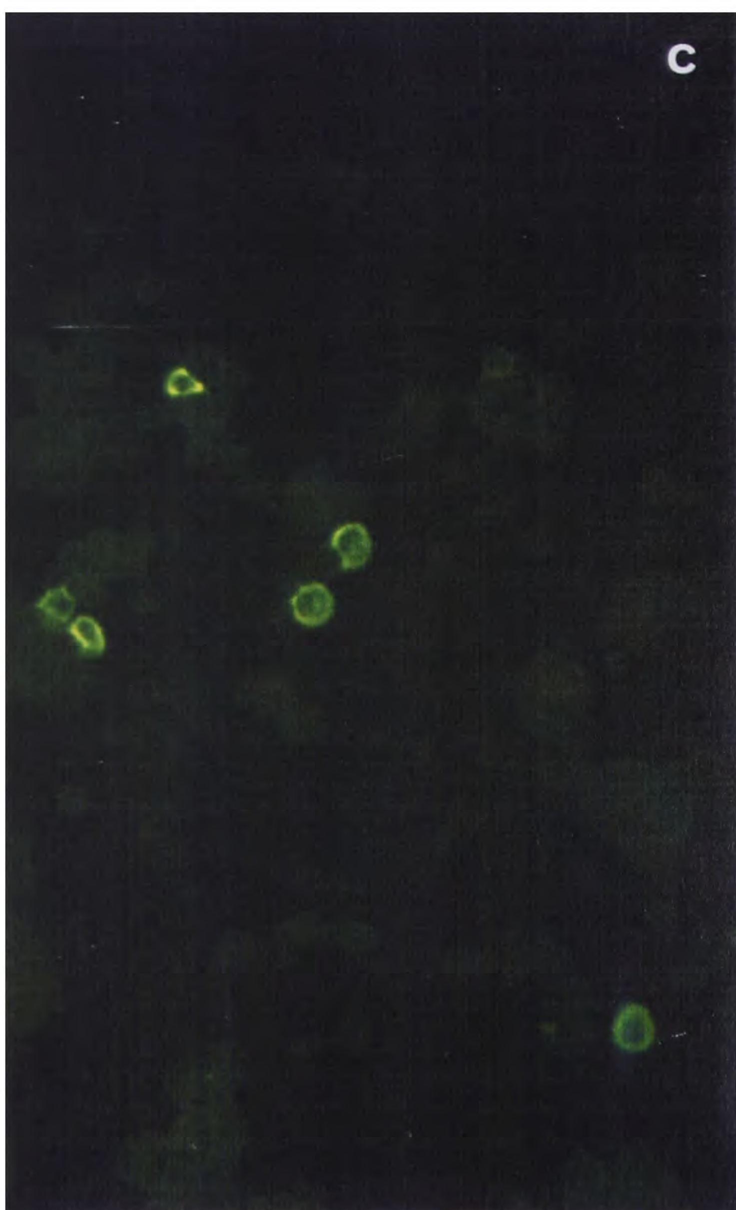
To ensure that the MAb MCA406 could be used to identify viral antigens, baby hamster kidney (BHK) cells, which are known to be permissive for HSV1 infection, were infected and labelled with this antibody. Figure 3.3 shows the very clear punctate labelling. The same method was applied to infected EC. Infected cells labelled brightly with MCA406 also (Figure 3.4 (a)). These cells were often associated in clumps, possibly in the process of virally-induced fusion. A higher percentage of cells were labelled with MCA406 than with B21-2 indicating that cell types in addition to LC, possibly keratinocytes, expressed virus antigen in the EC population. There were some cells in this labelled population the morphology of which could be described as "dendritic" (Figure 3.4 (b)). These were possibly infected LC but in the absence of labelling with B21-2 it is not possible to make a definitive statement as to the nature of these cells.

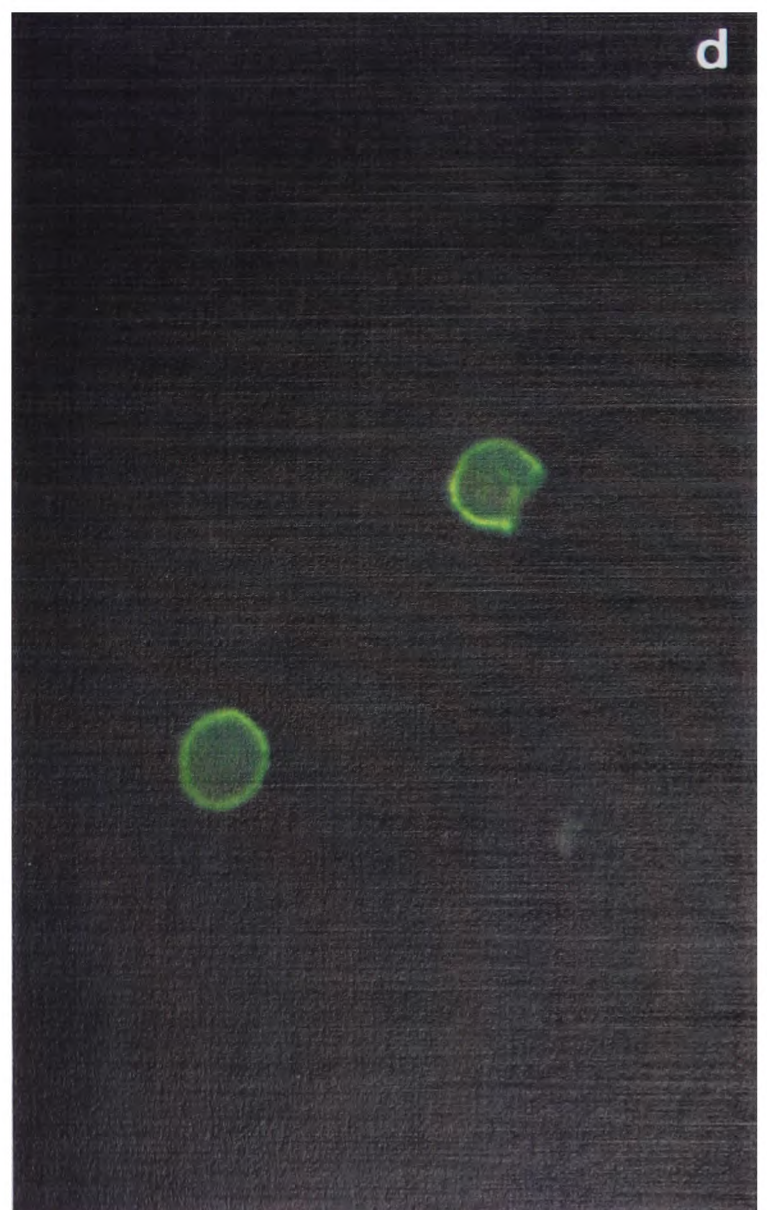
Having shown that EC could be labelled for MHC Class II and virus antigen it was necessary to stain these cells for both markers, by two-colour fluorescence, to assess the number of HSV1-positive LC. LC were labelled with B21-2 and the fluorochrome phycoerythrin (PE) which fluoresces red, and virus antigen was identified with MCA 406 and FITC which fluoresces green. Due to the overlap of the FITC and PE spectra, double-labelled cells would be seen under the FITC filter as 'orange' fluorescent cells. Infected EC were labelled according to this protocol (Figure 3.5). Uninfected LC were present (red) as were other EC, presumably keratinocytes, which expressed virus antigen alone (green). LC which expressed virus antigen were clearly present (orange). Thus at least some infected LC did express IPP but it was difficult to make an accurate assessment of the exact percentage of double labelled cells.

Figure 3.2









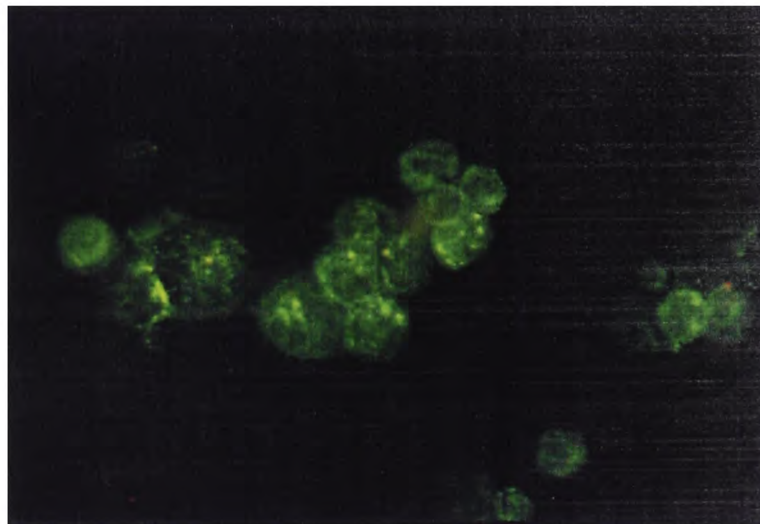


Figure 3.3 *BHK cells infected with HSV1 label brightly with the anti-HSV MAb, MCA406 (x200). BHK cells were exposed to HSV1/34 at an MOI=1.0 for 1h (Control cells were left uninfected). After 1h, cells were washed and incubated for 18h at 37oC. Cytospins were prepared and virus antigen was detected by indirect immunofluorescence using MCA 406 and FITC conjugated anti-mouse IgG.*

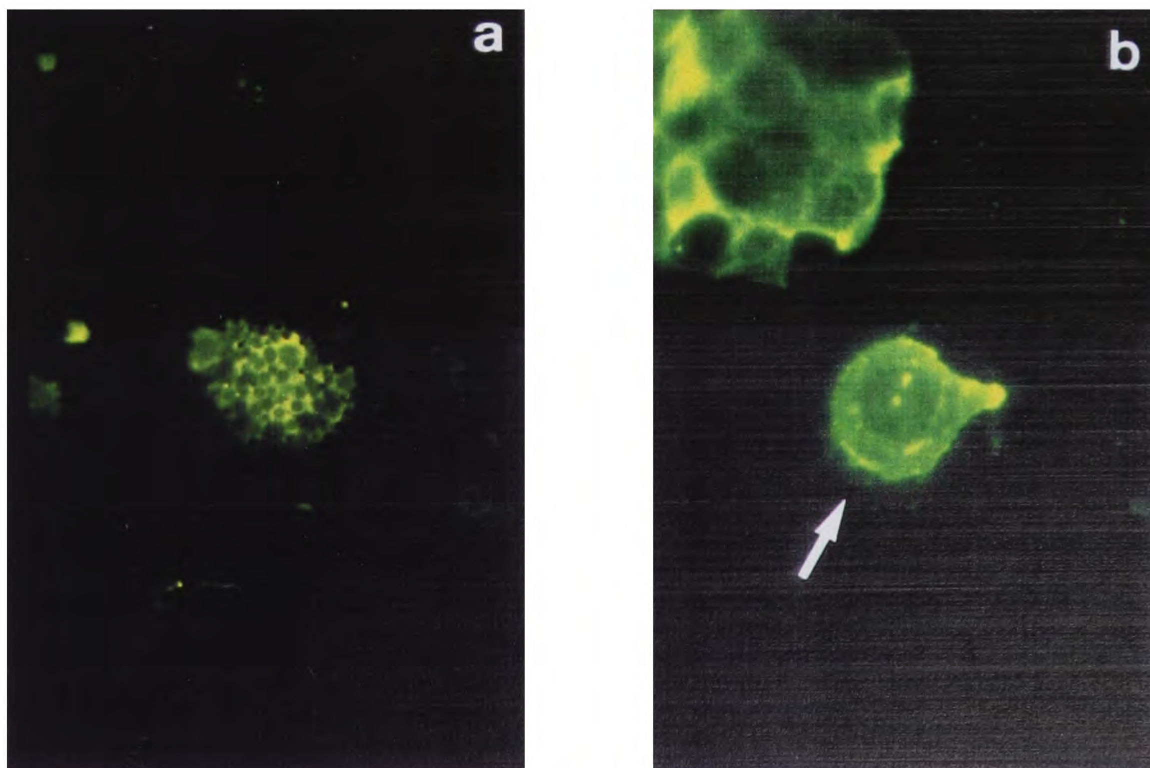


Figure 3.4 *EC, infected with HSV1, also labelled brightly with MCA406 (a) (x200). Some cells in this labelled population could be described as "dendritic" ((b)-arrowed) (x400). Cytospins of control (uninfected) and HSV1/34 infected EC were prepared and stained in a manner similar to that described for BHK cells in Figure 3.3 above.*

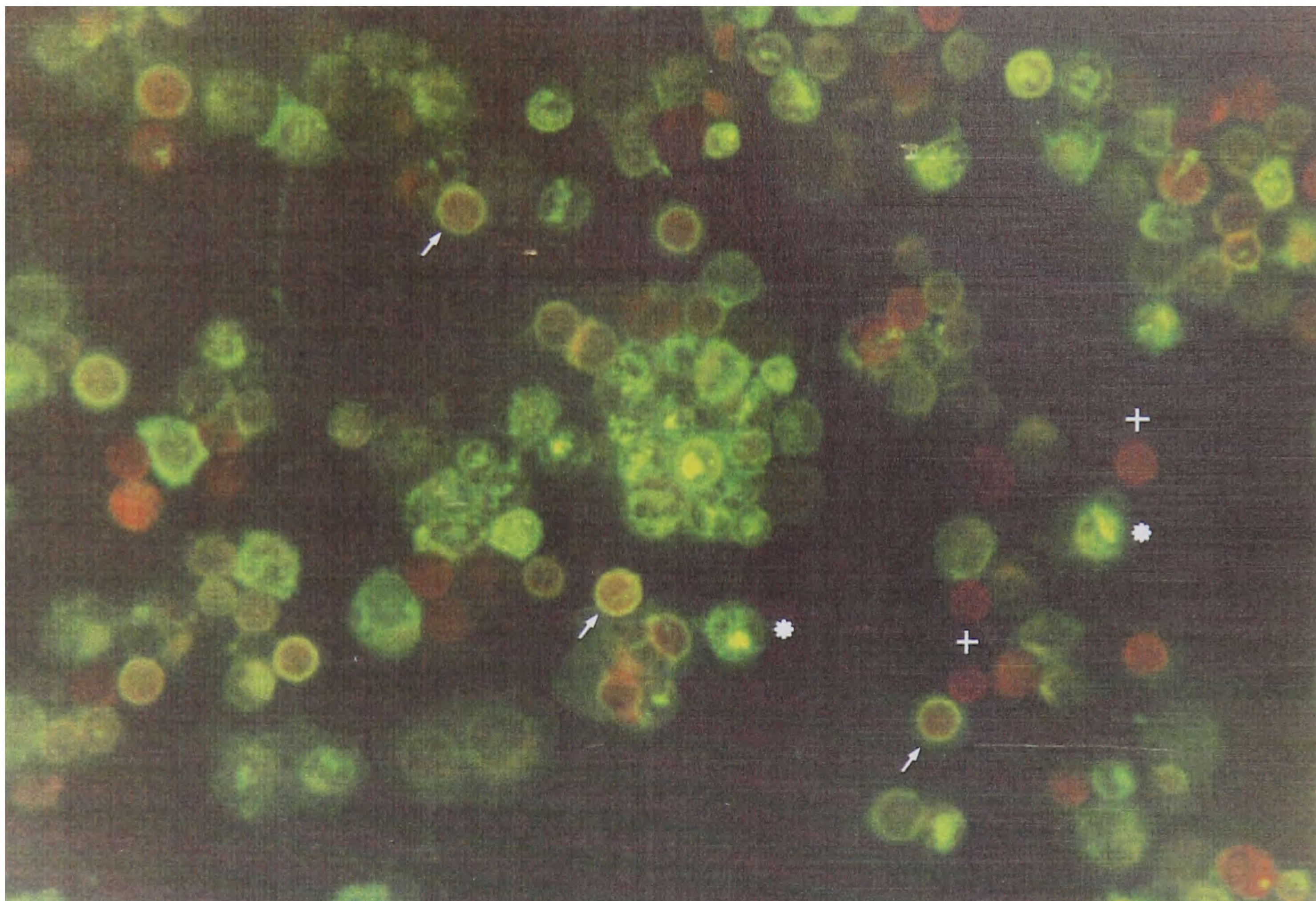


Figure 3.5 *Infected EC were double labelled for MHC Class II (B2-12) and HSV IPP (MCA406). Uninfected LC (cross); other EC which expressed virus antigen alone (star). LC which appeared to express virus antigen were also present (arrowed) (x200).*

3.3 Infection of LC determined by FACS Analysis

A protocol was developed whereby the number of LC expressing HSV1 antigens could be quantified by FACS analysis. Mean channel fluorescence (MCF) was used as an assessment of the brightness of individual cells.

3.3.1 Initial Observations

Initial experiments quantified by single-colour flow cytometry the apparent decrease in the number of LC in an infected EC population. Figure 3.6 shows the FACS profiles obtained when freshly isolated Balb/c EC were infected with HSV1/34 and assessed for LC levels using B21-2 (Note: unless otherwise indicated, infected (and control) EC populations were harvested and fixed for analysis at 18 hours post-infection). In this experiment cells were partially purified over BSA, and both LC-enriched low-density, and LC-depleted high-density, fractions were analysed. This was to ensure that any decrease in LC number in the low density fraction was not due to an HSV1-induced density change causing them to move into the high density fraction.

Infection with HSV1/34 resulted in a decrease in the percentage of LC ((a) 11.5%; (b) 5.3%), with an associated slight decrease in MHC Class II intensity (MCF (a) 194; (b) 165). No LC were detected in the high density fractions indicating that the decrease was not due to a change in LC density (Figure 3.6 (c) and (d)). This procedure was repeated at least twice and gave similar results.

It is worth re-emphasising that LC were defined for the purposes of FACS analysis as MHC Class II-positive^{bright} cells in EC populations, and all FACS analyses and interpretations are based on this population. Other cells in EC populations, such as keratinocytes, may be induced to express MHC Class II [138]. By gating on MHC Class II-positive^{bright} cells these are excluded from our analyses. B21-2 was chosen as the LC marker in skin for this study rather than NLDC 145, which specifically labels LC, since staining with B21-2 generates a distinct peak of LC, whereas NLDC145 often does not. This was important when it came to two colour analysis where it was necessary to gate on a well defined population.

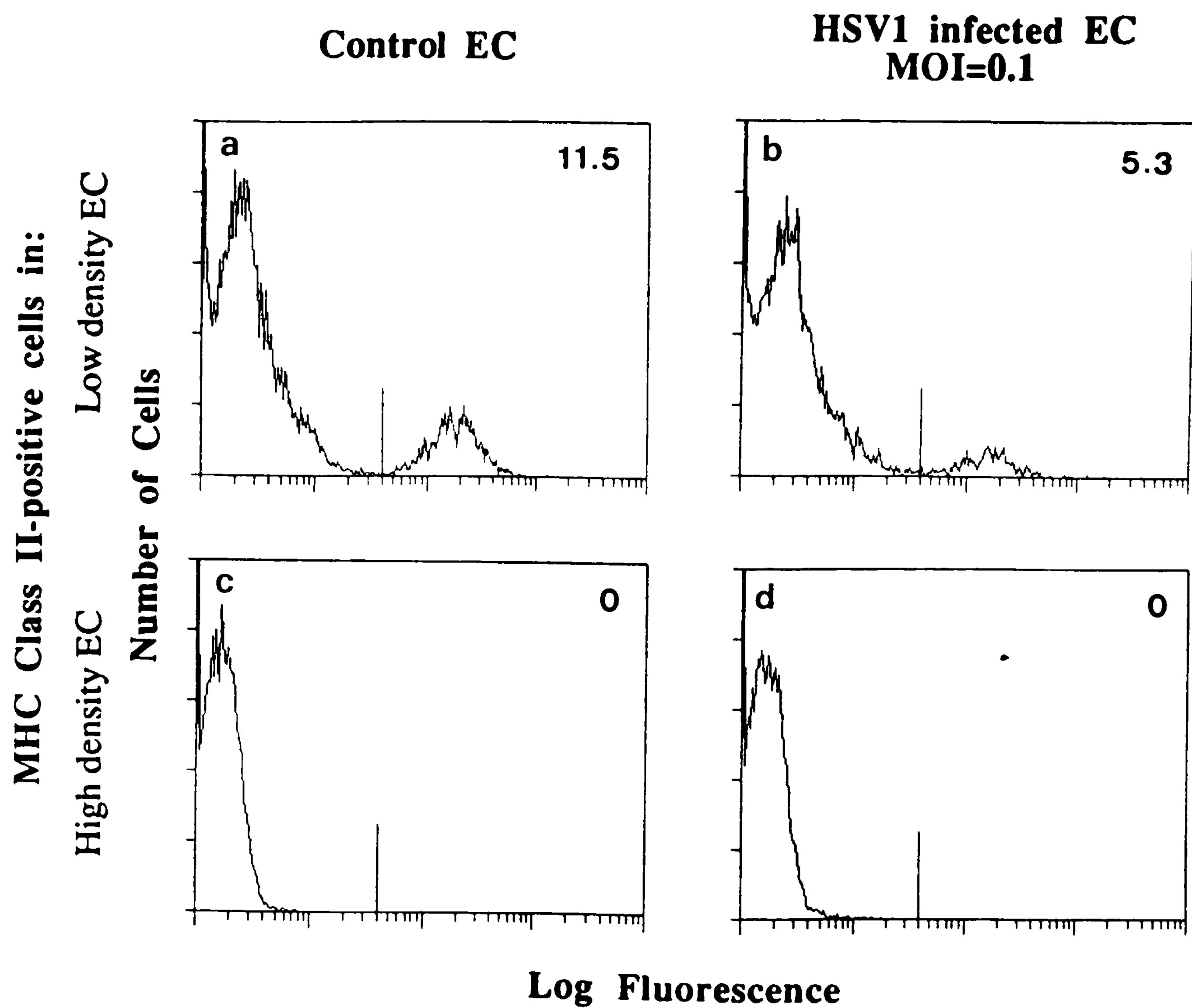


Figure 3.6 HSV1-infected EC (b),(d) and control EC (a), (c) were separated into high and low density fractions and stained for MHC Class II using B21-2 (Anti-Iab,d). See text for details.

Freshly isolated Balb/c EC were infected and labelled with B21-2 or MCA406 for single-colour flow cytometry. Infection resulted in a decrease in the number (Figure 3.7 (a) 9.0%; (b) 3.6%) and intensity (MCF (a) 85; (b) 42) of MHC Class II-positive LC as already noted (cf. Figure 3.6). However, whereas UV-microscopy of a preparation such as this revealed that approximately 20% of infected EC expressed IPP (MCA406-positive) (Figure 3.4), by single colour FACS analysis (Figure 3.7 (d)) only 2.9% of infected EC expressed IPP.

The extent of cell permeabilisation in both of these methods may explain this apparent discrepancy. Cells for FACS analysis are not intentionally permeabilised (although formaldehyde fixation may cause some degree of permeabilisation; personal observation) and therefore access of primary antibody to antigen is limited. Cells on a cytospin are fully permeabilised by acetone and intracellular antigens are readily available for labelling. If IPP is mainly intracellular then FACS analysis would detect lower levels than UV-microscopy.

Permeabilisation with buffered formal actetone (Bfa) of EC for FACS analysis resulted in an increased percentage of bulk EC labelled by MCA406 (Table 3.1; column 3), indicating that a considerable amount of IPP may be intracellular. Bfa permeabilisation also caused an apparent increase in the number of LC in the uninfected controls (Table 3.1; column 1). This was possibly due to the greater accessibility of intracellular MHC Class II to B21-2 in the permeabilised population.

Identification of a variety of HSV1 gene products which would be expressed on the infected cell surface was considered as an alternative approach to permeabilisation. A rabbit polyclonal Ab, F318 (Dako), which identifies all the major virus glycoproteins and one core protein, was tested. Infection of BHK cells with HSV1/34 and subsequent labelling showed that infected cells could be labelled very brightly with F318 (Figure 3.8).

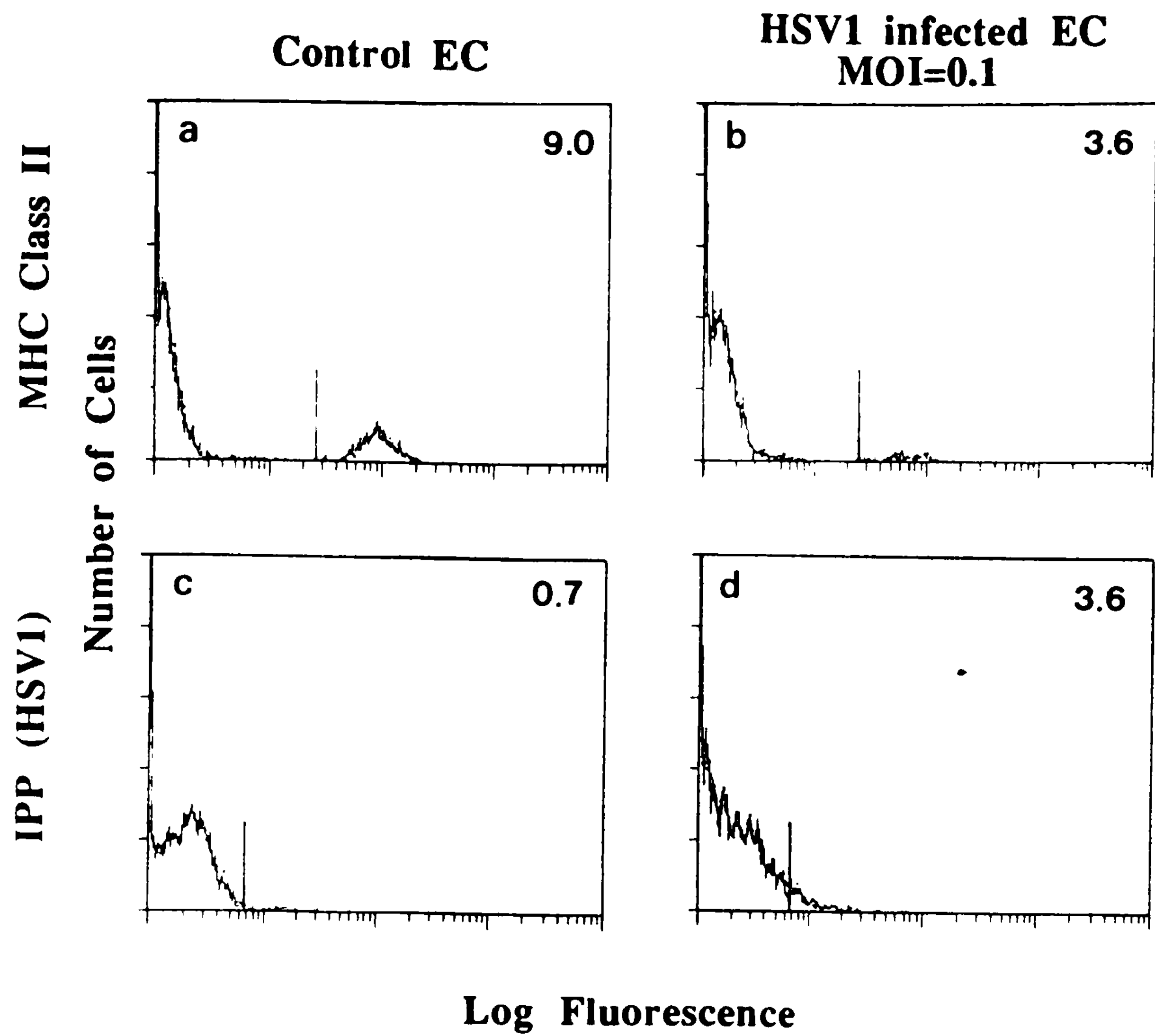


Figure 3.7 A small percentage of HSV1/34 infected EC were labelled with MCA406 (anti-HSV1 MAb) when analysed by single-colour flow cytometry (d). Upon infection, MHC Class II-positive cells are reduced in number (cf. (a) and (b)).

Table 3.1 *Permeabilisation increased the percentage of EC labelled with MCA406. Control and infected EC were permeabilised or left untreated prior to staining for single colour flow cytometry using B21-2 or MCA406. Key : LC in each population is shown as % MHC Class II LC; MCF MHC Class II represents the MHC Class II intensity of the LC populations.*

	%MHC Class II LC	MCF MHC Class II	% bulk EC expressing virus antigen
Unpermeabilised			
Control	4.3	124	2.3
Infected	2.6	95	***11***
Permeabilised			
Control	5.7	126	2.9
Infected	2.4	113	***27***

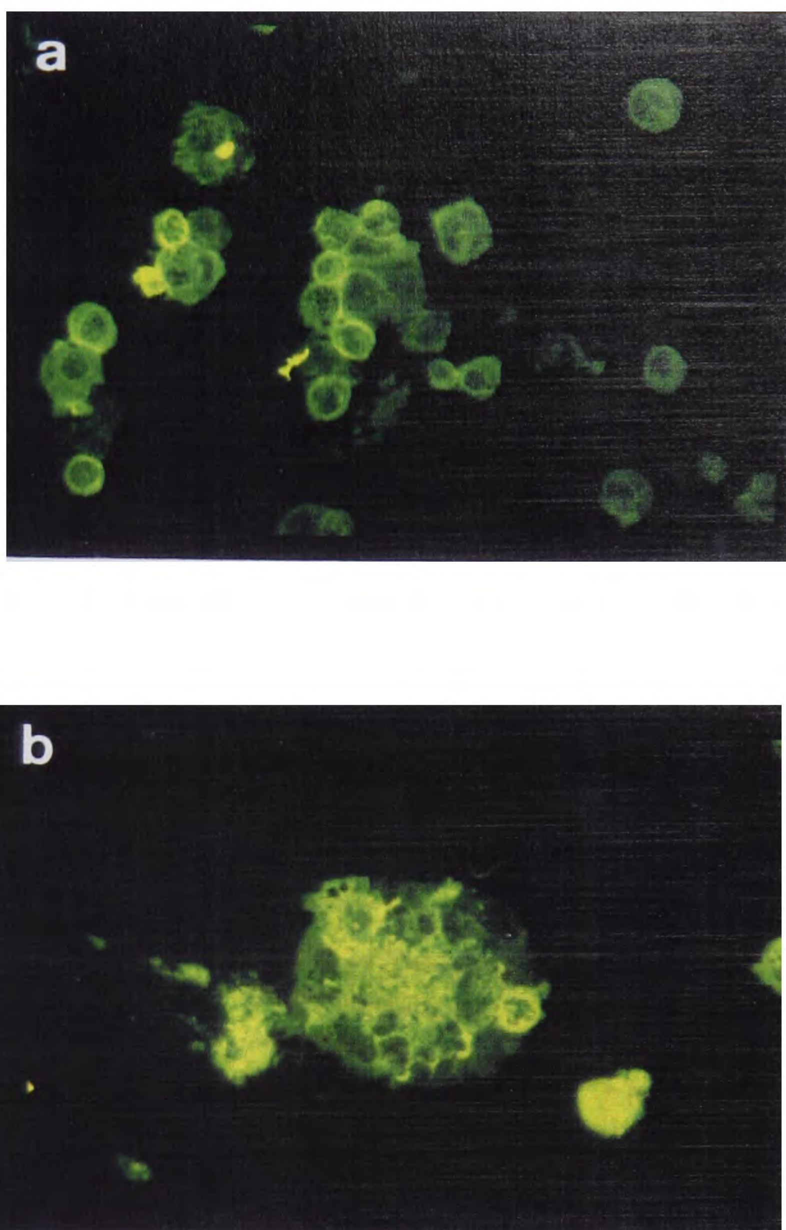


Figure 3.8 *HSV1/34 infected BHK cells were stained with F318, an anti-HSV1 polyclonal Ab, at a dilution of 1:30. Most cells labelled brightly with this antibody (a). Some cells appeared to be associated in clusters (b), possibly in the process of HSV1-induced fusion.*

F318 was chosen for the remainder of this study to identify HSV1 infected cells, for a number of reasons. Firstly, since it identified cell surface glycoproteins it avoided the need for permeabilisation prior to staining, and identified a much wider range of HSV1 proteins than MCA406. Secondly, being directly conjugated to FITC it required a simple one-step application, which eliminated the need for a secondary antibody. It therefore reduced potential cross reactivity problems with other reagents during two-colour analysis. Thirdly, and relatedly, a single step staining for virus products allowed rapid analysis of cells, and it also offered the possibility of amplification of the signal by using a second anti-rabbit FITC conjugate on weakly labelled cells.

3.3.2 Expression of HSV1 Antigens by Infected LC

In the following text we show that freshly isolated LC were susceptible to HSV1 infection as determined by their expression of virus glycoproteins. It was apparent that HSV1 infection had a variable effect on MHC Class II expression as indicated by the change in MCF upon infection, and HSV1 may act to down-regulate this antigen.

Freshly isolated Balb/c EC were infected with HSV1/34 at a multiplicity of infection of 0.1 (MOI=0.1 pfu/cell, i.e. 0.1 infectious units per cell), labelled with B21-2 and F318, and analysed by two-colour flow cytometry. One such experiment (Figure 3.9) showed that >70% of LC expressed some or all of the virus glycoproteins (Figure 3.9 (h), as defined by F318 labelling of gated infected LC. The results shown are typical of three experiments which indicated that an average of 65% (S.D.+/-4) LC expressed virus glycoproteins after infection with HSV1/34 at an MOI=0.1. This was often associated with a decrease in MHC Class II intensity.

3.3.3 Time course of HSV1 Antigen Expression

Infection of EC led to a decrease in MHC Class II-positive cells, either by cell death or by down regulation of MHC Class II expression. In order to assess the time course of MHC Class II expression in relation to virus antigen expression, Balb/c and C57/Bl/6 EC were infected with

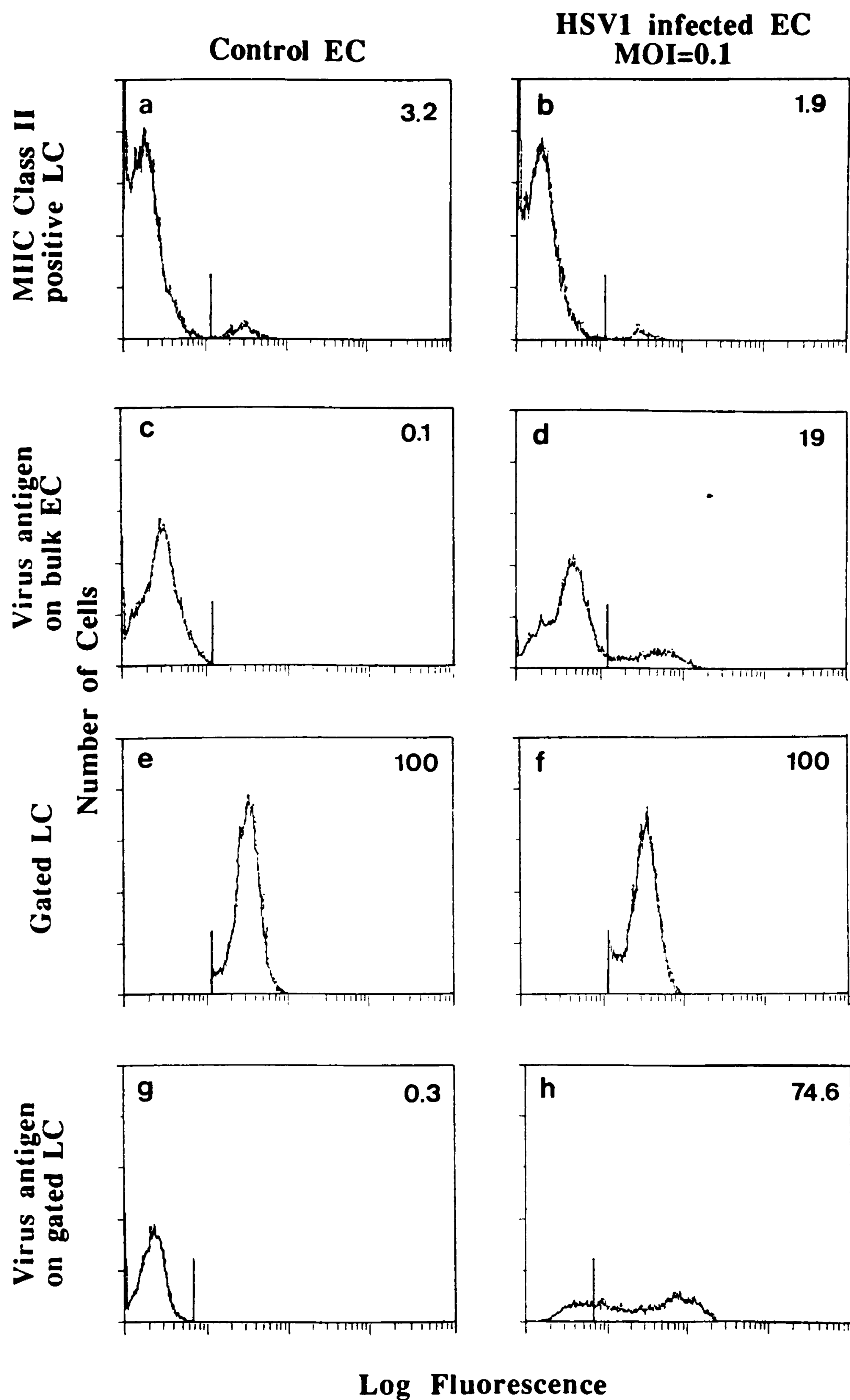


Figure 3.9 Acquisition of clearly defined populations of LC confirmed that a high percentage of LC expressed virus antigens post-infection (h). HSV1 antigens were also expressed on around 20% bulk EC (d). Uninfected EC were stained with F318 as the negative control (c). Uninfected (a) and HSV1-infected (b) EC were stained with B21-2 and the resulting MHC Class II-positive populations were acquired (e) and (f) respectively. The acquired uninfected population were used to set the control marker (g) and virus antigen expression was then assessed on acquired infected LC (h).

HSV1/34 at an MOI=0.1. Control cultures were left uninfected. At intervals shown in Figure 3.10 and Table 3.2, samples of cells were removed, fixed and analysed by two-colour flow cytometry. This procedure would also determine whether there were mouse strain differences in infection. The observed rise in control LC number with time was due to a progressive increase in MHC Class II intensity. While the overall percentage of LC differed between mouse strains it was obvious that around 8 h, infection with HSV1 resulted in an apparent decrease in the number of LC (Figure 3.10 (a) and (b)). A decrease in MHC Class II expression was also observed for both strains at a similar time point (Table 3.2, column 2). Glycoprotein expression was not greatly apparent until 6-8 h post-infection but continued to rise until 24 h. There appeared to be little strain difference in HSV1 antigen expression on bulk EC (c). Balb/c LC expressed higher percentages of glycoproteins than C57/Bl/6 LC at intermediate times, but by 24 h there was little detectable difference (d). It is interesting to note the correlation between the marked increase in virus glycoprotein expression and the effect in terms of LC number and MHC Class II intensity. Increased viral metabolic activity (de novo glycoprotein expression occurs around 8 h) may exert a more profound effect on cell antigens, in this case MHC Class II, ultimately resulting in cell death.

3.3.4 Mouse and Virus Strain Dependency

Down regulation of MHC Class II expression and/or cell death is associated with increased viral metabolic activity. To ensure that our observations were not restricted to a single isolate of HSV1, Balb/c and C57/Bl/6 EC were infected with increasing MOI of HSV1/34 and a second strain, HSV1 KOS. The results are presented in Figure 3.11 (a)-(f). Both virus strains caused a decrease in LC number in Balb/c EC (a) with increasing MOI. C57/Bl/6 LC were not as profoundly affected by HSV1/34 and the effects of HSV1 KOS were even more limited (b). Glycoprotein expression on EC by HSV1/34, was greater at higher MOI's in both strains of mice whereas with HSV1 KOS it tended to plateau above 0.5 MOI (c) and (d). Gated LC, at lower MOI, expressed higher levels of glycoproteins with HSV1 KOS in both strains of mice. At 1.0 MOI, a similar percentage of Balb/c and C57/Bl/6 LC expressed virus glycoproteins (e) and (f). Overall, C57/Bl/6 LC expressed lower levels of glycoproteins with both strains of

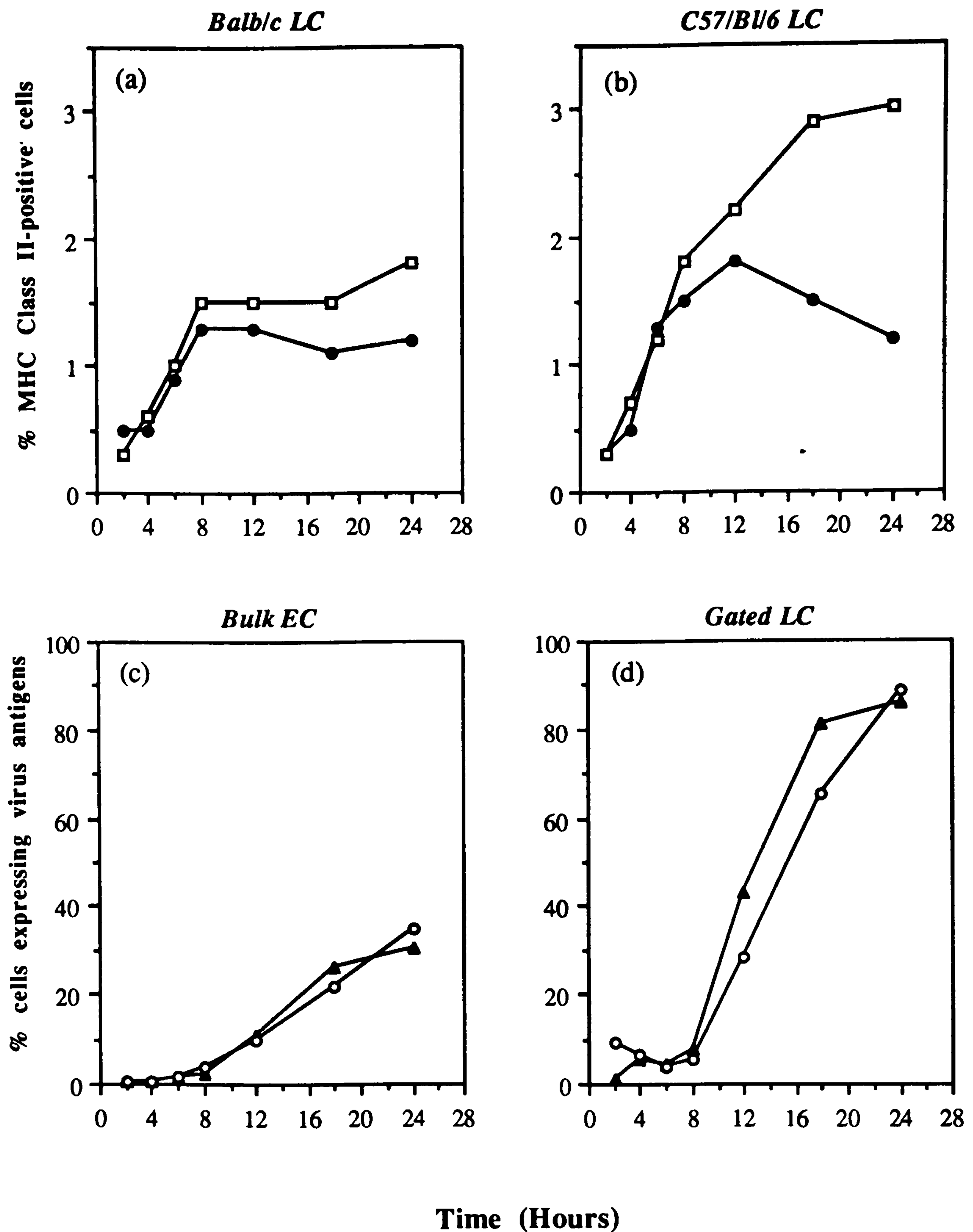


Figure 3.10 Time course of HSV1 infection of freshly isolated EC. Infection with HSV1 reduced the number of LC in Balb/c (a) and C57/Bl/6 (b) EC populations, but this effect was not seen until around 8 h post-infection. Virus antigen expression rose rapidly at this time on bulk EC (c) and gated LC (d) in both strains. In graphs (a) and (b) open squares represent the uninfected controls and closed circles represent the infected populations in terms of MHC Class II -positive cells. In graphs (c) and (d) expression of virus antigens is shown for Balb/c (triangles) and C57/Bl/6 (circles) strains.

Table 3.2 *Time course of HSV1 antigen expression. This table fully details the values obtained for the number of MHC Class II-positive LC in infected and control EC populations, the levels of MHC Class II expression on LC (MCF MHC Class II), and the percentage of bulk EC and gated LC expressing viral antigens, as defined by the anti-HSV1 PAb, F318.*

		% MHC Class II LC		*MCF MHC Class II*		*% cells expressing virus antigens: Bulk EC*		*Gated LC*	
Time		**Balb/c**	**C57**	**Balb/c**	**C57**	**Balb/c**	**C57**	**Balb/c**	**C57**
2h	**Control**	0.3	0.3	***24***	***33***	-	-	-	-
	Infected	0.5	0.3	***31***	***31***	0.3	0.3	1	9.4
4h	**Control**	0.6	0.7	***67***	***44***	-	-	-	-
	Infected	0.5	0.5	***55***	***40***	0.5	0.3	5.5	6.4
6h	**Control**	1	1.2	***88***	***49***	-	-	-	-
	Infected	0.9	1.3	***103***	***44***	1.4	1.9	4.6	4
8h	**Control**	1.5	1.8	***115***	***64***	-	-	-	-
	Infected	1.3	1.5	***114***	***61***	2.4	3.6	7.6	5.2
12h	**Control**	1.5	2.2	***156***	***89***	-	-	-	-
	Infected	1.3	1.8	***112***	***70***	11	10	43	29
18h	**Control**	1.5	2.9	***159***	***91***	-	-	-	-
	Infected	1.1	1.5	***71***	***53***	26	22	81	65
24h	**Control**	1.8	3	***140***	***79***	-	-	-	-
	Infected	1.2	1.2	***51***	***46***	30	35	86	88

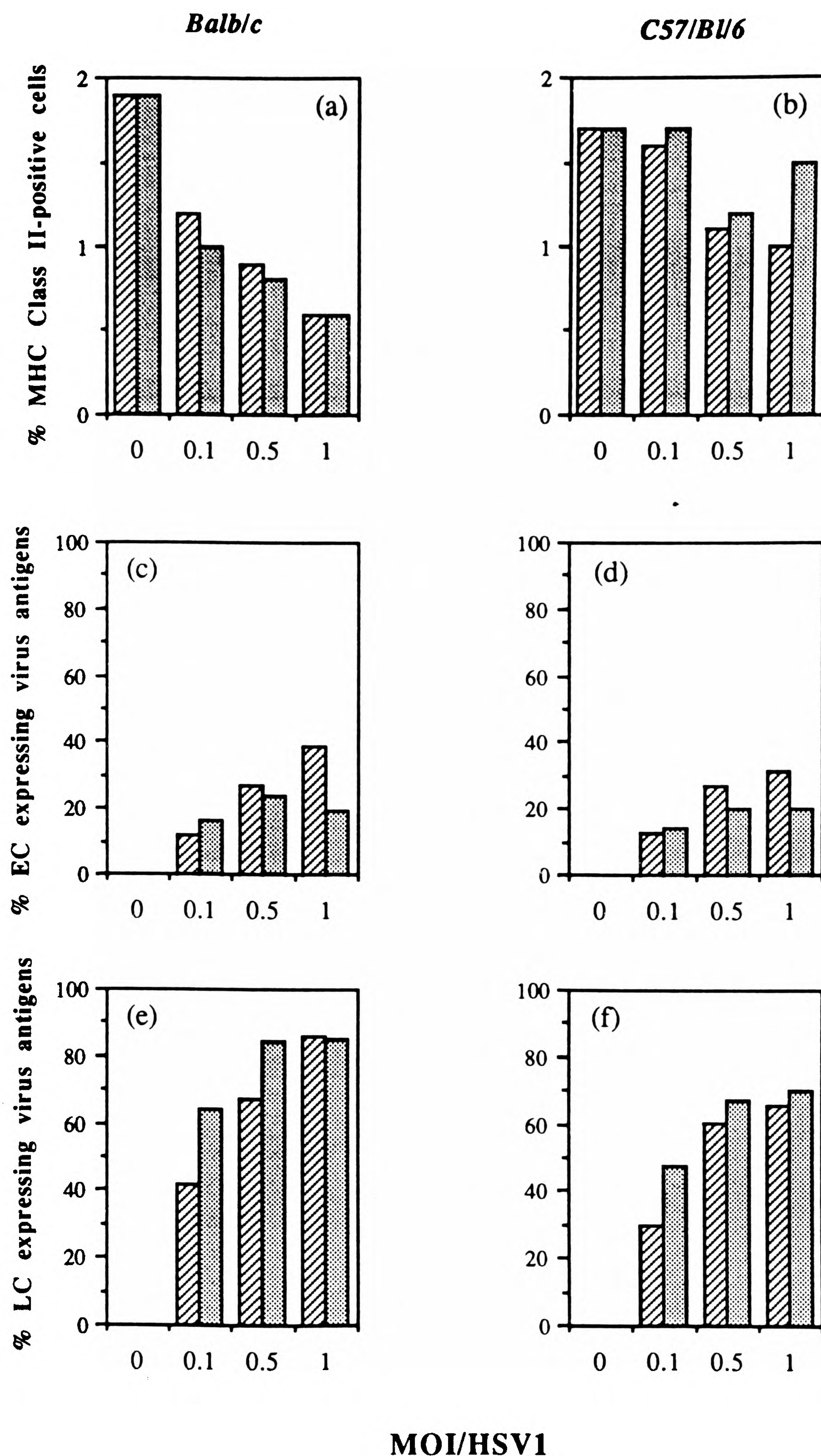


Figure 3.11 Balb/c and C57/Bl/6 EC were infected with two strains of HSV1 - HSV1/34 (hatched columns) and HSV1 KOS (dotted columns). After 18 h in culture the values detailed in the figures above and fully in Table 3.3 were generated by two-colour flow cytometry. MHC Class II LC are shown for Balb/c (a) and C57/Bl/6 EC (b). The percentage of cells expressing virus antigen is represented for bulk EC ((c), (d)) or gated LC ((e), (f)).

virus (f). Given the inter- and intra-mouse strain differences we have shown that it was possible to detect similar patterns of decrease in LC number, MHC Class II intensity and increase in glycoprotein expression with two strains of HSV1.

3.3.5 MHC Class II Expression on Infected LC

Table 3.3 primarily illustrates the reduction in MHC Class II expression with increasing MOI of HSV1. An increasing infectious dose of virus (0.05-1.0 MOI) was associated with a progressive decrease in the number of detectable LC (1.6-0.7%) and a reduction in their MHC Class II expression (MCF=1660-747). There is therefore an inverse relationship between the expression of virus antigens and the levels of MHC Class II on infected LC.

This phenomenon of MHC Class II reduction was suggested from our initial experiments with UV-microscopy (Section 3.2) and in most of the FACS analyses we have carried out, of which Table 3.3 is an example. However, this reduction in MHC Class II intensity was not observed in all experiments where we saw a reduction in LC number or expression of virus antigens on infected LC. From these results and those presented in the next section (Section 3.4, Table 3.4) we demonstrate that infection with HSV1 may kill LC while those that remain may show reduced levels of MHC Class II expression.

In control experiments β-propiolactone-inactivated HSV1/34 did not induce the changes observed with live infectious virus (data not shown), so these observations are not simply a consequence of exposure to viral antigens.

In conclusion, the results presented in this section (Section 3.3) indicate that infection of freshly isolated EC with HSV1 had various effects. It reduced the number of LC. HSV1 glycoproteins were detectable on infected bulk EC, but appear to be expressed on a much higher percentage of gated LC. A reduction in MHC Class II levels on the remaining LC was observed, but this was not found in all experiments. The ability of HSV1 to reduce cell surface levels of MHC Class II may be mouse, virus strain and virus dose dependent.

Table 3.3 *HSV1 infection of LC can reduce the levels of MHC Class II expression on infected LC (MCF MHC Class II) and this may be related to the dose of infecting virus. See text for details.*

pfu/cell	*% MHC Class II LC*	*MCF MHC Class II*	*% cells expressing virus antigens:* Bulk EC	Gated LC
Control	2.2	***1951***	-	-
0.05	1.6	***1660***	15	23
0.1	1.6	***1563***	16	27
0.2	1	***1227***	25	42
0.4	0.8	***1193***	24	56
1	0.7	*747*	39	68

3.4 Maturation of LC in culture, and the resultant effect on infection by HSV1

LC when cultured in vitro mature in phenotype and function such that they become virtually indistinguishable from splenic DC (sDC). Cultured LC show an increased ability to stimulate naive T cells in the context of the allogeneic mixed leucocyte reaction (MLR) and oxidative mitogenesis. Additionally, the ability of the cultured LC to endocytose and process antigens is down-regulated. In the previous section (Section 3.3) we demonstrated that freshly isolated LC appeared to be susceptible to HSV1 in vitro. In a direct comparison of freshly isolated LC with cultured LC in the following section we show that cultured LC were clearly susceptible to HSV1 infection and that maturation had a variable effect on this susceptibility.

EC were prepared and infected with HSV1/34 immediately after isolation or after culture for 48-96 h. In the first of these experiments cultured EC were incubated for 96 h prior to infection with HSV1 at an MOI=0.1 (Table 3.4; Expt.1). Detectable LC in both fresh and cultured populations decreased in number of LC and MHC Class II expression. Glycoprotein expression was much lower on cultured bulk EC than on fresh bulk EC. This was probably due to the relative absence of keratinocytes and other EC which die during the culture period prior to infection. Conversely, a higher percentage of cultured LC expressed virus glycoproteins than fresh LC. This indicated that culturing may render LC more susceptible. However, with long periods of culture, such as 96 h, LC may have begun to die and may be less able to control HSV1 infection. By reducing the culture period to 48 h this would allow sufficient maturation of LC without the possible, associated stresses of long term culture. The remainder of this section will deal solely with the infection of freshly isolated 0 h EC vs. cultured 48 h EC.

The results from the first set of data (Table 3.4, Expt.1) suggest, with qualification, that culturing rendered LC more susceptible to infection by HSV1. This procedure was repeated several times to establish a pattern in susceptibility. The results are summarised in Table 3.4 Expts. 2-4. LC, fresh or cultured, were reduced in number and MHC Class II intensity, as we

Table 3.4 Maturation had a variable effect on the susceptibility of LC to infection by HSV1/34, as determined by the ability of freshly isolated and cultured LC to express HSV1 antigens.

			% cells expressing virus antigens:		
	% MHC Class II LC	MCF MHC Class II	Bulk EC	Gated LC	Pattern
Expt. 1					
dO Control	1.6	151	-	-	
dO Infected	1.1	126	15	***30***	
d4 Control	1.4	89	-	-	
d4 Infected	0.8	54	4	***57***	***increase***
Expt. 2					
dO Control	2.6	4300	-	-	
dO Infected	2.2	3752	9	***20***	
d2 Control	1.9	3036	-	-	
d2 Infected	1.3	2252	3	***53***	***increase***
Expt. 3					
dO Control	3	2274	-	-	
dO Infected	2	1890	21	***46***	
d2 Control	2.1	1970	-	-	
d2 Infected	1.6	1456	1	***44***	***equal***
Expt. 4					
dO Control	1.4	2458	-	-	
dO Infected	1	1453	21	***47***	
d2 Control	0.8	506	-	-	
d2 Infected	0.8	336	0.7	***33***	***decrease***

have observed previously for freshly isolated LC alone (Section 3.3). We consistently observed fewer cultured bulk EC expressing virus antigens than fresh bulk EC. Despite this the effects of maturation on LC were highly variable. Maturation increased (Expt.2), decreased (Expt.4), or had little effect (Expt.3) on the susceptibility of LC to infection by HSV1.

The MOI of HSV1/34 used for infection determined the number of LC remaining in culture, either by killing them or inducing down-regulation of MHC Class II such that they are no longer identifiable. Because of this it seemed reasonable to suggest that when acquiring the FL2 (MHC Class II-positive) gated cells some infected LC were lost because they had fallen below the level of the FL2 marker. This may be influenced by the conditions of each individual culture and could explain the inconsistency of the patterns above. In an attempt to reduce this phenomenon EC were exposed to much lower MOI than previously used (i.e. MOI<0.1), so that cells could still be infected but may not be as profoundly affected in terms of MHC Class II expression. Freshly isolated and cultured EC were infected with HSV1 at an MOI= 0.025, 0.05 and 0.1 and the assessment is shown in Table 3.5. We were able to gate and acquire clearly defined peaks of LC and at lower MOI (0.025, 0.05) cultured LC expressed higher levels of virus antigen than freshly isolated LC. At MOI=0.1 this difference remained but was less marked. Maturation, in this instance, apparently *increased* susceptibility.

Having indicated that infection with HSV1 at a low MOI may enhance the acquisition of infected LC, Balb/c and C57/Bl/6 EC were compared using HSV1 at MOI=0.025 (Table 3.6). A lower percentage of C57/Bl/6 LC expressed virus antigens compared to Balb/c (as seen in Figure 3.10 and Figure 3.11). In this experiment, maturation, in both strains, *decreased* the susceptibility of LC to infection by HSV1/34.

In conclusion, we have shown that cultured LC were infected with HSV1/34; detectable LC and MHC Class II expression were reduced and >30-50% of gated cultured LC expressed virus glycoproteins. Therefore, maturation did not consistently or radically alter the susceptibility of cultured LC compared to fresh LC.

Table 3.5 *Maturation increased the percentage of LC which expressed HSV1 antigens post-infection. Infection of freshly isolated and cultured EC with HSV1/34 at low multiplicities for 18h indicated a difference in LC susceptibility which was less marked at higher infectious doses.*

			% cells expressing virus antigens:	
	% MHC Class II LC	*MCF MHC Class II*	*Bulk EC*	*Gated LC*
d0 Control	1.7	1580	-	-
d0 0.025	1.5	1771	9.7	***25***
d0 0.05	1.1	1439	21.5	***40.6***
d0 0.1	1	1578	30	***54.4***
d2 Control	3.3	1528	-	-
d2 0.025	2.9	1232	2.1	***50.2***
d2 0.05	2.5	1176	2.6	***60.6***
d2 0.1	2.5	1034	2.5	***64.3***

Table 3.6 *Virus antigen expression at 18h post-infection on freshly isolated and cultured Balb/c and C57/Bl/6 EC. Maturation reduced the percentage of LC expressing virus antigen in both strains of mice.*

	% MHC Class II LC		*MCF MHC Class II*		*% cells expressing virus antigens: Bulk EC*		*Gated LC*	
	Balb/c	**C57**	**Balb/c**	**C57**	**Balb/c**	**C57**	**Balb/c**	**C57**
d0 Control	1.2	2	190	114	-	-	-	-
d0 0.025	0.8	1.7	156	104	21.2	19.8	*83*	*57.8*
d2 Control	1.7	3.5	128	59	-	-	-	-
d2 0.025	0.7	3	80	49	1.9	2.7	*56.3*	*45.5*

3.5 Infection of Splenic DC (sDC)

Since cultured LC closely resemble lymphoid DC in phenotype and immunostimulatory function it was reasonable to propose that lymphoid DC might be similarly susceptible to HSV1. The following series of experiments demonstrated, using a variety of approaches, that only a very limited percentage of lymphoid DC isolated from spleen (sDC) expressed virus glycoproteins post-infection.

In the experiments so far detailed (sections 3.2 to 3.4), LC were infected by resuspending a pellet of known number of freshly isolated EC in a small volume of medium containing a given MOI of HSV1/34. With freshly isolated sDC this was not possible because they are an adherent population. In the following section, unless stated otherwise, freshly isolated EC were infected as previously as a resuspended pellet of cells. Using a variety of methods for infection for sDC (see sections 3.7.1-3.7.4) we attempted to directly compare LC and sDC in terms of their susceptibility to HSV1.

3.5.1 Method 1: Infection of sDC as low density pellicle cells

Initially, LC and sDC were infected in a resuspended pellet of cells (for detailed method, see section 3.7.1). At 18 h post-infection, all cells were analysed by two-colour flow cytometry by labelling for MHC Class II and virus antigens. Freshly isolated EC and LC expressed virus glycoproteins (see previous Figure 3.9; 19% (d) and 74.3% (h) respectively). There was a virus induced decrease in the number of LC (Figure 3.9; (a) 3.2% - (b) 1.9%). In contrast, an analysis of the sDC population (Figure 3.12) showed that only around 15% of gated sDC expressed virus glycoproteins (h). There was no decrease in the number of MHC Class II-positive sDC ((a) 62.5%; (b) 72.3%) and no change in the MCF (not shown).

There are at least two basic criticisms of the above technique. Firstly, HSV1 infection may alter the properties of sDC such that they become non-adherent or unable to adhere, and infected cells were discarded when the cells were removed after the plastic adherence step. Secondly, in the spleen, MHC Class II can also be expressed on mϕ and B cells as well as sDC.

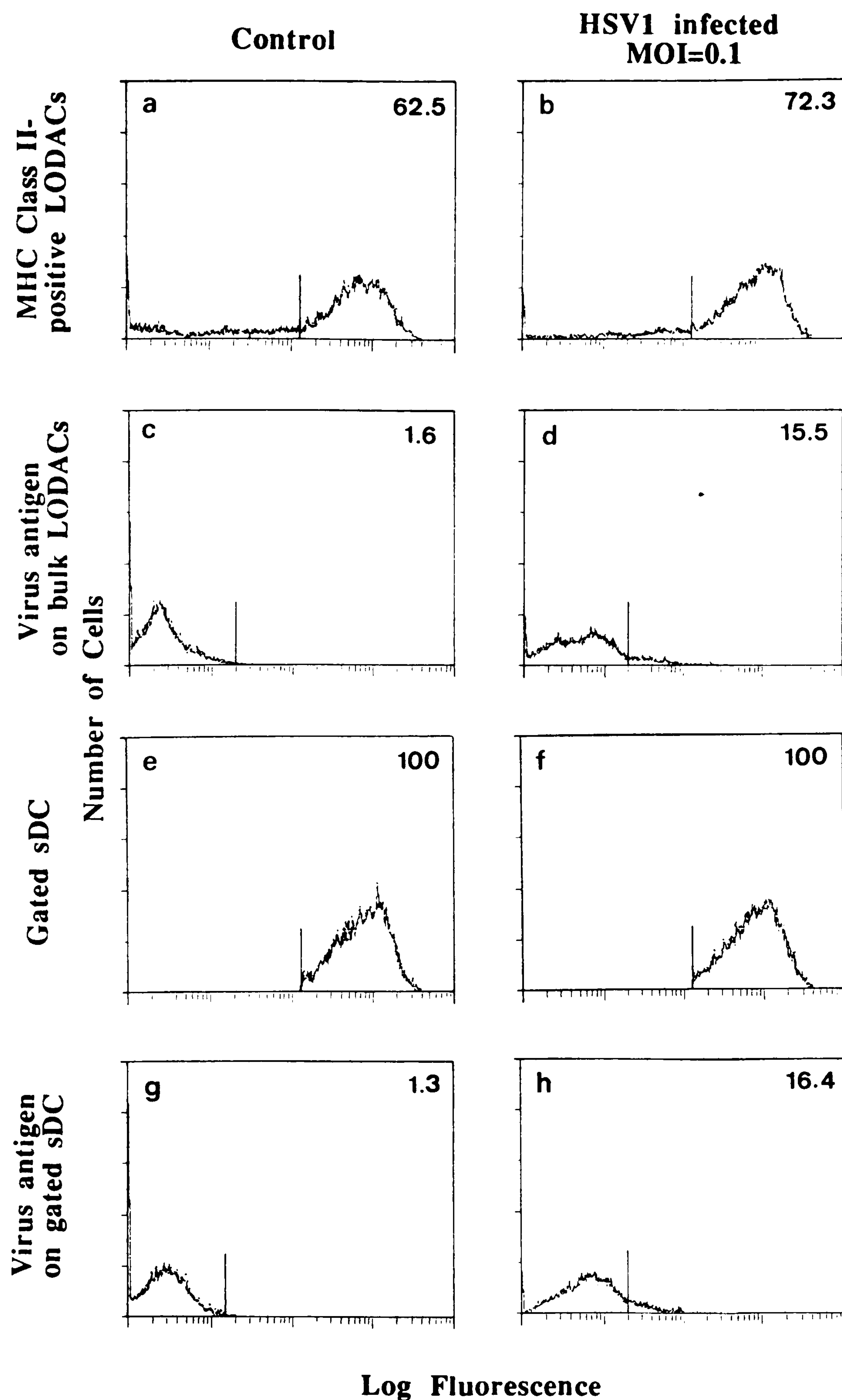


Figure 3.12 HSV1 antigens were expressed on a small percentage of gated B21-2-positive sDC. LODAC were labelled with B21-2 and F318 at 18 h post-infection. B21-2 expression on control (a) and infected sDC (b). Virus glycoprotein labelling of control (c) and infected sDC (d). Gated control (e) and infected sDC (f) were analysed for the expression of viral antigen (g) and (h).

Labelling for MHC Class II (B2-12) in the spleen is therefore not as exclusive as labelling for LC (the only cell type in a normal EC population which constitutively expresses MHC Class II). Our results may therefore be affected by the presence of other infected MHC Class II-positive cells.

The MAb N418 (anti-CD11c/CD18; Chapter 2, Table 2.4) specifically labels sDC. This antibody was tested on sDC as (i) LODACs (sDC, B cells and mϕ) and (ii) FcγRII-negative (FcR-ve) sDC (highly purified sDC). Both populations of cells were labelled for two-colour flow cytometry using B21-2 and N418 as reciprocal markers. Briefly, this showed that all sDC (N418-positive cells) expressed MHC Class II (Figure 3.13; (g) and (h)), but not all MHC Class II-positive cells in a LODAC population were necessarily sDC (cf. (e) and (f)). Purification of sDC significantly increased the percentage of cells which labelled for MHC Class II (cf. (a) and (b)) and N418 (cf. (c) and (d)). On this basis, B21-2 and N418 were used in parallel in the following experiments as markers for sDC, in conjunction with F318 defining virus antigen.

3.5.2 Method 2: Infection of sDC as low density adherent cells

In order to avoid the loss of cells due to HSV1-induced non-adherence, sDC were exposed to HSV1/34 at MOI=0.1, 0.5 and 1.0 in a small volume when the cells were freshly adherent (for detailed method, see section 3.7.2). The resulting populations were analysed by two colour flow cytometry (Table 3.7). For EC, the patterns were seen to be very similar to those previously noted (Sections 3.3 and 3.4). MHC Class II-positive sDC were slightly reduced in number (65%-51%) and MHC Class II expression (MCF 143-117); this was virus dose dependent (Table 3.7; columns 1 and 2). However, a much lower percentage of gated sDC (4%-24%) expressed virus antigens compared to LC (77%-91%). N418 expression was not reduced upon infection (MCF 53-58). Compared to the MHC Class II population, a higher percentage of gated N418-positive cells were found to express virus antigen. At least two points arose from this analysis. Firstly, labelling with B21-2 may underestimate the percentage of sDC infected (cf. the percentage of virus-positive MHC Class II-positive cells with virus-

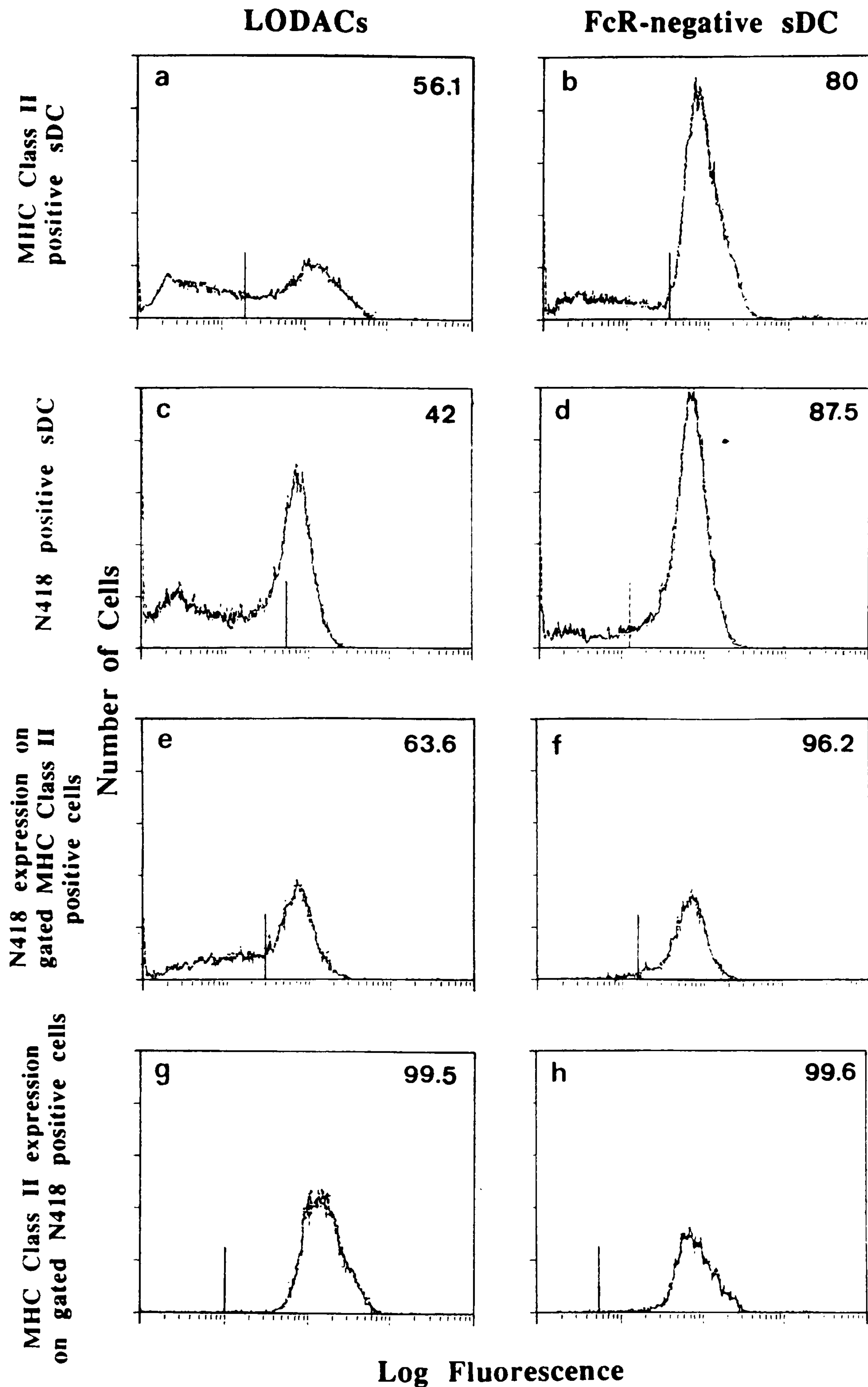


Figure 3.13 N418 can be used as a specific marker for sDC isolated in vitro. Two colour analysis was carried out on purified (LODAC) and unpurified (FcR-ve sDC) populations of sDC using N418 (anti-CD11c/CD18) and B21-2 (anti-Ia^{b,d}). MHC Class II expression and N418 expression are shown on ungated LODAC, (a) and (c) respectively, and on ungated FcR-ve sDC, (b) and (d) respectively. N418 expression (on gated B21-2-positive cells) is shown for LODAC (e) and FcR-ve sDC (f) and B21-2 expression (on gated N418-positive population) is shown for LODAC (g) and FcR-ve sDC (h).

Table 3.7 *Parallel infection of sDC and LC - a comparison of virus antigen-positive DC in both populations. Virus antigen expression at 18h post-infection was assessed on HSV1/34 infected sDC and LC by determining the number of DC which labelled with the anti-HSV1 PAb, F318. HSV1 antigens were found on a higher percentage of gated B21-2-positive LC than gated B21-2- or N418-positive sDC.*

EC	*% MHC Class II LC*	*MCF MHC Class II*	*Bulk EC*	*Gated LC*
Control	4.3	90	-	-
Infected MOI= 0.1	1.6	55	31	77
Infected MOI=1.0	0.5	25	56	91
LODACs	*% MHC Class II sDC*	*MCF MHC Class II*	*Bulk Lodacs*	*Gated sDC*
Control	65.3	143	-	-
Infected MOI=0.1	64.8	138	3.1	3.7
Infected MOI=0.5	58.3	120	12	12
Infected MOI=1.0	51.1	117	20	24
LODACs	*% N418 sDC*	*MCF N418*	*Bulk Lodacs*	*Gated sDC*
Control	47.3	53	-	-
Infected MOI=0.1	50.3	54	3	5.3
Infected MOI=0.5	46.3	56	10	20
Infected MOI=1.0	41	58	16	41

positive N418 cells). Secondly, while the comparison between EC and sDC indicated that LC were much more susceptible to HSV1 infection it is still possible that this is a consequence of the mode of infection (see next section).

3.5.3 Method 3: Parallel infection of sDC and LC with a fixed concentration of HSV1

While sDC and LC in section 3.5.2 were exposed to the same MOI of HSV1/34 the difference in inoculum volume (2ml for sDC vs. 100μl for EC) means that the local concentration of virus was higher for EC and could explain the apparent difference in susceptibility. To minimise this, sDC and LC were exposed to an equal concentration of HSV1 (10^7pfu/ml; for detailed method, see section 3.7.3). Figure 3.14 illustrates the outcome of this approach. Of the remaining LC, >75% (2.54% virus-antigen positive LC in a total of 3.14%) expressed virus glycoproteins (c) compared to 20-35% for sDC ((f) 16% virus-antigen positive MHC Class II sDC in a total of 89% and (h) 19% virus antigen positive N418 sDC in a total of 52%). These results further indicated that sDC were more resistant to HSV1 when compared to LC.

3.5.4 Method 4: Infection of 18 h cultured FcR-negative-sDC and LC

A final comparison infected EC and sDC with HSV1/34 in an identical manner - as 18 h EC and 18 h FcR-ve sDC in a pellet (for detailed method, see section 3.7.4). The results obtained are displayed in Figure 3.15 and are representative of two separate experiments. Detectable LC reduced in number and MHC Class II intensity (a-c), as previously observed. In contrast, there was clearly little change in the sDC population defined by MHC Class II expression (g-i) or N418 expression (m-o). Very few gated sDC expressed virus glycoproteins compared to gated LC. In fact, at an MOI=0.1 there was no glycoprotein expression on sDC (cf. k,q) above background ((j,p) respectively) while almost 60% LC expressed virus antigens (e). At the higher MOI=1.0, virus glycoproteins were detectable on 5-8% of sDC (l,r) but this compared with >80% on LC (f).

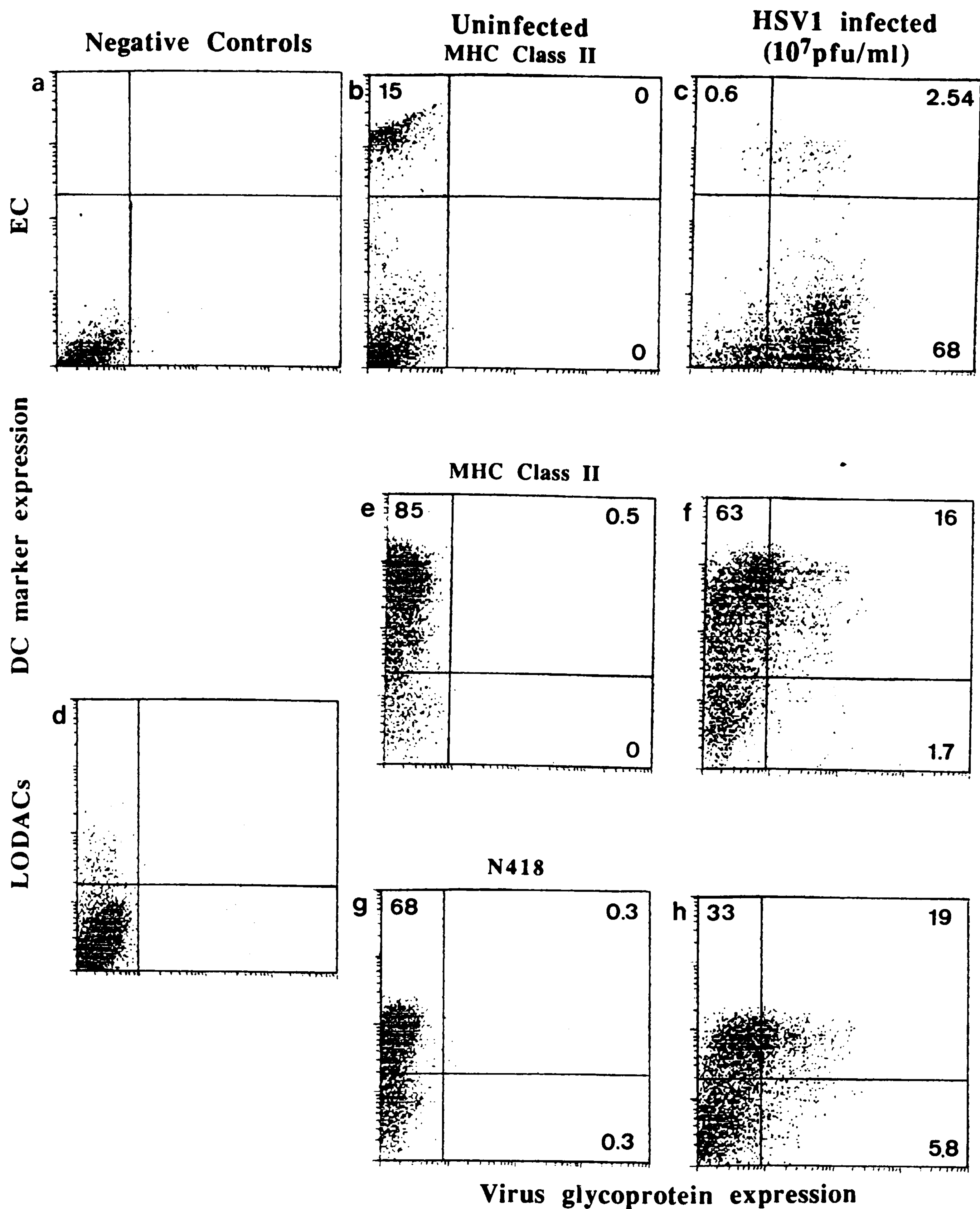


Figure 3.14 EC and LODACs were infected with equivalent concentrations of HSV1 and analysed by two colour flow cytometry. The results presented below are in the form of PE (DC marker) vs.FITC (virus glycoproteins) profiles and display MHC Class II expression on control LC (b) and control sDC (e), or N418 expression on control sDC (g). Infected populations were labelled for MHC Class II or N418 and virus glycoproteins and the number of double positive cells is located in the upper right hand quadrant of the scatter profiles for LC (c), N418 sDC (h) and MHC Class II-positive sDC (h). LC negative control (a) and sDC negative control (d).

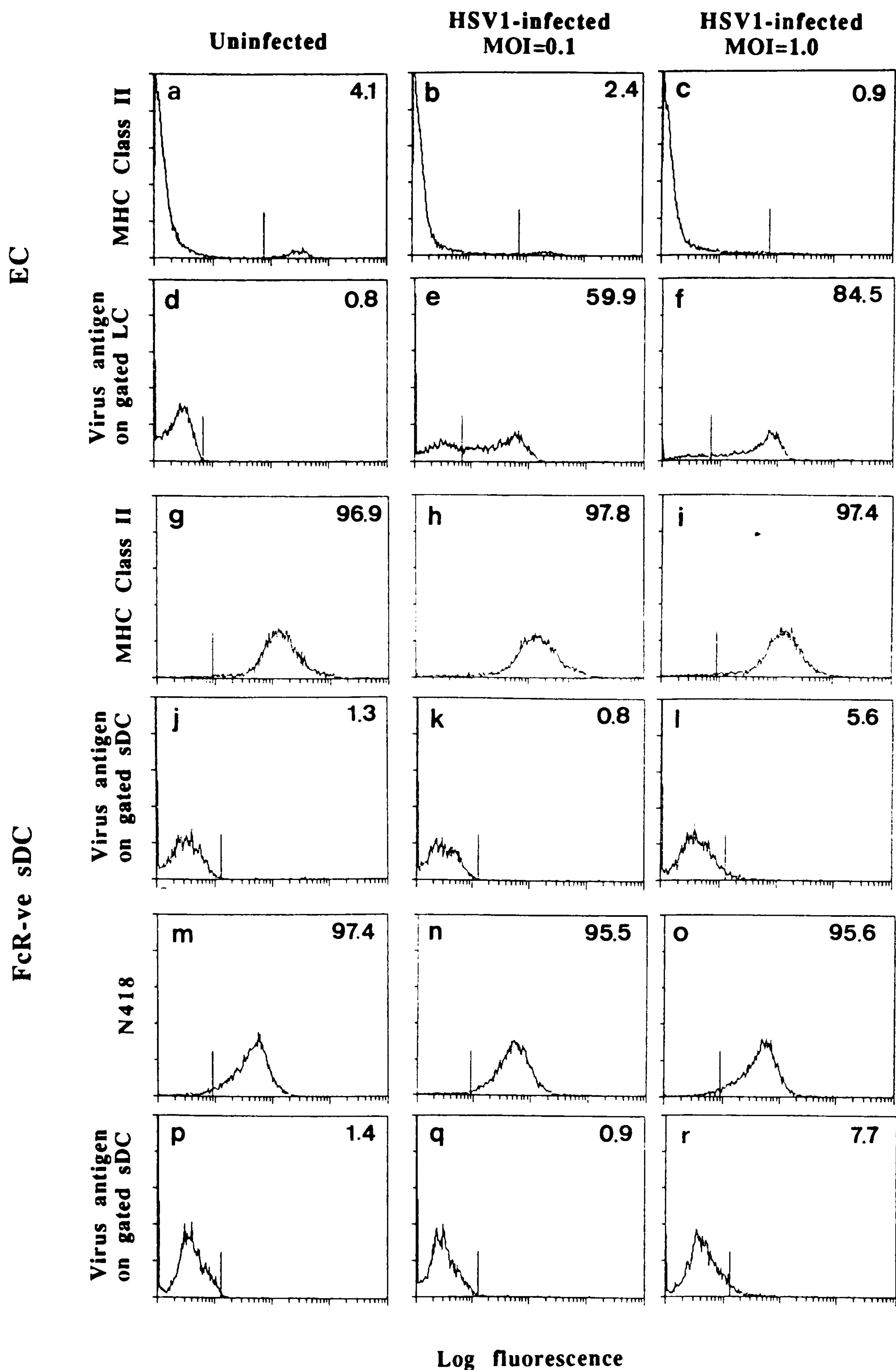


Figure 3.15 A small percentage of 18 h sDC expressed virus antigens when infected with HSV1/34 in parallel with 18 h EC. 18 h FcR-ve sDC and 18 h EC were infected and subsequently labelled for two colour flow cytometry. Control LC (a), control B21-2 sDC (g) and control N418 sDC (m) are compared with infected LC (b) and (c), infected B21-2 sDC (h) and (i) and infected N418 (n) and (o). Gated populations were analysed for their expression of virus antigens - for LC - control (d), infected MOI=0.1 (e) and infected MOI=1.0 (f); for B21-2 sDC - control (j), infected MOI=0.1 (k) and infected MOI=1.0 (l); for N418 sDC - control (p), infected MOI=0.1 (q) and infected MOI=1.0 (r).

In conclusion, using a variety of approaches we have compared the susceptibility of fresh LC and sDC to HSV1 and consistently found that fewer sDC expressed virus glycoproteins post-infection compared to LC. This was most clearly shown after 18 h in culture prior to infection when, using an identical method for infecting both cell populations, >80% of LC expressed virus glycoproteins compared with 5-8% sDC.

3.6 Chapter Summary

In this chapter we have shown by UV-microscopy that HSV1 infection of EC in vitro (i) apparently reduced the number of MHC Class II-positive LC, either by HSV1-induced cell death or profound down-regulation of MHC Class II, (ii) appeared to reduce the MHC Class II intensity on the remaining LC, and finally (iii) that a proportion of LC expressed virus antigen in the form of IPP. In order to quantify the number of virus positive LC we developed a two-colour FACS analysis protocol. By gating and acquiring LC from infected EC populations we observed that a considerable percentage of LC (from fresh or cultured EC) expressed HSV1 antigens post-infection. Our initial results demonstrated that 65-70% LC expressed virus glycoproteins. In a direct comparison with cultured LC this average decreased but still showed that HSV1 glycoproteins were expressed on cultured LC (30-50%) and freshly isolated LC (20-50%). HSV1-infection of LC also reduced the levels of MHC Class II (MCF) on the remaining LC in most, but not all, of our analyses. We have further indicated that sDC may be less susceptible than freshly isolated LC as judged by their apparently lower capacity to support the expression of HSV1 antigens post-infection. This may highlight a fundamental difference between sDC and cultured LC.

3.7 Specialised Materials and Methods: Infection of splenic DC

3.7.1 Method 1

Low density spleen cells, enriched for sDC, were isolated as a pellicle of cells on a BSA density gradient (Chapter 2, section 2.6.2). These cells were pelleted by centrifugation, counted and, in parallel with EC, were resuspended in 100μl R10 and exposed to HSV1/34 at an MOI=0.1. After 1 h at 37°C, excess virus was removed with two washes in R10 followed by centrifugation. Both EC and low density spleen populations were then resuspended in 10ml R10 and added to tissue culture plates. After 1-2 hours, all non adherent spleen cells were removed by lightly pipetting warm RPMI over the surface of the tissue culture plate. The adherent spleen cells, mainly sDC and macrophages, were returned to culture for a further 18 h. After this time, the EC and LODAC populations were removed for two-colour analysis.

3.7.2 Method 2

In this method, sDC were exposed to virus **after** the 1-2 h adherence step. EC and adherent spleen cells (mainly sDC and mϕ) were exposed to HSV1/34 at an MOI=0.1. A known number of EC were resuspended in 100μl of R10 containing the virus inoculum. The number of adherent spleen cells was estimated by counting the number of non adherent cells removed from each plate and subtracting this from the initial number added. Virus was added to the adherent spleen cells in 2ml R10. After a 1h exposure to the virus, EC were resuspended and washed to remove excess virus and plated on tissue culture plates for 18 h. For spleen adherent cells, the inoculum was removed from the plates and centrifuged to remove any cells which had become non adherent during this time. After washing, this pellet of cells was returned to the plates for 18 h, thereby permitting infection of the sDC without the significant loss of cells associated with the previous method.

3.7.3 Method 3

This method exposed resuspended EC and adherent spleen cells to HSV1/34 at pfu/ml not pfu/cell. Both EC and low density spleen populations were prepared to the point **prior** to incubation on tissue culture plates. For sDC this meant prior to the 1-2h adherence step. Both

populations of cells were then added to tissue culture plates, sDC for the 1-2 h adherence step, and EC in 3 ml R10 for a similar period of time. After 1-2 h the non adherent spleen cells were removed and 3 ml of R10 added to the sDC plates. At this point HSV1/34 was added to a concentration of 10^7pfu/ml to the EC and sDC plates. The cells were therefore exposed to equal local concentrations of virus, i.e. 3ml R10 at 10^7pfu/ml. After 1h, medium in each plate was made up to 10ml R10 and incubated for a further 18 h. Subsequent analysis assessed the percentage of N418 and MHC Class II-positive cells expressing virus glycoproteins.

3.7.4 Method 4

In method 3, although sDC and EC were exposed to the same concentrations of HSV1/34, sDC were an adherent population while EC were still mostly non adherent. This may increase the probability of EC coming into contact with HSV1 in the inoculum volume. In this last method, EC and LODACs were prepared as per protocol (Chapter 2, section 2.6.1 and 2.6.2) and incubated for 18 h *in the absence of virus*. After this time all the cells were removed from tissue culture plates. Contaminating cells such as B cells and mϕ were removed from the LODAC population by rosetting with antibody coated SRBC (Chapter 2, section 2.7.1) to give a population of highly purified sDC. A known number of purified sDC **and** EC were then resuspended in 100μl R10 containing the virus inoculum (HSV1/34 at an MOI of 0.1 and 1.0 pfu/cell). After 1h all cell populations were washed of excess virus and returned to culture at 37°C for a further 18 h. The cells were subsequently analysed by two-colour flow cytometry.

Chapter 4

HSV1 Impairment of DC function

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4.1 Introduction

LC are known to be accessory cells critical in the initiation of an HSV1 specific immune response in vivo (Chapter 1). The essential functions of such an accessory cell are two-fold. Firstly, it must present processed antigen in an immunologically relevant form as peptides bound to MHC Class I and/or II molecules. Secondly, it must be able to deliver the "second" or accessory signal(s) in the form of a secreted or membrane cellular molecule which, when presented together with processed antigen, will activate naive T cells. In order for LC to initiate an HSV1 specific response, for example in the draining lymph node, they must therefore deliver these two signals to the appropriate HSV1-specific, naive T cells. We have shown that fresh and cultured LC in EC bulk cultures are susceptible to infection by HSV1 in vitro (Chapter 3). Infection of cells by HSV1 also results in a shut down of host macromolecular synthesis [49]. In the following chapter we tested whether HSV1 could reduce or otherwise impair the immunostimulatory capacity of cultured LC. The immunostimulatory capacity of LC makes them potent stimulators of the allogeneic mixed leucocyte reaction (MLR) and this capacity is optimised by culturing the cells in vitro [223]. This maturation process is facilitated by the production of GMCSF by keratinocytes in a bulk EC culture, or by the addition of exogenous GMCSF [198]. The MLR and oxidative mitogenesis were used to test the immunostimulatory capacity of HSV1-infected LC.

4.2 LC in the Allogeneic Mixed Lymphocyte Reaction (MLR)

Bulk EC cultures from Balb/c (H-2^d) mice were cultured for 24 and 72 hours in vitro, and subsequently enriched for LC. These cells were titrated into C57/Bl/6 (H-2^b) T cells and proliferation was measured. Figure 4.1 shows that both populations of LC induced proliferation, but that 72 h LC were more potent. As has been previously reported [223], maturation of LC by culturing in vitro for 72h results in an increased ability to stimulate the MLR. On this basis, we decided to use 72h cultured LC in subsequent experiments.

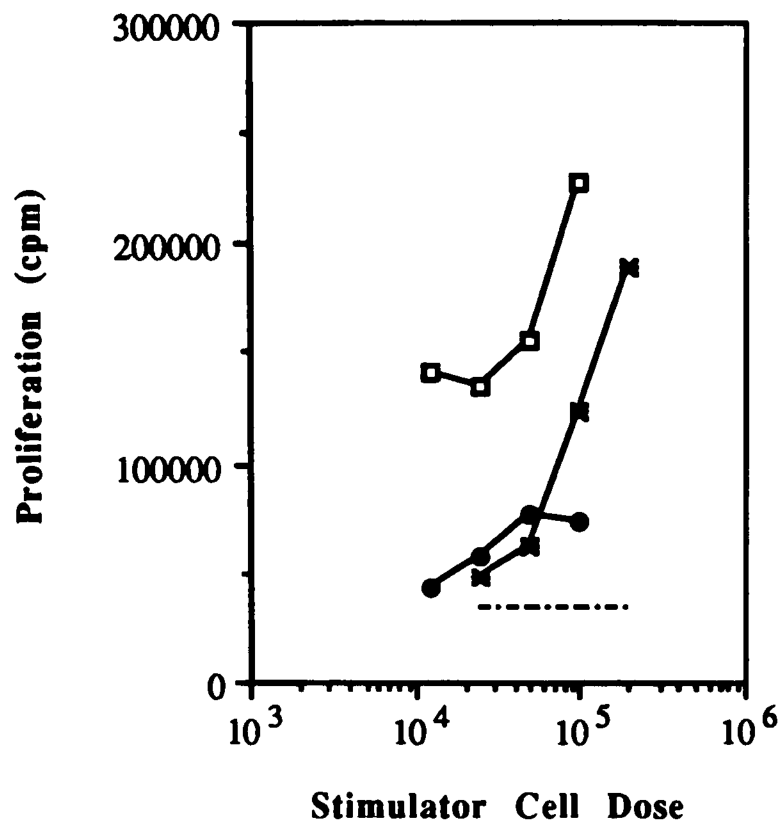
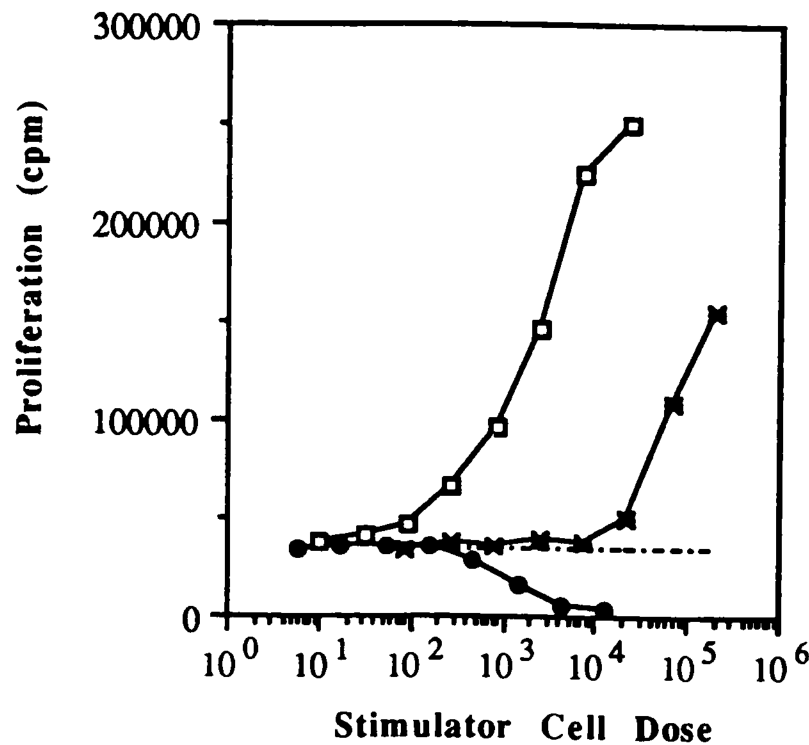


Figure 4.1 *72 h EC (open squares) are more potent stimulators than 24 h EC (closed circles) or bulk spleen (cross) when titrated into the MLR. See section 4.11.1 for method.*

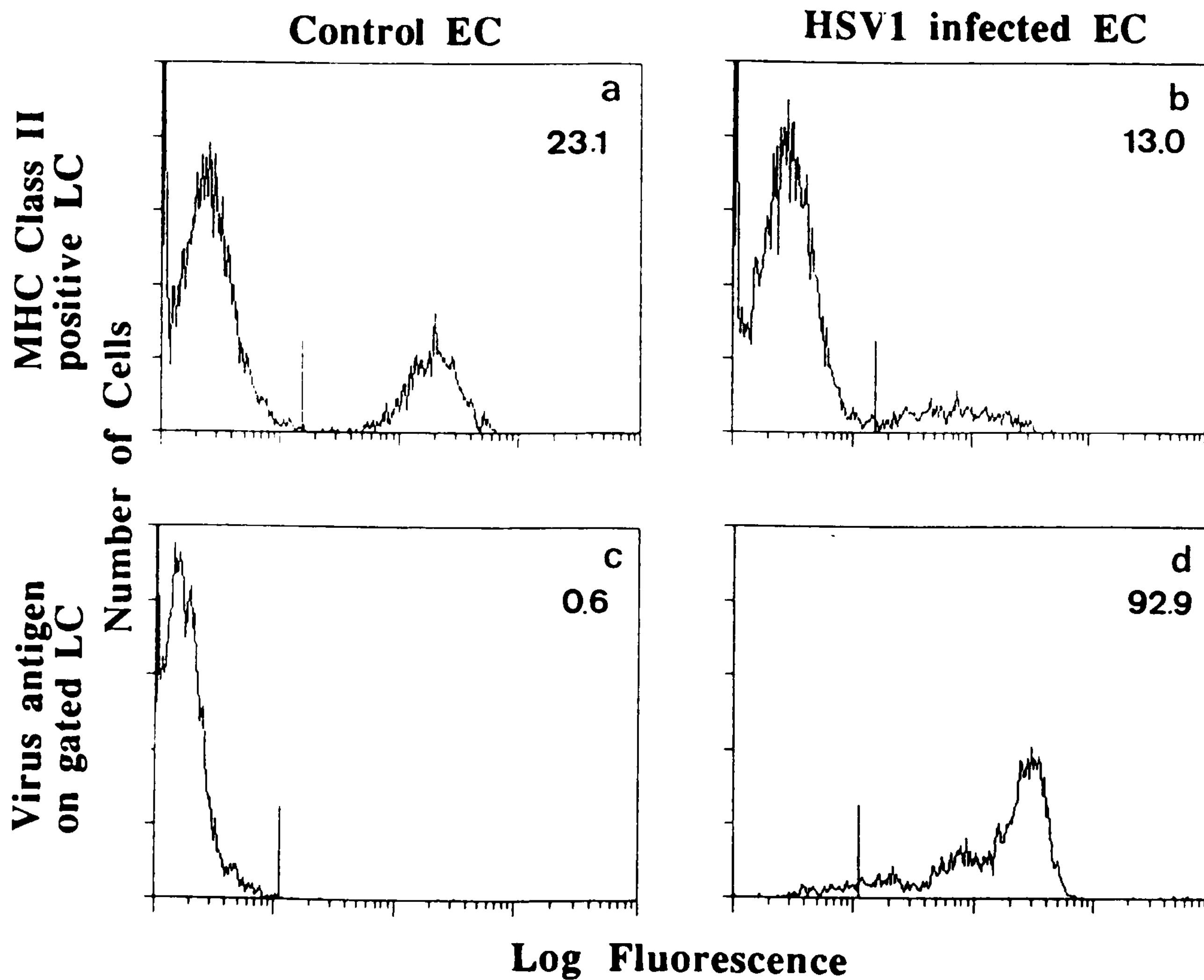
4.3 HSV1 Impairment of LC Function

HSV1 infection of LC in vitro resulted in a decrease in the number of LC, either by cell death or down-regulation of MHC Class II molecules (Chapter 3, section 3.3). To make any assessment of a difference in the stimulatory capacity between control and infected LC it was necessary to assess the number of input LC being titrated into the assay, and to correct for this difference when considering proliferation values. (This method, of course, did not consider the potentially continuing loss of LC during the course of the MLR or oxidative mitogenesis). Briefly, Balb/c EC were infected with HSV1/34 and the cells titrated into the MLR. A sample of each population - control (uninfected) and HSV1 infected - was kept for an assessment of MHC Class II-positive cells by FACS analysis. Figure 4.2 shows the resulting proliferation values and FACS profiles from a representative experiment. The addition of HSV1/34 infected LC to allogeneic T cells resulted in proliferation values which were negatively dose dependent, i.e. increasing the infected cell dose was associated with decreasing proliferation to even below

Figure 4.2 *Infection of EC resulted in a profound impairment of their ability to stimulate the alloMLR (a). Control, uninfected EC (open squares) and normal bulk spleen (crosses) were compared with HSV1/34 infected EC (closed circles) when titrated into the alloMLR.*



HSV1 infection also reduced the number of LC (compare (a) with (b)). Of the 13% LC remaining after infection, >90% expressed HSV1 glycoproteins (d). The marker for (d) was set using the profile generated from the gated, uninfected LC (c).



background values. Additional factors, other than the loss of LC upon infection, must therefore be acting to reduce proliferation to below background levels.

4.4 Infectious HSV1 in the MLR

Two possibilities were considered. Firstly, infected cells could produce progeny virus which may subsequently infect the responding T cells and impair their ability to proliferate. By titrating out the stimulator cell number we also titrated out the source of virus, resulting in a progressive return to background levels of T cell proliferation (see Figure 4.2). The second possibility is that infected cells may produce cytokines which are inhibitory for the MLR and, by the same reasoning as above, titrating out cell number also titrated out the 'inhibitory factor'. It was considered that the first option was the most reasonable and readily tested. A number of experiments centred around the possibility that HSV1/34 infected EC could produce infectious virus and have a resultant effect on the MLR. The first question to be addressed was whether or not infected EC in the absence of T cells could produce infectious virus over the time period used for the MLR. Figure 4.3 and Table 4.1 illustrate the yield of virus from EC incubated for 0-96 h. Virus is released from the cell fraction into the supernatant as the period of culture progresses from 0-24 h, but no infectious virus is detectable by 96 h. During the initial 24 h the net amount of virus does not appear to significantly increase indicating that little or no replication of the virus goes on after the initiation of culture, probably as a consequence of irradiating the cells prior to culture.

To initiate T cell proliferation in the MLR, DC, such as LC, cluster with T cells to deliver the appropriate signals. In other virus infections, for example HIV, this mechanism of clustering has been proposed as a means of rapid and efficient transfer of virus from infected DC to uninfected T cells [304]. It may be the case that productively infected LC in the EC population in our system can cluster with T cells, and by a mechanism similar to that for HIV, transfer HSV1 to uninfected T cells. LC were therefore tested for productive infection.

Figure 4.3

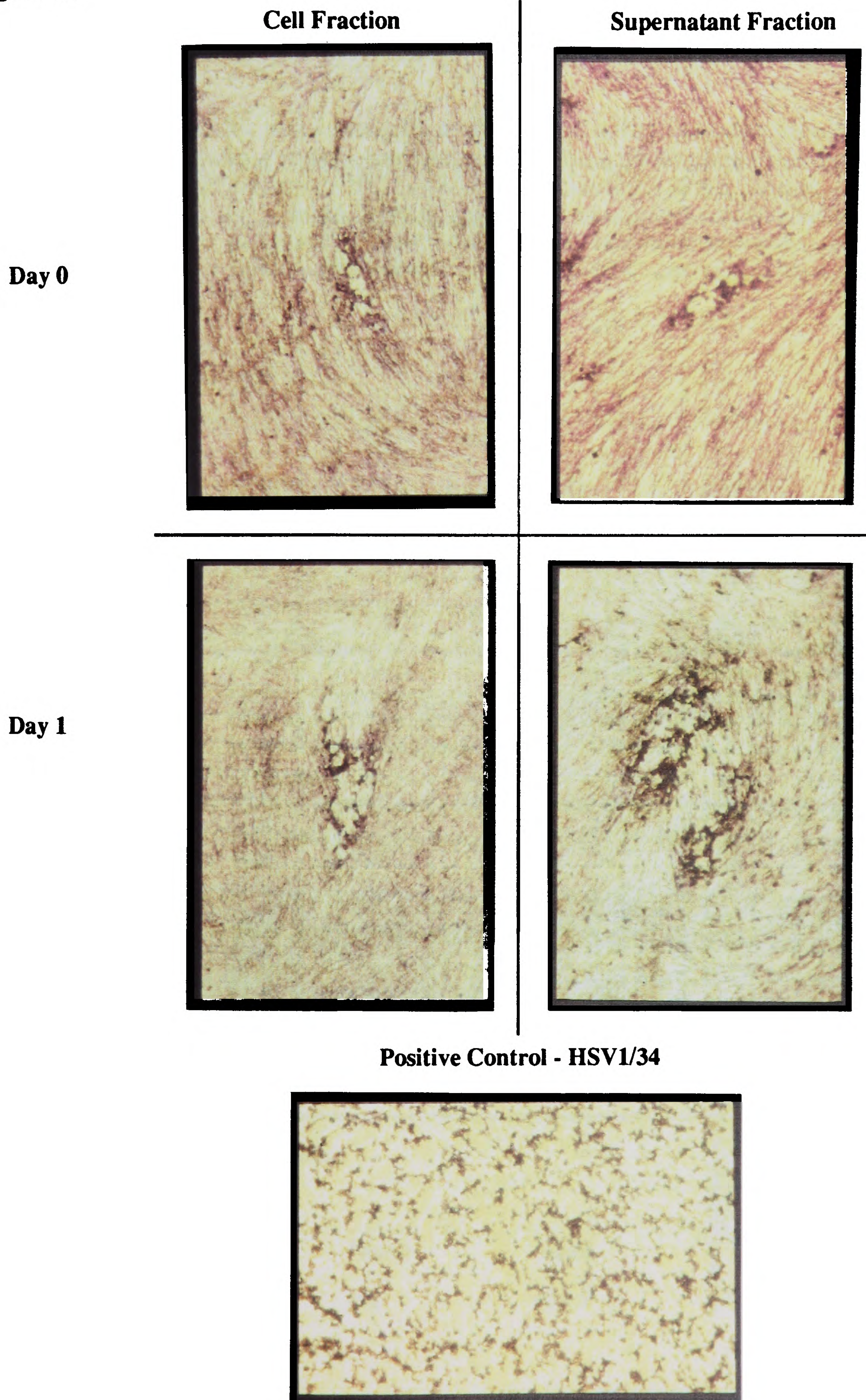


Figure 4.3 *(see previous page) Infectious virus is present in the cell and supernatant fractions of 0-24 h cultured HSV1/34-infected EC as measured by plaque assay (See section 4.12.11). The figure displays typical examples of the plaques (x100) which were produced from cell and supernatant fractions of HSV1/34 infected EC removed at 0-24h.*

Table 4.1 *details the plaque counts obtained from d0 (1 h) and d1 (24 h) cell and supernatant fractions. While the amount of virus in each fraction changed from d0-d1 (pfu/fraction (x 10^5)) the total infectious yield (total pfu (x 10^5) or pfu/cell) remained more or less constant. No HSV1 was detectable by d 4. Briefly, HSV1/34-infected EC were plated at 10^5 EC/well in a 96 well microtest plate. At 1 h (d0), 24 h (d1) and 96 h (d4), cell fractions (washed of free virus) and supernatant fractions were removed and stored at -70°C until assayed by plaque assay (See section 4.12.11).*

Time point	**pfu/fraction (x10^5)**	**total pfu in culture (x10^5)**	**pfu/cell**
Day 0			
Cell	1.32		
Supernatant	1.3	2.62	2.62
Day 1			
Cell	0.25		
Supernatant	2.84	3.1	3.1

Table 4.2 *The majority of LC sorted from an HSV1/34-infected EC population produced infectious virus as determined by infectious centre assay (Section 4.12.10) and plaque assay (Section 4.12.11).*

Infectious Centre Assay (ICA)			*Plaque Assay of ICA supernatant*
Number of infectious centres per plate:			
(500 sorted LC per plate)			
Plate 1	*Plate 2*	***Mean***	
447	492	*469.5*	*5.8 x 104 pfu/ml*

Because our EC population contains only 15-25% LC it was necessary to remove the contaminating keratinocytes etc. by cell sorting before any conclusions could be drawn about the production of infectious virus. HSV1/34 infected LC, labelled and purified on the basis of MHC Class II expression, were tested in an infectious centre assay and the resulting supernatants were tested by plaque assay. The majority of LC were shown to produce virus which could infect surrounding BHK cells (Table 4.2).

Any virus produced by LC and EC, although present, may not necessarily impair the MLR. To test this HSV1/34 was titrated into an MLR using bulk spleen cells as stimulators and responder T cells, or the latter alone, in an attempt to mimic the system using infected EC. The addition of HSV1/ 34 to the spleen MLR appeared to mimic that seen with EC, in that free HSV1/34 reduced proliferation to below background levels (Figure 4.4). The effect seen with responders alone was similar, if less profound (Figure 4.4), and indicated that the virus was capable of inhibiting background proliferation of T cells.

HSV1/34 may be released from infected EC and purified LC but it was still possible that T cells may be resistant to infection. Ideally, spleen stimulator and responder T cells should be co-cultured in the presence of virus followed by double labelling for FACS analysis for a cell marker and virus antigen. However, because of the strain combination used in our system (Balb/c (H-2^d) and C57/Bl/6 (H-2^b)) it was impossible to differentiate between cells in the responding vs. those in the stimulator population in our co-cultures. The anti-MHC Class II MAb, B21-2, labels $Ia^{b,d}$, and the T cell cocktail of anti-CD3,CD4 and CD8 identifies both strain haplotypes. Therefore, any estimation of the number of stimulator/T cells expressing virus glycoproteins in this system was only possible if the cell were incubated separately. Using a time course equivalent to that of the MLR, Balb/c bulk spleen cell stimulators and nylon wool passed C57/Bl/6 T cells were incubated separately in the presence (or absence) of HSV1/34, harvested and labelled for MHC Class II or CD3,CD4,CD8 cocktail vs. virus glycoproteins (Figure 4.5).

Figure 4.4 *Free HSV1 titrated into the bulk spleen alloMLR (open squares) or T cell responders alone (closed circles) resulted in a virus dose dependent inhibition of proliferation. Control alloMLR (irregular hatched line) and control T cells (regular hatched line) alone were incubated in the absence of HSV1/34.*

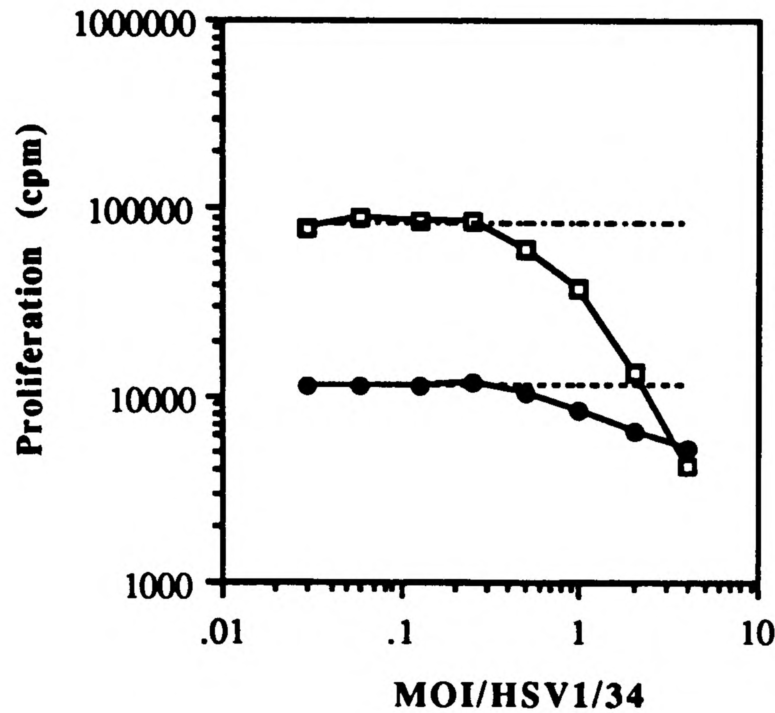
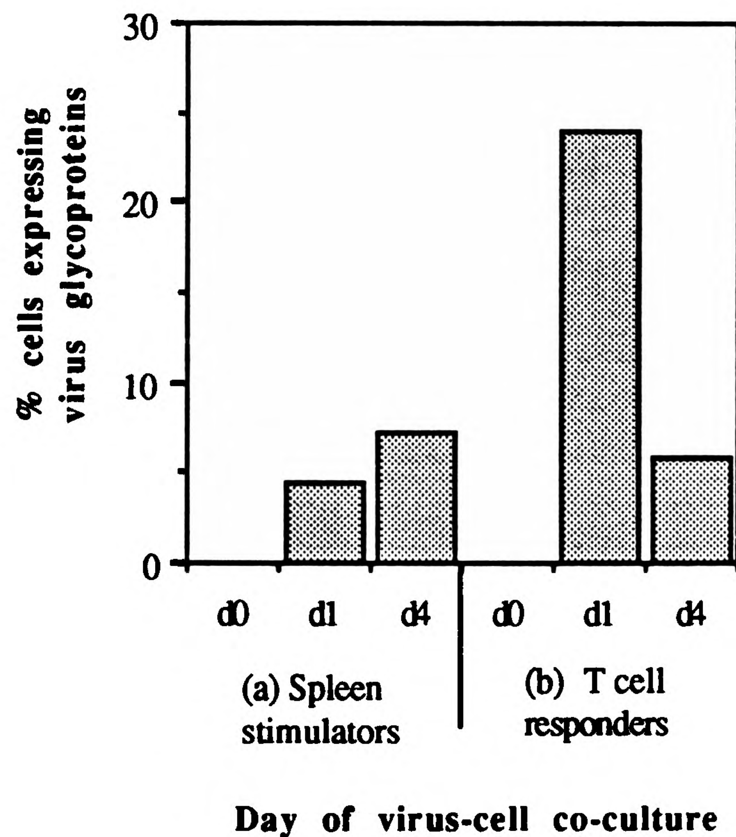


Figure 4.5 *Irradiated bulk spleen stimulators (a) and T cells responders (b) expressed HSV1 glycoproteins when incubated in the presence of HSV1/34 for 0-4 days.*



As shown by FACS analyses, T cells were, to some extent, susceptible to infection by HSV1. The low number of MHC Class II-positive cells in the stimulator population expressing virus glycoproteins may be a consequence of prior irradiation which may impair cell function and thereby hinder virus replication. Also, from evidence presented in Chapter 3, sDC, the critical immunostimulatory cells in a bulk spleen cell population, are relatively resistant to infection to HSV1 and we would therefore expect few to express glycoproteins.

In summary, the evidence presented suggests that the reduction in the MLR seen with infected EC may be a consequence of HSV1 transferred from LC or released infecting the responding T cell population. Therefore, if any further information about the functional impairment of infected LC was to be yielded from this system, the role of free virus infection of responding T cells needed to be ruled out as a mechanism of reducing proliferation in the MLR.

4.5 Heparin as an Inhibitor of HSV1 Infection

Heparin is known to reduce HSV1 infection of cell lines by preventing the attachment of the virus via gC and gB [1;13] and as such could act as an inhibitor of free HSV1 in our system. Porcine heparin (Sigma H5640) at different concentrations was initially tested for its ability to inhibit infection of (i) BHK cells (Figure 4.6) by two strains of HSV1 and (ii) EC/LC by HSV1/34, (Figure 4..7 (a) and (b)). It was shown to reduce virus glycoprotein expression in each population tested, especially over the concentration range used on BHK cells (Figure 4.6).

In control experiments, heparin, at the highest concentration of 10mg/ml, did not reduce the viability of BHK cells, as determined by trypan blue exclusion (data not shown).

***Figure 4.6** Heparin reduced the infection of BHK cells by HSV1/34 and HSV1 KOS. (see Section 4.11.6 for method).*

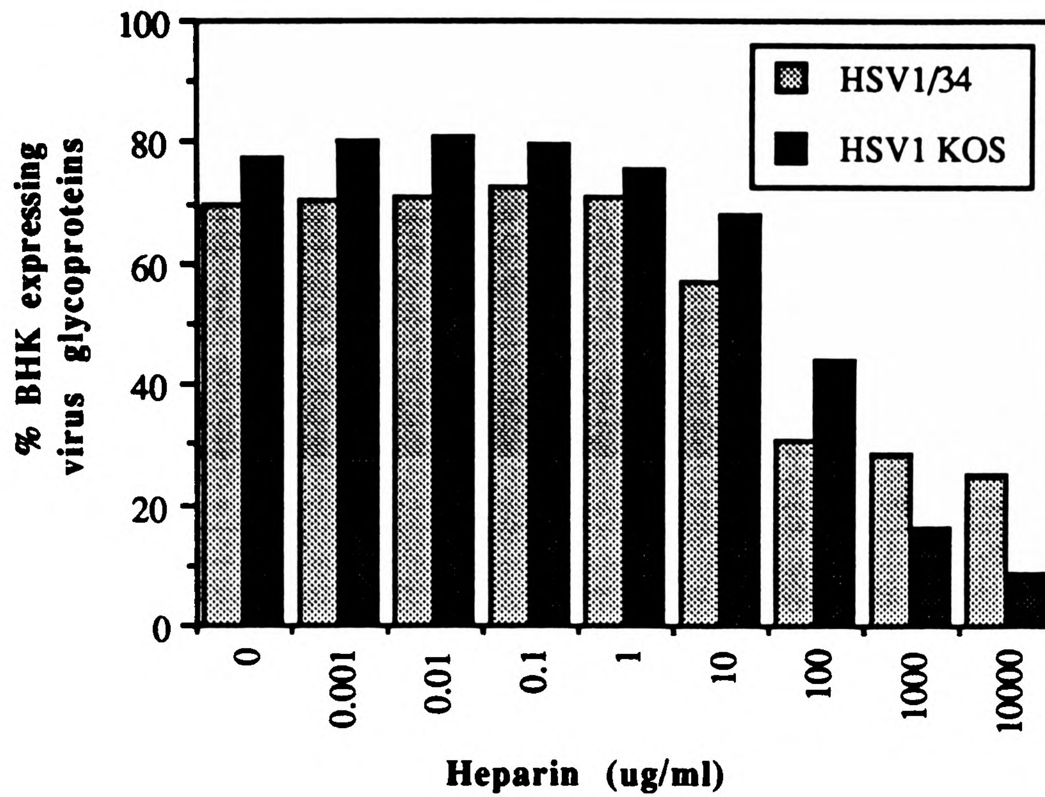
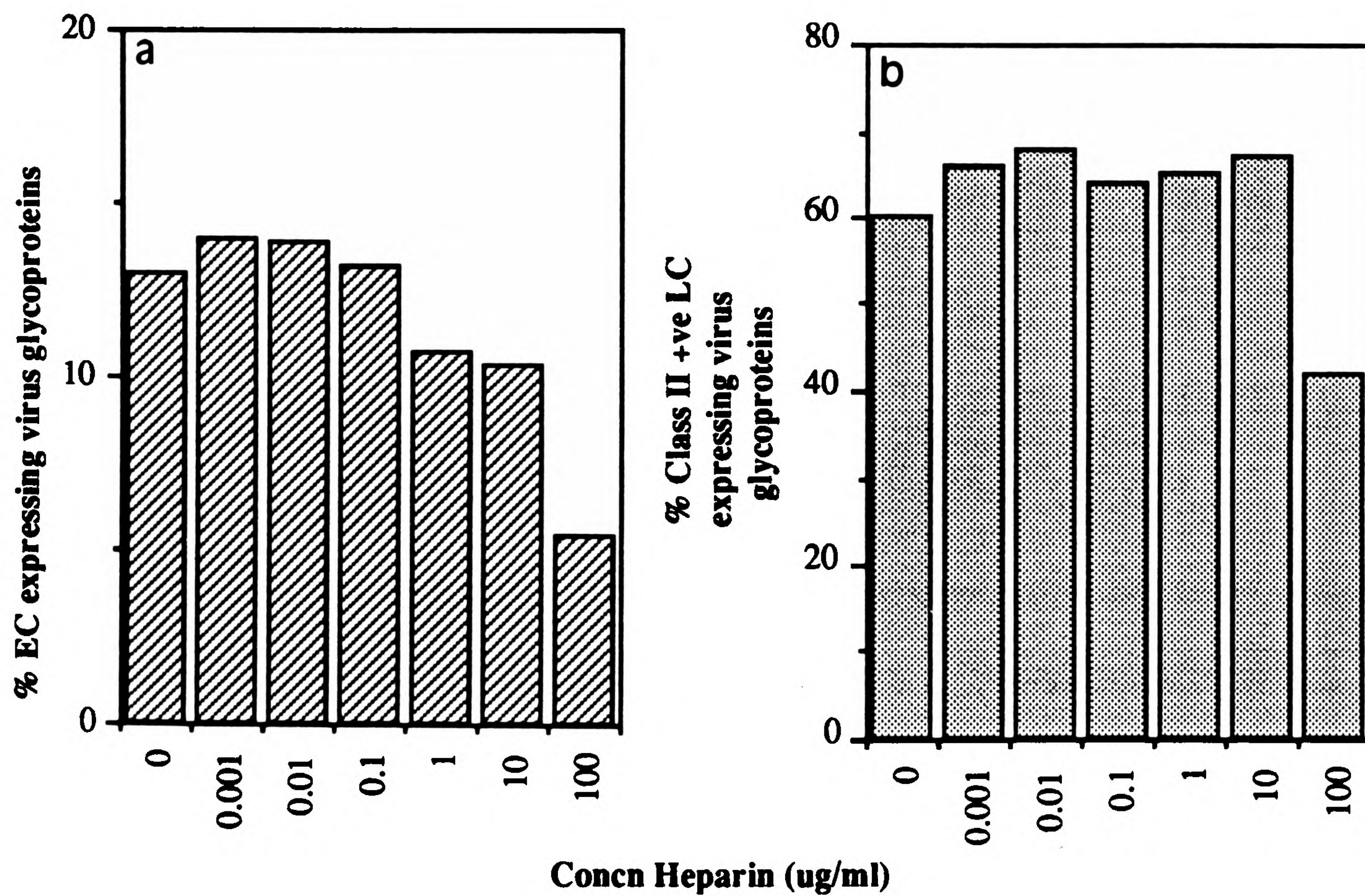


***Figure 4.7** Heparin reduced the infection of EC (a) and LC (b) by HSV1/34. (see Section 4.11.6 for method).*



4.6 Heparin as an Inhibitor of Vaccinia Virus Infection

A series of experiments was set up to show that heparin in our system was acting in a manner specific to HSV1. Vaccinia enters host cells by fusion, initially interacting with the epidermal growth factor receptor [307]. Heparin, which binds to HSV1 and blocks its interaction with cell surface heparan sulphate proteoglycans, should therefore have no effect on vaccinia infection of cells. BHK cells were infected with vaccinia virus and expression of virus antigen was detected using flow cytometry. Specific labelling of virus infected cells was possible at all concentrations of primary and secondary antibody tested but this was associated with a high background (data not shown).

Numerous methods were unsuccessfully applied to reduce this background such as end-point titration of primary antibody. Pre-treatment of vaccinia-infected and control cells with 5% normal rat or normal mouse sera prior to incubation with primary antibody was used to reduce this background staining (Figure 4.8). Normal mouse serum had little effect (b. NMS) relative to control cells (c. Control) which were not blocked. Normal rat serum only had a limited effect at a concentration of primary antibody at 1:20 (a. NRS).

We therefore assessed heparin inhibition of vaccinia infection of BHK cells, using the 5% normal rat serum preblocking step, which, as shown above (Figure 4.8) was at least partially effective.

The effect of heparin over and above background was assessed by comparing the overlapping FACS profiles (Figure 4.9 (c) and (d)). No reduction in the percentage of BHK cells expressing vaccinia antigens was seen with increasing concentration of heparin (b) and (d), compared with a >50% reduction in the expression of HSV1 glycoproteins, at a top dose of 10mg/ml heparin (a) and (c).

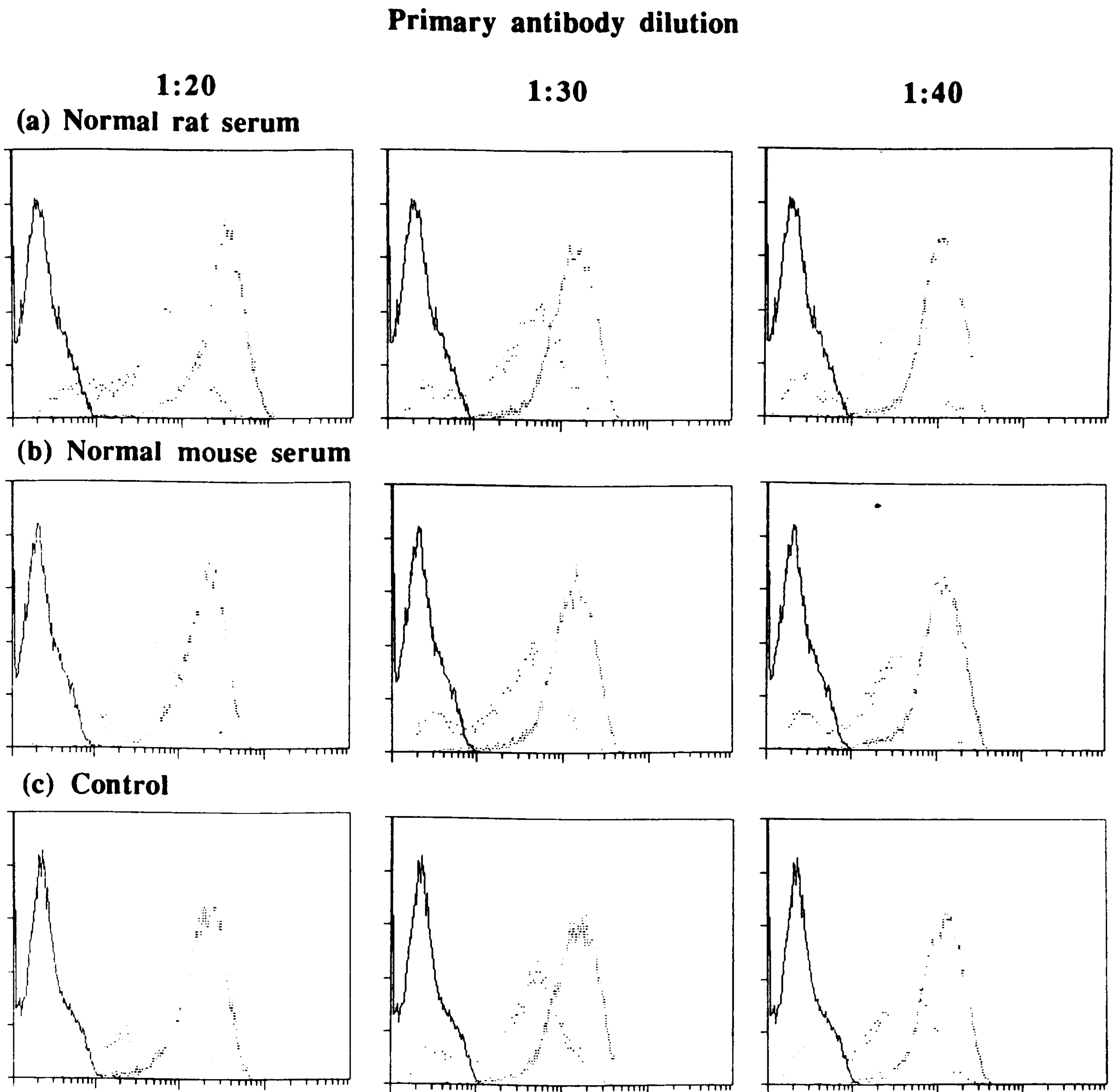
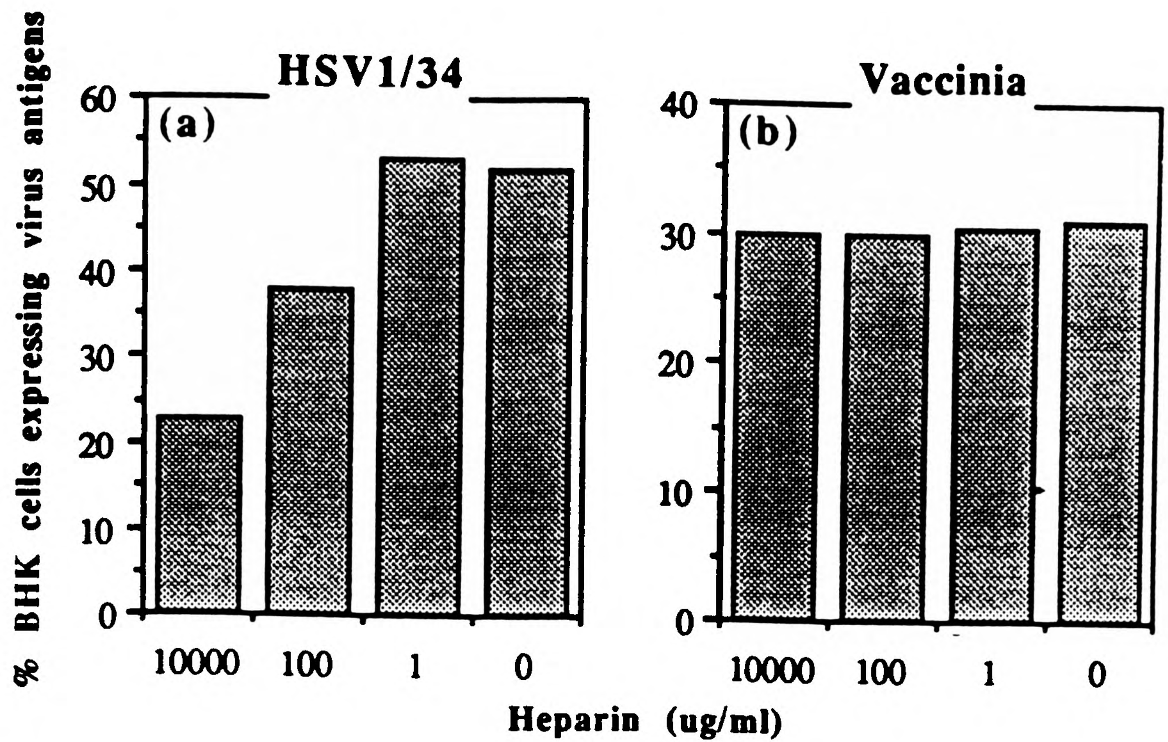


Figure 4.8 *5% normal rat serum (a) was only marginally effective at reducing the high background (· · · · ·) associated with three concentrations of the anti-vaccinia primary antibody (1:20; 1:30; 1:40). Normal mouse serum (b) was ineffective relative to the non-blocked, negative control (c).*

KEY: uninfected cells plus secondary antibody alone (———)
uninfected cells plus primary and secondary antibodies (· · · · ·)
infected cells plus primary and secondary antibodies (·······).



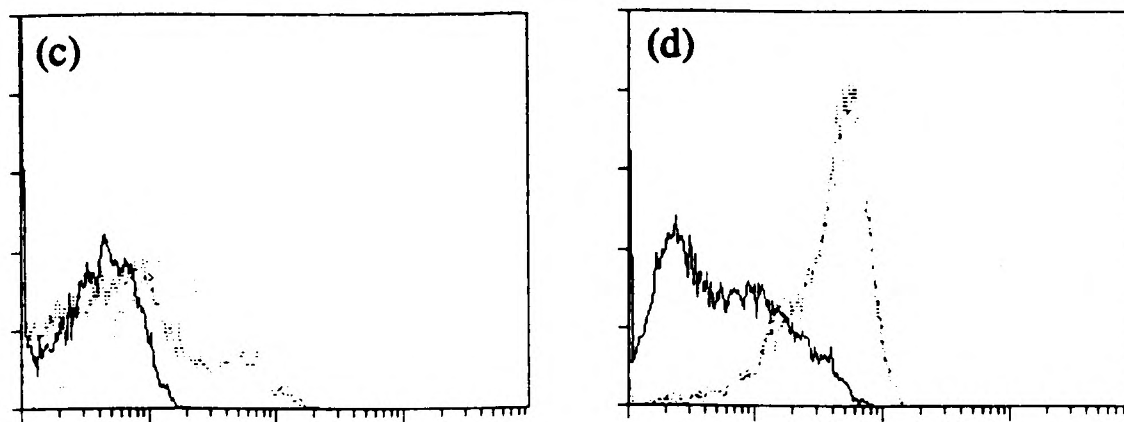


Figure 4.9 *Heparin reduced the expression of HSV1 antigens (a) and (c), but not vaccinia antigens (b) and (d), on virally infected BHK cells. (see Section 4.11.6 for method). Briefly, BHK cells were infected with HSV1 or vaccinia virus in the presence or absence of heparin. After 16 h, the cells were harvested and analysed using the appropriate primary antibodies by single-colour flow cytometry. (a) and (b) illustrate the expression of virus antigens over the range of heparin concentrations used. (c) and (d) display the overlapping FACS profiles for negative control (———), virus alone (· · · · ·) and virus plus 10mg/ml heparin (·······).*

4.7 Heparin Inhibition of Endocytosis

The possibility still remained that heparin was acting not only on the HSV1-host cell interaction by receptor-ligand interference but also by interfering with the normal functioning of the cell, thereby affecting virus uptake. The possibility that HSV1 enters the host cell by endocytosis cannot be entirely excluded as a mechanism leading to productive infection (Chapter 1).

The ability of heparin to inhibit endocytosis by BHK cells was therefore tested. Fluorescent 0.1 µm latex beads were used as a marker for endocytosis for two reasons. Firstly, they were of a size in the same order of magnitude as an HSV1 virion and as such would more closely mimic the uptake of HSV1. Secondly, cells which had bound and/or endocytosed these beads were readily identifiable, fluorescing in the FITC channel of the FACScan (Becton Dickinson, U.K.). The optimal dilution of latex beads was determined by incubating BHK cells at 37°C in the presence of increasing dilutions of latex beads. Figure 4.10 illustrates the fluorescence profiles of three different concentrations of latex beads, relative to the negative control. All concentrations below 1:200 overlapped to a greater or lesser extent with the negative control (b) and (c). The range chosen for subsequent experiments was 1:25-1:100 (a), specifically 1:50.

The effect of heparin was tested by incubating BHK cells with a 1:50 dilution of latex beads in the presence of increasing concentrations of heparin. This was carried out at 4°C and 37°C to measure binding and endocytosis respectively. Figure 4.11 is based on the measurements of mean channel fluorescence for each sample and is a measure of the proportion of latex beads associated with BHK cells.

Heparin should not affect latex bead binding unless they associate via a heparan sulfate moiety, but it does appear to impair the cell at the level of endocytosis. In conclusion, heparin acts to specifically inhibit HSV1, and not vaccinia, infection of cells mainly, it is assumed, in a manner analogous to that proposed by [1]. However, it may also act by reducing the cell's ability to endocytose, a mechanism of entry potentially available to HSV1.

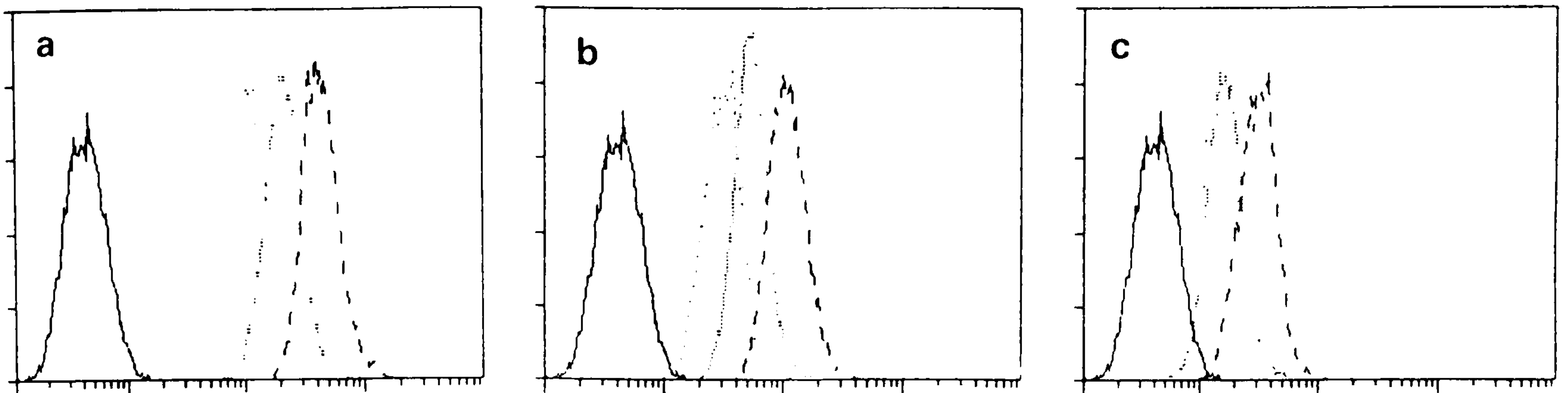


*Figure 4.10 Association of BHK cells with 0.1μm latex beads at 37°C. Latex beads at :
(a) 1:25 (- - - -), 1:50 (·······) and 1:100 (· · · ·);(b) 1:100 (- - - -), 1:200 (·······) and 1:400 (· · · ·)
(c) 1:400(- - - -), 1:800 (·······) and 1:1600 (· · · ·). Negative control - no beads (——).*

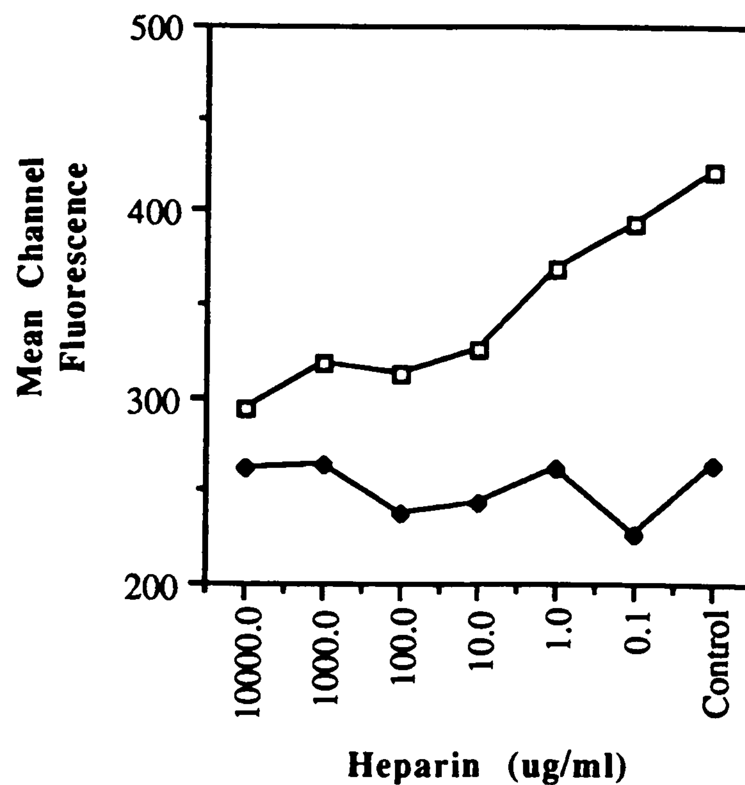


Figure 4.11 Increasing concentrations of heparin progressively reduced endocytosis at 37°C (open squares), but not binding at 4°C (diamonds), of 0.1μm latex beads.

4.8 Heparin Inhibition of Free HSV1 in the MLR

Heparin can inhibit HSV1 infection of cells. It was therefore tested as an inhibitor of free virus in the context of the MLR. Bulk Balb/c spleen cells were used to stimulate nylon wool passed C57/Bl/6 T cells in the presence or absence of HSV1/34. Varying doses of heparin were added to each well to assess its inhibitory potential for HSV1. Heparin at 1-1,000ug/ml was not inhibitory for the control MLR (Figure 4.12 (a)). Heparin at 1,000μg/ml partially restored proliferation in the virus treated MLR (b) but its effect was not complete. A tenfold increase in the top concentration of heparin to 10,000 ug/ml was found to be toxic for the control MLR (a).

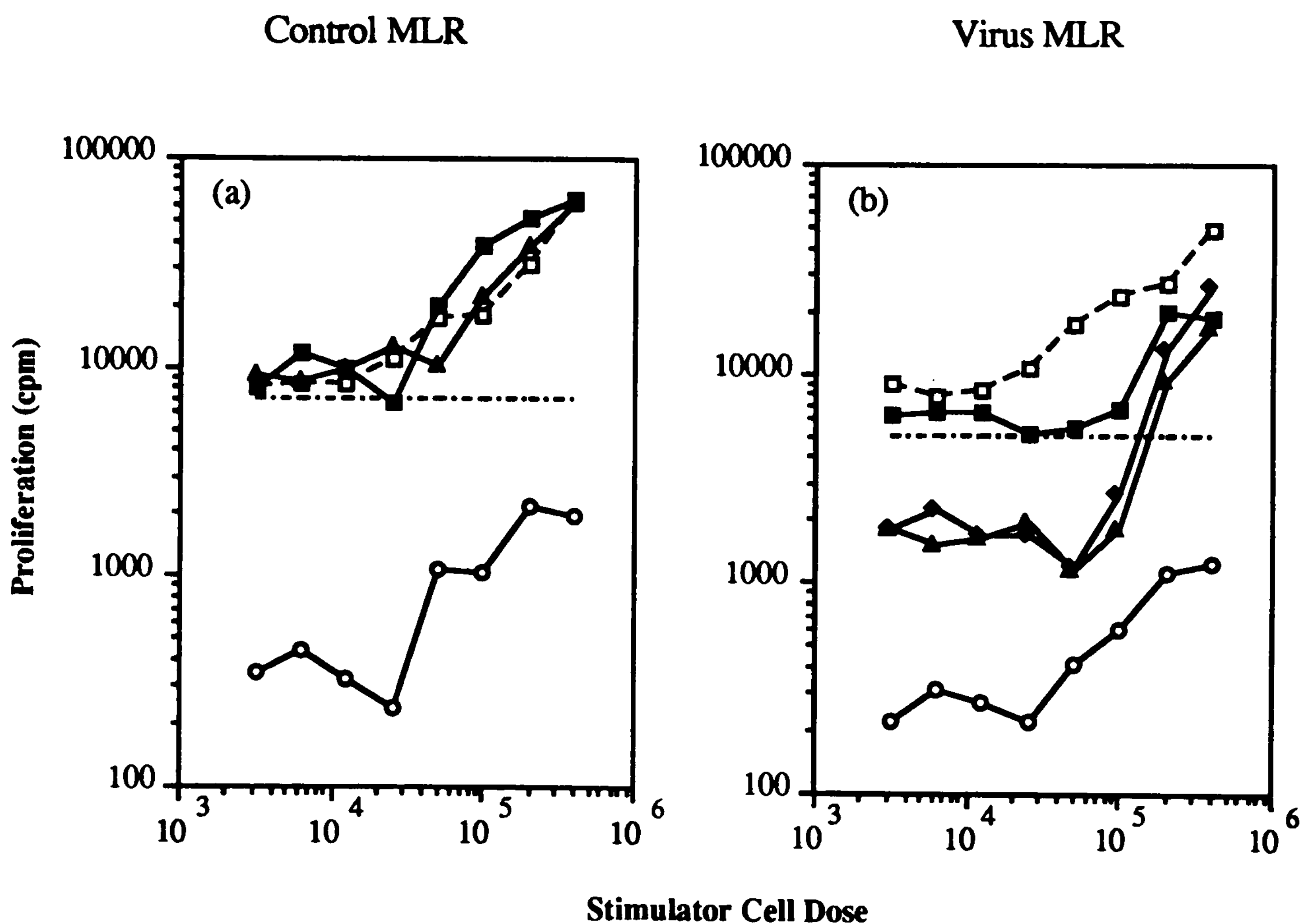


Figure 4.12 Heparin inhibition of free virus in the MLR. See text for details.
KEY: MLR: untreated (open squares) plus exogenous HSV1/34 (diamonds); 10,000ug/ml heparin (open circles); 1,000ug/ml heparin (closed squares); 1ug/ml heparin (triangles). Responders alone (hatched line)

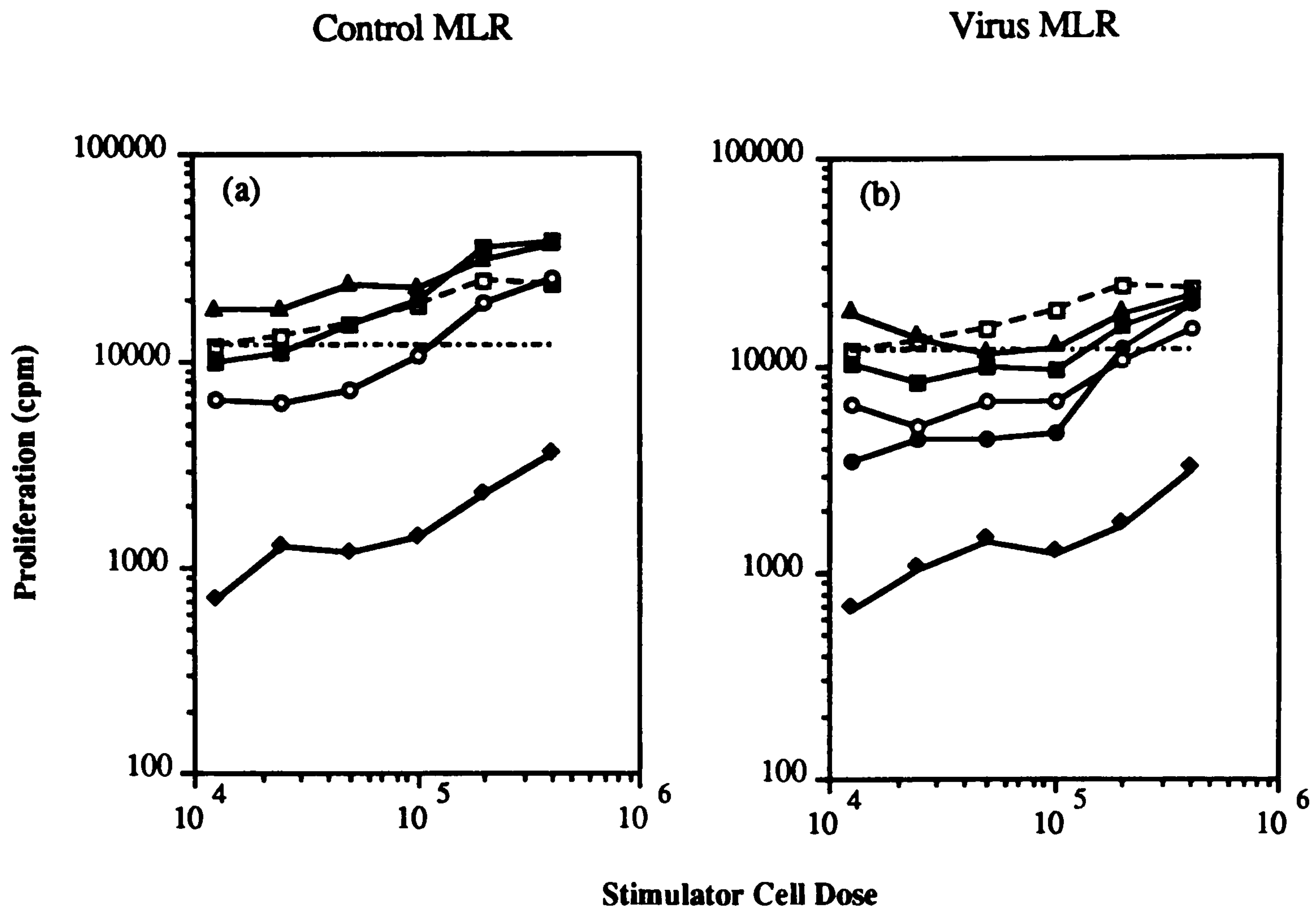


Figure 4.13 Increasing the concentration of heparin has only a limited effect on the virus-induced depression of the MLR. See text for details.
KEY: MLR: untreated (open squares) plus exogenous HSV1/34 (closed circles); 10,000ug/ml heparin (diamonds); 6,000ug/ml (open circles); 4,000ug/ml (closed squares); 1,000ug/ml heparin (triangles); Responders alone (hatched line).

Heparin was then employed at concentrations between 1,000-10,000 ug/ml to determine a dose which was fully inhibitory for virus in the MLR (>1,000 ug/ml) but not toxic for the control MLR (<10,000 ug/ml). Figure 4.13 (a) and (b) show the results obtained (not all concentrations shown). In the control MLR, concentrations >6,000 ug/ml were wholly or partially toxic whereas 4,000 ug/ml or below appeared to have little or no effect (a). Heparin at 1,000-4,000 ug/ml in the virus MLR reversed the effects of exogenously added HSV1, but this was incomplete (b). In conclusion, while being able to show that heparin reduced the effects of exogenously added HSV1 in the MLR we found that none of the concentrations tested were fully inhibitory for virus. It was therefore not considered further.

4.9 HSV1-Specific Antibody as an Inhibitor of Free HSV1 in the MLR

A rabbit polyclonal antibody to HSV1, B114, reported to be neutralising for HSV1, was tested. At a dilution of 1:10, B114 was as effective as heparin, at 10mg/ml, in reducing HSV1/34 infection of BHK cells (Figure 4.14).

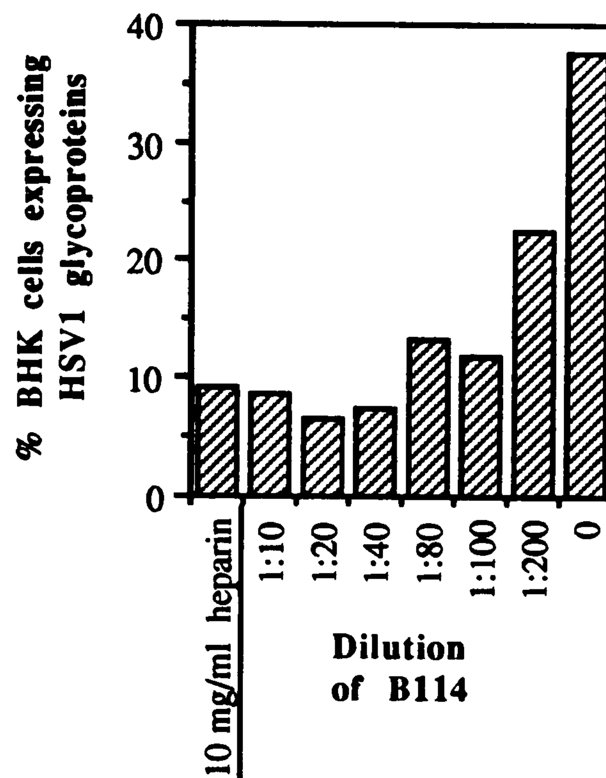


Figure 4.14 B114, a neutralising antibody for HSV1, will inhibit HSV1/34 infection of BHK cells. (see Section 4.11.7).

In the MLR, despite the fact that proliferation values were lower in this experiment than might be expected, B114 was found to completely neutralise the effects of exogenously added free virus at a concentration of 1:30, in the absence of any concomitant toxicity (Figure 4.15). Other experiments in the next section demonstrate that B114 was not toxic in the control MLR or oxidative mitogenesis. It therefore provided us with an ideal means of assessing the effects of HSV1/34 infected EC in the MLR without the associated impairment of responding T cells by infectious virus. B114 was utilised in a further series of studies which compared the effects of infectious HSV1/34 with replication defective mutants of HSV1 on LC in the MLR and oxidative mitogenesis.

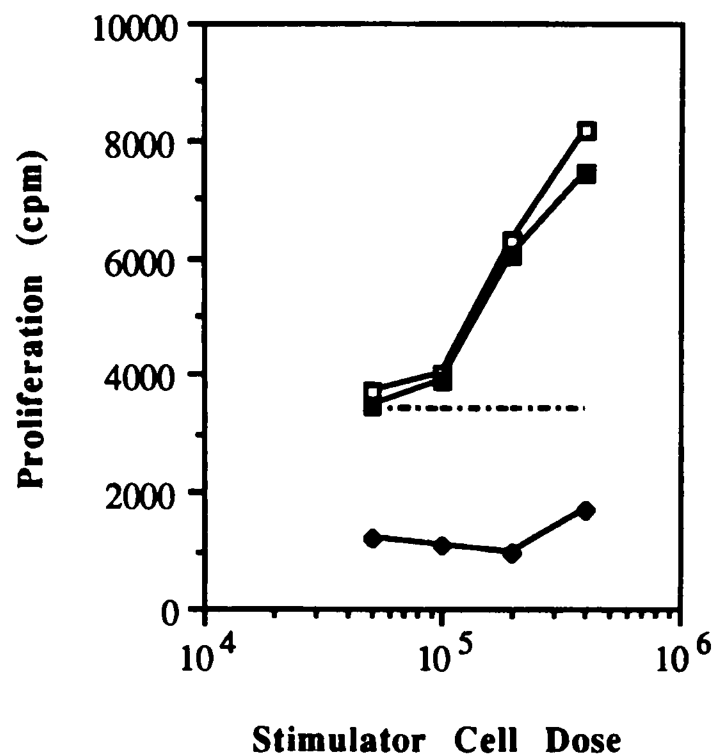


Figure 4.15 B114 completely reversed the effects of exogenously added free virus in the MLR (closed squares). Control MLR (bulk spleen stimulators) + B114 (open squares). Virus depression of the MLR (diamonds) (see Section 4.12.7).

4.10 A Mutant of HSV1 in the MLR and Oxidative Mitogenesis

Mutants of HSV1 are available which express all but one of the virus gene products. Some of these mutations have little or no effect on the normal replication of virus and the release of infectious progeny. It is also possible to generate mutants which cannot egress from the infected cell, or egress in a non-infectious form, or replicate and produce infectious progeny at a vastly reduced level. It was decided to test one such mutant of HSV1 in an effort to circumvent the need to inhibit virus production by infected EC. A mutant for gH (kindly supplied by Prof.A.Minson, Univ. of Cambridge) was employed. This was used in parallel with HSV1/34 infected EC, where the effects of free virus were inhibited by a neutralising concentration of B114.

The following studies were based around two methods of detecting the induction of proliferation. Firstly, the MLR utilising the Balb/c (H-2^d) vs. C57/Bl/6 (H-2^b), as before, and the Balb/c (H-2^d) vs. C3H (H-2^k) systems. Secondly, oxidative mitogenesis, which employs T

cells pre-treated with sodium periodate as responders. These are known to proliferate polyclonally in the presence of DC [308]. Oxidative mitogenesis provides a more rapid means of assessing immunostimulatory function, with an incubation time less than half that of the alloMLR.

Briefly, in several experiments, Balb/c (H-2^{d}) LC were prepared and infected with HSV1/34, and HSV1gH (gH-ve mutant) in parallel. They were then titrated (in duplicate, due to the limited numbers of stimulator cells available after purification) into C57/Bl/6 (H-2^{b}) or C3H (H-2^{k}) T cells (MLR) or periodate treated Balb/c T cells (oxidative mitogenesis). Figure 4.16 details the proliferation curves from representative experiments.

Infection of EC with HSV1/34 resulted in a profound loss of immunostimulatory function in oxidative mitogenesis (a). This loss was more evident in the MLR (b), probably as a consequence of the longer incubation period of this assay. HSV1gH infection of EC had a very similar effect to HSV1/34 (c) and (d).

From these results (reproduced in two further experiments) it is possible to suggest that, since infection of responding T cells by virus is no longer an issue with the use of B114 and the gH mutant of HSV1, infection of LC results in a significant loss of immunostimulatory function, as measured by oxidative mitogenesis and the MLR.

Assuming there is no continued loss of LC during the course of these assays this could be considered a reasonable conclusion. However, as we have already shown (Chapter 3, section 3.2 and 3.3), infection with HSV1 resulted in the death of LC or down-regulation of MHC Class II. There may be no reason to assume that this did not continue in the MLR or oxidative mitogenesis, unless co-culturing with T cells maintained the viability of these cells. It was therefore necessary to demonstrate the continued presence of LC during the time of culture and correlate this with any decrease in proliferation.

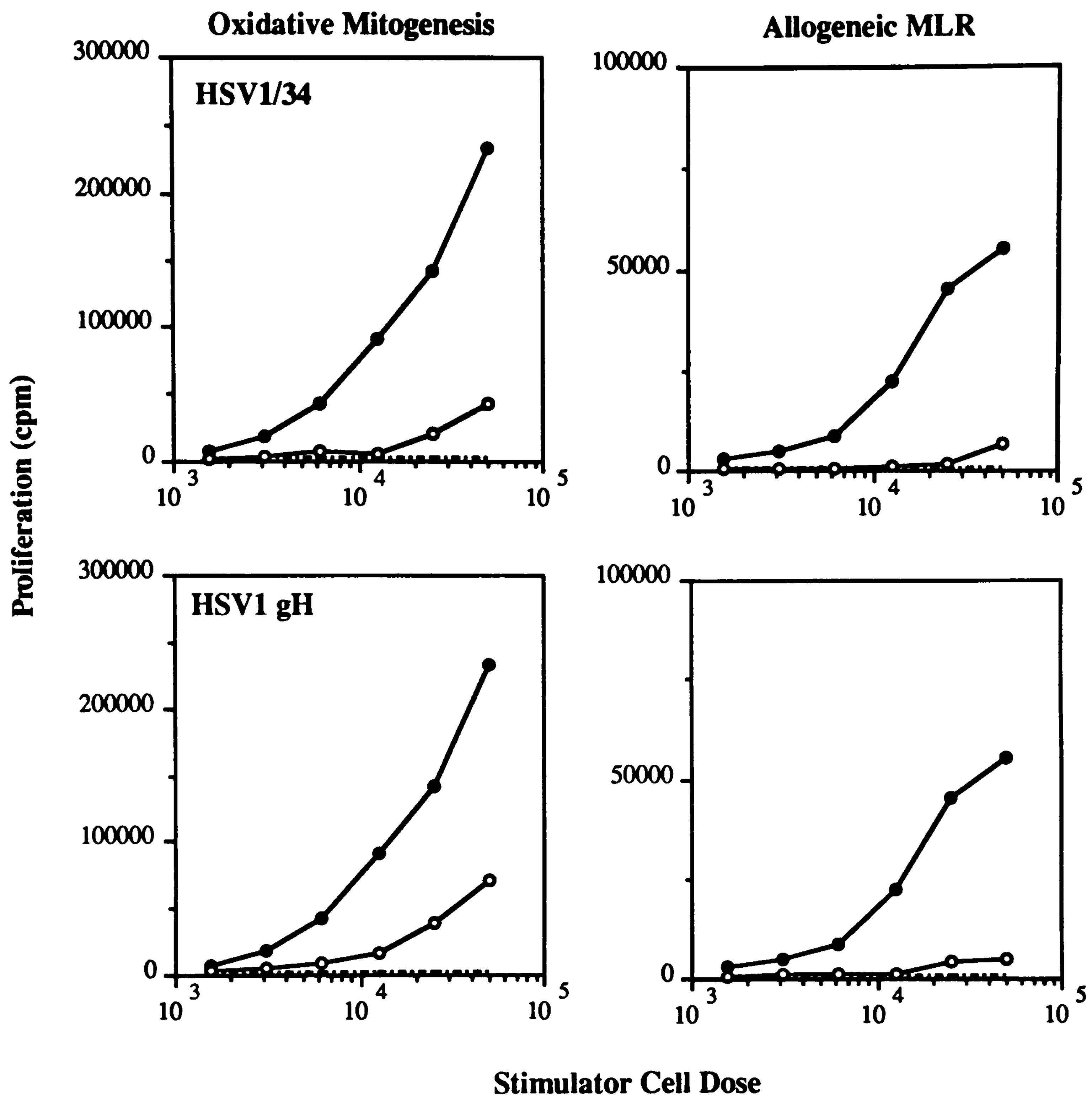


Figure 4.16 The capacity of graded doses of HSV1/34 and HSV1gH infected EC to stimulate the MLR and oxidative mitogenesis assays. Control (uninfected) EC (closed circles), infected EC (open circles), responders alone (hatched line). See text for details.

Given the right system, it could be possible to detect the loss of MHC Class II-positive LC in the co-culture. The anti-MHC Class II MAb, B21-2, identifies $Ia^{b,d}$, rendering an estimation of the loss of LC impossible in the MLR (Balb/c ($H\text{-}2^d$) vs. C57/Bl/6 ($H\text{-}2^b$) or oxidative mitogenesis (Balb/c vs. Balb/c) systems. This is because any contaminating MHC Class II-positive cells from the responding population not removed by nylon wool purification would

also be identified. Therefore C3H (H-2^k) T cells were employed in the MLR so that any remaining MHC Class II-positive cells in the responder population would not be detected by B21-2. EC were infected with HSV1/34 or HSV1gH, and co-cultured with C3H T cells for 96 h in an MLR. At 1 h (d0) and 96 h (d4) samples of cells were removed for analysis by flow cytometry. Proliferation was also measured at d3-4. The results in Table 4.3 show the combined FACS analysis values and proliferation values obtained.

Table 4.3 *The percentage of MHC Class II-positive LC in the virally infected populations do not correspond to the percentage levels of proliferation observed on d4 of the MLR.*

	Number and MHC Class II intensity of LC in co-culture					
Co-culture	Control		HSV1 gH		HSV1/34	
Day of co-culture	LC	MCF	LC	MCF	LC	MCF
d0	2	1281	0.8	933	0.9	1090
d4	1.3	542	0.6	520	0.6	780
	Proliferation (cpm x 10^{-3})					
d3-4	128		29		16	
	HSV1gH and HSV1/34 LC number and cpm as % of Control					
LC number	***100***		***46***		***46***	
cpm	***100***		***23***		***12.5***	

Incubation in the MLR resulted in a loss of MHC Class II-positive cells even in the control, uninfected samples (2-1.3%; MCF 1281-542). This may represent the generation of MHC Class II-specific CTL which subsequently kill LC. The number of LC on d0 and d4 was always lower in the cultures to which HSV1-infected EC were added. Control values for proliferation and MHC Class II-positive LC were taken as 100% and values for the HSV1-

infected EC co-cultures were calculated relative to this. Assuming that the percentage of MHC Class II-positive cells remaining at d4 was an approximate measure of the number of LC it appeared that the level of proliferation did not correlate with the remaining levels of LC in the co-culture (Table 4.3). For example, for HSV1gH infected EC proliferation = 23% while the % LC = 46%. This would indicate a loss of function, as well as a loss in number of the MHC Class II-positive population. This assumes that the MHC Class II-positive cells remaining are all leucocytes, namely LC, from the EC population.

This may not be the case. HSV1 infection of cells can result in an up regulation of MHC Class II on non-leucocytes, for example, keratinocytes [138]. There is also the possibility that γIFN produced by the responding T cell population in the co-culture would augment this phenomenon. It is conceivable that the numbers of MHC Class II-positive cells (see Table 4.3) may also represent the de novo expression on keratinocytes in the input population. These are non-stimulatory and do not accurately represent the levels of remaining LC, the stimulatory population. The above experiment was repeated but an estimation of the number of remaining MHC Class II-positive *leucocytes* was made by two-colour flow cytometry by staining for MHC Class II and CD45 (LCA). Figure 4.17 and Table 4.4 illustrate the relationship between proliferation and the level of MHC Class II+ve leucocytes; a repeat of this experiment generated profiles (not shown) with patterns almost exactly corresponding to those seen in Figure 4.17.

On d0, there was a significant difference in the number of LC in control vs. virally infected populations (cf. a,b and c) but virtually all of these cells expressed CD45 (cf. d,e and f). This emphasises that the MHC Class II-positive cells in the input population are all leucocytes, presumably LC. By d4, most of the LC in all the cultures had disappeared (cf. g,h and i). Of the remaining cells identified as MHC Class II-positive it is important to note that, in the control population, the majority of cells were still leucocytes (j). However, virus infection generated two distinct populations, as shown by the bimodality of the MHC Class II-positive cells on d4 (k and l). HSV1 infection resulted in the appearance of a significant population (39.9% for HSV1gH (k); 73.6% for HSV1/34 (l)) of MHC Class II+ve *non-leucocytes*. When the values

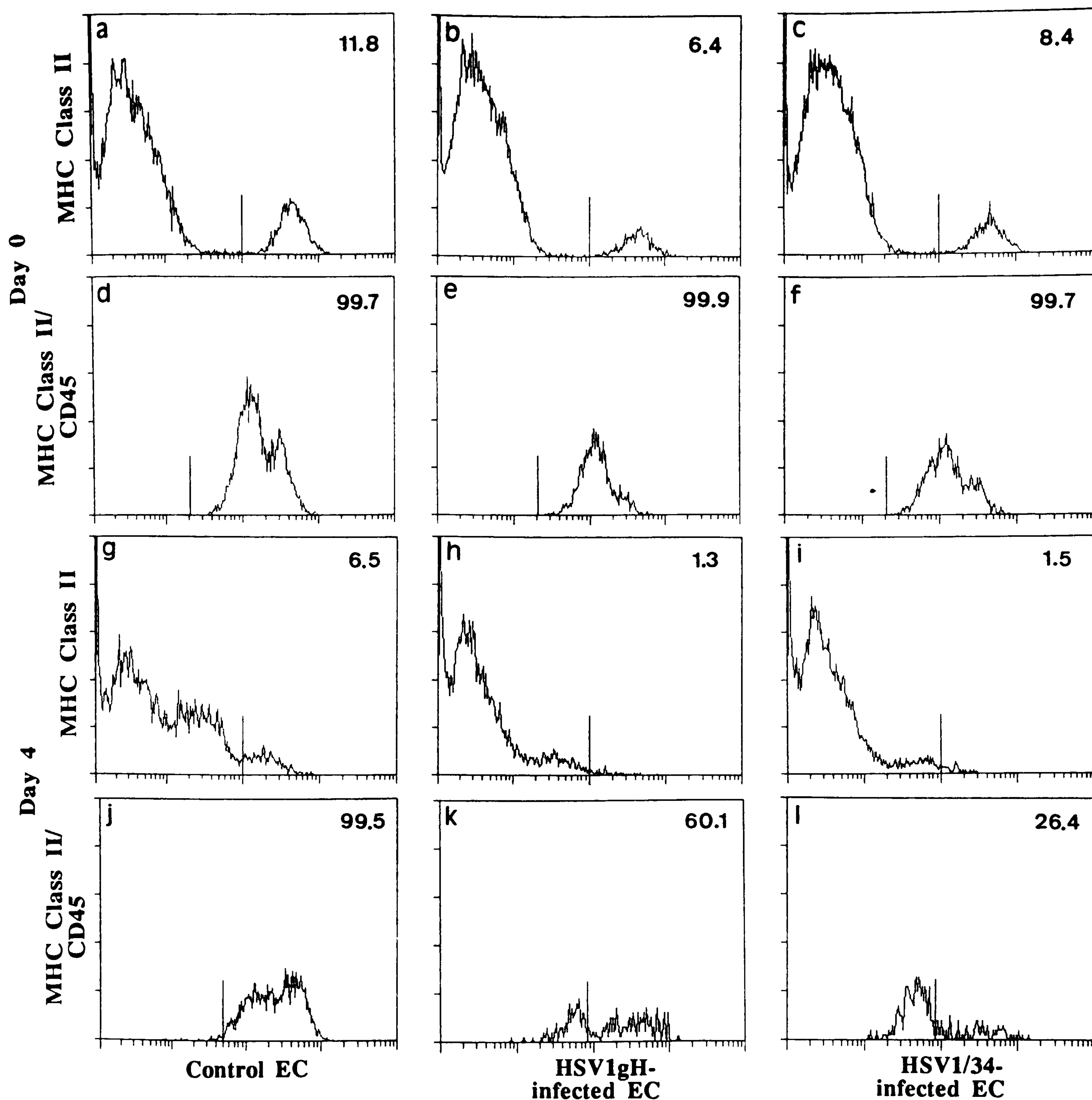


***Figure 4.17** HSV1gH and HSV1/34 infection of EC results in a profound loss in immunostimulatory function which is associated with a loss of MHC Class II-positive leucocytes, LC. The FACS profiles above illustrate that at the initiation of the MLR co-culture all MHC Class II-positive cells in the control (a) and virally infected (b,c) EC populations were leucocytes (d-f). By d4 the majority of the MHC Class-positive cells had gone (g-i) and while the remaining cells in the control population were all leucocytes (j) those in the HSV1-infected EC co-cultures contained a significant number of MHC Class II-positive non-leucocytes (k. 40%; l. 74%).*

were calculated and adjusted relative to the control values (as in Table 4.3) it appeared that there was a close correlation between the number of MHC Class II-positive leucocytes remaining and the level of proliferation (Table 4.4). This was most clearly seen in the results for HSV1gH infected LC, where the action of free virus on responding T cells can be ruled out. The level of proliferation (21%) corresponds closely to the number of remaining LC (16%). With HSV1/34, some free virus could have escaped neutralisation and infected the responding T cells giving the lower value for proliferation (2.5%) relative to remaining LC (23%).

Table 4.4 *The loss of proliferation observed with HSV1-infected EC as stimulators of the MLR corresponds closely to the percentage of remaining MHC Class II-positive leucocytes on d4. See Figure 4.17 and text for details.*

	d0 co-culture			d4 co-culture		
	Control	HSV1gH	HSV1/34	Control	HSV1gH	HSV1/34
% MHC Class II cells	11.8	6.4	8.4	6.5	1.3	1.5
% MHC Class II/CD45 cells	99.7	99.9	99.7	98.6	60.1	26.4
% Class II leucocytes				6.4 (100)	0.8 (12)	0.4(0.06)
Proliferation (cpm x 10-3)				76 (100)	12 (16)	2 (3)

In conclusion, infection of LC by HSV1/34 and HSV1gH resulted in a significant decrease in their ability to stimulate T cell proliferation in the MLR and oxidative mitogenesis. This decrease appeared to be associated with the continued loss of MHC Class II-positive leucocytes, namely LC, and not a functional defect induced by HSV1 infection of LC. Therefore, there is no evidence from this system that HSV1 induced a functional defect in LC in terms of their immunostimulatory capacity in vitro.

Splenic DC appear to be relatively resistant to infection by HSV1/34 (Chapter 3) Their function therefore should not be impaired. We tested this in the MLR and oxidative mitogenesis, using HSV1/34 and HSV1gH infected splenic DC as stimulators. The results obtained (Figure 4.18)

proved interesting. Infection with HSV1/34 did not impair the ability of DC to stimulate either proliferative assay. However, infection with HSV1gH had a profound effect. This may reflect a strain difference between HSV1/34 and HSV1gH (parental wild type HSV1 SC16) in terms of their ability to infect sDC. In control experiments, sDC were equally susceptible to four strains of HSV1 (HSV1/34, KOS, gH and its parent strain SC16) in terms of overall levels of virus glycoprotein expression post-infection (not shown). The ability of HSV1gH to affect sDC in the proliferative assays is therefore not reflected at the level of virus antigen expression post-infection. At the present time we cannot explain this curious phenomenon but the restrictions on time meant that we could not pursue this further.

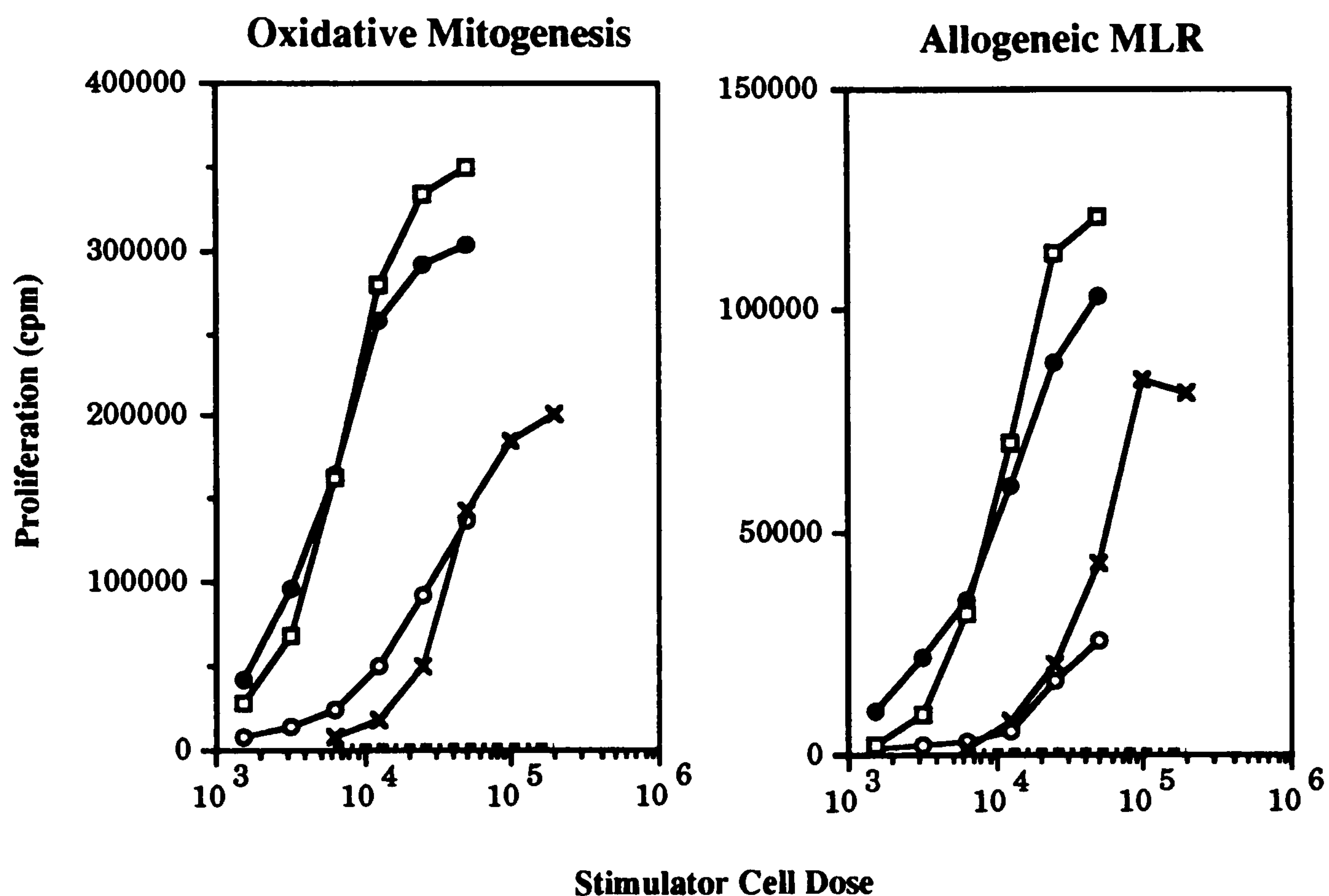


Figure 4.18 Infection of sDC with HSV1gH (open circles), but not HSV1/34 (open squares), reduced the immunostimulatory capacity of sDC (control sDC closed circles) in the MLR and oxidative mitogenesis. Control bulk spleen stimulators are also shown (crosses). See text for details..

4.11 Chapter Summary

We assessed the ability of HSV1 to functionally impair LC in the MLR and oxidative mitogenesis. Initial experiments were hindered by the release or transfer of virus by infected EC to the responding T cells. Heparin specifically inhibited HSV1 infection of cells by preventing virus attachment and possibly endocytosis. It partially reduced the effects of free virus in the spleen MLR. B114, an HSV1 specific neutralising antibody, was completely inhibitory for free virus. A combination of infectious virus and B114 with replication defective mutants of HSV1 showed that the reduction in proliferation in the MLR (and oxidative mitogenesis) was probably due to a loss of LC (MHC Class II-positive leucocytes) rather than an HSV1-induced loss of LC function.

4.12 Specialised Materials and Methods

4.12.1 Allogeneic Mixed Lymphocyte Reaction (MLR)

Graded doses of Balb/c (H-2d) stimulator cells - bulk spleen cells or uninfected/HSV1 BSA purified EC - were titrated into a constant dose of 2 x 10^5 nylon wool passed C57/Bl/6 (H-2b) or C3H (H-2k) spleen T cells in a 96 well flat bottom microtest plate. Plates were incubated for 72-96 h at 37°C/5%CO_2 in a humidified incubator. Each well was then pulsed with 50µl of {^{3}H} thymidine (^{3}H-TdR, Amersham UK, Cat.No. TRA310) 0.5-1µCi/50µl and incubated for a further 12-18 h at 37°C/5%CO_2 in a humidified incubator. Plates were harvested onto filter pads and ^{3}H-TdR incorporation was measured using a flat bed scintillation counter (1205 Betaplate™, Pharmacia-Wallac). Unless otherwise stated, results are presented as means of triplicate cultures and error bars have been omitted for the purposes of clarity (average SD=10-15% of mean value).

4.12.2 Periodate (NaIO4) treatment of T cells

Stock $NaIO_4$ solution was prepared by dissolving 50mg $NaIO_4$ in 1ml PBS and removing 100µl of this into 10ml PBS. This was filter sterilised and kept on ice. Nylon wool passed spleen T cells were washed twice in PBS and counted. The pellet of cells was resuspended to 2 x 10^7 cells/ml in PBS and an equal volume of stock $NaIO_4$ solution added - final concentration = 10^7 cells/ml. The cells were incubated on ice for 15 mins washed in R10 and counted for use as the responder population in oxidative mitogenesis.

4.12.3 Oxidative Mitogenesis

This assay was set up and analysed according to the protocol used for the MLR, except for a few essential differences. The responding population in oxidative mitogenesis was nylon wool passed, sodium periodate ($NaIO_4$) modified, spleen T cells which are syngeneic with the stimulator population, in this case, Balb/c (H-2d). Stimulator-responder cell co-cultures in oxidative mitogenesis were incubated for only 24-26 h prior to the addition of ^{3}H-TdR and then incubated for a further 12-18h at 37°C/5%CO_2. Apart from these noted differences, oxidative

mitogenesis assays and the MLR were carried out identically, using the same population of stimulator cells for both assays when set up in parallel.

4.12.4 Stimulator Cells for the MLR and Oxidative Mitogenesis

Irradiated bulk spleen cells were prepared as detailed in Chapter 2, section 2.6.3, and added in graded doses to the responding T cell population at a typical top dose of 5-10 x 10^5 cells/well.

EC were prepared as detailed in Chapter 2, section 2.6.1, and incubated on tissue culture dishes. After 24-72h the cell population was removed from all the dishes and the cells pooled from each time point. If no further manipulation was to occur, such as HSV1 infection, then each cell population was partially purified over a dense BSA gradient (Chapter 2, section 2.7.2). Alternatively, for HSV1 infection, for which non adherent 48 h EC were routinely used, the cells were counted and divided equally between individual conical tubes (polypropylene tubes were used to reduce adherence of cells during the incubation period for HSV1 infection) and spun down. Cell populations to be infected were resuspended in 100μl R10 plus the appropriate μl volume (typically 0.5-3μl) of the relevant HSV1 stock (Chapter 2, section 2.5.1); control cultures were resuspended in 100μl R10 alone and all populations were incubated for 1 h at 37°C/5%CO_2, during which time the tubes were regularly agitated. After 1 h, the cells were resuspended in 10ml R10 and immediately spun down. The resulting cell pellet was resuspended in a further 10ml of R10 and plated onto 100mm tissue culture dishes for an additional incubation period of 16-18 h at 37°C/5%CO_2. All cells were then removed from the dishes, similar populations pooled and individually purified over dense BSA gradients (Chapter 2, section 2.7.2). The low density pellicle cells were removed, counted, irradiated and titrated into the responder populations of the MLR and/or oxidative mitogenesis.

Low density, transiently adherent spleen cells were prepared according to the protocol previously discussed (Chapter 2. section 2.6.2) to the point where all non adherent cells were removed prior to overnight incubation of the adherent population. The non adherent cells were infected with HSV1 by the method previously detailed (Chapter 2, section 2.5.2 and Figure

2.3), in that the adherent population was exposed to HSV1 on the tissue culture dish, in this case at 10^7pfu/ml, in 2-3ml R10 for 1 h at 37°C/5%CO_2. 10^7pfu/ml HSV1 was chosen as the virus inoculum because when the effects of HSV1 infection on sDC were assessed by MLR and oxidative mitogenesis (Chapter 4, section 4.10.1) this was done in parallel with infected EC (protocol detailed above) which were exposed to 10^6 pfu in 100μl containing 10^7 48h EC, equivalent to 10^7pfu/ml. Both populations were therefore exposed to similar concentrations of virus. The virus inoculum was then removed together with any cells which had become non adherent and centrifuged to sediment the cells. Any resulting cell pellet was resuspended and returned to the appropriate tissue culture dishes and incubated overnight to allow the sDC to become non adherent. The non adherent population was then removed and depleted of FcR+ve cells using the antibody coated sheep erythrocyte method detailed in Chapter 2, section 2.7.1, to provide purified populations of sDC. All stimulator populations used were irradiated for 4.4 mins at 2000 rads from a ^{137}Cs source (Gammacell 1000) prior to use in functional assays.

4.12.5 Addition of free HSV1 to the MLR

HSV1/34 was added, unless otherwise stated, at a concentration of 6 x 10^5 pfu/well which is equivalent to 3 x 10^6 pfu/ml or an MOI=1 at a top stimulator cell dose of 4 x 10^5 bulk spleen cells and 2 x 10^5 responder cells. This is a concentration of virus known to reduce proliferation in the MLR and of responders alone (see Chapter 4, Figure 4.4).

4.12.6 Heparin inhibition of Virus infection

Porcine heparin was prepared and used from stock solution described in Chapter 2, section 2.2.7. Dilutions were prepared in R10 or αMEM+ and used to resuspend cell pellets or treat cell monolayers. A known dilution of HSV1 or Vaccinia virus was immediately added and the cells cultured for 1 h at 37°C/5%CO_2. Cells were then washed to remove excess heparin and unbound virus and then re-incubated for a further 16-18 h at 37°C/5%CO_2. Cell suspensions were stained with the appropriate anti-virus polyclonal antibody/FITC combination, and heparin

inhibition of infection was assessed by the percentage decrease in expression of virus antigens on heparin treated cells compared to untreated cells, as defined by flow cytometry. In the case of HSV1, it was obvious by eye that heparin clearly reduced the number of plaques formed on a BHK cell monolayer when compared with untreated cells (not shown).

4.12.7 Inhibition of free HSV1 in the MLR

Porcine heparin was prepared and used from stock solution described in Chapter 2, section 2.2.7. Dilutions were prepared in R10 and added to the appropriate wells of the MLR to give final concentrations of between 1-10,000μg/ml.

B114, the anti-HSV1 PAb (Dako, Cat.No. B114), was extensively dialysed against three changes of PBS to remove all traces of 15mM NaN_3, the buffer it is supplied in. NaN_3 would potentially have an inhibitory effect on the MLR and oxidative mitogenesis. Dialysed B114 was tested prior to use in a direct comparison with undialysed B114 to ensure its continued efficacy despite dialysis. The optical density (OD280nm) did not significantly change with dialysis indicating that the protein concentration was not altered (not shown). Neither was there any loss of binding as determined by staining of HSV1/34 infected BHK cells for UV microscopy and single colour flow cytometry (not shown). The final concentration used in the MLR and oxidative mitogenesis was 1:30.

Both inhibitors were added to free virus or infected cells, where appropriate, for 30 mins to 1 h to ensure the maximum amount of free or released virus was neutralised prior to the addition of responder cells.

4.12.8 Heparin Inhibition of Endocytosis

Fluorescent latex beads (Fluoresbrite™ fluorescent latex microspheres, Polysciences Inc., Cat. No.16662) with a diameter of 0.1μm were used to assess the ability of heparin to reduce endocytosis. Optimal dilution was assessed when BHK cells were resuspended in 100μl αMEM+ supplemented with increasing concentrations of latex beads, doubling from 1:25 to

1:1600. After 1 h the cells were washed of excess beads which did not sediment with the spun cells and the cell populations immediately fixed for analysis by single-colour flow cytometry. Heparin inhibition was assessed by repeating the above protocol using an optimal 1:50 dilution of latex beads on cells which were exposed to increasing tenfold dilutions of heparin, at 4°C (the addition of NaN_3 at 4°C had no effect on binding - data not shown) to measure binding, and 37°C to measure endocytosis. After 1 h the cells were washed of excess beads and the effect of heparin on binding and endocytosis was assessed. A comparison was made between groups (4°C vs. 37°C and heparin treated cells vs. untreated cells) of the mean channel fluorescence (MCF), as a measure of latex bead association.

4.12.9 Cell Sorting

HSV1/34 infected EC were prepared according to the method described in Chapter 2, section 2.6.1 and Chapter 4, section 4.11.3. The low density dense BSA fraction was removed and under sterile conditions, in the presence of NaN_3 to prevent capping and internalisation of antibody-antigen complexes, resuspended in 200µl sterile filtered B21-2 supernatant for 1 h on ice. Unbound primary was removed by washing and the cell pellet resuspended in 200µl of a 1:40 dilution of rabbit anti-rat FITC conjugate and incubated for a further hour. Cells were then washed and the FITC signal amplified using a 1:40 dilution of donkey anti-rabbit FITC. After washing the cells were pelleted and kept on ice until sorted on the basis of FL1 (FITC) fluorescence. The population contained approximately 15% LC on this basis and were sorted from the unlabelled cells using an Ortho Diagnostics Systems 50L Cytofluorograf interfaced with an Ortho 2150 computer which was kept at 4°C and operated by Mr. Z. Kruger-Gray (Division of Immunology, New Site, Addenbrookes Hospital, Cambridge).

4.12.10 Infectious Centre Assay

The population of cells resulting from cell sorting was pelleted in a 15ml conical tube. The cells were counted and 5 x 10^2 cells were added to each of two 60mm tissue culture dishes along with 2 x 10^6 BHK cells and mixed well by the addition of 3ml R10. Duplicate control plates received 2 x 10^6 BHK cells only. This was the only instance where BHK cells were not

incubated in αMEM+ but since the optimal survival of sorted LC was paramount R10 was used and found to have no deleterious effects on the growth of BHK cells. After 48 h the number of plaques was assessed by removal of tissue culture medium (into a sterile tube for subsequent analysis by plaque assay), fixation in 1% formaldehyde and staining with 10% Giemsa stain (Raymond A. Lamb Lab Supplies) for 20-30 mins. After washing off excess stain, the number of plaques was enumerated by assessment of the plates under a dissecting microscope.

4.12.11 Plaque Assay

The titre of cell free HSV1 was assessed using a conventional plaque assay. Briefly, BHK cells were allowed to grow in 6 well tissue culture plates to approximately 80% confluency before the culture medium was removed. 100μl of a dilution of samples was added and spread evenly over the cell monolayer. Virus was allowed to adhere for 1 h at 37°C, before 3-5ml of fresh αMEM+ was added. No addition to the medium was used to inhibit the effects of progeny virus. Addition of carboxymethlyl cellulose (CMC) appeared to have a deleterious effect on cell growth, making it impossible to assess plaque number. The effects, if any, of progeny virus was reduced by omitting any small plaques appearing in close proximity to large, fully formed plaques. Plaque formation was visualised after 48-72 h at 37°C/5%CO_2 using the method described for the infectious centre assay (section 4.11.9). Calculations were made from the dilution(s) which gave approximately 100 plaques and expressed as pfu/ml.

Chapter 5

Splenic DC as 'natural adjuvants' of the HSV1 immune response

5.1 Introduction

5.2 Splenic DC (sDC)-enriched Populations as APC for Ovalbumin-Specific Responses In Vitro.

5.3 In Vitro Proliferation Induced by HSV1-Pulsed sDC

5.4 In Vivo Initiation of an HSV1-Specific T Cell Proliferative Response by UV-HSV1-Pulsed sDC.

5.5 In Vivo Initiation of HSV1-Specific Antibody Responses by UV-HSV1-Pulsed sDC.

5.6 Chapter Summary

5.7 Specialised Materials and Methods

5.7.1 Generation of Ovalbumin Primed Lymph Node Cells

5.7.2 Preparation of Ova pulsed sDC-enriched APC

5.7.3 Ova-specific Proliferation Assay

5.7.4 Generation of HSV1-specific Responder Cells

5.7.5 HSV1 Antigen Preparations

5.7.6 Generation of HSV1-pulsed Purified sDC

5.7.7 Immunisation with Antigen-pulsed sDC and Control Cells

5.7.8 HSV1-specific Proliferation Assays

5.7.9 Preparation of Serum Samples

5.7.10 ELISA Buffers

5.7.11 Detection of HSV1 Antibody by ELISA

5.1 Introduction

Dendritic cells are known to be potent stimulators of antigen specific responses in vitro [309] and in vivo. The work in this chapter was based on studies detailing the potency of splenic dendritic cells (sDC) in initiating antigen specific responses in vivo. Two studies in particular show the efficacy of antigen-pulsed sDC in generating antibody *or* T cell proliferative responses to foreign proteins [262;266]. A third, much earlier work [283] suggested a role for sDC in the initiation of a virus specific antibody response. Although the experimental conditions were not identical in each of these studies, they all indicated the potential for sDC to function as 'natural adjuvants' [262], that is, DC pulsed in vitro with a foreign antigen can initiate immune responses in vivo. Their efficacy is dependent on, among other functions, their ability to select, process, and present the immunologically relevant determinants of an antigen.

As we have already stated (Chapter 1), HSV1 probably interacts initially with skin LC. It is conceivable that HSV1 may also encounter DC in the spleen if the virus is carried via the blood. It must be stressed that the results presented in the following chapter represent preliminary data. However, it is hoped that they will form the basis for further work on the capacity of LC (isolated from mouse skin and pulsed with HSV1 antigen in vitro) to initiate a primary HSV1-specific immune response in vivo. The reports detailed above provided a basis and precedent for the use of isolated splenic DC for the initiation of immunity in vivo. Previous studies have not tested the ability of pre-pulsed sDC to initiate virus-specific T cell and antibody responses in parallel.

We have presented results which indicate that sDC are relatively resistant to infection by HSV1 (Chapter 3). In the following chapter however we assessed the ability of sDC to act as highly efficient antigen presenting cells of UV-inactivated HSV1 in vitro and in vivo. Methods exist in this laboratory which enable purification of splenic DC to >95% purity, but as yet the best purification of LC using our BSA density gradient system is to 20-35% purity from epidermal cell (EC) suspensions. Thus, the capacity of splenic DC to initiate responses in vivo could be interpreted in the near complete absence of other contaminating cell types. The ability of EC

populations, depleted of LC, to induce immunity could have been tested given time, but the methods applied initially were those involving sDC.

5.2 Splenic DC (sDC)-enriched Populations as APC for Ovalbumin-Specific Responses In Vitro.

In the following section we show that spleen populations enriched for sDC are highly efficient at presenting ovalbumin (Ova) to Ova primed T cells. This enabled us to partially characterise the system for sDC presentation of UV-inactivated HSV1 in vitro.

Partially purified sDC (LODACs), pre-pulsed for 18 h with Ova (section 5.7.2), readily restimulated Ova primed lymph node cells in vitro (sections 5.7.1 and 5.7.3). This was reproducible over a number of experiments and Figure 5.1 shows a typical example. Occasionally, a high background was associated with control LODACs (pulsed in R10 alone; Figure 5.2). This background is most easily attributed to the ability of sDC to act as potent stimulators of the syngeneic MLR, in the apparent absence of specific antigen.

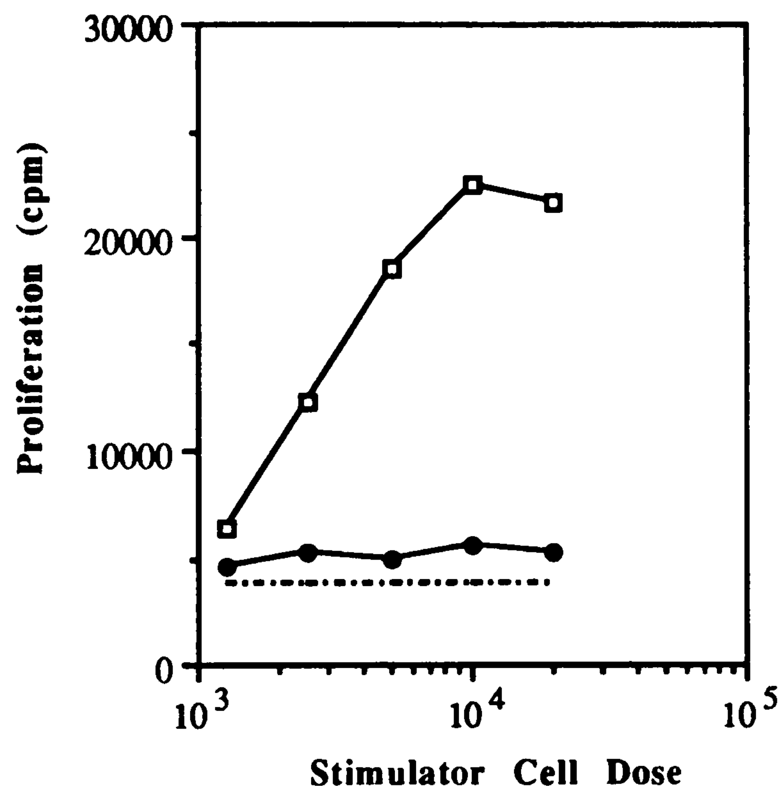


Figure 5.1 *The capacity of graded doses of sDC-enriched spleen cells (LODACs), pre-pulsed with Ova, to stimulate the proliferation of Ova-primed lymph node cells in vitro. Ova-pulsed LODAC (open squares); R10 (medium alone)-pulsed LODAC (closed circles); responders alone (hatched line).*

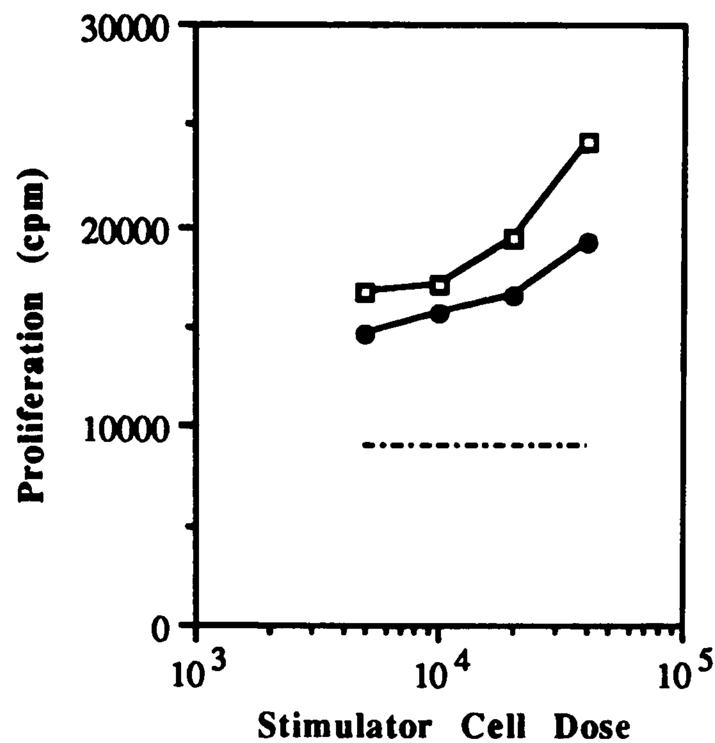


Figure 5.2 *LODACs pulsed in medium alone (closed circles) sometimes induced a high syngeneic MLR in Ova-primed lymph node cells, which almost equalled the proliferation induced by Ova-pulsed LODACs (open squares). Responders alone (hatched line).*

Pulsing control cultures with an irrelevant antigen, such as insulin, in some instances, significantly reduced this background proliferation (Figure 5.3). It is difficult to explain why this would have occurred unless insulin-derived peptides were able to replace peptides on the cell surface MHC molecules which stimulated the syngeneic MLR or Ova-primed T cells through cross reaction.

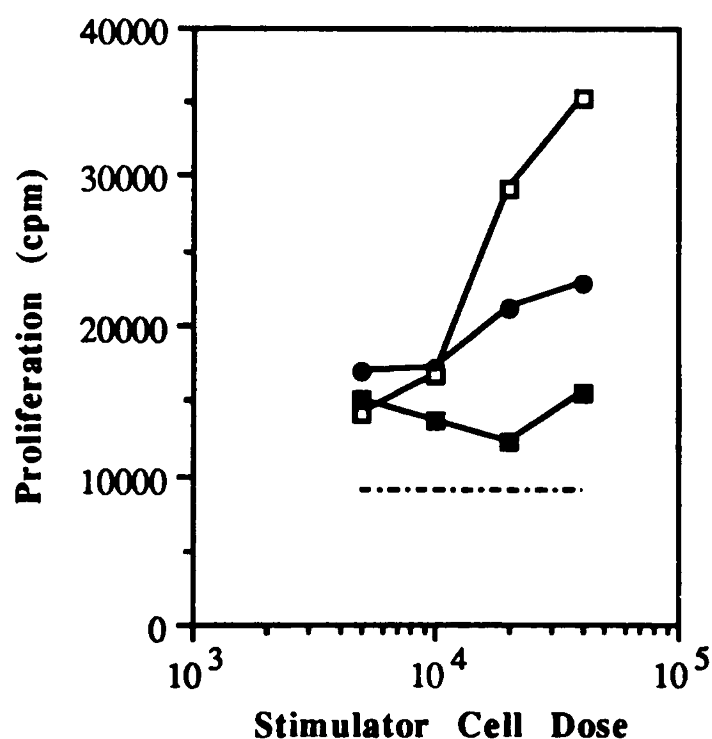


Figure 5.3 *Pulsing LODACs with insulin reduced the capacity of these cells to stimulate Ova-primed lymph node cells (crossed squares). R10-pulsed LODACs stimulated a higher proliferative response (closed circles) but this was lower than that induced by Ova-pulsed cells (open squares). Responder cells alone (hatched line).*

Using Ova we were able to show that sDC-enriched populations were capable of potently stimulating secondary proliferation of primed cells in vitro. This was utilised and developed in the following sections which specifically deal with the presentation of HSV1 by sDC. The primary goal of this next series of studies was to begin to assess the ability of purified sDC, pulsed in vitro with UV-inactivated HSV1 (UV-HSV1), to generate HSV1-specific antibody and T cell responses, when introduced into a naive animal.

5.3 In Vitro Proliferation Induced by HSV1-Pulsed sDC

To ensure that we could express antigens from HSV1 on sDC in an immunologically relevant form, HSV1-pulsed sDC were tested for their ability to stimulate virus-primed T cells in vitro. We show here that sDC efficiently presented UV-HSV1 and live HSV1 to HSV1-primed T cells. HSV1 MDK tk-, a thymidine kinase mutant, was used to prime mice and as the source of virus for UV-inactivated antigen (UV-HSV1). This strain is slightly impaired in its ability to replicate, is relatively apathogenic (depending on the route of inoculation) and generates good antibody and T cell proliferative responses in mice (A.A. Nash, personal communication).

Graded doses of sDC, pre-pulsed with UV-HSV1 (or in R10 medium alone; section 5.7.6), were used to restimulate lymph node and spleen cells isolated from mice primed subcutaneously with HSV1 (section 5.7.4). Figure 5.4 illustrates the proliferation values obtained. UV-HSV1 alone was used in all the proliferation assays detailed in this chapter to demonstrate the presence of HSV1-specific responders. (The proliferation induced by UV-HSV1 was not due to BHK cell contaminants in the antigen stock since BHK cell debris at an equivalent concentration failed to induce a response in primed cells - not shown). UV-HSV1-pulsed sDC were highly efficient stimulators of primed cells from the lymph nodes of these mice (Figure 5.4 (a)). At cell concentrations even as low as 4×10^3sDC/well, the proliferation induced by UV-HSV1-pulsed sDC was as high as that seen with the top dose of UV-HSV1 alone (equivalent of 10^6 pfu HSV1/well, a concentration which exceeds that available to sDC at the time of pulsing). Stimulation of spleen cells from the same animals was much lower

(Figure 5.4 (b) note the 10-fold difference in axes between (a) and (b)), probably represents the stimulation of recirculating memory cells.

Infectious HSV1 could also be used to pulse sDC. The response to infectious-HSV1 pulsed sDC was as high at top stimulator doses as that seen with its UV-inactivated counterpart (Figure 5.5). This demonstrates that while at least the bulk of sDC may be resistant to infection by HSV1 (Chapter 3), these cells can still internalise virus and generate antigenic peptides. While the proliferation levels using live-HSV1 pulsed, and UV-HSV1 pulsed, sDC were similar, these may represent the separate stimulation of the $CD8^+$ and $CD4^+$ T cell subsets due to endogenous presentation via MHC Class I and exogenous presentation via MHC Class II respectively.. In the absence of phenotypic analysis it was not possible to define the responding population in each of these cultures.

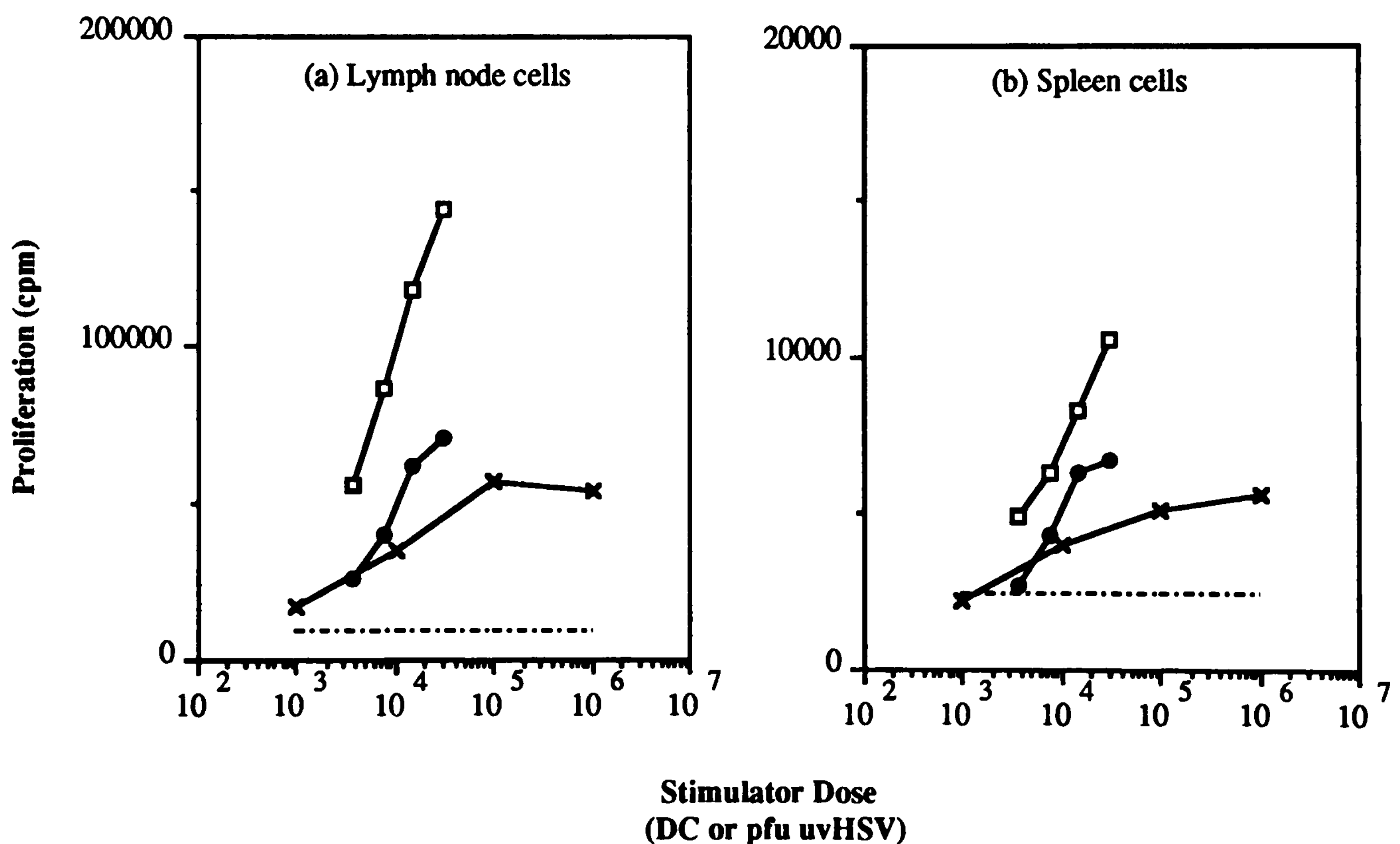


Figure 5.4 UV-HSV1 pulsed sDC (open squares) are potent stimulators of lymph node (a) and spleen (b) cells from mice immunised subcutaneously 14 days earlier with HSV1. UV-HSV1 antigen alone (crosses) was used to demonstrate the presence of HSV1 specific T cells in both populations. A high background was associated with R10 pulsed sDC (closed circles).

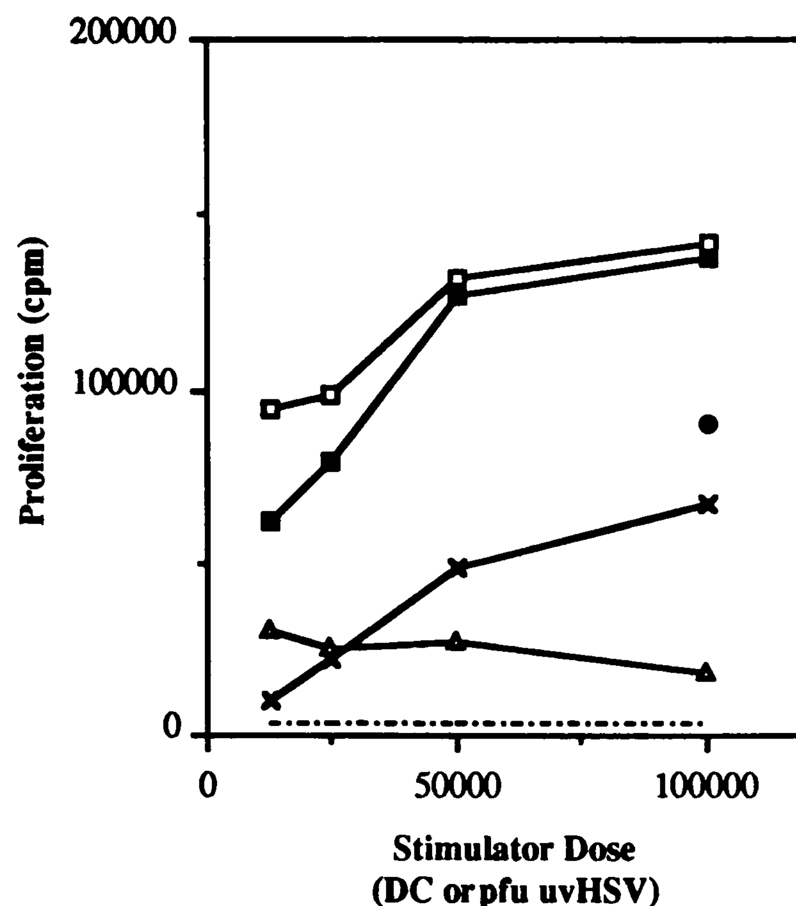


Figure 5.5 HSV1 primed lymph node cells were restimulated in vitro with UV-HSV1 alone (crosses) or purified sDC pre-treated with: UV-HSV1 (open squares), infectious HSV1 MDK tk- (closed squares), HGG (triangles) and R10 alone (closed circle). Responder cells alone (hatched line). See text for explanation.

As in section 5.2, high levels of proliferation were observed in many cultures when R10-pulsed sDC were used as stimulators (Figure 5.4-5.6). Figure 5.5 demonstrates the effectiveness of pulsing control sDC with an excess of human γ-globulin (HGG; Sigma G4386) in reducing this background. Due to limited cell numbers, a single dose of 10^5 sDC pulsed in R10 alone was compared with a titration of sDC pulsed in HGG (1 mg/ml). Proliferation induced by 10^5 R10-pulsed sDC was approaching 10^5 cpm, whereas that induced by HGG-pulsed sDC was only 2-4×10^4 cpm maximum.

Naive lymph node cells responded poorly to sDC (Figure 5.6). A syngeneic MLR was detectable with naive lymph node cells (b) but this was an order of magnitude lower than that seen with primed cells (note the 10-fold difference in axes between (a) and (b)). The background response of primed cells to R10-pulsed sDC was very high in this instance (a).

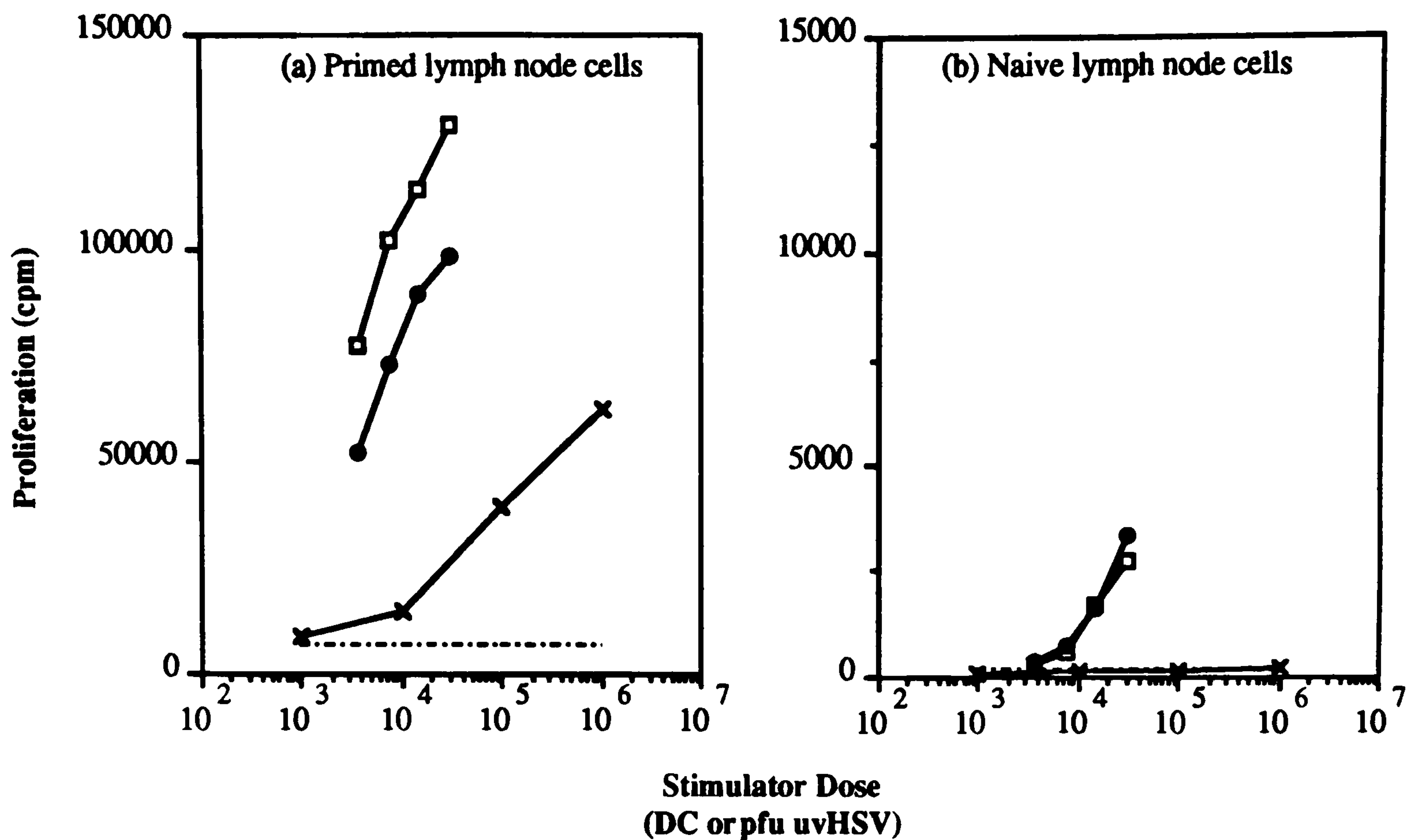


Figure 5.6 Naive lymph node cells (b) did not respond to stimulation with UV-HSV1 (crosses). A syngeneic MLR was detectable using R10-pulsed sDC (closed circles) and UV-HSV1-pulsed sDC (open squares) in naive lymph node cells (b). Primed cells (a) responded in a manner similar to that described in Figures 5.4 and 5.5, but R10-pulsed sDC induced a much higher response here (closed circles). Responder cells alone (hatched line).

In summary, we have shown that sDC pulsed with UV-HSV1 or live HSV1 of the same strain were capable of inducing high levels of proliferation in lymph node and spleen cells from HSV1-primed mice. In contrast, naive lymph node cells responded poorly to UV-HSV1 pulsed and control sDC. The proliferation of primed T cells by UV-HSV1-pulsed sDC vastly exceeded that which would have occurred if sDC had simply regurgitated antigen, acquired during pulsing, on co-culture with T cells. We concluded from this that our conditions of culture were appropriate for the generation of sDC which expressed HSV1 peptide in the context of the cells MHC Class I and/or II molecules.

5.4 In Vivo Initiation of an HSV1-Specific T Cell Proliferative Response by UV-HSV1-Pulsed sDC.

We attempted to test whether or not sDC pre-pulsed in vitro with UV-HSV1 could initiate an HSV1-specific primary response in vivo (see section 5.7.7 for detailed immunisation protocol). Briefly, sDC incubated in the presence of UV-HSV1 were used to immunise mice intravenously via the tail vein, or subcutaneously in the footpad. Two control groups of animals were immunised by the same routes with live HSV1 or UV-HSV1 alone. After 15-16 days, lymphoid organs were removed from the animals and bleeds were taken from the tail vein for serum samples. Antigen-specific T cell proliferation was tested by restimulation with UV-HSV1 in vitro in the absence of exogenously added APC. A summary of the experimental protocols is given in Table 5.1.

Table 5.1

	Immunising Cell Type	Medium used for pulsing sDC RPMI + ?		T cell response tested/group*
		Serum Source	UV-HSV1 (6 x10^6 pfu/ml)	
Expt. A	sDC	10% FCS	Yes	Lymph node *and* spleen cells
Expt. B	sDC	0.75% NMS	Yes	Lymph node *or* spleen cells
		10% FCS	No	Lymph node *or* spleen cells
Expt. C	3T3 cell line	0.75% NMS	Yes	Lymph node *or* spleen cells

* In Expt. A, lymph node cells **and** spleen cells were isolated from each mouse within a group, whether immunised subcutaneously or intravenously. In Expt. B and C lymph nodes were removed from footpad-immunised animals only, and spleens were removed from intravenously-immunised animals only.

5.4.1 Experiment A: sDC pre-pulsed in FCS and UV-HSV1

The proliferation values from this experiment (Expt. A, Table 5.1) are detailed in Figure 5.7. Subcutaneous immunisation of sDC resulted in high proliferation values (>50K cpm) in T cells from draining lymph nodes (e). Curiously, there was no dose response, and proliferation

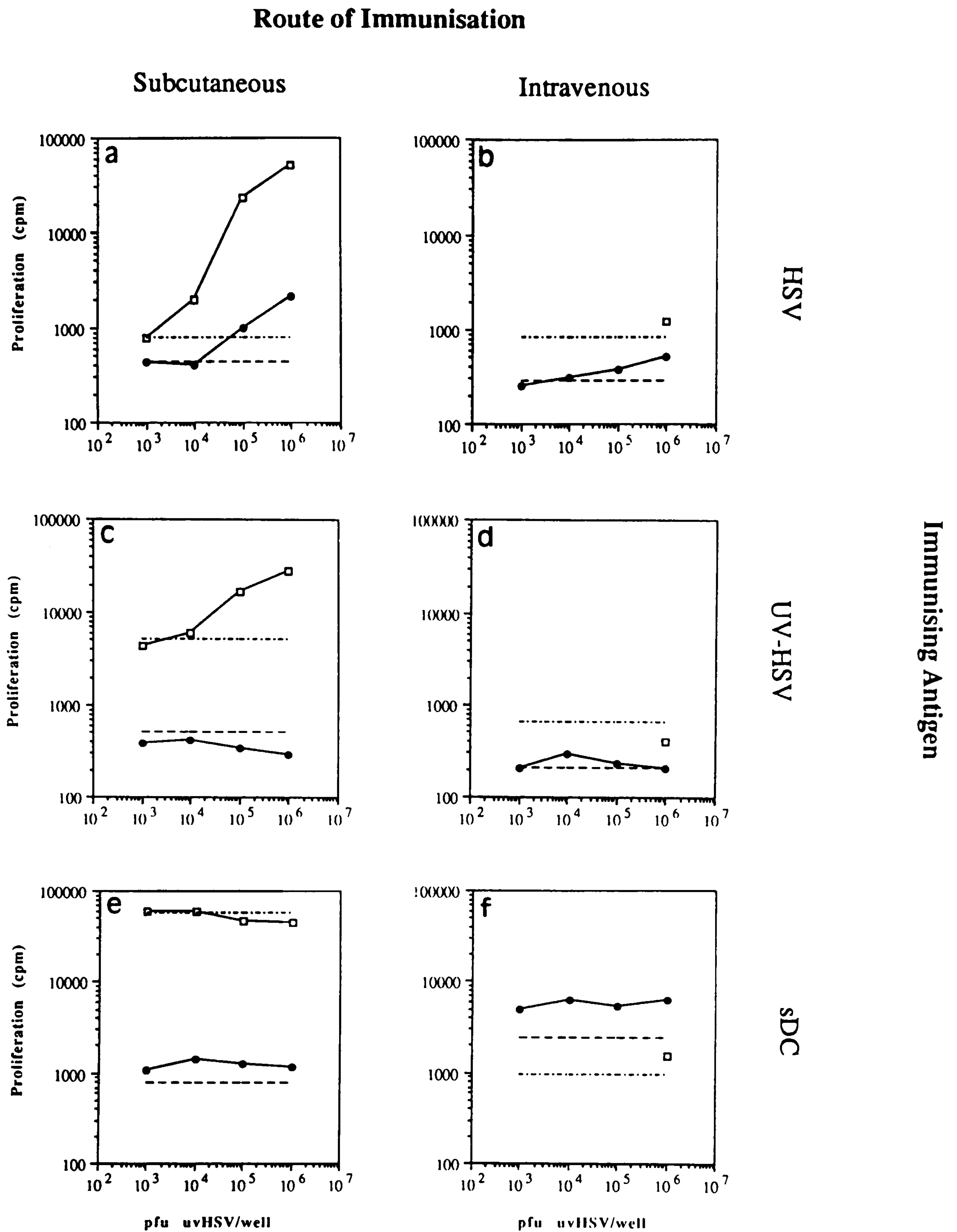


Figure 5.7 Lymph node and spleen cells from immunised mice (see Table 5.1, Expt. A) were restimulated in vitro with UV-HSV1. Route of immunisation - subcutaneous route (a) HSV1, (c)UV-HSV1, (e) sDC, or intravenous route (b) HSV1, (d) UV-HSV1, (f) sDC.

KEY: *lymph node cells plus restimulating antigen (open squares)*
spleen cells plus restimulating antigen (closed circles)
lymph node responders alone (irregular hatched line)
spleen responders alone (regular hatched line)

remained high even in the complete absence of restimulating antigen (e). Spleen cells from these mice did not respond much above background (e). Intravenous immunisation of sDC generated a slight response in the spleen (f). Again this did not titrate out but was slightly higher in the absence of restimulating antigen (f). In the control groups, subcutaneous administration of live HSV1 (a) and UV-HSV1 (c) primed mice for a T cell response in the draining lymph nodes. In contrast, neither of these antigens had any detectable effect on the spleen T cell populations when given intravenously ((b) and (d)).

In this first experiment, immunisation with sDC was associated with high levels of proliferation even in the absence of restimulating antigen. There are at least two possibilities which may account for this. Firstly, mice may have been primed to FCS acquired by sDC during the time of culture prior to immunisation. These FCS-primed T cells could then have been restimulated by cross reacting antigens in the horse serum-supplemented medium used in the proliferation assay. Secondly, the sDC used to immunise these animals may still have been resident in the lymphoid organs at the time of isolation and were thus capable of further stimulation in vitro. It seems unlikely, however, that sDC would survive for 16-20 days at a sufficiently high level to induce the observed levels of proliferation. We therefore tested the first option in the next experiment.

5.4.2 Experiment B: sDC pre-pulsed in NMS and UV-HSV1 or FCS alone

In this experiment (Expt. B, Table 5.1) two groups of sDC were used: (i) sDC pre-pulsed in 0.75% Balb/c normal mouse serum (NMS; a non-immunogenic source of serum) and UV-HSV1 (source of HSV1 antigen); and (ii) sDC pre-pulsed in FCS alone (to test the immunogenicity of FCS). Control groups of mice were immunised with infectious HSV1 or UV-HSV1 alone. The resulting proliferation values are detailed in Figure 5.8.

Immunisation with UV-HSV1/NMS-pulsed sDC generated a detectable HSV1-specific response when administered subcutaneously (b). The response was low, but significant (stimulation

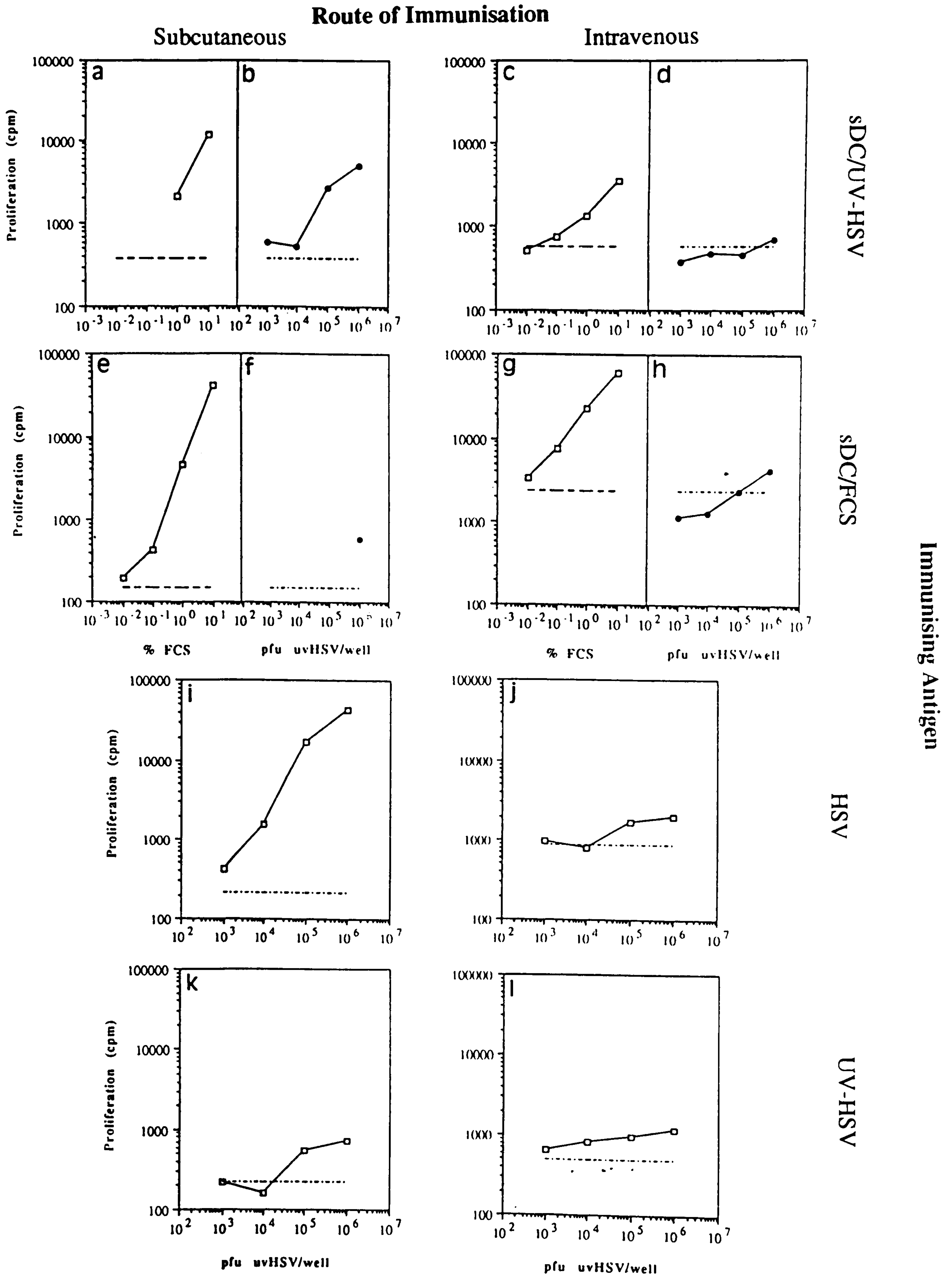


Figure 5.8 sDC, pre-pulsed with UV-HSV1, primed mice to HSV1 in vivo when given subcutaneously (b). sDC, pre-pulsed in FCS, were also able to specifically prime mice to FCS when administered intravenously (g) and subcutaneously (e). Control groups of animals were immunised with infectious HSV1 (i,j) or UV-HSV1 alone (k,l). See text for details.

Key: cells from the above animals were restimulated in vitro with graded doses of FCS (%FCS) or uv-inactivated HSV (UV-HSV).

index >13). However, intravenous immunisation with UV-HSV1/NMS-pulsed sDC did not induce a response in spleen cells (d).

In contrast, FCS-pulsed sDC stimulated a marked FCS-specific response when administered by either route (footpad (e), tail vein (h)). The proliferative response in both instances was high and exceeded the background proliferation induced by incubating responder cells from the UV-HSV1/NMS-sDC immunised animals in the same concentrations of FCS ((a) and (c)). Therefore, priming with FCS could have induced the high levels of proliferation seen in the previous experiment (Figure 5.7). In this experiment (Figure 5.8), however, we did observe lower levels of proliferation in the presence of horse serum alone, so priming with FCS may not entirely explain our previous results.

In summary from these preliminary data, we have shown that sDC pulsed with UV-HSV1 in normal mouse serum, generated an HSV1-specific response in subcutaneously immunised animals. This exceeded that seen with UV-HSV1 alone, which in this case gave little response (k).

5.4.3 Experiment C: 3T3 fibroblasts pre-pulsed in NMS and UV-HSV1

Splenic DC, pulsed with antigen in vitro, may prime animals simply by acting as a localised and persistent source of antigen in the spleen, lymph nodes or peripheral tissues. This therefore would not reflect any specialised ability of sDC to prime mice in vivo. To control for this, a group of animals was immunised with a cell line, Balb/c 3T3 A31 fibroblasts, pre-pulsed with NMS and UV-HSV1 (Expt. C, Figure 5.1). This would not control for presentation of antigen in the context of MHC Class II as Balb/c 3T3 A31 fibroblasts do not constitutively express these molecules. Figure 5.9 shows the results obtained. Whether administered intravenously or subcutaneously, 3T3 fibroblasts were unable to induce a detectable proliferative response ((a) and (b)). Control groups of mice gave detectable responses when immunised subcutaneously with infectious HSV1 (c) or UV-HSV1 (e). It seems less likely, therefore, that the production

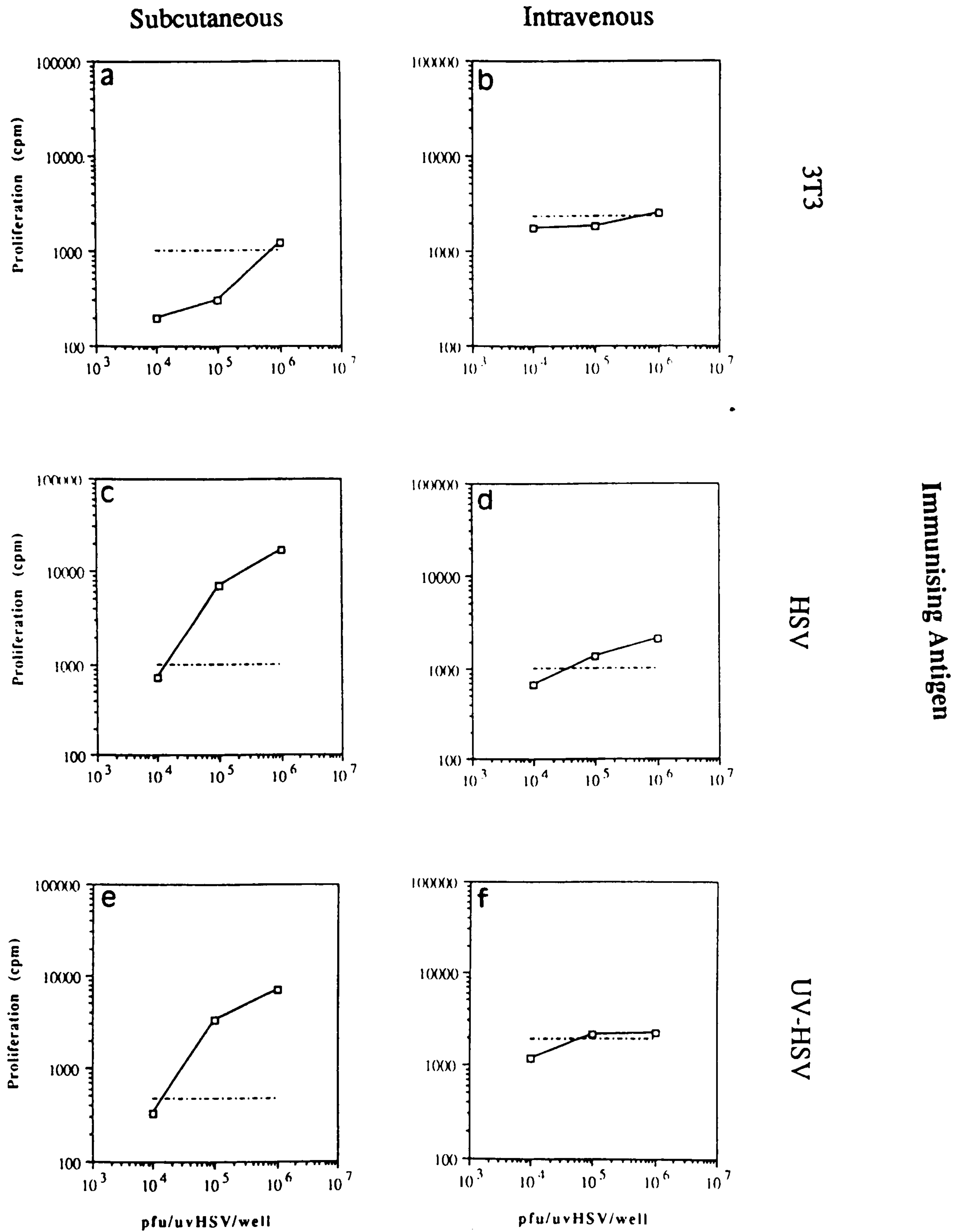


Figure 5.9 Lymph node and spleen cells from mice immunised with 3T3 fibroblasts pre-pulsed in UV-HSV1 ((a) and (b)) were restimulated in vitro with UV-HSV1. Control groups were immunised with live HSV1 ((c) and (d)), or UV-HSV1 ((e) and (f)). Responders alone (hatched lines). See text for details.

of an HSV1-specific response by antigen-pulsed sDC was simply a consequence of being a more persistent source of antigen in vivo. It would be interesting to test MHC Class II-transfected 3T3 cells in this system to control for presentation via MHC Class II.

5.5 In Vivo Initiation of HSV1-Specific Antibody Responses by UV-HSV1-Pulsed sDC.

Blood samples were taken from individual mice in Expts. A-C (Table 5.1) and the sera were tested at three different dilutions for the presence of HSV1-specific antibodies using an HSV1-specific ELISA (for detailed methods, see section 5.7.9-5.7.11).

Borderline levels of IgG were detected in a minority of the serum samples, at a dilution of 1:50, from sDC-immunised animals (Figure 5.10; (c)). UV-HSV1 alone also gave borderline levels (b). In contrast, IgG was readily detectable in all animals immunised subcutaneously and intravenously with infectious HSV1 (a). Figure 5.10 is typical of three separate experiments detecting whole IgG. In further experiments, it was demonstrated that UV-HSV1-pulsed 3T3 fibroblasts failed to prime for an antibody response (Figure 5.11).

Isotyping of the antisera demonstrated the presence of IgG2a in the majority of the samples from HSV1-immunised animals (Figure 5.12 (b)). IgG1 (a), IgG2b (c) and trace levels of IgG3 (d) were also present, but IgM (e) could not be detected. UV-HSV1 may induce low levels of IgG2a (b), but no other antibody was present. Sera from sDC immunised animals were negative for all the isotypes tested including IgM. Figure 5.12 is typical of two separate experiments.

In conclusion, we found no evidence that sDC could prime for an HSV1-specific antibody response.

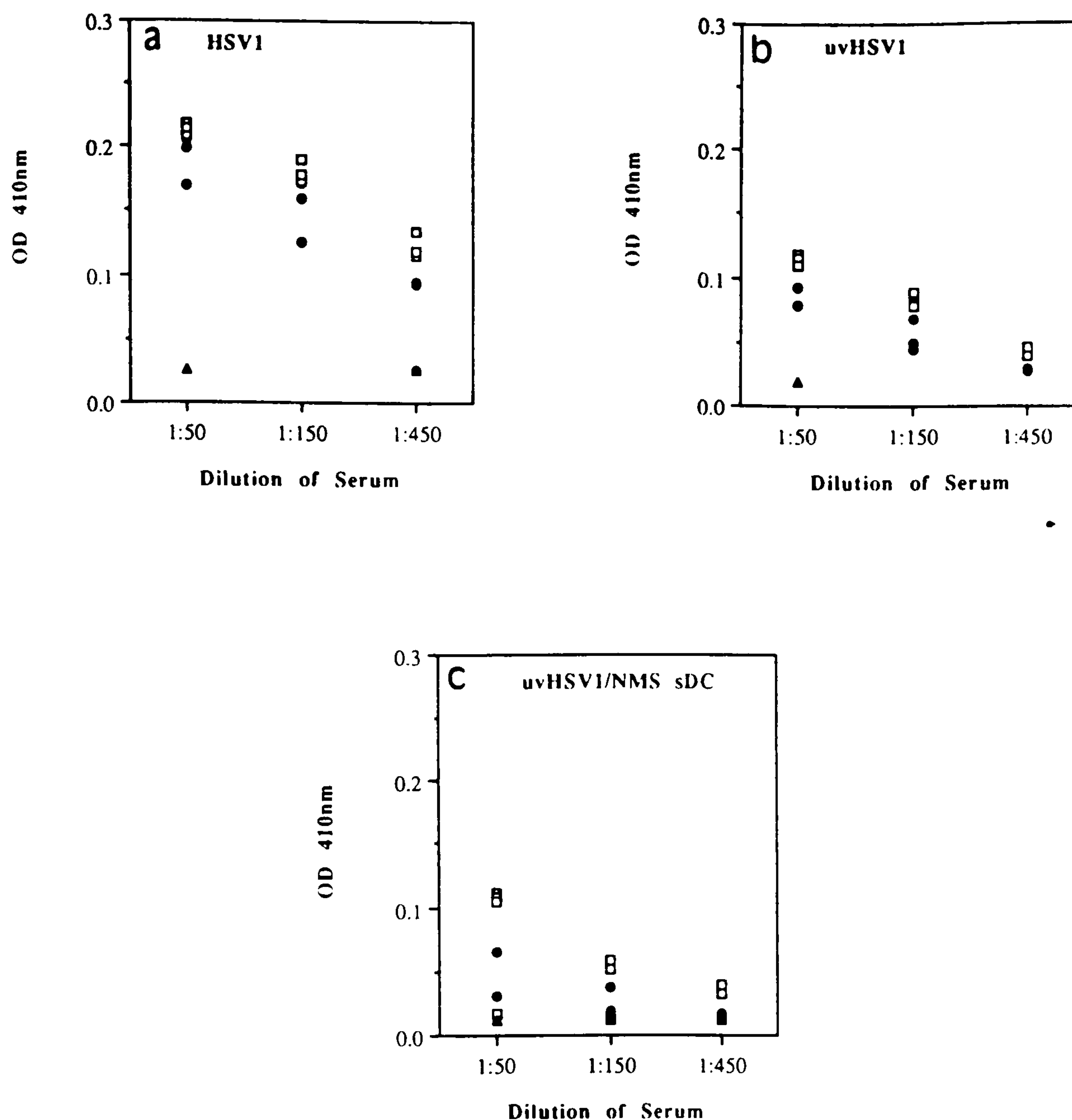


***Figure 5.10** displays typical values obtained when sera from three experimental groups of mice were tested for HSV1-specific IgG, after being immunised subcutaneously (open squares) or intravenously (closed circles). Typically, HSV1 produced high levels of antibody when mice were primed by either route (a), whereas UV-HSV1 gave only borderline levels at the highest dilutions of antisera (b). Immunisation with UV-HSV1-pulsed sDC failed to generate levels of antibody which even equalled that of UV-HSV1 alone (c). NMS controls are shown at the top dilution of 1:50 (closed triangles).*

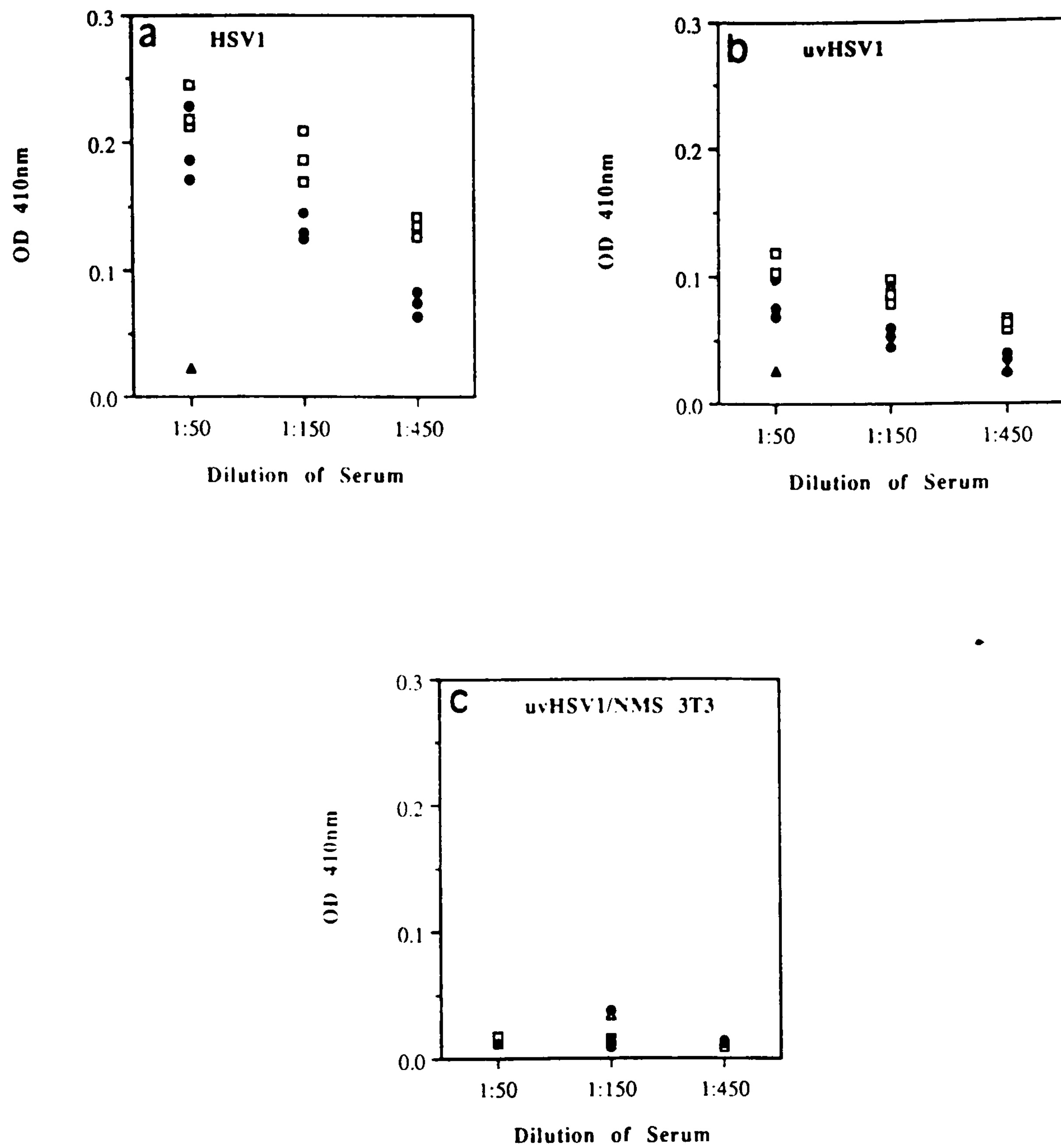


***Figure 5.11** 3T3 fibroblasts, pre-pulsed with UV-HSV1, were completely ineffective at producing HSV1-specific IgG (c). This compared with high levels of antibody in HSV1immunised mice (a), and borderline levels in UV-HSV1 primed mice (b). NMS controls (closed triangles).*

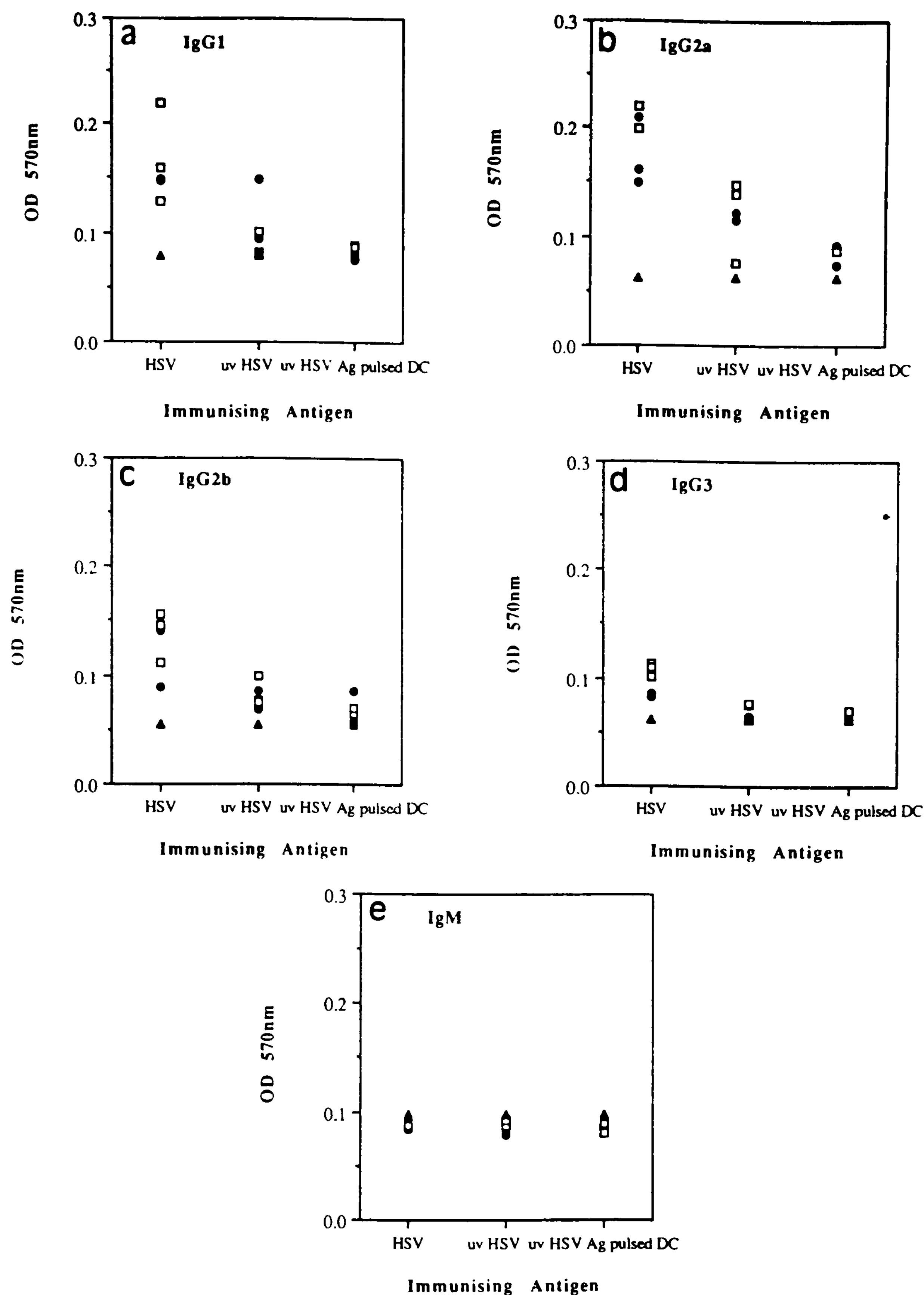


Figure 5.12 Isotyping of the antisera revealed that immunisation with HSV1 produced antibody of all IgG subclasses (a-d), with IgG2a being detected in the majority of samples tested. No IgM was detectable in any of the samples from any experiment (e). UV-HSV1 generated borderline levels of IgG1 (a), IgG2a (b) and IgG2b (c), but no IgG3 (d) or IgM (e) was detectable. UV-HSV1 pulsed sDC failed to produce any of the subclasses of antibody, including IgM (e). NMS controls (closed triangles).

5.6 Chapter Summary

We have shown that purified sDC, when pulsed with UV-inactivated HSV1, could induce high levels of proliferation of primed, but not naive, lymph node cells in vitro. The proliferation observed was as a consequence of pulsing with specific antigen, and not due to the stimulatory effects of sDC. Pulsing sDC with live HSV1 also induced high, in one case almost equivalent, levels of proliferation in the presence of polyclonal anti-HSV antisera to prevent infection of T cell responders (Chapter 4, section 4.9). The phenotype of the responding population was not determined and could represent the differential stimulation of separate T cell subsets. This needs to be examined in future studies.

Various protocols were then employed to test the efficiency of these uv-HSV1 pulsed sDC in generating HSV1-specific T cell proliferative and antibody responses in vivo (results summarised in Table 5.2). The results obtained only give an indication of the potential use of sDC in this context and have mainly served to begin the characterisation of such a system. Immunisation with antigen-pulsed sDC has been shown to be effective in initiating specific responses in vivo [262;266;283] around which these studies on HSV1 were based. Our studies have shown that it is possible to generate an HSV1-specific T cell response if UV-HSV1 pulsed sDC are given subcutaneously. Priming was not simply due to sDC acting as a cell source of antigen since no response was detected when 3T3 fibroblasts were used as the immunising cell. We were also able to prime mice with FCS-pulsed sDC when given subcutaneously and intravenously. This suggests that our immunisation protocol was effective and that the absence of a specific response when UV-HSV1 pulsed sDC were given intravenously may reflect a difference in the ability of certain antigens to prime by certain routes. Immunising mice with UV-HSV1, in the context of sDC, did not appear to induce an appreciable increase in HSV1 antibody levels, compared with using UV-HSV1 alone. Further studies will be needed to extend this work which has shown the possibilities and some of the potential problems inherent in such a system.

Table 5.2

	Route of immunisation and resulting response:			
	Subcutaneous		Intravenous	
Immunising Antigen	T cell	Antibody	T cell	Antibody
HSV1	+++	+++	-	+++
UV-HSV1	+/-	+/-	-	+/-
(UV-HSV1/NMS) sDC	+	+/-	-	+/-
(UV-HSV1/NMS) 3T3	-	-	-	-

5.7 Specialised Materials and Methods

5.7.1 Generation of Ovalbumin Primed Lymph Node Cells

C57/Bl/6 mice were immunised subcutaneously at the base of the tail with 50µg ovalbumin (Ova, Grade VII Sigma, Cat.No.7641) in PBS emulsified in Complete Freund's Adjuvant (CFA, Sigma, Cat.No.F4258) in a total volume of 50µl. Ova was prepared as a 10mg/ml stock solution in PBS and stored at -20°C until required. Emulsification of Ova and CFA was carried out in a glass bijoux by sonication or repeated syringing using a glass syringe. After 7-8 days the draining periaortic and inguinal lymph nodes were removed aseptically into a sterile universal containing 3-4ml RPMI-1640. The lymph nodes were decanted into a 60mm petri dish and excess fatty tissue was removed, before the RPMI-1640 was aspirated off. 3-4ml of 1mg/ml collagenase (Sigma, Cat.No.C2139) dissolved in R10 was immediately added and the nodes gently teased apart using watchmakers forceps. After pipetting with a pasteur to further dissociate the clumps of tissue the plate was incubated at 37°C/5%CO_2. After 20-30mins any residual, undigested tissue was broken up by aspirating with a pasteur and the cell suspension immediately transferred to 10ml R10 in a 15ml conical tube, to dilute out the collagenase, and centrifuged. Supernatant was discarded and the cells washed again in R10. Viability was assessed on the basis of trypan blue exclusion and was always >90%. This bulk lymph node population was used without further manipulation or partially purified by nylon wool passage to enrich for T cells as responders in the antigen specific proliferation assay.

5.7.2 Preparation of Ova pulsed sDC-enriched APC

Low density, transiently adherent spleen cells (LODAC) were prepared from C57/Bl/6 mice according to the method previously described (Chapter 2, section 2.6.2). Lodacs were incubated in R10 + 1mg/ml Ova, R10 + Insulin (Sigma, Cat.No.I5500) or in R10 alone. After overnight incubation all non adherent cells from these cultures were removed and washed of excess antigen. The resulting population contained sDC (70-75%) with contaminating B cells and mϕ. After irradiation for 4.4 mins with 2000 rads from a ^{137}Cs source these cells were used as stimulators of Ova primed lymph node cells in the antigen specific proliferation assay.

5.7.3 *Ova-specific Proliferation Assay*

Graded doses of Ova pulsed or control (R10 or insulin-pulsed) sDC-enriched APC (section 5.7.2) were added into a constant dose of 2 x 10^5 Ova primed bulk or nylon wool passed lymph node cells (section 5.7.1) all incubated in R10 in a 96 well flat bottom microtest plate. Plates were incubated for 72 h in a 37°C/5%CO_2 humidified incubator, after which time proliferation was assessed on the basis of ^{3}H-TdR incorporation as discussed in section 4.12.1. Unless otherwise stated, results are presented as means of triplicate cultures and error bars have been omitted for the purposes of clarity (average SD=10-15% of mean value).

5.7.4 *Generation of HSV1-specific Responder Cells*

For known HSV1 specific populations, Balb/c mice were primed by subcutaneous immunisation in the left ear flap with 8 x 10^5 pfu HSV1 MDK tk- in 20µl PBS. At day 7 the mice were challenged with the same dose of virus in both ear flaps. On day 14 the draining auricular lymph nodes and, where relevant, the spleens were removed from these animals. Naive, unprimed cells, as a control population, were isolated from the auricular lymph nodes of normal Balb/c mice. Bulk single cell suspensions were prepared from all isolated organs by mechanical dissociation of the tissue and pipetting of the resulting cell suspensions. All bulk populations were used without further purification as responders in the antigen specific proliferation assay.

5.7.5 *HSV1 Antigen Preparations*

To demonstrate antigen specificity, all bulk spleen or lymph node cells from Balb/c mice (naive or primed with the various antigen treatments described in the experimental text) were re-stimulated *in vitro* with tenfold dilutions of uv-inactivated HSV1 MDK tk- (UV-HSV1, supplied by P.Camboropolous, Division of Immunology, Old Site, Dept. of Pathology, University of Cambridge), typically from 10^6 to 10^3 pfu equivalent/well. Non-specific re-stimulation against FCS or BHK cell debris was tested using a mock infected BHK cell lysate prepared in the same manner and used at the same dilutions as UV-inactivated HSV1 MDK tk-

(supplied by P.Tonks, Division of Immunology, Old Site, Dept. of Pathology, University of Cambridge).

5.7.6 Generation of HSV1-pulsed Purified sDC

Low density, transiently adherent spleen cells (LODACs) were prepared from Balb/c mice according to the method previously described (Chapter 2, section 2.6.2). LODACs were then incubated in R10 alone (R10 control), R10 + 1mg/ml human gamma globulin (HGG control, Sigma, Cat.No. G4386) or R10 supplemented with (i) UV-inactivated HSV1 MDK tk- at an equivalent of 4 x 10^6 pfu/ml final concentration, or (ii) HSV1 MDK tk- at a final concentration of 4 x10^6 pfu/ml, all in a total of 10ml R10. After overnight incubation, all non-adherent cell were removed from tissue culture dishes and pooled where appropriate. Contaminating FcR-positive cells were then removed according to protocol (Chapter 2, section 2.7.1) and the resulting purified sDC populations were irradiated (section 5.6.2) and used as stimulators of lymph node or spleen cells from HSV1MDK tk- primed animals (section 5.6.6).

5.7.7 Immunisation with Antigen-pulsed sDC and Control Cells

Purified populations of UV-HSV1-pulsed sDC were prepared according to the method summarised in section 5.6.8. For the purposes of immunisation, these cells had been pre-pulsed in RPMI-1640 supplemented in the first instance with 10% FCS (R10) and subsequently with 0.75% heat inactivated Balb/c normal mouse serum (NMS), both with a higher dose of UV-HSV1 at an equivalent pfu/ml of 6 x 10^6. Control populations of cells were purified sDC pre-pulsed in R10 alone or Balb/c 3T3 A31 fibroblasts pre-pulsed with RPMI-1640 + 0.75% NMS + UV-HSV1 (6 x10^6 pfu equivalent/ml). Control antigens for immunisation were infectious HSV1 MDK tk- (4 x 10^6 pfu/animal) or UV-HSV1 (equivalent 4 x 10^6 pfu/animal). In all cases, animals receiving sDC were administered with 3x10^5 sDC/animal by either route. Groups of Balb/c mice, with a minimum of 3 mice per group, were immunised subcutaneously in the footpad (fp) or intravenously in the tail vein (iv) with the sources of antigen detailed above in this section in 20µl and 100µl of PBS respectively. After 15-16 days the mice were bled for serum samples into sterile penny bottles from an oblique incision in the tail vein, which

were kept separate for individual mice. The mice were then sacrificed and the draining popliteal lymph nodes and/or spleens removed aseptically into sterile bijoux containing 2-3ml R10 on ice. Bulk single cell suspensions were prepared from all isolated organs by mechanical dissociation of the tissue and pipetting of the resulting cell suspensions. All bulk populations were used without further purification as responders in the antigen specific proliferation assay.

5.7.8 *HSV1-specific Proliferation Assays*

Graded doses of HSV1 (UV-inactivated HSV1 MDK tk- or infectious HSV1 MDK tk-) pre-pulsed FcR-ve sDC, control (R10 or HGG pre-pulsed) FcR-ve sDC (section 5.6.2) or tenfold dilutions of UV-HSV1 were added into a constant dose of 4 x 10^5 primed bulk lymph node or spleen responder cells, all incubated in RPMI-1640 + 2% horse serum in a 96 well flat bottom microtest plate. Plates were incubated for 96 h in a $37^oC/5\%CO_2$ humidified incubator, after which time proliferation was assessed on the basis of ^3H-TdR incorporation as discussed in section 4.11.1. Unless otherwise stated, results are presented as means of triplicate cultures and error bars have been omitted for the purposes of clarity.

5.7.9 *Preparation of Serum Samples*

Blood samples were kept separate for individual mice and left at room temperature to clot. The clotted blood samples from all the mice were ringed to allow retraction of the clot and the samples were stored at 4^oC overnight to allow the release of serum. After overnight incubation, the serum was carefully removed using a pasteur pipette into individual, sterile 1.5ml eppendorfs and spun at 13,500rpm for 3 mins to remove any red blood cells and debris. The serum supernatant was removed and transferred to individual, sterile 0.5ml eppendorfs and stored at 4^oC until required.

5.7.10 *ELISA Buffers*

Carbonate buffer was used in the preparation of coating antigen and consisted of 1.6ml of Na_2CO_3 solution (21.2g/l), 3.4ml of $NaHCO_3$ (16.8g/l) and 15ml ddH_2O with a final pH=9.6. Washing buffer was PBS containing 0.05% Tween 20 (Sigma, Cat.No. P1379). Substrate

buffer was 0.02M NaPO4 at a pH=6.8. Immediately after adding a single tablet of 5-aminosalicylic acid (100mg/tablet, Sigma, Cat.No. A6178) to 100ml substrate buffer, 1ml of a 1% H_2O_2 solution was added and 100µl dispensed per well.

5.7.11 Detection of HSV1 Antibody by ELISA

Infectious HSV1 MDK tk- coating antigen was prepared. The required volume of HSV1 MDK tk- was thawed from -70°C, transferred into a sterile 1.5ml Eppendorf and microfuged at 13,500rpm for 5mins to remove BHK cell debris. The supernatant was added to carbonate buffer in the ratio of 200µl HSV1 stock to 5ml buffer, which is equivalent to 8 x 10^7 pfu/ml, and mixed well. 100µl of coating antigen was added to the inner 60 wells of a 96 well flat bottom ELISA plate (Immulon, No.1, Dynatech, Cat.No. M129/A) and incubated in a moist box for 18 h at 4°C. Excess antigen was removed after this time by 5 washes with PBS/Tween 20 into a 1:30 dilution of Savlon (ICI Pharmaceuticals, Cat.No.PL29/5012) all in a Class II sterile cabinet to screen out virus aerosols. Subsequent to these washes infectious virus was considered to be firmly bound to the plate and all further procedures were carried out on the open bench with the appropriate safety precautions. Samples of sera pre-diluted using PBS/1% Tween20/5% FCS to 1:50, 1:150 and 1:450 were added to the plates in triplicate for the initial analysis of sera from separate experiments. Subsequent analyses such as isotyping were carried out on dilutions of 1:50. Negative controls were heat-inactivated Balb/c normal mouse serum (NMS) at the appropriate dilutions or PBS/1% Tween20/5% FCS alone to measure non-specific binding of secondary antibody. Non-specific binding of elevated levels of immunoglobulin was controlled for by using an immune serum from Semliki Forest Virus (SFV) immunised animals (supplied courtesy of Dr. J. Fazakerley, Division of Immunology, Old Site, Dept. of Pathology, University of Cambridge). Serum was isolated from Balb/c mice taken 9 days after intraperitoneal immunisation with 5 x 10^3 pfu SFV, strain A774, and gave an ELISA reading of 0.6 at OD_{570nm} at a 1:500 dilution, so was considered to have high levels of immunoglobulin present. Positive control serum initially used was raised after CBA mice were immunised intravenously with HSV1 MDK tk-. In later instances, control sera took the form of a 1:50 dilution of randomly selected samples from each group - HSV1, UV-HSV1 and

uvHSV1/FCS sDC - in the first immunisation experiment, known to be positive, borderline and borderline respectively. This was intended to ensure, after initial problems with a secondary antibody giving spurious results, that subsequent analyses were consistent with known positive and borderline samples. Primary antisera were incubated at 37°C for 2h before excess antibody was removed with 3 washes.

Secondary antibodies employed in all instances were directly conjugated to horseradish peroxidase (HRPO). HRPO conjugates from two sources were employed, namely goat anti-mouse whole IgG (Fc specific) - HRPO (Serotec, Cat.No. AAC 01P) at 1:200, or goat anti-mouse Clonotyping System III (Seralab, Cat.No. SAB 5060 05) which contained antibody-HRPO conjugates to whole IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM and was used for all isotyping of antisera, all at 1:200. All HRPO conjugates were diluted in PBS/1% Tween20/5% FCS and 100μl was added to each well and incubated at 37°C for a further 2 h.

Substrate was prepared by adding a single tablet of 5-aminosalicylic acid (100mg/tablet, Sigma, Cat.No. A6178) to 100ml substrate buffer. 100μl was dispensed into each well and the colour allowed to develop at room temperature for 15-45mins on the open bench. If necessary, the reaction was stopped by adding 50μl of 3M NaOH to each well. OD levels were measured at 410nm using a Dynatech MR5000 ELISA reader.

Levels of HSV1 antibody were assessed at the 1:50 dilution of test antisera relative to the NMS control at 1:50. For the purposes of this analysis, antigen treatments said to generate positive sera showed, in the majority of samples tested, an OD clearly above the negative control. For example, immunisation with HSV1 by either route generated positive sera in all samples tested. Borderline levels are considered to be those which, in the majority of samples, yield OD elevated marginally above negative control levels.

Chapter 6

Discussion

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6.2 Experimental Findings and Future Directions

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6.1 General Overview

Our studies have primarily focused on the interaction of HSV1 with DC. DC are highly adapted, in function and phenotype, for their role as the APC which initiate immune responses. In the peripheral tissues they form a network of "sentinel" cells capable of sampling the microenvironment. Antigens, such as viruses, which are acquired by these cells can be processed and expressed as peptides bound to cell surface MHC Class I and II molecules, which DC express constitutively. Given the correct stimuli, such as those delivered during an ongoing infection, these cells migrate via the lymph or the blood to the lymphoid organs. Migration is accompanied by a functional maturation; the cells reduce their capacity for antigen processing and develop their immunostimulatory potential. In the T cell areas of the lymphoid organs they express antigen in an immunologically relevant form and can deliver the secondary signals necessary for the initiation of a primary immune response.

HSV1 is an ideal candidate for studying the interaction of viruses and DC. It almost certainly encounters DC in the mucocutaneous tissues of the oropharynx, its normal route of infection, and infection in the skin may be heavily influenced by the competence of the resident skin DC, the Langerhans cells (LC). In the murine experimental model, intradermal (subcutaneous) challenge with HSV1 most closely mimics the normal mucocutaneous route of infection in man. Many studies have focused on the LC in this system. In the absence of LC in the epidermis the proliferative response to HSV1 is abolished [229] and the pathogenicity of HSV1 is significantly enhanced [228]. LC have also been shown to act as highly efficient antigen presenting cells for in vitro secondary proliferative responses [228, 310] There is as yet no evidence which directly demonstrates their ability to induce a primary HSV1-specific immune response, indeed some evidence suggests they are incapable of doing so in vitro [275]. Few workers, if any, have directly studied the interaction of LC (or any other cells of the DC lineage) with HSV1 and its subsequent effects in the context of the primary immune response. This approach is important in two senses. Firstly, it may help to explain or expand our concepts on the generation of the HSV1-specific immune response. Secondly, in the context of DC biology, it would provide more detailed information on their ability to deal with viruses and the

consequences of that interaction. It was against this backdrop that we undertook a study of the interaction of DC with HSV1 and this chapter discusses our experimental findings.

6.2 Experimental Findings and Future Directions

6.2.1 HSV1 Infection of Dendritic Cells

LC are considered to be important for the initiation of immunity to HSV1 but little is known of the interaction of HSV1 with LC or other DC. Two previous reports have recorded the presence of HSV1-positive DC in the draining lymph nodes of HSV1 infected mice. Dickson [306] identified MHC Class II-positive cells in a DC-enriched population (40% DC) from the draining lymph nodes of mice infected subcutaneously with HSV1 SC16 24 h previously. These cells labelled with a rabbit anti-HSV1 polyclonal Ab, the specificity of which was not detailed, and while the cells also expressed MHC Class II, their morphology by EM was not classically dendritic. The author explained this by presuming that the "veils are easily lost during preparation". No infectious virus was detectable in the DC-enriched population, when tested in an infectious centre assay, so whatever population was HSV1-positive it was not productively infected. These observations, a lack of dendritic morphology of many of the virus-positive cells, expression of MHC Class II (possibly induced) and presence of virus antigen but lack of infectious virus in the DC-enriched fraction may indicate that some of the cells identified as virus-bearing DC were, in fact, mϕ. Macrophages (i) can be induced to express MHC Class II, (ii) may internalise and degrade infectious virus, and (iii) are non-permissive for virus replication [311]. Sprecher *et al* [229] did recover virus from the DC-enriched fraction (60-80% DC) of lymph node cells from HSV1-infected mice. They suggested from this that "LC might carry infectious virus from the site of infection in the skin when entering the lymph nodes". However, due to the presence of contaminating cells (20-40%) it is impossible to state that the DC are carrying the virus. There is, in summary, no clear evidence on the susceptibility of LC to infection by HSV1.

In our initial studies (Chapter 3) we isolated LC, in the form of bulk epidermal cell suspensions from mouse skin. We exposed these cells to low multiplicities of HSV1/34, a clinical isolate of

HSV1, and analysed the cells for expression of virus antigens at given times post-infection. By UV-microscopy and FACS analysis we were able to show that HSV1 infection reduced the number of LC in an freshly isolated EC population. In most of our experiments we observed that the remaining infected LC expressed lower levels of MHC Class II. This may be a natural consequence of HSV1 infection [47, 49] and a strategy to hinder the initiation of the immune response.

A significant percentage of LC (typically 40-90%) in infected EC populations expressed virus antigens. This percentage was high given the low multiplicities of HSV1 used for infection (MOI typically = 0.1), and it is our view that this cannot be entirely explained by the generation of progeny virus and infection of previously uninfected cells. The cell populations were typically analysed at 18 h post-infection around which time the first round of replication would be completing. For progeny virus to increase significantly the percentage of LC expressing virus antigens (mainly glycoproteins produced late in the replicative cycle), this would not only require infection of previously uninfected cells, but a sufficient time for these antigens to be expressed. Alternatively, the titre of infectious virus, conventionally determined by plaque assay on cell lines such as BHK cells, may underestimate the real number of infectious particles. LC may be exquisitely sensitive to HSV1 and therefore more accurately reflect the amount of infectious virus present. This is in part supported by our observations that infection of EC with HSV1gH (a gH-negative mutant which does not produce infectious progeny thereby ruling out infection of previously uninfected cells) at an MOI=0.1 (determined on BHK cells) resulted in >75% gated LC expressing virus antigens (unpublished observations).

LC cultured in vitro for 48-96h (cLC) did not show a consistent increase or decrease in susceptibility to HSV1 compared to fresh LC. In both cases the number of MHC Class II-positive cells was reduced and, in most instances, the level of expression of MHC Class II on the remaining cells was also reduced. Both freshly isolated and cultured EC populations contained considerable numbers of fLC (20-50%) and cLC (30-50%) which expressed virus antigens. While the phenotype [223] and function [198] of LC are profoundly affected by

culturing these cells in vitro, we would suggest that the changes which occur on maturation of LC do not profoundly affect the sensitivity of LC to HSV1.

Lymphoid DC, such as splenic DC (sDC), and cLC are virtually indistinguishable [198]. In a direct comparison of the infection of sDC with fLC we found a much lower percentage of sDC (15-35%) expressing virus antigens than fLC (60-85%). This may in part be explained by the different modes of infection (see Chapter 3, section 3.7). However, when we compared sDC and LC, which had been cultured for 18 h and infected in an identical manner, we again observed a profound difference in susceptibility.

In summary, we have indicated that LC and sDC may differ in their sensitivity to HSV1, as judged by their expression of virus glycoproteins post-infection. The difference we have observed may reflect a previously unidentified fundamental difference between cultured LC and sDC. It may also reflect the tissue of origin. This aspect is known to heavily influence the susceptibility of mϕ and T cells to infection by HSV1 [312]. In general, mϕ are highly resistant to HSV1 infection in vitro [313]. However, differentiated mϕ infected with HSV often show a severe cytopathic effect.

Productive infection of permissive cells by HSV1 invariably results in cell death. HSV1 infection of the skin may have several effects on the resident LC population, some of which we will now consider. (i) HSV1 may directly kill LC in situ. While this would be an effective means of ablating the resident APC population, it is difficult to reconcile with the proposed key role of LC in initiating immunity to HSV1 (see Chapter 1, section 1.5.8). (ii) Like mϕ, a subpopulation of LC may be highly susceptible to HSV1 in vivo and be efficiently killed. The resistant population of LC may in part be protected by cytokines released by other EC, especially in the course of an infection [277]. These LC carrying infectious virus or virus antigen could then migrate to the draining lymph node where the response is initiated. In our studies we saw a large population of LC expressing virus antigens post-infection, but it is possible that by removing LC from their normal microenvironment we may have removed the

locally-produced cytokines which restrict the number of LC which are susceptible to HSV1. (iii) All LC may be productively infected but are able to travel to the lymph node and initiate the HSV1 specific response before being disabled or killed. Local depletion of LC would prevent the initiation of the immune response by removing the cells which initiate it. HSV1-positive DC are detectable in the lymph node, peaking at 24 h [314]. At this time point, in our system, HSV1-positive LC were still present in bulk cultures, so it is conceivable that they could act to initiate the response before being killed.

Once in the lymph node, LC carrying infectious virus would cluster with T cells to initiate the HSV1-specific response. At this point virus may be transferred to T cells which would then be functionally impaired. As we have discussed (Chapter 1), this has been demonstrated for HIV, and may provide a highly efficient mechanism for impairing T cells. Firstly, we have shown that LC infected in our system produce infectious virus in an infectious centre assay (Chapter 4, section 4.4). Secondly, 25% of resting T cells expressed virus glycoproteins after 24 h in the presence of HSV1 (Chapter 4, section 4.4). It has been shown that T cells isolated from human peripheral blood are relatively resistant to HSV1 infection [312]. These cells are predominantly a resting population, but it may be that when T cells cluster with HSV1-infected LC, the lymphocytes are activated and their susceptibility to HSV1 is significantly enhanced. It remains to be established whether resting T cells differ in their susceptibility to HSV1 compared to activated T cells.

We cannot rule out the possibility that the patterns of LC susceptibility we have observed are confined to the in vitro situation. Removing LC from their microenvironment in the skin may deprive them of the cytokines which could effect the resistance of LC to HSV1 in situ. Further studies of LC in situ are required before we can assess their susceptibility to the virus in vivo. Methods are available for studying the expression of markers, such as virus antigens, on LC in epidermal and dermal sheets [315] and these methods could be applied to sections of skin previously exposed to HSV1 in vitro and in vivo.

6.2.2 HSV1 Impairment of DC Function

The interaction of HSV1 with LC reduced their number and intensity of MHC Class II expression. Freshly isolated LC weakly stimulate naive, resting T cells [223]. The ability of cultured LC, or other mature DC, to activate resting T cells is termed immunostimulation. The immunostimulatory capacity of DC is considered vital in the generation of a primary immune response. It is this ability of cultured LC and lymphoid DC which distinguishes them from other APC. We therefore assessed whether HSV1 affected the immunostimulatory function of LC in vitro.

The allogeneic mixed leucocyte reaction [316] and oxidative mitogenesis [308] are well characterised assays which are highly sensitive to the presence of immunostimulatory DC. Initially, HSV1/34 infected EC were used as stimulators in the MLR and we observed a significant depression of the proliferative response. We subsequently found that this was partly a consequence of free virus released by bulk EC. We would also suggest that purified LC, which produced infectious virus in vitro, were able to transfer HSV1 to resting, or partially activated, T cells in a manner analogous to that observed for HIV [304]. It was therefore necessary to prevent HSV1 infection of T cells, and for this we turned our attention to heparin, a specific inhibitor of HSV1 infection [2]. Heparin specifically inhibits the interaction of HSV1 gC and gB with cell surface heparan sulphate proteoglycans by binding and preventing their attachment to cell surface heparan sulphate [1, 13]. Heparin in our system reduced the percentage of BHK cells and EC expressing virus antigens when included during the time of infection. Its action was specific for HSV1 and it did not affect vaccinia infection of BHK cells.

The mechanism of entry of HSV1 is by no means clearly established. Initial studies favoured an endocytic mechanism [24] but this may not be supported by recent evidence. As we have already discussed (Chapter 1) fusion of the virion envelope with the cell plasma membrane is probably the main mechanism of entry. Endocytosis may in fact result in degradation of the virus [7] but these observations were restricted to one mutant cell type, the BJ cell line, the

characteristics of which may not reflect the normal situation. As we have already discussed, studies which concentrate on the interaction of HSV1 with one cell type may lead to biased conclusions [3]. From this, and our discussions on the role of endocytosis (Chapter 1; [31]), we would suggest that endocytosis cannot be ruled out as a mechanism of entry which leads to productive infection. It is therefore possible that the observation that heparin inhibited endocytosis, but not binding, of latex beads (Chapter 4) indicates that heparin may also reduce the cell's capacity to internalise virus as well as specifically hindering virus attachment.

In the control spleen MLR, heparin reduced the effects of free virus, restoring the MLR to almost 50% of the control values. However, it did not entirely prevent the depressive effects of free virus. This is perhaps not surprising given the fact that HSV1 may enter cells, albeit less efficiently, by a heparin sulphate independent mechanism when this moiety is not available on the cell surface or when heparin is employed as an inhibitor [1].

We have confirmed previous observations that heparin reduced the ability of HSV1 to infect cells (Chapter 4, [1]). We have now demonstrated that this phenomenon not only applies to continuous cell lines but is also effective in hindering infection of primary EC cultures and in the MLR. We have also been able to show that heparin reduced the endocytic capacity of BHK cells, and by this additional means it may hinder HSV1 infection.

Using B114, a neutralising antibody for HSV1, we completely inhibited the effects of HSV1 in the MLR. This was employed in conjunction with replication-defective HSV1 mutants. HSV1/34 and HSV1gH (mutant) suppressed the ability of LC to induce proliferation in the MLR and oxidative mitogenesis. This indicated to us that HSV1 not only reduced the number of LC but also reduced their capacity to stimulate a proliferative response. However, on further analysis, we were able to determine that a significant proportion of these MHC Class II-positive cells were in fact not leucocytes, i.e. not LC. Previous reports have indicated that in an ongoing viral infection MHC Class II can be expressed on keratinocytes [138] and we assume that this was happening in our cultures. Virus infection produced two very distinct populations

of MHC Class II-positive leucocytes and non-leucocytes. Taking this into consideration, it appeared that the percentage proliferation with HSV1gH-infected EC correlated with the percentage of MHC Class II-positive leucocytes remaining at the end of the assay. Infection with HSV1/34 lead to a lower percentage of proliferation relative to the percentage of remaining LC, but we cannot rule out the possibility that virus released by these cells was not entirely blocked by the neutralising antibody.

We have shown that infection of EC with HSV1 reduced their stimulatory capacity by killing, rather than functionally impairing, the LC sub-population. The ultimate consequence of a productive HSV1 infection is death of the susceptible cell. In vivo this could occur before the LC has travelled to the lymph node and interacted with T cells. Alternatively, the virus may impair its ability to prime T cells if it does reach the lymph node. We detected no functional impairment assuming that the percentage of LC remaining at 48 or 96 h co-culture reflected the potential for stimulating oxidative mitogenesis or the MLR. Inaba *et al* [316] have indicated that the vast majority of lymphoid DC have clustered with T cells by 20-40 h after the initiation of the MLR. Furthermore, Austyn *et al* [308] have shown that IL2 production by responding T cells in oxidative mitogenesis replaced the need for DC after 6-20 h. It may therefore be the case that the decisive, DC-dependent events in these proliferative assays occur within the first hours. We were not able to determine the reduction of LC number during the entire course of oxidative mitogenesis and the MLR. This was partly because the stimulator population in the oxidative mitogenesis assay was syngeneic with the responder population, making stimulators DC impossible to differentiate from residual DC in the responder population. We were also limited by numbers of EC available for setting up the co-cultures. Future studies may utilise stimulator populations from congenic strains, which differ at loci other than the MHC, and can be distinguished from cells of the responder population. This would allow the characterisation of the LC population during the entire course of oxidative mitogenesis and perhaps identify a functional impairment, if any.

6.2.3 DC as 'natural adjuvants'.

We were fascinated by the possibility of using cells of the DC family as "natural adjuvants" of the immune system. This refers to the apparent ability of antigen-pulsed DC to initiate primary immune responses in vivo [262, 263, 266, 283]. These studies, except one [283], have concentrated on the immune response to protein antigens such as myoglobin. No previous studies have been undertaken which simultaneously assess the cell-mediated and antibody response to immunisation with HSV1-antigen pulsed DC administered in vivo.

HSV1 naturally encounters LC in the skin and our ultimate aim is to develop a method which would assess the capacity of LC to initiate the primary response to HSV1 in vivo. Our initial studies concentrated on splenic DC (sDC).

Our control experiments showed that purified sDC pulsed with HSV1 could induce secondary proliferation of lymph node and spleen cells from HSV1-primed mice. The levels of proliferation were similar whether we used live or inactivated HSV1, of the same immunising strain, to pulse the sDC. It is possible that the proliferation observed with live HSV1-pulsed sDC is that of $CD8^+$ T cells in the responding population, and that seen with uv-HSV-pulsed sDC is due to $CD4^+$ T cell subset. A recent publication [281] has suggested such a difference in the ability of sDC to induce influenza-specific responses in vitro, in that sDC pulsed with live, infectious influenza virus were efficient generators of MHC Class I-restricted $CD8^+$ CTL's. In contrast, pulsing with inactivated influenza resulted in the marked proliferation of the MHC Class II-restricted $CD4^+$ T cell subset.

We were further able to demonstrate that sDC, pulsed with UV-HSV1, generated a proliferative response of T cells isolated from the lymph nodes of footpad immunised animals (S.I.=13). No response was detectable in the spleen T cells of intravenously immunised animals. We consider it unlikely that the immunised sDC are homing inefficiently to the lymph nodes or the spleen. The migration of DC to the lymph nodes and spleen when administered subcutaneously and intravenously is well documented [190, 263, 317]. Also we have observed that sDC pulsed in FCS alone can efficiently prime mice when administered by either

route. We would suggest that the efficient immunisation that we, and others, have observed for protein antigens (Chapter 5, FCS results, [262, 263]) reflects the high concentrations of single antigen(s) available for sDC to internalise and process. Inaba *et al* [262, 263] used purified protein antigens, such as myoglobin (Mb) and keyhole limpet haemocyanin (KLH). In our system we have used FCS which is composed primarily of albumins. HSV1, however, is composed of a variety of antigens most of which may be processed and presented, but none of which occur in such high concentrations. The effective concentration of any single immunogen would be higher in protein-pulsed than virus-pulsed cultures, and we would therefore expect a more marked proliferative response resulting from immunisation with the former.

Infectious virus injected subcutaneously and intravenously generated high levels of HSV1 specific antibodies, but we were repeatedly unable to detect significant levels in the serum samples of UV-HSV1-pulsed sDC immunised mice. This contrasts with the observations of others [266, 283] which demonstrated specific antibody after immunisation with antigen-pulsed sDC. In both of these studies it is difficult to reconcile the generation of antibodies, which requires native antigen, by DC directly, which present only processed antigen. We have already discussed the findings of the first of these reports [266] (Chapter 1) and concluded that DC may have acted to induce T_h cells which were then available for specific B cells when native antigen was subsequently administered. In the second report [283] the authors detail the generation of TMV-specific antibodies by TMV-pulsed sDC. No native antigen is given after the sDC so from this it appears that sDC alone may prime for an antibody response. We could postulate that virus replication in, and exit from, the DC generated native virus but TMV is a single stranded RNA plant virus so it is unlikely to replicate in these cells. The possibility that DC regurgitated TMV antigen in its native form is improbable but not impossible. Using our system it may be possible to detect, in a manner similar to Sornasse *et al* [266], enhanced levels of HSV1-specific antibody after immunisation with HSV1-pulsed sDC providing we subsequently challenge with HSV1 or HSV1 antigen. This remains to be tested.

In summary, we have clearly demonstrated the ability of sDC to induce an HSV1-specific T cell response if administered subcutaneously to naive mice. At present, we cannot explain why no

T cell response was generated when these cells were administered intravenously. Very low numbers of immunised sDC may have reached the spleen. Of the few cells which reached the spleen, UV-HSV1 peptides may have been expressed in too low a concentration to have primed the resident T cell population. The efficacy of these cells in immunising animals may be related to the concentration of antigen used for pulsing. We detected no HSV1 specific antibody.

Our studies in this area, while limited, have made us more aware of the questions and problems which need to be addressed in future studies. Several areas are open to further investigation. Firstly, the response induced by sDC may be optimised using higher concentrations and different forms of HSV1, e.g. different strains of live virus. Care would have to be taken in selecting the strains of live HSV1, since as we have shown (Chapter 4) some may impair sDC function. Experiments of this nature would also have to control for the response initiated by live HSV1 virus released by infected sDC. Secondly, future approaches could also take advantage of the many HSV1 immunopurified proteins and peptides which are available. These may provide antigen in high enough concentrations to enhance the limited response we have observed. Thirdly, as far as humoral immunity is concerned, the antibody response needs to be determined in mice primed with HSV1-pulsed sDC and subsequently challenged with native antigen. Fourthly, we would want to apply these procedures to an analysis of LC populations (fresh and cultured). This is the cell type HSV1 would typically encounter in vivo and one which appears intimately involved with the initiation of an HSV1 specific response.

6.2.4 Conclusions

We have shown that the interaction of HSV1 with cells of the DC lineage is complex and may impair normal cellular function. Whilst we cannot rule out the possibility that our initial studies (Chapter 3 and 4) are confined to in vitro observations it seems obvious that uptake of HSV1 may not have the expected or naturally desired consequences for the cell. How these cells interact in vivo with HSV1, or indeed any virus, and the consequences of that interaction, needs to be studied in further detail. Given the fact that many cell types are permissive for HSV1 it will be interesting to discover if DC have properties in vivo which protect them from infection.

Perhaps this will be so. Perhaps this will join the long list of properties which render DC the pivotal accessory cell of the immune system.

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