THE EFFECTS OF ULTRAVIOLET-B IRRADIATION ON THE IMMUNE RESPONSE TO VIRAL INFECTIONS

Thesis submitted for the Degree of Doctor of Philosophy by

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I dedicate this thesis to my parents,
family and friends,
whose constant support and
encouragement have made this possible

Declaration

I declare that the studies presented here are the result of my own independent investigation, with the exception of the immunohistochemical staining in chapter 5 and the cytotoxicity and NK assays and flow cytometry in Chapter 7, which were carried out with the assistance of Bill Neil.

This work has not been submitted for canditure for any other degree.

Katrina E. Halliday

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Abstract

The reported depletion of the stratospheric ozone layer, which serves as a protective shield against ultraviolet B (UVB) light reaching the Earth's surface, has focussed much attention on the harmful effects of UV radiation. Exposure to UVB radiation induces both local and systemic immunosuppression. Whether this modulation in immunity may lead to a decreased resistance to systemic, as well as to cutaneous infections, and ultimately to an increased incidence and/or severity of infectious diseases remains unknown.

The contact hypersensitivity (CH) response is a commonly used model of cutaneous immunity, in particular to explore the integrity of the cutaneous immune response following UVB exposure. Irradiation of mice prior to sensitisation is known to result in suppression of the induction phase of CH. Studying the effects of UVR on the elicitation phase of CH would appear to better represent the natural sequence of events in human contact sensitisation than traditional experiments. A mouse model of CH, using oxazolone as the contact sensitiser, was tested and the results provided limited evidence to support the previously reported enhancement of CH by exposure of mice to UVB prior to challenge.

Murine herpesvirus-68 (MHV-68) is a natural pathogen of mice which causes an acute lung infection and subsequently establishes latency in B lymphocytes of the spleen. A protocol was established for the induction and elicitation of a DTH response to MHV-68 in C3H/HeN mice. Exposure of mice to a single suberythemal dose of UVB (96mJ/cm^2) three days prior to sensitisation resulted in a suppression of the DTH response (by approximately 40%) upon ear challenge. This suppression was specific for MHV-68 and was long-lasting (tested up to one month). UVB exposure also suppressed the elicitation phase of DTH in mice infected several days before UVB exposure and in latently infected mice. Preliminary data suggest that

exposure of mice to suberythemal UVB has no effect on the replication of MHV-68 in the lungs following primary infection and did not reactivate latent virus.

While the systemic immune responses generated in herpes simplex virus type 1 (HSV-1) infections have been well documented, little is known about the cytokines induced locally in the epidermis in response to the virus. These may be critical in determining the outcome of the infection and the subsequent interaction of the virus with the host during latency and recrudescence. Cytokine mRNA profiles in both an *in vitro* and an *in vivo* model of HSV infection were studied. A mouse keratinocyte cell line (PAM-212) and mouse back skin were infected with HSV-1 and RNA was extracted at various times thereafter from lysed cells or skin homogenates. Interleukin-1 (IL-1), IL-10 and tumour necrosis factor-α (TNF-α) mRNAs were assayed by semi-quantitative RT-PCR, with normalisation relative to β-actin mRNA signals. Most notably, the expression of IL-10 mRNA *in vitro* peaked at 12 hours post-infection, and an increase in mRNA expression in the skin was seen from 96 hours post-infection. This would suggest that HSV-1 is inducing keratinocytes in the epidermis to produce IL-10 and this may represent a possible immune evasion strategy of HSV-1 by diverting the immune response away from a Th1 type response, known to be effective for viral clearance. UVB exposure of PAM-212 cells induced IL-10 mRNA by 24 hours. The effect of UVB exposure on the synthesis of IL-10 mRNA by HSV-infected PAM-212 cells was also examined.

To study the effects of UVB exposure on the functional activity of human epidermal cells (EC) to act as antigen presenting cells, the mixed skin lymphocyte reaction (MSLR) was used. EC were obtained from suction blister roofs of volunteers. *In vitro* irradiation of EC appeared to suppress the MSLR to near background levels. *In vivo* irradiation of subjects with 0.5 minimal erythemal dose (MED) for seven days on the subsequent skin blister site, also caused some suppression of the MSLR. Urocanic acid (UCA) has been proposed as a photoreceptor for UV radiation and *cis-*

UCA mimics some of the effects of UVB on the immune system. Treatment of EC with *cis*-UCA *in vitro* caused suppression of the MSLR by an average of 71%.

Normal subjects, known to be seropositive for HSV, underwent a standard course of broadband UVB phototherapy, as used in the treatment of psoriasis. They received whole body irradiation three times a week, with incremental doses depending on skin type. Blood samples were taken before the start of treatment, after one week and after four weeks. The ability of these individuals to mount an HSV-specific cytotoxic T cell response was examined, using autologous B cell lines (prepared by infection with Epstein-Barr virus), infected with HSV-1 as targets. The cytotoxic response was not altered in all four subjects examined to date. However, the natural killer cell activity was suppressed within the first week of phototherapy, further suppressed at four weeks, but returned to the pre-irradiation value within a week after the final UVB exposure .

List of Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
AOO	acetone: olive oil
APC	antigen presenting cell
ATP	adenine triphosphate
BCG	bacillus Calmette-Guerin
BCL	B cell line
cDNA	complimentary deoxyribonucleic acid
CFC	chlorofluorocarbon
CH	contact hypersensitivity
CLA	cutaneous lymphocyte-associated antigen
CMM	cutaneous malignant melanoma
CMV	cytomegalovirus
con A	concanavalin A
cpm	counts per minute
CSF	colony stimulating factor
CTL	cytotoxic T cell
DAB	3,3'-diaminobenzidine tetrahydrachloride
DC	dendritic cell
DETC	dendritic epidermal T cell
DLN	draining lymph node
DNA	deoxyribonucleic acid
DNFB	dinitrofluorobenzene
dNTP	dinucleotide triphosphate
DTH	delayed type hypersensitivity
DTT	dithiothreitol
EBV	Epstein-Barr virus
EC	epidermal cell
EDTA	ethylene diamine tetra acetic acid
ELAM-1	endothelial cell-leucocyte adhesion molecule-1
ELISA	enzyme-linked immunosorbent assay
ETAF	epidermal thymocyte activating factor
EV	epidermodysplasia verruciformis
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor

GRO-α	growth-related gene product-alpha
HETE	hydroxyeicosatetraenoic acid
HHV-4	human herpes virus-4
HIV	human immunodeficiency virus
HPV	human papilloma virus
HSV	herpes simplex virus
HVS	herpesvirus saimiri
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IL-1α Ra	interleukin-1 alpha receptor antagonist
IP-10	γ-interferon-inducible protein
IU	international units
KLH	keyhole limpet haemocyanin
LC	Langerhans cell
LFA-1	lymphocyte function-associated antigen-1
LIF	leukaemia inhibitory factor
LN	lymph node
LPS	lipopolysaccharide
LT	leukotriene
LTR	long terminal repeat
MAIDS	murine acquired immunodeficiency syndrome
M-CSF	macrophage-colony stimulating factor
MED	minimal erythemal dose
MELR	mixed epidermal lymphocyte reaction
MHC	major histocompatability complex
MHV-68	murine herpesvirus-68
MIP-2	macrophage inflammatory protein-2
MLR	mixed lymphocyte reaction
M-MLV	moloney murine leukaemia virus
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
MSLR	mixed skin lymphocyte reaction
NK	natural killer
NMSC	non-melanoma skin cancer
OD	optical density
OXA	oxazolone
PAGE	polyacrylamide gel electrophoresis

PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pfu	plaque-forming unit
PG	prostaglandin
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethane sulphonyl fluoride
PNK	polynucleotide kinase
PUVA	psoralen with ultraviolet A therapy
PWM	pokeweed mitogen
rIL	recombinant interleukin
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SALT	skin-associated lymphoid tissue
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SPF	sun protection factor
TBS	tris-buffered saline
TCR	T cell receptor
TGF-β	transforming growth factor-beta
Th	T helper cell
Th1	T helper cell type-1
Th2	T helper cell type-2
TMAC	tetramethylammonium chloride
TNCB	trinitrochlorobenzene
TNF-α	tumour necrosis factor-alpha
T4N5	T4 endonuclease V
UCA	urocanic acid
UV	ultraviolet
UVB-R	UVB-resistant
UVB-S	UVB-susceptible
UVR	ultraviolet radiation
VCAM-1	vascular cell adhesion molecule-1
VEH	vehicle
VLA-4	very late antigen-4

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Chapter 1
Introduction

1.1 Skin Immune system

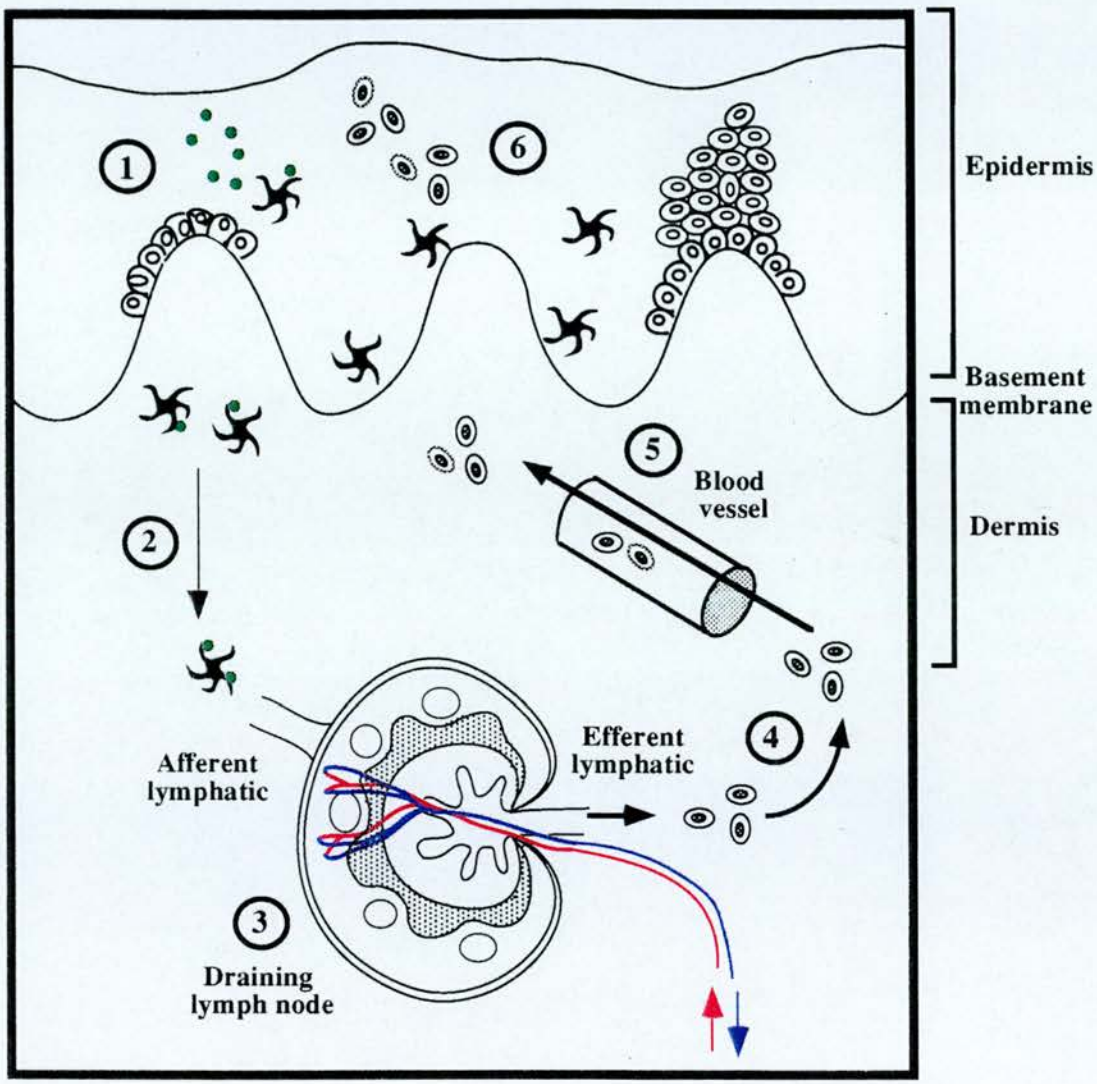
1.1.1 The concept of skin-associated lymphoid tissue

The skin is the largest organ of the body. It is in continual contact with the external environment and therefore, in addition to its roles in controlling water loss, and in temperature regulation, it encounters a wide range of potentially damaging factors such as ultraviolet radiation (UVR), chemicals and microorganisms. An extensive immune system is known to exist within the skin and this responds to a variety of stimuli including the presence of malignancies or microorganisms in the cutaneous microenvironment and exposure to certain chemicals. A comprehensive model for this skin immune system, known as skin-associated lymphoid tissue (SALT), has been proposed involving a network of antigen-presenting cells and cytokine-producing keratinocytes, poised to encounter foreign substances within the cutaneous compartment and then engage more central components of the immune system to provide specific immunity (Bos and Kapsenberg, 1986; Bos and Kapsenberg, 1993; Streilein, 1978; Streilein, 1991). SALT therefore provides the skin with the ability to detect and eliminate both neoplastic cells and exogenous pathogens. A schematic representation of the skin immune system , during the course of a cutaneous immune response is shown in Figure 1.1.

1.1.2 Langerhans' cells

Langerhans' cells (LC) were first observed in 1868 and are now known to be the major antigen-presenting cell of the skin (Langerhans, 1868). They are of bone-marrow origin and are defined by their dendritic morphology and possession of a unique intracytoplasmic organelle called a Birbeck granule, the function of which remains unclear

Figure 1.1 Schematic diagram of the skin immune system during the course of an immune response



Langerhans cells (LC) reside in the epidermis where they take up and process foreign antigen ( )(1). Antigen-loaded LC then migrate via the lymphatic vessels (2) to the draining lymph node, where antigen presentation to CD4+ T cells ( ) takes place in the paracortical (shaded) area (3). Antigen-specific T cells then leave the lymph node through the efferent lymphatic vessel, enter the bloodstream through the thoracic duct and migrate via the blood (4) to the site of antigen invasion. The T cells extravasate through high endothelial venules (5) and participate in the immune response to eliminate the antigen (6). Keratinocytes ( ), once activated, are able to secrete immunomodulatory cytokines and affect the immune response. Adapted from Henry & Tschachler (1996).

(Birbeck *et al.*, 1961). They are also distinguished by phenotype, being major histocompatability complex (MHC) class II^+, $CD45^+$, Thy-1^-, $CD3^-$, $CD11b^+$ and they possess Fc receptors for IgG and IgE. Langerhans cells were first thought to be involved in cutaneous immunity when studies of contact hypersensitivity responses, in which haptens were applied through sites naturally deficient in LC, such as mouse tail skin and hamster cheek pouch epithelium, revealed that specific unresponsiveness to the applied hapten occurred upon challenge (Bergstresser *et al.*, 1980; Streilein and Bergstresser, 1981; Toews *et al.*, 1980). The phenotype, function and role of LC in cutaneous immunity are discussed further in section 1.2 and in detail elsewhere (Lappin *et al.*, 1996b; Teunissen *et al.*, 1997a).

1.1.3 Dendritic cells other than Langerhans cells

Dermal dendritic cells (DC) comprise a mixed group of cells. Several populations of MHC class II^+ cells have been described in both murine and human skin, defined according to their phenotypes and it remains to be shown whether these cells represent separate antigen-presenting cell populations or migratory populations of DC trafficking to or from the epidermis (reviewed in Lappin *et al.*, 1996b). In human skin, dermal dendrocytes are factor $XIII^+$. This factor is involved in scab formation by the cross-linking of fibrin with structural proteins and therefore dermal dendrocytes may be important in wound healing. It has also been suggested that these cells are immature precursors of LC. The DC of the skin, as well as $CD1b^+$ macrophages, have been extensively reviewed elsewhere (Rowden, 1997).

1.1.4 Lymphocytes

Dendritic epidermal T cells (DETC) comprise between 0.8% to 2% of murine epidermal cells (Bergstresser *et al.*, 1993; Tigelaar *et al.*, 1990). They reside in the basal layer of the epidermis and express Thy-1 cell surface glycoprotein, CD3 and CD45, but lack class II MHC antigen (Bergstresser *et al.*, 1983; Tschachler *et al.*, 1983). The T cell lineage of

these cells is confirmed by evidence that the numbers of DETC are significantly reduced in athymic nude mice (Bergstresser *et al.*, 1983; Tschachler *et al.*, 1983). DETC do not express CD4 or CD8 and the CD3 antigen is predominantly associated with T cell receptor (TCR) γ/δ heterodimers. These cells do not participate in the recirculation pathways described for α/β T lymphocytes (see below). Their specific function remains unclear but it has been suggested that they are involved in the induction of tolerance (Welsh and Kripke, 1990). It has recently been suggested that cytokines such as interleukin-2 (IL-2), IL-7 and IL-15, secreted by neighbouring cells, promote the residence of DETC and regulate their immune function (Takashima and Bergstresser, 1996). Conversely, DETC are thought to regulate the function of neighbouring keratinocytes and LC by elaborating other growth factors and cytokines. The molecular and cell biology of DETC has been recently reviewed (Boismenu *et al.*, 1996).

To date no human T cell population comparable to the DETC has been identified. Most T cells of human skin reside in the dermis rather than the epidermis and predominantly express TCR α/β rather than γ/δ (Bos *et al.*, 1987; Foster *et al.*, 1990). Intradermal T cells consist of both $CD4^+$ and $CD8^+$ populations and are found clustered around postcapillary venules and skin appendages (Bos *et al.*, 1987). The vast majority of T cells in human skin are $CD45RO^+$, suggesting that they are memory cells (Foster *et al.*, 1990).

1.1.4.1 T helper cell subsets

The division of T helper (Th) cells into Th type 1 (Th1) and Th type 2 (Th2) on the basis of cytokine production profiles was originally described among mouse $CD4^+$ T cell clones (Mosmann *et al.*, 1986) and has since been described in human T cells (Del Prete *et al.*, 1988), although the synthesis of certain cytokines is not as tightly restricted to a particular subset in humans. Mouse Th1 cells produce IL-2, interferon-γ (IFN-γ) and tumour necrosis factor-β (TNF-β), whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10. IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α are secreted by

both Th1 and Th2 clones. The existence of Th1 and Th2 cytokine producing $CD8^+$ T cells is now recognised both in mice (Salgame *et al.*, 1991) and in humans (Croft *et al.*, 1994). At present no definitive cell surface marker exists to allow separation of Th1 and Th2 cells, although they do exhibit preferential expression of some activation markers. For example, CD30 is mainly expressed in Th2 and cytotoxic T cell clones, whereas lymphocyte activation gene 3 (LAG-3) preferentially associates with Th1-like cells (Romagnani, 1997). The cytokine profiles of these Th cell subsets correlate well with their function. Th1 cells are involved in cell-mediated responses such as delayed-type hypersensitivity (DTH) and cytotoxic responses, while Th2 cytokines induce antibody production, in particular IgE responses (Mosmann and Coffman, 1989).

Although many T cell clones and *in vivo* immune responses demonstrate a dramatic dichotomy in cytokine secretion patterns, it is now clear that the situation is of greater complexity than was first suggested. Other patterns of cytokine secretion are known to exist, for example cells designated Th0, which secrete both Th1 and Th2 cytokines, and Th3 cells which produce large amounts of transforming growth factor-β (TGF-β) (Mosmann and Coffman, 1989; Romagnani, 1991; Romagnani, 1996). This subset complexity may be due to quantitative differences in cytokine production at different developmental stages, or due to the existence of a continuum of possible cytokine secretion patterns with the Th1 and Th2 profiles as the extreme situations.

It is currently thought that both Th1 and Th2 cells derive from a common IL-2-producing precursor cell and that differentiation may proceed through an intermediate state in which multiple cytokines are expressed. Costimulatory molecules have also been suggested to selectively influence differentiation and antibodies against B7-1 and B7-2 have been shown to selectively inhibit the development of Th1 and Th2 cells respectively (Thompson, 1995), although the situation now appears to be more complex than this simple relationship suggests. The process of differentiation is also believed to be

controlled by cytokine secretion, probably by other cell types, such as macrophages and natural killer (NK) cells, especially in a primary immune response during which antigen-specific T cells will be present at low frequencies. For example, IL-4 is known to stimulate Th2 cell differentiation while, IFN-γ, IL-12 and TGF-β all enhance Th1 cell development (Muller *et al.*, 1995; Seder and Paul, 1994).

Cross-regulation is known to occur between the two subsets, with cytokines from each subset being inhibitory for the differentiation and effector function of the reciprocal subset. For example, IFN-γ selectively inhibits proliferation of Th2 cells and IL-10 inhibits Th1 cell cytokine synthesis (Mosmann and Coffman, 1989). Therefore once induced, the cytokines produced by each subset act to increase the number of effector cells committed to that particular subset, while acting in an antagonistic manner towards the induction of effector cells of the other Th subset.

1.1.4.2 Lymphocyte trafficking

Circulating skin-homing lymphocytes are crucial to the initiation and execution of cutaneous immune responses. Intercellular adhesion molecules expressed on dermal endothelial cells may mediate leucocyte adhesion, migration through vessel walls and trafficking into the dermis. T lymphocytes can adhere to endothelial cells and to keratinocytes specifically through lymphocyte function-associated antigen (LFA-1; CD11a/CD18) and intercellular adhesion molecule-1 (ICAM-1; CD54) binding. Endothelial cells constitutively express low levels of ICAM-1 but can be induced to express increased levels by IL-1, TNF-α and IFN-γ (Detmar *et al.*, 1992), as can keratinocytes by TNF-α and IFN-γ (Barker *et al.*, 1990). Cutaneous lymphocyte-associated antigen (CLA) is a carbohydrate epitope present on memory/effector T cells that infiltrate inflammed skin. E-selectin (CD62E), formerly termed ELAM-1 (endothelial cell-leucocyte adhesion molecule) is the ligand for CLA and is induced by inflammation on endothelial cells. Memory, but not resting T cells adhere to E-selectin (Shimizu *et al.*,

1991). This interaction may therefore also direct the movement of specific T lymphocytes into the skin (Fresno *et al.*, 1997; Picker *et al.*, 1991). It has been suggested that CLA has a homing function in directing the T cell to subsequently interact with LFA-1/ICAM-1 and/or very late antigen-4 (VLA-4)/vascular cell adhesion molecule-1 (VCAM-1), which results in enhanced adhesion and migration across cytokine-activated endothelial cells (Santamaria Babi *et al.*, 1995). Recently it has been demonstrated that Th1 but not Th2 cells are able to bind to E-selectin resulting in the selective recruitment of Th1 cells into inflammed skin, providing the first evidence of differential trafficking of these T helper cell subsets (Austrup *et al.*, 1997).

1.1.5 Keratinocytes

Keratinocytes comprise over 80% of epidermal cells, forming a stratified multilayered epithelium. Keratinocytes adjacent to the basement membrane are predominantly rapidly proliferating stem cells and, as they become committed to differentiation migrate through the suprabasal layer towards the stratum corneum, losing their capacity to proliferate as they do so. Terminally differentiated keratinocytes are shed from the stratum corneum as dead cornified squames. It is now known that in addition to their structural role, keratinocytes are immunologically active cells, being an important source of immunomodulatory cytokines.

Before the ability of LC to process native antigen was recognised, it had been suggested that keratinocytes were the source of processed antigen in the epidermis (Luger *et al.*, 1983; Streilein, 1991). It now seems unlikely that epidermal LC require such peptides, but the knowledge that keratinocytes can be induced to express class II MHC molecules by IFN-γ (Basham *et al.*, 1985; Nickoloff and Turka, 1994) and that they are able to provide accessory cell function to T cells previously stimulated with superantigens or anti-CD3 monoclonal antibody (Nickoloff and Turka, 1994), means that these cells are likely to play an important role as antigen presenting cells in secondary, but not primary immune

responses. The importance of keratinocytes as antigen-presenting cells stems from their sheer number in the epidermis. They are likely to play a role in situations such as that following UV exposure when LC are lost from the epithelium. The resulting environment is one in which epithelial cells may predominate in the initial immune response. Keratinocytes, when used as accessory cells, have been shown to induce T cells defective in IFN-γ production (Goodman *et al.*, 1994). This lack of IFN-γ production in keratinocyte-supported cultures is thought to be due to the failure of keratinocytes to produce IL-12 (Goodman *et al.*, 1994; Nickoloff and Turka, 1994). In the skin, failure of keratinocyte-supported responses to induce IFN-γ in T cells, resulting in the generation of Th2 cytokines, may play a role in the termination of cell-mediated immune responses such as the challenge phase of contact hypersensitivity (Nickoloff and Turka, 1994).

Keratinocytes constitutively secrete, or can be induced to secrete, a large number of cytokines that can affect the immune response, including both stimulatory and inhibitory molecules. One of the first pro-inflammatory factors shown to be produced by keratinocytes was originally described as epidermal thymocyte activating factor (ETAF) (Luger *et al.*, 1981), and is now known to be composed of a mixture of low molecular weight cytokines, namely IL-1, IL-8 and TNF-α. Keratinocytes produce mRNA for IL-1α and IL-1β but only IL-1α activity has been identified in culture. This is explained by the fact that keratinocytes lack a specific protease necessary to cleave the biologically inactive IL-1β into the active form (Mizutani *et al.*, 1991). In addition to pro-inflammatory cytokines such as IL-1 and TNF-α, keratinocytes are also able to produce growth factors for immunocompetent cells, chemoattractants, and also suppressive cytokines such as IL-10, the production of which remains controversial in human keratinocytes but which has been implicated in UV-induced immunosuppression (see section 1.5.3.2). The currently available data about keratinocyte-derived cytokines are summarised in Table 1.1.

Table 1.1 Keratinocyte-derived cytokines

Cytokine	[a]Constitutive expression		Expression after stimulation / activation of keratinocytes		Effect of UVB	References
	Murine	Human	Murine	Human		
ETAF	+	+	+ PMA; muramyl dipeptide		Increase in mouse and human protein *in vitro*; increase in murine protein *in vivo*	(Ansel *et al.*, 1983; Kupper *et al.*, 1986b; Luger *et al.*, 1981; Luger *et al.*, 1983b; Sauder *et al.*, 1982)
IL-1 α	+	+	+ LPS; (no production once terminally differen-tiated)	+ IL-1α; PMA; TGFα; contact allergen	Transient increase in human mRNA expression and protein; increase in murine protein at 24-48 hrs after low dose UVB	(Ansel *et al.*, 1988; Bell *et al.*, 1987; Enk and Katz, 1992c; Kondo *et al.*, 1994b; Kupper *et al.*, 1986b; Kupper *et al.*, 1987; Lee *et al.*, 1991; Partridge *et al.*, 1991)
IL-1 β		+ (inactive form)			Transient increase in human mRNA expression	(Kondo *et al.*, 1994b; Kupper *et al.*, 1986b; Kupper *et al.*, 1987; McKenzie and Sauder, 1990; Mizutani *et al.*, 1991)
IL-1 RA		+ intra-cellular		+ Ca^{2+}		(Bigler *et al.*, 1992)
IL-3	+/-		+/- PMA; conA; LPS		Increase in murine mRNA in sub-confluent cultures; decrease in mRNA in confluent cultures	(Danner and Luger, 1987; Gallo *et al.*, 1991; Luger *et al.*, 1986; Luger *et al.*, 1988; Peterseim *et al.*, 1993)
IL-6		+	+	+ TGF-β; TNF-α; GM-CSF; IL-6; IL-13; IL-1 α/β; PMA	Increase in human mRNA stability following UVB	(de Vos *et al.*, 1994; Derocq *et al.*, 1994; Kirnbauer *et al.*, 1989; Kupper *et al.*, 1989; Partridge *et al.*, 1991)
IL-7	+	+	+ LPS			(Heufler *et al.*, 1993; Matsue *et al.*, 1993)

continued overleaf

Table 1.1 continued

Cytokine	[a]Constitutive expression		Expression after stimulation / activation of keratinocytes		Effect of UVB	References
	Murine	Human	Murine	Human		
IL-8		+		+ IL-1, TNF-α	Induction of human mRNA and increase in protein production	(Kondo *et al.*, 1993; Larsen *et al.*, 1989; McKenzie *et al.*, 1991; Venner *et al.*, 1995)
IL-10	+	+/-	+ contact allergen	+/-	Upregulation of murine and human mRNA and protein	(Enk and Katz, 1992c; Enk *et al.*, 1995; Grewe *et al.*, 1995; Rivas and Ullrich, 1992; Teunissen *et al.*, 1994; Teunissen *et al.*, 1997)
IL-12		+ p35 chain mRNA only		+ p40 induced by contact allergens		(Aragane *et al.*, 1994; Muller *et al.*, 1994)
IL-13				+ IL-6		(Michel *et al.*, 1994)
IL-15		+		+ DNFB	Decrease in human mRNA in dose and time-dependent manner; increase in human mRNA and protein	(Barbulescu *et al.*, 1995; Blauvelt *et al.*, 1996; Mohamadzadeh *et al.*, 1995)
TNF-α			+ contact allergen	+ LPS; staphylococcal protein A and toxins	Increase in human mRNA and protein following *in vitro* UVB and *in vivo*	(Enk and Katz, 1992b; Ezepchuk *et al.*, 1996; Kock *et al.*, 1990)
TGF-α		+		+ IL-1α; TGF-β		(Lee *et al.*, 1991)
TGF-β		(+)			Increase in human mRNA and protein expression	(Lee *et al.*, 1997; McKenzie *et al.*, 1991)
IFN-α		+				(Yaar *et al.*, 1988)
IFN-γ				+ contact allergen		(Enk and Katz, 1992b; Howie *et al.*, 1996)

continued overleaf

Table 1.1 continued

Cytokine	[a]Constitutive expression		Expression after stimulation / activation of keratinocytes		Effect of UVB	References
	Murine	Human	Murine	Human		
G-CSF		+				(Denburg and Sauder, 1986; Sauder *et al.*, 1988)
M-CSF	+	+	+ LPS	+ LPS		(Chodakewitz *et al.*, 1990)
GM-CSF	+	+	+	+ IL-1	Increase in murine mRNA and protein	(Ansel *et al.*, 1990; Chodakewitz *et al.*, 1988; Denburg and Sauder, 1986; Gallo *et al.*, 1991; Kapp *et al.*, 1987; Kupper *et al.*, 1986a; Kupper *et al.*, 1988b; Nozaki *et al.*, 1991)
GRO-α		+		+	Increase in human mRNA and protein	(Venner *et al.*, 1995)
PDGF		+		+ TGF-β; IFN-γ		(Ansel *et al.*, 1990)
IP-10		-	+ contact allergen			(Enk and Katz, 1992b; Luster and Ravetch, 1987)
LIF		+		+ Ca^{2+}	Increase in human mRNA	(Ng *et al.*, 1992; Paglia *et al.*, 1996)
MIP-2			+ contact allergen			(Enk and Katz, 1992b)

[a] Constitutive protein production unless entry in brackets which indicates mRNA expression only

Abbreviations: conA, concanavalin A; DNFB, dinitrofluorobenzene; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IL-1α RA, interleukin-1 alpha receptor antagonist; IP-10, γ-interferon-inducible protein-10; LIF, leukaemia inhibitory factor; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein-2; M-CSF, macrophage colony-stimulating factor; PDGF, platelet-derived growth factor; PMA, phorbol-12-myristate-13-acetate; TGF, transforming growth factor; TNF, tumour necrosis factor.

1.1.6 Melanocytes

Melanocytes are neural crest-derived cells which are present in the basal layer of the epidermis and their primary function appears to be protection of the host from the detrimental effects of UV irradiation. This is mediated by the production and transfer of melanin to keratinocytes. Melanocytes can respond to a number of cytokine-mediated signals and once stimulated thay are able to secrete cytokines such as IL-1α, IL-1β, TGF-β and IL-8 (reviewed in Armstrong and Ansel, 1994).

1.1.7 Mast cells

These bone-marrow-derived cells are located in the skin in the vicinity of blood vessels but are rarely found in the epidermis. Mast cells store pre-formed enzymes, glycosaminoglycans and mediators of inflammation such as TNF-α and histamine. They secrete cytokines in a pattern similar to that of Th2 cells (see section 1.1.4.1), which favour the induction of IgE-dependent immune processes, and express the high affinity IgE receptor on their surface.

1.1.8 Humoral constituents of the skin immune system

In addition to the keratinocyte-derived cytokines previously discussed, other humoral factors, particularly eicosanoids, are thought to play a role in cutaneous immune responses. Eicosanoids are generated from arachidonic acid which is released from membrane phospholipids, either directly by phospholipase A activity or indirectly by phospholipase C. Arachidonic acid can then be metabolised either by the cyclooxygenase pathway to give prostaglandins (PG) and thromboxanes, or by the lipoxygenase pathway to give leukotrienes. Since the skin lacks the capacity to synthesise arachidonic acid, it must obtain it from endogenous sources (Iversen and Kragballe, 1997). The leukotriene LTB_4, prostaglandin PGE_2 and 12-HETE (12-hydroxyeicosatetraenoic acid) have all been demonstrated to have proinflammatory activity (Iversen and Kragballe, 1997) and in general all appear to modulate inflammatory response by potentiating the effects of other mediators.

Histamine is another important inflammatory mediator. It is involved physiologically in such processes as allergic responses, vasoconstriction and vasodilation but is also important in immunoregulation. Histamine influences the cytokine network, both by acting as a modulator of certain cytokine-receptor interactions and by being released as a target of cytokine action (Falus and Meretey, 1992). Large amounts of histamine are stored in mast cells located in the superficial dermis of the skin and are released upon appropriate stimulation (Benyon, 1989). However, human epidermal cell cultures have also been shown to produce significant amounts of histamine (Malaviya *et al.*, 1996). It was found that approximately 40% of subjects demonstrated high basal histamine levels and that histamine was induced in the skin of some individuals after exposure to UVB. These observations suggest that histamine release by epidermal keratinocyte may contribute significantly to skin inflammatory responses.

1.2 Cutaneous Immune Responses

1.2.1 Contact hypersensitivity

Induction of a contact hypersensitivity (CH) reaction follows application of a sensitising chemical to the skin (Streilein, 1991). These contact allergens are generally small, structurally simple and consequently not generally recognised by the immune system in their native form. However, the haptens are reactive with protein in the skin which they bind to, producing hapten-derivatised self-proteins. These act as epitopes and a hapten/protein-specific immune response is generated against them (reviewed inBour *et al.*, 1997). In order for such an immune response to be generated, the antigen must be taken up and processed by antigen-presenting cells, primarily LC, which migrate to the lymph node(s) draining the site of antigen encounter.

They present the antigen to antigen-specific $CD4^+$ T cells, which are induced to proliferate and differentiate into an effector population, which travels via the peripheral blood system to the initial site of infection (see Figure 1.1). These T cells then extravasate into the skin site where they serve to orchestrate the effector response and eliminate the antigen. It is believed that previously encountered antigens may evoke a response involving peripheral antigen presentation to recirculating memory T cells.

The CH response can be divided into the sensitisation (afferent) phase and the elicitation (efferent) phase. The former occurs at the first contact of the skin with a particular hapten and leads to the generation of hapten-specific T cells in the lymph node and their migration back into the skin. This sensitisation phase lasts 10-15 days in man and 5-7 days in the mouse. The elicitation phase occurs as a result of challenge with the same hapten and comprises of an early (1-2 hours) and a late (24-48 hours) phase of tissue swelling in mice (MacKenzie and Hillier, 1981; van Loveren *et al.*, 1983). The early phase is thought to involve the degranulation of mast cells and the release of vasoactive amines such as histamine and serotonin, which induce vasodilation and other physiological changes. These changes facilitate the entry of effector T lymphocytes which mediate the classical delayed type hypersensitivity reaction. This late efferent phase of CH peaks at 72 hours in man.

The antigen-processing pathway for CH responses is thought to model the mechanisms of antigen-processing for other antigens and CH responses are therefore commonly utilised as models in which to investigate the integrity of cutaneous immune responses. In most experimental protocols, challenge of the animal involves the application of a sub-inflammatory concentration of the same chemical used for sensitisation, to either the dorsum of the ears or to the footpad. These sites allow for easy quantification of the inflammatory response by measurement of the increase in thickness of the site. In humans

the CH response is generally measured by scoring features of inflammation such as erythema, oedema and epidermal necrosis (Tie *et al.*, 1995).

1.2.2 Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) responses are similar to CH responses, except that the antigen is usually a protein rather than a hapten and is administered intradermally. DTH inflammatory reactions are characterised by the infiltration of mononuclear cells into the dermis, with less cellular infiltration into the epidermis compared with CH responses.

1.2.3 Antigen processing

Langerhans cells are the main antigen-presenting cell (APC) of the cutaneous immune system. In order to be an effective APC, LC must have the ability to internalise and process antigen in the epidermis. This process takes place intracellularly in acidified endosomes / lysosomes. Freshly isolated murine LC are known to have phagocytic activity and have been shown to process and present the protein ovalbumin and *L.major* amastigotes, but this ability is lost during culture (Moll, 1993; Streilein and Grammer, 1989).

1.2.4 Langerhans' cell migration and differentiation

For effective induction of a primary immune response, antigen processed in the epidermis must be transported to the local draining lymph node (DLN) and be presented to antigen-specific T cell clones in order to stimulate proliferation and differentiation of these cells. LC are known to be able to migrate out of the skin in response to a number of stimuli such as UVB and skin-painting with contact sensitisers (Bergstresser *et al.*, 1980). Depletion of murine LC by such treatments results in a subsequent accumulation of DC in the lymph node draining the treated site (Kinnaird *et al.*, 1989; Moodycliffe *et al.*, 1992), attributed to an influx of LC and/or DC from the skin. The most compelling evidence for epidermal LC migration to the DLN after antigen challenge, comes from a model in which nude mice

were sensitised with the contact sensitiser fluorescein isothiocyanate (FITC) on an allogeneic skin graft. Birbeck granule-containing DC bearing the hapten could be recovered from the DLN of the nude mice and these cells were able to induce sensitisation to FITC upon injection into naive mice of the same haplotype as the graft donor (Kripke *et al.*, 1990). This confirmed that at least some of the antigen-presenting cells in the DLN are derived from the skin and are LC in origin.

A signal is required to initiate the migration of hapten-loaded LC to the DLN. Contact sensitisers, as well as UVB, cause the upregulation of cytokines including IL-1α, IL-3, IL-6, IL-8, GM-CSF, TNF-α and TGF-β by keratinocytes (Bos and Kapsenberg, 1993; Enk and Katz, 1992b; Koch *et al.*, 1990). Topical application of contact sensitisers to mice was shown to result in increased epidermal expression of IL-1α, IL-1β, GM-CSF, TNF-α, macrophage inflammatory protein-2 (MIP-2) and interferon-inducible protein-10 (IP-10) mRNA (Enk and Katz, 1992a; Enk and Katz, 1992b).

Message for TNF-α is also upregulated after exposure to contact sensitisers and UVB. Additionally, intradermal injection of TNF-α causes an accumulation of DC in the DLNs of mice (Cumberbatch and Kimber, 1995) and a decrease in epidermal LC numbers (Cumberbatch *et al.*, 1994), implicating TNF-α as an important signal for LC migration after sensitisation. Also pre-treatment of mice with neutralising antibodies to TNF-α before UV exposure, prevented the normal UV-induced accumulation of DC in the DLN (Moodycliffe *et al.*, 1994).

IL-1β has recently been implicated in the migration of LC from the epidermis and their accumulation in draining lymph nodes as DC (Cumberbatch *et al.*, 1997). Depletion of epidermal subsets revealed that IL-1β is mainly LC-derived and this cytokine was demonstrated to be an important initiator signal for the induction of contact sensitisation (Enk *et al.*, 1993a). IL-1β injected intradermally resulted in a similar cytokine profile to

that which occurred following sensitisation and a neutralising antibody to IL-1β was able to block sensitisation (Koch *et al.*, 1990).

The most notable difference between the effects of treatment of mouse ears with recombinant TNF-α and IL-1β, is the kinetics of the induced changes in LC frequency and DLN DC numbers. The delayed responses seen after IL-1β treatment have led to the hypothesis that IL-1β stimulates migration of LC secondary to a paracrine induction of increased TNF-α production and that it is this latter cytokine that acts directly upon local LC to stimulate their movement from the skin (Cumberbatch *et al.*, 1997). This hypothesis is supported by the fact that intradermal injection of mice with IL-1β results in a rapid and marked transcriptional activation of cutaneous TNF-α (Enk *et al.*, 1993a).

LC undergo morphological, phenotypical and functional changes as they migrate to the DLN, with molecules essential for antigen-presentation being upregulated as they mature. This process is believed to be mimicked by *in vitro* culture of LC and MHC class I and II are upregulated on cultured LC (Schuler and Steinman, 1985; Shimada *et al.*, 1987). MHC expression is increased on lymph node-isolated DC in comparison to epidermal LC (Cumberbatch *et al.*, 1991). ICAM-1 is also upregulated on cultured LC (Aiba *et al.*, 1993) and is thought to be important in providing the initial antigen-independent interaction between DC and T cells.

LC migration is thought to come about as a result of phenotypic alteration. For example, epithelial (E)-cadherin is expressed at high levels by LC (Blauvelt *et al.*, 1995; Tang *et al.*, 1993) and is believed to be the molecule through which LC bind to keratinocytes (Tang *et al.*, 1993). E-cadherin expression is down-regulated on LC during culture (Tang *et al.*, 1993) and on the sub-population of LC, determined morphologically and phenotypically to be activated, following epicutaneous application of contact allergen to murine skin (Schwarzenberger and Udey, 1996). Hence a signal such as TNF-α may cause down-

regulation of E-cadherin, reducing the adhesion between LC and keratinocyte, allowing the LC to move out of the epidermis. ICAM-1 and LFA-1 may also be involved in this process since intravenous injection of monoclonal antibodies against these molecules caused a reduction in the number of hapten-bearing DC found in the DLN after skin painting of the hapten, and an inhibition of the induction of CH resulted (Ma *et al.*, 1994). The expression of molecules such as CD44, VLA-4 and ICAM-1 on differentiated LC may also play a role in LC migration by their interaction with ligands expressed for example on high endothelial venules (Aiba *et al.*, 1993; Omary *et al.*, 1988; Springer, 1990).

1.2.5 Antigen presentation and induction of a cutaneous immune response

Once LC have migrated into the afferent lymphatics they are known as veiled cells (Knight *et al.*, 1982). They drain into the lymph node and localise in the paracortical region where they are called interdigitating cells because of the extensive contact of their dendritic projections with surrounding cells (reviewed inLappin *et al.*, 1996b). They present antigen in an MHC-restricted manner to T cells and upon receiving signals, both soluble and via the costimulatory molecules of the APC, antigen-specific T cells are induced to proliferate and differentiate (Budjoso *et al.*, 1989; McKeever *et al.*, 1992).

Antigen-independent adhesion takes place between T cells and APC prior to antigen-dependent clustering of these cells and this process may allow 'sampling' of different T cells (Inaba and Steinman, 1986). The interaction is thought to involve LFA-1 and ICAM-1 binding, since in a mouse model in which mutant APC expressed reduced levels of ICAM-1, these cells had a greatly impaired ability to present antigen to T cells (Dang *et al.*, 1990). This was restored upon reconstitution of ICAM-1 by transfection of the gene into the APC.

Functional effector cells are only generated from naive T cells if the cell receives signals, not only through the antigen-restricted TCR/MHC pathway, but also through non-antigen specific costimulatory pathways. Such pathways proposed to date include the interactions between CD28/CTLA-4 and molecules of the B7 family; CD2 and LFA-1 and heat stable antigen and its ligand. Soluble signals mediated by cytokines such as IL-1 and IL-2 are also important in T cell activation.

CD28 is expressed widely on both human and mouse resting T cells, while CTLA-4 expression is limited to activated T cells (June *et al.*, 1994). B7-1 (CD80) is not expressed constitutively on epidermal LC but is induced during culture (Inaba *et al.*, 1994; Larsen *et al.*, 1992; Symington *et al.*, 1993). B7-2 (CD86) is found constitutively at low levels on murine epidermal LC and is greatly up-regulated after 24 hours in culture (Inaba *et al.*, 1994). Expression of B7-2 is also found on peripheral blood DC (Young *et al.*, 1992), amongst other cells. Freshly isolated LC are less able to stimulate allo-responses than are cultured LC (Freeman *et al.*, 1991; Larsen *et al.*, 1992; Symington *et al.*, 1993). The ability of cultured LC to induce these responses is blocked using CTLA-4-Ig (Symington *et al.*, 1993). This is a fusion protein with the extracellular portion of CTLA-4 spliced to the constant region of human IgG1 and acts as a soluble ligand for B7-1 and B7-2 (Lane *et al.*, 1993). There is some evidence that B7-1 signalling induces Th1 type response, while B7-2 mediates Th2 response, although this hypothesis is still controversial (Corry *et al.*, 1994; Sayegh *et al.*, 1995).

After being stimulated to proliferate and differentiate, antigen-specific T cells leave the lymph node in the efferent lymph, enter the bloodstream via the thoracic duct and migrate preferentially to the skin where they orchestrate adaptive immune responses such as CH and DTH. A schematic representation of such a primary cutaneous immune response is shown in Figure 1.1.

Once the immune system has been exposed to a particular hapten, a pool of activated or semi-activated T cells specific for the antigen will exist. Therefore in a secondary response, for example upon subsequent hapten challenge, T cells could encounter the antigen in the periphery, presented by LC or by other APC populations such as B cells, macrophages or keratinocytes. Memory T cells (CD45RO-expressing) can be activated by a wide range of APC in contrast to naive T cells which require DC (Bradley *et al.*, 1993). It is thought that antigen-specific skin-homing T cells, once activated in the periphery, could initiate a cascade response, perhaps through cytokine production, resulting in the migration of further effector cells into the site of antigen challenge

1.2.6 Effector cells of CH/DTH

The effector phase of hypersensitivity is associated with the infiltration of T cells, as well as cells of the macrophage/monocyte lineage, into the dermis and epidermis (reviewed inBour *et al.*, 1997; Streilein, 1991). T cell-derived IFN-γ induces the activation of macrophages, causing them to produce pro-inflammatory cytokines and increase their phagocytic activity. Therefore the infiltration of T cells and macrophages into the skin is the main effector response and is responsible either directly or indirectly for the clincial features associated with these types of immune response.

The classical effector cell of CH, induced 24-48 hours after challenge is a $CD3^+$, $CD4^+$, IL-$2R^+$, TCRα/β^+ T cell (Ptak *et al.*, 1996). Experiments using CD4 knockout mice, in which reduced CH responses to DNFB were observed, confirmed the role of $CD4^+$ T cells in the CH response (Cavanagh *et al.*, 1996).

1.3 Ultraviolet (UV) radiation

1.3.1 UV wavelengths and sources

UV radiation (UVR) is commonly divided, depending on wavelength, into three parts, UVC (200-290 nm), UVB (290-315 nm) and UVA (315-400 nm). The division is somewhat arbitrary, although roughly corresponds to the behaviour of the UVR in the atmosphere. Approximately 9% of the solar radiation is in the UV waveband, although this is not what reaches the Earth, as UVR of wavelengths shorter than 280 nm are effectively filtered out by the atmospheric ozone. As the wavelengths increase more photons pass unhindered and UVR of wavelengths greater than 320 nm are hardly affected at all by ozone and follow the same path as visible light. The UV spectrum of the sun at ground level is dependent on geographical location, the time of day, month of year and also on weather, air pollution and reflection from the Earth's surface. Exposure to UVR for the majority of individuals is primarily from the sun, although the use of sunbeds and the phototherapy treatment of some skin conditions mean that artificial sources are also relevant. Throughout this study Philips TL-20 W/12 lamps are used as a source of broadband UV-B, the output spectrum of which is shown in Figure 2.1.

1.3.2 Environmental considerations

Since 1985 seasonal fluctuations in the ozone layer have been observed regularly in Antarctica and in some parts of the northern hemisphere where there is minimal air pollution (Farman *et al.*, 1985). These measurements, together with the environmental politics surrounding the discovery that chlorofluorocarbons (CFCs) were causing depletion of the ozone layer, focussed attention on the harmful effects of UV light. It is believed that any reduction in the atmospheric ozone layer will lead to increased environmental exposure, in particular to UVB which reaches the Earth's surface in amounts inversely proportional to the concentration of atmospheric ozone (Kerr and McElroy, 1993). The Convention for the Protection of the Ozone Layer, adopted in

Vienna in 1985, as well as the Montreal Protocol and the subsequent Copenhagen amendments of it, resulted in an international commitment to reduce the production and emission of ozone-damaging chemicals. As a result the increase in CFCs in the atmosphere appears to be levelling off, which may eventually lead to a recovery of the ozone layer.

1.3.3 Biological effects

Wavelengths in the UVB range are present in sunlight at a lower intensity than UVA (100-1000 times less), but have the largest biological effect per unit of irradiation. Concern is growing about the impact of increased UVR exposure on animal and plant ecosystems, as well as on humans. Investigations are currently being carried out on populations as diverse as Antarctic phytoplankton, thought to have decreased photosynthetic capability; Patagonian sheep suffering from UV-related eye diseases; and frog populations in Oregon, believed to be declining due to a combination of increased UV exposure and low photolyase activity (Baustein *et al.*, 1994; Nilsson, 1996). A direct link between increased UV exposure as a result of ozone depletion and any of these observations has not been made, but such studies continue in an attempt to identify any causal links and assess any potential risks.

In humans the effects of UVR exposure are known to be both positive and negative. UVR is critical for the photochemical formation of vitamin D3 in the skin, which is necessary for bone formation. In addition, as mentioned above, UVR has beneficial effects in the treatment of certain skin diseases such as psoriasis. In addition, tanning for cosmetic reasons is promoted in our society as a symbol of health and sun exposure is linked to a general feeling of well being. In contrast the sun can cause the development of erythema or 'sunburn', with redness resulting from vasodilation of the blood vessels in the skin. More seriously UV exposure has been linked to the development of both melanoma and non-melanoma skin cancers (discussed in section 1.4.1.1), cataract

formation and is known to contribute to a range of photoaging effects (Reviewed in Browder and Beers, 1993; Goettsch, 1995).

1.4 UVB and Immunosuppression

1.4.1 Role of UVB in photocarcinogenesis

1.4.1.1 Epidemiology of skin cancer

The incidence of both melanoma and non-melanoma skin cancers has been rising for the past 20 years with epidemiological evidence suggesting an association between exposure to sunlight and the development of cutaneous malignancies of all types (Last, 1993; McKenzie and Sauder, 1994). Attempts have been made to estimate future risks of skin cancer development, as a result of current ozone depletion, and to compare the effects of the restrictions specified under the Montreal Protocol and the Copenhagen Amendments (section 1.3.2) on this risk (Slaper *et al.*, 1996). Without the legislation of the Copenhagen Amendments, the risk model predicts a runaway increase in skin cancer, providing some evidence for the importance of such international measures.

Non-melanoma skin cancers (NMSC; basal cell carcinomas and squamous cell carcinomas) are the most common skin tumours in white populations. Sun exposure is one of the most important risk factors for NMSC, especially squamous cell carcinomas, as they occur predominantly on chronically exposed body regions. Fair skin is also a significant risk factor, probably due to a lack of protective pigment (Green and Battistuta, 1990; Vitaliano and Urbach, 1980).

Although cutaneous malignant melanoma (CMM) is a relatively rare tumour in comparison to NMSC, the incidence of CMM has increased rapidly in all white populations and in some countries, including Scotland, the incidence has doubled or tripled during the past thirty years (MacKie *et al.*, 1992; MacKie and Hole, 1996; Nilsson, 1996). However, a

less well established relationship exists between sun exposure and the development of CMM (Elwood, 1992). Immunocompetent hairless mice, if chronically exposed to UVB, never develop melanoma and outdoor workers are less at risk from melanoma than indoor 'white-collar' workers (de Gruijl, 1993). However, epidemiological evidence supports an association between episodes of childhood sunburn as well as occasional high exposures, for example from summer holidays abroad, with the development of melanoma later in life (Goettsch, 1995).

1.4.1.2 The role of UVB in tumourigenesis

The phenomenon of UV-induced modulation of tumour immunity was first identified by Kripke and co-workers who demonstrated that UV-induced skin tumours, transplanted to normal syngeneic mice, were rejected by these immunocompetent hosts. However the tumours grew progressively if the recipient had been UV-irradiated, with subcarcinogenic doses of UV radiation, prior to transplantation (Fisher and Kripke, 1977; Kripke, 1974; Kripke, 1976).

The majority of experimental models of UV-induced carcinogenesis have been carried out using the hairless mouse, in which irradiation induces mainly squamous cell carcinomas (de Gruijl and Forbes, 1995). Studies of melanocytic tumour development have been extremely limited since these tumours have only been induced by UV in fish (hybrids of the genus Xiphophorus) (Setlow *et al.*, 1989) and opossums (*Monodelphis domestica*) (Ley *et al.*, 1989). Kripke and coworkers have however recently developed a murine melanoma model which has allowed the role of the local microenvironment in the immunological control of tumour development to be examined (Donawho *et al.*, 1996).

The action spectrum for carcinogenicity in mice, depicting the wavelength dependence of squamous cell carcinoma induction in the hairless mouse (de Gruijl *et al.*, 1993), as well as that for non-melanoma skin cancer development in human skin, closely resemble the

human erythemal action spectrum (Parrish *et al.*, 1982). These action spectra show that UVB radiation is the most effective wavelength (the peak carcinogenicity per unit of ultraviolet exposure (J/m^2) occurs at 293nm). UVA has also been demonstrated to play a role in carcinogenesis and in fact there is a second peak in the action spectra for SCC induction in the UVA range (de Gruijl *et al.*, 1993). It is therefore an important factor to consider because of the high intensity of UVA in sunlight and the inability of certain sunscreens to block UVA wavelengths effectively.

1.4.1.3 UVB induction of tumours by DNA damage

DNA is known to absorb UV radiation in the range 230-300 nm (Kochevar, 1995). Damage can be caused as a result, including single strand breaks and most commonly, the formation of cyclobutyl pyrimidine dimers between adjacent pyrimidines on the same DNA strand (Beukers and Berends, 1960). These distort the secondary helical structure of the DNA molecule, blocking the processes of replication and transcription (reviewed inJung and Bohnert, 1995). Mutations result from transcriptional errors introduced during the course of repairing these alterations in DNA. The most superficial cells of the epidermis, which receive the greatest UV exposure, are committed to terminal differentiation as they move into the stratum corneum and therefore injury of these cells is not highly significant. However, mutation of the basal-layer stem cells may result in the development of malignancy. The process of excision repair is essential for the repair of UV-induced dimer photoproducts within cellular DNA.

Patients with the hereditary condition xeroderma pigmentosum can develop skin tumours at an early age by exposure to sunlight (Daya-Grojean *et al.*, 1995; Lambert *et al.*, 1995). Research has revealed that cells derived from these individuals have defective repair of UV-induced damage in cellular DNA (Cleaver, 1968). This indicates that DNA damage produced by sunlight is directly involved in the process of carcinogenesis. DNA absorbs UV light and the role of DNA damage in UVB-induced immunosuppression will be

discussed in section 1.5.1.1. However, the importance of UVB as an initiator of the process of tumourigenesis is thought to result from its ability to cause mutations in genes crucial for cell cycle regulation such as proto-oncogenes like *ras* and tumour suppressor genes such as p53. Cells in the epidermis will accumulate DNA errors replicated over years of sun exposure and these dysfunctional genes can eventually cause a malignant transformation. For example, without functional p53, cells are unable to arrest in the G1 phase of the cell cycle. G1 phase arrest is critical for DNA repair and cells unable to carry out these repair processes will accumulate DNA damage and subsequently have an increased risk of transformation. A high percentage of sunlight-induced human skin cancers and UV-induced murine skin cancers contain unique p53 mutations (Dumaz *et al.*, 1993; Kress, 1992; Sato *et al.*, 1993). Such mutations are thought to be an early event in UV-induced carcinogenesis, being detected in mouse skin months before the appearance of skin tumours (Ananthaswamy *et al.*, 1997). Examination of p53 gene sequences in individual basal and squamous cell carcinomas reveals that dimers of the same base composition at different sites can have quite different mutagenic potentials. Different regions of the p53 gene were found to be repaired at different rates in cultured human cells, with the sites of mutations observed in human cutaneous cancers being poorly repaired (Tornaletti and Pfeifer, 1994).

1.4.1.4 UVB promotion of tumours by suppression of immunosurveillance

It is thought that the skin immune system is important in the control of certain tumours by performing an immunosurveillance role, monitoring for changes associated with cellular transformation. In order for this surveillance mechanism to be broken, a transformed cell would need either to outgrow the immune response or the immune response itself would need to be weakened in some way. Some evidence that UV-induced immunosuppression plays a role in carcinogenesis comes from data collected from renal allograft recipients receiving immunosuppressive drugs. These individuals have a highly increased risk of developing squamous cell carcinomas, which are generally found on sun-exposed skin

sites (Hartevelt *et al.*, 1990). In addition over 92% of individuals with a previous history of basal/squamous cell carcinomas show susceptibility to UVB, as defined by a suppressed CH response following UVB exposure (Streilein *et al.*, 1994), compared to a 40% susceptibility frequency in the normal adult caucasian population (Yoshikawa *et al.*, 1990). Therefore UVB appears to have the potential to act both as a tumour initiator, by causing DNA damage, and as a tumour promotor by inhibiting tumour surveillance and hence has been described as a 'complete carcinogen'.

1.4.2 Suppression of CH and DTH

1.4.2.1 Contact hypersensitivity

Probably the most extensively studied immune reaction in the skin is that of contact hypersensitivity, described in section 1.2.1. In animal models, chemical allergens such as dinitrochlorobenzene (DNCB) and oxazolone (OXA) are used to induce contact sensitivity and these systems have been used to try to elucidate the mechanisms involved in UV-mediated immunosuppression.

UVR is known to cause suppression of CH responses both in mice (Moodycliffe *et al.*, 1994; Shimizu and Streilein, 1994a; Toews *et al.*, 1980) and in humans (Tie *et al.*, 1995). From early murine studies it was shown that exposure to sub-erythemal doses of UVB on four consecutive days resulted in a significant decrease in the number of Langerhans' cells in the exposed skin (Toews *et al.*, 1980). Exposed sites and also sites naturally deficient of LC such as tail skin, were unable to support the induction of CH. A direct correlation between LC numbers in the epidermis and the ability to induce CH was found. The immunosuppression induced in this system was confined to the irradiated site, a phenomenon known as local immunosuppression. In addition, the immunosuppression generated was demonstrated to be highly specific for DNCB, the sensitiser used. Since mice failed to respond to DNCB following resensitisation through unirradiated skin 14 days later, but retained the capacity to become sensitised to unrelated molecules, specific

tolerance was said to have been induced, rather than a general unresponsiveness. A similar situation has been demonstrated in humans, with UVB able to suppress CH to DNCB in certain individuals for up to 4 months. Tolerance was induced in approximately 30% of subjects who were exposed to DNCB through irradiated skin (Cooper *et al.*, 1992).

UVB-induced immunosuppression of the CH response is known to result in the generation of hapten-specific T suppressor cells in the spleens of various strains of mice, irrespective of whether they demonstrate suppressed CH responses following UV exposure or not (Glass *et al.*, 1990). This cell population, when adoptively transferred into naive syngeneic mice, induces suppression of the elicitation phase of CH responses (Elmets *et al.*, 1983; Glass *et al.*, 1990).

1.4.2.2 Local and systemic immunosuppression

Irradiation of mice with UVB suppresses CH either 'locally', when the contact sensitiser is applied to the UV-irradiated site (Streilein and Bergstresser, 1988; Toews *et al.*, 1980), or 'systemically', when the sensitiser is applied to a site distant from the site of UVB exposure (Noonan *et al.*, 1981). The timing of exposure to UVB in relation to sensitisation is now known to be a critical factor in determining whether local or systemic immunosuppression occurs. It was originally thought that this distinction was dependent on the UVB dosage, with high doses resulting in systemic suppression, but it has since been demonstrated that for a given strain of mouse, the UV dose-response for local and systemic immunosuppression is identical (Noonan and De Fabo, 1990). It was found that local suppression was initiated if the sensitiser was applied immediately, or up to two days after UV exposure, whilst systemic suppression was initiated only if sensitisation was delayed until three days after the last irradiation (Kripke and Morison, 1986; Noonan and De Fabo, 1990; Shimizu and Streilein, 1994b). It is thought that the mediators of systemic and local immunosuppression may be different. For example, treatment of mice with antiserum to TNF-α was protective against local CH impairment (Moodycliffe *et al.*,

1994) but not against systemic suppression of CH (Shimizu and Streilein, 1994a). Other mediators which have been implicated in the systemic suppression induced by UVB include *cis*-urocanic acid (UCA) and IL-10 which will be discussed in sections 1.5.1.2 and 1.5.3.2 respectively.

1.4.2.3 Resistant and susceptible phenotypes

Strain differences in the susceptibility to UVB induced immunosuppression of CH responses have been reported in mice (Noonan and Hoffman, 1994; Streilein and Bergstresser, 1988). There appear to be similar differences in susceptibility in humans (Cooper *et al.*, 1992; Tie *et al.*, 1995). In mice two main models have been used and various anomalies exist which result from the different protocols employed.

A model of local immunosuppression in which the mice are sensitised immediately following irradiation on the exposed site (Streilein and Bergstresser, 1988) identified two groups; UVB susceptible strains (UVB-S; C57BL/6 and C3H/HeN) that showed over 80% suppression of CH responses following UVB, and resistant mice (UVB-R; BALB/c) which showed less than 10% suppression. Matings of BALB/c with C3H/HeN mice produced F1 hybrids which proved to be UVB-S and so this trait appeared to be dominant. It was then demonstrated that C3H/HeJ mice were UVB-R. C3H/HeN and C3H/HeJ mice differ only at the *Lps* locus which regulates the production of proinflammatory cytokines (IL-1, IL-6 and TNF-α). It was therefore hypothesised that polymorphisms at genes regulating cytokines might be the basis of UVB-S.

Analysis of the effects of UVB radiation on CH induction in H-2 congenic mice suggest that the H-2 complex contains one or more loci governing UVB-S (Kurimoto and Streilein, 1994; Streilein and Bergstresser, 1988; Vincek *et al.*, 1993). Molecular genetic studies identified a series of alleles at the *Tnfα* locus and there is correlation between the *Tnfα* allele and the UVB-R trait. These polymorphisms reside at regulatory regions of the

Tnfα gene and it has therefore been hypothesised that UVB, either directly or indirectly, promotes activation of the *Tnfα* gene in UVB-exposed cutaneous cells. Therefore, UVB impairs CH induction in mice with a genetic predisposition. Susceptibility to this deleterious effect of UVB radiation appears to be dictated by alleles at polymorphic loci that regulate the production of proinflammatory cytokines, especially TNF-α. However, despite wide acceptance of this proposal that differences in the *Tnfα* locus confer resistance and susceptibility to UVB-induced immunosuppression, the evidence is not definitive and the mechanism remains speculative.

A model of systemic immunosuppression in which trinitrochlorobenzene (TNCB) was used as a sensitiser reported three subsets of mice; a high susceptible group, a group showing intermediate susceptibility and a low susceptible group (Noonan and Hoffman, 1994). This model employed much higher doses of UV and comparison of the results from the local and systemic studies showed a number of differences in the allocation of mouse strains between the groups.

Outbred human populations also seem to show differing susceptibilities to UVB-induced immunosuppression. Normal individuals were subjected to low-dose UVB and were then immediately sensitised with DNCB on the UVB-exposed site. Approximately 40% of the total population show a suppression of the CH to DNCB upon challenge (Tie *et al.*, 1995). Interestingly, a similar outcome was observed when subjects with deeply pigmented skin were subjected to an identical experimental protocol (Vermeer *et al.*, 1991). Similar findings have been reported by others (Cooper *et al.*, 1992), although the sensitising dose of DNCB utilised varied beween these studies, as did the UV irradiation regimen employed. Susceptibility to UVB-induced immunosuppression is in fact a risk factor in the development of cutaneous cancers (section 1.4.1.4). Among basal and squamous cell cancer patients, more than 90% displayed the UVB-S phenotype and all the

patients studied with malignant melanoma were judged to be UVB-S (Yoshikawa *et al.*, 1990).

1.4.3 Photoprotection from immunosuppression

Extensive research has led to the development of highly effective sunscreens, which are assessed by their ability to inhibit human erythema, expressed as the sun protection factor (SPF). Action spectra for human erythema indicate that UVB is 3-4 times more effective than UVA, and so despite the high UVA content of solar UVR, solar UVB is primarily the cause of sun-induced erythema. The majority of sunscreens therefore contain UVB blocking agents, although some do include UVA-absorbing molecules as well. Effective use of high SPF sunscreens can result in substantial changes in sun-exposure behaviour, particularly of those individuals with sun-sensitive skin types (I and II) who are enabled to receive prolonged solar exposure without burning. In addition, the use of sunscreens will alter the spectral distribution of UVR to which the skin is exposed. For example, use of UVB protection alone will allow individuals to become exposed to higher doses of UVA than they would normally, the consequences of which are unknown.

There are currently no reliable data on the long-term effects of the use of sunscreens on human health, and the scientific justification for their use comes from mouse models. Such studies have demonstrated that sunscreen application prior to UVR exposure can reduce photocarcinogenesis and photoaging (Harrison *et al.*, 1991; Wulf *et al.*, 1982). However, these experiments do not take into account any alterations in sun-exposure behaviour which may result from the use of sunscreens.

In order to protect against skin cancer, sunscreens should offer effective protection against DNA damage. This has a critical role in the development of skin cancer, as demonstrated by the propensity of subjects with xeroderma pigmentosum, who have a deficiency in excision repair, to develop squamous cell carcinoma (Kraemer, 1980; Lambert *et al.*,

1995). In addition, the incidence of skin cancer in mice is reduced by the topical application of a dimer-specific repair enzyme, T4 endonuclease V (T4N5), contained in liposomes, post-irradiation (Yarosh *et al.*, 1992). Current data, although limited, show that sunscreen application inhibits UVR-induced DNA damage (Ananthaswamy *et al.*, 1997; Freeman *et al.*, 1988; Wolf *et al.*, 1993c). However, there are conflicting results regarding the capacity of sunscreens to protect against the immunosuppressive effects of UVR. While some authors have reported that various sunscreens offer minimal protection against local and systemic suppression of CH in mice after chronic UV irradiation (Bestak *et al.*, 1995; Wolf *et al.*, 1993b), others have claimed that the immunoprotective capacity of some sunscreens exceeded their SPF with respect to local (Roberts and Beasley, 1995; Roberts and Beasley, 1997) and systemic (Roberts and Beasley, 1997) suppression of CH in mice or epidermal alloantigen-presenting capacity in a human skin explant system (Davenport *et al.*, 1997). The current literature is reviewed extensively elsewhere (Young and Walker, 1995). It appears that the ability of sunscreens to provide immunoprotection depends critically on the dose and wavelength(s) of UVR used, as well as the model system. Ultimately, the ability of sunscreens to protect against environmental UVR must be assessed in humans exposed to solar UV, using biologically relevant endpoints (Wolf and Kripke, 1996). In the meantime fundamental questions remain to be answered. In particular, how different wavelengths of UVR alter immune function and how best to assess immunoprotection. It is clear that sunscreens effective in preventing erythema are either ineffective or less effective at preventing UVR-induced immunosuppression, in spite of apparent protection against LC damage.

1.5 Mechanisms of UVB-induced Immunosuppression

1.5.1 Photoreceptors for UVB

1.5.1.1 DNA

In addition to its role as a tumour-inducer, in particular of non-melanoma skin cancer (section 1.4.1.3), there is evidence that DNA damage causes suppression of the immune response necessary for tumour surveillance, as discussed in section 1.4.1.4. When used to treat UV-irradiated mouse skin, T4N5 increased the rate of dimer removal. This treatment was able to prevent the UVB-induced suppression of CH and DTH following a single exposure to $10kJ/m^2$ of UVB (Kripke *et al.*, 1992). Spleen cells taken from these T4N5-treated, UVB irradiated mice failed to transfer immunosuppression of CH responses. It is currently not fully understood how DNA damage modulates immune responses at a distant site. However it has been proposed that DNA damage induces the release of immunoregulatory cytokines (Kripke *et al.*, 1992). Application of T4N5 in liposomes onto UV-irradiated skin of C3H mice also prevented the loss of LC from the skin and reduced the local immunosuppression caused by UVB (Wolf *et al.*, 1995). The T4N5 was detected in LC and DC in the DLN as well as in keratinocytes (Wolf *et al.*, 1995). Using a monoclonal antibody against cyclobutyl thymine dimers, it was demonstrated that cells containing these dimers were present in the DLN following a single dose of UVB irradiation, and were believed to have originated from the skin (Sontag *et al.*, 1995). These findings led to the hypothesis that immune cells contain DNA damage and serve as cellular targets of UV-induced local immunosuppression (reviewed in Vink *et al.*, 1996). It is hypothesised that UVR damages DNA in APC resulting in altered antigen presentation, while DNA damage in keratinocytes causes the release of immunomodulatory cytokines (O'Connor *et al.*, 1996), resulting in local and systemic immunosuppression respectively.

1.5.1.2 UCA

Urocanic acid (UCA) is synthesised as the *trans*-isomer from histidine, by the enzyme histidase, and is predominantly located in the stratum corneum of the skin where it accumulates. *Trans*-UCA represents 0.7% of the dry weight of the epidermis (Norval, 1996) and following irradiation it converts to the *cis*-isomer in a dose-dependent manner

until the photostationary state is reached with about equal quantities of the two isomers (Norval *et al.*, 1989). Once formed, *cis*-UCA persists in the epidermis for several weeks. It is found in sweat and suction blister fluid in humans following UV phototherapy treatment. After a single UVB exposure it is present transiently in the serum of mice (Moodycliffe *et al.*, 1993) and in the urine of human subjects for at least a week (Kammeyer *et al.*, 1997).

Urocanic acid was originally proposed as a photoreceptor for UV irradiation and as a mediator of the immunosuppressive effects of UV exposure when the absorption spectrum of *trans*-UCA was found to match the action spectrum for the inhibition of CH in mice, with a peak at 270 nm (De Fabo and Noonan, 1983). However, it has since been demonstrated that the *trans*- to *cis*- isomerisation actually occurs in the UVA range and at these wavelengths CH is not suppressed (Reeve *et al.*, 1994). More recently the action spectrum for the isomerisation of UCA in mouse skin *in vivo* has been produced which peaks at 300-310 nm (Gibbs *et al.*, 1993). Further evidence for the involvement of *cis*-UCA in UV-induced immunosuppression came from mice which congenitally lack the histidase gene and hence have only 10% of the normal UCA levels (De Fabo and Noonan, 1983). The parent strain mice develop UV-induced immunosuppression, whereas the histidase-deficient mice were found to be resistant to such suppression. Indeed, increased dietary levels of histidine, resulted in an increase in mouse skin UCA levels and enhanced UVB-induced immunosuppression of CH (Reilly and DeFabo, 1991).

A body of evidence exists which demonstrates that *cis*-UCA mimics some of the effects of UVB. These effects include depletion of LC from the epidermis (Kurimoto and Streilein, 1992; Moodycliffe *et al.*, 1993; Norval *et al.*, 1990) and suppression of the HSV-specific DTH response (Ross *et al.*, 1986; Ross *et al.*, 1988) as well as suppression of CH responses (Kondo *et al.*, 1995; Kurimoto and Streilein, 1992).

The production of a monoclonal antibody against *cis*-UCA has been useful in providing clearer evidence for the role of *cis*-UCA in immunosuppression (Moodycliffe *et al.*, 1993). For example, the reduction in LC number achieved by UVB exposure or topical application of *cis*-UCA was shown to be abrogated by pretreatment of mice with this monoclonal antibody (El-Ghorr and Norval, 1995). In addition the *cis*-UCA monoclonal antibody blocked the suppression of the HSV-specific DTH response induced by UVB (El-Ghorr and Norval, 1995) and the CH response to DNFB (Kondo *et al.*, 1995).

In contrast to UVB exposure, painting of *cis*-UCA onto mouse ears does not induce accumulation of DC in the DLN (Moodycliffe *et al.*, 1992) nor does intra-peritoneal injection of *cis*-UCA block UVB-induced DC accumulation (El-Ghorr and Norval, 1995). Therefore it would seem that although *cis*-UCA mimics many of the *in vivo* effects of UVB, the relationship between the photoisomerism of UCA and UVB-induced immunosuppression does not appear to be a direct or simple one.

To date there is no evidence to suggest the possibility of an inter-relationship between DNA and *cis*-UCA as chromophores, although there may be a common immunosuppresive mediator downstream in the immunosuppressive pathway that links the chromophores and the molecular events triggered by UVB exposure (Simon *et al.*, 1994b; Vink *et al.*, 1996).

1.5.2 Effect of UVB on cutaneous immune cells

1.5.2.1 Langerhans' cells

Both sub-erythemal and erythemal doses of UVB cause a decrease in the number of LC within human skin (approximately 20% and 70% respectively) (Cooper *et al.*, 1992). In addition, morphological changes of LC take place, such as loss of dendricity, as well as alterations in cell surface marker expression. UVB exposure of 5-10 mJ/cm^2 *in vitro* was demonstrated to inhibit the upregulation of ICAM-1 expression on LC which normally

takes place on culture (Tang and Udey, 1991). UVB has also been reported to inhibit the functional expression of B7-1 and B7-2 in a dose-dependent manner (Weiss *et al.*, 1995). These molecules are known to deliver important co-stimulatory signals through their interaction with CD28 and CTLA-4 on T cells. In contrast, other authors have demonstrated that the cell surface expression of membrane determinants necessary for effective antigen presentation, including ICAM-1 and B7-2 on DC in DLN, remains unaffected by *in vivo* immunosuppressive doses of UVB given prior to hapten sensitisation (Lappin *et al.*, 1996a).

UVB exposure also has effects on the functional abilities of epidermal LC. *In vitro* irradiation (10-20 mJ/cm^2) of both human epidermal cell suspensions and purified LC populations resulted in a significant inhibition of the ability of these cells to stimulate primary alloresponses and proliferation in response to mitogens and recall antigens (Rattis *et al.*, 1995). The exposure of murine epidermal cells to 2.5 mJ/cm^2 UVB *in vitro* reduced anti-CD3 induced T cell proliferation (Tang and Udey, 1991). However, contradictory evidence also exists with regards UVB effects on LC function. For example, the ability of DC, taken from the DLN of contact sensitised mice, to induce hapten-specific secondary T lymphocyte proliferative responses or mixed lymphocyte reactions *in vitro* were unaffected by the treatment of mice, prior to sensitisation, with immunosuppressive doses of UVB (Lappin *et al.*, 1996a).

The division of Th lymphocytes into Th1 and Th2 subsets was discussed in section 1.1.4.1. Briefly, Th1 cells are capable of stimulating cell-mediated inflammatory responses through the production of cytokines such as IL-2 and IFN-γ, while the activation of Th2 cells favours the induction of humoral responses, by way of Th2 cytokines such as IL-4, IL-5, IL-6 and IL-10. There is now evidence to suggest that UVB irradiation of LC results in an inability of these cells to stimulate Th1 cells. A schematic diagram of the effect of UVB on the antigen-presenting ability of LC is shown

in Figure 1.2. Purified LC were exposed to 20 mJ/cm^2 UVB and their ability to present keyhole limpet haemocyanin (KLH) to Th cell clones was studied. Irradiated LC induced tolerance (for up to 16 days) in antigen-specific Th1 cell clones but activated Th2 cells, specific for KLH (Simon *et al.*, 1992; Simon *et al.*, 1991).

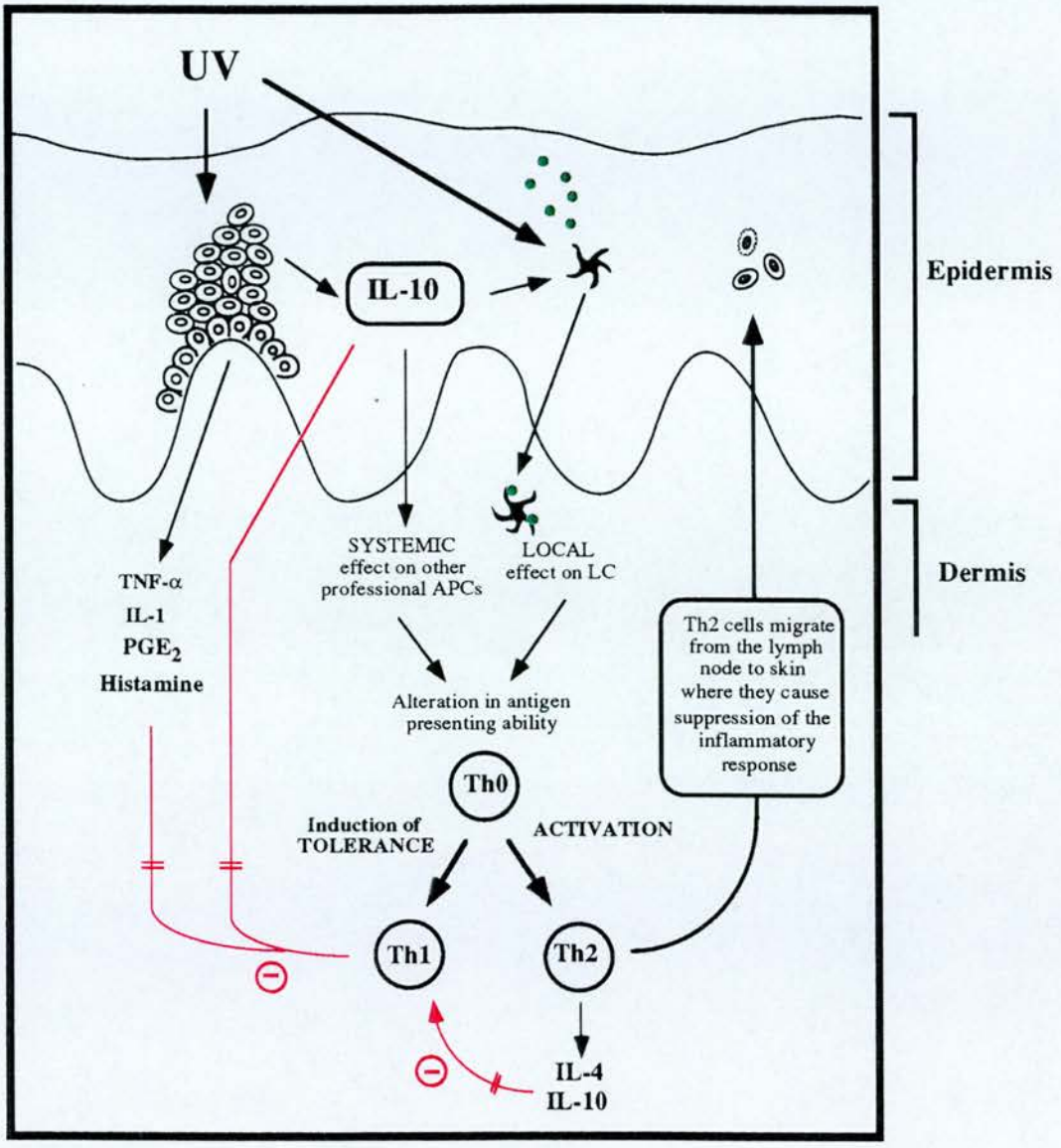
A study of cytokine production by lymph node cells following UVB irradiation has provided some *in vivo* evidence for UVB-mediated alterations of APC function. Mice were exposed to 20 mJ/cm^2 UVB on four consecutive days and were then sensitised with DNFB on the final day and 24 hours later. UVB-treated mice demonstrated suppressed CH responses, as well as reduced proliferative responses of lymph node cells to DNFB *in vitro* in comparison to unirradiated control mice (Simon *et al.*, 1994a). Most significantly, lymph node cells from UVB exposed mice produced lower levels of the Th1 cytokines IL-2 and IFN-γ when stimulated with DNFB *in vitro*.

1.5.2.2 Lymphocytes

Local UV irradiation of human skin has been shown to cause a decrease of intraepidermal CD3$^+$ T lymphocytes and an increase in dermal CD3$^+$ T cells by 24 hours post-irradiation. This infiltration reached a maximum at 48 hours and the CD3$^+$ cells were demonstrated to coexpress CD4 (Di Nuzzo *et al.*, 1996).

UV irradiation of mice for six consecutive days induces an alteration in the distribution of lymphocytes, with an increased migration of lymphocytes to the peripheral lymph nodes in UV-irradiated mice compared with unirradiated control mice (Spangrude *et al.*, 1983). Lymphocytes are retained for a prolonged period in the lymph nodes possibly as a result of PG released following UV irradiation, since PGs are known to induce efferent lymphatic blockage (Chung *et al.*, 1986). UV exposure may also affect the homing patterns of lymphocytes by altering the expression of adhesion molecules such as ICAM-1 which will affect the normal balance of cell recirculation (Norris *et al.*, 1990).

Figure 1.2 The effect of UV exposure on the generation of an immune response



UV is absorbed by cutaneous chromophores such as *trans*-UCA and DNA. It can alter an immune response either by direct action on LC or by activating keratinocytes causing the release of immunomodulatory cytokines or through other molecules like prostaglandins (PGE_2) and histamine. UV-exposed LC are able to activate Th2 cells but induce tolerance or clonal anergy in Th1 cells. Keratinocyte-derived cytokines, in particular IL-10, can affect the function of LC locally but can also act systemically to inhibit the antigen presentation ability of other professional antigen presenting cells (APCs). Suppression of Th1 cell function and the activation and expansion of Th2 cells result. The production of IL-4 and IL-10 by Th2 cells serves to further suppress Th1 cell function and hence suppress the induction of inflammatory immune responses. (Abbreviations and symbols as in Figure 1.1).

Finally, the effect of UVR on the function of circulating lymphocytes remains a controversial issue. A transient decrease in function has been reported following UVR irradiation or PUVA (8-methoxypsoralen (8-MOP) plus UVA light) photochemotherapy (Morison *et al.*, 1979; Rivers *et al.*, 1989). A significant suppression in lymphoproliferative responses has also been shown in patients after four weeks of TL-01 (narrow-band UVB) phototherapy (Jones *et al.*, 1996). Others have observed no significant alteration in the numbers and function, including lymphoproliferative response, of circulating lymphocyte subsets in patients with psoriasis undergoing broad-band UVB phototherapy (Gilmour *et al.*, 1993b; Jones *et al.*, 1996). In contrast, depressed NK cell activity was exhibited in psoriatic patients during treatment with broad-band UVB, narrow-band UVB and PUVA (Gilmour *et al.*, 1993a). The narrow-band UVB caused a significant reduction in NK cell activity after one week of treatment (Guckian *et al.*, 1995). The apparently conflicting data may result from variations in UV source, dose and timing of both the UV exposure and sample collection.

1.5.2.3 Keratinocytes

At the level of the single cell, UVB can induce the formation of apoptotic keratinocytes or 'sunburn cells', which appear in the epidermis. However, it would appear that keratinocytes are capable of playing a major role in UVB-induced immunosuppression, by means of the production of immunosuppressive soluble factors (see Table 1.1 and Figure 1.2). For example, sera taken from UVB-irradiated mice and also culture supernatants from UVB-irradiated epidermal cells have been shown to suppress CH when infused into normal recipients (Schwarz *et al.*, 1986; Swartz, 1984).

One of the first factors released by keratinocytes into the epidermis upon UV exposure is IL-1 which is stored in vast amounts in keratinocytes but is only released in response to a stimuli such as tissue injury or exposure to UVB (Ansel *et al.*, 1988; Kupper *et al.*, 1987; Murphy *et al.*, 1989). The fever response following sunburn has been attributed to UV-

induced increases in keratinocyte IL-1 production (Granstein and Sauder, 1987; Kupper *et al.*, 1987) and IL-1 has also been implicated as a suppressive factor for the induction of CH responses in mice (Schwarz *et al.*, 1986). In addition, murine keratinocytes have been shown to produce an inhibitor of IL-1, known as contra-IL-1. (Schwarz *et al.*, 1987) This inhibitor has been found in serum following UV exposure of mice (Schwarz *et al.*, 1988) and is thought to be important in limiting inflammatory responses. Although contra-IL-1 has not been sequenced to date, it is believed to be a soluble type II IL-1α receptor, based on its molecular weight. Type II receptors can bind IL-1α, Il-1β and IL-1 receptor antagonist (IL-1 Ra), which is structurally related to IL-1β.

IL-6 serum levels are elevated in individuals with sunburnt skin and this IL-6 is believed to be keratinocyte-derived (Urbanski *et al.*, 1990). Human keratinocyte IL-6 mRNA stability is increased by UVB irradiation suggesting possible post-transcriptional regulation of IL-6 gene expression after UV exposure (de Vos *et al.*, 1994). Keratinocyte-derived IL-6 is believed to partially mediate the 'sunburn' reaction including the acute phase response and is induced in response to pro-inflammatory cytokines such as TNF-α.

IL-8 production by human keratinocytes has been shown to be up-regulated immediately after UV-exposure *in vitro* (Kondo *et al.*, 1993), with significant levels found in cell supernatants 24 hours after irradiation. Evidence has since been provided that IL-8 is rapidly upregulated in human skin after UVB irradiation (Strickland *et al.*, 1997). Both mRNA and protein increased at 4 hour post-irradiation and reached a maximum between 8 and 24 hours. IL-8 is a pro-inflammatory cytokine and additionally is a chemoattractant for neutrophils. Neutrophil infiltration into UVB-irradiated human skin was first observed at 8 hours and then progressively increased (Strickland *et al.*, 1997). This provides some evidence that IL-8 may be acting as a chemoattractant for neutrophils following UVB irradiation

UVB radiation has also been shown to induce secretion of TNF-α and IL-10 by keratinocytes (Enk *et al.*, 1995; Grewe *et al.*, 1995; Kock *et al.*, 1990). Both of which are also induced in response to contact allergens (Enk and Katz, 1992b; Enk and Katz, 1992c). They have therefore been implicated as important mediators of UVB-induced immunosuppression and are discussed in sections 1.5.3.1 and 1.5.3.2 respectively.

Finally, keratinocytes demonstrate enhanced PG secretion following UVB exposure, in particular of PGE_2 which is also believed to contribute to some extent to UVB-induced immunosuppression. For example, treatment of mice with the prostaglandin inhibitor, indomethacin, has been shown to abrogate the capacity of UVB radiation to induce suppression of contact allergy in mice (Robertson *et al.*, 1987).

1.5.2.4 Macrophages

$CD11b^+$ macrophages are stimulated to appear in the epidermis of human skin between 2 and 3 days following *in vivo* exposure to 2-4 minimal erythemal doses (MED) UVB (Cooper *et al.*, 1985; Cooper *et al.*, 1986; Cooper *et al.*, 1992). They are ultrastructurally and phenotypically distinct from LC and have been shown to be potent producers of IL-10 in the epidermis (Kang *et al.*, 1994). The ability of murine splenic and peritoneal macrophages to phagocytose and inactivate bacteria following high-dose *in vivo* UVB exposure was found to be significantly suppressed when tested (Jeevan *et al.*, 1995).

Recently the action spectra for UV-induced inhibition of the mixed lymphocyte reaction (MLR) and mixed epidermal cell lymphocyte reaction (MECLR) were determined (Hurks *et al.*, 1995). Human peripheral blood mononuclear cells (PBMC) or epidermal cells were irradiated with monochromatic light of various wavelengths and then used as stimulator cells in the MLR or MECLR. The maximum of both spectra was found to lie at 254 nm, but both the MLR and MECLR were also sensitive to irradiation in the UVB range.

1.5.2.5 Endothelial cells

There is evidence to suggest that E-selectin expression is upregulated on blood vessels of the superior plexus venosus in humans by 6 hours following UV exposure of skin (Norris *et al.*, 1991). This upregulation peaks at 24 hours after irradiation and correlates with the accumulation of inflammatory neutrophils and macrophages.

1.5.2.6 Mast cells

Mast cells play an important role in the generation of the 'sunburn' response through the UV-mediated release of vasoactive substances. Mast cell degranulation is known to be induced by UVA and high dose UVB (Danno *et al.*, 1980) and histamine levels are elevated in venous blood taken from UV-exposed skin (Beissert and Granstein, 1997). Mast cells are located in the dermis of the skin and since UVB is thought to penetrate the skin poorly, it is likely that the effects of UVB on mast cells are mediated through the release of soluble mediators in the epidermis, such as IL-1 and PGs.

1.5.3 Soluble suppressor factors

1.5.3.1 TNF-α

Exposure to UVB radiation results in depletion of epidermal Langerhans' cells and an impairment of CH (Aberer *et al.*, 1982; Toews *et al.*, 1980). As described in section 1.4.2.3, TNF-α has been implicated as the key to the phenomenon of UVB susceptibility and resistance with respect to CH responses and hence the primary mediator of these deleterious effects (Piguet *et al.*, 1991). Intradermal injection of recombinant murine TNF-α has been shown to reproduce the effects of UVB described above and these effects can be abrogated by systemic treatment with anti-TNF-α antibody (Yoshikawa and Streilein, 1990). It is thought that TNF-α impairs CH induction by interfering with the ability of hapten-bearing Langerhans' cells to move to the DLN and hence stimulate activation of hapten-specific T cells. Streilein has hypothesised that epidermal

Langerhans' cells are immobilised in the epidermal compartment, based on evidence that TNF-α profoundly alters the functional properties of these cells, and hence they fail to arrive in the DLN in sufficient numbers during the 24 hours after irradiation (Streilein, 1991). The cellular source of this mediator of UVB immunosuppression is thought to be epidermal keratinocytes, which in culture produce large amounts of TNF-α following *in vitro* UVB exposure (Kock *et al.*, 1990). Increased TNF-α levels have been detected in the serum of humans 12 and 24 hours after a single total body UVB exposure, providing evidence for the *in vivo* release of TNF-α after UV exposure (Kock *et al.*, 1990).

1.5.3.2 IL-10

1.5.3.2 (i) Sources and function of IL-10

IL-10 was originally identified as 'cytokine synthesis inhibitory factor' because of its ability to inhibit the production of IFN-γ produced by Th1 cell clones (Fiorentino *et al.*, 1989). IL-10 is produced by B cells (Go *et al.*, 1990), mast cells (Thompson-Snipes *et al.*, 1991) and monocytes (de Waal Malefyt *et al.*, 1991), as well as by Th2 cell clones (Fiorentino *et al.*, 1989). It enhances B cell class II MHC expression and viability (Go *et al.*, 1990) and prevents antigen-specific T cell proliferation by inhibiting monocyte antigen-presenting capacity through down regulation of MHC class II expression (de Waal Malefyt *et al.*, 1991). In synergy with IL-2 and IL-4 it acts as a growth cofactor for mature and immature T cells (MacNeil *et al.*, 1990) and with IL-3 and IL-4, IL-10 induces rapid proliferation of mast cells (Thompson-Snipes *et al.*, 1991).

Both murine and human keratinocytes have been demonstrated to produce IL-10 (Enk and Katz, 1992c; Enk *et al.*, 1995; Rivas and Ullrich, 1992), although its production by human keratinocytes remains controversial (Jackson *et al.*, 1996b; Teunissen *et al.*, 1997b). The mRNA for IL-10 is detectable in human skin in Th2-like skin conditions such as poison ivy dermatitis, and also following tape-stripping (Nickoloff *et al.*, 1994). Throughout the epidermis, IL-10 mRNA was identified, predominantly localised within

the cytoplasm of keratinocytes. In contrast, no IL-10 mRNA was detected in normal or psoriatic plaque keratinocytes (Nickoloff *et al.*, 1994). Another study could not detect IL-10 gene expression in human keratinocytes either constitutively or following stimulation with IL-1β, IL-6, IL-8, TNF-α IFN-γ, retinoic acid, osmotic stress or UV irradiation (Ried *et al.*, 1994). Extensive investigation by Teunissen (Teunissen *et al.*, 1994; Teunissen *et al.*, 1997b) also failed to detect either IL-10 mRNA or protein in human keratinocytes or in the keratinocyte cell lines HaCaT and A431. It has been argued that contaminating melanocytes in human epidermal cell cultures are responsible for the production of IL-10 mRNA (Teunissen *et al.*, 1997b).

The ability of murine keratinocytes to synthesise IL-10 is generally accepted. Cell depletion studies have been used to implicate keratinocytes as the main source of IL-10 mRNA in the murine epidermis following application of contact allergens (Enk and Katz, 1992c) and IL-10 mRNA expression has been shown to occur late in the induction phase of CH in mice (Enk and Katz, 1995) . IL-10 protein was detectable in keratinocyte cultures derived from skin of mice of different strains, and also from the mouse keratinocyte cell line PAM-212 following hapten stimulation (Enk and Katz, 1992c).

1.5.3.2 (ii) Induction of IL-10 by UV irradiation

Both murine and human keratinocytes have been shown to produce IL-10 in response to UVB irradiation (Enk *et al.*, 1995; Rivas and Ullrich, 1992). Supernatants from UV-irradiated keratinocytes were shown to contain IL-10 bioactivity as determined by their ability to suppress IFN-γ production by antigen-stimulated Th1 cells and anti-IL-10 monoclonal antibodies were able to block these suppressive effects.

Following UVB irradiation, $CD11b^+$ macrophages infiltrating the epidermis, have also been demonstrated to produce both IL-10 mRNA and protein and were shown to secrete 200-400 fold higher levels of protein than the ($CD11b^-$) keratinocytes (Kang *et al.*, 1994).

Since the cellular infiltration of macrophages into the epidermis is one of the major events in CH and DTH responses, the production of IL-10 by these cells, in addition to the keratinocyte, may play an important role in the down-regulation of the inflammatory response.

1.5.3.2 (iii) The role of IL-10 in suppression of CH and DTH responses

IL-10 has been implicated as a mediator of UVB-induced suppression of CH and DTH responses. IL-10 gene-targetted (IL-10T) mice, lacking expression of IL-10, exhibit a normal DTH response following UVB treatment in contrast to the suppressed response seen in normal mice (Beissert *et al.*, 1996). However, UVB exposure could suppress the induction of CH to a hapten applied at a distant non-irradiated site in these IL-10 T mice, supporting the concept that the regulatory pathways of CH and DTH responses which UV affects differ in some way.

Recombinant IL-10 (rIL-10), when injected intra-peritoneally into mice, suppressed their ability to be sensitised to trinitrophenyl-coupled spleen cells for a DTH response (Schwarz *et al.*, 1994). Furthermore, administration of neutralising antibodies to IL-10 largely, but not completely, inhibited the ability of UVB irradiation to suppress sensitisation to alloantigens (Rivas and Ullrich, 1994). Treatment of mice with rIL-10 resulted in the blocking of the elicitation phase of DTH as well as the sensitisation phase (Schwarz *et al.*, 1994).

In contrast to the DTH response, injection of rIL-10 into mice blocks the effector phase but not the induction phase of the CH response (Schwarz *et al.*, 1994). This effect is only seen if IL-10 is administered at least 12 hours before hapten challenge, which suggests that IL-10 works indirectly through the action of other mediators. An exaggerated CH response was mounted by IL-10T mice, in terms of both magnitude and duration of the response (Berg *et al.*, 1995). Conversely, exogenous IL-10, when injected into mouse

ears was found to be effective in reducing ear swelling, leukocyte infiltration and IFN-γ mRNA expression in DNFB-sensitised and challenged mice (Kondo *et al.*, 1994).

A recent study has implicated IL-10 as an important mediator in hapten-specific tolerance induced by acute low-dose UVB exposure (Niizeki and Streilein, 1997). While no role was found for IL-10 as a mediator of the deleterious effects of UVB on CH, using this model system, UVB-dependent IL-10 production in the skin was found to be effective in promoting tolerance to a contact sensitiser. Neutralising antibodies to IL-10 significantly reversed the tolerance induced by UV irradiation.

Production of IL-10 by keratinocytes is markedly increased by application of contact sensitisers, but irritants and tolerogens do not have the same effect (Enk and Katz, 1992c). The signal strength of IL-10 mRNA in response to antigen was found to increase at 4-6 hours, reaching maximal strength 12 hours after allergen application (Enk and Katz, 1992c). This increase in IL-10 mRNA signal strength was accompanied by abrogation of T cell-derived IFN-γ and IP-10 mRNA signals suggesting a possible counter-regulatory role for IL-10 in skin DTH reactions.

In a separate study IL-10 protein production was found to peak at 10-14 hours after antigenic challenge (Ferguson *et al.*, 1994). This would seem to indicate that in such secondary responses, IL-10 production corresponds with the late induction of, or peak in the inflammatory response, providing an anti-inflammatory effect to limit the response. A neutralising IL-10 antibody prolonged the CH response to 72 hours which upholds a role for IL-10 in the down-regulation of CH responses. This anti-inflammatory activity of IL-10 may occur through the down-regulation of macrophage-derived pro-inflammatory and chemotactic cytokines and probably serves to minimise inflammatory tissue damage. IL-4 has also been implicated as an important down-modulator of inflammation. Sensitised mice have been shown to have significantly elevated IL-4 mRNA levels compared to naive

mice, after challenge with a contact sensitising agent (Asada *et al.*, 1997). Treatment of mice with anti-IL-4 antibody before challenge resulted in increased ear swelling 24 hours after challenge compared to mice pretreated with isotype control antibody.

UVB exposure has been shown to induce IL-10 mRNA transcription in keratinocytes 8 hours after irradiation (Enk *et al.*, 1995). If sensitisation takes place through an irradiated site, the presence of UVB-induced IL-10 means that LC take up and process antigen in an IL-10 rich environment which can affect the antigen-presenting cell function of the LC (see below). The role of UVB-induced IL-10 in a secondary response seems clearer. Here IL-10 is present 4-24 hrs after challenge and therefore this anti-inflammatory signal will prevent the inflammatory response from starting, hence causing the characteristic immunosuppression.

1.5.3.2 (iv) The effect of IL-10 on antigen presentation

Pretreatment of LC with UV-irradiated PAM-212 supernatants, but not with mock-irradiated cell supernatants inhibited the ability of LC-enriched epidermal cells to elicit a DTH response to tumour-associated antigens (Beissert *et al.*, 1995). The role of IL-10 in this keratinocyte-mediated suppression was tested using neutralising IL-10 antibodies which confirmed that IL-10 in the UV-irradiated supernatants was responsible for the inhibition of antigen presentation both for the induction and elicitation of immunity. Other experiments involving the pre-treatment of APC with IL-10 showed that IL-10 acts principally on the APC to inhibit IFN-γ production by Th1 clones (Fiorentino *et al.*, 1991). A more recent study has been carried out in which it was found that both IL-10-pretreated LC and untreated LC were able to induce Th2 cell proliferation equally well, while IL-10-treated LC were essentially unable to induce Th1 cell proliferation in response to native protein or peptide antigen and instead induced anergy in these Th1 clones (Enk *et al.*, 1993b). From studies of LC it now seems likely that IL-10 prevents upregulation of costimulatory molecules necessary for T cell activation and so tolerance results (Th1-

specific), although there is no evidence of this to date. Since IL-10 is induced late in the cytokine cascade initiated during the generation of a primary immune response in the skin, it is likely that IL-10 exerts inhibitory effects late in the inflammatory response. It may convert LC migrating to the DLN late in the inflammatory response to tolerising cells and so potentially limit the number of antigen-specific T cells activated (Enk *et al.*, 1993b). The IL-10-mediated effects of UVR on the generation of a cutaneous immune response are shown schematically in Figure 1.2.

1.6 Herpes Simplex Virus

1.6.1 Herpes simplex virus and its pathogenesis

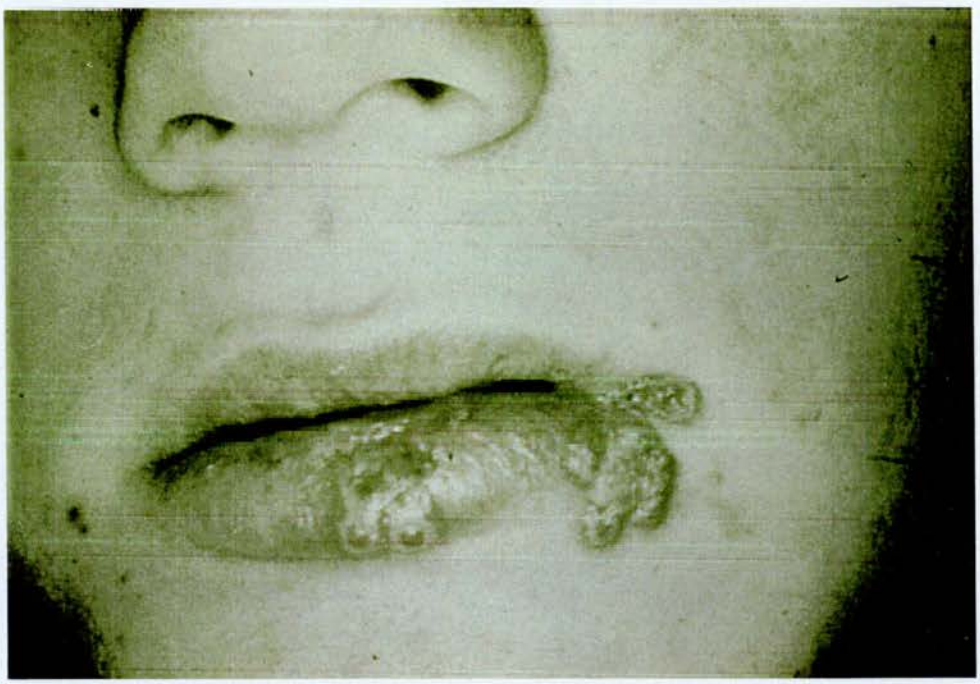
Herpes simplex virus (HSV) has all the unifying features of the herpes virus family, including an enveloped icosahedral capsid of approximately 100 nm in diameter, containing a linear double-stranded DNA genome of approximately 152 kilobase pairs. This genome encodes approximately 80 proteins, including mRNA/DNA synthesis regulatory proteins and enzymes that promote viral DNA replication. HSV is a prototype member of the α-herpesvirinae subgroup which are able to replicate in a wide variety of cultured cells and to form infective virions rapidly.

Herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are closely related viruses which are morphologically identical and have approximately 46% DNA homology (Kieff *et al.*, 1972). Both initially infect and replicate in mucoepithelial cells and then establish latent infection of the innervating neurons. HSV-1 is usually associated with orolabial infections, while HSV-2 is, in general, associated with genital infections. The natural history of a particular HSV infection depends on a complex interaction between virus and host.

The primary exposure to HSV-1 generally occurs at an early age, with the virus acquired from infectious saliva. Commonly an asymptomatic infection results from the interaction between host and virus, as the high prevalence of HSV antibodies in individuals with no history of clinical symptoms illustrates. This suggests that host responses are generally effective in controlling the primary disease (Gibson *et al.*, 1990; Johnson *et al.*, 1989). Only a minority of individuals present with clinical symptoms during primary infection and, of these, most develop relatively mild and localised vesicular lesions on the mucous membrane ('cold sores') which ulcerate liberating progeny virus and then heal after several days. It is a localised process but the virus very rapidly finds sensory nerve terminals which have virus receptors and penetrates into the nerve axon. In this site the virus was previously thought to be inaccessible to the immune system. However, despite obvious constraints on the immune system within such tissue, there is increasing evidence that an active immune response exists in latently infected ganglia (Shimeld *et al.*, 1995) Recovery from the primary infection is associated with the establishment of viral latency in the dorsal route ganglia of the nerves which serve the site of the original lesion (Stevens and Cook, 1971).

At intervals there may be reactivation of the virus in the nervous tissue. The virus then moves to the peripheral site, either initiating a lesion in the neurodermatome relating to the particular ganglion (recrudescence) or not causing an observable clinical lesion (recurrence). Certain common triggering factors for recrudescence are recognised in human subjects including exposure to UV light (see section 1.8.1.1), fever, skin trauma and immunosuppression. A typical human recrudescent orofacial HSV-1 lesion is shown in Figure 1.3. Recrudescent herpetic infections are generally mild and limited to the same anatomical location as the primary infection. Exceptions are the comparatively rare severe and/or disseminated HSV disease, mainly restricted to individuals who are in some way immunocompromised. For example, neonates, patients with Hodgkin's lymphoma, individuals suffering from severe burns (Kohl and Erisson, 1982) and those undergoing

Figure 1.3 A typical human recrudescent orofacial HSV lesion



the immunosuppressive regimen associated with organ transplantation (Ho, 1977; Pass *et al.*, 1979; Rand *et al.*, 1976). Such observations naturally lead to the conclusion that the control of HSV disease, including periodic recrudescence, is a function of the immune system.

1.6.2 Immune Response to HSV

Observations from patients with a range of clinical conditions have given insight into the immune mechanisms involved in HSV infections. For example, patients with depressed T cell function may develop severe HSV disease, whereas individuals with an immunoglobulin defect appear competent to control HSV infections (Corey and Spear, 1986). Also human immunodeficiency virus-1 (HIV-1) infected individuals, in which $CD4^+$ T cells are depleted as a result of this infection, frequently develop severe HSV lesions (Siegal *et al.*, 1981). There is a high dependency on animal models to provide further information about immune mechanisms that act against HSV infection, based on these clinical observations (Wildy and Gell, 1985). They have confirmed that cell-mediated immune responses are crucial in the clearance and recovery from HSV infection, while antibody responses seem to be less important.

1.6.2.1 Local immune responses

HSV infections are, in general, confined to the epidermis of the skin and therefore the local elements of the skin immune system are critical in control of the infection and elimination of the virus. It is likely that LC are involved in initiating the immune response to the virus. In fact, the pathogenicity of HSV-1 in mice has been shown to be dependent on epidermal LC density (Sprecher and Becker, 1987; Sprecher and Becker, 1989). It has been shown that the epidermis is unable to initiate a rapid specific T cell response to HSV *in situ* (Williams *et al.*, 1991). However, LC are able to efficiently present antigen to HSV-specific primed T cells upon secondary challenge, for example during a recurrent infection. Therefore in a primary response, LC are thought to take up the viral antigens

and travel to the DLN where presentation to T cells takes place. However, there is evidence that LC can become infected with HSV which has implications for the systemic spread of the virus.

The following events are then believed to occur following sensitisation, starting with the recirculation of activated T cells to the skin (reviewed inNash and Cambouropoulos, 1993). The infiltrating T cells secrete IFN-γ which results in the expression of MHC class II antigens and ICAM-1 on keratinocytes, and the release of IL-1 from LC and keratinocytes. Expression of adhesion molecules on keratinocytes may enhance T cell/keratinocyte interactions and the migration of $CD8^+$ lymphocytes into the epidermis found late in HSV lesions. Within the epidermis there is a release of inflammatory mediators and cytokines, such as IL-1, which attract effector cells and amplify the immune response. It is known that both cytotoxic and DTH responses are required for the efficient clearance of HSV from the skin (Martin and Rouse, 1987; Wildy and Gell, 1985). Helper T cells may act by producing lymphokines which attract and activate cytotoxic T lymphocytes (CTL), NK cells and macrophages. Infected cells can be lysed by CTL which may produce TNF-β. Macrophages are found in advanced necrotic lesions and are important in the control of infection (Morahan *et al.*, 1985). They exhibit intrinsic resistance to HSV replication and function to ingest and inactivate HSV, destroy infected cells and inhibit viral spread between cells. In addition they produce mediators which serve to enhance inflammatory responses to HSV, as well as TNF-α, thought to cause lysis of virally-infected cells and inhibit viral growth. NK cells and neutrophils are also found in the epidermal infiltrate, while few B cells have been observed (Heng *et al.*, 1989).

1.6.2.2 Systemic immune responses

Most human subjects develop high antibody titres to the virus during a primary infection, which remain elevated throughout the life of the subject, and vary little even with

recrudescences (Vestey *et al.*, 1989). Although high antibody titres do not appear to prevent recrudescence (Douglas and Couch, 1970; Zweerink and Stanton, 1981), there is evidence that antibody-dependent cell-mediated cytotoxicity (ADCC) may play a role early in HSV infections (Kohl, 1991). In addition, studies in mice have suggested that spread of virus from epidermal sites to neuronal tissue, and the reverse spread upon reactivation of latent virus, may be contained to some extent by the presence of antibodies (Simmons and Nash, 1985).

With regard to cell-mediated responses to HSV, both Th responses, as determined by *in vitro* lymphoproliferative responses to HSV antigens, during primary infections and CTL responses occur. The degree of lymphoproliferative response varies between individuals but is not related to the severity or likelihood of subsequent recrudesences. Both DTH responses to intra-epidermal HSV challenge and HSV-specific CTL responses are detectable. These responses both persist, indicating the establishment of immunological memory. Studies involving the analysis of HSV-expanded lymphocyte clones from patients have revealed the main CTL subset to be $CD4^+$ MHC class II-restricted, although $CD8^+$ MHC class I-restricted CTL have also been reported (Schmid, 1988).

Analysis of NK cell activity, HSV-specific lymphoproliferation and IFN production suggests that a temporary depression in immune responses may occur just before or during a recrudesence (Vestey *et al.*, 1989). It is possible that triggering factors for recrudescent HSV, such as UVR, may play a role in the suppression or delay in the normal cell-mediated immune response resulting in recrudescence. This may allow an asymptomatic recurrent infection to progress to replication in the epidermis and result in the development of a lesion.

1.7 Murine Herpes Virus-68

1.7.1 Murine gammaherpesvirus : the virus

Murine gammaherpesvirus-68 (MHV-68) was originally isolated from *Clethrionomys glareolus* (bank vole) in Slovakia (Blaskovic *et al.*, 1980). Initially thought to be an alphaherpesvirus, MHV-68 was reclassified because its genome structure and organisation were found to share homology with those of other gammaherpesviruses such as Epstein-Barr virus (EBV; recently renamed human herpes virus-4 (HHV-4)) and herpesvirus saimiri (HVS) (Efstathiou *et al.*, 1990a; Efstathiou *et al.*, 1990b). In fact MHV-68 is the first natural pathogen of murine rodents to be classified as a gammaherpesvirus. Gammaherpesviruses are a subset of the Herpes Virus Family, classified by their ability to grow in lymphoblastoid cells. As with all herpesviruses, gammaherpesviruses are double-stranded DNA viruses which have the capacity to establish life-long latent infections from which virus may be reactivated. These viruses share the capacity to establish latent infections in lymphocytes, with EBV adapted to infect and persist in B lymphocytes and HVS able to persist in T lymphocytes. They also have the ability to induce lymphoproliferative disease in the infected host. There is growing interest in gammaherpesviruses, EBV in particular, because of their association with cell proliferation and with neoplasia.

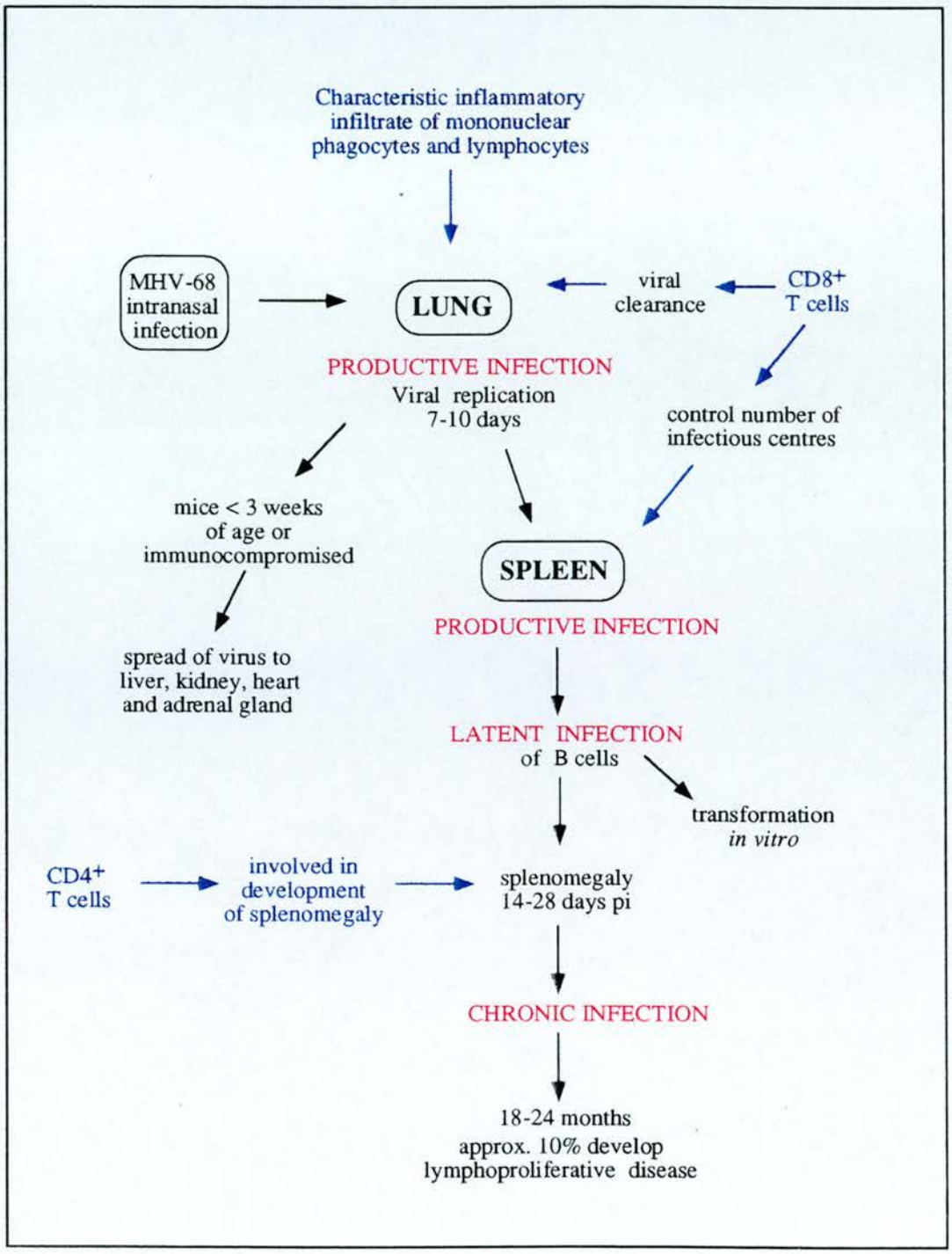
MHV-68 replicates in epithelial cells *in vitro* and has also been demonstrated to infect cell lines of B cell origin but not of T cell origin *in vitro* (Sunil-Chandra *et al.*, 1993). Infection of myeloma cells induces persistence without any apparent cytopathic effect but with the production of infectious virus. Following treatment of these cells with the anti-herpetic agent acyclovir, the productive infection is abolished while a large number of cells harbour virus in a latent form, providing some evidence that MHV-68 can latently infect B lymphocytes (Sunil-Chandra *et al.*, 1993). In fact B lymphocytes have been demonstrated to be necessary for establishment of splenic latency *in vivo* (Usherwood *et*

al., 1996b). Some contradictory evidence exists (Weck *et al.*, 1996) which may reflect the different routes of viral inoculation used in these studies.

1.7.2 Pathogenesis of MHV-68 infection

The pathogenesis of MHV-68 is represented diagrammatically in Figure 1.4. Briefly, upon intranasal administration of MHV-68 to mice, the virus establishes a productive infection in the lung, localised to alveolar epithelial and mononuclear cells (Rajcani *et al.*, 1985; Sunil-Chandra *et al.*, 1992a). Infectious virus can be detected in the lungs between days 1 and 7 post-infection and then declines to undetectable levels by days 10 to 15. Following the acute respiratory infection, the virus spreads to the spleen where a productive infection also results. The pathogenesis of the primary infection closely resembles that of EBV in infectious mononucleosis. Subsequently, the virus is found in a latent state in B lymphocytes in the spleen (Sunil-Chandra *et al.*, 1992b; Sunil-Chandra *et al.*, 1993). Latent virus is not present in any organs other than the spleen and lungs, suggesting these to be the only sites of virus persistence (Rajcani *et al.*, 1985; Sunil-Chandra *et al.*, 1992a). Viral DNA has been observed in the lung long after primary infection, but it remains unclear whether this represents a latent infection or a low level chronic productive infection with virus shedding. This occurs even without B cells and so represents a difference between the model of persistence of MHV-68 and that proposed for EBV. Splenomegaly has been observed to occur from 14 days post-infection and then gradually decline (Sunil-Chandra *et al.*, 1992a; Usherwood *et al.*, 1996a). During the splenomegaly there is a large increase in the number of latently infected spleen cells (Sunil-Chandra *et al.*, 1992b). In addition, inbred mice chronically infected with MHV-68 develop lymphoproliferative disease at a frequency of 9% of all infected animals (Sunil-Chandra *et al.*, 1994). The lymphoproliferative disease is similar to that seen in patients infected with EBV and 50% of affected mice display high grade lymphomas.

Figure 1.4 Schematic of the pathogenesis of MHV-68 and the immune response following a primary infection of young mice



1.7.3 Immune response to MHV-68

1.7.3.1 Cellular immune response

During the first three days of an MHV-68 infection in the lung, the host response is characterised by an infiltration of monocytes/macrophages. This initial response declines as the number of T lymphocytes in the lung, which are almost exclusively $CD8^+$, increases. Lymphocyte depletion studies revealed that removal of $CD4^+$ T lymphocytes had relatively little impact on clearance of the virus from the lung (Ehtisham *et al.*, 1993). In contrast, the absence of $CD8^+$ T cells allowed the continued replication of the virus instead of the usual elimination of infectious virus by 10-12 days. It was also observed that, although $CD8^+$ T cells do not prevent the establishment of latency (Ehtisham *et al.*, 1993; Weck *et al.*, 1996), they do play a role in the control of latently infected B lymphocytes in the spleen (Nash *et al.*, 1996). This $CD8^+$ T cell-mediated control of persistent MHV-68 infection was shown to be lost in the absence of the $CD4^+$ T cell subset, using $CD4^-$ T cell (MHC class II -/-) deficient mice (Cardin *et al.*, 1996). This is in contrast to the situation with HSV in which CD8-deficient mice cleared an infection normally while CD4-deficient mice showed a markedly delayed clearance and a worse infection of the nervous system (Nash *et al.*, 1987). With regard to the development of lymphoproliferative disease in chronically infected mice, it appears that there is a role for T cells in controlling the emergence of tumours since cyclosporin A treatment of mice, which interferes with T cell function, resulted in an increase in the number of animals with lymphomas (Sunil-Chandra *et al.*, 1994).

During splenomegaly the numbers of B lymphocytes and both $CD4^+$ and $CD8^+$ T lymphocytes increase (Usherwood *et al.*, 1996a). Depletion of $CD4^+$ T cells prevents the splenomegaly, but has no effect on the long-term level of latently-infected cells in this model. It is thought that as well as the presence of $CD4^+$ T cells, infected B cells are also required for splenomegaly to occur (Usherwood *et al.*, 1996a; Usherwood *et al.*, 1996b).

Cells from the spleen and lung-draining lymph nodes produce high levels of IL-6 and IFN-γ and lower levels of IL-2 and IL-10 following MHV-68 infection (Sarawar *et al.*, 1996). A similar pattern of cytokine production is also seen in the bronchoalveolar lavage fluid from infected mice. While IFN-γ is known to play a role in clearance of other severe herpes infections (Lucin *et al.*, 1992; Smith *et al.*, 1994), IL-6 and IL-10 have been implicated in the establishment and maintenance of latent EBV infections (Miyazaki *et al.*, 1993; Tanner and G, 1991) and it will therefore be interesting to further study the role of cytokines in this gammaherpesvirus model.

1.7.3.2 Humoral immune response

The antibody response to a primary MHV-68 infection is delayed, with antibody production occurring between days 15 and 20 (Nash and Sunil-Chandra, 1994). Recently the ability of MHV-68 to establish latency in mice deficient in B lymphocytes has been studied using transgenic mice in which there is a disruption in the μ immunoglobulin chain gene, resulting in an inability of pre-B cells in the bone marrow to develop successfully (Usherwood *et al.*, 1996b). In these mice little change was seen in the acute lung infection or virus clearance, indicating that antibody does not contribute significantly to recovery from the infection. Since a slight delay in viral clearance has been observed in both CD4 T cell and B cell-deficient mice, it is possible that the removal of $CD4^+$ T cells deprives B cells of immunological help, resulting in a weaker antibody response to the virus. No splenomegaly was observed in these mice and neither free nor latent virus could be detected in their spleens. However, MHV-68 genome was detected in the lungs of both control and the transgenic mice by PCR analysis. These data would seem to suggest that although cells in the lung may act as a reservoir of latent virus which is independent of the B cell infection, the splenic B lymphocytes are the major reservoir for latent MHV-68.

1.7.4 MHV-68 as a model for Epstein-Barr virus

EBV is the best known human virus in the gammaherpesvirus sub-family and humans are the exclusive natural host. EBV is generally transmitted by saliva and approximately 90% of the human population are latently infected (Wolf *et al.*, 1993a). The virus is most commonly involved in asymptomatic infections early in childhood or in the classical infectious mononucleosis (glandular fever) of adolescents in the developed world. However, the virus was first described by Epstein, Barr and Achong in cells from a common lymphoma in African children studied by Burkitt (Burkitt, 1962). A viral aetiology for the tumour is now well-established (Epstein *et al.*, 1965; Magrath *et al.*, 1992) and an association between EBV and a number of lymphoproliferative diseases exists (Rickinson and Kieff, 1996; Young *et al.*, 1989). EBV has a very limited tissue tropism, defined by the expression of CD21, the receptor for the virus which is expressed on mature resting B lymphocytes and on cells of the basal layer of stratified squamous epithelium (Nemerow *et al.*, 1985). Virus production is restricted to the differentiated cells of the granular layer and above (Sixbey *et al.*, 1983). Since it is technically very difficult to grow differentiating epithelia in culture, studies of the replication of EBV have been limited. It is known that cell-mediated immunity is essential for controlling EBV-associated disease since individuals with either acquired or congenital immunodeficiency have difficulties in resolving EBV infections (reviewed in White and Fenner, 1994). The lack of an effective model for EBV infections means that relatively little is known about the interactions of EBV with the host immune system. The known association of EBV with tumours such as nasopharyngeal carcinoma and Burkitt's lymphoma (Magrath, 1990; reviewed in Rickinson and Kieff, 1996; zur Hausen *et al.*, 1970), together with the facts that the only natural host for EBV is man and that there is no completely productive or permissive system for culture of the virus, emphasise the need for an animal model to study the natural history of this virus. MHV-68 infects a wide variety of cell types from a number of different species. It has similar coding sequence organisation and related proteins to both EBV and HVS. MHV-68 latently infects B lymphocytes and several

features of the primary and persistent infection resemble EBV infection in man. *In vitro* studies with MHV-68 and the mouse model of infection with this virus therefore provide invaluable tools with which to study detailed events in the life cycle of gammaherpesviruses.

1.8 Suppression of immune responses to infectious diseases by UV exposure

The evidence presented in section 1.4 demonstrates the role of UVB in the suppression of cutaneous immune responses. There is growing concern about the implications of UV-induced immunosuppression for the pathogenesis of infectious disease. The currently available data, although limited, would seem to suggest that UVB exposure can indeed affect the immune response to pathogenic organisms and is discussed below.

1.8.1 Human infections modulated by UV radiation

Three viral infections of human subjects provide evidence for the influence of UVR, in the form of natural exposure to sunlight, on viral pathogenesis; namely infections caused by HSV, human papillomavirus (HPV) and HIV.

1.8.1.1 Herpes simplex virus

HSV and its pathogenesis have been discussed more fully in section 1.6.1. Briefly, the virus replicates in epidermal cells and then is capable of establishing latency in the trigeminal ganglia and upon appropriate stimulation is able to travel back to the periphery, where it can replicate and cause a clinically apparent lesion. One of the commonest and most recognisable triggers for this phenomenon of recrudescence is exposure to UV and, in a study group in Edinburgh, 30% of the subjects who suffered from frequent orolabial lesions identified sunlight as a stimulus for the virus to recur (Vestey *et al.*, 1989).

It is not known how UV acts as a stimulating factor although a temporary suppression in various immune responses at the time of a recrudescence has been described, such as NK activity, HSV-specific lymphoproliferation and IFN production (Norval, 1992). It is possible, therefore, that an asymptomatic recurrent infection, where the virus reaches the epithelial cells from the neurons, could progress to a clinically apparent lesion if there is a delay in, or down-regulation of, the normal cell mediated immunity (Yasumoto *et al.*, 1987). Certainly UV has been shown to reduce the capacity of epidermal cells to present HSV antigens at the site of exposure (Howie *et al.*, 1986). In addition, some inflammatory mediators released in skin following UV radiation, such as prostaglandins, may enhance HSV replication and increase the number of virus particles released per infected cell. However, whether UV-induced immunosuppression in the local cutaneous site is critical to the development of the recrudescence or whether this modulation is connected to some, as yet, undefined neurotransmitter is not known.

1.8.1.2 Human papillomaviruses

Cutaneous and mucocutaneous infections with various types of HPV are associated with the development of several tumours. With regard to UV, the most relevant of these are the squamous cell carcinomas (SCC) found in immunosuppressed individuals and in those subjects with the rare genetic disease, epidermodysplasia verruciformis (EV). In the former group, there is a high incidence of warts compared with the general population and the number rises in proportion to the duration of graft survival. Similarly the incidence of SCC is high and rises with graft survival so that, for example, in South-East Scotland 20% of renal allograft recipients had HPV infection and 2% skin cancers up to 5 years post-transplantation; from 5-22 years post-transplantation these figures increased to 77% and 13% respectively (Barr *et al.*, 1989). The development of SCC is almost totally on areas of the body exposed naturally to sunlight, such as the backs of the hand and the face, and in addition, the prevalence of these tumours is highest in sunny climates. Recent

studies have shown that at least 65% of such SCC contain HPV DNA covering a variety of types, including the EV types (Proby *et al.*, 1996). In EV itself, there is the development of multiple flat warts and macular lesions induced by at least 20 HPV types, and an underlying defect in cell-mediated immunity which has not been defined. During the third decade of life in one-third of the patients, some of the warts progress to SCC, most frequently on sun-exposed areas of the body (Majewski and Jablonska, 1995).

The precise roles of UV and HPV in the oncogenic process have not been defined and a complex relationship is envisaged. It is believed that HPV alters the local cutaneous immune environment to enable it to persist. For example, decreased LC numbers and altered cytokine patterns have been observed in association with HPV infections (Jackson *et al.*, 1994; Jackson *et al.*, 1996a). Exposure to the sun may induce changes in epidermal DNA which could be perpetuated and amplified by the cellular proliferation stimulated by HPV infection. In addition the irradiation could alter local immune responses leading to lowered surveillance. This could be significant even if the individual is already immunosuppressed as a result of taking drugs, as in the renal allograft recipients, or due to a genetic defect, as in EV.

1.8.1.3 Human immunodeficiency virus

There is a major cutaneous involvement in HIV infections with more than 90% of subjects developing disorders of the skin or mucous membrane. This can occur at any stage of the disease, not only during the terminal immunodeficiency. Indeed in many cases the skin is the first organ affected and the ability to generate DTH responses has been suggested as a more accurate indicator of disease progression than CD4 counts in the blood (Henry and Tschachler, 1996).

Several *in vivo* and *in vitro* investigations have shown that exposure to UV can lead to activation of HIV and its promoter. For example, latent HIV in chronically infected

cultured monocytes was activated by UVB (Stanley *et al.*, 1989), and HIV gene expression was induced by sunlight in transgenic mice carrying the long terminal repeat (LTR) and *tat* gene elements (Vogel *et al.*, 1992). The mechanisms whereby UV radiation promotes LTR activation have been studied and there is evidence for the involvement of DNA damage and of cellular transcription factors.

Data on the situation during natural HIV infection are lacking, especially at the early stages of the disease. Patients infected with the virus have a variable, but frequently prolonged, latent period before symptoms of AIDS develop. Activation of transcription of viral structural genes is thought to represent a critical step in this process. As many HIV-infected individuals use sunlamps cosmetically or have holidays in the sun, and, as treatment of their skin conditions may involve phototherapy (Adams *et al.*, 1996), it is important to establish whether UV exposure could lead to disease progression or to a drop in CD4 T cell numbers. There is the additional worry that, as HIV infection is thought to cause a shift from a type 1 to a type 2 profile of cytokine production (Clerici and Shearer, 1993), this may be accentuated by UV irradiation.

1.8.2 Animal models of infectious disease

A variety of mouse and rat models have been developed in which the effect of UV on the course of infection and on resistance can be assessed. An outline of the results obtained from these models is shown in Table 1.2. As indicated, the protocols involve different organisms administered by different routes and, in addition, the doses and wavelength of UV, the lamps, the timing of the irradiation with respect to infection and whether the infection occurs at an exposed or unexposed site vary between one model and another. An additional complication arises from the different parameters employed to determine any effect of UV on the outcome of infection, ranging from clinical symptoms and counting the number of organisms to measurement of antigen-specific immune responses, such as DTH and lymphoproliferation. However, in many instances, suppression of immunity can

Table 1.2 : The effect of UV exposure on animal models of infection

Micro-organism	Route of infection	Species	Timing of UV exposure	Antigen-specific effects of UVR	Reference
herpes simplex virus	subcutaneous or epicutaneous	mouse	before infection	lesions unaffected, suppressed DTH	(Norval and El-Ghorr, 1996)
	epicutaneous following immunisation	mouse	before challenge	lesions more severe, DTH unaffected, decreased lymphoproliferation	(El-Ghorr and Norval, 1996)
murine leukaemia virus	intra-peritoneal	mouse	before and after infection	decreased lymphoproliferation, greater spleen histopathology, decreased MLR	(Brozek *et al.*, 1992)
reovirus	intra-peritoneal or intragastric	mouse	before infection	suppressed DTH, decreased lymphoproliferation, decreased cytotoxic T cell activity, clearance of virus unaffected	(Letvin *et al.*, 1981)
rat cytomegalovirus	intra-peritoneal	rat	before infection	decreased viral clearance, increased tissue necrosis	(Goettsch *et al.*, 1994a)
Mycobacterium bovis BCG	sub-cutaneous	mouse	before or after infection	decreased clearance of bacteria, decreased phagocytic function, suppressed DTH (temporary)	(Jeevan and Kripke, 1989; Jeevan *et al.*, 1996)
Mycobacterium lepraemurium	sub-cutaneous	mouse	before infection	decreased clearance of bacteria, increased local inflammatory response, suppressed DTH (temporary)	(Jeevan *et al.*, 1992a)

(continued overleaf)

Table 1.2 continued

Mycobacterium lepraemurium	sub-cutaneous	mouse	chronic before infection	DTH unaffected, clearance of bacteria unaffected	(Jeevan and Kripke, 1990)
Listeria monocytogenes	intravenous	rat	before infection	decreased clearance of bacteria, decreased lymphoproliferation suppressed DTH	(Goettsch *et al.*, 1996)
	sub-cutaneous	rat	before infection	decreased DTH, decreased macrophage phagocytic activity	(Goettsch *et al.*, 1996)
Borrelia burgdorferi	sub-cutaneous (inactivated)	mouse	before immunisation	suppressed DTH, suppressed IgG2a and 2b, elevated IgG1 (after secondary challenge)	(Brown *et al.*, 1995)
Candida albicans	sub-cutaneous (inactivated)	mouse	before or after immunisation	suppressed DTH	(Denkins *et al.*, 1989)
	iv following immunisation	mouse	before challenge	decreased survival time	(Denkins and Kripke, 1993)
Leishmania major	intradermal	mouse	before and after infection	less severe lesions, no effect on numbers of organisms, suppressed DTH	(Giannini, 1986)
Trichinella spiralis	oral	rat	before infection	decreased clearance of larvae, suppressed DTH, suppressed lymphoproliferation	(Goettsch *et al.*, 1994b)
Schistosoma mansoni	per-cutaneous	mouse	before infection	no effect on clearance of virus, no effect on tissue necrosis	(Jeevan *et al.*, 1992c; Noonan and Lewis, 1995)

be demonstrated and, in addition, a decreased ability to clear the pathogen. It is interesting to note that UV exposure can influence both skin-associated infections, such as HSV, and others which are systemic with no cutaneous involvement. One example of the latter category is *Trichinella spiralis* infection in rats where the worms are administered by mouth, with subsequent spread of the larvae from the gut to muscle tissue (Goettsch *et al.*, 1994). In this case the antigen presenting cells will be totally different from the Langerhans' cells in the epidermis, but UV radiation is still capable of suppressing immunity to the parasite and increasing the number of larvae in the muscle compared with unirradiated control rats.

Recently an attempt was made to develop a quantitative risk assessment for lowered resistance to infections in human subjects due to solar exposure (Garssen *et al.*, 1996). Using data generated for Listeria infection in the rat model and other results which compared the effects of UV on immunological parameters in human and rat skin, it was calculated that exposure to about 100 minutes of sunlight at noon in Italy or Spain would suppress the lymphoproliferative response to Listeria by 50%.

1.8.3 Differential effects of UV radiation on T cell subsets in infectious disease

Recent evidence has been obtained from murine studies *in vitro* and *in vivo* that UV radiation may lead to the promotion of Th2 type responses with anergy of Th1 responses. These events are summarised in Figure 1.2.

Most work so far on the UV effects on Th subset induction has not involved microorganisms except for two cases. In the first, mice were immunised with inactivated *Borrelia burgdorferi*, a protocol which preferentially elicits a Th1 response. The effect of UV-B irradiation before immunisation on DTH, as a measure of T cell activity, and on antibody subclasses as a means of distinguishing Th1 and Th2 activity was assessed

(Brown *et al.*, 1995). In addition to stimulating cell-mediated inflammatory responses, such as DTH and CH, Th1 cells stimulate the production of IgG2a, IgG2b and IgG3 (opsonising and complement-fixing antibodies). In contrast, activation of Th2 cells favours the induction of humoral responses, in particular the production of IgG1 and IgE. Irradiated animals demonstrated suppressed DTH to Borrelia on challenge. In addition the IgG2a and IgG2b primary responses were reduced while the IgG1 secondary response was slightly elevated in the exposed mice. Thus these results indicate a depressed Th1 response but without a dramatic shift to a Th2 response. In the second study, it was found that UV irradiation prior to intradermal infection of mice with HSV-1 resulted in a marked suppression of IFN-γ production and enhancement of IL-4 synthesis by lymph node and spleen cells compared with unirradiated controls (Yasumoto *et al.*, 1994). In addition the lesions were more severe in the irradiated animals. Thus a link between the UV-induced alteration in cytokine profile and clinical symptoms is substantiated.

It is becoming clear that some microorganisms induce a predominant Th1 or Th2 response in mice and human subjects, and alteration of these profiles by cytokines or cytokine antibodies can change host resistance or susceptibility. If the cytokine profile is characterised, it may be possible to predict which particular microbial infections might be affected adversely by UV exposure. Thus UV radiation could alter the outcome where a Th1 reponse is preferentially induced and is protective, but may not have measurable effects in the case of a Th2 response unless this is associated with disease progression.

Studies regarding the effects of UV radiation on microbial infections are only just beginning but it is important to recognise that, in addition to acting as a mutagenic agent, exposure also modulates immunity. In three natural infections of human subjects and in several animal models of infection, resistance is impaired together with suppressed antigen-specific immune responses, and, in some cases, decreased ability to clear the microorganisms. It is important to note that in many of the studies small doses of UV

were employed, frequently suberythemal, and ones which would be encountered in natural sunlight. In addition, while the incidence of skin cancer is known to be higher in fair-skinned individuals than in those with highly pigmented skin (Cooper, 1996), there is evidence that susceptibility with respect to infectious disease may affect people with darker skins too (Oberhalman *et al.*, 1994; Scheibner *et al.*, 1987; Vermeer *et al.*, 1991). This would suggest that pigmentation provides no protection and hence the susceptibility group for UV suppression in relation to infectious disease can be extended to the general population (Cooper, 1996).

1.9 Aims

1) To study the effects of UVB on the elicitation phase of CH and to try to repeat the findings of others, which indicate that exposure of mice to UVB prior to elicitation results in an enhancement of the CH response. Such experiments better represent the natural sequence of events in human contact sensitisation than traditional CH experiments.

2) To establish an effective protocol for the induction and elicitation of an MHV-68-specific DTH response in C3H/HeN mice. It was anticipated that the effect of a single sub-erythemal dose of UVB, or a series of such exposures on both the induction and elicitation phases of this immune response would be examined.

3) To develop and characterise *in vitro* and *in vivo* murine models of HSV infection and to study cytokine profiles, (IL-1α, TNF-α and IL-10) at the mRNA level, in these models following HSV infection, using semi-quantitative RT-PCR. The effect of UVB irradiation on expression of these cytokines was also to be examined in a mouse keratinocyte cell line.

4) To carry out a preliminary investigation into the effects of UVB exposure on the functional activity of human EC from skin blister roofs. The effect of *in vitro* and *in vivo* UVB exposure on the alloantigen presentation by epidermal LC, as measured in a MSLR were to be examined, as well as the effects of pre-treating EC with *cis*- and *trans*-UCA to identify any role for UCA as a mediator of the observed UVB effects .

5) To examine the effects of a standard course of UVB phototherapy on two parameters of systemic immunity, namely HSV-specific T cell cytotoxicity and NK cell activity, in normal individuals. To date no studies have identified effects of broadband UVB phototherapy on CTL activity directed at viral antigens. In addition, the phenotypes of PBMC were to be monitored by flow cytometry to identify any UVB-induced alterations.

Chapter 2
Materials and Methods

2.1 General

2.1.1 Media and supplements

RPMI-1640 (Flow Laboratories, Irvine, Ayrshire, UK) was supplemented with 100 Iu/ml penicillin, 200μg/ml streptomycin, 2mM L-glutamine, 100μg/ml gentamicin, 10μg/ml fungizone and 10% heat inactivated foetal calf serum (FCS; Gibco BRL, Paisley, UK), designated RPMI-FCS throughout. Dulbecco's Modified Eagles Medium (DMEM; Gibco) was supplemented as for RPMI, but without the addition of L-glutamine since it contained glutaMAX^{TM} (580μg/ml).

2.1.2 Cells

Baby hamster kidney (BHK-21) cells were obtained from Prof.A.A.Nash (Department of Veterinary Pathology, University of Edinburgh). They were cultured in DMEM supplemented with 7% FCS and were used for growing MHV-68 viral stocks and initially for plaque assays. Vero cells were cultured in RPMI-FCS and were used for HSV plaque assays and for some MHV-68 plaque assays as described. PAM-212 cells (Yuspa *et al.*, 1980) were obtained from Dr.R.McKenzie (Department of Dermatology, University of Edinburgh) and were cultured in DMEM-FCS, supplemented with 25mM HEPES buffer (Gibco). L929 cells were cultured in RPMI-FCS and were used as target cells in the murine cytotoxic T cell assay. K562 cells were grown in RPMI-FCS and were used as target cells in the NK cell assays. Cells were harvested using a 0.1% trypsin / 0.53mM ethylene diamine tetra acetic acid (EDTA) solution.

2.1.3 Viruses

2.1.3.1 Herpes simplex virus

The virus used throughout these experiments was a plaque-purified isolate of herpes simplex virus type 1 (HSV-1) from a clinical case (Howie *et al.*, 1986a). It was passaged *in vitro* in Vero cells at a low multiplicity of infection (moi; 0.2 pfu/cell). The titre of stock virus was determined by plaque assay (section 2.1.3.3) and the virus was stored at -70°C in small aliquots for up to one year, after which the infectivity of the virus diminished. Three different viral stocks were used throughout the study which varied in titre from $2x10^6$ to $1x10^7$ plaque forming units (pfu)/ml.

2.1.3.2 Murine Herpes Virus-68

The GII sub-stock of MHV-68 was obtained from Prof.A.A.Nash (Department of Veterinary Pathology, University of Edinburgh). Viral working stocks were prepared from this by infection of BHK-21 cells at a low moi of 0.1 pfu/cell. Virus suspensions in DMEM were clarified by centrifugation, and the supernantant, as well as the pellet were sonicated and the titre of each stock determined by plaque assay (section 2.1.3.3). Viral stock titres varied from $2x10^7$ to $4x10^8$ pfu/ml.

2.1.3.3 Determination of viral titre

Plaque assays for infectious virus were performed using monolayers of BHK or Vero cells in 96-well flat-bottomed plates (Nunclon, Roskilde, Denmark) with 0.25% Seaplaque agarose (FMC Bioproducts, Rockland, ME, USA) in the overlay medium. After 2-3 days incubation for HSV, and 4 days incubation for MHV (Sunil-Chandra *et al.*, 1992) at 37°C, the cells were fixed in formal saline, stained with 10% Giemsa, and the plaques counted under the low power of a light microscope.

2.1.3.4 Preparation of viral antigen

UV-inactivated viral stocks were prepared by exposing thin films of sonicated virus to approximately 300 mJ/cm^2 UVB. UV-inactivated MHV-68 antigen was used in DTH experiments and proliferation assays (sections 2.3.2 and 2.3.4). Sonicated BHK cells

were used as control antigen in these experiments. UV-inactivated HSV was used in the HSV-specific cytotoxicity assays (section 2.6.2).

2.1.4 Mice

C3H/HeN (H-2^k) female mice were obtained from the specific-pathogen-free animal breeding facility at the Department of Medical Microbiology Transgenic Unit, University of Edinburgh. Mice were aged 6-10 weeks at the start of each experiment, except in the case of some experiments involving infection with MHV-68 (see section 2.3.1), and mice did not differ in age by more than 2 weeks within any experiment. The mice were housed in a room where ambient light was regulated on a 12 hour light/dark cycle, and had free access to food and water.

2.1.5 UVB Irradiation

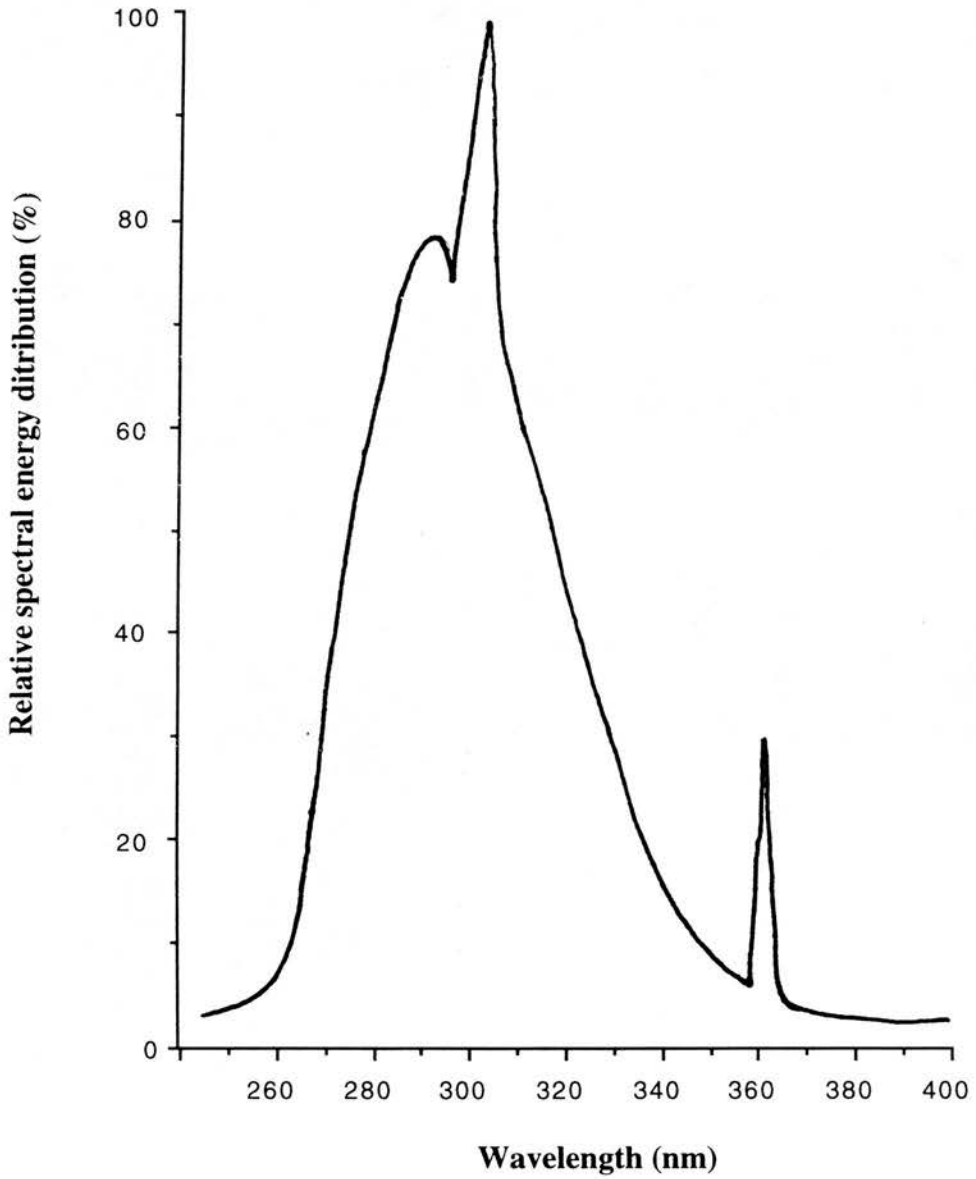
2.1.5.1 UVB source

Two Philips TL-20W/12 bulbs with an output range of 270-350nm (peak 305nm), emitting 80 mW/cm^2 were used throughout these studies (see Figure 2.1). The output of this source was determined by Dr. Neil Gibbs (Photobiology Unit, University of Dundee) using a filtered photodiode meter, calibrated against measurements made with a UV-visible spectroradiometer (model 742, Optronic Laboratories) across the spectral range 250-400nm. The tube to target distance was 16cm.

2.1.5.2 UVB irradiation of cells

PAM-212 cells, used for cytokine mRNA analysis (section 2.4.1), were irradiated as monolayers in culture plates, prior to HSV infection. Medium was removed immediately prior to UV exposure and the cells were bathed in phenol red-free phosphate-buffered saline (PBS). The PAM-212 cells received 10-20 mJ/cm^2 UVB. Epidermal cells, undergoing *in vitro* UVB irradiation prior to use in mixed skin lymphocyte reactions (section 2.5.1), were resuspended in 500 µl of PBS and transferred to a sterile petri dish prior to UVB exposure. These cells received between 5-60 mJ/cm^2 UVB as stated.

Figure 2.1: Relative spectral energy distribution of a Philips TL-20W/12 UVB lamp



2.1.5.3 UVB irradiation of mice

Mice were irradiated on the ears or shaved dorsum and were contained in a perspex box, with a maximum of four mice per box to prevent shielding by littermates. Control mice were dorsally shaved where apropriate. One MED for C3H/HeN mice under these conditions was 150 mJ/cm^2. Where avoidance of irradiation of the ears was necessary, mice were anaesthetised by intra-peritoneal injection of 0.1ml sterile distilled water containing 0.83mg/ml Hypnovel (Roche, Welwyn Garden City, UK) and 1.67mg/ml Hypnorm (Janssen Pharmaceutical, Oxford, UK) and their heads were covered with aluminium foil to protect them from UVB exposure.

2.2 Contact Hypersensitivity Experiments

2.2.1 Contact Sensitiser

4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone; OXA) was obtained from Sigma-Aldrich (Poole, UK). Oxazolone was dissolved in a 4:1 acetone : olive oil (AOO) vehicle at 0.25% (w/v) for challenge and at 1% (w/v) for sensitisation.

2.2.2 UVB Irradiation

Mice were exposed on shaved dorsal skin to UVB irradiation doses equivalent to 70 mJ/cm^2, which corresponds to 0.44 MED for this mouse strain. They were irradiated daily for four days either on days -3 to 0 or days 4 to 7 of each experiment, as described.

2.2.3 Measurement of contact hypersensitivity

2.2.3.1 Effect of UVB prior to sensitisation on the CH response

The CH reaction to OXA was measured 24 hours after challenge and the protocol was based on that of Polla Polla, 1986 #264 . Briefly, 7 mice per group received a sensitising dose of 50µl 1% OXA in AOO topically, on their abdomen, on day 0, having been UVB irradiated on days -3 to 0 as described. Abdominal fur was removed prior to sensitisation using electric clippers and the abdomen was then shaved lightly with a razor blade to remove all traces of hair. CH was elicited on day 7 by

challenging the dorsum of both ears of each mouse with 25µl of either 0.25% OXA or AOO vehicle (VEH). Control groups of mice were either sensitised but not irradiated or neither sensitised nor irradiated. Ear thickness was measured using a spring-loaded micrometer (Draper Precision Instruments, Japan), as shown in Figure 2.2. Measurements were made 24 hours after application of the challenge dose and compared with ear thickness prior to challenge. The increment in ear swelling was used as a measure of the development of CH. Mean values for incremental ear swelling and standard errors of the mean (SEM) were calculated for each group. The student's *t* test was used to compare the differences between mean values. Results are expressed as the mean challenge-induced increase in ear thickness in $mm^{-2} \pm$ SEM.

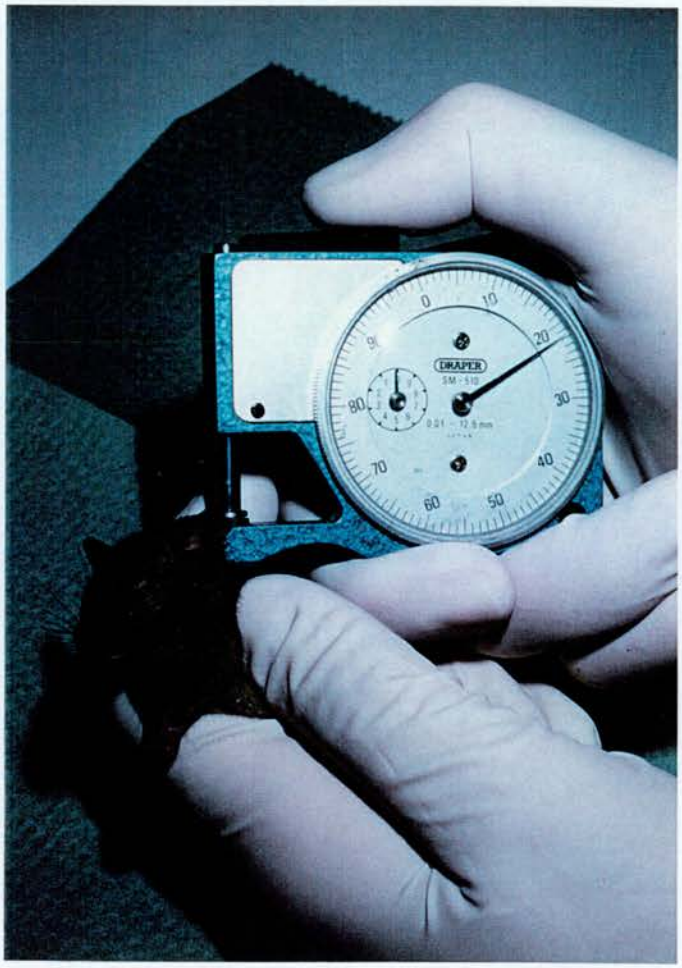
2.2.3.2 Effect of UVB after sensitisation on the CH response

The experimental protocol was as described in section 2.2.3.1 except that mice were UVB irradiated on days 4 to 7 after sensitisation and CH was elicited four hours after the final irradiation on day 7. Additional control groups were included in which mice were sensitised with VEH, UVB irradiated as described on days 4 to 7 and then challenged with OXA. The mean response for this group, in which ear swelling is assumed to be due to the UVB treatment alone, was used to control for this swelling in the experimental group, in order to determine whether any enhancement in the specific CH response was elicited. In later experiments, mice were UVB irradiated on days 3 to 6 so that challenge took place 24 hours after the final UV exposure, by which time UV-induced swelling of the ears was negligible. In experiments designed to study the systemic effects of UVB on this system, mice were anaesthetised and their ears covered during UV exposure as described in section 2.1.5.3.

The percentage suppression was calculated as described (Noonan *et al.*, 1984), according to the formula :-

$$\% \text{ suppression} = 100 - \left\{ \frac{100 \times \text{net increase in UV-irradiated mice}}{\text{net increase in control mice}} \right\}$$

Figure 2.2 Measurements of mouse ear thickness made using a spring-loaded micrometer



2.3 MHV-68 Experiments

2.3.1 MHV-68 infection of mice

Mice were inoculated intranasally with $4x10^5$ pfu MHV-68. The volume of the inoculum varied from 20-40µl per mouse, depending on the viral stock used. Mice were lightly anaesthetised with halothane before administration of the virus, as this aided inhalation of the inoculum. Control mice were infected with an equivalent number of sonicated BHK-21 cells ($1x10^4$ per mouse), suspended in the same volume of PBS and sonicated prior to use. In certain experiments latently infected mice were required and these were infected intranasally when 3-4 weeks old as described, at least three weeks before the start of the experiment to allow latency to be established.

2.3.2 Delayed-type hypersensitivity (DTH) assay

Mice were infected intranasally at 6 weeks of age, as described above and eight days after this primary infection, they were individually tail-marked within each group, and their ear thicknesses were measured using a micrometer gauge, as shown in Figure 2.2. The mice were then challenged by injection of 10µl containing $4x10^6$ pfu UV-inactivated MHV-68, using a 31 gauge needle, in both ear pinnae. Twenty-four hours after challenge the ear thicknesses were again measured and the increase in each ear thickness was calculated. A mean and SEM for each experimental group were calculated and the student's *t* test was used to compare the differences between mean values.

2.3.2.1 The effects of UVB on the DTH response to MHV-68

2.3.2.1 (i) The effect of UVB on the sensitisation phase of DTH

To identify any effects of UVB irradiation on the generation of DTH to MHV-68, mice were irradiated with 96 mJ/cm^2 UVB (corresponding to 0.6 MED in these mice) as described in section 2.1.5.3, three days prior to initial infection with MHV-68. The DTH response was elicited as described eight days later and the mean increase in ear

thickness per group was calculated as well as the SEM. Percentage suppression was calculated according to the formula shown in section 2.2.3.2.

Any subsequent challenges of the mice, used to investigate the establishment of tolerance, were made at 2 week intervals and no additional UV exposures of the mice were carried out.

2.3.2.1 (ii) Attempt to transfer MHV-68-specific tolerance into syngeneic mice

The protocol employed was based on that of Howie (Howie *et al.*, 1986b). Briefly, mice were sensitised as described for a DTH experiment, with either BHK-21 cell sonicate ($1x10^4$ cells/mouse) or MHV-68 ($4x10^5$ pfu/mouse) intranasally. Eight days later single cell suspensions of spleen cells were prepared from untreated control mice, and from mice exposed to 96 mJ/cm^2 UVB prior to sensitisation with MHV-68 and demonstrated to exhibit a depleted DTH response. Red blood cells were lysed by brief exposure to sterile distilled water. The remaining splenocytes were counted and resuspended at $2x10^8$ cells/ml in PBS. The cell suspension was allowed to warm to room temperature and $2x10^7$ control or UV-spleen cells were transferred intravenously (i.v), by tail vein injection, into MHV-68-sensitised mice (eight per cell type). Twenty-four hours later the recipient mice, along with mice sensitised with BHK-21 cell sonicate and MHV-68 to act as negative and positive controls for the DTH response respectively, were challenged in the dorsum of each ear. The mean increase in ear thickness was calculated for each group, as well as the SEM.

2.3.2.1 (iii) The effect of UVB on the elicitation phase of DTH

Groups of mice were either infected with MHV-68 on day 0 or were latently infected (see section 2.3.1) with MHV-68 at the time of UVB exposure. They were exposed to one of two UVB regimes; either 96 mJ/cm^2 per day on days 6, 7 and 8, or 144 mJ/cm^2 once, on day 8. Groups of positive control, unirradiated animals were included which were either infected on day 0 or were latently infected at this time. All mice were challenged on day 11.

2.3.3 The effects of UVB on viral titre

Mice were either exposed to 144mJ/cm^2 UVB on their shaved dorsum or were shaved but unexposed to UVB. Twenty-four hours later all mice were infected with MHV-68. On days 1, 3, 5 and 10 after infection, mice were killed and their lungs and spleens removed aseptically and stored in PBS at -70°C. Organs from 3 mice per group were pooled for each time-point. Organs were homogenised in 500µl PBS in a 1ml glass homogeniser (Jencons, Leighton Buzzard, UK). Tissue suspensions were sonicated before being centrifuged at 10,000g for 10 mins and the supernatants collected. The supernatants were assayed for infectious virus particles as described in section 2.1.3.3.

To determine whether a sub-erythemal dose of UVB irradiation was able to cause reactivation of latent virus in mice, latently infected mice (section 2.3.1) were exposed to 144 mJ/cm^2 UVB on shaved dorsal skin. Spleens from these mice were collected on days 2, 4 and 6 after UV-exposure and homogenised as described above. The supernatants were assayed for infectious viral titre by plaque assay and compared with homogenate supernatants of organs taken from latently infected but unexposed mice.

2.3.4 Lymphoproliferative response to MHV-68

At various time intervals after either primary or secondary MHV-68 infection, mice were killed by cervical dislocation and lymph node or spleens were removed aseptically and collected in PBS containing 10% FCS. Single-cell suspensions were prepared by mechanical disaggregation of tissue through 200-mesh stainless steel gauze (J.Stanier and Co, Manchester, UK). Red blood cells in the spleen cell suspensions were lysed by brief exposure of the cell suspension to sterile distilled water. The cells were washed twice in RPMI-FCS and viable cells counted by exclusion of 0.5% trypan blue. Cells were plated out into 96 well round-bottomed plates at a concentration of 2x10^5 cells/well. They were stimulated in triplicate wells with either concanavalin A (con A; 2.5µg/ml), UV-inactivated MHV (equivalent to moi of 1, 0.5 and 0.25) or BHK cell sonicate (2x10^2 cells/well) in a total volume of 200µl.

Control wells were also included containing lymphocytes and RPMI-FCS only. Cultures were incubated for 4 days at 37°C before being pulsed with 0.7μCi [^{3}H]-methyl thymidine (Amersham Life Sciences, Little Chalfont, UK) per well over the final 24 hour culture. The cells were then harvested onto glass fibre filters using an Automash 2000 cell harvester (Dynatech, UK). One ml of hydro-lumo scintillant (Luman, Belgium) was added to each vial before counting and the [^{3}H]-methyl thymidine incorporation measured in counts per minute (cpm) using a liquid scintillation counter (Canberra Packard, Zurich, Switzerland). The results are expressed as cpm ± SEM.

2.4 Cytokine profiles following HSV infection of keratinoctyes *in vitro* and *in vivo*

2.4.1 *In vitro* infection of PAM-212 cells

The spontaneously transformed mouse keratinocyte cell line PAM-212 was used and cells were plated out at cell density of approximately 1x10^5 cells per well in 6 well culture plates (Nunc, Gibco, Paisley, UK) or in 25cm^2 cell culture flasks (Falcon, Oxnard, USA). Once 80% confluent the cells were infected with HSV at moi of 0.5. For RNA extraction, cells were washed in PBS and then lysed at various time-points after infection in ice-cold reagent D (4M guanidinium isothiocyanate, 25mM sodium citrate pH7, 0.5% sodium lauryl sarkosinate, 0.1M 2-mercaptoethanol) and stored at -70°C. Time-courses were also carried out in which PAM-212 cells were either UVB-irradiated alone (10mJ/cm^2), as described in section 2.1.5.2, or UVB treated immediately prior to identical HSV infection. Within each experiment three wells per time-point for each treatment were used. Identical experiments were set up for determination of cytokine protein production. Culture supernatants were collected and centrifuged at 1000g for 5 mins before use. Cells were lysed in a triple detergent lysis buffer (50mM Tris-HCl (pH 8.0), 150mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulphate (SDS), 1% NP-40, 100μg/ml phenylmethanesulphonyl fluoride (PMSF), 1μg/ml aprotinin, 0.5% sodium deoxycholate) at 0, 12 and 24 hours after

infection/UVB treatment. These supernatants and lysates were used in an IL-10 ELISA (section 2.4.8).

2.4.2 *In vivo* infection

C3H/HeN mice were shaved dorsally and then anaesthetised with halothane before their dorsal skin was lightly tape-stripped approximately 6-8 times, and infected epicutaneously with HSV (approximately $1x10^5$ pfu per mouse in a 20µl volume) by rubbing the virus into this site with a plastic pippette tip. Mice were killed at 0, 3, 6, 12, 24, 48, 72, 96 and 120 hours after treatment and 1-1.5 cm^2 pieces of back skin were removed. Any sub-cutaneous fat was carefully removed and the skin was cut into pieces using a sterile scalpel blade, before being immediately homogenised on ice in 4M guanidinium isothiocyanate using an Ultra-Turrex T8 hand-held electric homogeniser (IKA Laboratories, Stafen, Germany). The homogeniser was decontaminated between samples by soaking in a 1% Virkon solution. A time-course following tape-stripping alone, without infection, was also carried out to control for this procedure. Three mice were used per time-point for each treatment where possible. Samples of skin were also collected and frozen at -70°C for cryostat sectioning. All mice developed lesions between days 3-8 following infection. In a few instances mice developed zosteriform lesions which resulted in hind-leg paralysis and these mice were killed immediately.

2.4.3 Immunohistochemistry

PAM-212 cells were cultured in eight-chamber slides for immunocytochemical staining (Life Technologies, Glasgow, UK). Cryostat sections (5µm) were cut from the mouse back skin samples and these were air-dried overnight before being fixed in acetone for 20 mins at room temperature. Slides were air-dried and stored at -20°C until required. Indirect immunoperoxidase staining was then performed on the PAM-212 cells and frozen tissue sections using an anti-HSV type-1 purified immunoglobulin fraction of rabbit antiserum (DAKO, Glostrup, Denmark), known to react with all the major HSV glycoproteins and at least one core protein. Staining was carried out in a Sequenza

(Shandon Life Science International, Basingstoke, UK), a semi-automated histochemical staining unit. Slides were placed in plastic disposable cassettes which allow a controlled flow of solution over the sample. Briefly, monolayers or sections were washed in Tris-buffered saline (TBS) and endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide in methanol for 10 mins. Sections were incubated for 20 mins in normal rabbit serum (1:5 dilution in TBS) to block non-specific background staining. They were then incubated for 30 mins with the rabbit anti-HSV type 1 antibody diluted optimally to 1:100 in TBS. Sections were then exposed to the secondary antibody, biotinylated goat anti-rabbit immunoglobulins (DAKO, High Wycombe, UK) at a 1:400 dilution in TBS. After a 20-30 min incubation with avidin/horseradish peroxidase (HRP; Vector Laboratories, Peterborough, UK), sections were incubated for 3-5 mins with the peroxidase substrate, 3,3'-diaminobenzidine terahydrachloride (DAB; Sigma), made up by dissolving a 10mg DAB tablet in 10ml of distilled water with the addition of 14µl hydrogen peroxide. Sections were counter-stained with haematoxylin and Scott's tap water substitute, dehydrated by passing through increasing concentrations of solvent and then mounted in DPX (BDH, Poole, UK). Using this technique, positive staining appeared as a brown colour. Control sections were stained using an anti-cytomegalovirus (CMV) isotype control antibody (DAKO).

2.4.4 RNA extraction

Total RNA was extracted from cell and tissue lysates using a phenol-chloroform procedure (Chomczynski and Sacchi, 1987). Briefly, the samples were extracted twice with phenol / chloroform / isoamylalcohol (25:24:1 v/v) and precipitated overnight at -70°C in 0.6 volume of ethanol to selectively precipitate RNA (Jackson *et al.*, 1996). The RNA was then washed in 70% ethanol, dried and dissolved in 20-40µl distilled water. Samples were then quantitated spectrophotometrically (Philips spectrophotometer model PU8625) and stored at -70°C.

2.4.5 Reverse transcription

2µg total RNA was used as a template to generate complementary DNA (cDNA). The volume was made up to 9µl and the RNA denatured at 65°C for 2 mins, and then immediately quenched on ice. A reverse transcription (RT) cocktail was then added to each sample which consisted of : 1 x RT buffer (50mM Tris-HCl, 75mM KCl, 3mM $MgCl_2$); 1.25mM each dinucleoside triphosphate (dNTP); 30 units human placental RNase inhibitor; 200 units Moloney murine leukemia virus reverse transcriptase (M-MLV RT); 2µg oligo $dT_{(18)}$; 0.01M dithiothreitol (DTT) to give a total volume of 20 µl per reaction. After incubation for 1 hour at 37°C, the reaction was terminated by heating to 95°C for 5 mins. The M-MLV RT, 5x RT buffer and DTT were obtained from Gibco and the dNTPs, RNA guard and oligo dT were obtained from Pharmacia Biotech (St. Albans, Herts, UK).

2.4.6 Semi-quantitative PCR

2.4.6.1 Primer labelling

Each primer was $\gamma\{^{32}P]$-dATP -end labelled in a 30µl reaction mix consisting of 5µM primer, 5mM DTT, 30 units T4 polynucleotide kinase (PNK) (New England Biolabs, Beverly, MA, USA,) 1x PNK buffer (70mM Tris-HCl, 10mM $MgCl_2$) and 5µl 0.08mCi/ml $\gamma\{^{32}P]$-dATP (ICN Biochemicals, Thame, Oxfordshire, UK) at 37°C for 1 hour before denaturing at 95°C for 5 mins.

2.4.6.2 PCR amplifications

PCR was performed using 2µl RT-reaction in a PCR cocktail containing 0.08mM dNTP mix (Pharmacia Biotech); 0.5units thermally stable "Red-hot" DNA polymerase (Advanced Biotechnologies Ltd, Leatherhead, Surrey, UK); 1 x PCR buffer (20mM $(NH_4)_2SO_4$, 75mM tris-HCl, 0.01% Tween) and 1µM tetramethylammonium chloride (TMAC; ICN Biochemicals). For each primer set the optimum Mg^{2+} concentration of the PCR mix was determined to eliminate non-specific signals and is shown in Table 2.1. Reactions were overlayed with 20µl of mineral oil to prevent evaporation. All PCR reactions were 'hot-started' (D'Aquila *et al.*, 1991) by addition

of 0.375μM of each primer in a 1.5μl mix at 85°C and amplified on a Techne PHC-3 or Gene-E dri-block cycler (Techne, Cambridge, UK) at 94°C for 1 min (denaturation), 65°C for 1 min (annealing) and 72°C for 1 min (polymerisation). β-actin, IL-1α and IL-10 primer sets were purchased from Pharmacia Biotech and TNF-α primers were obtained from Clontech (Palo Alto, CA, USA). Primer sequences and the size of predicted cDNA amplification product are listed in Table 2.1. The optimum number of cycles was determined for each set of primers by plotting product accumulation against cycle number, following autoradiography (section 2.4.6.3). The cycle number for further amplifications was chosen from a point within the linear exponential portion of the plot to ensure that the PCR reaction for each cytokine was not saturated.

2.4.6.3 SDS-PAGE and autoradiography

Radioactive γ{^{32}P]-dATP-labelled PCR products were resolved on 12% SDS-polyacrylamide electrophoresis (SDS-PAGE) at 150 volts for 1.5 hrs. PCR products were identified on the basis of their predicted size. The gels were exposed to Kodak X Omat AR autoradiography film (Sigma). Autoradiographs were developed using AGFA developer and fixative (HA West Ltd, Edinburgh, UK) and were visualised on the Seescan system image analyser (Seescan, Cambridge, UK) and integrated optical density (OD) units were obtained using gel analysis software v1.0 1D (Seescan). The house-keeping gene β-actin was used to control for differences in abundance of cDNA resulting from variation in the efficiancy of each RT reaction. This was done by normalising the densitometry values for each sample to that of the β-actin gene signal for that sample. The ratio of integrated OD units for each cytokine : β-actin, was used to compare relative cytokine mRNA expression at time-points after treatment. All samples and controls in a particular experiment were amplified within the same run, subjected to SDS-PAGE and autoradiographed at the same time. All PCR reactions for each cell / tissue sample used cDNA from identical RT reactions. All PCR reactions were performed at least twice.

Table 2.1 PCR Primer pairs and optimal conditions used in the PCR analysis

Cytokine	Primer sequence	Product size (bp)	Mg^{2+} (mM)	Annealing temp. (^{o}C)	Cycle number (PAM cells)	Cycle number (mouse skin)
β-actin	5' 5'-GTGGGCCGCTCTAGGCACCAA-3' 3' 5'-CTCTTTGATGTCACGCACGATTTC-3'	540	0.5	65	28	28
IL-10	5' 5'-ACCTGGTAGAAGTGATGCCCCAGGCA-3' 3' 5'-CTATGCAGTTGATGAAGATGTCAAA-3'	237	2.0	65	36	32
IL-1α	5' 5'-ATGGCCAAAGTTCCTGACTTGTTT-3' 3' 5'-CCTTCAGCAACACGGGCTGGTC-3'	625	2.0	65	32	32
TNF-α	5' 5'-ATGAGCACAGAAAGCATGATCCGC-3' 3' 5'-CAAAAGTAGACCTGCCCGGACTC-3'	692	1.0	65	36	34

Primers spanned at least one intron to distinguish cDNA product from genomic DNA.

2.4.7 Controls

RNA from phorbol-12-myristate-13-acetate (PMA)-stimulated EL-4 cells (mouse T cell line) was used as a positive control for β-actin and IL-10 mRNA expression. For TNF-α mRNA expression, RNA from mouse spleen cells stimulated with 5 μg/ml con A for 24 hours was used as a positive control RNA source and for IL-1α, RNA extracted from tape-stripped mouse ear homogenate was used. Negative controls consisted of cDNA synthesis reactions of RNA from positive controls without the reverse transcriptase enzyme, in order to rule out the possibility of amplifying genomic DNA. PCR reactions with distilled water instead of cDNA were also used to check for contamination of PCR reagents.

2.4.8 IL-10 specific ELISA

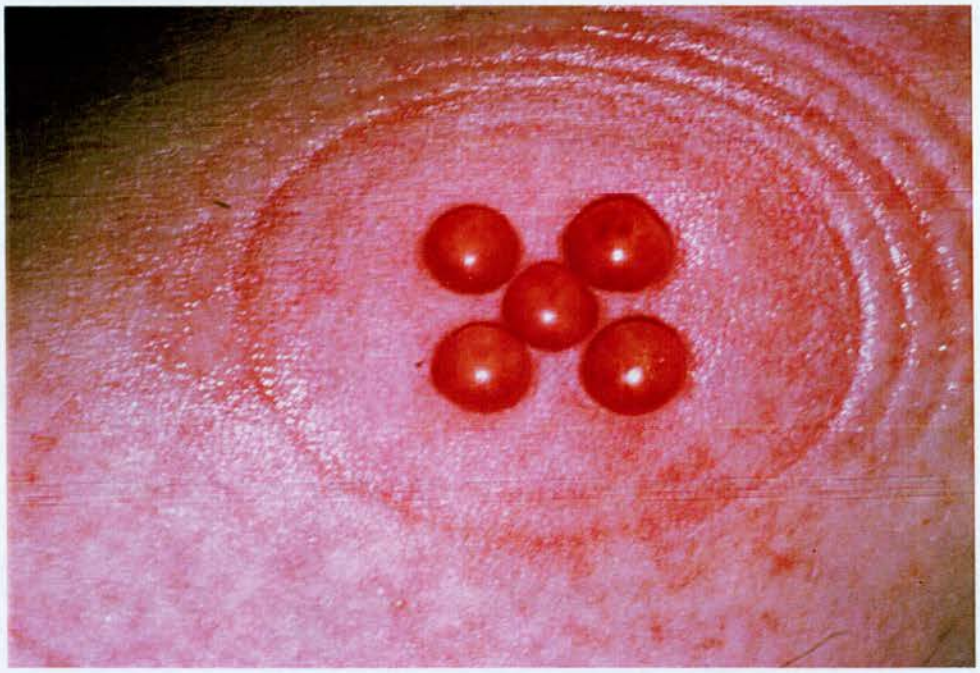
A mouse IL-10 ultrasensitive Immunoassay kit was purchased (Biosource International, Camarillo, CA, USA) and used according to the manufacturer's instructions to detect IL-10 protein in the range of 0.62-80 pg/ml.

2.5 Mixed skin lymphocyte reaction (MSLR)

2.5.1 Preparation of epidermal cell suspensions

The method used was as that of Vestey *et al* (1990). Briefly, suction blisters were raised under negative pressure from UV-unexposed areas of either the lower forearm or upper, inner arm. A set of raised suction blisters are shown in Figure 2.3. The roofs were cut off and placed in 0.5% trypsin (Sigma). They were incubated at 37°C for one hour with regular agitation and then gently pipetted to disaggregate the cells and give a single cell suspension. 15% heat-inactivated pooled human serum was then added before the cells were washed twice in PBS and then resuspended in RPMI supplemented with 10% human serum at concentrations of $6x10^4$, $1x10^5$ and $3x10^5$ live cells/ml.

Figure 2.3 Skin blister roofs



Suction blister cups were held in place using an elasticised bandaged. An negative pressure of -300mmHg was applied using a hand-operated pump. Suction blisters were raised in 45-60 minutes and the blister roofs were removed using sterile scissors. Epidermal cell suspensions were prepared as outlined in section 2.5.1.

2.5.2 *Cis*- and *trans*-UCA treatment of epidermal cells

Epidermal cells were resuspended in 100µl of either *cis*- or *trans*-UCA at a concentration of 100 µg/ml, or PBS for 1 hour at 37°C. The cells were then washed and resuspended at the appropriate concentration. Trans-UCA was purchased from Sigma and cis-UCA was obtained by preparative thin layer chromatography of UVB-irradiated trans-UCA as previously described [Norval, 1989 #930]

2.5.3 UVB treatment of epidermal cells

For *in vitro* exposure suction blisters were raised from an unirradiated site, disaggregated as described and then the cell suspension irradiated with 5-60 mJ/cm^2 UVB, as described in section 2.1.5.2.

For *in vivo* exposure of epidermal cells, the MED of each donor was determined using a Philips 20W T12 bulb housed in a plastic tube with 10 apertures, the light from which was attenuated to varying degrees by metal foil, to give a range of known UV irradiances (Dept. of Medical Physics and Medical Engineering, University of Edinburgh). The tube was held against (unexposed) back skin for 1 minute and the lowest amount of UVB which produced a visible erythema 24 hours after exposure was recorded as the MED for that individual. A previously UV-unexposed area of the upper fore-arm, sufficiently large to cover the area of the suction blister cup, was exposed each day for 7 days with 0.5 MED UVB. On day 8, suction blisters were taken from the irradiated and also an unirradiated control site.

2.5.4 Preparation of peripheral blood mononuclear cells (PBMC)

Venous blood from an individual, assumed to be allogeneic, was collected into preservative-free heparin (10 U/ml final concentration) and PBMC were isolated by centrifugation (1500g for 20 min) on Lymphopaque (Nyegaard Ltd. Birmingham, UK). Cells were washed three times before the viable cell number was determined by trypan blue exclusion. Cells were resuspended in RPMI containing 15% heat-inactivated pooled human serum, at a concentration of 1x10^6 cells/ml. In some experiments Mini-MACS separation of PBMC was carried out to obtain a CD3-purified cell population which was used as responder cells.

2.5.5 MSLR

$1x10^5$ PBMC were incubated in 96 well round-bottomed plates (Nunc) with epidermal cells, at various responder to target cell ratios, at 37°C for 4 days. Control wells were set up containing PBMC or epidermal cells alone. All tests were done in triplicate. Cells were pulsed with 0.7μCi [^{3}H]-methyl thymidine (Amersham Life Sciences, Little Chalfont, UK) added to each well over the final 18 hour culture. The cells were then harvested and the [^{3}H]-methyl thymidine incorporation measured as described in section 2.3.4. The data are represented as the mean cpm ± SEM from triplicate cultures. Some results are expressed as the mean stimulation index ± SEM. Stimulation indices were calculated as:

$$\text{stimulation index} = \frac{\text{mean cpm test}}{\text{mean cpm control}}$$

The percentage suppression of the MSLR for the experiments in which EC were treated with *cis*- and *trans*-UCA, were determined using the equation in section 2.2.3.2.

2.6 Effect of phototherapy on systemic immune function

2.6.1 Broadband UVB phototherapy

Subjects known to be seropositive for HSV were exposed to whole-body broad-band UVB in a Waldmann 1000 cabinet containing 26 Sylvania UV6 fluorescent tubes (approximate spectral output 280-400 nm, peak output 323 nm; 0.3% UVC, 58% UVB and 42% UVA) at the Department of Dermatology, Royal Infirmary of Edinburgh. Subjects were treated three times a week for four weeks, with incremental doses of UVB, depending on skin type, as previously described (Gilmour *et al.*, 1993). The study protocol was approved by local ethics committees. Blood samples were taken from subjects before treatment started and at 10, 20 and 30 days after the start of treatment.

2.6.2 Human HSV-specific T cell cytotoxicity assay

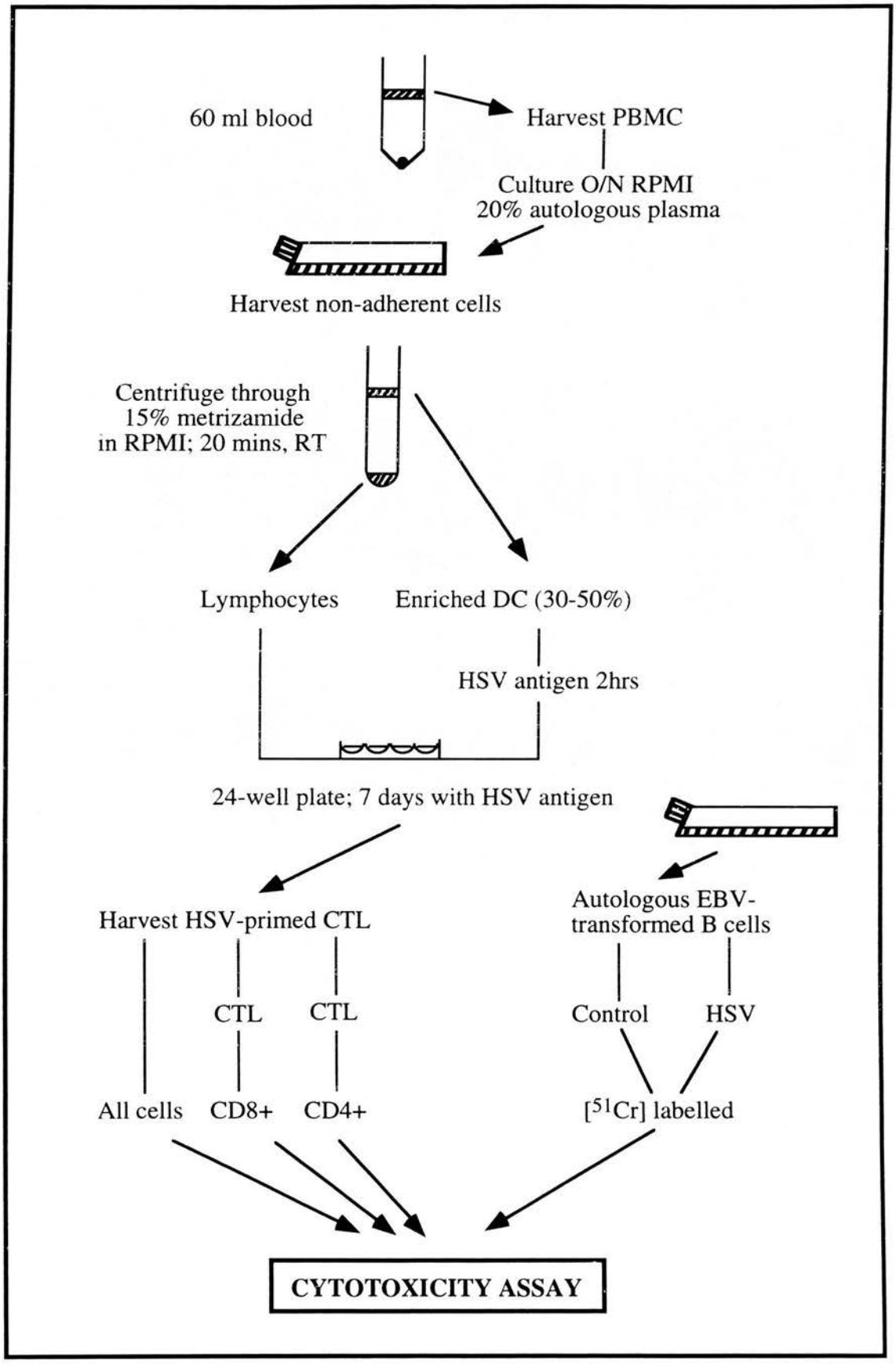
2.6.2.1 Generation of cytotoxic T lymphocytes

In order to generate cytotoxic T lymphocytes (CTL) *in vitro,* a method described by Tarpey *et al.* (1994), based on the protocol outlined by Macatonia *et al* (1991) was employed and is summarised in Figure 2.4. Venous blood was collected from an HSV-seropositive subject and PBMC were separated from whole blood by centrifugation on Lymphopaque as described in section 2.5.4. Adherent and non-adherent cells were separated by incubation of PBMC in a T75 culture flask for 1 hour at 37°C. Non-adherent cells (population A) were removed from the flask in the supernatant, collected as a pellet by centrifugation at 600g and resuspended in fresh RPMI containing 10% autologous plasma. Dendritic cell (DC)-enriched cell populations were then prepared by density gradient centrifugation on metrizamide Nygaard, Oslo, Norway) as described by Macatonia (1986). Briefly, 5mls of non-adherent cell suspension was carefully layered onto 5 mls of 14.5% metrizamide, made up just prior to use, in RPMI containing autologous serum (RPMI-serum). This gradient was then centrifuged for 20 mins at 600g at room temperature. The low buoyant density population (DC^+) that accumulated at the interface was collected and washed twice in medium. DC were resuspended in 2mls RPMI-serum and were incubated with 100µl UV-inactivated HSV (equivalent to $1x10^6$ pfu) at 37°C for 2 hours. They were than washed and recombined with washed population A cells at a ratio of between 40:1 and 10:1, depending on DC yield. Cells were cultured in 24 well plates (Nunclon) in RPMI-serum at approximately $2x10^6$ cells per well for 6 days prior to the cytotoxicity assay.

2.6.2.2 MiniMACS cell separation

The *in vitro* stimulated cells were harvested and used as the CTLs in the cytotoxicity assay. Purified populations of CD4 and CD8 expressing cells were prepared from these CTLs using a MiniMACS cell separation column (Miltenyi Biotec Inc, Bisley, Surrey, UK) according to the manufacturer's protocol. Briefly, enrichment for $CD4^+$ cells was carried out directly using MACS microbeads coated with CD4 antibody and

Figure 2.4 Schematic of the protocol for the generation of CTL and HSV-specific cytotoxicity assay



enrichment for $CD8^+$ cells was carried out indirectly using a mouse anti-CD8 monoclonal antibody, followed by goat anti-mouse IgG microbeads.

2.6.2.3 Preparation of target cells

The target cells for the cytotoxicity assay were autologous B cell lines (BCL) prepared for each subject by transformation with Epstein-Barr virus. The transformation was carried out by Sarah Lockett, Centre for HIV Research, ICAPB, University of Edinburgh. One million BCLs were resuspended in 100µl of UV-inactivated HSV ($1x10^6$ pfu equivalent) and incubated for 2 hours at 37°C. Control cells were incubated for 2 hours in RPMI only. BCLs were then washed and labelled by incubation with 100µCi of $Na_2[^{51}Cr]O_4$ ($[^{51}Cr]$, Amersham Life Sciences) for 2 hours at 37°C. The cells were again washed twice in RPMI-FCS and resuspended in medium at $5x10^4$ cells/ml. Two round-bottomed 96 well plates (Nunc) were prepared with wells containing 100µl of either HSV-target cells or control target cells.

2.6.2.4 Cytotoxicity assay

CTLs and the $CD4^+$ and $CD8^+$ enriched cell populations were washed twice and resuspended at $2x10^6$ cells/ml. A doubling dilution of each cell type was then carried out and 100µl of each cell suspension was added to wells, in triplicate, in order to give final effector to target cell ratios of between 40:1 and 1.25:1. Identical plates were set up using the control and HSV-target cells. Spontaneous $[^{51}Cr]$ release was determined by addition of 100µl of RPMI-FCS to triplicate wells of each set of target cells. Maximum release was determined by addition of 100µl of 2% acetic acid to triplicate wells of the target cells. The assays were incubated at 37°C for approximately 18 hours. To harvest the assay, the plates were centrifuged at 500g for 10 minutes before 100µl of the supernatant from each well was removed for determination of radioactivity (mean cpm) using a Packard liquid scintillation counter.

Mean percentage release values were calculated according to the formula :-

$$\% \text{ specific } [^{51}Cr] \text{ release} = \frac{[\text{test release - spontaneous release}]}{[\text{maximum release - spontaneous release}]} \times 100$$

These assays were carried out with the assistance of Bill Neil.

2.6.3 Determination of Natural Killer Cell Activity

K562 erythroleukaemic cells, which are sensitive to lysis by NK cells were used as target cells in this assay (Lozzio and Lozzio, 1975). One million cells were labelled by incubation with 100µCi of sodium chromate (Amersham Life Sciences) for 2 hours at 37°C. The cells were washed twice in RPMI-FCS and resuspended in RPMI-FCS at $5x10^4$ cells/ml. 100µl of these [^{51}Cr]-labelled cells ($5x10^3$ cells) were placed in each well of a round-bottomed 96 well plate (Falcon).

PBMC prepared from venous blood of human subjects (see section 2.5.4) were resuspended at $2x10^6$ cells/ml and doubling dilutions were carried out in RPMI-FCS. 100µl of each dilution was added to triplicate wells of the labelled target cells, to give effector to target cell ratios of 40:1 to 2.5:1. Spontaneous [^{51}Cr] release was determined by adding 100µl of RPMI-FCS to triplicate wells of target cells. Maximum release was determined by addition of 100µl of 2% acetic acid to triplicate wells. The cells were incubated for 18 hours at 37°C, 5%CO_2. The plates were then centrifuged at 500g for 10 minutes before removing 100µl of the supernatant for determination of radioactivity using a Packard liquid scintillation counter. The percentage specific [^{51}Cr] release was determined using the equation shown in section 2.6.2.4.

2.6.4 Phenotypic analyses

Cell populations ($5x10^5$ cells) were incubated on ice for 30 minutes with 10µl of either neat primary monoclonal antibody (see table 2.2) or the appropriate isotype control. Monoclonal antibodies recognising CD3, CD4, CD8, HLA-DR and CD14 were

obtained from the Scottish Antibody Production Unit (SAPU, Carluke, Scotland, UK). Monoclonal antibodies recognising CD56, as well as a triple colour reagent containing monoclonal antibodies against CD3, CD4 and CD8 were obtained from DAKO. Cells were washed once with 2mls of PBS and then incubated with a sheep anti-mouse IgG FITC conjugate (Serotec, Oxford, UK) for 30 minutes on ice. Negative control cells were incubated with this secondary antibody only. Finally the cells were washed once more in PBS, fixed in 1% formal saline and analysed using a Coulter XL flow cytometer.

Cells were identified first using their forward scatter (FS) and single angle light scatter (SS) characteristics to quantify their size and granularity. Regions were placed around the cell population of interest and around the total cell population. Ten thousand events were accumulated and the events within each region were then displayed on histograms of log fluorescence intensity (x-axis) against cell count (y-axis). Negative controls were routinely set at 1%. The percentage of cells expressing each marker and the density of expression (mean fluorescent intensity) of the marker were recorded.

Table 2.2: Antibodies used in flow cytometric analysis

Specificity	Target cell	Isotype	Clone	Supplier
CD3	T cells	IgG1	UCHT1	SAPU
CD4	$CD4^+$ T cells	IgG1	LT4	SAPU
CD8	$CD8^+$ T cells	IgG1	LT8	SAPU
CD3, 4 & 8		IgG1 kappa	UCHT1 (CD3) MY310 (CD4) DK25 (CD8)	DAKO
HLA-DR	Monocytes/macrophages B cells Activated T cells	IgG2a	LDR	SAPU
CD14	Monocytes	IgG1	BA8	SAPU
CD56	NK cells	IgG1 kappa	MOC-1	DAKO

Chapter 3

The Effect of UVB on the Contact Hypersensitivity Response to Oxazolone in C3H/HeN mice

3.1 Introduction

Allergic contact dermatitis is characterised clinically by an eczematous reaction at the site of contact with an allergen, for example nickel found in some jewellery. This CH response only occurs in individuals whose skin has previously been sensitised by contact with the allergen. The CH response to a chemical allergen is commonly used as a model of cutaneous immunity and in particular to explore the integrity of cutaneous immune responses following UV exposure.

As described in section 1.2.1, the CH response can be divided into two distinct phases (reviewed inBour *et al.*, 1997). The sensitisation, induction or afferent phase occurs at the initial contact of the skin with a specific hapten and leads to the generation of hapten-specific T cells in the lymph node. The second phase is known as the elicitation or efferent phase and occurs upon challenge of the skin with the same hapten. A number of chemicals have been used as experimental contact sensitisers in animal models, including dinitrofluorobenzene (DNFB) (Elmets *et al.*, 1983; Toews *et al.*, 1980), trinitrochlorobenzene (TNCB) (De Fabo and Noonan, 1983) and oxazolone (OXA) (Moodycliffe *et al.*, 1994).

Experiments to investigate the effects of UVB exposure on CH have, in general, targetted the sensitisation phase of the response (Moodycliffe *et al.*, 1994; Toews *et al.*, 1980). The

literature reveals that *in vivo* exposure prior to sensitisation results in a suppression in the CH response upon challenge, both in mice (Moodycliffe *et al.*, 1994; Shimizu and Streilein, 1994; Toews *et al.*, 1980) and humans ((see section 1.4.2) Cooper *et al.*, 1992; Tie *et al.*, 1995). This suppression can be a systemic effect, where suppression occurs even if the sensitiser is applied at a site distinct from that exposed to UVB. Following certain UVB exposure regimens the suppressive effect is a local one, that is sensitisation only at the site of irradiation results in hyporesponsiveness. Local and systemic suppression are discussed more fully in section 1.4.2.2. Throughout this chapter the classical definitions of local and systemic immunosuppression will be used. These refer to the effect of irradiation on the sensitisation phase of CH, irrespective of the site of UVB exposure in relation to challenge.

The initial studies of the effects of UVB exposure on the elicitation phase of CH were carried out in guinea-pig models. A decreased intensity of the elicitation response of CH in sites irradiated prior to challenge with UVB, compared with non-irradiated sites, was reported (Haniszko and Suskind, 1963; Morison *et al.*, 1981). This finding led Polla and coworkers to study the effects of UVB exposure on the elicitation phase of the CH response using a mouse model with TNCB and DNFB as contact sensitisers (Polla *et al.*, 1986). In contrast they found that local exposure of the site of elicitation of CH prior to challenge, in sensitised mice, resulted in a significant enhancement of the CH response. The same results were observed in two strains of mice and with both contact sensitisers. The enhancement was demonstrated to result from a local rather than systemic effect of the UVB radiation.

The aim of this work was to try to repeat these findings using our mouse model of CH, with oxazolone (OXA) as the contact sensitiser. Experiments such as these, to study the effects of UVB on the elicitation phase of CH, rather than on the initial sensitisation to a particular allergen, would appear to better represent the natural sequence of events in

human contact sensitisation. In other words, once an individual has been sensitised to a particular contact allergen, such as nickel, will exposure to sunlight result in a more severe or prolonged hypersensitivity response upon subsequent contact with the allergen? This is of consequence to the 1-2% of the general population affected by allergic contact dermatitis, but in particular to those individuals with a higher incidence, such as workers exposed to common sensitisers in the building and chemical industries.

The experiments discussed in this chapter are of three types and are designed to study the following:-

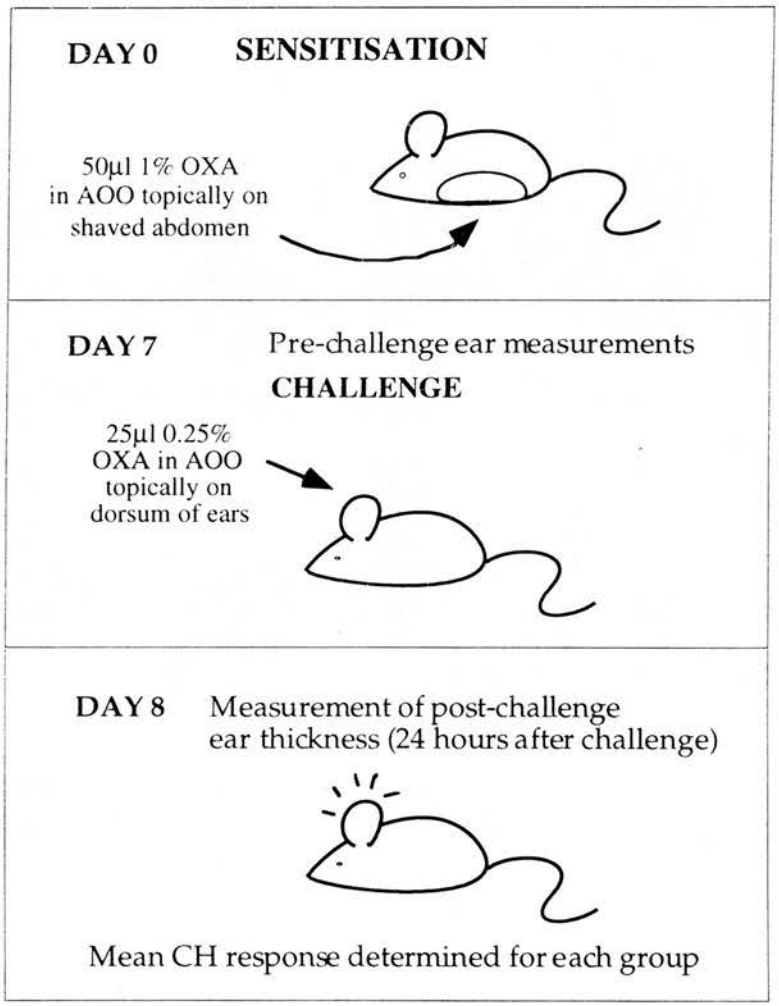
(i) The effect of UVB on the sensitisation phase of CH (section 3.2.2.1).

(ii) The effect of UVB on the elicitation phase of CH (section 3.2.2.2).

(iii) The effect of local and systemic UVB exposure on the sensitisation and elicitation phase of CH (section 3.2.3).

The basic protocol employed throughout these experiments to measure the CH response of C3H/HeN mice to OXA is outlined in Figure 3.1. Mice were sensitised with OXA, applied to shaved abdominal skin on day 0 and were then challenged on day 7 by application of OXA to the dorsum of each ear. Ear thickness was measured using a spring-loaded micrometer before and twenty-four hours after challenge, when the CH response is maximal. The increment in ear swelling was used as a measure of the development of CH.

Figure 3.1 **Protocol employed to assess the contact hypersensitivity response to oxazolone**



3.2 Results

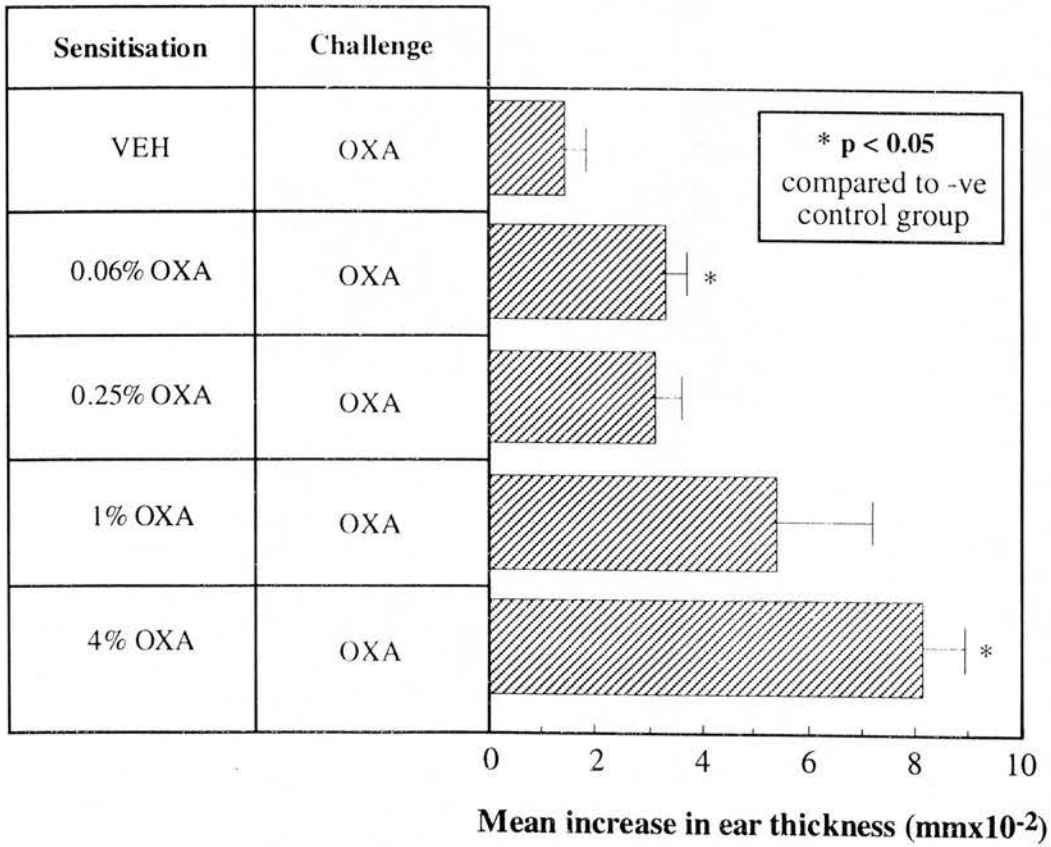
3.2.1 Dose response of oxazolone concentrations for sensitisation

Preliminary dose response experiments were carried out to establish a suitable OXA concentration to use for sensitisation in this model. Figure 3.2 shows such a dose response experiment, with OXA concentrations of between 0.06% and 4% (w/v) used for abdominal sensitisation. These solutions were made up in a vehicle (VEH) of acetone: olive oil (AOO; 4:1 ratio) to aid penetration of the contact sensitiser, and this vehicle was used as a control solution with which to sensitise a group of mice in each experiment. Mice were challenged in all experiments with 0.25% OXA, known to be a sub-inflammatory concentration. The CH response elicited, even using the higher OXA doses, were less than those obtained using similar OXA concentrations applied to dorsal skin. In all future experiments the abdominal skin was shaved using electric clippers followed by removal of any remaining fine hairs with a razor blade, as described by Polla *et al* (1986). Figure 3.3 shows the effect of this shaving procedure on the CH response elicited. Mice sensitised with both 1% and 4% OXA mounted a good CH response upon challenge, which was found to be significantly different from that made by the negative control (VEH-sensitised) group of mice ($p<0.001$). A 1% OXA solution was therefore used to sensitise mice in all subsequent experiments, since increasing the concentration above 1% does not appear to result in a greater CH response, based on this experiment.

3.2.2 The effect of UVB exposure on the sensitisation and elicitation phases of the CH response to OXA in C3H/HeN mice

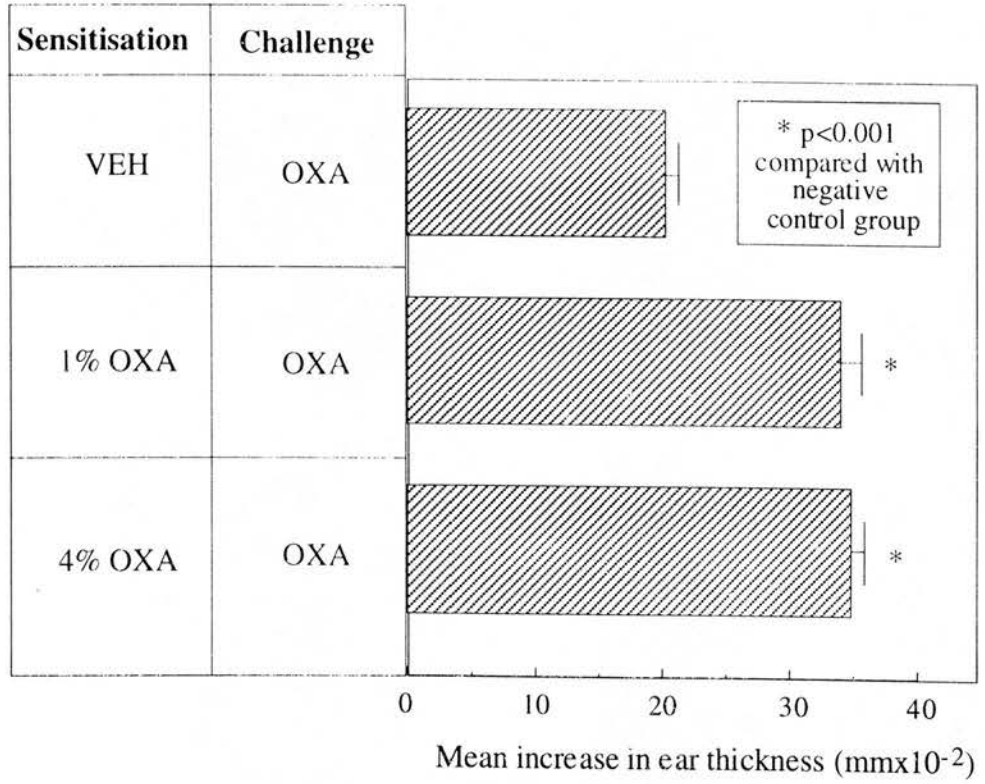
To establish the effect of UVB exposure in this CH model, mice were exposed daily to 70 mJ/cm^2 UVB either for four days prior to sensitisation (days -3 to 0) or for four days prior to challenge (days 4 to 7). Figure 3.4 shows a representative experiment demonstrating the effects of these UVB exposure regimens on the CH response. It is important to note that the experiments discussed in this section involve a systemic model

Figure 3.2 Dose response for oxazolone sensitisation of C3H/HeN mice



Mice (n=5) were sensitised on shaved abdominal skin with either AOO vehicle (VEH) or OXA, at the concentrations stated (w/v). Seven days later mice were challenged by application of 0.25% OXA to the dorsum of each ear. Pre- and post-challenge measurements of ear thickness were made and the mean increase in ear thickness used as a measure of the CH response, expressed as mean ± SEM. Significant differences between the negative group and the experimental groups were determined using the student's *t*-test.

Figure 3.3 Dose response for oxazolone sensitisation of C3H/HeN mice through hairless abdominal skin



Mice (n=5) were sensitised on abdominal skin which had been shaved with electric clippers and fine hair removed with a razor blade. They were sensitised with either AOO vehicle (VEH) or OXA at the concentrations stated (w/v). Seven days later mice were challenged by application of 0.25% OXA to the dorsum of each ear. Pre- and post-challenge measurements of ear thickness were made and the mean increase in ear thickness used as a measure of the CH response, expressed as mean increase ± SEM. Significant differences between the control and experimental groups were determined using the student's *t*-test.

of CH, ie. a model in which the UVB exposure occurs on a distinct site from that of sensitisation.

3.2.2.1 The effect of UVB exposure on the sensitisation phase of CH

The effects of UVB exposure prior to sensitisation are shown in Figure 3.4. The positive control group (group B) mounted a good CH response which was significantly greater than that of the negative control group (group A; $p<0.001$). The CH response of mice exposed to UVB prior to sensitisation, was shown to be significantly suppressed ($p<0.001$) compared to positive control group. This UV-induced suppression was calculated to be a 71% suppression of the normal CH response.

In all subsequent experiments mice in group C were irradiated on days -4 to -1, with all mice being sensitised on day 0 and challenged on day 7 (see explanation in section 3.2.2.2). Figure 3.5 shows a representative experiment using the amended protocol. A suppressed CH response of approximately 19% was seen in the group of mice exposed to UVB before sensitisation in this experiment.

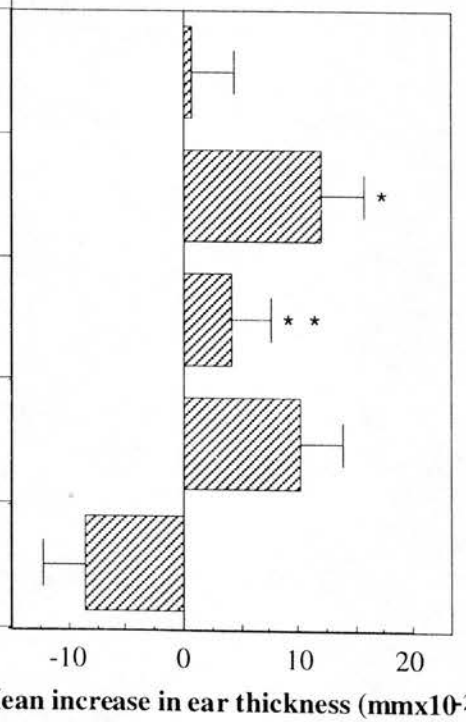
3.2.2.2 The effect of UVB exposure on the elicitation phase of CH

Figure 3.4 shows a representative experiment demonstrating the effects of the UVB exposure regimen described, on the elicitation phase of the CH response. Three identical experiments were carried out and similar results were obtained each time. Mice were challenged four hours after the final UVB exposure of group D on day 7 (see Figure 3.4) and therefore an additional control group of mice was required, which received the same UVB regimen but were challenged with OXA (group E), in order to control for UVB-induced ear swelling.

On initial inspection, there appears to be no enhancement of the CH response as a result of UVB exposure prior to challenge. However, since the mice were challenged just four

Figure 3.4 **Systemic effect of UVB exposure on the sensitisation and elicitation phases of the contact hypersensitivity response to oxazolone in C3H/HeN mice**

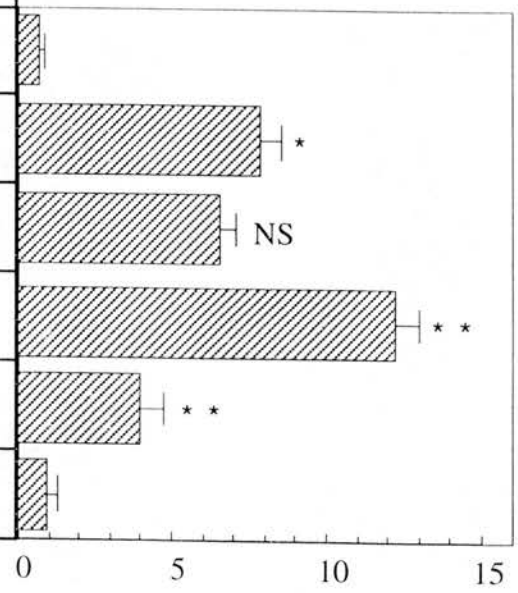
Group	UVB irradiation	Sensitisation	UVB irradiation	Challenge
A		VEH		OXA
B		OXA		OXA
C	UVB	OXA		OXA
D		OXA	UVB	OXA
E		VEH	UVB	OXA



Mice (n=8) were sensitised on shaved hairless abdominal skin with either AOO vehicle (VEH) or 1% OXA on day 0. On day 7 mice were challenged by application of 0.25% OXA to the dorsum of each ear. Pre- and post-challenge (24 hours later) measurements of ear thickness were made and the mean increase in ear thickness used as a measure of the CH response. Group C mice were exposed to 70 mJ/cm^2 UVB on four consecutive days prior to sensitisation (days -3 to 0). Groups D and E were exposed to 70 mJ/cm^2 UVB on four consecutive days prior to challenge (days 4 to 7). Results are expressed as the mean increase in ear thickness ± SEM. Significant differences between groups were determined using the student's t-test.
* positive control group B was significantly different from the negative control group A ($p<0.001$)
** group C was significantly different from group B ($p<0.001$).

Figure 3.5 Systemic effect of UVB exposure on the sensitisation and elicitation phases of the contact hypersensitivity response to oxazolone in C3H/HeN mice

Group	UVB irradiation	Sensitisation	UVB irradiation	Challenge
A		VEH		OXA
B		OXA		OXA
C	UVB	OXA		OXA
D		OXA	UVB	OXA
E		VEH	UVB	OXA
F		OXA	UVB	VEH



Mice (n=8) were sensitised on hairless abdominal skin with either AOO vehicle (VEH) or 1% OXA on day 0. On day 7 mice were challenged by application of 0.25% OXA to the dorsum of each ear. Pre- and post-challenge (24 hours later) measurements of ear thickness were made and the mean increase in ear thickness used as a measure of the CH response. Group C mice were exposed to 70 mJ/cm^2 UVB on four consecutive days prior to sensitisation (days -4 to -1). Groups D and E were exposed to 70 mJ/cm^2 UVB on four consecutive days prior to challenge (days 3 to 6). Results are expressed as mean increase in ear thickness ± SEM. Significant differences between the groups were determined using the student's t-test. *Group B was significantly different from group A ($p<0.001$). **Groups D and E were both significantly different from group B ($p<0.01$ and $p<0.05$ respectively), while group C was not significantly different from group B (NS; $p=0.20$).

hours after the final UVB exposure, it seems likely that much of the ear swelling in these mice (group D) was due to the irradiation rather than as a direct result of the challenge procedure. UV-induced ear swelling was compensated for using the results from control mice (group E).These mice received an identical UVB regimen to those in group D and were challenged with OXA, but VEH sensitised so that they did not respond to the elicitation procedure itself. Compensating for UV-induced oedema in this way, an enhancement of the CH response could be seen and was calculated to be a 67% enhancement of the normal CH response in this experiment.

This experimental protocol was used in a number of experiments, and although the same trend was seen after compensation for UVB-induced ear swelling, the enhancement of CH elicitation by UVB in this system remained to be firmly established as a phenomenon. The protocol was therefore altered slightly to try to clarify the situation. In all subsequent experiments mice received the same total dose of UVB as before, but with no irradiation being given on the same day as either sensitisation or challenge, allowing twenty-four hours for the UV-induced oedema to reduce before challenge. Mice in groups D, E and F were irradiated on days 3 to 6, with all mice being sensitised on day 0 and challenged on day 7.

The experiment represented by Figure 3.5 followed this amended protocol and shows the CH response to be significantly enhanced ($p<0.01$) following UVB irradiation prior to challenge, in comparison to that of unirradiated mice. In this experiment an additional control group was included (group F), which was sensitised with OXA, UVB exposed as group D but challenged with VEH. This group was thought to serve as a more appropriate control for UVB-induced ear swelling in the experimental group D. The enhancement was calculated to be 15% using the mean increase from group E and 56% using the mean from group F.

3.2.3 Local / systemic effects of UVB exposure on the sensitisation and elicitation phases of the CH response to OXA in C3H/HeN mice

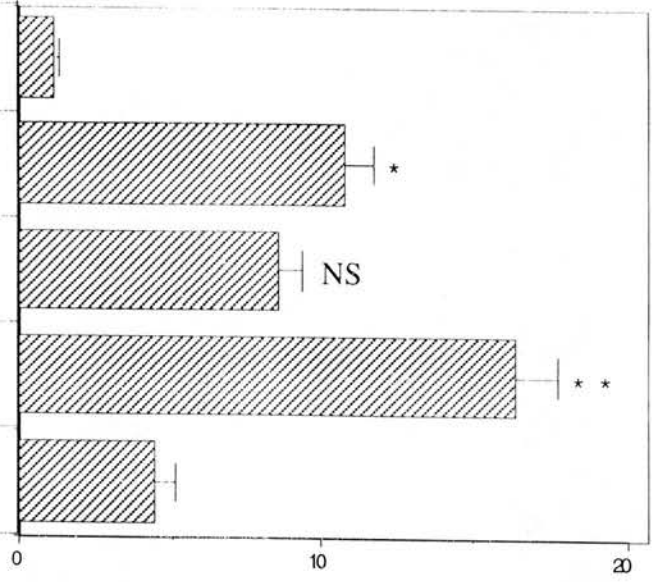
In order to determine whether the effects of UVB exposure on the CH response to OXA previously described are local or systemic, two experiments were carried out. To examine the local effects of the UVB exposure regimen employed, mice were exposed as before, either on days -4 to -1 (before sensitisation) or on days 3 to 6 (before challenge). The mice were sensitised on day 0 on the dorsum of each ear rather than on abdominal skin, and were then challenged as before on day 7 on each ear. To verify the systemic effects of the UVB exposure regimen previously demonstrated, mice were shaved dorsally before UVB exposure and had their ears covered during the irradiation. These mice were sensitised on abdominal skin and challenged on the ears as in previous experiments. This experiment was designed to ensure that the UVB regimen was not having any direct effect on the ears of the mice. The protocols for these experiments are outlined in Figure 3.6 and Figure 3.7 respectively.

Figure 3.6 shows the results following sensitisation, UVB irradiation and challenge of mice, all on the same site and therefore provides information about the local effects of the UVB exposure regimen employed. Exposure of mice before sensitisation resulted in a reduced CH response, although this was not significant ($p=0.095$) compared with unirradiated mice. The percentage suppression was calculated to be 23.2%. Exposure of mice before challenge resulted in a statistically significant increase in the CH response in comparison with unirradiated mice ($p<0.01$). The percentage enhancement, allowing for UVB-induced ear swelling (control group E), was calculated to be 23.8%.

Figure 3.7 shows the results following UVB exposure of mice through shaved dorsal skin, with their ears covered throughout the irradiations. Mice were sensitised on the abdomen and challenged on the dorsum of each ear and both of these sites were therefore unirradiated.UVB exposure of mice to 70 mJ/cm^2 on four consecutive days before

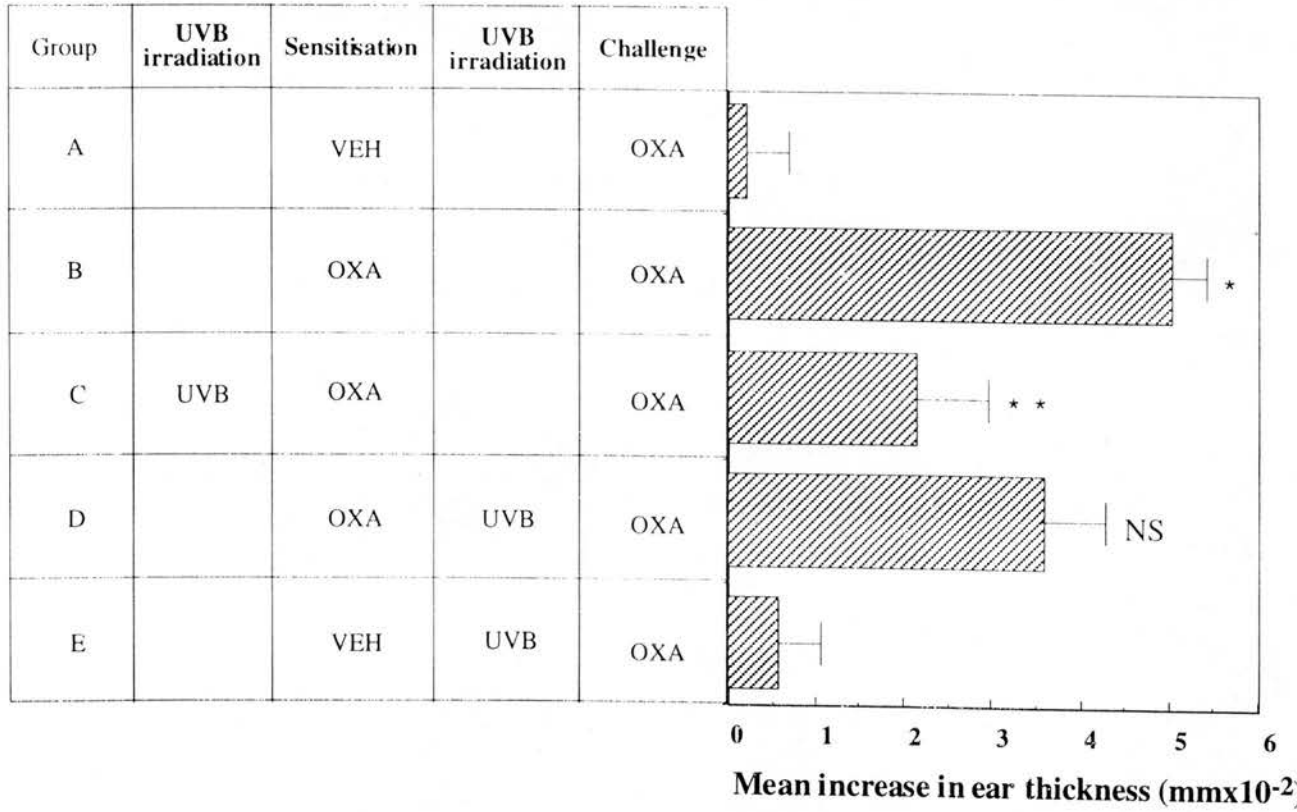
Figure 3.6 **Local effects of UVB exposure on the sensitisation and elicitiation phases of the contact hypersensitivity response to oxazolone in C3H/HeN mice**

Group	UVB irradiation	Sensitisation	UVB irradiation	Challenge
A		VEH		OXA
B		OXA		OXA
C	UVB	OXA		OXA
D		OXA	UVB	OXA
E		VEH	UVB	OXA



Mice (n=8) were sensitised on day 0 on the dorsum of both ears with VEH or 1% OXA and challenged on day 7 on the dorsum of each ear with 0.25% OXA. Groups of mice were exposed to 70 mJ/cm^2 UVB either on days -4 to -1 (before sensitisation) or on days 3 to 6 (before challenge). Pre- and post-challenge measurements of ear thickness were made and the mean increase in ear thickness used as a measure of the CH response, expressed as the mean ± SEM. Significant differences between the groups were determined using the student's *t*-test . *Group B was significantly different from group A ($p<0.001$). Group C was not significantly different from group B(NS; $p=0.095$). ** Group D was significantly different from group B ($p<0.01$).

Figure 3.7 **Systemic effects of UVB exposure on the sensitisation and elicitation phases of the CH response to oxazolone in C3H/HeN mice**



Mice (n=8) were sensitised on day 0 on hairless abdominal skin with either VEH or 1% OXA and challenged on day 7 on the dorsum of each ear with 0.25% OXA. Groups of mice were exposed to 70 mJ/cm^2 UVB either on days -1 to -4 (before sensitisation) or on days 3 to 6 (before challenge). Before these irradiations, mice were shaved dorsally and their ears were covered during each exposure. Pre- and post-challenge measurements of ear thickness were made and the mean increase in ear thickness used as a measure of the CH response, expressed as the mean ± SEM. Significant differences between the groups were determined using the student's *t*-test. * Group B was significantly different from Group A ($p<0.001$). ** Group C was significantly different from group B ($p<0.05$), but Group D was not (NS; $p=0.16$).

sensitisation was found to cause a statistically significant suppression of the CH response compared with unirradiated mice ($p<0.05$). This suppression was calculated to be 59.9%. In contrast to the previous experiment, no enhancement of the CH response was seen following UVB exposure of mice before challenge, at a site distant from that of challenge.

3.3 Discussion

3.3.1 Effect of UVB on the sensitisation phase of CH

The basic protocol employed in the early experiments in this study was identical to that of Polla and coworkers, with mice exposed on four consecutive days to 70 mJ/cm^2 UVB. These sub-erythemal doses have been demonstrated to cause local suppression of the induction phase of CH and result in the production of hapten-specific suppressor T cells (Elmets *et al.*, 1983). The UVB exposure regimen employed here would not be expected to induce systemic suppression of the CH induction. As described in section 1.4.2.2, systemic suppression is usually only induced following an acute, low dose regimen if sensitisation is delayed until at least twenty-four hours after the last irradiation. However, the experiment shown in Figure 3.4 demonstrates a significant suppression (71%) of the sensitisation phase of CH using this UVB regimen. In contrast, in the next experiment shown in Figure 3.5, a suppressed CH response was observed (21%), but this was not significantly different from the positive control response. It is possible that this discrepancy results from the slight differences in the timing of the final UVB irradiation in relation to sensitisation between these experiments. However, based on the findings of Shimizu and Streilein (1994), a greater suppressive effect would have been anticipated to occur in the second of these two experiments. Other workers have also reported that immunosuppression was enhanced by increasing the interval between irradiation and sensitisation (Miyauchi-Hashimoto and Horio, 1996). It may be that the slightly higher doses of UVB employed in this study compared to others, can partly explain the high level of suppression seen in the experiment represented in Figure 3.4 (Miyauchi-Hashimoto and Horio, 1996; Shimizu and Streilein, 1994b).

However, a significant suppression of the CH response following UVB exposure of mice prior to sensitisation is shown in Figure 3.7, supporting the fact that systemic immunosuppression is being induced in this system. The induction of systemic immunosuppression in this model would suggest the involvement of soluble

mediators, rather than a mechanism in which the primary effect of irradiation is on LC since the UVB exposure is at a distant site to that of sensitisation.

3.3.2 Effect of UVB on the elicitation phase of CH

The results shown here demonstrate that exposure of mice to 70 mJ/cm^2 UVB on four consecutive days before challenge caused an enhancement of the elicitation of the CH response to OXA in C3H/HeN mice. This result was confirmed in later experiments in which the UVB-induced ear swelling was greatly reduced by leaving 24 hours between the final UVB exposure and challenge. Any increase in ear thickness in irradiated mice was therefore due to the challenge procedure, as well as any UVB-induced immunological effects, rather than as a direct consequence of the irradiation.

The results shown in Figures 3.5 and 3.6 are very similar. In contrast, no enhancement of the elicitation response is seen in the experiment represented in Figure 3.7. Since UVB irradiation and challenge were both through the ears in Figure 3.6, while UV irradiation was through shaved dorsal skin in Figure 3.7, it seems reasonable to conclude that the enhancement of the CH response to OXA in this system is a local effect. This finding is in agreement with Polla *et al* (1986) who demonstrated that the enhancement effect seen in their system resulted from a local and not a systemic effect of UVB radiation. They demonstrated this by covering the left ear of one group of previously sensitised mice during each pre-challenge irradiation. On challenge the exposed right ears of these mice had significant enhancement of the elicitation response compared to the covered left ears.

3.3.3 Comparison with other studies

Both Haniszko *et al* (Haniszko and Suskind, 1963) and Morison *et al* (Morison *et al.*, 1981) used guinea-pigs in their experiments rather than mice and it may be that there are differences in the immune responses between the two species, which explain the differences in the results obtained. Additionally, these earlier studies used erythema as an end point, rather than oedema, with a categorised scoring system which is open to

more subjectivity than direct measurement of ear swelling. Finally, the dose of UVB radiation and the exposure regimen employed varied between studies, making direct comparisons difficult.

Polla *et al* (1986) demonstrated an enhanced CH response following UVB exposure prior to challenge, in both C3H/HeJ and A/J inbred mice. It has been demonstrated here that this enhancement of CH is also seen in C3H/HeN mice. Yoshikawa and Streilein categorise C3H/HeJ and A/J mouse strains as UVB-resistant, and C3H/HeN as UVB-susceptible (Yoshikawa and Streilein, 1990). In contrast, Noonan and Hoffman categorise all three mouse strains as being of the intermediate susceptibility phenotype (Noonan and Hoffman, 1994). These classifications are explained in detail in section 1.4.2.3. A study by Yoshikawa and coworkers supports the fact that UVB irradiation amplifies the expression of CH in both UVB-resistant and UVB-susceptible mice (Yoshikawa *et al.*, 1992). They demonstrated this phenomenon in C57BL/6 (UVB-susceptible) and BALB/c (UVB-resistant) mice, using DNFB as the contact sensitiser.

In a human study, UVB radiation impaired CH expression among volunteers originally sensitised through UVB-treated skin, but had the opposite effect among individuals originally sensitised via normal, unirradiated skin (Tie *et al.*, 1995). To study the effect of UVB exposure on the sensitisation phase of CH, twenty-four volunteers were subjected to an acute low dose UVB radiation protocol of 144 mJ/cm^2 on four consecutive days. The UVB exposure was on two buttock sites (previously unexposed skin) and within 30 minutes of the final irradiation, the individuals were sensitised through one irradiated site with DNCB. Individuals were designated to be either UVB-resistant or UVB-susceptible, depending on their ability to make a CH response upon challenge 30 days after sensitisation. Seventeen subjects (71%) responded to challenge (UVB-resistant), while seven were found to be UVB-susceptible.

In contrast it was found that when the CH response was induced through normal human skin, its expression was enhanced, in some individuals, when they were challenged on skin previously exposed to UVB radiation. Sixteen individuals received a UVB exposure of 144 mJ//cm^2, on four consecutive days, to a single buttock-skin site. After the final irradiation, each subject was sensitised with DNCB, applied to an unirradiated buttock site. Eleven days later each individual received two DNCB challenges, one applied to the forearm (unirradiated), the other applied to the previously irradiated buttock site. Eight of these subjects (50%) displayed more intense CH reactions at the UVB-exposed site than at the unirradiated site. Although the timing of UVB exposure with respect to elicitation differs from murine experiments, this result implies that the phenomenon observed in mice also occurs in certain humans. It would be interesting to see such a human study extended to try to identify any correlation between those individuals in which an enhanced response is seen and their skin type or other phenotypic characteristics.

3.3.4 Possible mechanisms to account for the enhancement of the elicitation of CH

Several mechanisms may account for the enhancement of elicitation of CH. Firstly, UVB is known to stimulate the production and release of a number of inflammatory mediators, including histamine (Malaviya *et al.*, 1996) and prostaglandins (DeLeo *et al.*, 1985; Pentland *et al.*, 1990), irrespective of the timing of the exposure. It is thought that while these mediators have been shown to cause suppression, they may serve to amplify the elicitation response under certain conditions. It is likely that the local micro-environment induced by UV exposure is the critical factor in determining the response mediated by UVB-induced soluble factors. For example, the presence of suppressor T cells may influence the local microenvironment following UVB irradiation. Secondly, recruitment of immunological competent cells to the site of irradiation recurs, ie an increase in the number of effector T cells or cells with antigen-presenting ability. For example, an influx of T6(CD1a)$^-$ HLA-DR$^+$ cells, thought to be macrophages, into the epidermis as reported by Cooper (Cooper *et al.*, 1985).

Finally, an increase in the local capacity of epidermal cells (EC) to present antigen to sensitised T cells may account for the enhanced CH response seen. It was found that EC obtained from human skin immediately after *in vitro* or *in vivo* exposure to UVR had a decreased ability to present alloantigen in the allogeneic epidermal cell lymphocyte reaction (ELR). In contrast, EC harvested 24 hours or more after UV exposure *in vivo* exhibited a marked dose-dependent enhancement of allostimulation in the ELR. The time course of this enhancement coincided with the reported appearance of the macrophage population in the epidermis, as well as with a decrease in the percentage of LC. A similar response to UV exposure is believed to occur in mice, with increased accessory cell activity of murine EC being reported for cells prepared from sites exposed to UVB 3 days earlier (Lynch *et al.*, 1983). It is therefore possible that the enhancement of the elicitation phase of CH may result from increased antigen presentation by recruited macrophages.

An important question is why the same UVB exposure protocol employed in this study, and in that of Polla *et al*, resulted in a suppression of the induction phase of CH but an enhancement of the elicitation phase. If the enhancement is a result of the antigen presenting ability of recruited cells, then a possible explanation would be a qualitative difference between the ability of LC and these macrophages to induce or elicit immunity. The induction phase of CH represents a primary immune response and so LC are likely to be the principle APC. The elicitation phase represents a secondary immune response, in which antigen-specific primed T cells exist and non-professional APCs, such as MHC class II^+ keratinocytes, may be sufficient to stimulate a specific immune response.

3.3.5 Role of TNF-α in the regulation of CH

Streilein's group has confirmed the findings of Polla *et al* and suggest that local production of TNF-α is responsible for the increase observed in the elicitation phase of CH (Yoshikawa *et al.*, 1992). The dichotomy between the effect of TNF-α on elicitation, as compared to the induction of immunity, is consistent with observations

reported by Granstein and coworkers, examining the effect of TNF-α on LC (Grabbe *et al.*, 1992).

Yoshikawa *et al* (1992) reported that exposure of mouse ear skin to acute low dose UVB (40 mJ/cm^2 on four consecutive days), exaggerated the expression of the CH response to DNCB. Irradiations were given on days 4, 5, 6 and 7 after sensitisation on the abdomen, and mice were challenged one hour after the final UV exposure, by application of DNCB to the UVB-exposed dorsal surface of the ears. Intradermal injection of TNF-α at the site of challenge was also found to enhance the expression of CH in sensitised mice. Whereas the suppression of the induction of CH by acute low dose UVB exposure only occurs in UVB-suscepible strains of mice, the enhancement of CH expression by injection of TNF-α occurred in both UVB-susceptible and UVB-resistant mice. Interestingly, while TNF-α amplified the expression of CH in the ears of all mouse strains, anti-TNF-α antibodies neutralised UVB-enhanced CH only in UVB-susceptible mice. The observation that DNFB painted on UVB-exposed or TNF-α-treated skin was much less effective at eliminating LC from the epidermis than epicutaneous application of DNFB on untreated skin led to the hypothesis that TNF-α caused LC to be transiently immobilised within the epidermis. UVB exposure was hypothesised to cause this apparent blocking of LC migration indirectly by stimulating keratinocytes to produce TNF-α. The evidence presented by Streilein and colleagues support the proposition that TNF-α, released from UVB-exposed EC, is a crucial mediator of the effects of UVB radiation on both the induction and elicitation of CH.

In contrast however, Moodycliffe *et al* have reported that UVB enhances migration of LC and accumulation of DC in LN draining the site of irradiation (Moodycliffe *et al.*, 1992). It has also been reported that intra-dermal injection of homologous recombinant TNF-α resulted in a reduced frequency of LC in the epidermis at the site of injection (Cumberbatch *et al.*, 1994). In addition, an increase in the number of DC within DLN was reported (Cumberbatch and Kimber, 1992). Further evidence was provided by experiments in which mice were treated systemically with neutralising

anti-TNF-α antibody. This treatment resulted in a marked inhibition of DC accumulation in DLN induced by contact allergens and caused a significant inhibition of CH when the antibody was administered two hours prior to sensitisation (Cumberbatch and Kimber, 1995). It has also been demonstrated that treatment with the same antibody significantly inhibited the accumulation of DC in DLN, resulting from local UVB irradiation (Moodycliffe *et al.*, 1994). TNF-α is therefore believed to provide an important signal for the migration of LC from the skin and consequently is an important mediator for CH.

The role of TNF-α in the regulation of CH, and in particular in the elicitation phase, therefore remains a controversial issue. However, the fact that anti-TNF-α antibodies neutralised UVB-enhanced CH in UVB-susceptible mice, suggests that TNF-α may play a role in the phenomenon of UVB enhancement of the elicitation phase of CH described here in C3H/HeN mice.

Returning to the paradoxical situation of UVB causing exaggerated expression of CH, while also being able to impair the induction of CH to epicutaneously applied hapten. It was suggested above that enhanced expression of CH may result from an increase in the number of effector T cells recruited to the challenge site. It has been reported that UVB radiation as well as TNF-α and IFN-γ induce the expression of ICAM-1 on keratinocytes (Norris *et al.*, 1990). Such upregulation of cell adhesion molecules may result in an increased migration of leukocytes into the epidermis. In addition, TNF-α is known to have the capacity to alter the endothelial surfaces of vessels by upregulating expression of cell adhesion molecules and therefore allowing increased recruitment of effector T cells and other inflammatory cells into the dermis (Pober *et al.*, 1986). However, a more recent study demonstrates that although TNF-α mRNA and protein in human skin are upregulated by *in vivo* UVB exposure, no significant alteration in ICAM-1 or VCAM-1 expression was involved (Strickland *et al.*, 1997). In addition TNF-α did not appear to be involved in the early induction of E-selectin.

3.3.6 Criticisms of experimental design and suggestions for further work

The experimental protocols employed in this study were necessarily complex but made interpretation of the results difficult at times. Although Polla *et al* did not make any calculations of percentage enhancements, it was felt important to use control groups of mice to compensate for UVB-induced ear swelling, to distinguish this from antigen-specific effects. Since mice in the experiments of Polla and coworkers were challenged four hours after the final UVB irradiation, and in some experiments UVB doses of up to three times the magnitude used in our experiments were employed, it seems unlikely that direct UVB-induced ear swelling would not need to be taken into account. The percentage enhancement of the CH response to TNCB in C3H/HeJ mice, using data from this paper and taking into account UVB-induced ear swelling, was calculated to be 31%, following the same UVB exposure protocol employed in our experiments (Polla *et al.*, 1986).

Ideally, the antigen-specificity of suppression and enhancement of CH should have been investigated. This could be carried out by including controls in which the sensitiser or challenge hapten was a different chemical, such as DNCB. However, it is important to note that the inclusion of the vehicle only at the sensitisation stage does go some way to answer this point. In addition, the observation that OXA challenge, after vehicle and subsequent UVB exposure, lead to an increase in CH, argues that the UVB treatment was itself causing damage to the skin. This may have been aggravated in a non-specific way by the addition of chemical and independently of specific sensitisation.

In order to elucidate the mechanism involved in the UVB-mediated enhancement of CH, it would be interesting to utilise the techniques of Yoshikawa *et al* (1992) to determine whether, for example, anti-TNF-α antibodies were able to neutralise the UVB-enhanced CH in our system. It would also be interesting to study the cytokines produced during the different phases of CH, and following the UVB exposure

regimen employed in this study, either at the protein level or mRNA level using RT-PCR. Finally, a phenotypic analysis of the cell populations within the epidermis during the elicitation phase of CH would be of interest. Such a study would identify any particular subset of cells responsible for the enhancement observed, comparable perhaps with the suppressor T cells thought, at least in part, to be responsible for the hapten-specific unresponsive state induced by UV irradiation.

3.3.7 Summary

The aim of this study was to optimise a protocol for the establishment and elicitation of CH using the contact sensitiser OXA in C3H/HeN mice. The UVB exposure regimen used by Polla *et al* (1996) was then employed to try to repeat their finding of an enhancement in the elicitation response of CH, as well as to examine the effects of UVB on sensitisation, in this model system. Exposure of mice to 70 mJ/cm^2 UVB on four consecutive days prior to challenge was found to result in an increased CH response in comparison to unirradiated mice. This finding supports the results of Polla *et al* (1986) and Yoshikawa *et al* (1992) in murine models. In addition an observation in humans made by Tie *et al* (1995) suggests that a similar phenomenon occurs in certain humans. This has implications for individuals who suffer from contact dermatitis reactions and may be exposed to UVB, either through environmental exposure or through the use of sunbeds or a course of phototherapy treatment, before subsequent exposure to the contact sensitiser.

Chapter 4

The Effect of UVB on the Immune Response to Murine Herpesvirus-68

4.1 Introduction

The ability of low doses of UVB to induce suppression of cell-mediated immunity suggests that UVB-modulation of such immune responses to infectious agents may occur. In fact there is now a body of evidence, obtained from rodent models, to suggest that UVB can indeed affect immune responses to a range of pathogenic organisms (section 1.8.2).

The delayed-type hypersensitivity (DTH) response is one of the classical immune reponses used in experimental models as a measure of a specific inflammatory response. UVB-induced immunosuppression of the DTH response to a range of agents have been recorded to date. A number of groups have demonstrated the suppression of DTH responses to sheep red blood cells, alloantigens and protein antigen with single UVB exposures (Molendijk *et al.*, 1987; Mottram *et al.*, 1988; Ullrich, 1986; Ullrich *et al.*, 1986).

To date the DTH response to a range of pathogenic organisms, namely HSV-1 (Howie *et al.*, 1986a; Norval and El-Ghorr, 1996), reovirus (Letvin *et al.*, 1981), *Mycobacterium bovis* BCG (Jeevan and Kripke, 1989), *Mycobacterium lepraemurium* (Jeevan *et al.*, 1992a), *Listeria monocytogenes* (Goettsch *et al.*, 1996)*Borrelia burgdorferi* (Brown *et al.*, 1995), *Candida albicans* (Denkins *et al.*, 1989), *Trichinella spiralis* (Goettsch *et al.*, 1994b), and *Leishmania major* (Giannini, 1986) has been shown to be suppressed by

UVB exposure in animal models. The degree of suppression as well as the persistence of the suppressed state vary greatly in these models. These parameters are likely to be dependent on the UVR regime used, ie the dose and numbers of exposures, as well as the nature and route of infection.

Human HSV-1 infection has acted as a prototype for the study of the effects of UVB on the immune response to viral agents. The virus can recrudesce from latency at intervals and exposure to sunlight is a common stimulus for this (Norval, 1992) as described in section 1.8.1.1. In the mouse model, a single exposure to UVB prior to subcutaneous infection with HSV, induces suppression of the DTH response to the virus when the mice are subsequently challenged (Howie *et al.*, 1986a). The knowledge that UVB exposure can cause systemic immunosuppression, for example to contact sensitisers, presents the possibility that exposure to UVB may affect the immune response to more systemic viral infections too.

The gene homology and *in vivo* observations described in section 1.7 would seem to indicate that murine herpesvirus-68 (MHV-68) may act as a model for EBV, providing a unique opportunity to study the pathogenesis of and immune response to gammaherpesviruses in general. Unlike HSV, MHV-68 has no cutaneous involvement and establishes a persistent infection in the spleen. Therefore it provides an opportunity to examine the effects of UVB exposure on a pathogen which is entirely systemic.

T lymphocytes are important in the immune response against MHV-68, with $CD8^+$ T cells playing a critical role in the control of viral clearance in the lung and the control of latently infected B cells in the spleen (Ehtisham *et al.*, 1993; Nash *et al.*, 1996). $CD4^+$ T cells also appear to have some function since the kinetics of viral clearance are delayed in mice deficient in these cells (Ehtisham *et al.*, 1993). In addition, the infiltration of mononuclear

phagocytes, as well as T lymphocytes, into the site of infection is characteristic of the immune response to MHV-68. The DTH response to MHV-68 was therefore used as a measure of the cell-mediated immune response to this virus and the effects of UVB on this immune parameter were studied.

The primary aim of this study was to establish an effective protocol for the induction and elicitation of an MHV-68-specific DTH response in C3H/HeN mice. It was anticipated that the effect of a single sub-erythemal dose of UVB, or a series of such exposures on both the induction and elicitation phases of this immune response would be examined.

4.2 Results

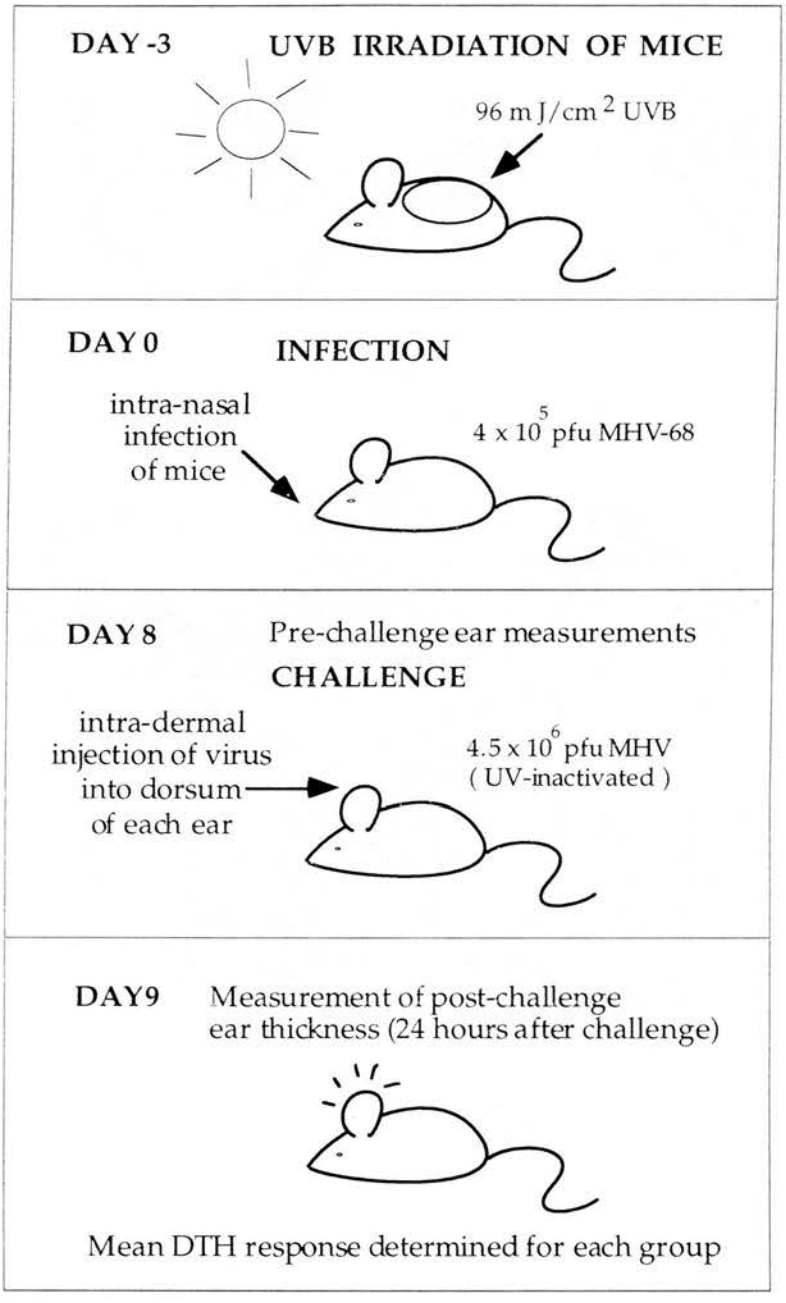
4.2.1 Establishment of MHV-68-specific DTH response

The first experiment was to establish that MHV-68 was able to infect C3H/HeN mice when administered intranasally using halothane anaesthesia. Previous investigations of the pathogenesis of MHV-68 and the immune responses to it used BALB/c mice and ether anaesthesia for intranasal administration of the virus. The use of ether is now prohibited in University of Edinburgh animal facilities and halothane does not cause the characteristic inhalations thought to aid uptake of the virus. However, plaque assays carried out using homogenates of lung tissue following intranasal infection of mice with halothane, demonstrated that the mice had been successfully infected. Infectious virus titres were comparable, although slightly lower than those reported following infection using ether anaesthesia in BALB/c mice (Sunil-Chandra *et al.*, 1992a). Representative viral titres following intranasal infection of 4 week old mice are shown in Table 4.3.

The ability of adult mice to generate a specific DTH response to MHV-68 was established in subsequent experiments. Previously only mice of 3-4 weeks of age were known to be successfully infected with the virus using the intranasal route (Sunil-Chandra *et al.*, 1992a). However, at this age the immune response of the mice is not fully developed and so a balance had to be reached between successful infection and the ability of the mice to generate the necessary cell-mediated immune response. The protocol adopted is shown in outline in Figure 4.1 (days 0-9). Briefly mice were infected by intranasal infection with $4x10^5$ pfu MHV-68, followed by elicitation of the DTH response 8 days later. This was done by injection of UV-inactivated virus equivalent to $4.5x10^6$ pfu into the dorsum of each ear.

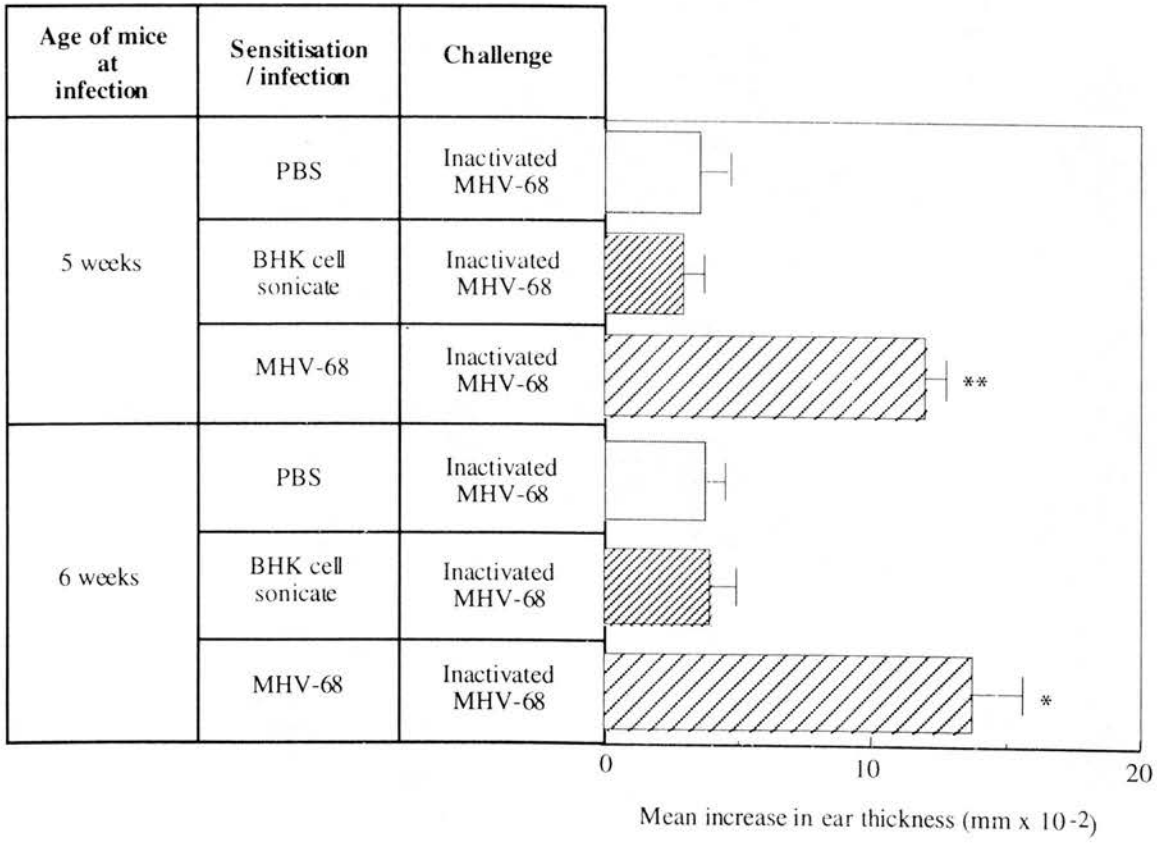
It was established that mice of 5 weeks and older could generate an MHV-68-specific DTH response, as shown in Figure 4.2, while mice of 4 weeks and younger could not. The DTH response to the virus was directed against MHV-68 antigens rather than BHK

Figure 4.1 Protocol employed to assess the DTH response to MHV-68



Mice were sensitised by intra-nasal infection with MHV-68. Control mice were sensitised with BHK-21 cell sonicate. Eight days later the ears of each mouse were measured before challenge of each mouse by intra-dermal injection of inactivated MHV-68 into the dorsum of each ear. Twenty-four hours later the increase in ear thickness was assessed and used as a measure of the DTH response. To determine the effects of UVB (96 mJ/cm^2) on the induction phase of DTH, groups of mice were exposed to UVB on shaved dorsal skin three days prior to sensitisation.

Figure 4.2 The delayed-type hypersensitivity response to MHV-68



Eight mice were used per group and were sensitised either with PBS, BHK-21 cell sonicate (approximately $1x10^4$ cells/mouse) or $4x10^5$ pfu MHV-68 intra-nasally. Eight days later mice were challenged by injection of $4.5x10^6$ pfu equivalents of UV-inactivated MHV-68 into each ear. Post-challenge measurements of ear thickness were made 24 hours after challenge and the mean increase in ear thickness for each group was calculated and is shown as mean ± SEM. Significant differences between the positive and negative groups were determined using the student's *t*-test.

** $p<0.001$ compared to 5 week BHK cell sonicate negative control group; * $p<0.01$ compared to 6 week BHK cell sonicate control group.

cell antigens. Since no significant difference was seen between the negative control groups sensitised with PBS or with BHK-21 cell sonicate, only the BHK-21 cell sonicate control was used in all subsequent experiments since it provides a more stringent control for the specificity of the DTH assay. In addition, mice of at least six weeks of age, at the time of sensitisation, were used in all subsequent experiments, unless otherwise stated.

4.2.2 Suppression of MHV-68-specific DTH response by UVB exposure

To examine the effect of pretreatment of mice with UVB on the generation of DTH to MHV-68, the protocol outlined in Figure 4.1 was employed. Figure 4.3 demonstrates that a single exposure to a suberythemal dose of UVB irradiation induced a significant (41%) suppression of the DTH response to MHV-68 ($p<0.05$). A repeat experiment showed a 40.1% suppression of the DTH in the UVB group.

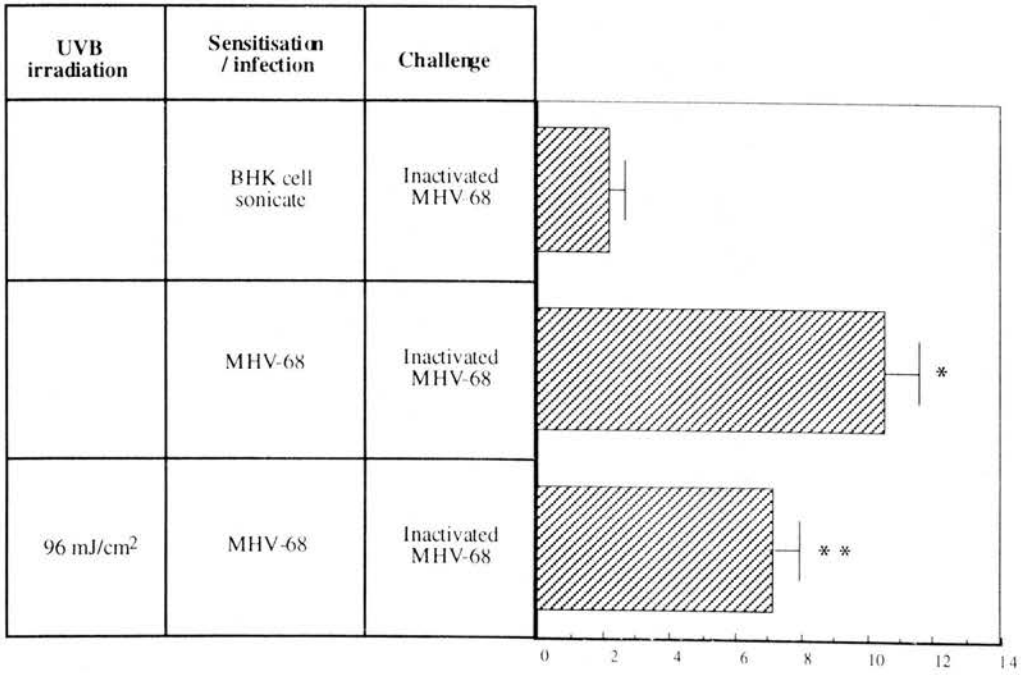
4.2.3 Induction of MHV-68-specific tolerance

To determine the duration of the suppressive effect of a single exposure to UVB, mice previously UVB-exposed (96 mJ/cm^2) and demonstrated to have a suppressed DTH response, were re-challenged at two week intervals, without being subjected to any further UVB exposure. The UVB-irradiated group showed a reduced DTH reponse at each challenge and therefore the MHV-68-specific immunosuppression was found to be long-lasting (Table 4.1), as tested (up to one month) after the initial challenge. It would therefore appear that specific tolerance to MHV-68 has been induced in these mice. The negative control response from challenge one was used to calculate the percentage suppression at each challenge. These percentage suppressions are shown in Table 4.1.

4.2.4 Attempt to transfer MHV-68-specific tolerance into syngeneic mice

The suppression of delayed and contact hypersensitivity reactions by UV irradiation is frequently associated with the appearance of antigen-specific T suppressor lymphocytes in the spleen (Kripke, 1984), as reported, for example, for the DTH response to HSV

Figure 4.3 The effect of UVB on the induction phase of the DTH response to MHV-68



Eight mice were used per group and were sensitised either with PBS, BHK-21 cell sonicate (approximately $1x10^4$ cells/mouse) or $4x10^5$ pfu MHV-68 intra-nasally. One group of mice were exposed to 96 mJ/cm^2 UVB on shaved dorsal skin 3 days before sensitisation with MHV-68. Eight days after sensitisation, all mice were challenged by injection of $4.5x10^6$ pfu equivalents of UV-inactivated MHV-68 into each ear. Post-challenge measurements of ear thickness were made at 24 hours after challenge and the mean increase in ear thickness for each group was calculated and is shown as mean ± SEM. Significant differences between the negative and positive control groups, and between the positive control group and the UVB-exposed group were determined using the student's *t*-test. * $p<0.001$ compared to BHK cell control group. ** $p<0.05$ compared to positive control group.

Table 4.1 Suppression of DTH to MHV-68 following repeat challenge without further UVB exposure

UVB irradiation	Sensitisation / infection	Challenge	Challenge 1		Challenge 2		Challenge 3	
			Increase in ear thickness (mm x 10^{-2} ± SEM[a])	% suppression of DTH	Increase in ear thickness (mm x 10^{-2} ± SEM[a])	% suppression of DTH	Increase in ear thickness (mm x 10^{-2} ± SEM[a])	% suppression of DTH
	BHK cell sonicate	Inactivated MHV-68	3.00±0.67					
	MHV-68	Inactivated MHV-68	10.94±0.86		9.44±1.09		13.19±0.71	
96mJ/cm^2	MHV-68	Inactivated MHV-68	8.06±0.68[b]	36.3%	6.38±1.11	47.5%	9.81±1.0[b]	33.2%

One group of mice were exposed to 96 mJ/cm^2 UVB on shaved dorsal skin three days prior to the initial sensitisation. Mice were sensitised intra-nasally on day 0 with either $1x10^4$ sonicated BHK-21 cells or $4x10^5$ pfu MHV-68. Mice were challenged on day 8 (after sensitisation) with UV-inactivated MHV-68 equivalent to $4.5x10^6$ pfu in both ear pinnae and then rechallenged at two week intervals, without further UVB exposure of mice.

[a] Eight mice per group

[b] Significantly different from the experimental group sensitised with MHV-68 without prior UVB exposure, $p<0.05$ (Student's *t*-test)

(Howie *et al.*, 1986b). To determine whether UV-induced suppression of the DTH response to MHV-68 was also transferable with spleen cells, an identical experimental protocol was employed to that of Howie (Howie *et al.*, 1986b). Spleens from mice with suppressed DTH responses were removed and single cell suspensions prepared. Normal syngeneic mice were infected with live virus ($4x10^5$ pfu/mouse) and then eight days later $2x10^7$ spleen cells from UVB-suppressed mice were transferred intra-venously by injection into the tail vein. Twenty-four hours later the recipient mice were challenged as usual. A control group of mice received $2x10^7$ control spleen cells from untreated mice intra-venously. As shown in Table 4.2, recipients of spleen cells from mice irradiated before sensitisation (group D), showed a reduced MHV-68-specific DTH response upon challenge, in comparison to mice sensitised with MHV-68 but which did not receive any spleen cells i.v (group B). Although the mean DTH response of group D was not found to be statistically significantly different from the control group (group C; $p=0.087$), as determined by the student's *t*-test, the suppression was calculated to be 28.8%. The group of mice (group C) which had $2x10^7$ control spleen cells transferred prior to challenge did not demonstrate a DTH response that was statistically different from the control group (group B; $p=0.47$). This would seem to indicate that the transfer of spleen cells itself had no effect on the subsequent elicitation of the DTH response.

4.2.5 Suppression of the elicitation phase of the MHV-68-specific DTH response by UVB exposure

A more natural sequence of events, with respect to the human situation, might involve the exposure of individuals to UV at a time point after primary infection. Experiments were therefore carried out to identify any effects of UVB exposure, subsequent to primary infection, on the DTH response to MHV-68 in mice. Mice infected with MHV-68 on day 0 were then exposed to one of two UVB regimens prior to challenge. The regimen consisted of either a single 144 mJ/cm^2 exposure (day 8) or exposure to 96 mJ/cm^2 UVB on three consecutive days (days 6, 7 and 8). The DTH response was elicited as before, on

Table 4.2 Transfer of suppression of DTH response to MHV-68

Group	Sensitisation / infection (Day 0)	No. spleen cells transferred i.v. (Day 8)	Increase in ear thickness ($mmx10^{-2} \pm SEM^a$)	% suppression of DTH
A	BHK cell sonicate		2.19 ± 0.38	
B	MHV-68		7.50 ± 0.80	
C	MHV-68	$2x10^7$ (control)	6.56 ± 0.98	
D	MHV-68	$2x10^7$ (UV)	5.30 ± 0.88	28.8%[b]

Mice were sensitised with either BHK-21 cell sonicate or MHV-68 ($4x10^5$pfu) intra-nasally on day 0. Eight days later mice in group D were injected i.v with spleen cells from mice previously exposed to 96 mJ/cm^2 UVB prior to sensitisation and demonstrated to have a suppressed DTH response on three subsequent challenges (Table 4.1). Mice in group C had the same number of control spleen cells transferred by i.v injection. All mice were challenged on Day 9 with UV-inactivated MHV-68 equivalent to $4.5x10^6$ pfu in both ear pinnae.

[a] Eight mice per group

[b] % suppression calculated in comparison to group C

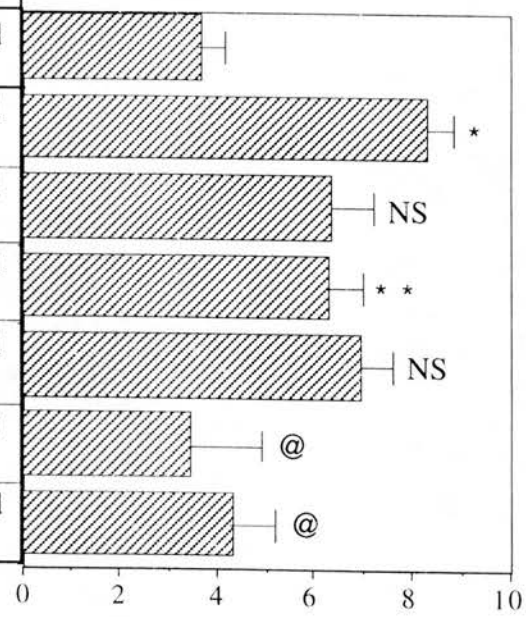
day 11. Since mice in group C (see Figure 4.4) were exposed to a cumulative dose of 288 mJ/cm^2 UVB, which would result in UV-induced damage and swelling of the ears, making measurements difficult, all mice were anaesthetised with Hypnovel/Hypnorm and their ears covered with foil during the irradiations.

Exposure of mice to one sub-erythemal dose of UVB (144 mJ/cm^2), three days prior to challenge of mice during primary infection (group D), was found to significantly suppress the DTH response ($p<0.05$), as shown in Figure 4.4. Exposure of mice to three sub-erythemal doses of UVB on consecutive days, up to three days before challenge (group C) also resulted in a reduced, although not significantly so, DTH response. The percentage suppression for groups C and D were calculated to be 41.4% and 43.7% respectively, in comparison with group B.

The ability of post-sensitisation UVB exposure of mice to alter the elicitation phase of the DTH response to MHV-68 was also studied in mice latently infected with the virus at the time of exposure to UVB. Latency has been shown to be established in mice 2-3 weeks after intranasal infection with MHV-68 (Nash *et al.*, 1996; Sunil-Chandra *et al.*, 1992a), provided the mice are less than three weeks of age at the time of infection. Latently infected mice (infected 3 weeks previously) were exposed to UVB of the same doses as previously described, either 3 days prior to challenge (144 mJ/cm^2), or 3, 4 and 5 days prior to challenge (96 mJ/cm^2 per day). The results are also shown in Figure 4.4. Latently infected mice were demonstrated to be able to mount a good DTH response to MHV-68 and this response was shown to be significantly suppressed ($p<0.05$) by either three sub-erythemal doses given on consecutive days before challenge (group F) or one sub-erythemal dose of UVB three days before challenge (group G). The percentage suppression of the DTH response induced by these UVB regimens was calculated to be 104.8% and 78.7% respectively, in comparison with group E.

Figure 4.4 The effect of UVB on the elicitation phase of the DTH response to MHV-68

Group	Sensitisation / infection	UVB irradiation	Challenge
A	BHK cell sonicate		Inactivated MHV-68
B	MHV-68		Inactivated MHV-68
C	MHV-68	3 x 96 mJ/cm^2	Inactivated MHV-68
D	MHV-68	1 x 144 mJ/cm^2	Inactivated MHV-68
E	Latent		Inactivated MHV-68
F	Latent	3 x 96 mJ/cm^2	Inactivated MHV-68
G	Latent	1 x 144 mJ/cm^2	Inactivated MHV-68



Eight mice were used per group. Mice in groups B, C and D were infected with $4x10^5$ pfu MHV-68 on day 0. Additional mice were sensitised with $1x10^4$ sonicated BHK-21 cells at the time of infection (group A). Mice in groups E, F and G were infected with $4x10^5$ pfu MHV-68 three weeks previously so that they were latently infected by day 0. Mice were exposed to two UVB exposure regimen as described either on days 6, 7 and 8 (96 mJ/cm^2) or on day 8 only (144 mJ/cm^2). All mice were then challenged on day 11 by injection of $4.5x10^6$ pfu equivalents of UV-inactivated MHV-68 into the dorsum of each ear. Post-challenge measurements of ear thickness were made at 24 hours after challenge and the mean increase in ear thickness for each group was calculated and is shown as mean ± SEM. Significant differences between the groups were determined using the student's *t*-test.

* Group B is significantly different from group A ($p<0.001$).

NS Groups C and E are not significantly different from group B ($p=0.068$ and $p=0.071$ respectively)

** Group D is significantly different from group B ($p<0.05$).

@ Groups F and G are significantly different from group E ($p<0.05$).

4.2.6 Effect of *in vivo* UVB exposure on viral replication

Experiments were carried out to determine whether UVB exposure of mice had any direct effect on viral replication following infection of 4 week old mice with MHV-68. Control, unirradiated mice and mice exposed to 96 mJ/cm^2 UVB on shaved dorsal skin, were infected intranasally with 4x10^5 pfu MHV-68 per mouse 24 hours later. The lungs from three mice from each group were collected at 1, 3, 5 and 10 days after infection. The viral titres were determined by plaque assay of tissue homogenates and the results are represented in Table 4.3 as $\log_{10}$pfu/organ. The figures represent the mean viral titre for the pooled homogenate from three mice per time-point. These results, although preliminary, would seem to suggest that exposure of mice to 96 mJ/cm^2 UVB, one day before infection with MHV-68, has no effect on viral replication in the lungs during a primary infection, since the viral titre results do not differ greatly between the two groups. The only possible difference between the groups is that the viral replication in the UVB irradiated group of mice appears to be slightly delayed in comparison to the unirradiated control group, with the peak titre reached at 5 days rather than 3 days post-infection.

Table 4.3 Viral titres of lung homogenates from UVB-irradiated and unirradiated mice, as determined by plaque assay

Time after infection (days)	Viral titre ($\log_{10}$pfu/organ)[a]	
	Control mice[b]	Irradiated mice[b]
1	3.45	3.45
3	4.20	3.39
5	3.45	3.72
10	ND[c]	1.00

[a] Limit of detection of plaque assay was 10 p.f.u/organ (($\log_{10}$pfu/organ=1.00)
[b]Mean infectious virus titre for pooled homogenate from three mice (age 4 weeks at time of infection) per time-point following inoculation with 4x10^5 p.f.u. of virus /mouse
[c] ND= below detectable level of this assay system

4.2.7 Inability of *in vivo* UVB exposure to reactivate latent virus

MHV-68 establishes latency in the spleens of the majority of mice, several weeks after primary infection. UVB exposure is known to trigger the reactivation of HSV. Experiments were therefore carried out to determine whether UVB exposure had any effect on latent MHV-68 infection. More specifically, the question was asked whether exposure of mice to a single sub-erythemal dose of UVB could lead to the reactivation of latent virus. Two groups of mice were infected with MHV-68 intranasally using a protocol known to result in latent infection 2-3 weeks after primary infection. Three weeks after primary infection, the mice were shaved on their dorsums and one group of mice was exposed to 96 mJ/cm^2 UVB. Spleens were then collected 1, 3 and 5 days after irradiation. The spleens were homogenised and the viral titre for each pooled homogenate (spleens from 3 mice per time-point) was determined by plaque assay. No plaques could be seen for any of the spleen homogenates. This indicates that if there was any infectious virus in any of the organs then it was below the limit of detection of this assay since positive control virus stocks were used to confirm the validity of the assay. The plaque assays were repeated using different aliquots of the homogenates but no plaques could be seen. This experiment therefore does not provide any evidence that exposure of mice to a single sub-erythemal dose of UVB causes reactivation of latent MHV-68.

4.2.8 Other immune responses to MHV-68

In order to investigate the effects of UVB exposure on other immune response to MHV-68, attempts were made to set up assays for other immune parameters. These included a lymphoproliferative assay and an enzyme-linked immunoassay (ELISA) to test for MHV-68-specific antibody in the serum of mice.

4.2.8.1. MHV-68-specific lymphoproliferation assay

Initial experiments involved the infection of mice intranasally with MHV-68 or control inoculation with either BHK-21 cell sonicate or PBS. Mice were reinoculated sub-cutaneously two weeks later and then lymph nodes were collected at time intervals and single cell suspensions were prepared. Lymph node cells ($2x10^5$ per well) were set up in quintuplicate wells and were stimulated with con A, UV-inactivated MHV-68 and BHK-21 cell sonicate, as described in section 2.3.4. Table 4.4 shows the proliferative responses induced in such an experiment. The con A-induced proliferation of lymph node (LN) cells from all three groups of mice peaked at 5 days after the secondary inoculation. In this experiment the proliferation of LN cells from mice inoculated with MHV-68 in response to con A was consistently less than that of cells from either control group. Normal mouse serum (NMS) was used instead of FCS in the medium control wells since this has previously been demonstrated to give lower background proliferations (Williams *et al.*, 1991). At both 3 and 5 days after secondary inoculation, an MHV-68-specific proliferative response can be seen in cells from mice inoculated with MHV-68, but not in cells from mice inoculated with either PBS or BHK-21 cell sonicate. The MHV-68-specific response was significantly different from both the BHK-21 sonicate and PBS-induced proliferative responses at both 3 and 5 days, as determined by a student's *t*-test ($p<0.001$). This MHV-68-specific response appears to be lost by 8 days post-inoculation. Cells from BHK-inoculated mice proliferate in response to *in vitro* stimulation with BHK-21 cell sonicate by 3 days post-inoculation but were not seen to do so at 5 days. Lymph node cells from MHV-68-inoculated mice did not respond to BHK-21 cell sonicate, demonstrating that the proliferation induced by the MHV-68 antigen is a specific response.

Although an MHV-68-specific proliferative response was observed in this experiment, such a response could not be consistently induced. Attempts were made to simplify the experimental protocol by collecting LN cells for *in vitro* stimulation after only one inoculation of the mice. However, no MHV-68-specific proliferative response by LN cells

Table 4.4 Lymphoproliferative response of lymph node cells to various stimulants

Stimulant	Concentration	C.p.m.± SEM								
		3 days p.i.			5 days p.i.			8 days p.i.		
		PBS	BHK	MHV	PBS	BHK	MHV	PBS	BHK	MHV
Con A	2.5 µg/ml	53126±2600	51255±2791	35406±1993	788852±2721	70617±1227	44637±923	16579±1116	28842±1553	9578±654
RPMI	(10% NMS)	499±89	220±7	246±22	67±8	126±27	1601±322	217±49	262±49	148±15
Inactivated MHV	moi = 1	491±83	636±85	3376±188	134±11	326±44	3657±192	157±20	173±17	180±21
Inactivated MHV	moi = 0.5	311±70	1144±209	3372±358	174±16	400±29	3991±391	318±37	206±26	202±20
Inactivated MHV	moi = 0.25	350±82	827±116	ND	227±29	444±48	4274±287	214±39	208±31	343±62
BHK cell sonicate	$2x10^2$ cells/well	277±110	4524±237	ND	121±7	342±65	927±66	ND	ND	ND

Mice were inoculated intra-nasally with either PBS, BHK-21 cell sonicate ($1x10^4$ cells/mouse), or MHV-68 ($4x10^5$ pfu/mouse). Two weeks later mice were reinoculated by sub-cutaneous injection of PBS, BHK-21 cell sonicate ($5x10^4$ cells/mouse) or MHV-68 ($2.7x10^6$ pfu/mouse). All LNs were removed from four mice per group at time-points of 3, 5 and 8 days after boosting. Pooled single cell suspensions were prepared from each group of inoculated mice and $2x10^5$ cells per well were incubated with either conA, UV-inactivated MHV-68 or BHK-21 cell sonicate as indicated, with quintuplicate wells set up for each. LN cells were also incubated with RPMI alone as a measure of background proliferation. LN cells were stimulated *in vitro* for 5 days at 37°C and each well was then pulsed with 0.7mCi [^{3}H]-thymidine for the final 24 hours. The cells were harvested and the cpm measured. The results are expressed as the mean cpm per quintuplicate set of wells ± SEM.

was observed at any time point tested after a single inoculation. No MHV-68-specific lymphoproliferative response was generated by spleen cells from MHV-68-infected mice either.

The primary productive infection of MHV-68 in mice is localised to the lung, following intranasal administration of the virus (Sunil-Chandra *et al.*, 1992a). Attempts were therefore made to isolate lymphocytes from lung tissue and identify any MHV-68-specific lymphoproliferative responses in these isolated cells. Lungs from infected mice were homogenised and lymphocytes isolated by density gradient centrifugation on Lympholyte-M. An additional step in which lung homogenates were exposed to collagenase-dispase (0.1 U/ml and 0.8 U/ml respectively) was also employed to try to disaggregate the lung tissue further before isolation of lymphocytes as described by van Ginkel (van Ginkel *et al.*, 1995). This technique yielded fairly low numbers of lymphocytes (approximately $5x10^4$ cells per lung) and although the cells isolated were viable, as determined by trypan blue exclusion, and were able to proliferate at low levels in response to stimulation with conA, no MHV-68-specific proliferation could be induced by *in vitro* stimulation with inactivated virus.

In summary, no consistent MHV-68-specific lymphoproliferative response was found using LN cells, spleen cells or lymphocytes isolated from infected lungs. It was therefore not possible to determine whether UVB exposure of mice had any effect on this cell-mediated response.

4.2.8.2 MHV-68-specific ELISA

Attempts were also made to establish an ELISA to detect the presence of MHV-68-specific antibodies in the serum of infected mice. As a positive control, a hyperimmune serum was made by infecting a group of mice subcutaneously with an emulsion of MHV-68 and Freund's complete adjuvant. These mice were subsequently boosted twice, at 4 week

intervals, with an emulsion of virus in Freund's incomplete adjuvant. The mice were then bled and the serum pooled. This positive immune serum and control mouse serum were used to optimise the ELISA system. However, it proved difficult to produce hyper-immune serum for MHV-68 which was sufficiently different in titre from the control serum to allow an effective test to be evaluated.

4.3 Discussion

4.3.1 Suppression of the DTH response to microorganisms by UVB exposure

4.3.1.1 Nature and dose of UVB exposure

Recent studies have addressed the significance of UVB-induced suppression of cell-mediated immune responses with respect to infectious disease. UVB irradiation has been demonstrated to cause abrogation of the DTH response to a number of microorganisms, as previously described. However, these studies vary greatly in terms of the nature and dose of UVB exposure employed. Systemic suppression has been demonstrated for the DTH response to *M. bovis* BCG and *C. albicans* (Denkins *et al.*, 1989; Jeevan and Kripke, 1989), as well as for CH (Morison and Kripke, 1984; Noonan *et al.*, 1981a), by exposure to a single high dose of UV radiation of approximately 45 kJ/m^2 (4500 mJ/cm^2). However, this dose greatly exceeds a typical dose encountered in a single exposure to solar irradiation and therefore the relevance of such experiments in relation to environmental exposure is not clear. Studies by researchers in Kripke's group have since utilised smaller doses given over a protracted period of time. For example, mice exposed to 2.26 kJ/m^2 UVB (226 mJ/cm^2; approximately 1 MED for the BALB/c mice used) on between one and fifteen occasions prior to sensitisation, demonstrated an impaired ability to mount a DTH response to *M.bovis* BCG (Jeevan and Kripke, 1990). Later in the course of this chronic UVB exposure regimen, mice recovered the ability to mount an effective DTH response.

In contrast, the suppression of the DTH response to HSV was seen following a single sub-erythemal exposure to UVB (Howie *et al.*, 1986a). Few studies to date have examined the potential of such sub-erythemal doses of UVB to affect the DTH response to viral agents which do not have any cutaneous manifestations. It has been demonstrated here that a similar sub-erythemal dose UVB exposure can suppress the DTH response to a respiratory viral pathogen. The possible mechanisms for this suppression are discussed below.

4.3.1.2 Effect of UVB on induction/elicitation phases of DTH

It was found that a single dose of UVB could suppress both the induction and elicitation phases of the DTH response to MHV-68. The UVB-induced suppression of the DTH responses to *M. bovis* BCG and *C. albicans* also occur both at the induction and elicitation stages (Denkins *et al.*, 1989; Jeevan and Kripke, 1989). The results from the *C. albicans* study in particular suggest that the suppression of DTH occurs by two different mechanisms, depending on the timing of UV exposure relative to sensitisation (Denkins *et al.*, 1989). Irradiation before sensitisation is known to lead to the appearance of splenic suppressor cells (see below). However, irradiation after sensitisation appears to interfere with the elicitation phase of the DTH response by a mechanism not involving splenic suppressor cells.

4.3.1.3 The development of splenic suppressor cells

It has been reported that the suppression of the DTH response to HSV-1 induced by exposure to 96 mJ/cm^2 UVB three days before sensitisation (Howie *et al.*, 1986a) can be transferred by injecting spleen cells from mice irradiated with UVB before immunisation with live virus, into syngeneic animals (Howie *et al.*, 1986b). This suppression of DTH to HSV was shown to be due to two distinct T cell subsets, namely Ly1$^+$2$^-$ and Ly1$^-$2$^+$ (Howie *et al.*, 1986b). Ultraviolet irradiation before sensitisation also leads to the appearance of suppressor cells for the DTH response to *C. albicans* (Denkins *et al.*, 1989), as well as for other antigens that induce DTH (Mottram *et al.*, 1988; Ullrich, 1986; Ullrich *et al.*, 1986) The suppressor cells specific for *C.albicans* were identified as having the phenotype Thy1.2$^+$, Lyt2$^+$, L3T4$^-$, Lyt1$^-$ (Denkins *et al.*, 1989). It should be noted that different protocols for the transfer of suppression were employed in these studies. The ability to transfer UV-induced suppression of DTH to *C. albicans* with spleen cells was demonstrated by transfer of spleen cells from UV exposed mice, known to have a suppressed DTH response, into naive mice. These recipients were immediately sensitised with *C. albicans* antigen and their DTH response measured six days later. In contrast, Howie

et al demonstrated the transfer of suppression to HSV by injecting spleen cells from suppressed mice, into syngeneic mice that had been infected with live virus eight days previously (Howie *et al.*, 1986b). This latter protocol allowed the examination of the cells responsible for the efferent suppression of DTH to HSV and was the protocol employed to investigate the possibility of transfer of suppression in the MHV-68 system.

The transfer experiment, carried out to test whether suppressor spleen cells are induced upon UVB-induced suppression of the DTH response to MHV-68, was inconclusive because the group of mice which received spleen cells from mice known to be suppressed did not demonstrate a statistically significant reduction of the DTH response upon challenge. However, there was a 41.4% suppression in the DTH response of this group and it would therefore be worth repeating the experiment several times to establish whether the suppression observed following spleen cell transfer was a genuine result, indicating the presence of MHV-68-specific suppressor cells. It is possible that insufficient spleen cells were transferred into the recipient mice, although suppression of the DTH response to HSV-1 was transferable with between $1x10^7$ and $3x10^7$ spleen cells (Howie *et al.*, 1986b).

4.3.1.4 Effect of UVB on antigen-presenting capacity of cells

UVB irradiation is known to affect the antigen-presenting capacity of the skin (Perry and Greene, 1982; Stingl *et al.*, 1981) which can partly explain the suppression of immune responses to cutaneous viruses such as HSV. It has been demonstrated that the functional ability of epidermal cells to present HSV is altered following *in vivo* UVB exposure (Howie *et al.*, 1986c). It was also shown that suppression of the DTH response occurred if mice were inoculated at the site of HSV infection with epidermal cells from irradiated mice (Howie *et al.*, 1987). This suppression was shown to be specific and to be associated with splenic T cells. It is therefore believed that suppression of the DTH response to HSV is mediated by splenic suppressor cells and

that the initial presentation of antigen by epidermal cells determines whether the DTH response itself or suppressor cells are generated (Norval and El-Ghorr, 1996).

It is possible to imagine that the same mechanisms involved in the UVB-induced suppression of DTH to HSV are also responsible for the suppression induced to other cutaneous pathogens. It is more difficult to imagine how exposure of the the skin to UVB can result in suppression to a pathogen administered directly into the respiratory tract. There is some evidence that UVB irradiation can affect the antigen-presenting cell function in the spleen and lymph node (Gurish *et al.*, 1982; Simon *et al.*, 1990) and it is possible that the suppression is generated in such lymphoid tissue.

4.3.1.5 The role of cytokines in the UVB-induced suppression of DTH responses

Keratinocyte-derived IL-10 is an essential mediator for the UV-induced suppression of DTH to alloantigen (Rivas and Ullrich, 1992; Ullrich *et al.*, 1990). In a mouse model of infection with *B. burgdorferi*, the DTH response was diminished by exposure of mice to 10 kJ/m^2 (1000 mJ/cm^2) UVB 4 days before immunisation (Brown *et al.*, 1995). Administration of antibody to IL-10 at 4 and 24 hours after UV irradiation abrogated this effect of UVB. Injection of rIL-10 (10 ng/mouse) into mice 4 days prior to sensitisation was found to reduce the DTH response by approximately 50%. These results confirm the importance of IL-10 as a mediator of UVB-induced immunosuppression of DTH responses.

Experiments to investigate the role of keratinocyte-derived soluble mediators in the UVB-induced suppression of DTH to *M.bovis* BCG have also been carried out. Supernatants from UV-irradiated keratinocytes were injected intra-venously into mice and were able to suppress both the induction and elicitation phases of the DTH response (Jeevan *et al.*, 1992b). The results suggest that soluble mediators released from keratinocytes may act by interfering with certain macrophage functions. This provides one explanation for the systemic impairment of the immune response caused by exposing the dorsal skin of mice to UV radiation. To further characterise the

cytokines involved, UV irradiated mice were injected with antibodies to various cytokines. Injection of mice with anti-IL-10 immediately after UV exposure restored the DTH response and reversed the observed UV-induced inhibition of bacterial clearance (Jeevan *et al.*, 1996). Injections of anti-TGF-β1 only partially restored the DTH response. Cytokines and in particular IL-10, therefore appear to play a role in the UVB-induced suppression of the DTH response to several microorganisms. It is possible that such soluble mediators are involved in the suppression of the DTH response to MHV-68, although this hypothesis remains untested.

4.3.2 Relationship between suppression of DTH response and pathogenesis of the infectious disease

The demonstration of UVB-induced suppression of a relevant immune response gives cause for concern, particularly when the suppression results from an environmentally relevant UVB dose. However, a more important consideration is whether this suppression can affect the pathogenesis of the infectious disease. For example, exposure to UVB has been shown to trigger the recrudescence in mice with latent HSV infection (Yasumoto *et al.*, 1987).

Attempts have been made in a number of animal models of infectious disease, to investigate any effect of UVB on pathogenesis. One such study was carried out to determine whether UV irradiation, known to inhibit the DTH response, also affects the pathogenesis of systemic *C. albicans* infection. It was found that the survival time of mice exposed to UVB one day before challenge of immunised mice with a lethal dose of viable yeast, was reduced by more than 50% (Denkins and Kripke, 1993).

The study in which the DTH response to *M.bovis* BCG was suppressed early in the course of chronic UVB exposure, also showed an increase in the number of bacterial colony-forming units in the spleen and LN of the irradiated animals compared with unirradiated controls (Jeevan and Kripke, 1990). With continued exposure, the mice regained the ability to mount a DTH response and there was no longer an increase in

the number of viable bacteria in the lymphoid organs. In a model of *L.major* infection, although the DTH response was depressed in UV-irradiated mice, the appearance of lesions was actually improved by exposure of the mice to UVB prior to infection; however, UV irradiation did not reduce the number of parasites in the infected skin (Giannini, 1986).

The experiments involving the effects of UVB on MHV-68 viral replication and latency are preliminary and so it is not yet clear whether the UV regimen used to impair the DTH response to MHV-68 is associated with an increase in susceptibility to infection. However, the fact that exposure of mice to UV radiation alters their ability to generate and elicit an immune response to MHV-68 raises the possibility that increased pathogenicity, or a decrease in the ability of mice to prevent the establishment of latency may result. This has possible implications for the human situation and EBV infections. Further work is therefore required to determine whether there is a risk of increased pathogenicity in the mouse model of MHV-68 and the potential therefore of such a detrimental effect on human health.

Exposure to UVB is believed to cause reactivation of latent HSV by causing a transient suppression of the local immune response at the site of reactivation, which allows latent virus to replicate and a productive infection may result. It is thought that while antibody-mediated immune responses contribute little to recovery from primary HSV infections, they are important in terms of preventing the establishment of latency (Nash and Cambouropoulos, 1993). For example, agammaglobulinaemic mice suffer a more severe HSV infection of the nervous system and have a higher incidence of latent infection (Kapoor *et al.*, 1982). It is therefore unlikely that UVB-mediated suppression of cell-mediated immune responses will aid the establishment of HSV latency. However, since T cells are known to be important in the regulation of B cells latently infected with MHV-68, it is possible that such suppression may enable latency to be established when it otherwise wouldn't be as a result of the regulatory immune response, or a greater degree of latency to be established. In addition, while there is

not currently any available information as to the predominance of a Th1 or Th2 type response to MHV-68, it may be that the reported selective suppression of Th1 type responses by UVB, allows MHV-68 to establish a latent infection in the spleen more readily.

4.3.3 Criticisms of experimental design and suggestions for further work

Since only sub-erythemal doses of UVB were employed in the experiments to study the effects of such exposure on the induction phase of the DTH response to MHV-68, it was originally felt that there was no need to cover the ears of the mice as they underwent UVB exposure, as no UV-induced ear swelling was anticipated. However, since the virus was administered intranasally and not through a cutaneous route, the suppression induced by UVB exposure can be said to be systemic. It would however have been interesting to have shielded the ears during irradiation to see if this affected the amount of suppression of the DTH response compared to that observed in unshielded ears, in order to eliminate the possibility that local irradiation of the test site causes the suppressive effect seen.

It would also be interesting to determine the duration of the suppressive effect of a single exposure to UV radiation. To do this mice should be UV irradiated, and sensitised at several timepoints thereafter, ie do mice exposed to UVB earlier than 3 days prior to sensitisation all exhibit a markedly depressed DTH response. Studies with *C. albicans* show that exposure to 46.8 kJ/m^2 between 3 and 14 days prior to sensitisation resulted in a 64-100% suppression of the DTH response (Denkins *et al.*, 1989). Exposure to the same UVB dose 21 days before sensitisation did not result in an impaired DTH response. Similar studies were carried out using HSV and it was found that if irradiation took place 7 days before inoculation with virus, suppression of the DTH response occured. However, if irradiation took place 14 days before, no suppression was found (Howie *et al.*, 1986a).

The preliminary experiments to identify any possible effects of low dose UVB on viral latency should have included the co-cultivation techniques employed by Sunil-Chandra (Sunil-Chandra *et al.*, 1992a). This involves the co-cultivation of tissue with permissive cells for 5 days prior to the plaque assay and allows the isolation of latent/persistent virus. This technique would have allowed absolute verification that the mice were latently infected. It would also have shown any effects of UVB irradiation of mice, prior to sensitisation, on the establishment of latency.

The inability to generate hyper-immune serum to MHV-68 may reflect the technique used as others have produced such serum by intramuscular inoculation of live virus in Freund's complete adjuvant, followed by booster injections in incomplete adjuvant (Sunil-Chandra *et al.*, 1992a). The poor hyper-immune serum generation may also be an indication of the non-immunogenic nature of this virus. Since a strong virus-specific immunoproliferative response could not be consistently induced either, it seems probable that the virus is relatively non-immunogenic, being a poor inducer of both cell-mediated and antibody responses. It is possible that this is a contributory factor in the ability of MHV-68 to establish latency.

A protocol for an ELISA for MHV-68-specific antibody has recently been published (Usherwood *et al.*, 1996b). It may therefore be interesting in the future to use this protocol to identify any effects of UVB irradiation on the generation of a humoral immune response to MHV-68. However, anti-viral antibody is of relatively little importance in the resolution of the primary lung infection (Usherwood *et al.*, 1996b).

Although the mice used throughout these studies were successfully infected with MHV-68, by determination of infectious viral titre in the lungs, the titres obtained were lower than the mean titres reported following intranasal infection of BALB/c mice using ether anaesthesia (Sunil-Chandra *et al.*, 1992a). Approximately 50% of the BALB/c mice developed clinical signs and symptoms 7 to 9 days post-infection. In severe cases, these consisted of ruffled fur, hunched stance, severe weakness and

emaciation. Some severely sick mice died 10 to 12 days post-infection, whereas those with mild clinical symptoms recovered during this period. Comparable symptoms were not seen in the C3H/HeN mice used in this study, although a few mice appeared to have some of the signs described. This could be an indication that either the infection procedure using halothane anaesthesia or the different mouse strain, or a combination of both factors, result in a less severe infection.

4.4 Summary

The primary aim of this study was to establish a protocol for the induction, elicitation and measurement of the DTH response to the systemic gammaherpesvirus MHV-68 in C3H/HeN mice. The effect of a single sub-erythemal dose of UVB on the induction phase of DTH was investigated. Mice irradiated with 96 mJ/cm^2 on shaved dorsal skin, three days before intranasal injection of MHV-68, showed suppressed DTH responses (by approximately 40%) to challenge with inactivated virus, compared with non-irradiated control animals. This suppression was specific for MHV-68. Further studies found the suppression to be long-lasting, as tested up to one month after the first challenge, which would suggest that tolerance to MHV-68 antigen was induced. UVB exposure was also shown to suppress the elicitation phase of DTH in both productively and latently infected mice. Preliminary data suggest that exposure of mice to sub-erythemal doses of UVB has no effect on the replication of MHV-68 in the lungs following primary infection or on latent virus.

Chapter 5

Expression of Cytokine mRNA in Herpes Simplex Virus Type 1-Infected Keratinocytes *in vivo* and *in vitro*

5.1 Introduction

Herpes simplex virus (HSV) infects its host at a mucosal or epithelial surface. It replicates in the epidermis, inducing a vesicular lesion, and may then establish latency in the cell bodies of peripheral nerves innervating this site. Reactivation of the virus can occur following certain triggering factors which stimulate new viral synthesis in the sensory ganglion followed by travel of the virus down the sensory axons and infection of epidermal cells at the site of first exposure. During primary infection the immune response serves to control the production of infectious virus and to localise the lesion. However, while a great deal is known about the immune responses generated systemically to HSV, relatively few investigations have been carried out to identify the local immune responses initiated by infection with HSV. These may be critical in determining the outcome of the infection and the subsequent interaction of the virus with the host during latency and recrudescence.

A range of cellular immune components have been implicated in local anti-HSV immunity (see section 1.6.2.1). A study in which sections of skin biopsies from patients with recurrent peripheral HSV lesions were immunoperoxidase stained with a variety of antibodies has been carried out (Cunningham *et al.*, 1985). It revealed a great deal about the types of cells recruited into HSV lesions and hence the relative importance of these cells in the local immune response against HSV. Helper T lymphocytes were the predominant cell type in subepidermal and perivascular regions

of early lesions (12-24 hrs), but this Leu3 $(CD4)^+$ Th cell predominance over Leu2 $(CD8)^+$ cells was reduced in later lesions. From two days onwards, as seen in these later lesions, monocytes/macrophages became more prominent in the inflammatory infiltrate, particularly adjacent to vesicles. Since no B cells and very low numbers of NK cells were detected in the subepidermal infiltrate, it was suggested that T cells and monocytes migrated selectively into the lesions. A more recent report of the immune cell infiltration into corneal tissue following UV-induced reactivation of HSV-1 in the trigeminal ganglion, also reported the predominant early infiltrating cells to be T cells, although these were both $CD4^+$ and $CD8^+$ cells (Shimeld *et al.*, 1996). In addition large numbers of B cells subsequently appeared, suggesting a role for local antibody production in the control of reactivated infection in this model.

An important consideration is the antigen-presentation of viral antigen necessary for the initiation of effective primary and secondary anti-HSV immune responses. Studies have revealed that while spleen cells are capable of presenting HSV to non-immune virus-specific T cells *in vitro*, epidermal cells do not share this ability (Williams *et al.*, 1991). Epidermal cells were however capable of restimulating the HSV-specific response of *in vivo* primed mouse T cells. Cell depletion studies revealed the HSV-specific responder cells to be mainly Thy-1^+, Lyt-1 $(CD5)^+$, L3T4 $(CD4)^+$, Lyt-2 $(CD8)^-$ and I-A^-, which agrees with the predominant T cell type observed in the immunohistological study. The activation of HSV-specific T cells does not therefore appear to take place locally in the epidermis during the initial encounter with the virus. However, during secondary challenge, for example upon reactivation of latent virus, LC are able to present antigen efficiently to primed T cells.

In addition to the infiltrating inflammatory cells, keratinocytes are likely to play an important role in the establishment of the local immune environment. Throughout its natural infection, HSV interacts with epithelial cells, known to be predominantly keratinocytes, at the entrance of the infectious agent through the skin and during recurrent infections. Based on the fact that keratinocytes can present HSV-1 antigen to

T cells (Cunningham and Noble, 1989) and that keratinocytes are capable of both transcribing and producing a range of cytokines (see table 1.1), a crucial role has been suggested for keratinocytes in the immune response to HSV. It is thought that infiltrating macrophage and T cell-derived IFN-γ induces MHC class II expression on epidermal keratinocyes, allowing these cells to present antigen directly to T lymphocytes in recurrent HSV infections (Cunningham *et al.*, 1985). It is believed that the enhancement of keratinocyte-derived cytokines *in vivo* may also have effects both locally and systemically, allowing the epidermis to have a considerable regulatory role during immune responses.

Exposure to sunlight is one of the precipitating factors involved in the recurrence of HSV infections (see section 1.8.1.1). A number of studies have been carried out to investigate the effects of UVB irradiation on immunity to HSV infection in mice. UVB irradiation of mice prior to infection with HSV either sub-cutaneously (Howie *et al.*, 1986a) or intradermally (Yasumoto *et al.*, 1987), resulted in a suppressed DTH response when mice were subsequently challenged. Antigen-specific T lymphocytes were found to have been generated in the spleens of UVB-irradiated mice, which were capable of mediating suppression upon transfer into mice already infected with HSV (Howie *et al.*, 1986b; Yasumoto *et al.*, 1987). This T cell-dependent phenomenon was demonstrated to be due to two phenotypically distinct subsets; one $Ly1^{-}2^{+}$ ($CD4^{+}$) and the other $Ly1^{+}2^{-}$ ($CD8^{+}$) (Howie *et al.*, 1986b). The suppression of DTH could also be induced by the transfer of epidermal cells from UV-irradiated mice, as long as these cells were injected into the recipients at the same time and into the same site as the virus (Howie *et al.*, 1987). Additionally, antigen-presentation by epidermal cells was shown to be impaired by UVB (Howie *et al.*, 1986c). Irradiation of mice prior to a secondary HSV infection resulted in an increased severity of infection which may be due to modulation of local antigen presentation (El-Ghorr and Norval, 1996). Further studies have tried to elucidate the possible mechanisms which might lead to recrudescence (Aurelian *et al.*, 1988; Yasumoto *et al.*, 1987). UVB exposure of the

site of viral infection has been shown to result in a higher incidence of zosteriform lesions in mice.

Araneo and co-workers (Araneo *et al.*, 1989) reported that UV exposure depresses IL-2 and enhances IL-4 production by activated T lymphocytes in mice. They suggested that UV irradiation may induce promotion of cytokines of a Th2 type with concomitant down-regulation of Th1 cells. This concept of preferential activation of Th2 cells after irradiation may be important in the context of cutaneous HSV infection, because clearance of HSV is known to primarily depend on DTH responses, mediated by Th1 cells. A delay in the clearance of HSV from the infected cutaneous site could potentially lead to the development of more severe skin lesions. Similarly, altered local cytokine profiles could lead to a higher frequency of recrudescence.

The aim of this study was to develop and characterise *in vitro* and *in vivo* models of HSV infection. The *in vitro* model consisted of infection of a murine keratinocyte cell line, PAM-212, with HSV-1. In the *in vivo* model C3H/HeN mice were infected epidermally with HSV-1. Using these models, cytokine profiles, at the mRNA level, were examined at time intervals following HSV infection. The cytokines examined were IL-1α, TNF-α and IL-10.

IL-1 is a pro-inflammatory cytokine secreted by many cell types. The major cell source of IL-1 in the epidermis is keratinocytes which produce active IL-1α and the inactive IL-1β precursor. The production and regulation of IL-1α are described in section 1.5.2.3. IL-1α has a pivotal role in epidermal inflammation. It induces adhesion molecules on endothelial cells and stimulates the secretion of secondary cytokines such as IL-6 (Partridge *et al.*, 1991), IL-8 (Larsen *et al.*, 1989) and GM-CSF (Kupper *et al.*, 1988) from many cells including fibroblasts and keratinocytes.

The pro-inflammatory cytokine TNF-α induces the secretion of IL-1 (Kutsch *et al.*, 1993), IL-6, IL-8 and GM-CSF by KC. TNF-α also stimulates the expression of

MHC class II, IL-2 receptor and the adhesion molecules ICAM-1 and E-selectin on endothelial cells. TNF-α is expressed at low levels in normal skin (Oxholm *et al.*, 1991) . TNF-α is implicated in the migration of LC from the epidermis (Cumberbatch and Kimber, 1992) and is thought to be one of the key soluble mediators of UVB-induced immunosuppression (see section 1.5.3.1).

The sources of IL-10 and its function as an inhibitory regulator of the immune system have previously been described in section 1.5.3.2(i). IL-10 regulates the inflammatory immune response by inhibiting the synthesis of IL-1α, TNF-α, GM-CSF and CSF (de Waal Malefyt *et al.*, 1991) and IFN-γ production by T cells (Enk, 1994; Fiorentino *et al.*, 1991) . The latter may be partially mediated by the down-regulation of IL-12 p40 by IL-10 (D'Andrea *et al.*, 1993). IL-10 directs the immune response towards a Th2 type response by inhbiting Th1 cell function and augmenting Th2 cell growth (Gajewski and Fitch, 1988).

5.2 Results

5.2.1 Infection of PAM-212 cells with HSV-1

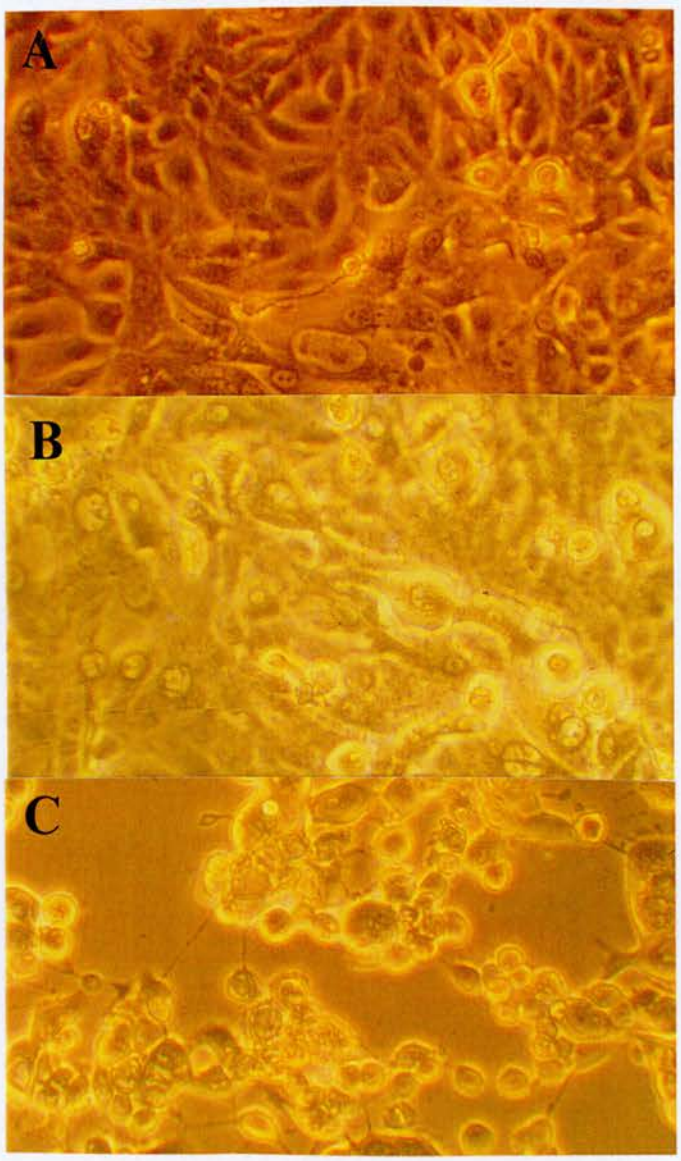
PAM-212 cells were infected with HSV-1 at a m.o.i. of 0.5 and, by 12 hours post-infection, a characteristic cytopathic effect (c.p.e) was visible microscopically, presenting as rounded, ballooned cells in foci. Figure 5.1 shows uninfected PAM-212 cells and cells at 12 and 24 hours post-infection.

5.2.2 Infection of mouse skin with HSV-1

Preliminary studies were carried out to establish a suitable site for HSV infection of C3H/HeN mouse skin. Initially the ears were lightly tape-stripped and infected with the virus, since they provide a localised site for cutaneous infection. However, no lesions developed on any ears infected in this way. In addition, when cryostat sections were made of the ears, and these sections were examined following immunoperoxidase staining with anti-HSV antiserum as described in section 2.4.3, no positive staining could be identified. This suggested that mouse ears were not a suitable site for the study of localised HSV infection. It is possible that the tape-stripping procedure necessary to allow penetration of the virus, does not leave sufficient epidermal cells for establishment of a cutaneous infection at this site.

Subsequent infections of mice with HSV were made on dorsal skin. The dorsum was shaved and tape-stripped, as described in section 2.4.2. All mice developed lesions between 3 and 5 days post-infection and mice with typical lesions are shown in Figure 5.2. Cryostat sections of skin samples taken at intervals after infection, were made and stained using an immunoperoxidase method with anti-HSV antiserum. Examples of such immunohistochemical staining of mouse back skin sections are shown in Figure 5.3. Viral antigen could be detected in these sections from 3 days after infection. Positive staining was largely localised to the epidermis. The lesions were self-healing and had generally resolved by 10-12 days post-infection.

Figure 5.1 HSV-infected PAM-212 cells



Uninfected PAM-212 cells (A) and at 12 hours (B) and 24 hours (C) after infection with HSV-1 at moi of 0.5, demonstrating viral-induced cytopathic effect. Magnification x100.

Figure 5.2 Mice with dorsal HSV-1 lesions

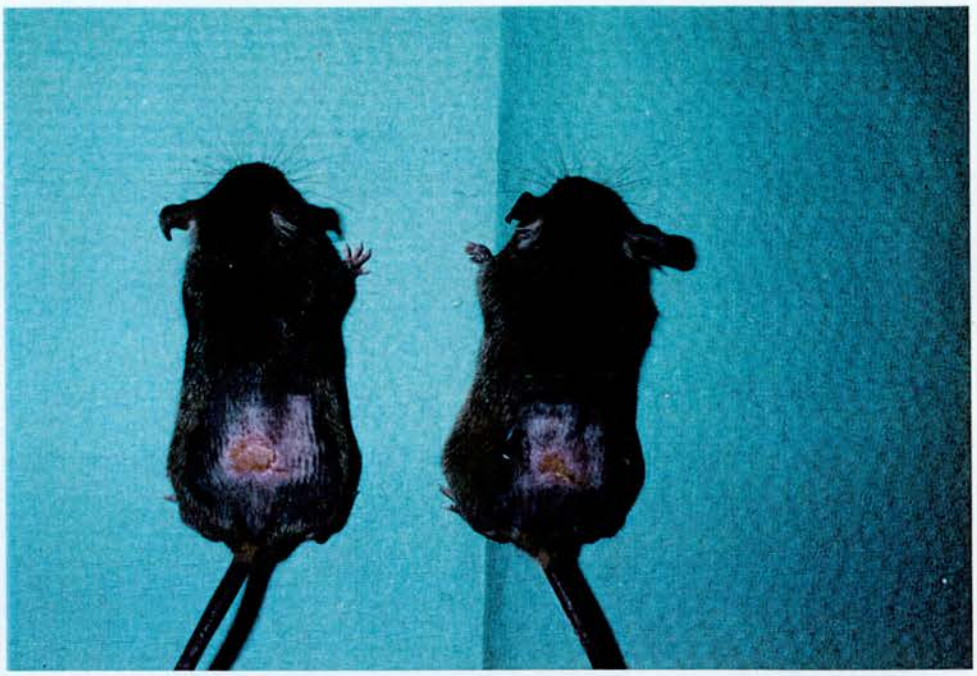
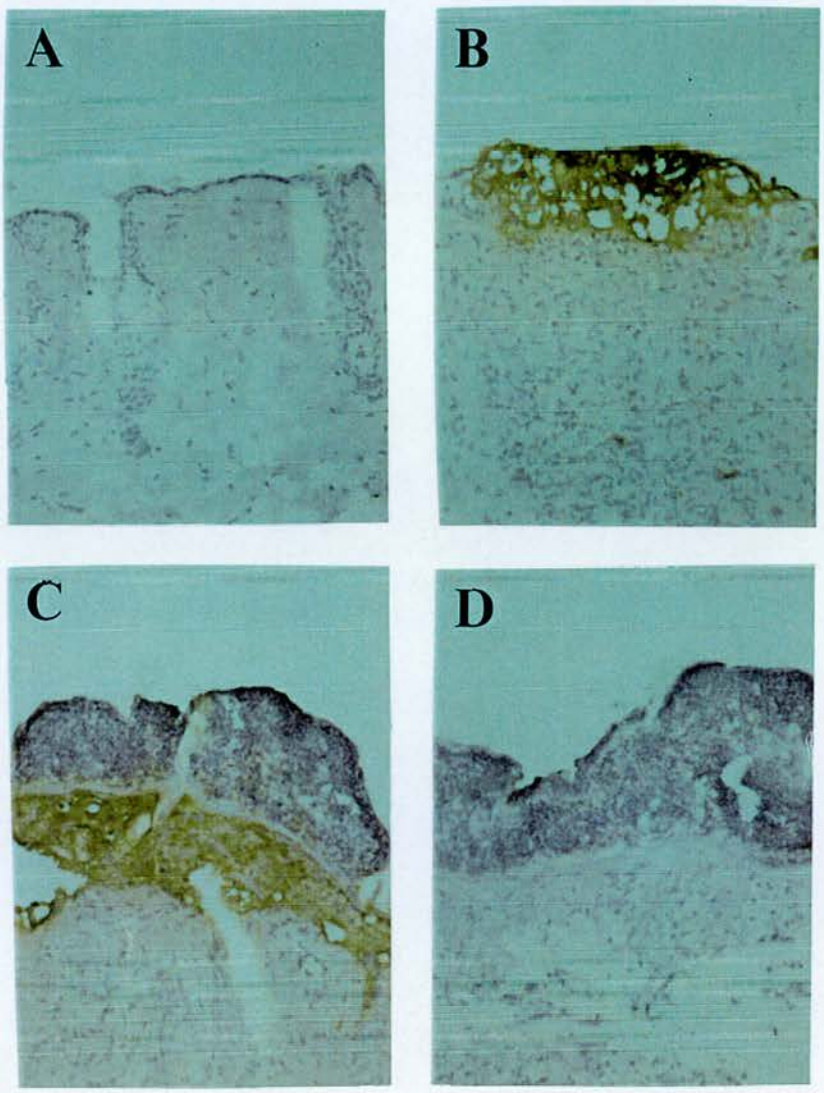


Figure 5.3 Sections of HSV-1-infected mouse back skin



Immunoperoxidase staining of mouse back skin sections at (A) 0 hours (B) 48 hours and (C) 96 hours post-infection with HSV-1 using an anti-HSV polyclonal antibody, and a control section stained using an anti-cytomegalovirus primary antibody (D) 96 hours post-infection. (All at x110 magnification).

5.2.3 Semi-quantitative PCR

5.2.3.1 Optimum cycle number determination

In order to analyse cytokine mRNA expression semi-quantitatively, the optimum cycle number for each PCR product was determined. A pool of cDNA taken from PAM-212 cell and one from mouse skin RT reactions, from all stages of a HSV timecourse of infection experiment were used as target sequences. Figure 5.4 shows the autoradiographs for these cycle experiments. Forty cycles was the maximum cycle number used in this study since above this number the reaction is likely to plateau due to exhaustion of DNA polymerase and other PCR reagents. Integrated OD units of PCR products from the cycle experiments were derived and the linearity of PCR product accumulation versus cycle number was determined (Figures 5.5 and 5.6). Optimum cycle numbers for detection of each cytokine mRNA determined from Figure 5.5 for PAM-212 cell cDNA were 28 cycles for β-actin, 32 cycles for IL-1α and 36 cycles for IL-10 and TNF-α. Optimum cycle numbers for mouse skin cDNA samples determined from Figure 5.6 were 28 cycles for β-actin, 32 cycles for IL-1α and IL-10, and 34 cycles for TNF-α.

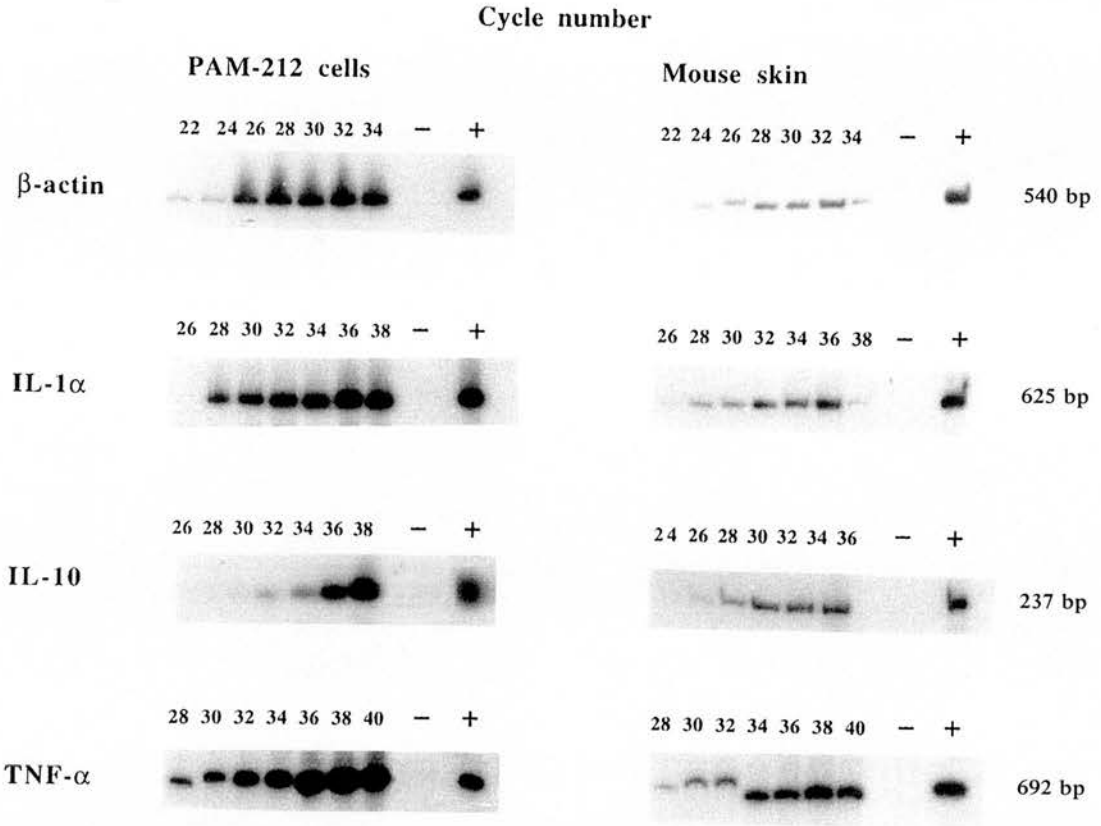
5.2.3.2 Semi-quantitative expression of cytokines following HSV infection

5.2.3.2 (i) *In vitro* model of HSV infection

The expression of IL-1α and IL-10 mRNA was analysed in PAM-212 cells at intervals after infection with HSV-1. Representative autoradiographs of PCR products for these cytokines at the optimum cycle number are shown in Figure 5.7(a). Comparable observations to those described here were seen upon repeat PCR of the same samples and also following RT-PCR of a second set of samples.

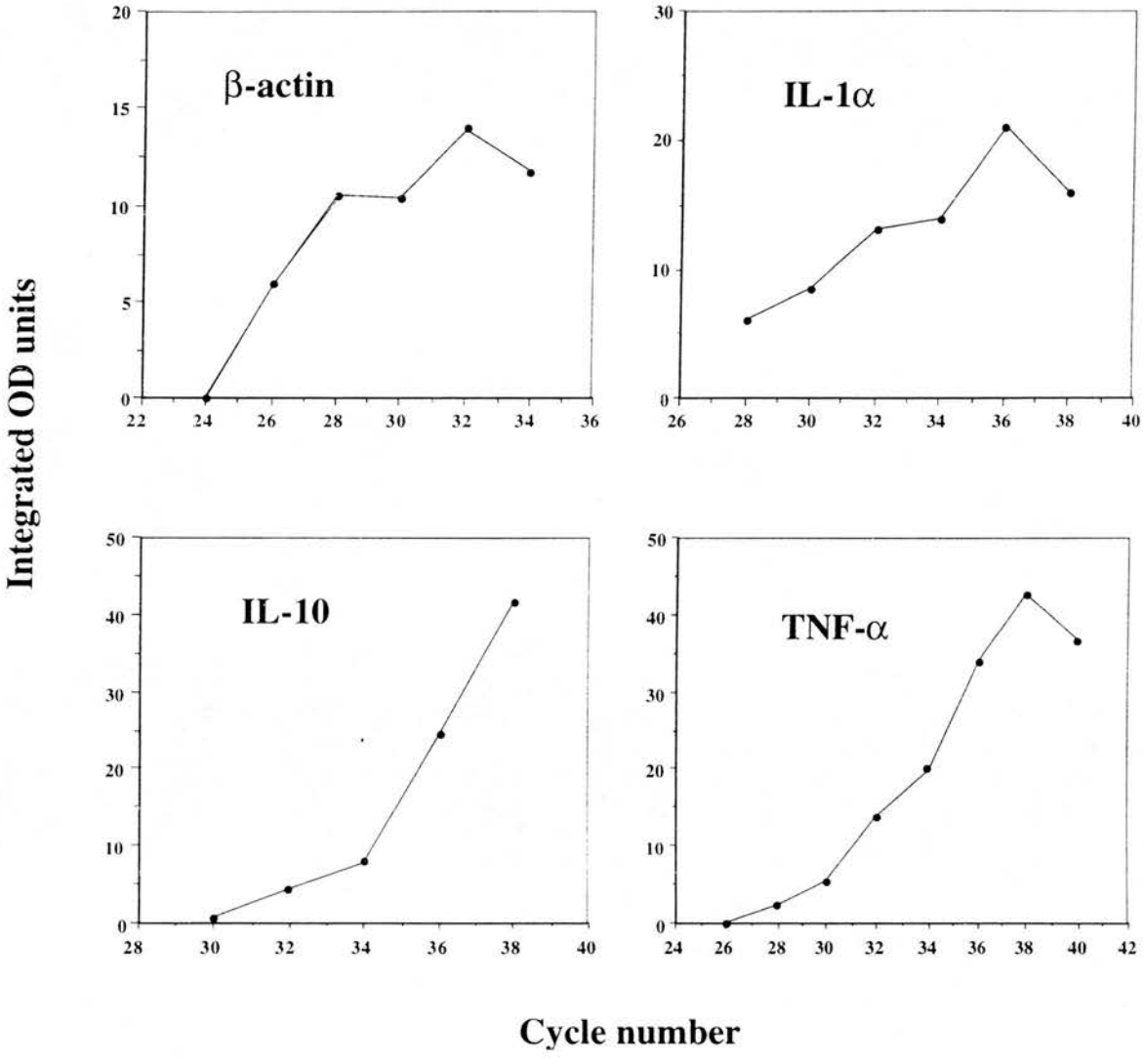
Figure 5.8(a) represents the expression of IL-1α mRNA after HSV infection in PAM-212 cells. HSV infection induced a decrease in the level of mRNA expression of this cytokine between 0 and 6 hours post-infection. After this time, the level of IL-1α gene expression gradually increased towards the initial level by 24 hours post-infection.

Figure 5.4 Determination of optimum cycle numbers



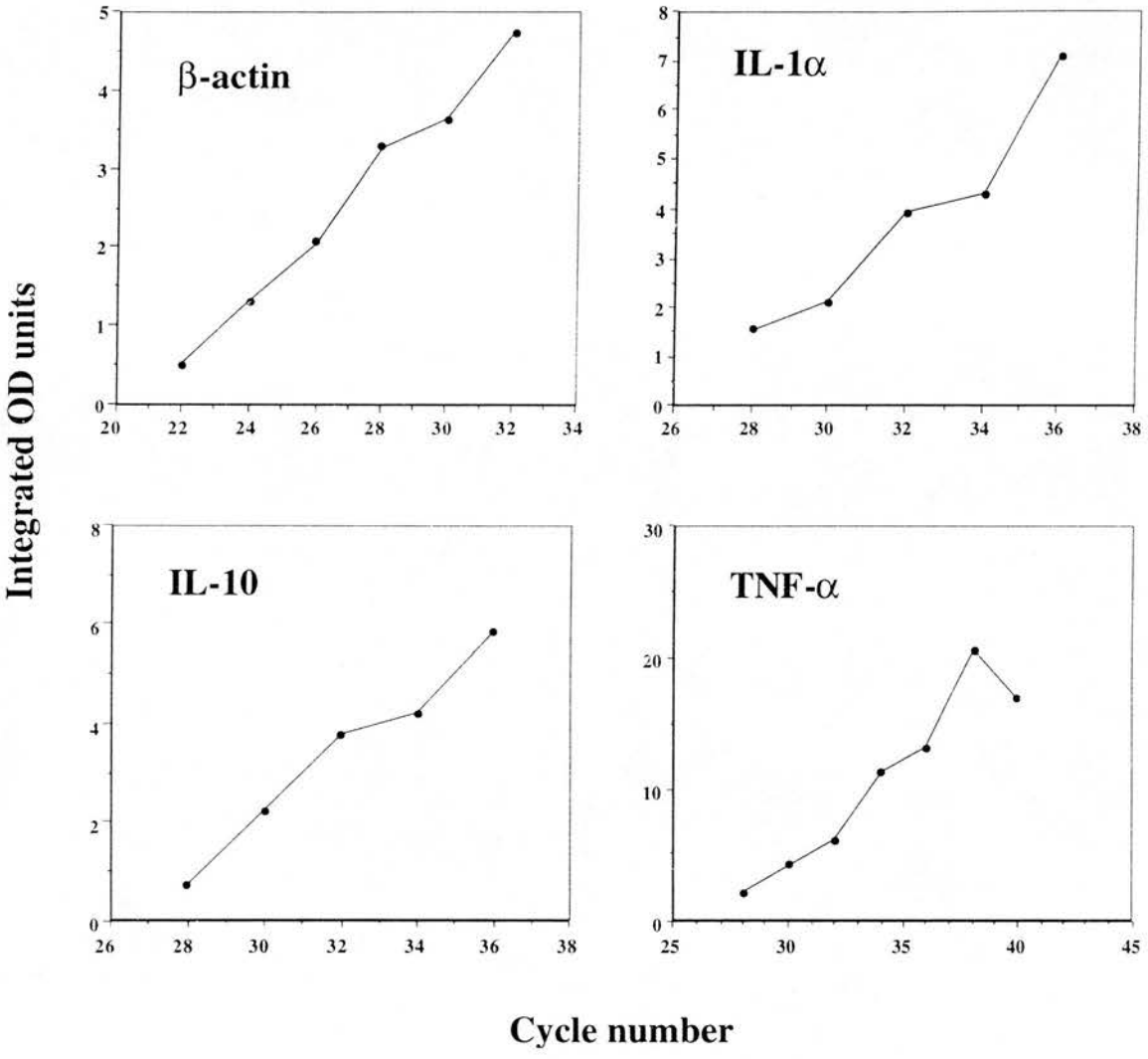
PCR reactions were performed using pooled RT reactions, as described in section 5.2.3.1. Reaction tubes were removed at determined cycle number. Products were subjected to SDS-PAGE and autoradiographs of these gels are shown.

Figure 5.5 **Linearity of PCR product accumulation with increasing cycle number for PAM-212 cells**



Densitometry of PCR products shown in Figure 5.4 was performed and accumulation of PCR product versus cycle number is shown.

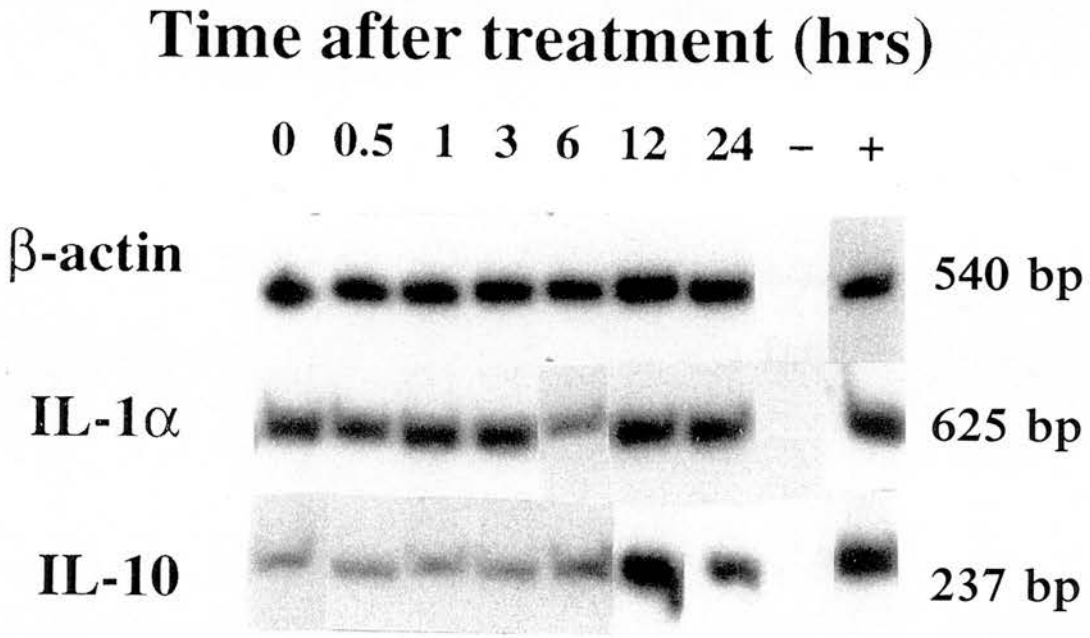
Figure 5.6 Linearity of PCR product accumulation with increasing cycle number for mouse skin samples



Densitometry of PCR products shown in Figure 5.4 was performed and accumulation of PCR product versus cycle number is shown.

Figure 5.7 **Cytokine expression following infection with HSV-1**

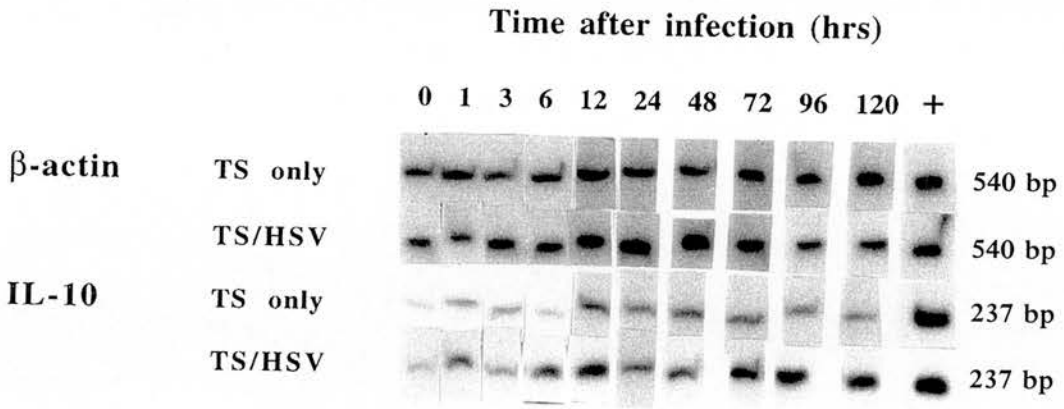
(a) In PAM-212 cells



Autoradiographs of representative [^{32}P]-labelled cytokine PCR products of PAM-212 cells subjected to SDS-PAGE.

Figure 5.7 **Cytokine expression following infection with HSV-1**

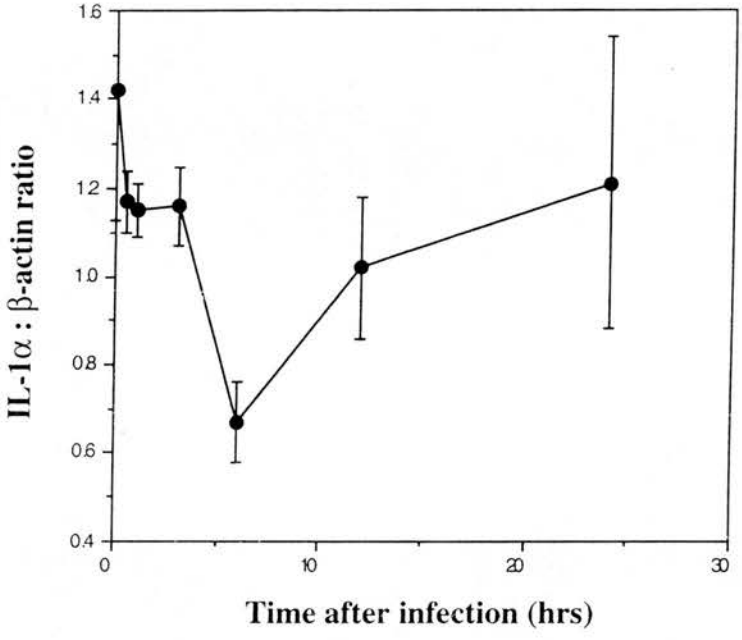
(b) In mouse back skin



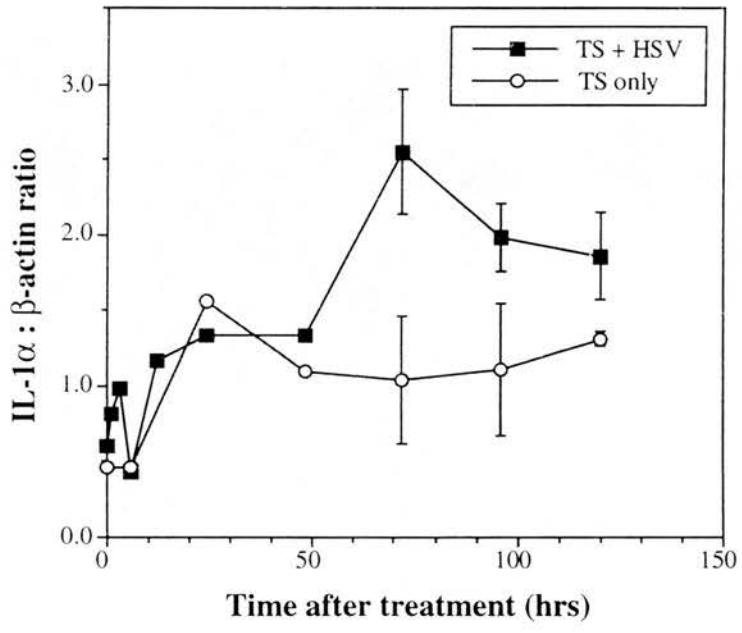
Autoradiographs of representative [^{32}P]-labelled cytokine PCR products of mouse back skin, subjects to SDS-PAGE. The mouse skin samples were either taken from tape-stripped skin (TS only) or skin that was epicutaneously infected with HSV-1 following tape-stripping (TS/HSV).

Figure 5.8 Expression of IL-1α mRNA

(a)



(b)



(a) In PAM-212 cells following HSV-1 infection. The data represent the mean ± SEM of triplicate samples at each time-point
(b) In mouse back skin following tape-stripping alone (TS) or with subsequent HSV infection (TS + HSV). The data represent the mean ratio for skin samples from three mice per time-point, where possible. Mean ± SEM shown from 72 hours after treatment.
Integrated optical density units were obtained by image analysis of autoradiographs. Relative expression of IL-1α is shown as a ratio of the cytokine : β-actin.

As shown in Figure 5.9(a), HSV infection induced an upregulation in the production of IL-10 mRNA in the keratinocyte cell line PAM-212, with a peak at 12 hours post-infection. At this time in the course of infection, signs of viral cytopathic effect were visible microscopically. Between 12 and 24 hours post-infection, the level of mRNA expression for IL-10 began to decrease.

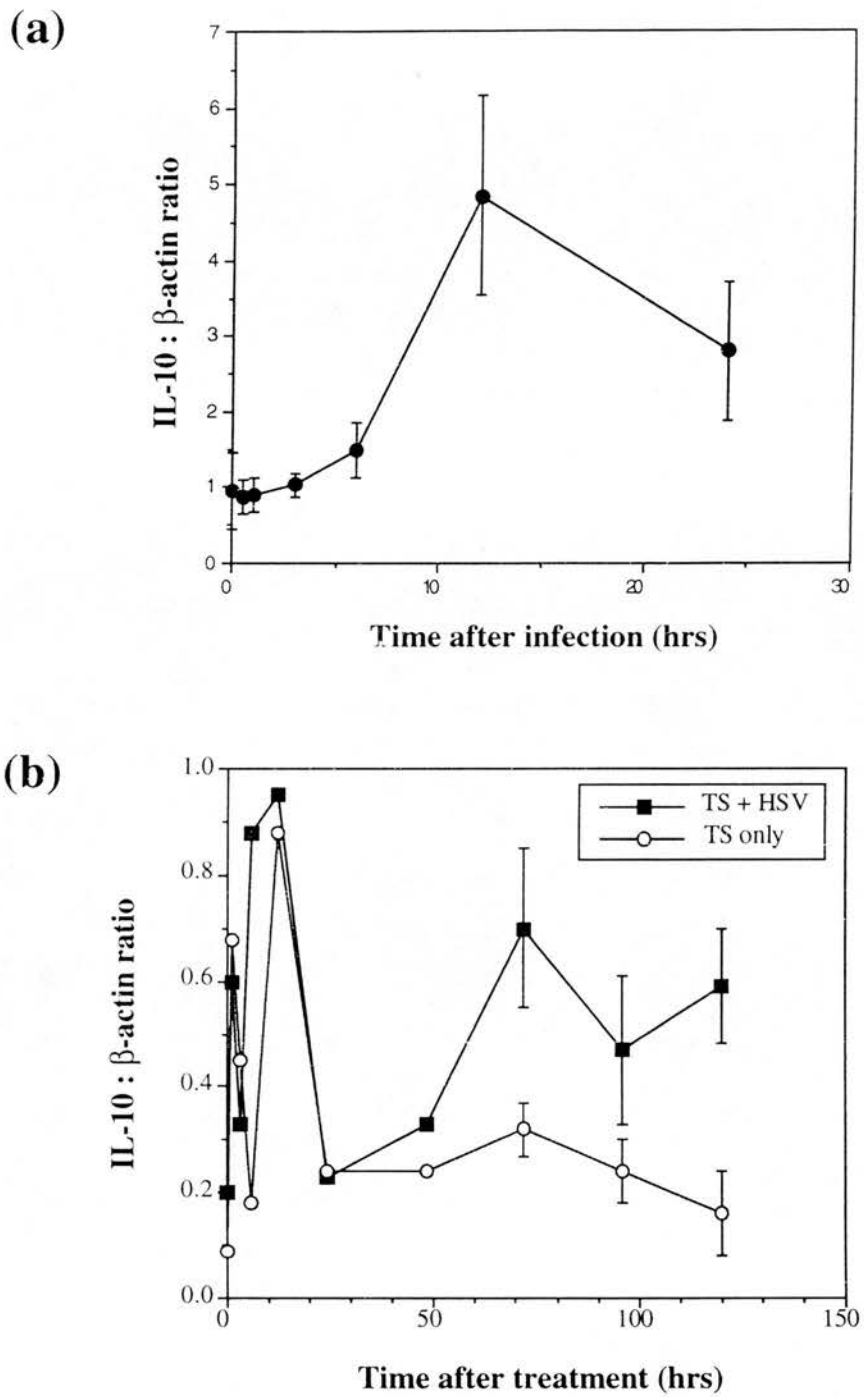
5.2.3.2 (ii) *In vivo* model of HSV infection

Cytokine profiles induced *in vivo* were studied following epicutaneous infection of shaved, lightly tape-stripped dorsal skin with the same strain of HSV-1. Back skin was removed at intervals after infection, immediately homogenised and RNA extracted as described in section 2.4.4. A time-course following tape-stripping of skin alone without infection was also carried out to control for this procedure.

Figure 5.8(b) shows the data for IL-1α mRNA expression, in the *in vivo* model of HSV infection, represented graphically. Both the control mice which had been tape-stripped only (TS) and the experimental mice which had been tape-stripped and then subsequently infected with HSV (TS + HSV), showed an initial trend of increasing IL-1α mRNA expression up to 24 hours post-infection. After this time, the HSV-infected mice displayed a gradual increase in mRNA expression for this cytokine, with peak expression at 72 hours (3 days) post-infection. However, this increase was not found to be significantly different from the control group between 72 and 120 hours post-treatment, as determined by the student's *t*-test. In contrast the uninfected mice did not show this late peak in IL-1α gene expression.

As in the *in vitro* model, IL-10 mRNA production was up-regulated in the *in vivo* model of HSV infection, as shown in Figure 5.9(b). Autoradiographs of representative PCR products for IL-10 at the optimum cycle number are shown in Figure 5.7(b). The tape-stripping procedure itself caused an early increase in IL-10 mRNA up to 12 hours post-infection but, from approximately 4 days post-infection,

Figure 5.9 Expression of IL-10 mRNA



(a) In PAM-212 cells following HSV-1 infection. The data represent the mean ± SEM of triplicate samples at each time-point
(b) In mouse back skin following tape-stripping alone (TS) or with subsequent HSV infection (TS + HSV). The data represent the mean ratio for skin samples from three mice per time-point, where possible. Mean ± SEM shown from 72 hours after treatment.
Integrated optical density units were obtained by image analysis of autoradiographs. Relative expression of IL-10 is shown as a ratio of the cytokine : β-actin.

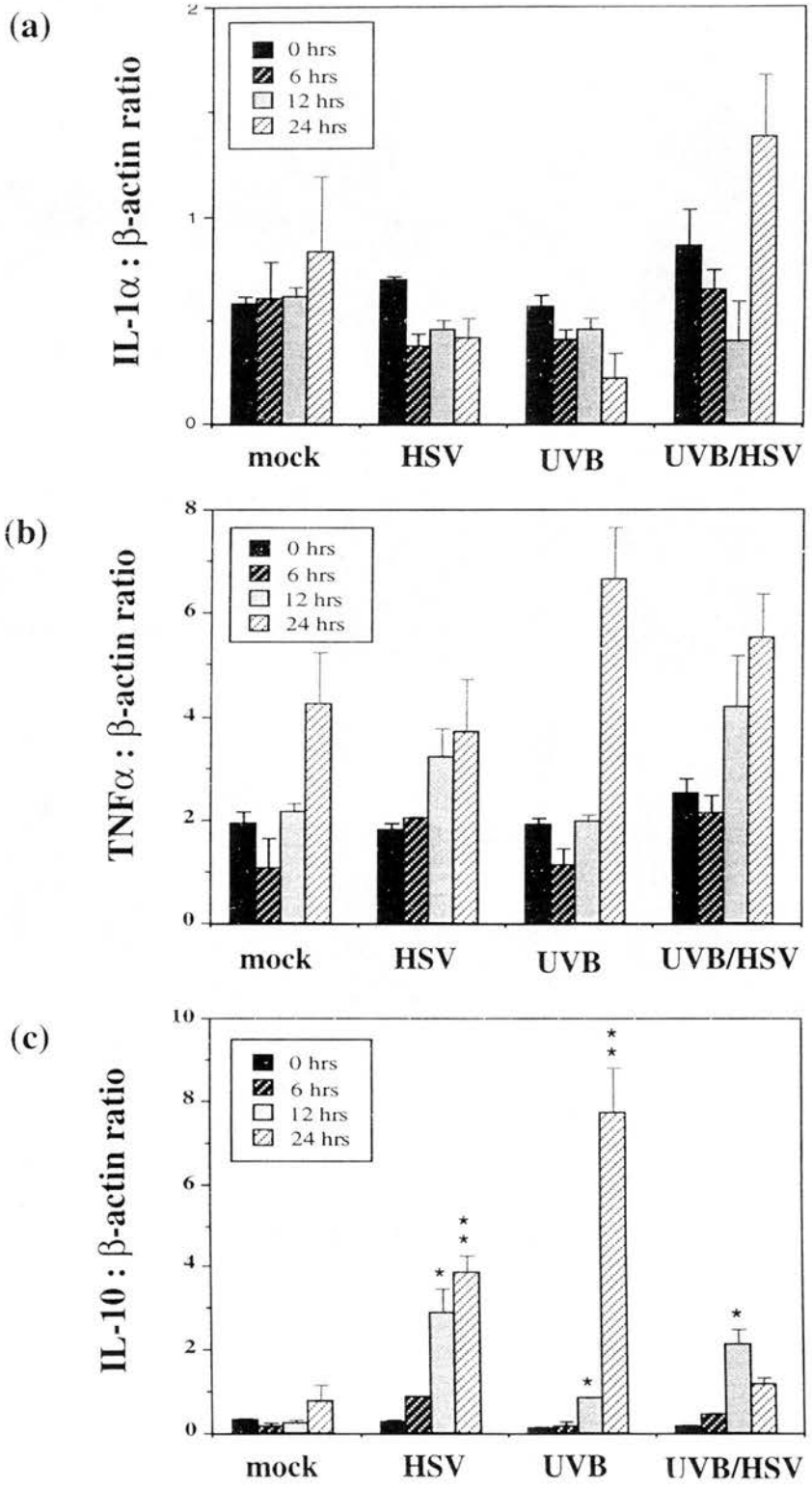
there was also an HSV-induced increase in IL-10 mRNA. This increase seen in the HSV-infected mice, was found to be statistically different from the control mice at 120 hours post-treatment ($p<0.05$), but not at 72 or 96 hours ($p=0.3$ and 0.2 respectively), as determined by the student's *t*-test. In this model, visible lesions appeared at approximately 4 days post-infection and viral antigen could be detected by immunoperoxidase staining of skin sections from 3 days after infection.

5.2.3.3 Semi-quantitative expression of cytokines following UVB irradiation

The effect of UVB exposure of keratinocytes *in vitro* was examined. Time-course experiments were carried out using PAM-212 cells to identify the effects of UVB exposure and HSV infection, both independently and together on this keratinocyte cell line. Problems with sample degradation prevented a parallel study from being carried out using the *in vivo* model and this is discussed further in section 5.3.4.1.

Figure 5.10(a) represents the data obtained from such an *in vitro* experiment with respect to IL-1α mRNA expression. The mock-infected PAM-212 cells exhibited a stable level of expression. The HSV-infected cells showed a viral-induced decrease in expression of IL-1α mRNA at 6 hours post-infection. This agrees with the previously reported data shown in Figure 5.8(a). UVB treatment of the PAM-212 cells (10 mJ/cm^2) also resulted in a decline in IL-1α mRNA levels, in comparison with the initial level, at 6, 12 and 24 hours post-treatment. The lowest expression level for IL-1α mRNA was seen at 24 hours post-UV exposure. This may reflect the slight decrease in viable cell numbers at this time-point, as determined by trypan blue exclusion (shown in Figure 5.12). The PAM-212 cells which were exposed to UVB and infected with HSV showed a decreasing level of IL-1α mRNA expression between 0 and 12 hours after treatment. The level of expression then increased substantially at 24 hours. Statistically significant differences between the mock-infected group and the other treatment groups at each timepoint were determined using the student's t-test and are shown in Figure 5.10.

Figure 5.10 Cytokine mRNA expression in PAM-212 cells, following HSV infection and/or UVB exposure



Three wells of a 6-well cell culture dish containing $5x10^5$ PAM-212 cells were used per treatment per time-point. HSV-infected cells (HSV) were infected with virus at moi=0.5. Mock-infected cells (mock) were incubated with a volume of PBS equivalent to the viral inoculum used. UVB-treated cells (UVB) were exposed to 10 mJ/cm^2 UVB and the final group (UVB/HSV) were exposed to 10 mJ/cm^2 UVB immediately prior to infection with HSV. Following RNA extraction, semi-quantitative RT-PCR was carried out using primers for IL-1α, TNF-α and IL-10. Integrated optical density units were obtained by image analysis of autoradiographs. Relative expression of each cytokine is shown as a ratio of cytokine : β-actin. The data represent the mean ratio ± SEM of the triplicate samples. Significant differences between the mock-infected group and other treatment groups at each timepoint were determined using the student's t-test. *$p<0.01$; **$p<0.05$

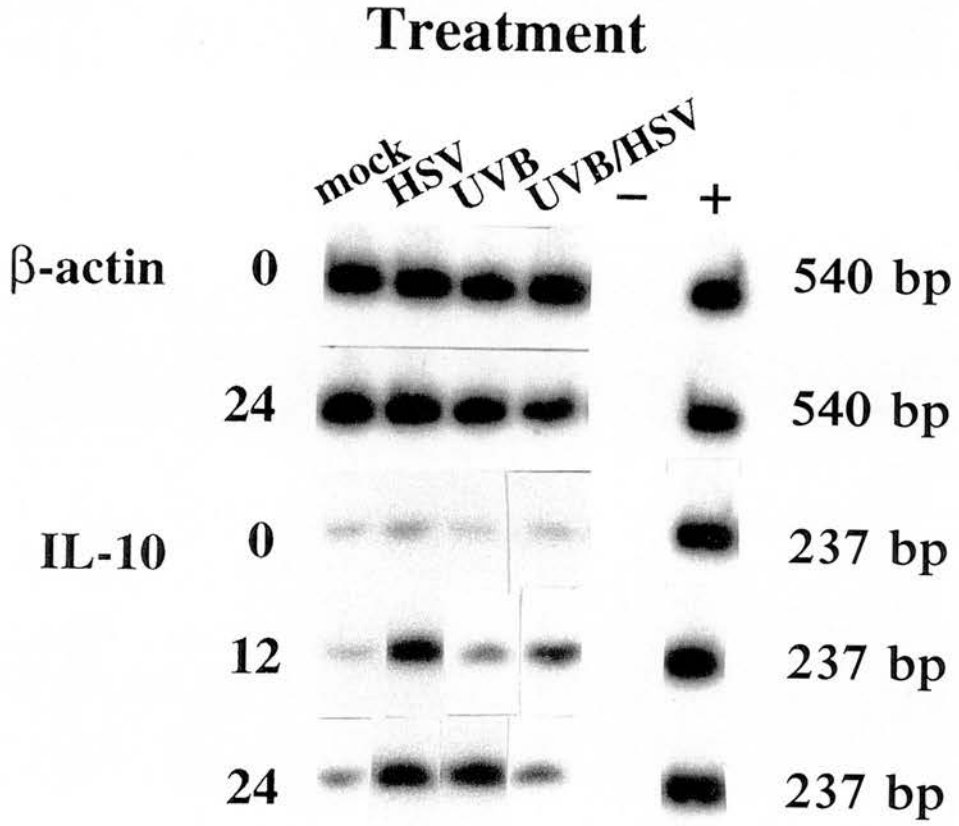
The results of TNF-α mRNA expression in this experiment are shown in Figure 5.10(b). Expression of the gene for this cytokine appeared to be upregulated, with time, in mock-infected cells, HSV-infected cells and UVB-exposed, HSV-infected cells. The greatest expression was seen at 24 hours after treatment in the UVB-exposed group, which did not demonstrate an enhancement of expression until this time.

Figure 5.10(c) shows the expression of IL-10 mRNA in PAM-212 cells treated as previously described. Representative autoradiographs of PCR products from 0, 12 and 24 hour post-treatment samples are shown in Figure 5.11. Very low levels of cytokine message were observed in mock-infected cells and in the 0 and 6 hour time-points for all treatments. HSV-infection of PAM-212 cells induced a time-dependent increase in mRNA expression for this cytokine. UVB exposure also induced such an increase, with maximal expression at 24 hours post-treatment. The combination of HSV infection and UVB exposure in fact resulted in a decrease in the cytokine expression in this experimental group at 24 hours post-treatment.

5.2.4 Detection of IL-10 protein

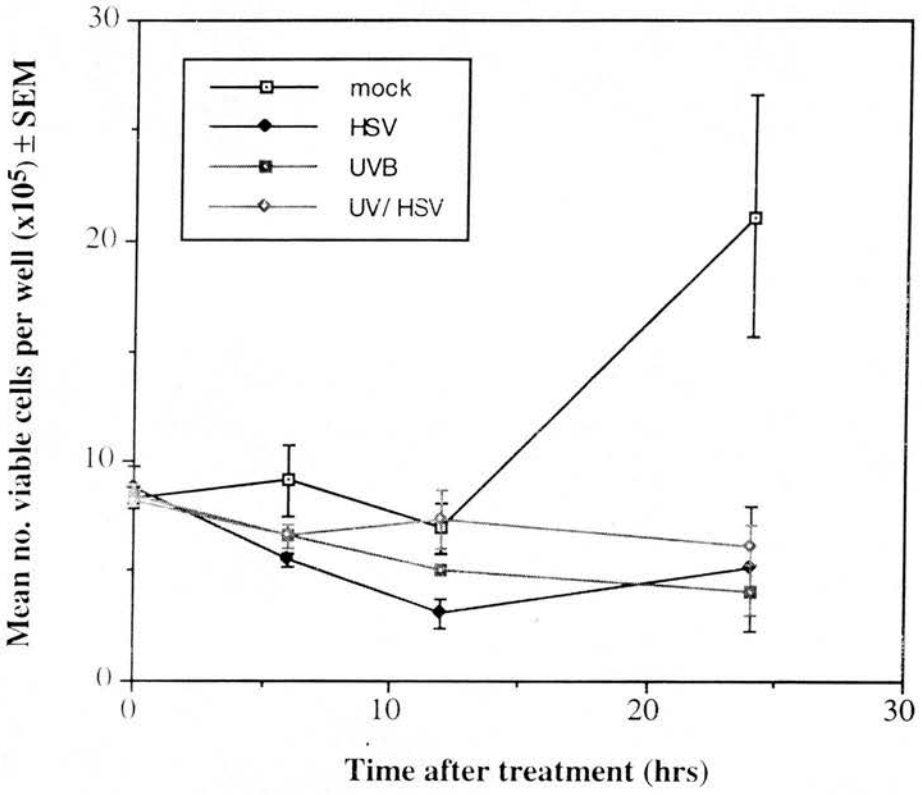
The presence of IL-10 protein in PAM-212 cells was investigated using an ultra-sensitive ELISA kit (see section 2.4.8). No IL-10 protein could be identified in supernatants of untreated PAM-212 cells, or of UVB-exposed or HSV-infected cells. However, PAM-212 cell lysates were demonstrated to contain significant amounts of IL-10 protein (results not shown).

Figure 5.11 Expression of IL-10 mRNA in PAM-212 cells following HSV infection and/or UVB exposure



Autoradiographs of representative [^{32}P]-labelled IL-10 PCR products from PAM-212 cells, subjected to SDS-PAGE. The PAM-212 cells were either mock infected, HSV-infected (moi=0.5), UVB-exposed (10 mJ/cm^2) or HSV-infected and UVB-exposed.

Figure 5.12 Viability of PAM-212 cells following HSV infection and/or UVB exposure



Three wells of a 6-well culture dish containing 5x10^5 PAM-212 cells were used per treatment for each time-point. Cells were treated as in Figure 5.9. At each time-point cells were trypsinised and the total number of viable cells per well determined using trypan blue exclusion. The data are represented as the mean cell number ± SEM.

5.3 Discussion

5.3.1 Models of HSV infection

Since HSV recurrence in humans is often unpredictable, there is a high dependency on animal models to provide information about the immune mechanisms that act against HSV. Indeed the majority of current knowledge has been gleaned from mouse models, since mice are relatively inexpensive, genetically well-defined and easily manipulated. However, in many cases, studies of HSV disease in the mouse do not serve as a true analogue for human disease. The routes of infection used are frequently different and in particular recrudescent infection in mice surviving primary infection cannot consistently be induced. With respect to the model of infection used in this study however, efforts were made to mimic the natural human infection by infecting the mice epidermally with a clinical isolate of HSV-1. No attempt was made here to study any aspect of recrudescence, although it is anticipated that immune mechanisms identified as being important in the primary infection will also be relevant to recurrent infections. Although variations between human and murine skin would be expected, it is anticipated that parallels can be drawn between the two systems and therefore both the murine model and the *in vitro* model can provide useful information, relevant to the human situation.

A characteristic of herpesvirus-infected cells is the rapid shut-off of host macromolecular metabolism, early in infection. This includes host DNA synthesis shut-off, and a decline in host protein synthesis. The products of the HSV gene *vhs* are responsible for the destabilisation and degradation of host mRNA leading to host shut-off (Roizman and Sears, 1996). In addition, destabilisation of viral mRNA occurs, which allows a rapid transition from one regulatory class to the next. For the *in vitro* infection of keratinocytes, a moi of 0.5 was used and no host shut-off of mRNA was seen during the time period of 24 hours examined in this study. It is possible however that infected cells are not synthesising mRNA and the production seen is actually from neighbouring, uninfected

keratinocytes. It is likely that these cells will receive signals, for example through cytokine production, from infected cells early on. It would be interesting to further dissect the production of cytokines by infected and uninfected cells. This could be done by comparing results from experiments in which different moi were used.

5.3.2 Expression of cytokine mRNA by keratinocytes

5.3.2.1 IL-1α

Since keratinocytes release stored IL-1α in response to cell damage, it could be anticipated that infection with HSV would provide such a 'damage' signal. However, following *in vitro* infection of keratinocytes with HSV, a decrease in IL-1α mRNA was seen initially (Figure 5.8(a)). This is unexpected as IL-1α acts as a signal for the production of both secondary inflammatory cytokines and adhesion molecule expression necessary for the characteristic infiltration associated with a HSV lesion. However, this early depression in gene expression was also seen in a subsequent experiment (Figure 5.10(a)). It is possible that this decrease in IL-1α mRNA expression is a result of viral shut-off as an immune evasion strategy to permit easier infection. Although not known for HSV, some viruses encode homologues of the extracellular binding domain of cellular cytokine receptors. These proteins are secreted, bind to the cytokine and interfere with its activity by preventing interaction with cellular receptors. Examples of such soluble receptors are the TNFR and IL-1βR encoded by vaccinia virus (Alcami and Smith, 1992; Alcami and Smith, 1995). Vaccinia and cowpox viruses also encode a serine protease inhibitor which inhibits the IL-1β-converting enzyme (ICE) which cleaves pro-IL-1β to give active IL-1β (Ray *et al.*, 1992).

In contrast to the *in vitro* situation, IL-1α mRNA increased in the *in vivo* model of infection from approximately 48 hours post-infection. By this time viral replication, which is known to take between 18-20 hours in fully permissive tissue culture cells, is well underway. While the sheer numbers of keratinocytes make them the likely source of

the IL-1α mRNA detected, it is possible that some may be being produced by activated monocytes or macrophages, another major cellular source of this cytokine. However, this is unlikely since the clonal expansion and activation of specific $CD4^+$ T cells, between 4-12 days after infection, is a necessary prerequisite for the recruitment and activation of macrophages in any significant number. Immunohistochemical studies verify that a significant number of macrophages are only seen in the inflammatory infiltrate of late lesions, ie from 48 hours onwards (Cunningham *et al.*, 1985).

5.3.2.2 TNF-α

From Figure 5.10(b) it can be seen that although HSV infection of PAM-212 seems to induce the up-regulation of TNF-α mRNA by 24 hours post-infection, a similar increase was also seen in mock-infected cells, suggesting that the increase in gene expression may be a time-dependent rather than an HSV-specific phenomenon. It would be interesting to repeat this experiment to verify any HSV-induced TNF-α production at either the RNA or protein level. *In vitro* infection with other DNA and some RNA viruses have been demonstrated to induce the production of TNF-α and TNF-β and these cytokines were shown to have anti-viral activity. They were able to synergise with interferons in the induction of resistance to both RNA and DNA virus infections, including HSV-2 (Wong and Goeddel, 1986).

5.3.2.3. IL-10

HSV infection induced an upregulation in the production of IL-10 mRNA in the keratinocyte cell line PAM-212, with a peak at 12 hours post-infection (Figure 5.9(a)). Although murine keratinocytes and the PAM-212 cell line have been reported to secrete IL-10 protein as well as synthesise IL-10 mRNA (Enk *et al.*, 1993b; Nash and Cambouropoulos, 1993; Rivas and Ullrich, 1992; Teunissen *et al.*, 1997b), secretion of IL-10 protein into the culture supernatant by this strain of PAM-212 cells could not be detected by us or others (N.Gibbs, Photobiology Unit, University of Dundee; personal

communication) by ELISA. Preliminary studies did however reveal these cells to be synthesising IL-10, since cell lysates were found to be positive for IL-10 protein by ELISA. It is possible that this phenotype is specific to the particular PAM-212 cells used in these experiments and selective pressures over time may have resulted in a particular non-IL-10-secreting clone predominating in the cultures.

The production of IL-10 mRNA was similarly up-regulated in the *in vivo* model of HSV infection. It is likely that HSV is inducing keratinocytes in the epidermis to produce IL-10, as supported by the *in vitro* cell line result. Alternatively, it is possible that the IL-10 mRNA found in this model may be being produced by other cells infiltrating the dermis and epidermis, such as Th2 lymphocytes. However, immunohistochemical studies suggest that while T cells are the predominant early infiltrating cell type, from two days monocytes/macrophages are more prominant and $CD4^+$ T cells no longer selectively migrate into infected areas (Cunningham *et al.*, 1985).

IL-10 has been implicated as an important suppressor factor in both T lymphocyte and antigen presenting cell effector functions. It is known to affect the antigen presenting ability of Langerhans cells, allowing them to stimulate Th2 cells, but induce only tolerance or anergy in Th1 cells (Moore *et al.*, 1990). IL-10 also acts on macrophages to down-regulate MHC class II expression which may result in the inhibition of cytokine synthesis by activated Th1 cells and NK cells. This could result *in vivo* in a switch in the immune response from a delayed type hypersensitivity-type response (Th1) to an antibody-type response (Th2). In terms of HSV infection, an antibody response is believed to contribute minimally to recovery from a primary infection and is known to be ineffective once latency is established. Therefore HSV-induction of IL-10 may be important in aiding the infectivity of the virus and in evasion of the host immune response sufficiently to allow latency to be established. Indeed a viral analogue of IL-10 is known to exist within the

genome of some Herpesviruses, including the product of the BCRF1 gene of Epstein Barr virus (Moore *et al.*, 1990), although such an analogue has not been reported in HSV.

In order to persist in their host it is essential that viruses have mechanisms of evading the host's immune response. HSV has evolved several such mechanisms and it is quite possible that it has other as yet unknown evasion strategies. Firstly, HSV establishes latency in neural cells and during this period only a region of the HSV genome encoding latency-associated transcripts are transcribed and these do not encode any known protein products. In addition, neural cells do not express either class I or class II MHC molecules and therefore avoid cell-mediated immune recognition should any viral antigens be produced. A second viral strategy employed by HSV involves the interaction of viral proteins with components of the humoral immune system. Two examples of this are known and they are virally-encoded Fc and complement receptors. Finally, it was recently reported that the HSV immediate-early protein, ICP47, blocks the transporter associated with antigen presentation (TAP) which translocates antigenic peptides across the endoplasmic reticulum membrane (Hill *et al.*, 1995). This inhibits the association of viral peptides with MHC class I molecules, so that peptides are not presented to anti-HSV $CD8^+$ T cells (York *et al.*, 1994).

5.3.3 The effects of UVB on cytokine mRNA expression

The study involving the infection and/or UVB exposure of PAM-212 cells was not carried out in the *in vivo* infection model and therefore the extrapolation of results to the cutaneous system must be limited. None the less, the study contains some interesting findings and provides a preliminary insight into the possible interactions between HSV infections and UVB.

An increase in TNF-α mRNA was seen following UVB exposure. A smaller but more gradual increase was seen after HSV infection and a similar pattern of expression was

seen in the infected and UVB exposed group. Strickland *et al* found that TNF-α mRNA was increased by 8 hours in human skin following exposure to 2.5 MED, and to reach a maximum by 24 hours post-exposure (Strickland *et al.*, 1997). In addition, UVB irradiation of human keratinocytes has been shown to induce production of TNF-α (Kock *et al.*, 1990).

The expression of IL-10 mRNA increased with time following HSV infection and to a greater extent following UVB exposure. However, only a very small increase was seen at 12 hours post-treatment in the infected and UVB exposed cells and no increase was seen at 24 hours. This is difficult to explain, particularly because no significant difference was seen in the viabilities between the control, mock-infected cells and any of the experimental groups of cells ($p<0.05$; student's *t*-test), other than the mock-infected cells which had proliferated by 24 hours.

5.3.4 Criticisms of experimental design

5.3.4.1 Problems with sample degradation

Comparable experiments to those depicted in Figure 5.10, using mouse skin samples rather than PAM-212 cells were not completed because of problems of sample degradation. It is believed that the skin contains RNase enzymes which are responsible for the degradation of samples, since RNA samples do not normally degrade if stored at -70°C. RNA usually degrades from the ends inwards and hence it may not be possible to carry out RT reactions with oligo dT priming if samples are slightly degraded and hence have lost the poly-A tail. Attempts were made to carry out RT reactions using random hexamer priming but no more samples were successfully reverse-transcribed than using oligo dT.

5.3.4.2 Use of β-actin as a house-keeping gene

The house-keeping gene β-actin was chosen for this study because of its wide usage. However, there have been some reports that in certain cell types the expression of the gene is dependent on the differentiation state of the cell. The literature reveals that this phenomenon has only been reported for adipocytes (Spiegelman and Farmer, 1982). It is unlikely to pertain to keratinocytes since very little variability in the β-actin signal was seen throughout the samples used in these experiments. Attempts were however made to use another set of house-keeping gene primers, namely primers for ribosomal RNA, s18 (Hamilton *et al.*, 1995). These proved difficult to use and the results were not consistent. Therefore, although ideally several house-keeping genes should be used to verify the results obtained, this was not practically possible.

5.3.4.3 Possible viral DNA contamination of samples

The possibility of viral DNA contamination of RNA preparations had to be considered, although viral DNA is likely to move into the phenol phase of the RNA extraction mixture with cellular DNA and hence be removed. However, the consequences of any such contamination should be examined.

The purity of RNA samples was estimated by measuring the optical density (OD) of each sample at 260 and 280 nm, which gives an indication of purity. Pure DNA and RNA preparations have expected ratios of ≥1.8 and ≥2.0 respectively. Any contaminating viral DNA could potentially be included in the OD measurements and affect the correct estimation of the concentration of RNA in each sample. This could mean that the amounts of RNA used for each sample for an RT reaction could vary, depending on the amount of contaminating viral DNA. However, since the procedure of semi-quantitative RT-PCR is dependent on generating ratios of cytokine expression to house-keeping gene expression, variations of this type will be taken into account at this stage.

The second possible effect would be contaminating fragments of viral template DNA hybridising to the RNA and serving as primers during reverse transcription. However since specific primer pairs are used for the PCR reaction, any such transcriptions made would not be successfully primed.

Attempts were made to confirm the conclusions that viral DNA contamination would not affect the final result. Oligoribonucleotides were removed from the cellular RNA preparation by treatment of each sample with DNase, after initial extraction. It was demonstrated that the protocol employed resulted in effective degradation of DNA. However, when used on skin samples it was found that the DNase treatment rendered it impossible to reverse transcribe the RNA samples, probably due to the presence of endogenous RNases in the skin which cause degradation of the RNA during the incubation period at 37°C necessary for effective DNase treatment.

5.3.5 Comparison of results with other studies

Several investigators have studied the effect of HSV infection on cytokine production by a range of cell types. For example, the induction of cytokine mRNA in splenocytes, following infection with HSV, has been reported (Kita *et al.*, 1993). After HSV infection IFN-α, IFN-β, IFN-γ, IL-1β, IL-4, IL-6 and TNF-α mRNA were significantly induced. This study demonstrated that cytokine mRNA, other than IFNs, may play a role in the defence mechanism against HSV infection.

A separate study investigated the effects of three herpesviruses, including HSV, on the synthesis of cytokines by infected peripheral blood mononuclear cells (Gosselin *et al.*, 1992b). It was found that HSV infection caused the up-regulation of IL-6 and TNF-α protein, which are both immunoregulatory cytokines secreted mainly by activated monocyte/macrophages. In addition HSV-induced alterations in IL-6 and TNF-α were

also seen at the transcriptional level in human mononuclear blood cells (Gosselin *et al.*, 1992a).

The effect of HSV-1 infection of keratinocytes, both *in vitro* and *in vivo*, on the gene transcription of various cytokines has also been examined by others. A marked reduction of IL-1α gene expression was noted 6 hours after HSV-1 infection of murine primary keratinocytes *in vitro*, and almost total shut-off was noted after infection for 24 hours (Enk *et al.*, 1991). In contrast, HSV-1 infection of mouse skin was found to induce IL-1β, TNF-α and IL-6 gene transcription in keratinocytes at 24 hours post-infection (Sprecher and Becker, 1992). Induction of IL-1β and TNF-α but not of IL-6 gene transcription was detected in Langerhans cells obtained from infected mice at 24 hours post-infection.

Differences between the results shown here and those of Enk (Enk *et al.*, 1991), with regard to IL-1α production by HSV-1-infected murine keratinocytes, may reflect a number of differences in experimental protocol. Firstly we used a mouse keratinocyte cell line rather than the primary cells used by Enk. Secondly, the fact that Enk *et al* observed host cell shut-off of gene transcription is likely to reflect the fact that they infected cells at a moi of 5, whereas we used a moi of 0.5.

Yasumoto and colleagues studied the production of cytokines by lymph node and spleen cells taken from UVB irradiated, HSV-1-infected mice (Yasumoto *et al.*, 1994). They found that exposure of shaved abdominal skin to 120 mJ/cm^2 UVB prior to HSV infection of the same site caused a marked suppression in IFN-γ production in comparison to that of non-irradiated controls .

Finally, ocular infection of mice with HSV-1 induced IL-12 (p40) mRNA in the cornea and DLN (Kanangat *et al.*, 1996). Protein was also detected in the cornea and it is

thought that infiltrating inflammatory cells are the main source of this IL-12. Since this cytokine is known to stimulate the proliferation and differentiation of Th1 cells, it may induce a protective Th1-type response.

An interesting possibility is the direct interaction between the immune system and the nervous system, mediated by cytokines. The role of cytokines in the latency and reactivation of HSV is of particular interest. Following primary infection with HSV-1 of the eye or oral mucosa, antigen expression has been shown to peak in the trigeminal ganglion at 3 days post-infection. An inflammatory response is induced and elevated cytokine expression in the trigeminal ganglion was seen. By 10-14 days post-infection, HSV replication ceases as a host immune response is mounted and latency is established. If true latency of HSV-1 is established, cytokine expression in the trigeminal ganglion should cease in the absence of viral antigen. However, several studies have found evidence of lingering cell-mediated immunity in the trigeminal ganglion of latently infected mice (Shimeld *et al.*, 1995). Recently it was reported that TNF-α and T cell-associated transcripts (eg IL-10 and IFN-γ) were consistently present in latently-infected trigeminal ganglia at 1-2 months after infection, suggesting T cell cytokine expression to persist well into latency (Halford *et al.*, 1996). Low level expression of HSV proteins during latency could account for lymphocyte infiltration and cytokine expression in the latently infected trigeminal ganglion. However, neurons do not express class I MHC molecules and are therefore incapable of presenting endogenous antigens to CD8+ T cells. This data suggests that viral antigens are present in sufficient quantities during latency to stimulate an ongoing immune response, although the mechanism of antigen presentation remains unknown.

5.3.6 Suggestions for further work

The first and simplest way to continue this work would be to extend the range of cytokines investigated and to carry out *in vitro* studies using a wider range of cells,

including other keratinocyte cell lines, as well as primary keratinocyte cultures. Human keratinocytes could therefore be included in the study to establish similarities in the cytokine profiles obtained after HSV infection.

It would be interesting to investigate further which cytokines are expressed locally by keratinocytes following HSV infections and in skin lesions. In addition, it would be extremely useful to identify the cytokines expressed in the lymph nodes draining an HSV-infected site. Such studies would ideally include the identification of cytokine protein as well as mRNA. Studies in which phenotypic changes in both epidermal cells and DLN cells were monitored would also be of great interest . This could identify whether the down-regulation of IL-2 and upregulation of IL-4, described by Araneo *et al* (1989), is directly relevant *in situ* to HSV infection. These studies could be extended to look at secondary and possibly recrudescent HSV infections.

Further work would be necessary to ascertain whether UV-induced alterations in the cytokine profiles leads to more severe symptoms or to recrudescences in the case of latency. Also studies to compare cytokine expression during HSV infections in normal and single cytokine knock-out mice would help to further a knowledge of local immune responses to HSV. These experiments will clarify the sequence of local events which is required to control HSV infections of the epidermis and will help to resolve the mechanism of action of UV in altering the balance between the virus and the host in such cutaneous sites. The results will be of relevance in understanding the pathogenesis of HSV infections of human subjects and in the development of possible treatment strategies such as the local administration of IFN-γ.

5.4 Summary

In this study *in vitro* and *in vivo* HSV infections have been established and characterised, and the mRNA expression of several cytokines in these systems have been studied. In the case of *in vitro* infection of keratinocytes, HSV infection was confirmed microscopically by the appearance of the characteristic HSV c.p.e. In the *in vivo* model, in which mice were infected epidermally with HSV-1, vesicular lesions usually developed by 3 days post-infection. Immunohistochemistry of sections of infected skin revealed HSV antigen to be detectable from 3 days post-infection. The HSV-positive staining was localised within the epidermis. Semi-quantitative RT-PCR using primers for IL-1α, TNF-α and IL-10 revealed several HSV-induced effects. Most importantly, an increase in IL-10 mRNA expression was observed both *in vitro* and *in vivo*. This suggests that epidermal keratinocytes are induced by the virus to produce IL-10, a strategy which may serve to minimise host immune recognition of HSV.

Chapter 6

The Effect of UVB Exposure and UCA Treatment on the Functional Activity of Epidermal Cells in the Mixed Skin Lymphocyte Reaction

6.1 Introduction

Langerhans cells are vital for the initiation of skin-associated immune responses and are known to play a key role in UVB-induced immunosuppression. It has been suggested that the decrease in epidermal LC number (Morison *et al.*, 1984; Noonan *et al.*, 1984; Toews *et al.*, 1980), the alteration in LC morphology (Noonan *et al.*, 1992) or changes in the antigen-presenting function of LC (Simon *et al.*, 1990; Simon *et al.*, 1991), seen following UVB irradiation, may be responsible for UVB-induced immunosuppression.

Since LC are vital for the initiation of CH responses, it has been hypothesised that there is a correlation between UVB-induced depletion of epidermal LC and local immunosuppression (Toews *et al.*, 1980). However, as described in section 1.4.2.3, local suppression only occurs in certain mouse strains, whereas UVB-induced depletion of LC occurs in all strains (Yoshikawa and Streilein, 1990). It therefore seems unlikely that UVB-induced depletion of epidermal LC is causally related to local immunosuppression.

The currently favoured hypothesis is that UVB-induced alterations in the antigen-presenting function of LC are responsible for immunosuppression. It is thought that UVB converts the antigen-presenting function of LC for Th1 cells from immunogenic to tolerogenic (Simon *et al.*, 1991). In essence, Th1 cells become specifically tolerant to the antigen presented by the affected LC. The most recent hypothesis is that non-

MHC-associated receptors are responsible for the UVB-induced effects on the antigen-presenting capacity of LC, for example adhesion molecules such as ICAM-1 and B7/BB1 may be involved (Tang and Udey, 1992; Young *et al.*, 1993). Alternatively, or perhaps additionally, soluble keratinocyte mediators such as IL-10 and TNF-α may be responsible for this effect of UVB exposure (Streilein, 1993).

One way to study the antigen-presenting abilities of epidermal LC is by a mixed skin lymphocyte reaction (MSLR). This is an adaptation of the mixed leucocyte reaction, an *in vitro* model of T cell recognition of foreign MHC gene products which is induced by culturing mononuclear leucocytes from one individual or strain with mononuclear leukocytes from another individual or strain. Recognition of allelic differences, both MHC class I and II, between the two cell populations will result in proliferation, measured by incorporation of [^{3}H]-thymidine into DNA during cell replication. In the MSLR the stimulator cells are epidermal cells (EC) which proliferate very little in comparison to the mononuclear cells and therefore do not need to be γ-irradiated. It has been shown, in murine skin, that between 2 and 5% of an EC suspension consists of LC and a similar figure is likely for human skin (Stingl *et al.*, 1981). Suspensions of EC were therefore used in this study, but the principal cell required for alloantigen-presentation and hence for the proliferation of responder cells is the LC.

The aim of this study was to carry out a preliminary investigation into the effects of UVB exposure on the functional activity of human EC. Epidermal cells from skin blister roofs were used to investigate the effect of *in vitro* and *in vivo* UVB exposure on the alloantigen presentation by LC, as measured in a MSLR. In addition, the effects of pre-treating EC with *cis*- and *trans*-UCA on their antigen-presenting capacity was examined to identify any role for UCA as a mediator of the observed UVB effects. The evidence for *cis*-UCA as a possible photoreceptor for the effects of UVB on the immune system is discussed in section 1.5.1.2.

6.2 Results

6.2.1 The effect of *in vitro* UVB exposure on the functional activity of human epidermal cells

Epidermal cells were obtained by raising suction blisters from the forearm of normal individuals and then disaggregating the cells with trypsin. These cells were exposed in suspension to broadband UVB, prior to being used in a MSLR as stimulator cells. Allogeneic PBMC were used as responder cells (see section 2.5.1).

The effect of *in vitro* irradiation of EC on their functional activity in a MSLR was examined in five responder/stimulator pairs. In each case UVB exposure of EC suppressed the MSLR to near background levels and these results are shown in Table 6.1. An initial experiment (Table 6.1, experiment 1) showed responder and stimulator cells, when incubated separately for 5 days, to give low counts for [^{3}H]-thymidine incorporation. This confirmed that irradiation of stimulatory cells was not necessary and that non-specific proliferation of responder cells was acceptably low.

In experiments one and two (Table 6.1), EC were exposed to 20, 40 and 60 mJ/cm^2 UVB. The results of experiment one are shown in Figure 6.1. These irradiated EC showed reduced counts compared with the appropriate non-irradiated cells in the MSLR. This suppression was found to be significant using EC exposed to 40 mJ/cm^2 in experiment one, and 20 and 60 mJ/cm^2 in experiment two, as determined using a student's *t*-test. ($p<0.05$). Since all three *in vitro* UVB doses used in these initial experiments reduced the [^{3}H]-thymidine incorporation to near the responder background level, it was decided to use lower doses of UVB, namely 5, 10 and 20 mJ/cm^2, in subsequent experiments (experiments 3-5, Table 6.1). It was anticipated that this would allow a range of suppression to be observed, dependent on UVB dose. In fact these reduced UVB doses also reduced the alloresponse to background responder levels.

Table 6.1 Effect of *in vitro* UVB exposure on the functional activity of epidermal cells in the MSLR

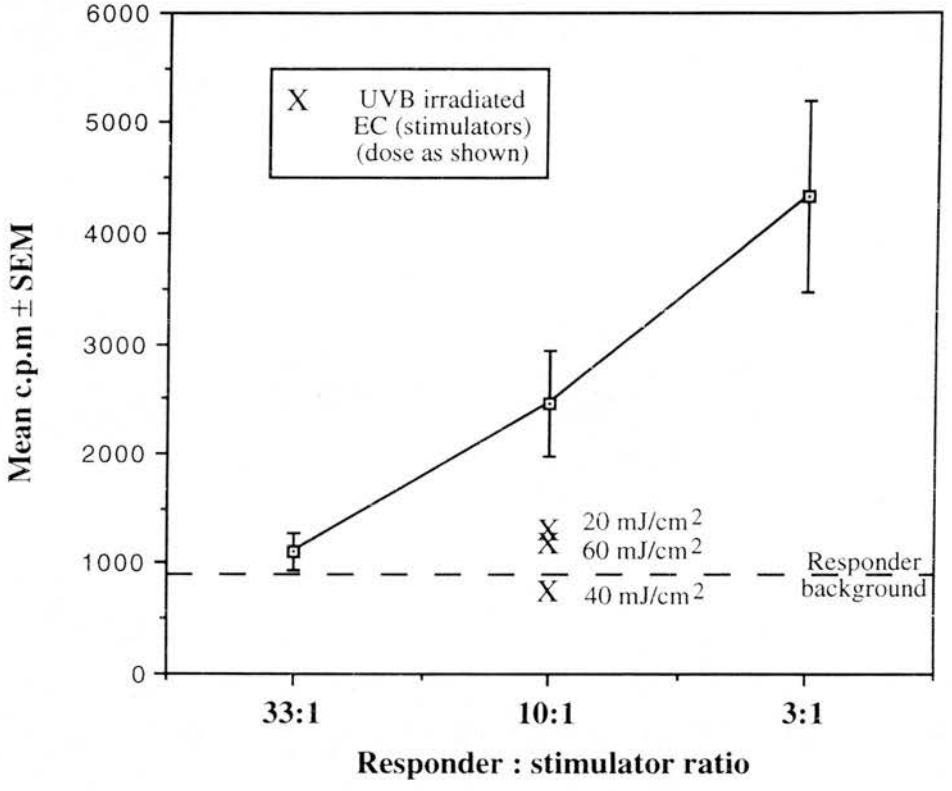
Experiment	EC source	PBMC source	Responder cells only[a] Mean cpm ± SEM	MSLR using unirradiated EC[b] Mean cpm ± SEM	UVB dose (mJ/cm^2)	MSLR using irradiated EC[b] Mean cpm ± SEM	p value[c]
1	MN	WN	927.7 ± 37.6	2467.7 ± 481	20	1233.0 ± 99.2	NS
					40	812.0 ± 74.9	p<0.05
					60	1226.7 ± 228.9	NS
2	MN	WN	188.0 ± 9.1	318.7 ± 44.6	20	178.3 ± 10.8	p<0.05
					40	213.0 + 27.4	NS
					60	188.3 + 6.4	p<0.05
3	KH	GK	189.7 ± 11.4	549.3 ± 62.9	5	127.3 ± 5.2	p<0.01
					10	130.7 ± 3.3	p<0.01
					20	140.3 ± 18.9	p<0.01
4	MG	WN	189.0 ± 29.9	245.7 ± 15.2	5	161.0 ± 8.9	NS
					10	158.7 ± 30.0	NS
					20	127.0 ± 16.3	NS
5	ML	WN	498.3 ± 54.7	4787.3 ± 462.9	5	465.0 ± 96.4	p<0.01
					10	320.0 ± 15.3	p<0.01
					20	341.0 ± 9.5	p<0.01

[a] PBMC at $1x10^5$ cells per well were used to determine the responder background proliferation

[b] $1x10^4$ unirradiated or *in vitro* irradiated EC and $1x10^5$ PBMC were used per well to give a responder to stimulator cell ratio of 10:1 All assays were carried out in triplicate.

[c] The student's *t*-test was used to determine any significant differences between the results of the MSLR using irradiated EC and unirradiated EC. NS; not significant.

Figure 6.1 Effect of *in vitro* UVB exposure on the functional activity of human epidermal cells in the MSLR
(Experiment 1 in Table 6.1)



Epidermal cells were prepared from suction blister roofs and single cell suspensions were exposed to 20, 40 or 60 mJ/cm^2 UVB *in vitro*. Irradiated and unirradiated, control EC were used as stimulator cells. Allogeneic PBMC were used as responder cells and responder to stimulator ratios of 33:1, 10:1 and 3:1 were used for the MSLR. The assays were incubated at 37°C for 5 days, with 0.7μCi [^{3}H]-thymidine added to each well for the final 24 hours of culture. The mean c.p.m. was determined for each treatment and the results are expressed as the mean c.p.m. ± SEM. Responder cells alone were used to determine the background proliferation level, which is indicated by a dashed line on the graph.

Therefore exposure of EC to as little as 5 mJ/cm^2 UVB *in vitro* can reduce the functional activity of these cells.

In some of the experiments in this study, the allogeneic proliferative responses were fairly low and so pokeweed mitogen (PWM) was added to some control responder cell cultures. This acted as a control to demonstrate that these cells were capable of proliferating effectively. The mean cpm for 1x10^5 PBMC ranged from 2.1-3.7x10^4.

6.2.2 The effect of *in vivo* UVB exposure on the functional activity of human epidermal cells

For the *in vivo* UVB exposure experiments, the MED of each subject was determined (see section 2.5.3) and then an area of the forearm was irradiated each day for 7 days with 0.5 MED. On day 8, suction blisters were taken from both the irradiated site and a non-irradiated, control site. The EC were disaggregated and used in a MSLR. The total UVB dose received by an individual of skin type II was typically 105 mJ/cm^2.

The effect of *in vivo* irradiation on EC has to date only been studied using the EC of four individuals. These four experiments are shown in Table 6.2 and the results of one representative experiment (experiment 1, Table 6.2) are depicted in Figure 6.2. Although the statistical differences between the assays using irradiated EC and control unirradiated EC were only significant in experiment one ($p<0.05$), a consistent decrease in the response was seen in all assays. The data therefore suggests that a course of sub-erythemal UVB exposure can cause suppression of the MSLR, by affecting the functional activity of EC.

6.2.3 The effect of *cis*- and *trans*-urocanic acid on the functional activity of human epidermal cells

In order to try to identify the mediator responsible for the functional suppression induced in UVB-irradiated EC, further MSLR assays were carried out using EC which had been treated with either *cis*- or *trans*-UCA. In these five experiments, CD3-positive and negative populations from PBMC were separated using a MiniMACS

Table 6.2 Effect of *in vivo* UVB exposure on the functional activity of epidermal cells in the MSLR

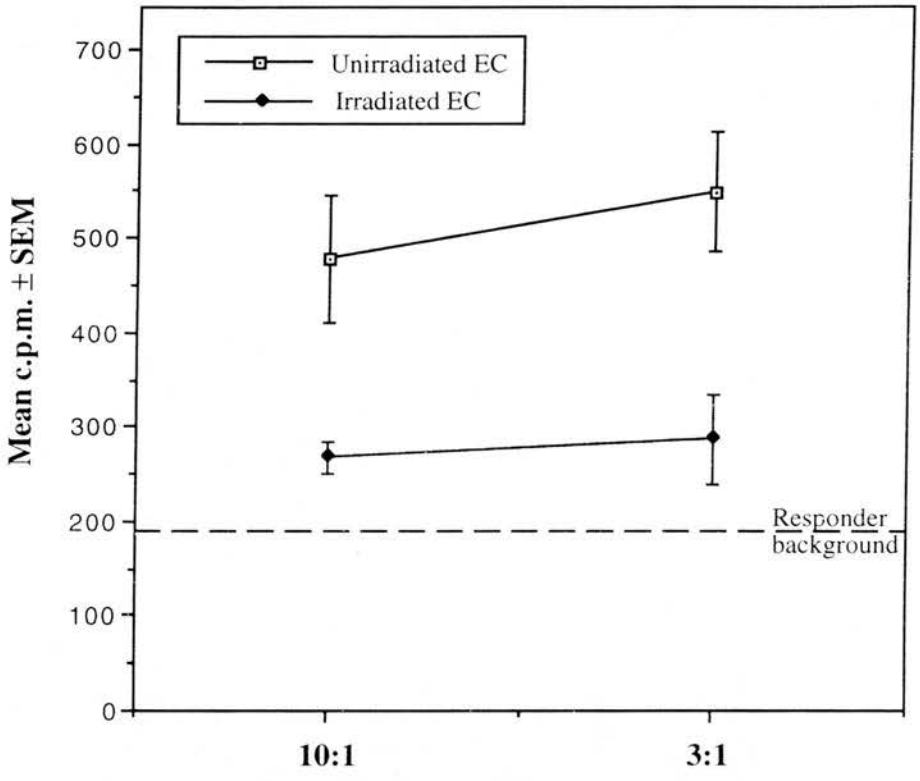
Experiment	EC source	PBMC source	Responder cells only[a] Mean cpm ± SEM	Responder : stimulator cell ratio	MSLR using unirradiated EC Mean cpm ± SEM	MSLR using irradiated EC[b] Mean cpm ± SEM	p value[c]
1	KH	GK	189.7 ± 11.4	10:1	549.3 ± 62.9	287.0 ± 47.0	$p<0.05$
				33:1	477.3 ± 68.0	267.3 ± 16.8	$p<0.05$
2	MG	WN	189.0 ± 29.9	3:1	767.3 ± 41.7	522.7 ± 160.1	NS
				10:1	245.7 ± 46.6	174.3 ± 29.5	NS
				33:1	156.7 ± 15.2	146.8 ± 18.0	NS
3	ML	WN	498.3 ± 54.7	3:1	7255.0 ± 334.6	7058.3 ± 808.3	NS
				10:1	4787.3 ± 462.9	3829.7 ± 463.5	NS
				33:1	1688.0 ± 212.0	1089.7 ± 365.7	NS
4	SS	WN	338.4 ± 48.8	3:1	3596 ± 938.6	2592.9 ± 531.6	NS
				10:1	2021.3 ± 469.4	983.5 ± 173.9	NS
				33:1	946.3 ± 166.6	463.9 ± 77.8	NS

[a] Allogeneic PBMC at $1x10^5$ cells per well were cultured for 5 days at 37°C and used to determine the responder background proliferation.

[b] An area of each subject's forearm was irradiated each day for 7 days with 0.5 MED. On day 8, suction blisters were taken from both the irradiated and a non-irradiated site. The EC were disaggregated with trypsin and used in a MSLR at cell concentrations to give the stimulator to effector cell ratios stated.

[c] The student's t-test was used to determine any significant differences between the results of the MSLR using irradiated EC and unirradiated EC. NS; not significant.

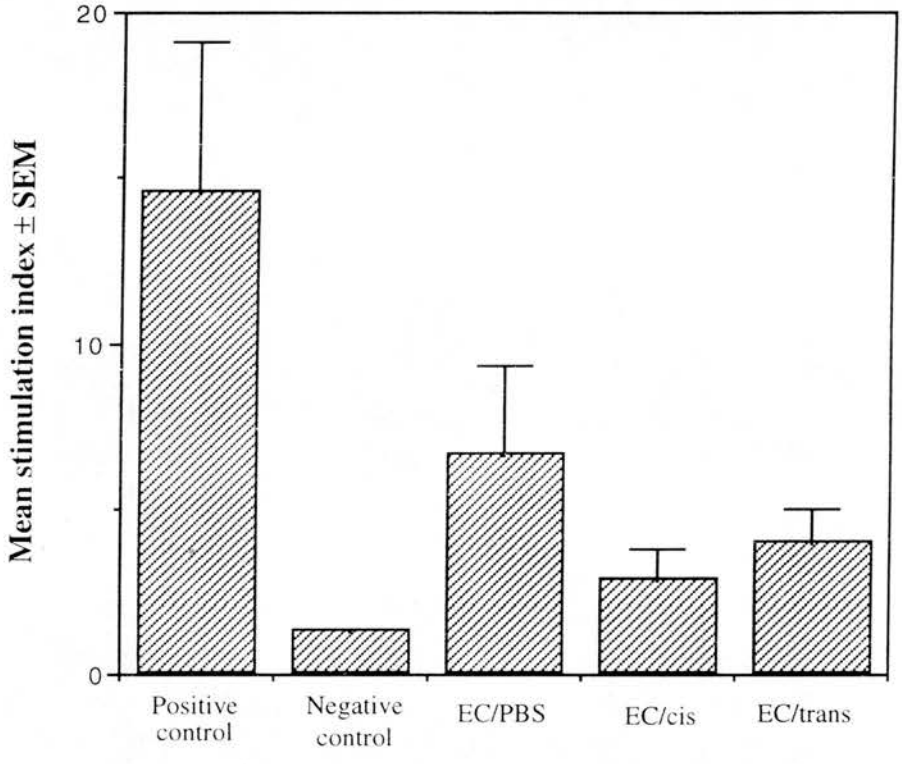
Figure 6.2 Effect of *in vivo* UVB exposure on the functional activity of human epidermal cells in the MSLR



An area on the forearm of a subject (KH) was irradiated each day for 7 days with 0.5 MED. The total dose of UVB was 105 mJ/cm^2. On day 8 suction blister were taken from both the irradiated and a non-irradiated, control site. Epidermal cells were prepared from these blister roofs and were used in a MSLR. Allogeneic PBMC were used as responder cells to give responder to stimulator cell ratios of 10:1 and 3:1. The assays were incubated at 37°C for 5 days, with 0.7μCi [^{3}H]-thymidine added per well for the final 24 hours of this incubation period. The mean c.p.m. was determined for each treatment and the results are expressed as the mean c.p.m. ± SEM. Responder cells alone were used to determine the background proliferation level, which is indicated by a dashed line on the graph.

separation procedure to remove any potential APC. These populations were used as responder cells. EC were obtained from suction blister roofs as before and were suspended in 100 μg/ml *cis*- or *trans*-UCA or PBS for one hour at 37°C. Following this treatment, MSLR responses were carried out using these EC and allogeneic CD3-enriched cell populations at a responder to effector cell ratio of 10:1. As a positive control unseparated PBMC were incubated for 5 days with inactivated HSV (all the subjects were seropositive for HSV). A population of PBMC enriched for CD3-expressing cells was incubated with inactivated HSV, but with no EC, as a negative control. Stimulation indices were calculated as a ratio of mean experimental c.p.m. to the positive control value for each experiment. The mean stimulatory index for the five experiments were calculated and are shown in Figure 6.3. These experiments were identical apart from the EC and PBMC donors used. The results show that pre-treatment of EC with *cis*-UCA suppresses the ability of these cells to induce a proliferative response in a MSLR assay. Although this suppression was not found to be statistically significant as determined by the student's *t*-test, the percentage suppression induced by *cis*-UCA treatment of EC was calculated to be 71%, using the equation shown in section 2.2.3.2. *Trans*-UCA treatment also reduced the allo-response by 50%.

Figure 6.3 **Effect of *cis*- and *trans*-UCA treatment on the functional activity of human EC in the MSLR**



Epidermal cells were prepared from suction blister roofs and single cell suspensions were treated with either *cis*-or *trans*-UCA (100μg/ml) or PBS for one hour. These treated EC were used as stimulator cells in a MSLR assay. Allogeneic PBMC were used as responder cells in the positive control and were incubated for 5 days at 37°C. A MiniMACS separation procedure was carried out to give an enriched $CD3^+$ population which was used as responder cells in all other wells. Negative control wells contained $CD3^+$ responder cells and inactivated HSV only. Experimental assay wells contained $CD3^+$ responder cells and treated EC (EC/PBS; EC/cis; EC/trans), at a responder to stimulator cell ratio of 10:1. All assays were carried out in triplicate and were incubated at 37°C for 5 days. For the final 24 hours of culture 0.7μCi [^{3}H]-thymidine was added to each well. The cells were then harvested and the [^{3}H]-thymidine incorporation measured in c.p.m. using a liquid scinilation counter. The results are expressed here as the mean stimulation index for five identical experiments ± SEM.

6.3 Discussion

6.3.1 Effects of *in vitro* and *in vivo* UVB exposure on the functional activity of human epidermal cells

Exposure of EC to UVB both *in vitro* and *in vivo*, caused suppression of the alloreactive capacity of these cells in the MSLR, to some extent. Under *in vitro* conditions, doses as low as 5 mJ/cm^2 UVB were able to suppress the stimulatory activity of EC. This is in agreement with the results obtained by Goettsch *et al* who also found 5 mJ/cm^2 UVB *in vitro* to be suppressive (Goettsch, 1995). In this study they also found that twenty-fold higher doses were needed for significant suppression of the MSLR after *in situ* exposure to UVB. The *in situ* exposure involved irradiation of intact skin sheets, prior to the disaggregation of EC. It may therefore be that the doses employed in our study were on the border-line for suppressive activity and this is one reason why the MSLR was not consistently suppressed in all experiments. However, it is important to remember that the subjects in the *in vivo* studies described here had 7 days of irradiation, rather than the single irradiation of the EC *in vitro*. In the *in vivo* experiments, the migration of cells, both into and out of the irradiated epidermis, must be considered.

A study which examined the effect of UVB exposure on LC in mice, also found *in vitro* UVB exposure of EC to have a marked and dose-dependent effect on the MSLR response (El-Ghorr *et al.*, 1994). The ability of EC to stimulate a MSLR response was reduced immediately following *in vivo* broadband UVB irradiation, with a dose of 96 mJ/cm^2 (0.6 MED for C3H/HeN mice). This is similar to the doses employed in the *in vivo* UVB exposure study reported here and since Goettsch *et al* found human EC to be less susceptible to UVB than rodent EC (see below), this data also suggests that the doses we employed were border-line suppressive (Goettsch, 1995).

The doses of UVB for the *in vitro* exposures of EC were selected as doses higher than 20 mJ/cm^2 are likely to kill the cells. The *in vivo* doses were chosen to be sub-

erythemal and the subjects's MED was used to take skin type into consideration. In one subject undergoing the localised UVB treatment (Experiment 4, Table 6.2), the exposed area was observed to be more highly pigmented than the surrounding skin by the end of the seven day treatment. In fact the response of EC from this individual in a MSLR was not significantly suppressed and it is possible that the response of tanning is protective against the functional suppression seen in this study. This observation is interesting and deserves further investigation as it suggests that higher doses of UVB which induce a tanning response may not, as a result, be able to cause suppression, while lower doses could be harmful.

One of the problems encountered in this study was the low [^{3}H]-thymidine incorporation counts and hence the low proliferative responses obtained in some experiments. Since the PBMC responder cells proliferated greatly in response to PWM, the low counts obtained were not due to a functional problem with these cells. The proliferative response induced in the responder cells is likely to be related to the difference in MHC antigens between the EC and PBMC donors and therefore cannot be improved upon. Attempts were however made to achieve higher counts by incubating the assays for an additional twenty-four hour period but this did not greatly affect the final results obtained.

It was initially anticipated that this study would form part of a larger collaboration aimed at assessing differences in UV-susceptibility in various species. Statistical regression models have been used to compare data from different species (human, rat and mouse) and these studies may be useful in the long-term to gain a greater understanding of the risk assessment on the UVB-induced suppression of the immunological resistance to infections in humans (Goettsch, 1995). In this study it was found that mouse EC were the most susceptible to both *in situ* and *in vitro* UVB exposure and that human cells were least susceptible. It was also found that the UVB doses needed to impair the alloreactivity, were lower than the doses necessary to alter the morphology of the LC *in situ*. This would suggest that the UVB-induced

suppression in EC function is not a result of morphological changes. In contrast, it has been shown by others that ICAM-1 expression on LC, which is important for (allo)antigen-presentation, is very sensitive to UVB radiation (Austad and Braathen, 1985; Streilein, 1993). Such evidence suggests that low UVB doses are able to alter the membranes of LC and through doing this, alter the (allo)antigen presenting function.

A study of the effects of *in vitro* UVB irradiation using the MSLR, has been carried out using both enriched LC suspensions (8-20% LC) and purified LC suspensions (70-90% LC) (Rattis *et al.*, 1995). UVB doses of between 2.5 and 20 mJ/cm^2 were employed and suppressed the proliferative response of allogeneic T cells in a dose-dependent manner. These experiments suggest that keratinocytes do not play a major role in the UVB-induced inhibition of the MSLR. This finding would indicate that the functional suppression induced by UVB is not mediated by soluble factors released by keratinocytes, although the 10-30% possible contamination by other cells may be sufficient for the production of inhibitory factors.

6.3.2 Effect of urocanic acid isomers on the functional activity of human epidermal cells

The data presented presented here indicate that *cis*-UCA treatment of EC, prior to use as stimulator cells in a MSLR, causes a functional suppression in the alloantigen-presenting capacity of these cells. This would suggest that UCA acts as a mediator in the UVB-induced suppression of LC function, through its isomerisation from *trans*- to *cis*- UCA. However, this result in in contrast to that obtained by Rattis *et al* (1995) who showed that the addition of *cis*-UCA, at a concentration of 2.5 mg/ml, had no effect on the allostimulatory function of human LC in a MSLR. This difference may result from the protocols employed in these studies, since EC were pre-incubated with UCA in the study described here.

A murine study showed that broadband UVB exposure caused a dose-dependent conversion of *trans-* to *cis*-UCA in mouse ear skin (El-Ghorr *et al.*, 1994). A maximum of 45% cis-UCA was recorded which was thought to represent the photostationary state (Gibbs *et al.*, 1993). However, no correlation was detected between the reduction in the MSLR response seen and the formation of *cis*-UCA following UVB irradiation (El-Ghorr *et al.*, 1994). This implies that, at least in the murine system, *cis*-UCA is not acting as the main mediator in the UVB-induced suppression of the alloantigen-presenting function of epidermal LC.

It is anticipated that the number of epidermal LC will decrease during the 7 days of *in vivo* irradiation of human subjects. It is known that a sub-erythemal dose of UVB can cause at least a 20% decrease in the number of LC in human skin (Cooper *et al.*, 1992). The antigen-presenting function of EC collected at the end of the irradiation period will therefore be poor since the LC population will have been greastly reduced. A study by Hurks *et al* (1997) demonstrated that following exposure of human skin to erythemal UVB doses of 160 mJ/cm^2 for four consecutive days, there was an influx of $CD36^+DR^+$ macrophages into the epidermis (Hurks, 1997). An enhancement of the MSLR was observed when EC from irradiated skin were used and this was associated with the influx of the macrophage population. However, no $CD36^+$ cells appeared in the epidermis in a separate study, following four weeks (thrice weekly) of sub-erythemal UVB treatment (Hurks, 1997). Since the *in vivo* exposures used in the study described here were sub-erythemal and irradiation was over a period of just one week, it is likely that no influx of macophages occurs. The lack of replacement of APC in the epidermis following LC migration is therefore likely to explain the suppression in allo-presenting capacity of EC in the MSLR following *in vivo* irradiation.

6.3.3 Criticisms of experimental design

The process of taking suction blisters is a mild procedure, in comparison for example to taking biopsies. Although it involves pressure being applied to the skin and hence to

the epidermal layer, no damage to the cells is believed to be caused by this, other than vacuolation. In fact the proliferation seen by the responder cells in the MSLR shown here demonstrate that the functional capacity of the EC must remain intact following suction blister treatment.

The use of localised UVB was to see any effects of *in vivo* UVB exposure in a preliminary study, without the need for individuals to undergo a course of whole-body irradiation. The use of the MED as a method of determining the relative doses of UVB that individuals receive is a fairly effective one as it takes into consideration certain characteristics of the individual's skin. This is better than simply exposing individuals to a particular UVB dose which may be sub-erythemal in individuals of certain skin types while erythemal in others.

It is possible that unirradiated control skin sites, if near to the UVB-exposed site, may be affected by any soluble mediators released by UVB irradiated EC. Additionally, the level of previous UVB exposure by the EC donors may affect the results obtained.

6.3.4 Suggestions for further work

This study is only a preliminary one and therefore expanding the number of subjects providing EC would be a priority. This is particularly important with respect to examining the effect of *in vivo* UVB exposure. The use of slightly higher *in vivo* doses of UVB would also be informative, in the light of the findings of Goettsch *et al* (1995). An interesting expansion of the study might be to include *in situ* irradiation of EC, ie irradiating blister roofs before the disaggregation of EC. By comparison with the other protocols, this may reveal details of the interactions of EC following irradiation, perhaps through the release of soluble mediators. In addition, the use of skin blisters taken from individuals undergoing UVB phototherapy, would further add to the information it may be possible to acquire through experiments involving only localised exposure.

6.4 Summary

Alterations in the function of EC, especially LC, were studied using the MSLR. *In vitro* UVB exposure of EC, derived from human skin blisters, revealed that low doses of UVB radiation impaired the alloreactive capacity of these cells. For suppression of the alloreactive capacity of EC after *in vivo* UVB exposure of skin, higher doses of UVB radiation were required. Pre-treatment of EC with *cis*-UCA, and to a lesser extent *trans*-UCA, caused a suppression of the functional capacity of EC in the MSLR. This suggests a possible role for UCA as a mediator of the UVB-induced suppression of EC (allo)antigen-presentation.

Chapter 7

The Effect of UVB Phototherapy on HSV-Specific T Cell Cytotoxicity and NK Cell Activity

7.1 Introduction

Exposure to UVB has been demonstrated to result in the suppression of selected immune responses to a range of antigens. In general, evidence for this has come from animal models, as discussed in section 1.4.2. The immunological consequences of UV irradiation of humans are much less well characterised and studies have mainly been confined to suppression of CH responses (Baadsgaard, 1991; Cooper *et al.*, 1992; Morison, 1989; Tie *et al.*, 1995). The effect of UVB on human LC, such as the transient alterations in the numbers, ultrastructure and morphology, is also well-documented (Reviewed in Lappin *et al.*, 1996). These changes are associated with suppressed alloactivation by epidermal cells *in vitro* (Cooper *et al.*, 1985) and reduced antigen presentation (Austad and Braathen, 1985). However, relatively few studies have examined the effects of UVB exposure on other immune parameters in humans.

7.1.1 Cytotoxic T lymphocytes

Cytotoxic T lymphocytes (CTL) kill target cells expressing specific antigen. They are important effector cells in terms of viral infection, acute allograft rejection and rejection of tumours. The phenotype and functions of CTLs have been reviewed elsewhere (Berke, 1991; Kupfer and Singer, 1989; Nabholz and MacDonald, 1983). The majority of CTLs express the CD8 molecule, use an $\alpha\beta$ TCR and recognise peptide associated with class I MHC molecules. MHC class II-restricted, $CD4^+$ CTLS are less prevalent but may come to dominate the cytotoxic response against some viral infections (Schmid, 1988). T cells using the $\gamma\delta$ TCR may also be cytotoxic but they

are not restricted by conventional polymorphic MHC molecules. The appearance of functional CTLs depends upon the process of differentiation from pre-CTLs. These precursor cells have already undergone thymic maturation and are specific for a particular foreign antigen, but lack cytolytic function. Two separate signals are required for the differentiation of pre-CTLs to functional CTLs. The first is specific recognition of antigen on a target cell. The second is provided by $CD4^+$ T cell-derived cytokines, such as IL-2, IFN-γ and IL-6. The involvement of $CD4^+$ T cells indicates that maximal CTL responses depend upon antigen presentation, utilising both class I and class II MHC molecules.

Differentiation of pre-CTLs involves the acquisition of the machinery necessary to perform cell lysis. CTL killing is antigen-specific and requires cell contact. The CTL binds to the target cell through the specific antigen receptor and through other accessory cell molecules, such as CD8, CD2 and LFA-1. The CTL is activated by cross-linking of its antigen receptor and then delivers a lethal hit by several mechanisms, the relative importance of each mechanism being dependent on the cytotoxic cell and the target. The granules of cytotoxic lymphoid cells contain perforin and granule enzymes (granzymes). Upon activation, the cytoskeleton of the CTL rearranges and the granules are reorientated towards the target before exocytosis. Perforin polymerises on the target cell membrane to create holes, which act as ion-permeable channels in the target cell plasma membrane (Krahenbuhl and Tschopp, 1991). In sufficient numbers, such channels lead to osmotic swelling of the target cell and lysis. The function of the granzymes is less clear, although they are thought to enter the target cell via perforin pores to activate endogenous killing mechanisms, such as apoptosis. The second mechanism of target cell lysis involves the secretion of a cell toxin which activates target cell enzymes that cleave DNA in the target cell nucleus. Once the target cell DNA is fragmented, apoptosis of the target cell results (Cohen, 1991). The cytotoxic cell is protected from its own killing mechanisms and can subsequently kill further targets.

Classical CTL are $CD8^+$ in humans, $Lyt2^+$ in mice and MHC class I-restricted. However, in humans, $CD4^+$, MHC class II-restricted rather than $CD8^+$T cells are responsible for the main CTL activity of HSV antigen-expanded clones obtained from seropositive donors (Schmid, 1988; Yasukawa and Zarling, 1984). Known targets for anti-HSV CTL include glycoproteins gB, gC, gD and the immediate-early protein ICP27 (Banks *et al.*, 1991; Zarling *et al.*, 1986). It is conceivable that these $CD4^+$ CTLs function in the skin to destroy infected keratinocytes, induced to express MHC class II molecules by IFN-γ (Cunningham and Noble, 1989). Immunocytochemical studies indicate that MHC class II expression on keratinocytes from HSV-1 cutaneous lesions begins within 24 hours of infection and by 48 hours the expression is uniform on all keratinocytes within and adjacent to the lesion (Cunningham *et al.*, 1985). This study also demonstrated that during the first 48 hours, only $CD4^+$ CTLS infiltrate into the lesion site, together with macrophages and then $CD8^+$ T cells infiltrate, until eventually they are present in similar proportions. Since typical HSV lesions begin to resolve by 48 hours (Cunningham *et al.*, 1985), a central role for $CD4^+$ T cells in the control of recurrent lesions would appear likely, with $CD8^+$ late infiltrating cells probably helping in the final stages of resolution.

7.1.2 Natural killer cells

Natural killer (NK) cells represent an extremely heterogeneous population of predominantly large granular lymphocytes. NK cells can spontaneously kill certain susceptible target cells in a manner which, unlike CTLs, is not MHC-restricted. They have a relatively limited specificity repertoire and therefore the frequency of any given specificity is high. NK cells are able to kill target cells at first contact and these characteristics enable them to act as a first line of defence, while specific CTLs are developing from precursors. They are therefore particularly important in natural resistance against neoplasia and infectious agents, particularly in limiting viral infection during the first few days of infection (Biron, 1997). The function and mechanism of action of NK cells are reviewed in Trinchieri (1989).

NK cells express both T cell markers, eg CD2, and myeloid markers such as CD11b and CD16. The latter is the low affinity Fcγ receptor (FcRIII) which allows NK cells to mediate ADCC directed to antibody-coated target cells. NK cells also express CD56, a member of the neural adhesion molecule (NCAM) family of adhesion molecules. The majority of NK cells in peripheral blood in humans are $CD56^+$ $CD16^+$ $CD2^+$ $CD11b^+$ $CD3^-$ (Trinchieri, 1989). NK cells are directed by both positive and negative interactions with their targets. They express receptors for MHC class I molecules and activation of these receptors results in inhibition of their effector cell function (Lanier and Phillips, 1996; Raulet, 1996). In the mouse these receptors belong to the Ly49 protein family. Similar, although structurally distinct, receptors have been identified on human NK cells (Moretta *et al.*, 1996). These receptors are believed to be critical for the mechanism that explains why for example, NK cells kill virally infected cells, but not normal self-cells . The lack of expression of one or more class I alleles on a target cell is thought to lead to NK-mediated target cell lysis.

Unlike CTLs, NK cells do not appear to require prior contact with target antigens to develop cytolytic capacities. A protein, NK-TR, has been postulated to be part of the NK target-recognition/triggering complex (Chambers *et al.*, 1994). NK cells can be activated to increase their ability to lyse target cells by treatment with type I IFN, IFN-γ, TNF-α or IL-2. IL-12, produced mainly by phagocytic cells, induces IFN-γ production by T cells and NK cells and is known to enhance NK cell activity (Trinchieri, 1995). Killing of targets by NK cells involves similar mechanisms as killing by CTLs, namely granule exocytosis and secretion of a cell toxin.

A variety of target cell types, with varying degrees of susceptibility have been employed to measure NK cell cytotoxicity. The K562 cell line employed in this study was derived from a patient with chronic myeloid leukemia and is highly sensitive to NK cell lysis. This cell line lacks both MHC class I and II antigens.

7.1.3 UVB Phototherapy

A wide variety of skin diseases are currently being treated with UVB phototherapy, including psoriasis and atopic dermatitis. Importantly, an increasing number of AIDS patients are having their AIDS-associated dermatoses treated with phototherapy. The use of UVB phototherapy is largely empirical and the complete mechanism of action remains unknown, although it is thought to act, at least in part, by modulation of immune responses. There is growing concern about the side effects of UVB phototherapy in terms of the potential risk of developing skin cancer, as well as the implications of immune modulation. The majority of studies of the effects of UVB exposure in humans have employed UV regimens which bear little resemblance to those used therapeutically. In addition, previous studies of the effects of broadband UVB phototherapy on human immunological parameters have mainly involved psoriatic individuals. These studies have demonstrated that while phototherapy was therapeutically successful, no measurable changes were observed in the phenotypes of PBMC subsets or in the lymphoproliferative responses to HSV antigen and con A (Gilmour *et al.*, 1993b; Guckian *et al.*, 1995). However, psoriatic individuals may have a number of underlying abnormalities in their immune function, making it difficult to extrapolate from them to normal individuals. The NK cell activity of subjects, both psoriatic and normal individuals, during and after PUVA and UVB phototherapy have been reported to be suppressed (Gilmour *et al.*, 1993a).

The aim of this study was therefore to examine the effects of a standard course of UVB phototherapy on two parameters of systemic immunity, namely HSV-specific T cell cytotoxicity and NK cell activity, in normal individuals. To date no studies have identified effects of broadband UVB phototherapy on CTL activity directed at viral antigens. In addition, the phenotypes of PBMC were monitored by flow cytometry to identify any UVB-induced alterations.

7.2 Results

7.2.1 HSV-specific cytotoxicity assay

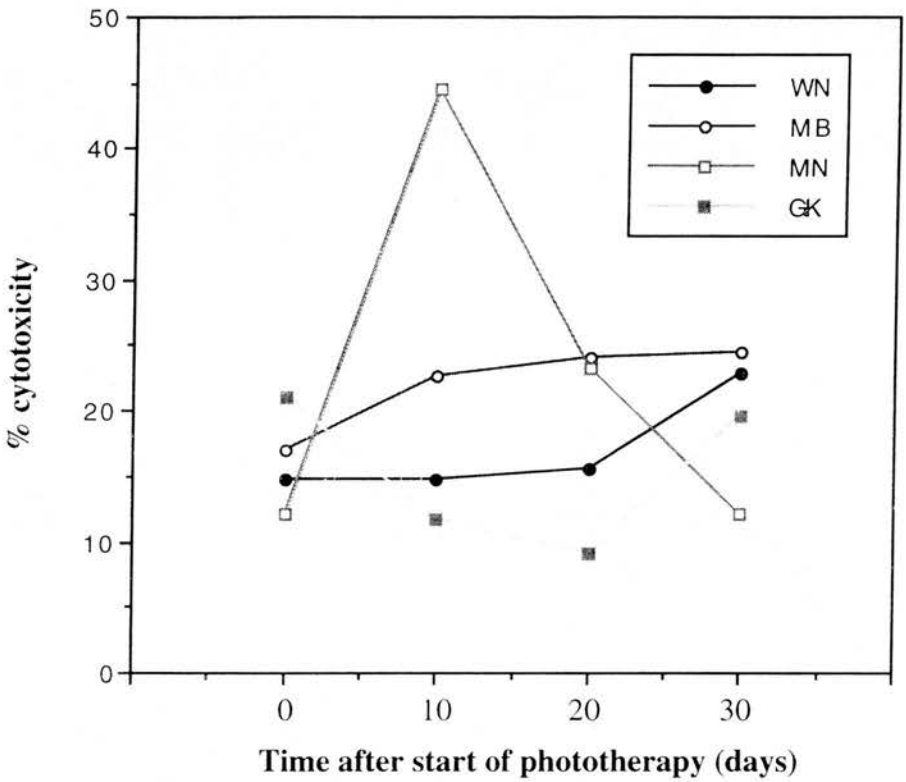
Normal individuals, known to be seropositive for HSV, underwent a standard course of broadband UVB phototherapy, as used in the treatment of psoriasis. They received whole body irradiation three times a week, for four weeks, with incremental doses dependent on skin type. Blood samples were taken before the start of treatment and at 10, 20 and 30 days from the start of phototherapy. Samples were also taken from two subjects (WN and MB) at 37 days after the start of treatment.

Table 7.1 Details of the subjects and UVB doses employed

Subject	Age	Sex	Skin type	Dose of UVB (mJ/cm^2)	Frequency of HSV recrudescence per year
WN	57	M	II	2100	3
MB	40	M	II	3200	4
MN	52	F	I	1300	1
GK	34	F	II	2100	2

As shown in Figure 7.1, two of the four individuals examined to date, showed no real change in the cytotoxicity response elicited throughout the experimental period, using unseparated CTL. However, the cytotoxicity response of one subject (GK) declined from the start of phototherapy to 20 days, before returning to near basal level by the end of treatment. The fourth individual (MN) showed a peak in cytotoxic response after 10 days of UVB phototherapy. However, this response was found to have decreased towards pre-UV levels in the two subsequent time-points. It is therefore possible that the early-UV time-point for this individual (day 10) is an anomaly as it does not reflect the general trend seen throughout the other time-points and individuals. The data represented in Figure 7.1 is for an effector to target cell ratio of 40:1. However, similar results were observed at lower ratios. Table 7.2 shows the

Figure 7.1 HSV-specific cytotoxicity assay using unseparated CTLs during broadband UVB phototherapy



Four individuals known to be seropositive for HSV underwent a standard course of broadband UVB phototherapy. They received whole body irradiation three times a week for four weeks, with incremental doses depending on skin type.
The ability of these individuals to mount an HSV-specific cytotoxic T cell response was examined. Cytotoxic T lymphocytes (CTLs) were generated by *in vitro* incubation of non-adherent cells from PBMC with HSV primed dendritic cells for 6 days at 37°C.
Target cells were autologous B cell lines (BCLs) prepared for each subject by transformation with Epstein-Barr virus. BCLs were incubated with inactivated HSV for two hours prior to being used as target cells in the cytotoxicity assay.
The results shown here are for cytotoxic cell assays set up for the four subjects, with a final effector to target cell ratio of 40:1. Spontaneous [^{51}Cr] release was determined by incubation of target cells with RPMI-FCS and maximum release by incubation with 2% acetic acid. The SEM for each assay was always within 10% of the mean value. The percentage specific release was determined according to the formula shown in section 2.5.2.1.
Blood samples from each individual were taken prior to UVB exposure (pre-UV), and after 10 (early-UV), 20 (mid-UV) and 30 (end-UV) days after the start of phototherapy.

Table 7.2 Titration of effector cells for HSV-specific cytotoxicity assay

Effector cell[a]	Target cell[b]	Effector : target cell ratio	Mean cpm ± SEM	% cytotoxicity[c]
CTL	Control BCL	40 : 1	3449.0 ± 143.4	17.39
		20 :1	3214.0 ± 95.8	13.44
		10 : 1	2971.7 ± 42.1	9.36
CTL	HSV BCL	40 : 1	5161.3 ± 265.0	37.12
		20 : 1	4678.3 ± 435.2	30.56
		10 : 1	3793.7 ± 179.6	18.54
$CD4^+$ CTL	Control BCL	40 : 1	4094.7 ± 56.2	28.23
		20 : 1	3651.3 ± 112.1	20.78
		10 : 1	3218.7 ± 281.8	13.51
$CD4^+$ CTL	HSV BCL	40 : 1	4998.3 ± 585.7	34.90
		20 : 1	4511.0 ± 253.1	28.29
		10 : 1	3589.0 ± 176.8	15.77
$CD8^+$ CTL	Control BCL	40 : 1	3159.0 ± 118.6	12.51
		20 : 1	3545.3 ± 115.7	18.67
		10 : 1	2678.0 ± 250.8	4.43
$CD8^+$ CTL	HSV BCL	40 : 1	4432.3 ± 41.0	27.22
		20 : 1	4246.7 ± 101.6	24.69
		10 : 1	4079.7 ± 46.8	22.42

The results of HSV-specific cytotoxicity assays for one subject (GK) are shown. The assays were set up using CTLs generated from a blood sample taken after completion of the course of phototherapy (end-UV; day 30).

[a] Cytotoxic T lymphocytes (CTL) were generated by *in vitro* incubation of non-adherent cells from PBMC with HSV-primed dendritic cells for 6 days at 37°C. Populations of these CTLs enriched for CD4 and CD8 expressing cells were prepared using a separation column and used as effector cells in separate assays.

[b] Autologous B cell lines (BCL) were prepared for each subject by transformation with EBV and used as target cells. BCLs were incubated with inactivated HSV for two hours prior to being used as target cells. BCLs incubated with RPMI only were used as control target cells.

[c] The percentage specific [^{51}Cr] release was determined according to the formula shown in section 2.5.2.1. Spontaneous [^{51}Cr] release was determined by incubation of target cells with RPMI-FCS and maximum release by incubation with 2% acetic acid.

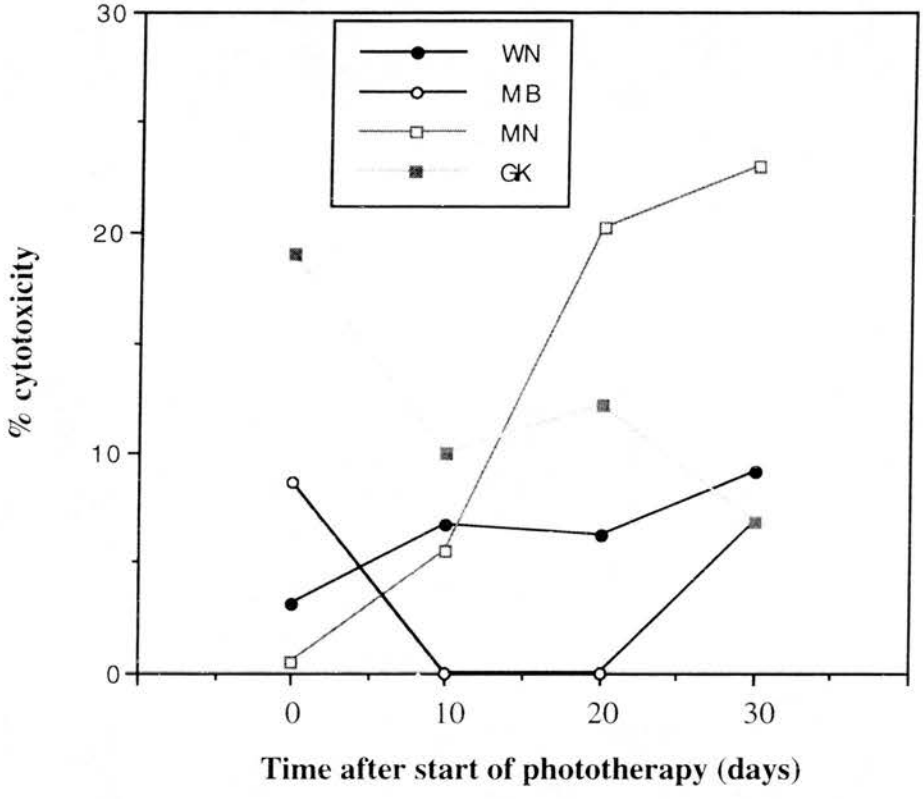
percentage cytotoxicity for one individual (GK; end-UV) for three effector to target cell ratios. It can be seen that as the number of effector cells are reduced, so the percentage cytotoxicity reduces. This titratable response was demonstrated for all four individuals studied.

The HSV-specific CTL activity was further studied by the purification of the CTL population for $CD4^+$ and $CD8^+$ cells (see section 2.5.2.1). These purified cell populations were used as effector cells in the cytotoxicity assay, in the same way as the whole CTL population. Figure 7.2 shows the results of the cytotoxicity assay using $CD4^+$ cells as effector cells and Figure 7.3 shows the results obtained using $CD8^+$ cells as the effector population. No consistent trend as to the effect of phototherapy on these cytotoxicity assays was seen between the four individuals. These results do not conclusively indicate a unique role for either $CD4^+$ or $CD8^+$ lymphocytes in the CTL response to HSV, as neither MiniMACS-purified cell population is solely responsible for the cytotoxic response. It also appears that UVB exposure does not preferentially affect the activity of either one of these T cell populations. The SEM at each effector to target cell ratio and within each experiment were always less than 10% of the mean value.

7.2.2 NK cell assay

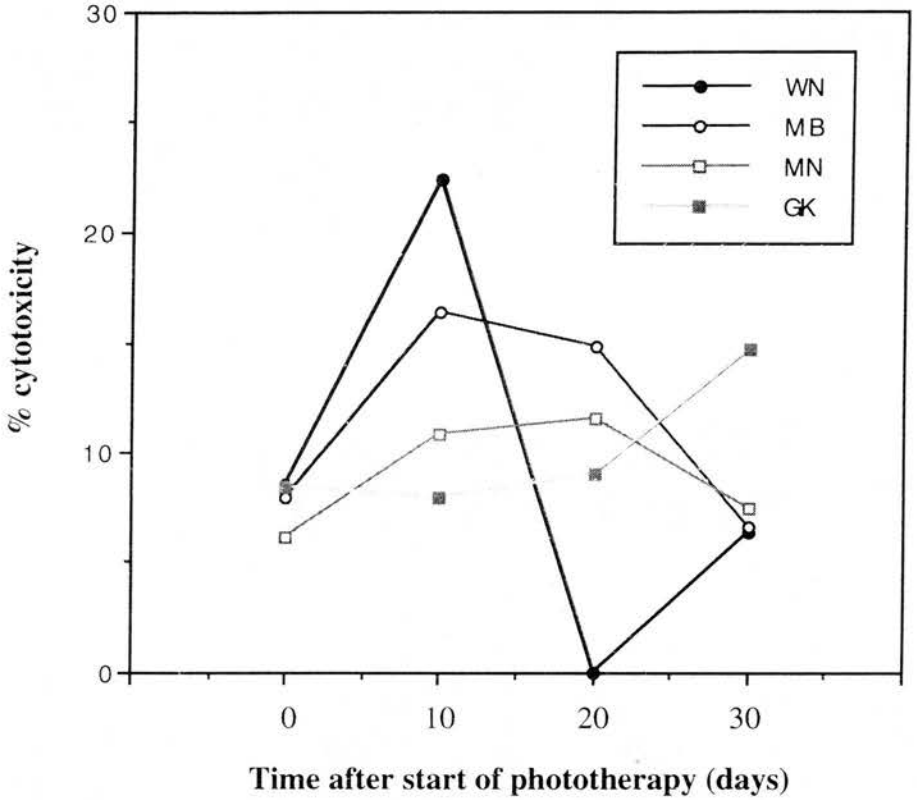
As shown in Figure 7.4, all four individuals subjected to a standard regimen of UVB phototherapy demonstrated suppressed NK cell activities. The SEM within each experiment were less than 10% of the mean value. The initial percentage cytotoxicities demonstrated by the subjects ranged from 44.9% to 70.2%. This NK cell activity was significantly reduced in all four individuals at the first time-point after the initiation of phototherapy (day 10). The NK cell activity continued to decline in all four subjects until the end of phototherapy, when the percentage cytotoxicity values ranged from 12 to 25%. However, the NK response rapidly returned after the cessation of phototherapy, in the two individuals (WN and MB) examined at this time (day 7 after

Figure 7.2 HSV-specific cytotoxic assay, using CD4-purified CTLs, during broadband UVB phototherapy



Four individuals known to be seropositive for HSV underwent a standard course of broadband UVB phototherapy. They received whole body irradiation three times a week for four weeks, with incremental doses depending on skin type.
The ability of these individuals to mount an HSV-specific cytotoxic T cell response was examined. Cytotoxic T lymphocytes (CTLs) were generated by *in vitro* incubation of non-adherent cells from PBMC with HSV primed dendritic cells for 6 days at 37°C. A population of these CTLs purified for CD4 expressing cells was prepared and used as effector cells in the cytotoxicity assay.
Target cells were autologous B cell lines (BCLs) prepared for each subject by transformation with Epstein-Barr virus. BCLs were incubated with inactivated HSV for two hours prior to being used as target cells in the cytotoxicity assay.
The results shown here are for cytotoxic cell assays set up for the four subjects, with a final effector to target cell ratio of 40:1. Spontaneous [^{51}Cr] release was determined by incubation of target cells with RPMI-FCS and maximum release by incubation with 2% acetic acid. The SEM for each assay was always within 10% of the mean value. The percentage specific release was determined according to the formula shown in section 2.5.2.1.
Blood samples from each individual were taken prior to UVB exposure (pre-UV), and after 10 (early-UV), 20 (mid-UV) and 30 (end-UV) days after the start of phototherapy.

Figure 7.3 HSV-specific cytotoxicity assay, using CD8-purified CTLs, during broadband UVB phototherapy



Four individuals known to be seropositive for HSV underwent a standard course of broadband UVB phototherapy. They received whole body irradiation three times a week for four weeks, with incremental doses depending on skin type.

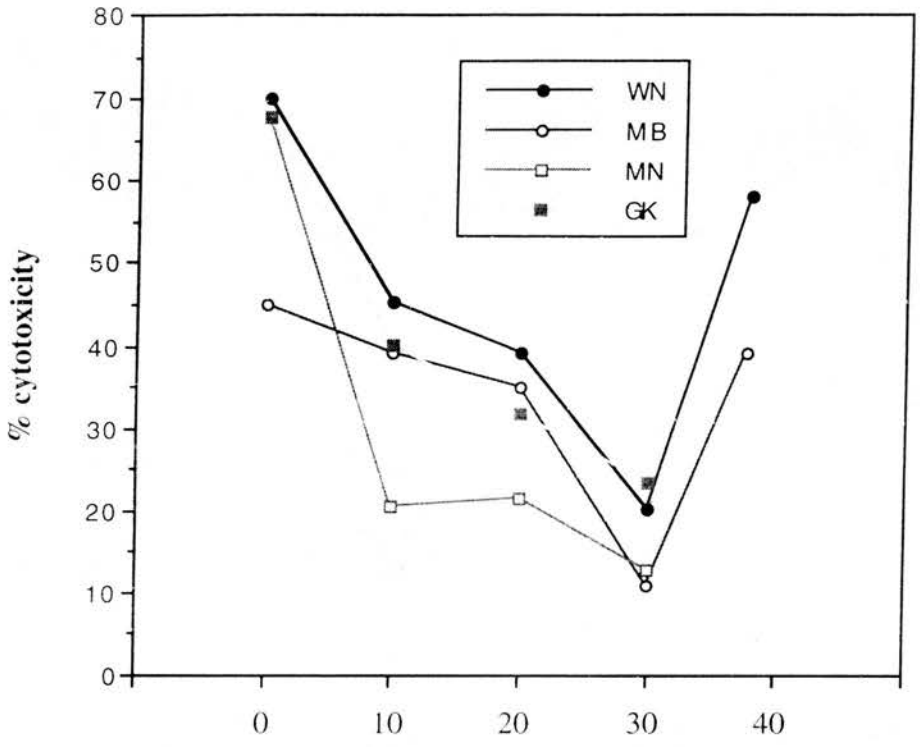
The ability of these individuals to mount an HSV-specific cytotoxic T cell response was examined. Cytotoxic T lymphocytes (CTLs) were generated by *in vitro* incubation of non-adherent cells from PBMC with HSV primed dendritic cells for 6 days at 37°C. A population of these CTLs purified for CD8 expressing cells was prepared and used as effector cells in the cytotoxicity assay.

Target cells were autologous B cell lines (BCLs) prepared for each subject by transformation with Epstein-Barr virus. BCLs were incubated with inactivated HSV for two hours prior to being used as target cells in the cytotoxicity assay.

The results shown here are for cytotoxic cell assays set up for the four subjects, with a final effector to target cell ratio of 40:1. Spontaneous [^{51}Cr] release was determined by incubation of target cells with RPMI-FCS and maximum release by incubation with 2% acetic acid. The SEM for each assay was always within 10% of the mean value. The percentage specific release was determined according to the formula shown in section 2.5.2.1.

Blood samples from each individual were taken prior to UVB exposure (pre-UV), and after 10 (early-UV), 20 (mid-UV) and 30 (end-UV) days after the start of phototherapy.

Figure 7.4 NK cell activity during broadband UVB phototherapy



Four individuals underwent a standard course of broadband UVB phototherapy. They received whole body irradiation three times a week for four weeks, with incremental doses depending on skin type. The NK cell activity of these individuals was before the start of treatment (pre-UV) and after 10 (early UV), 20 (mid UV), 30 (end UV) and 37 (post-UV) days after treatment began. K562 cells were used as targets and assays were carried out at various effector (PBMC) to target cell ratios. The assays represented here are for ratios of 20:1. Spontaneous [^{51}Cr] release was determined by incubation of target cells with RPMI-FCS and maximum release by incubation with 2% acetic acid. The SEM for each assay was always within 10% of the mean value. The percentage specific release was determined according to the formula shown in section 2.5.2.1.

the end of phototherapy). The NK cell response in these subjects returned almost to the respective pre-UVB treatment levels.

The results shown in Figure 7.4 represent NK cell assays in which an effector to target cell ratio of 20 to 1 was employed. Table 7.3 shows the results of NK assays for two individuals prior to phototherapy, using a range of effector to target cell ratios. Although the maximum cytotoxic response obtained, ie at a 20:1 ratio, differs between the two subjects, the percentage cytotoxicity is titratable in both individuals, as the number of effector cells used is reduced. The NK cell response seen for all four individuals at all time-points was titratable in this way.

7.2.3 Phenotypic analysis of PBMC

Flow cytometry was used to monitor any phenotypic changes in the subjects' PBMC, following staining of the cells with monoclonal antibodies to various cell-surface markers, as described in section 2.5.4. Phenotypic analysis was carried out at 0, 10, 20 and 30 days after the initiation of treatment, as for the functional assays. No variation in the percentage of positive CD3, CD4 or CD8 PBMC was seen in any of the subjects throughout phototherapy (results not shown). Figure 7.5 shows a typical forward-angle versus side-angle light scatter plot of unseparated PBMC and metrizamide-separated PBMC. Approximately 90% of the unseparated PBMC were in the region with size and granularity that is characteristic of lymphocytes. The metrizamide-enrichment gave two populations, with approximately 50% of cells being lymphocytes and 50% DC, as determined by their size and granularity. The DC population were demonstrated to be 100% HLA-DR$^+$, and the lymphocyte population was HLA-DR$^-$. Figure 7.5 also shows representative graphs of CD8 expression on unseparated PBMC and on a typical MiniMACS-purified CD8$^+$ population. All MiniMACS purified populations used were greater than 97% pure, as determined by flow cytometry.

Table 7.3 Titration of effector cells in NK cell assay

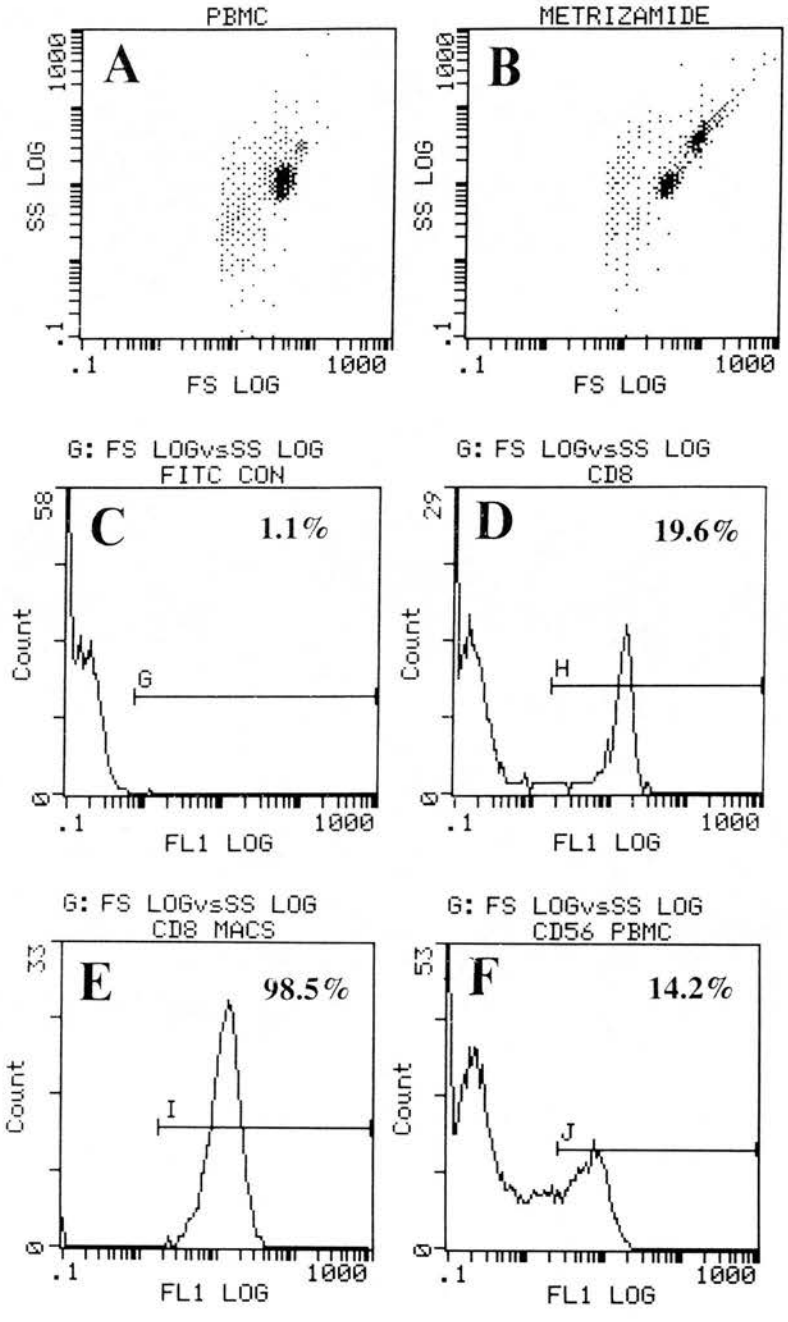
Effector : target cell ratio[a]	WN (pre-UV)		MB (pre-UV)	
	Mean ± SEM	% cytotoxicity[b]	Mean ± SEM	% cytotoxicity[b]
20 : 1	4777.3 ± 187.8	70.2	3371.7 ± 103.5	44.9
10 : 1	4184.7 ± 16.3	59.5	1950.7 ± 129.2	19.4
5 : 1	2993.0 ± 228.5	38.1	1665.7 ± 27.1	14.3
2.5 : 1	2253 ± 110.9	24.8	1301.3 ± 25.5	7.7
1.25 : 1	1719.7 ± 61.6	15.2	1094.3 ± 27.2	4.0

Results are shown for the NK cell assays of two subjects (WN and MB) using blood samples taken prior to commencement of phototherapy (day 0).

[a] K562 cells were used as targets and assays were carried out at the various effector (PBMC) to target cell ratios indicated.

[b] The percentage specific release was determined according to the formula shown in section 2.5.2.1. Spontaneous [^{51}Cr] release was determined by incubation of target cells with RPMI-FCS and maximum release by incubation with 2% acetic acid.

Figure 7.5 Flow cytometry



The size and granularity of (A) PBMC and (B) a metrizamide-enriched DC population. Representative CD8 expression on (D) unseparated human PBMC and (E) a MiniMACS-purified CD8 population. The isotype control staining is shown in (C). Representative CD56 expression on unseparated human PBMC (F). The line on each of graphs C-F represents the region within which fluorescence is considered positive. These regions were set using the appropriate isotype controls. The percentage of positive cells in this region is stated on each histogram.

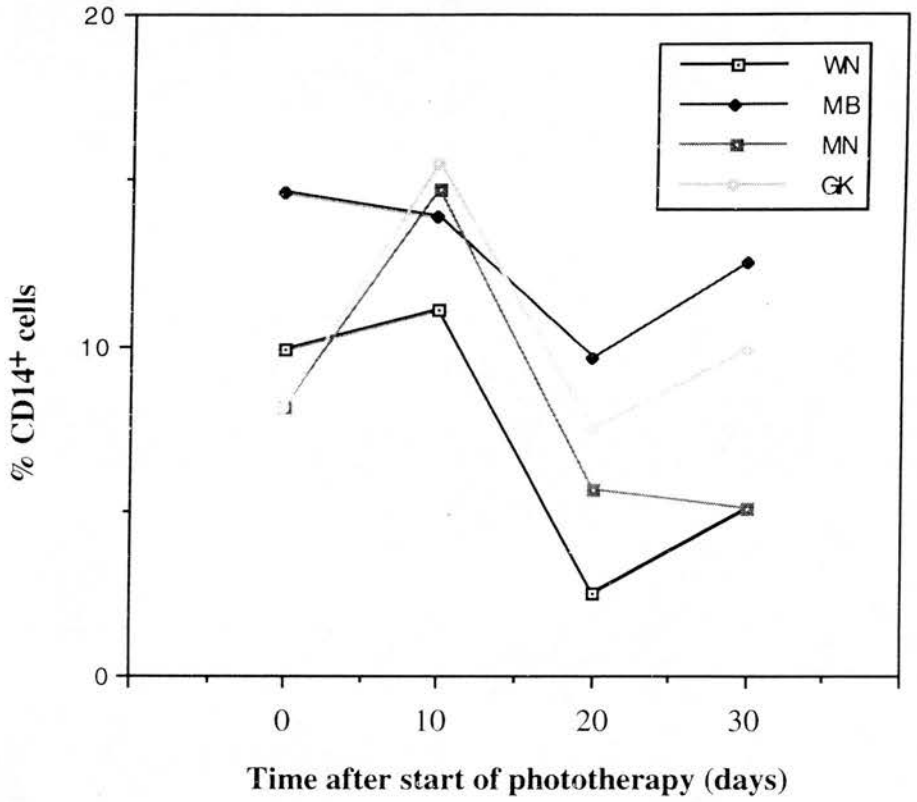
7.2.3.1 Expression of CD14

In contrast the percentage of cells expressing the monocyte marker CD14 varied during photherapy in all the subjects, as shown in Figure 7.6. The general trend appears to be an initial increase in the percentage of CD14-positive cells at 10 days after the start of treatment. This increase was seen prominantly in two of the four subjects. Between 10 and 20 days a decrease in the percentage of CD14-positive cells was observed, with the 20 day (mid-UV) expression below the initial levels in three of the subjects. From day 20 to day 30 three of the subjects showed an increased percentage of positive cells, towards the basal level.

7.2.3.2 Expression of CD56

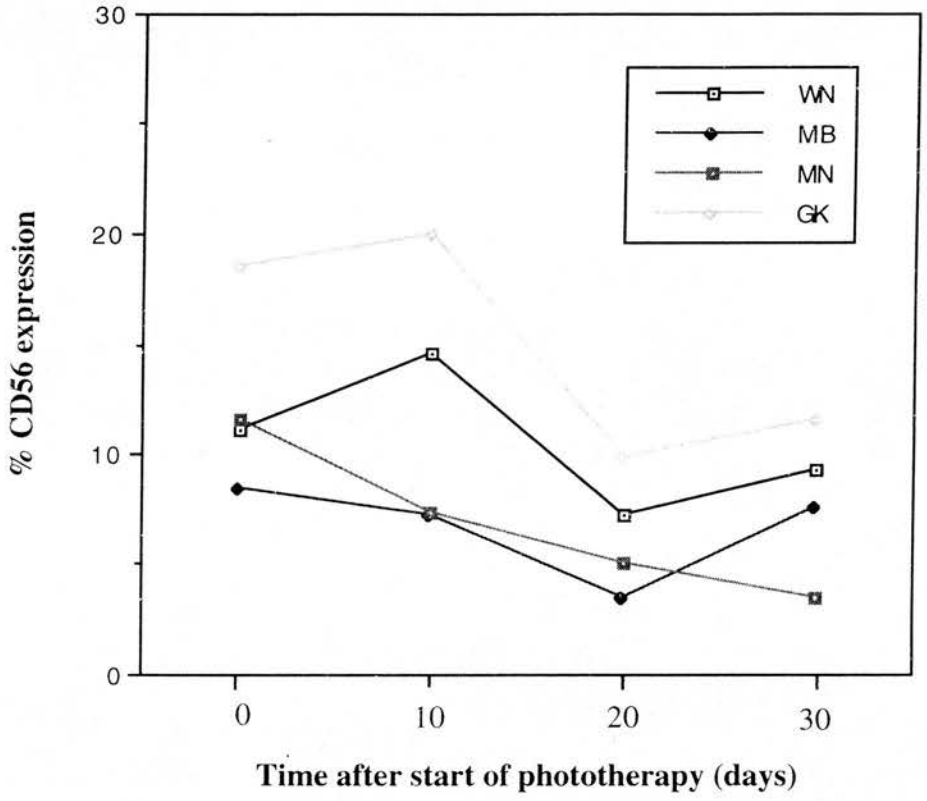
The percentage of CD56-positive cells (an NK cell marker), also varied throughout phototherapy, as shown in Figure 7.7. From 10 days after the start of phototherapy, the percentage of CD56 expressing cells decreased in all four subjects, to below the initial level. Between 20 days and the end of treatment, the CD56-positive cells increased slightly in three of the individuals. Figure 7.5 includes a representative graph showing CD56 expression on unseparated PBMC.

Figure 7.6 Percentage of CD14+ cells during broadband UVB phototherapy



Four individuals underwent a standard four week course of broadband UVB phototherapy. Phenotypic analysis of PBMC from each subject was carried out using flow cytometry, following staining of cells with monoclonal antibodies against cell surface markers. Samples were collected before the start of treatment (day 0), and at 10, 20 and 30 days after the start of phototherapy. The percentage of CD14-positive PBMC is shown for each individual at the four time-points.

Figure 7.7 Percentage of CD56+ cells during broadband UVB phototherapy



Four individuals underwent a standard four week course of broadband UVB phototherapy. Phenotypic analysis of PBMC from each subject was carried out using flow cytometry, following staining of cells with monoclonal antibodies against cell surface markers. Samples were collected before the start of treatment (day 0), and at 10, 20 and 30 days after the start of phototherapy. The percentage expression of CD56 on PBMC is shown for reach individual at the four time-points.

7.3 Discussion

7.3.1 Effects of UVB on CTL activity

The currently available data regarding the effect of UVB exposure on CTL generation and activity are very limited and on the whole restricted to studies of cytotoxicity against syngeneic tumours and alloantigens. In studies in which mice were irradiated *in vivo*, no effect of the UV exposure was reported on either the primary *in vitro* or *in vivo* induction of T cell cytotoxicity against UV-induced syngeneic tumour cells or alloantigens (Spellman *et al.*, 1977; Thorn, 1978). Since these initial studies, exposure of mice to $4.5x10^3$ mJ/cm^2 UVB, before sensitisation with a contact sensitiser, was demonstrated to cause depressed levels of priming for a secondary *in vitro* cytotoxic response against haptenated cells (Jensen, 1983). A defect in antigen-presenting cells was found to be largely responsible for the reduced priming response. Additionally the irradiated, immunised mice possessed suppressor cells, capable of blocking priming for cytotoxic responses against haptenated cells in normal mice. Thorn showed that chronically exposed mice had depressed secondary cytotoxic responses, generated *in vitro* or *in vivo*, against UV radiation-induced syngeneic tumour lines (Thorn, 1978). Suppressor cells from these chronically irradiated mice were able to block the cytolytic memory response against UV radiation-induced tumours (Thorn *et al.*, 1981). It remains unknown whether the UV-induced alteration in APC function is the primary defect leading to the production of suppressor cells specific for UV radiation-induced tumour antigens, as well as for haptens.

UVB-induced cytotoxic unresponsiveness is not observed with all antigens. Evidence for this comes from the findings that chronically irradiated mice, as well as mice exposed on only one occasion, make normal cytotoxic responses against allogeneic cells (Jensen, 1983; Thorn, 1978). Such findings imply that differences exist in the way different antigens are processed and presented to the precursor cells involved in CTL function.

The evidence currently available from mice suggests that UV irradiation exerts its effects on at least two components of the immune system, namely induction of a defect in APC function and generation of suppressor cells. Since there appears to be some specificity in the CTL function defect, there may be antigen selectivity in the antigen-presenting cell defect induced by UV radiation. Finally, because primary *in vitro* responses in spleen cultures from UV-irradiated mice are normal, cells from UV-irradiated mice must be able to present hapten in a recognisable manner to precursor CTLs.

In addition there is some evidence that *in vitro* exposure of human target cells (Sa; an EBV-transformed B cell line) to 10 kJ/m^2 UVB induces resistance of these target cells to alloreactive CTL (Kobata *et al.*, 1993a). This UVB-induced resistance was correlated with a deficient conjugate formation between target cells and CTL. Decreased surface expression of CD54 (ICAM-1), CD58 (LFA-3) and HLA were reported following UVB exposure, although these changes were not demonstrated to be critical for increased resistance. The same UVB-exposed target cells were shown to induce *in vitro* clonal anergy in alloreactive CTLs (Kobata *et al.*, 1993b). It is thought that while the target cells can be recognised by CTL and can induce high affinity IL-2 receptor expression on them, they do not provide sufficient signals to induce CTL proliferation. This may in part be due to the down-regulation of the TCR on the anergic CTL.

7.3.2 Effects of UVB on NK cell activity

UV irradiation results in a transient suppression of immune responses to a number of agents against which NK cell activity may play a role. These include animal models of HSV (Howie *et al.*, 1986a), MAIDS (Brozek *et al.*, 1992), reovirus (Letvin *et al.*, 1981) and CMV (Goettsch *et al.*, 1994a), as well as tumour antigens and allografts. *In vitro* irradiation of human PBMC results in a dose-dependent suppression of NK cell activity (Elmets *et al.*, 1987; Schacter *et al.*, 1983; Weitzen and Bonavida, 1984; Yaron *et al.*, 1995). In addition there is an indication that exposure to UV *in vivo* may

also result in a suppression in NK cell activity (Gilmour *et al.*, 1993a; Hersey *et al.*, 1987). In fact, the NK cell activity of subjects, both psoriatic and normal individuals during PUVA and narrow band UVB phototherapy, and after broadband UVB phototherapy, have been reported to be suppressed (Gilmour *et al.*, 1993a). The timing of this suppression appears to be critically dependent on the lamps used, the dose employed and the timing of the study (Gilmour *et al.*, 1993a; Jones *et al.*, 1996). It was also found that *in vitro* treatment of PBMC with *cis*-UCA induced a dose-dependent suppression of NK cell activity (Gilmour *et al.*, 1993a). In contrast *trans*-UCA had almost no effect. These results suggest that there may be a correlation between the formation of *cis*-UCA in the epidermis and the modulation of NK cell activity following UVB phototherapy. However, more recent evidence is in direct contradiction to this. It was found that *trans*-UCA was a strong inhibitor of the NK cell activity of human PBMC against K562 targets *in vitro,* whereas *cis*-UCA had no effect on this response (Uksila *et al.*, 1994). In addition, while the percentage of *cis*-UCA in the epidermis of patients receiving narrow-band UVB phototherapy increased, no correlation was found between this and the observed decrease in NK cell activity (Guckian *et al.*, 1995).

7.3.3 Effect of broadband UVB phototherapy on cellular immune function

The course of broadband UVB phototherapy did not have any measurable effect on HSV-specific T cell-mediated cytotoxicity. In addition, the results shown here did not conclusively indicate a unique role for either $CD4^+$ or $CD8^+$ lymphocytes in the CTL response to HSV, as neither enriched cell population was solely responsible for the cytotoxic response. This may reflect contamination of the separated populations, although phenotypic analysis using a flow cytometer, revealed the MiniMACS-purified populations to be greater than 97% pure (see Figure 7.5 for representative CD8-purified population).

However, broadband UVB phototherapy did cause a suppression of the NK cell activity. This finding is in agreement with those of others, who also demonstrated that *in vivo* exposure of individuals to UVB resulted in a suppression of NK cell activity (Gilmour *et al.*, 1993a; Hersey *et al.*, 1987). The suppression in this study was evident within the first week of treatment, when very small, sub-erythemal doses of UVB were employed. In terms of environmental exposure, a standard course of UVB phototherapy, as used in the study in chapter 7, has been calculated to be equivalent to approximately two weeks of solar exposure in the summer in Spain (40°N). The implications of such suppression must therefore be considered with respect to normal individuals exposed to environmental UVB, as well as patients undergoing phototherapy and individuals increasing their exposure through the use of sunbeds. Since NK cells are known to provide a first line of immunological defence against a range of infectious agents, any suppression of this function could render individuals more susceptible to infection, particularly from viral agents. Such suppression may also increase the possibility of developing malignancies, since NK cells are important in natural resistance against neoplasia.

7.3.4 Relationship between phenotype and function

The main cellular expression of CD14 is on monocytes. The slight decrease in expression of CD14 seen in all four subjects between 10 and 20 days from the start of phototherapy is likely to represent a UVB-induced decrease in the total number of CD14-positive monocytes, rather than a decrease in the cell surface expression of this molecule. Since UVB can only penetrate the epidermis and the upper layers of the dermis, it seems likely that any direct effect of UVB will be on circulating cells as they migrate through the dermal vasculature. A second possibility is that UVB-induced soluble factors, such as cytokines, are mediating the effects of UVB. Since monocytes mediate NK cell activation through the production of IL-12, a relationship may exist between the decrease in the number of $CD14^+$ cells and the suppression of NK cell activity. However, no direct evidence exists to this effect. It is interesting to

note such a potential UVB-induced effect on the numbers of certain cell types, as this will affect the relative proportions of cell types in peripheral blood.

In contrast, the percentage of $CD56^+$ PBMC appears to directly relate to the NK cell activity during phototherapy. The CD56 expression data implies that the decrease in NK cell activity is not a result of a loss of function but of decreased NK cell numbers. This is particularly evident between 10 and 20 days after the start of treatment, when the percentage of $CD56^+$ cells in all four subjects and the NK cell activity in three of the subjects decreased. The situation in the initial 10 day period, when the NK cell activity decreased, but the percentage of $CD56^+$ cells increased in two individuals, may be explained if the increase in $CD56^+$ cells is due to repopulation of the $CD56^+$ population by precursor cells, unaffected by the UVB. During the next 10 days of treatment, there will be no such unaffected precursor cells available to repopulate the $CD56^+$ compartment and hence the decrease in the number of circulating $CD56^+$ cells seen at this time. Between 20 and 30 days there appears to be a recovery of CD56-expressing cells, although to pre-UVB levels in only two of the subjects. This increase in the $CD56^+$ population would suggest a recovery of precursor cells, allowing repopulation. This may be due to adaptation of such cells to the UVB exposure, although no mechanism is proposed at this time. It is more likely that adaptation of the epidermis occurs by tanning and epidermal thickening, thereby protecting the CD56 population.

7.3.5 Limitations of the study

The main limitation of a study such as this one, is the relatively small number of subjects which can realistically be recruited and undergo the treatment at any one time. In particular, the subjects must be known to be seropositive for HSV and must live within reach of the phototherapy facilities to be able to attend on a regular basis. The assays used in this study are fairly time-consuming, especially the cell-separation procedures, making it difficult to process samples from several individuals at once. This problem is accentuated with the cytotoxicity assay since the target cells used are

unique to each individual. The generation of B cell lines for use as targets is itself a lengthy process which is not always successful and is therefore another factor in the recruitment of subjects.

7.3.6 Suggestions for further work

Obviously the first priority in developing this study would be to increase the size of the sample group by recruiting more individuals, in order to clarify some of the trends seen during phototherapy. Since the suppression of NK cell cytotoxicity was a consistent finding, it would be interesting to test whether particular sunscreens could protect individuals against such immunosuppression and hence prevent any potential increase in susceptibilty to infection. With respect to immunity to HSV, it would be interesting to determine the Th cell subset which predominates in HSV seropositive individuals before and during phototherapy. A preliminary study, using cell depletion of responder cells, suggests that EC present HSV almost exclusively to $CD4^+$ cells (W.Neil, Dept. of Medical Microbiology, University of Edinburgh; personal communication). To date no studies have been undertaken to ascertain whether a systemic Th1 to Th2 switch, reported to occur following UVB exposure, may occur in HSV-infected subjects as a result of such exposure. It would be very interesting to find out whether individuals who suffer from frequent recrudescences are induced to have a more prominant Th2 response to HSV than those subjects who are seropositive for HSV but who never, or rarely develop recrudescences.

To complement the human HSV cytotoxicity assay, attempts were made to establish a suitable protocol for assaying HSV-specific T cell cytotoxicity in mice. Mice were infected intra-peritoneally and at subsequent time-points lymph node and spleen cells were removed and cultured *in vitro* with inactivated HSV for 5 days to generate CTLs. A chromium release assay was then carried out as described in section 2.6.2, using HSV-infected L929 cells as targets. However, HSV-specific cytotoxicity could not be detected using either lymph node or spleen cells. The protocol was altered by giving mice booster injections of HSV, increasing the amount of HSV used for both the

infection of mice and for the *in vitro* incubation and attempting to increase the effectiveness of the *in vitro* culture stage. Although such cytotoxicity assays are characteristically difficult to establish, it would prove to be a very valuable tool in further characterising the specific effect of UVB on anti-viral immune mechanisms. To date the effect of UVB exposure on virus-specific cytotoxicity has not been studied in a murine model which has obvious potential advantages in terms of manipulation. The recent introduction of a MiniMACS system for the purification of murine dendritic cells may make it possible to isolate these cells for use as antigen presenting cells in the *in vitro* CTL-generating culture stage.

7.4 Summary

In this study four normal subjects, known to be seropositive for HSV, underwent a standard course of broadband UVB phototherapy, as used in the treatment of psoriasis. Before the start of phototherapy and at 10, 20 and 30 days, blood samples were taken and phenotypic analysis, as well as functional assays were carried out. It was found that the course of phototherapy had no measurable effect on HSV-specific T cell-mediated cytotoxicity, using autologous B cell lines, infected with HSV as targets. In contrast, the NK cell activity of all four subjects was suppressed within the first week of phototherapy and further suppressed by 30 days. The percentage of PBMC expressing CD56 decreased between 10 and 20 days of treatment in all four individuals and this would suggest that the decline in NK cell activity results from a reduction in the number of NK cells rather than a functional defect. The NK cell activity of two of the subjects was studied a week after the end of phototherapy, by which time the activity had returned towards the pre-irradiation values. The UVB-mediated suppression of NK cell activity, although transient, has implications with respect to infectious agents, as reducing an individual's innate immunity may result in an increased susceptibility to infectious disease.

Chapter 8

Final Discussion and Summary

There are mounting environmental concerns about the global depletion in stratospheric ozone which will lead to a corresponding increase in the quantity of UVR reaching the surface of the earth. In addition, individuals are becoming exposed to increased natural sunlight through foreign travel and to artificial UVR through the use of sunbeds and phototherapy. It is therefore imperative to understand the biological effects of such increased exposure.

Since the field of photoimmunology was created twenty years ago, a growing body of evidence indicates that exposure of human subjects and experimental animals to UVB irradiation can modify some immune responses. However, to date, investigations into the impact of UVR on the susceptibility to infection are relatively few.

A series of events occurs when the skin is UV-irradiated. The first is absorption of the radiation by a cutaneous chromophore, the most likely candidates being DNA and *trans*-UCA. Various mediators are then implicated, including TNF-α, IL-10 and PGs. The outcome is complex, with multiple cellular changes, both phenotypic and functional.

Interest has centered on the role of keratinocytes in cutaneous immune responses. The production of cytokines by keratinocytes is believed to be critical in determining the local microenvironment, which in turn determines the type of immune response elicited. The importance of the cutaneous microenvironment in terms of the immune response elicited, has been highlighted in a recent paper (Kitagaki *et al.*, 1997). It was found that the local cytokines present at the time of antigen presentation to T cells in

the elicitation phase of a CH response, altered the time-course of antigen-specific hypersensitivity from a typical delayed-type to an immediate-early type response. Chronic exposure to an antigen (ie. repeated elicitation), was found to alter the balance of locally released cytokines, with a shift toward a predominant Th2 response, rather than the characteristic Th1-type response associated with a classic CH response. It is believed that this shift in response represents an evolutionary adaptation to reduce the deleterious Th1 response elicited in response to chronic antigenic challenge.

A single exposure, or several sub-erythemal exposures, prior to sensitisation results in suppressed CH on subsequent challenge. However, exposure of sensitised mice to sub-erythemal doses of UVB on four consecutive days prior to challenge was found to result in an enhanced CH response in comparison to unirradiated mice (chapter 3). With respect to alterations of CH responses, it is quite possible that the cutaneous cytokine milieu induced by UVB exposure plays a key role in the magnitude of the subsequent immune response induced. It may be that the type of APC involved will affect the local cytokines produced by keratinocytes, infiltrating inflammatory cells and T cells, and hence a particular microenvironment will be generated, be that suppressive or enhancing. Another possibility is that the keratinocytes themselves act as APC during the elicitation phase of the CH response, or that other novel APC, such as dermal dendrocytes, are involved.

The effect of UVB exposure of mice prior to infection with MHV-68, a gammaherpesvirus with no cutaneous involvement and which establishes a persistent infection in the spleen, was examined (chapter 4). It was found that a sub-erythemal dose of UVB caused suppression of the DTH response. This finding adds to the accumulating evidence demonstrating the suppressive effects of UVB exposure on the immune responses to infectious agents, which include not only epidermal pathogens but also ones which cause systemic infections.

A preliminary characterisation was carried out of the changes in cytokine expression associated with HSV infection, both *in vitro* and *in vivo* (chapter 5). It was found that HSV infection induced an upregulation of IL-10 mRNA expression both in a keratinocyte cell line and in mouse skin. Since IL-10 is produced by Th2 cells and by murine keratinocytes following appropriate stimulation and blocks activation of cytokine synthesis by Th1 cells, its production could result in a switch in the local immune response from a protective Th1-type to a Th2 response. It is believed that such viral-induced changes in the local immune microenvironment may be important in aiding the infectivity of the virus and in evasion of the host immune response sufficiently to allow latency to be established. The expression of IL-10 mRNA was also enhanced following UVB exposure of keratinocytes, although no increase in expression was seen following irradiation and HSV infection together, in the preliminary *in vitro* study. Evidence obtained from murine studies *in vitro* and *in vivo* shows that UVR may lead to the promotion of Th2 responses with anergy of Th1 responses (Araneo *et al.*, 1989; Simon *et al.*, 1991). It remains to be seen whether the combination of these UVB and HSV-induced effects on local cytokine production can explain the occurrence of HSV recrudescent lesions following exposure to sunlight. Such a link between the UV-induced alteration in cytokine profile and clinical symptoms is substantiated by experiments in which the cytokine production by LN and spleen cells of mice were examined following intradermal infection with HSV-1 (Yasumoto *et al.*, 1994). In mice exposed to UVB prior to infection, a marked suppression of IFN-γ synthesis was found, and an enhancement of IL-4 synthesis. An associated increase in the severity of lesions compared with unirradiated control animals was observed.

Animal models are essential in order to assess the effects of UVB exposure during infections *in vivo*, since it would be unethical to carry out such experiments on humans. However, some experiments, such as those described in chapters 6 and 7 of this study, provide valuable evidence that similar UVB-induced immunosuppressive

effects occur in humans as have been reported in mice. It was found that while a standard course of broadband UVB phototherapy had no measurable effect on HSV-specific T cell-mediated cytotoxicity, the NK cell activity was suppressed within the first week of irradiation. In addition, both *in vitro* and *in vivo* irradiation of human EC suppressed the alloreactive capacity of epidermal LC. Suppression of the APC function of EC in the MSLR by UVB exposure has also been demonstrated in mice and rats. These rodent models along with data obtained from human experiments have been used to try to develop a quantitative risk assessment of lowered resistance to infections in humans due to solar UVB exposure (Garssen *et al.*, 1996). It has been calculated that exposure to about 100 minutes of sunlight at noon in Italy or Spain would suppress the human lymphoproliferative response to *Listeria* by 50%. The same degree of suppression is predicted as a result of 88 minutes of exposure in Queensland, Australia in the summer, (or 55 hours in Shetland in January!).

A recent publication revealed that the antarctic ozone hole covers a larger area and begins to form much earlier in the year than was previously thought (Roscoe *et al.*, 1997). Such findings are important because they are thought to reflect the world-wide situation and because ozone-poor air from the edge of the ozone hole regularly passes over S.America, exposing populations to larger than normal doses of damaging UVR. The questions which remain to be answered are whether UVB-induced reductions in protective immune responses are likely to exacerbate disease processes, resulting in more prolonged or more severe infections. The accumulating data from animal models, such as those shown in Table 1.2, suggest that they will. However, there is considerable redundancy in the immune system so, even if one particular parameter of immunity is down-regulated by UV exposure, another may compensate without an exacerbation of disease symptoms. Whether an individual's resistance to reinfection will decrease or whether an increased frequency of reactivation will occur in the case of persistent organisms, remain to be seen. Although preliminary data from the study of the effects of UVB exposure on MHV-68 infection did not demonstrate any effect

of irradiation on reactivation of latent virus, the knowledge that UVB can cause recrudescence of HSV in human subjects suggests that further investigation of this phenomenon should be carried out.

UVB-induced immunosuppression may prove to be particularly important with respect to diseases such as tuberculosis, once considered to be under control in industrialised countries. These diseases are rising in incidence and are becoming a major public health problem, not only in developing nations, but also in industrialised countries because of an increased prevelance of mycobacterial infections in AIDS patients, immigration and the emergence of drug-resistant strains. Since DTH responses are important in controlling such diseases, an increase in solar UVR could exacerbate this problem. Historical treatment of tuberculosis patients using sun exposure often took place in cold climates, with the patient covered, except for their face. Immunosuppressive doses of UVB were not therefore thought to have been encountered and the occasional successful treatment is thought to be due to the fresh air and increased standard of patient care.

The implications of UVB-induced immunosuppression must also be considered with respect to vaccination. If a particular immunisation strategy depends upon the induction of a cell-mediated response, there is a risk that immunosuppression might make the vaccine less effective. Current research on this subject is limited, but a scenario where UV-induced suppression allows the immune response to develop a specific unresponsiveness or tolerance to a particular pathogen rather than defend against it, has potentially disastrous consequences. Ethical permission has recently been obtained by a group in Utrecht, to carry out a study of the effects of sub-erythemal doses of UVB on the subsequent administration of a sub-unit hepatitis B vaccine to human subjects. The results of this study will provide valuable information with which to begin to assess potential risks.

The experiments described in this study, although fairly diverse in nature, all share the common aim of elucidating the modulatory effects of UVR on immune responses, in particular to viral infections. The animal models and human studies described have all been carried out using sub-erythemal doses of UVB, indicating that the sunburn response, usually thought of as a warning sign for the effects of sun exposure, comes too late for the effects of UVB on the immune system. In addition, the potential for protection from a tan seems to be minimal, with immunosuppression having been reported in both dark-skinned Australian aborigines and in fair-skinned people of Celtic descent. This may be because the photoreceptors for UVB, such as UCA are found nearer to the surface of the skin than the melanin-producing cells. It is likely that adaptation will take place in response to UVB exposure in the short-term, with tanning and epidermal thickening responses, but also possibly at the evolutionary level, if increased environmental UVR becomes a selection pressure. The ability of sunscreens to protect against immunosuppression remains controversial. In addition sunscreen use can result in changes in sun-exposure behaviour, as well as altering the spectral distribution of UVR to which the skin is exposed, the consequences of which remain unknown. Finally, it has been shown that human subjects and certain mouse strains fall into the categories of UV-susceptible and UV-resistant as defined by contact hypersensitivity following irradiation. Individuals with skin cancer are almost always of the susceptible type. However, it is not known at the present time whether the susceptibility/resistance division is applicable to the infectious diseases situation, as well as to cutaneous oncogenesis.

The down-regulation of the immune system by UVB is likely to have an evolutionary origin, in order to prevent a continuous immune response against UV-induced damage to skin cells occurring. However, an evolutionary balancing act must exist so that the down-regulation of the cutaneous immune system does not allow neoplasia or infections to develop and progress undetected. It is of course important to appreciate that UVR has many beneficial effects and that the effects of UV irradiation on the

pathogenesis of disease can differ dramatically, depending on the infectious agent, its route of entry into the host and the immune mechanisms involved in resistance to infection. It is therefore difficult to generalise about the effects of UVB on infectious disease and in particular, to extrapolate from animal models to disease processes in humans. More information is therefore required to be able to assess the full impact of increased solar UVB radiation on human health.

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Publications and abstracts arising from this work

1. The effect of ultraviolet radiation on infectious diseases.
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EXPRESSION OF CYTOKINE mRNA IN HERPES SIMPLEX VIRUS-INFECTED KERATINOCYTES *IN VIVO* AND *IN VITRO*

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While the sytemic immune responses generated in herpes simplex virus (HSV) infections have been well documented, little is known about the cytokines induced locally in the epidermis in response to the virus. These may be critical in determining the outcome of the infection and the subsequent interaction of the virus with the host during latency and recrudescence. A mouse keratinocyte cell line (PAM-212) was infected with HSV type 1, and ears of C3H mice were infected epidermally after tape-stripping the dorsal surface. RNA was extracted at various times thereafter from lysed cells or whole ear homogenates, and the mRNA of various cytokines assayed by semi-quantitative reverse transcriptase-polymerase chain reaction, with normalisation relative to β-actin mRNA signals. In the cell line, no change in the expression of IL-1α mRNA was apparent up to 24 hours post-infection, by which time viral antigens could be detected by immunoperoxidase staining. In contrast, an up-regulation in IL-10 mRNA expression was seen, starting at 3 hours post-infection.