LOCAL EFFECTS OF ULTRAVIOLET RADIATION ON CUTANEOUS IMMUNE RESPONSES

Thesis submitted for the Degree of Doctor of Philosophy by

Malcolm Scott Duthie

University of Edinburgh

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Declaration

I declare that the studies presented here are the result of my own independent investigation, with the exception of some ELISA in chapter 4 which were carried out by Dr. RJ Dearman, AstraZeneca, and immunoperoxidase staining of PAM-212 cells in chapter 6 which were carried out with the assistance of Dr. G Zak-Prelich.

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

Malcolm S Duthie

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Chapter 2 - Materials and Methods

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List of Abbreviations

ADP	adenine diphosphate
AIDS	acquired immunodeficiency syndrome
AOO	acetone: olive oil
APC	antigen presenting cell
ATP	adenosine triphosphate
BCC	basal cell carcinoma
BSA	bovine serum albumin
CH	contact hypersensitivity
conA	concanavilin A
cpm	counts per minute
DAB	3,3'-diaminobenzidene tetrahydrachloride
DC	dendritic cell(s)
DLN	draining lymph node
DLNC	draining lymph node cell(s)
DNCB	1-chloro-2, 4-dinitrobenzene
DTH	delayed type hypersensitivity
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FITC	fluorescein isothiocyanate
FCS	foetal calf serum
HIV	human immunodeficiency virus
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
ICE	IL-1β converting enzyme

IFN	interferon
IGIF	IFN-γ inducing factor
IL	interleukin
IMMA	imidazole-4(5)-methylidene malonic acid
IRAK	IL-1 receptor-associated kinase
KLH	keyhole limpet haemocyanin
LC	Langerhans cell(s)
LPS	lipopolysaccharide
MECLR	mixed epidermal cell: lymphocyte reaction
MED	minimum erythemal dose
MeIMMA	methylated imidazole-4(5)-methylidene malonic acid
MLR	mixed lymphocyte reaction
MM	malignant melanoma
MMLV RT	Moloney murine leukemia virus reverse transcriptase
Ox	4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E_2
PMA	phorbol myristate acid
PNK	T4 polynucleotide kinase
PUVA	psoralen with UVA
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SALT	skin-associated lymphoid tissue
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate

SDS-PAGE	SDS-polyacrylamide electrophoresis
SEM	standard error of the mean
SLE	systemic lupus erythematosus
T4N5	T4 endonuclease V
T_{50}	50% probability of tumour development
TBARS	thiobarbituric acid reactive substances
Th	T helper cell ($CD4^+$)
TGF	transforming growth factor
TNCB	2, 4, 6-trinitro-1-chlorobenzene
TNF	tumour necrosis factor
TPA	phorbol 12-myristate 13-acetate
Ts	T suppressor cell
UCA	urocanic acid
UV	ultraviolet
UVA	ultraviolet A (315-400 nm)
UVA1	ultraviolet A (340-400 nm)
UVA2	ultraviolet A (315-340 nm)
UVB	ultraviolet B (280-315 nm)
UVC	ultraviolet C (200-280 nm)
UVB-R	UVB-resistant
UVB-S	UVB-susceptible

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Abstract

Depletion of stratospheric ozone and changes in lifestyles have led to increased concern about the harmful effects of ultraviolet radiation (UVR). Exposure to UVR has been shown to induce both local and systemic suppression of immune responses initiated through the skin. The mechanism of dysregulation of immune responses following UV exposure is complex, and is initiated by chromophores. One such chromophore is urocanic acid (UCA), which is isomerised from the *trans*- to the *cis*-isoform by UVR, in the epidermis. The primary antigen presenting cell (APC) of the skin, the Langerhans cell (LC), plays a critical role in cutaneous immune responses. UVR alters LC frequency within the irradiated epidermis, and also exerts effects on the mature form of LC, the dendritic cells (DC), within lymph nodes draining irradiated sites. These changes appear to be concurrent with an alteration in T cell cytokine profiles. UVR suppresses immune responses which are normally characterised by Th1-like cytokine profiles, possibly by immune deviation to Th2-like cytokine profiles.

The contact hypersensitivity (CH) response is commonly used to demonstrate cutaneous immunity. A model of CH in C3H/HeN mice was established. This model was utilised to examine the effects of local UVB irradiation before induction of contact sensitisation. Cytokine profiles within lymph nodes draining the site of elicitation of CH, or the site of contact sensitisation were investigated. Results suggest that UVB has no effect on cytokine production during the sensitisation phase of CH, but that it downregulates Th1-like cytokine production upon elicitation of CH.

Effects of UVR and UCA application were examined in relation to LC frequency within the epidermis. UVA1 (340-400nm) irradiation, or *trans*-UCA application, did not alter the LC numbers within the exposed site. UVB (280-315nm) irradiation and *cis*-UCA application depleted LC, and timecourses for LC depletion were established for both treatments. Injection of antibodies against either IL-1β or

TNF-α before UVB irradiation or *cis*-UCA treatment completely abrogated their effects on LC numbers. Thus, the UVB-mediated reduction of LC is dependent on the cytokines IL-1β and TNF-α. Despite reports that UVA1 irradiation protects mice from subsequent immunosuppression by UVB exposure, UVA1 irradiation did not affect the decrease in LC numbers induced by UVB.

Despite the differences in effects on LC frequency, both UVA1 and UVB induced an accumulation of DC within lymph nodes draining the site of irradiation. For both irradiation regimens, the accumulation of DC was dependent on IL-1β. This was identified by injecting neutralising IL-1β antibodies before irradiation, which inhibited the accumulation of DC within draining lymph nodes following irradiation. Similar experiments indicated that accumulation of DC following UVB irradiation, but not following UVA1 irradiation, was also dependent upon TNF-α.

Induction of cytokines within irradiated skin was examined. UVB exposure increased the expression of IL-10 and TNF-α proteins at the irradiated site. Attempts to identify the source of these cytokines were inconclusive, as both keratinocyte (PAM-212) and melanocyte (B16) cell lines failed to secrete these cytokines following UVB irradiation. Intracellular stores of TNF-α decreased as the dose of radiation increased. The technique of reverse transcription-polymerase chain reaction (RT-PCR) was established to examine expression of cytokine mRNA in irradiated skin. Following UVB irradiation, TNF-α mRNA was upregulated and there was induction of IL-10 mRNA. UVA1 irradiation did not result in such changes. There were also differences in the timecourse of IL-1β mRNA upregulation. IL-1β mRNA expression peaked transiently 4h after UVB irradiation. Following UVA1 irradiation, IL-1β mRNA expression did not peak until 24h, and remained upregulated at 48h, after exposure.

Chapter 1

Introduction

1.1. Environmental ultraviolet radiation

Ultraviolet radiation (UVR) is commonly subdivided into three categories, depending on wavelength; UVC (200-280 nm), UVB (280-315 nm) and UVA (UVA2; 315-340 nm, UVA1; 340-400 nm). Of the solar radiation that reaches Earth, 9% is in UV waveband. Ozone in the stratosphere effectively filters out all the UVC and much of the UVB wavelengths, whilst wavelengths above 320 nm pass through relatively unhindered. Around 1000-fold more UVA reaches the Earth's surface in comparison with UVB. Between 1970 and 1987, a substantial decrease in the protective ozone layer occurred (Frederick and Snell, 1988; Kerr, 1988), and steps such as the Vienna Convention, Montreal Protocol, and Copenhagen Amendments are being taken to redress the situation.

While reductions in ozone have been most publicised over the polar caps, measurements in more populated regions also suggest a pattern of decline. Indeed, radiative transfer analyses of satellite based O_3 measurements between 1979 and 1992 have shown that surface UVB increased substantially at all latitudes except the tropics, if other factors such as cloud cover and local pollutant levels remained constant (Madronich and de Gruijl, 1992).

Hawaii is the most UV-intense location on the planet, with the lowest ozone thickness values ever recorded outwith the Antarctic region (Bergmanson and Sheldon, 1997). Relative to values in the 1970s, by 1998 surface UV radiation had increased 7% at Northern hemisphere mid-latitudes in Winter/ Spring and around 4% in Summer/ Autumn, and about 6% at Southern hemisphere mid-latitudes on a

year-round basis. In the polar regions, increases were 130 and 22% in Antarctic and Arctic springs respectively (Madronich *et al* 1998). Biologically damaging radiation at Palmer Station (64^0S) was found to be comparable or even exceed the maximum summer values of San Diego (32^0N) (Madronich *et al* 1998). A study conducted in Glasgow revealed that there was an increase in UVB between 1995 and 1996. This was not due to clearer local conditions, as UVA levels were comparable on the days studied (Moseley and Mackie, 1997). Long-term predictions are that there will be a slow recovery (decline in UVB) to pre-ozone depletion levels over the next 50 years.

The amount of UVB reaching the Earth's surface in specific regions is dependent on other factors, such as weather conditions and the degree of pollution. UV measurements are routinely higher (by ground base data) in the less industrialised and less densely populated Southern hemisphere (Madronich *et al* 1998). After volcanic eruptions, ozone depleting aerosols are injected into the stratosphere and a delicate balance is struck between ozone depletion and increased reflective properties due to these particles. Estimates of the biologically effective UVR at the land surface after the El Chichon (1982) and Mount Pinatubo (1991) eruptions suggested that levels increased, as the effect of ozone depletion outweighed that of increased UV scattering (Vogelmann *et al* 1992).

UVR plays an important role in environmental considerations outwith direct effects on health. The cyanobacteria that play a key role in maintaining an ecological balance are dramatically affected by UV. Not only are cyanobacteria primary sources of the marine food web, but they are also important biofertilisers. Exposure of three cyanobacteria species (*Nostoc*, *Anabena* and *Scytonema*) to UVB corresponding to a 20% increase in its environmental flux has drastic effects on biomass production, photosynthetic rate and nitrogen fixation (Babu *et al* 1998).

Although most UVR that people experience is derived from sunlight, particularly with increased UVB due to ozone depletion, exposure can occur via other sources.

These include both cosmetic and medical use of sunlamps. Industrial UV exposure can also occur, as is the case with welding arcs or tungsten halogen lamps.

UV exposure can mediate alterations of immune responses. This chapter will describe generally cells of the skin, and immune responses activated by antigen exposure in the cutaneous environment. It will then detail the mediators involved in translating UVR into an effective modulator of such responses. Current understanding of the UV-induced alterations in cells of the immune system will be outlined. Effects at a cellular level will bestudied, including the involvement of cytokines. The effect of UV irradiation on disease states will be summarised, and finally, potential mechanisms to reduce UV-mediated immune suppression will be discussed. Although the experiments conducted in this thesis are entirely based in mice, within this introduction there will be an emphasis on UV effects on human systems. A general discussion will provide comparison of the results discussed herein with those found by previous studies on mice.

1.2. Skin physiology

The skin is the largest tissue of the body, providing a relatively impermeable barrier to the external environment. Skin is made up from two distinct layers; the dermis and the epidermis. The dermis is the thickest and deepest layer, comprised of connective tissue with a network of capillaries and lymphatics. The dermis is overlaid by the epidermis, with the majority of cells being keratinocytes at different stages of terminal differentiation. The epidermis also contains melanocytes and Langerhans cells (LC). Finally, the *stratum corneum* is the 'dead' layer on the epidermal surface, comprised of dead cells and molecules sloughing off the body.

1.2.1. Cells of the skin immune system

The skin is readily accessible to infectious organisms. Like the gut and respiratory tract, the skin has its own associated immune system. The skin-associated lymphoid tissue (SALT) (Streilein, 1993) was recognised because lymphoid malignancies which showed cutaneous tropism were always T cell derived, and a population of 'passenger leukocytes' within the skin was identified.

Keratinocytes comprise around 90% of epidermal cells. When adjacent to the basement membrane they are predominantly proliferating stem cells. As they mature, keratinocytes move through the suprabasal membrane toward the *stratum corneum*. Keratinocytes are capable of secreting cytokines and expressing MHC class II molecules. Mast cells are resident within the skin, and as described later (see section 1.3.6), have an important role in merging innate and specific, adaptive immune responses. Langerhans cells (LC) also play a key role, as the cells resident in the epidermis with the ability to initiate specific immune reponses upon presentation of antigen to T cells within DLN (described in section 1.3.1).

The cells involved in immune responses within the skin are detailed in table 1.1.

	resident	recruited	recirculating
Innate	Keratinocytes	Monocytes	Natural killer
	Endothelial cells	Granulocytes -	cells
	Mast cells	basophils	Dendritic cells
	Tissue	eosinophils	
	macrophages	neutrophils	
	(Melanocytes)	Mast cells	
		Epitheloid cells	
Adaptive	T cells	T cells	T cells
	Langerhans cells	B cells	
	Dendritic cells		

Table 1.1. Innate and adaptive cells of the skin immune system (adapted from Bos *et al* 1997).

1.3. Cells of the immune response

1.3.1. Langerhans cells

The primary antigen presenting cell (APC) in the epidermis are LC, which form a semi-continuous network comprising around 3% of epidermal cells. They are defined by their dendritic morphology, unique organelles named Birbeck granules within the cytosol, and membrane expression of ATPase, class II MHC, and CD1a (humans).

LC act as sentinel cells within the epidermis and are important in initiating cutaneous immune responses (Toews *et al* 1980). In the course of a normal cutaneous immune response, LC internalise and process antigen, with changes in cytokine production by keratinocytes and LC themselves leading to their migration to the draining lymph node (DLN). During migration via the afferent lymphatics, LC mature, as revealed by the altered expression of various adhesion and costimulatory molecules. Once in the paracortical region of the lymph node, where they are known as dendritic cells (DC), they present antigenic peptides to specific T cells, which are stimulated to proliferate and produce a particular cytokine profile.

It was first observed that application of a contact sensitiser to a body site deficient in LC, such as the hamster cheek pouch or mouse tail skin, led not to contact sensitisation, but rather to a specific unresponsiveness upon later challenge with that sensitiser. This observation suggested a key role for LC in presentation of antigens encountered through the skin. In guinea pigs, application of a contact sensitiser (DNCB) caused LC numbers, as assessed by ATPase, to drop in the first 24h. ATPase density reached its lowest level by 24h, with fewer dendritic processes and rounding of cells. Morphology and density slowly recovered to normal levels by 96h (Hanua *et al* 1989). Loss of ATPase activity correlated with an "activated" state, and application of a second contact sensitiser 24h after the first resulted in tolerance to the second.

The key migratory signals are believed to be provided in an autocrine fashion by interleukin (IL)-1β, and in a paracrine fashion by tumour necrosis factor (TNF)-α (Wang *et al* 1999; Kimber *et al* 1998). LC express only the type 2 (p75) TNF-α receptor (TNFR2; CD120b), and mice deficient in expression of TNFR2 have significantly impaired LC migration (Wang *et al* 1997). Blocking of TNF-α activity by neutralising antibodies revealed that TNF-α acts early in the response, as injection of antibodies prior to contact sensitisation, but not 18h after, inhibited contact sensitisation and the subsequent contact hypersensitivity (CH) response upon challenge (Cumberbatch and Kimber, 1995). IL-1β is produced solely by LC within the murine epidermis. Topical exposure of mice to chemical allergens stimulates increased production of IL-1β by LC (Enk and Katz, 1992a). Intradermal injection of this cytokine stimulates LC migration, at a slower rate than TNF-α injection (Cumberbatch *et al* 1997b). It is therefore postulated that IL-1β acts as the initial signal to induce TNF-α production and release from keratinocytes, and also delivers an autocrine signal for LC migration (Kimber *et al* 1998).

Although freshly prepared or 12h-cultured LC actively present antigen to primed T cells, they are weak or inactive at stimulating a primary allogeneic proliferation response, with a 12- to 16-fold lower stimulatory capacity compared with 72h LC. In contrast, 72h-cultured LC are extremely active stimulators of primary responses. Fresh LC do not initiate the formation of cell aggregates, in spite of their ability to present antigen in some assays. Cultured LC and splenic DC do induce clustering of lymphocytes. Freshly isolated LC lack the ability to cluster with T cells by an antigen-independent pathway, whereas mature LC can initiate clustering by an antigen-independent mechanism. LC cultured for 72h have functions that fully resemble those of lymphoid DC (Inaba *et al* 1986). The surface density of Ia molecules increases 4-fold during culture. This maturation can be seen in serum free cultures, indicating that factors responsible for maturation are not limited to, or present, in serum. Cell free medium from keratinocyte cultures do not alter

maturation, indicating that keratinocytes are not solely responsible for maturation either (Picut *et al* 1988).

1.3.2. Dermal dendritic cells

The classification of dermal DC comprise a diverse number of cell types that exhibit dendritic morphology, including monocytic/ macrophagic cells, fibroblasts, dermal APC and stromal cells. Due to the wide variety of cells forming this population, definitive cell markers are limited and often not exclusive. In humans, most dermal DC are factor XIII$^+$, a molecule involved in wound repair, CD1a$^+$, CD1b$^+$ and CD1c$^+$ (Bergstresser *et al* 1995) and sometimes MHC class II$^+$ (Braverman and Keh-Yen, 1992). A human LC-like dermal APC has been identified, expressing the LC-associated markers MHC class II, CD45 and CD1a, but also markers not expressed by LC (CD1b, CD11b, and factor XIII). This unique population comprises approximately 2.7% of total dermal cells.

In mice, similar dermal DC populations can be found, with 2.5% dermal cells being Ia$^+$. Ia$^+$ DC, present in the perivascular region of the mouse dermis, when hapten-derivatised, are capable of inducing CH in naive mice (Tse and Cooper, 1990). There also exists a population of Thy-1$^+$, Ia$^-$ cells, exhibiting a dendritic morphology, within the murine epidermis. It has been suggested that the relative frequency of these cells to LC influence the intensity of CH (Bigby *et al* 1987). Adoptive transfer of hapten-derivatised Thy-1$^+$ DC specifically downregulate contact sensitisation (Sullivan *et al* 1986).

1.3.3. Monocyte/macrophage cells

Monocytes leave the circulation by binding to the endothelial leukocyte adhesion molecule (ELAM)-1 (now known as E-selectin; CD62E). This is induced on the vascular endothelium in the proximity of a UV-irradiated site, or after injection with purified protein derivative (PPD) which elicits delayed type hypersensititvity (DTH)

(Norris *et al* 1991). ELAM-1 is first detectable at 6 hours, and maximally at 24 hours, in both cases. Infiltration by macrophages into the treated site has a similar time-course. By three days however, ELAM-1 is more strongly expressed in DTH sites than in UV-exposed skin. This difference may be due to local cytokine activity. Thus, following UV, ELAM-1 could act by recruiting antigen presenting macrophages to temporarily replace the LC which had migrated from the skin. Other adhesion molecules induced by intradermal injection of PPD to elicit DTH, such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, are unaltered in UV-exposed skin (Norris *et al* 1991).

Investigation of epidermal cell suspensions, derived from skin which had been subjected to erythemagenic UVB each day for 4 days, showed a doubling in numbers of $CD36^+$ macrophages compared with unirradiated controls (Hurks *et al* 1997). This increase correlated with recovery, and enhancement, of mixed epidermal cell lymphocyte reaction (MECLR) responses (Hurks *et al* 1997; Cooper *et al* 1985). MECLR is used as a measure of immunostimulatory activity; epidermal cells act as stimulators to induce proliferation of allogeneic peripheral blood responder cells. *In situ* immunohistochemistry and flow cytometric analyses of cell supensions revealed that UVR induced not only infiltration of $CD36^+$ monocyte/macrophage cells into the epidermis, but also expansion of the dermal macrophage subset, which is phenotypically identical (Meunier *et al* 1995).

1.3.4. $CD4^+$ T cells (and cytokine dichotomy)

The cytokine microenvironment in which an antigen is encountered determines the type of immune response initiated against it. $CD4^+$ T cells are commonly divided into Th1- and Th2-type subsets, as determined by the cytokine profiles they produce. Evidence for these profiles is strong in the mouse, where immune responses can be driven by repeated antigenic stimulation to levels at which clear and distinct cytokine profiles can be determined (Mosmann *et al* 1991; Mosmann and Sad, 1996). There is

evidence for Th1- and Th2-type cytokine profiles in humans, particularly in long-lived disease states such as malaria, but under experimental conditions polarisation is usually weaker due to fewer exposures to antigen (Romagnani, 1991; Romagnani, 1997). Evidence for subsets is also available from *in vitro* manipulations to produce T cell lines. Th1-like cytokines include IL-12 (although not produced by T cells themselves) and IFN-γ i.e. those promoting cell-mediated immune responses (Trinchieri, 1993). Th2-like profiles promote humoral immune responses, and include IL-4, IL-5 and IL-10. Cytokines of both subsets can interact with and inhibit the function and activation of cells from the opposite subset (see fig. 1.1), thereby promoting outgrowth of their own subset. For instance, IFN-γ can inhibit the proliferation of Th2 cells, and IL-10 can block cytokine synthesis by Th1 cells. IL-4 has dominant effects over those of IL-12 (Hsieh *et al* 1993).

CD4+ T helper cell subsets

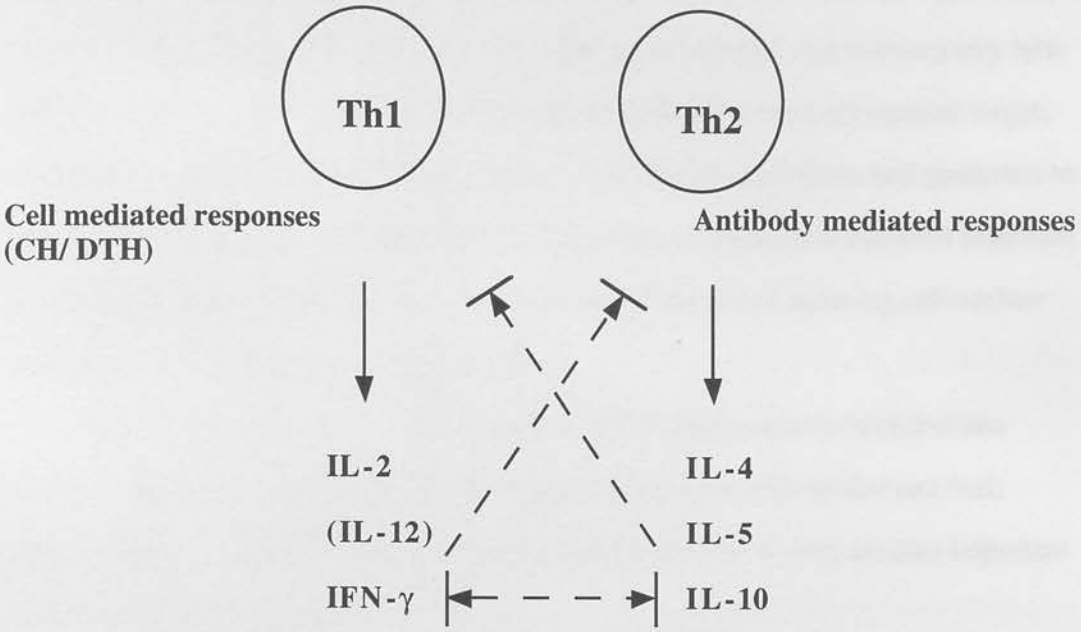


Figure 1.1. The subsets of $CD4^+$ T cells, the cytokines they produce and the cross-regulatory mechanisms they use.

1.3.5. $CD8^+$ T cells

$CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTL), are class I MHC-restricted cells. Upon activation $CD8^+$ T cells kill infected or abnormal cells by either of two mechanisms. These are a secretory lytic pathway or a nonsecretory lytic pathway (Berke, 1995). The first of these involves binding to an appropriate target, activation of a Ca^{2+}-dependent degranulation, with release of perforin and granymes to cause lysis of the target cell. The second, nonsecretory mechanism involves induction of apoptosis in the target, following cross-linking of apoptosis inducing cell-surface molecules on the target, such as Fas (CD95).

Recent investigations are showing that $CD8^+$ T cells can be subdivided into distinct subgroups on the basis of their cytokine production (Mosmann and Sad, 1996). Similar to $CD4^+$ T cells, cytokines secreted by $CD8^+$ T cells are also important in directing immune responses.

1.3.6. Contact hypersensitivity

Allergic contact dermatitis is a common ailment, characterised by an eczematous reaction at the site of contact with a specific sensitiser. CH responses are reactions to small, structurally simple haptens. There are nearly 3000 chemical agents which can result in allergic contact dermatitis and common sensitisers include nickel in jewellery, compounds in plants, and many industrially relevant compounds. The sensitisers alone are generally incapable of generating an immune response, but react with proteins in the skin to create immunologically relevant hapten-derivatised proteins. These can be presented by APC, primarily LC, inducing their migration to the lymph node(s) draining the site of encounter. The CH response only occurs in individuals who have been sensitised to the chemical allergen by previous exposure. Around 1-2% of the general population have some form of contact sensitivity.

CH is widely used to elicit and examine immune responses in the skin of experimental animals. As alluded to above, CH responses can be divided into two

phases. The first of these is the sensitisation (afferent) phase. During contact sensitisation, hapten-carrier complexes are taken up by LC in the epidermis, carried to the DLN and presented to specific T cells, as shown in fig. 1.2. In the lymph node, the hapten conjugate is presented on MHC molecules to antigen-specific T cells, inducing their proliferation and differentiation into an effector population. This T cell population then leaves the lymph node and circulates to the site of sensitisation via the blood. The T cells extravasate into the skin with the aid of adhesion molecules on the epithelium, and promote an inflammatory reaction.

Upon rechallenge with the specific contact sensitiser (elicitation), T cells can be activated *in situ*, so that the reaction occurs at a quicker rate (see fig. 1.3). Additionally, circulating T cells generated during the sensitisation phase can migrate into the site of contact and release cytokines, leading to infiltration of more cells into the area and a measurable inflammation. The majority of contact sensitisers elicit an immune response characterised by a Th1-like cytokine profile during both phases of the reaction (Kimber and Dearman, 1997). CD4 gene targeted mice ($CD4^-$) demonstrate hyporesponsiveness in CH (Fujisawa *et al* 1996).

Destruction of afferent lymphatics draining the exposed skin prevents contact sensitisation taking place. Congenitally athymic (nude) mice do not show a lymphoproliferative response to oxazolone (Ox) and contact sensitisation does not develop. The degree of DLNC proliferation to the sensitiser correlates with the level of sensitisation and the severity of elicitation. Indeed, the local lymph node assay is able to identify at least moderate and strong skin allergens (Kimber, 1992; Kimber *et al* 1990b). Antigenic competition in CH is associated with, and probably a consequence of, a depressed primary proliferative response to the second chemical (Kimber *et al* 1990a). Therefore, modulation of the primary response may directly affect the degree of sensitisation, and the magnitude of response upon challenge.

In the secondary, elicitation, phase, classical 24- to 48h CH skin reactions are preceded by an early swelling reaction that peaks 2h after challenge. The ability to

initiate this early event is T cell dependent (van Loveren *et al* 1983), and also relies on release of serotonin (Gershon *et al* 1975) and participation of mast cells (van Loveren *et al* 1983; Askenase *et al* 1983). Pharmacological agents that blocked the activity of serotonin not only inhibited the early phase of the CH response upon elicitation, but also the late stage. This only occurred when serotonin was blocked at the time of challenge but not later, indicating that serotonin is not involved in the late component (van Loveren and Askensase, 1984). The early CH response is easily compressed, indicating that it is an oedematous response. The late response is more robust, indicating that it is a cellular infiltrate (van Loveren *et al* 1984b).

Two functionally distinct, antigen specific T cells populations mediate the different components of CH elicitation. The T cell population responsible for the early reaction is induced within 24h of sensitisation. Another population mediates the late 24-48h swelling reaction, with the cells being induced 3-4 days after sensitisation, and requiring much more antigen for immunisation (van Loveren *et al* 1984a).

It is thought that antigen specific T cells activate mast cells to release serotonin. Serotonin then opens gaps between adjacent epithelial cells and allows extravasation of more T cells into the site, which in turn release chemokines and attract mononuclear cells. However, CH responses could be elicited in mast-cell deficient mice by some groups (Galli and Hammel, 1984; Thomas and Schrader, 1983), questioning the contribution of these cells in serotonin release. Local injection of serotonin alone could initiate CH. When serotonin was injected into the ears of mice that had been intravenously injected with only late-acting CH T cells (i.e. T cells involved in the late component of ear swelling), a 2h ear swelling could be measured (Ptak *et al* 1991). It was postulated that only a small number of mast cells may be required to initiate the response, as mast cells are still around 1% of normal numbers in mast-cell deficient mice. More recent data suggest that there may be redundancy in the source of serotonin, and platelets may also contribute (Geba *et al* 1996).

Complement may play a role in elicitation and development of the CH response. Complement-depleting cobra venom and antibodies against the C5 component inhibited CH responses. Locally increased macrophage chemotactic activity was lost from skin extracts of mice depleted of complement, as was the late elaboration of IFN-γ (Tsuji *et al* 1997). It is thought that C5 attracts macrophages into the site of elicitation, with subsequent attraction of circulating T cells, and IFN-γ production at the site producing a Th1-like cytokine microenvironment.

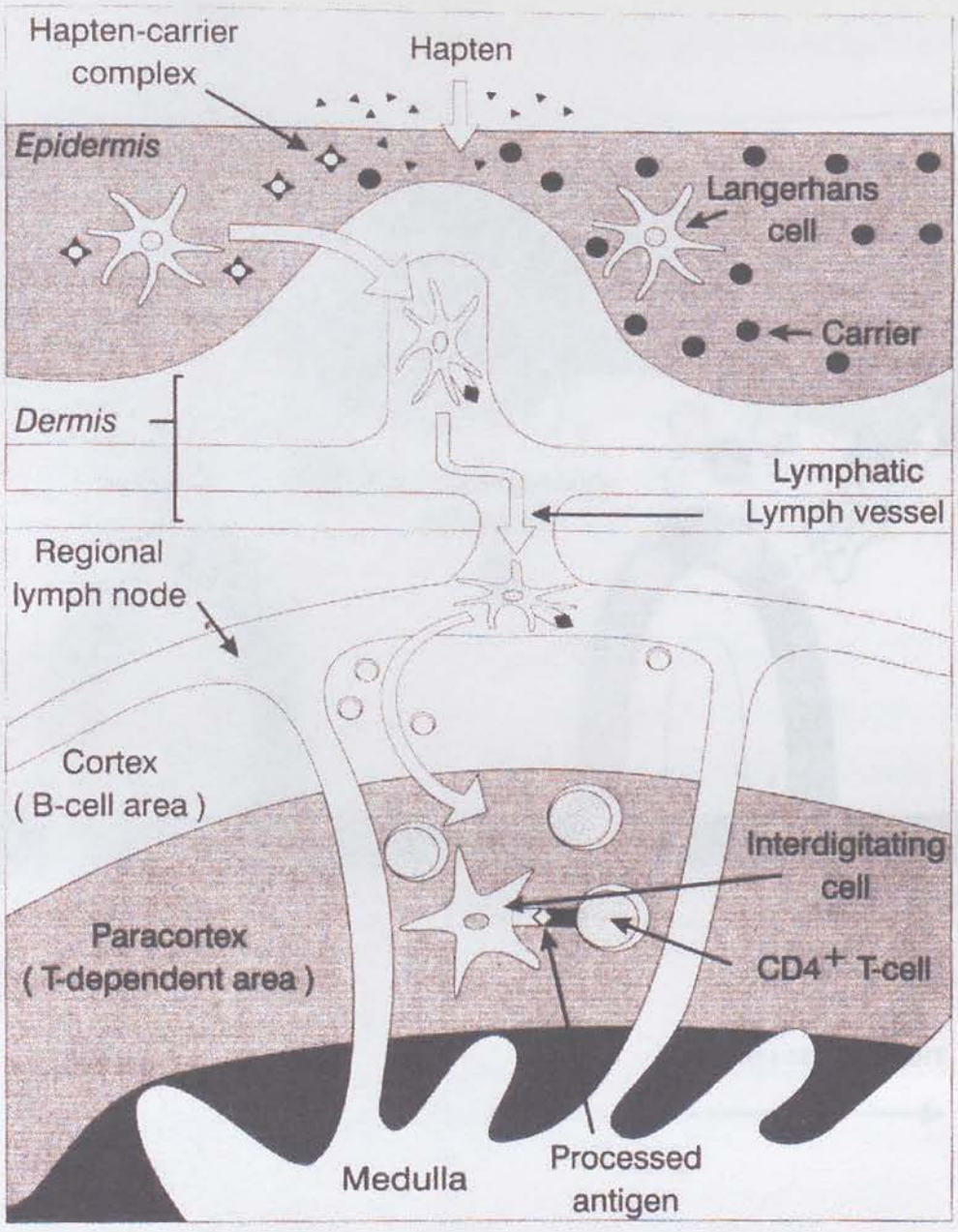


Figure 1.2. The sensitisation phase of contact hypersensitivity. The contact sensitiser forms a hapten-carrier complex in the epidermis. Langerhans cells internalise the antigen and migrate via the afferent lymphatics to the paracortical region of the draining lymph node, where, as dendritic cells, they present antigen to $CD4^+$ T cells (taken from National Radiological Protection Board, NRPB-R297).

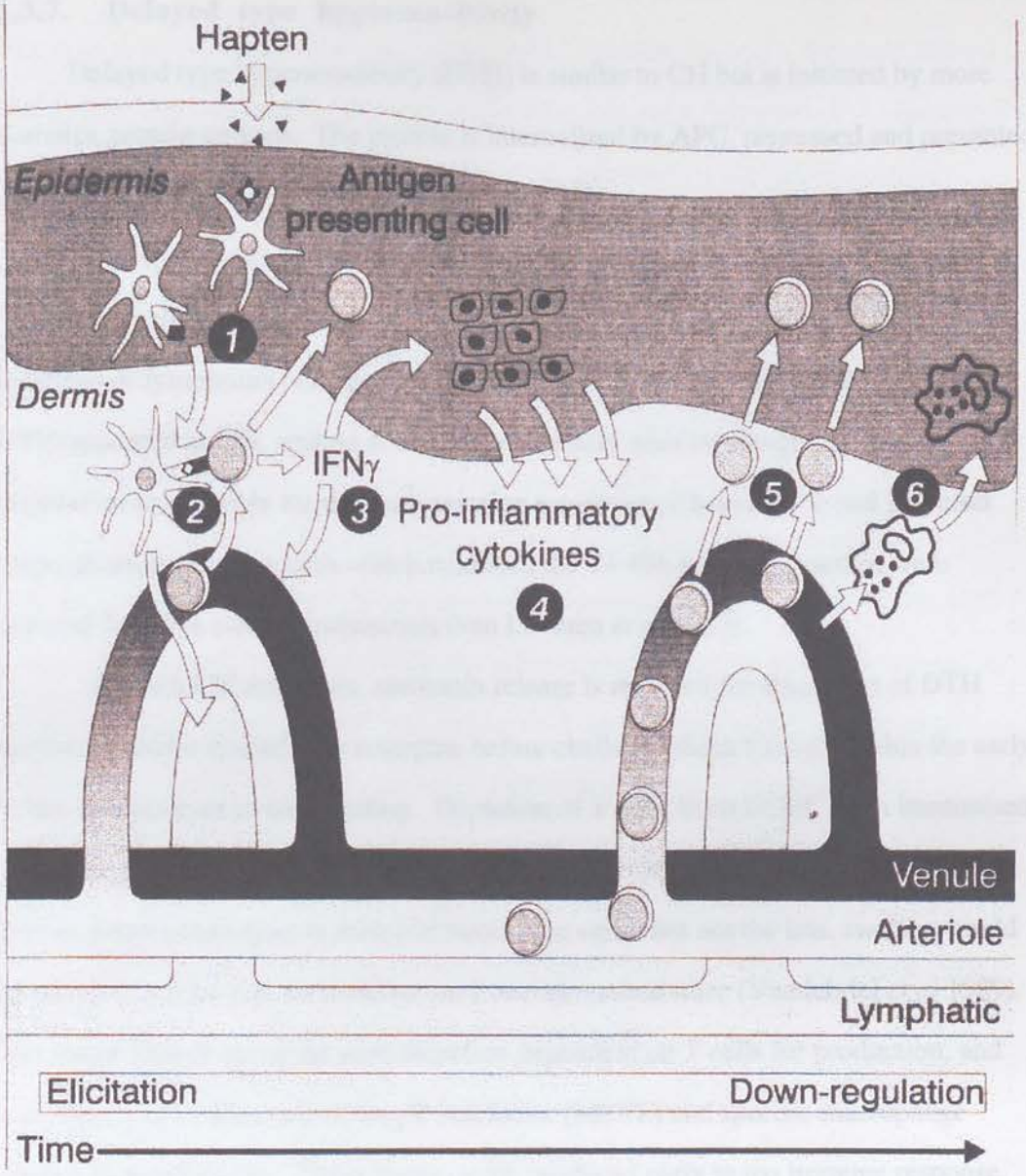


Figure 1.3. Elicitation phase of contact hypersensitivity. Antigen presenting cells (Langerhans cells) carrying the hapten-carrier complex (1) migrate from the epidermis into the dermis, where they present antigenic peptides to $CD4^+$ memory T cells (2). Activated $CD4^+$ T cells release pro-inflammatory cytokines (eg. IFN-γ) (3), which activate keratinocytes to release other pro-infammatory cytokines (4). Non-antigen specific $CD4^+$ T cells are attracted to the site (5), along with activated macrophages (6). The reaction is downregulated thereafter (taken from National Radiological Protection Board, NRPB-R297).

1.3.7. Delayed type hypersensitivity

Delayed type hypersensitivity (DTH) is similar to CH but is initiated by more complex protein antigen. The protein is internalised by APC, processed and presented on MHC molecules to specific T cells in the DLN.

In experiments by van Loveren, classical 24- to 48h DTH responses to lymphoma cells were preceded by an early swelling reaction that peaked 2h after injection of lymphoma cells into mouse footpads. As in CH, the late response of DTH appeared at 18h, peaked at 24h, then declined, after challenge. The T cell population responsible for the early reaction was induced between 12 and 24h after immunisation, whereas cells which mediated the 24-48h swelling reaction were induced 3-4 days after immunisation (van Loveren *et al* 1983).

As with CH responses, serotonin release is required for elicitation of DTH responses. Mice treated with reserpine before challenge did not exhibit either the early or late components of ear swelling. Depletion of T cells from DLNC from immunised donor animals, to be used in adoptive transfer experiments, removed the potential to initiate either component in recipient mice. The early, but not the late, swelling could be induced in mice that received serum from immunised mice (Vandebriel *et al* 1989). The serum factors involved were therefore dependent on T cells for production, and were found to be mast cell arming T cell factor (MCTF) and specific macrophage arming factor (SMAF). These factors were produced early in the immune response, becoming detectable in serum 2-3 days after immunisation (Vandebriel *et al* 1989). MCTF and SMAF 'arm' their respective cell types to recognise specific antigen. C5 dependent mechanisms also operate in sites of DTH elicitation (Tsuji *et al* 1997).

1.3.8. General immunological tolerance

Tolerance is generally associated with abnormal antigen presentation, failing to activate APC to release cytokines (Weigle and Romball, 1997). "Split tolerance" can develop, where, by preferential Th subset activation, the 'incorrect' arm of the immune

response is activated due to alterations in the cytokine milieu. The use of adjuvants can also lead to preferential subset activation, with Complete Freund's Adjuvant (CFA) inducing a Th1-like profile, whereas Incomplete Freund's Adjuvant (IFA) induces Th2 cells (Mauri *et al* 1996; Forsthuber *et al* 1996). Similarly, a Th2 profile can be achieved by targeting of autoantigens to B cells (Weigle and Romball, 1997). The ability of lipopolysaccharide (LPS), TNF-α and IL-1 to interfere with tolerance suggests a role for pro-inflammatory (Th1-like) cytokines.

Both B cells and DC have been strongly implicated in tolerance induction *in vivo*. Mice deficient in B cells can still become tolerant in both Th1 and Th2 compartments to the peptide and superantigen, human IgG. In the human IgG tolerance model, tolerance induction may be correlated to the manner in which APC prepare the antigen. Aggregated human IgG is aggressively degraded, processed to peptides, and activates the APC to produce IL-1 and other cytokines. Monomeric human IgG remains basically intact, and fails to induce cytokine production.

Alongside cytokines, costimulatory molecules appear to play a key role in tolerance. Blocking of B7-CD28 interaction has been suggested as a tolerance inducing mechanism. However, *in vitro* upregulation of B7 expression on B cells does not convert a tolerogenic to an immunogenic signal.

Investigation of suppressor T cell-cell interactions by Lowy and colleagues revealed I-J-encoded determinants to be restrictive elements (Lowy *et al* 1983). Depletion of I-J$^+$ cells with specific monoclonal antibody and complement before immunisation with azobenzenearsonate-coupled APC removed the APC required for Ts but not CTL induction. Granstein and collegues found tolerance inducing cells in the high density, hapten-coupled epidermal cell (HD-EC) fraction (Granstein *et al* 1987), with the property confined to the adherent population. After UVB, electron microscopy of this HD-EC population revealed two cell types - keratinocytes and macrophage-like cells. Pretreatment of recipient mice with cyclophosphamide abolished the ability to induce Ts formation upon HD-EC transfer. Further

experiments indicated that the epidermal cells that activate suppressor T cells were high-density, Ia^+, I-J restricted, glass adherent and UVR resistant, as well as cyclophosphamide sensitive (Granstein *et al* 1987).

1.4. UV and chromophores in the skin

UVR has been shown to have immunosuppressive effects, with the greatest effect within the UVB spectrum (280-315 nm). *Ex vivo* experiments indicated that these wavelengths penetrate only as far as the epidermis (see fig 1.4), where it is thought that initiation of immunosuppressive effects occurs. More recent data from irradiation of human skin with monochromatic light *in vivo* indicates that wavelengths as short as 300 nm can penetrate as far as dermal cells (Young *et al* 1998). Within the epidermis, chromophores for UV-radiation include *trans*-urocanic acid (UCA) (Norval *et al* 1989b; Noonan and De Fabo, 1992), found within the *stratum corneum*, and DNA (Applegate *et al* 1989). Upon exposure to UVB, one and/or other of these chromophores appears to initiate a cytokine cascade, leading to suppression of T cell mediated immune responses, such as DTH and CH.

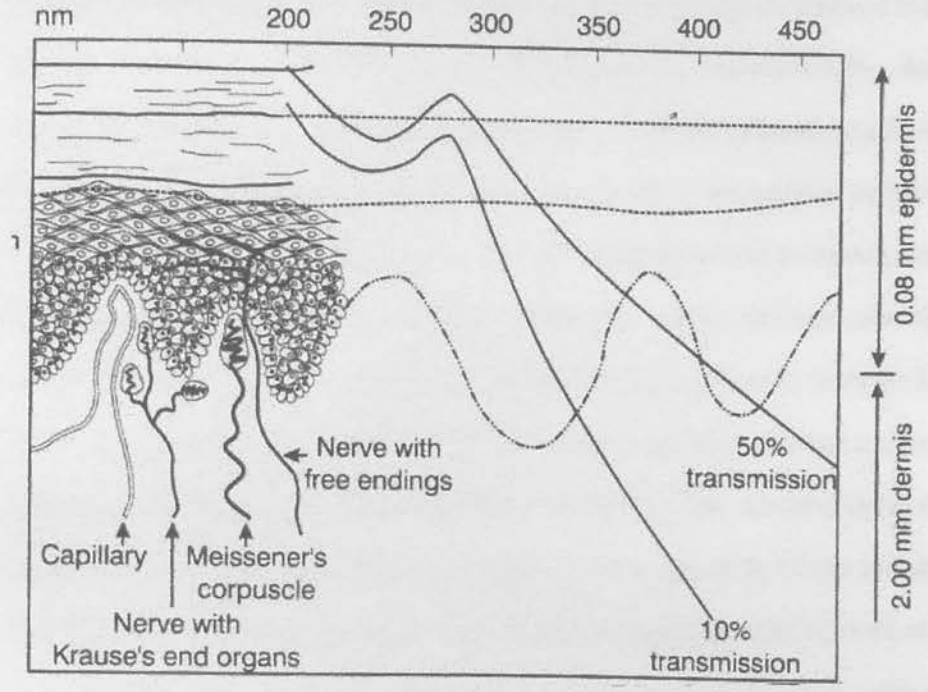


Figure 1.4. Penetration of human skin by UVR. Shorter wavelengths are absorbed in the epidermis, whilst longer wavelengths (UVA) can penetrate as far as the dermis (taken from National Radiological Protection Board, NRPB-R297).

1.4.1. Urocanic acid

One of the major chromophores for UVR within the epidermis is UCA. UCA is formed by the action of the enzyme histidine ammonia-lyase during keratinisation. It is found naturally as the *trans*-isomer within the *stratum corneum* and is a major absorber of UV in the skin. Upon UVR, *trans*-UCA isomerises to *cis*-UCA in a dose-dependent fashion until a photostationary state is reached when approximately equal quantities of the two isomers are present. It then takes around two weeks for *cis*-UCA to return to a baseline level. Broad-band UVB is more potent in formation of *cis*-UCA in mouse skin than narrow-band UVB (Gibbs *et al* 1993). In Caucasian skin the photoisomerisation of UCA occurs between 305-341 nm (i.e. in both the UVB and UVA wavebands) (Kammeyer *et al* 1995; Gibbs *et al* 1997) and has a maximum efficiency at 290-310 nm *in vitro* (Gibbs *et al* 1997). This red shift between isomerisation *in vitro* and that *in vivo* may be due to the 10 to 12 nm red shift in the absorption of UCA in association with stratum corneum proteins (Jones *et al* 1996).

Untreated murine flank skin contains around 5 $\mu g/cm^2$ UCA, with 4% in the *cis* isoform (0.2 $\mu g/cm^2$) (Norval *et al* 1988). A 500 kJ/m^2 dose of UVA1 led to UCA isomerisation, with 17% in the *cis* isoform in C3H/HeN mice (El-Ghorr and Norval, 1999). UVA lamps have also been shown to cause isomerisation of UCA in BALB/c mouse skin (Webber *et al* 1997). Significantly elevated levels of *cis*-UCA were found after as little as 100 J/m^2 UVB (broadband, TL-12 bulbs), with over 40% formation after a 1000 J/m^2 exposure. Narrow-band UVB (from a TL-01 bulb) was 5-10 fold less efficient compared to the broad-band UVB source, with a dose of 10 kJ/m^2 TL-01 needed to reach 40% *cis*-UCA formation (El-Ghorr and Norval, 1999).

The rate of *cis*-UCA formation can be reduced by topical application of sunscreens. Formation rate is reduced with increasing sun protection factor (SPF), and only broad-spectrum, highly protective sunscreens offer protection after multiple exposures (Krien and Moyal, 1994). There is a three-fold greater amount of *cis*-UCA in the sunburned *stratum corneum* than in normal skin. *Cis*-UCA can be formed in

human skin with common exposures (1 and 0.5 minimum erythemal dose (MED)) over a broad spectrum range of UVB and UVA, up to at least 363 nm (Kammeyer *et al* 1995). The photoisomerisation rate decreased as wavelength increased, with the dosage of UVA1 needed to reach the photostationary point much higher than that with UVA2 (Mohammad *et al* 1999). A theoretical approach to estimate the distribution of *cis*-UCA after irradiation indicated that *cis*-UCA may diffuse into the deeper layers of the epidermis at a rate of $10^{-17}m^2/s$ (Krien and Moyal, 1994). The percentage of *cis*-UCA in suction blister fluid 24 hours after 3 MED of either UVB or UVA1 (340-400 nm) is increased over that found in fluid from unirradiated skin (Skov *et al* 1998a). Analysis revealed that *cis*-UCA was maintained for several weeks following irradiation (Gilmour *et al* 1993b), in the dermis or near the dermo-epidermal junction (Norval *et al* 1995). UCA was transiently detectable in the serum of individuals given a single suberythemal dose of UVB, reaching a maximum at 24h after exposure. UCA has been detected in urine 5-7h after 1-1.5 MED total body exposure (Norval *et al* 1995).

There is a large inter-individual variation in UCA concentrations but little difference between body sites within one person (Kavanagh *et al* 1995; de Fine Olivarius *et al* 1997b). No correlation between the total UCA concentration and skin type, degree of pigmentation, MED or *stratum corneum* thickness was found (Kavanagh *et al* 1995; de Fine Olivarius *et al* 1997b). A study of seasonal variation in UCA isomers in human skin has revealed that the total UCA content was lower in the summer months in all 6 body sites tested, of which some were normally exposed and others not exposed to sun (de Fine Olivarius *et al* 1997a). The percentage of *cis*-UCA was close to the maximum obtainable during the summer months, except in the unexposed buttocks, and it decreased to below 7% in the winter months in all body sites apart from the forehead. A recent study of patients who had been treated for basal cell carcinoma and malignant melanoma concluded that there was no difference in the absolute amount of UCA isomers in the skin of the two cancer groups and normal

controls (de Fine Olivarius *et al* 1998). Irradiating these subjects with a single test UV dose resulted in a relatively higher production of *cis*-UCA in both cancer groups compared with the controls. The significance of this change, if any, is not known at present.

UCA was regarded initially as a potential natural sunscreen, but tests to evaluate its SPF have shown that this is not the case. Addition of *trans*-UCA to human skin in amounts around 200 times greater that those occurring naturally afforded a SPF of only 1.58 (de Fine Olivarius *et al* 1996). Rodent experiments have suggested that *cis*-UCA can initiate at least some of the events leading to immunosuppression (reviewed in Norval *et al* 1989b; Norval *et al* 1995), and the pioneering work of De Fabo and Noonan showed that CH suppression has a similar action spectrum to the absorption spectrum of UCA (De Fabo and Noonan, 1983; De Fabo *et al* 1983). Evidence to corroborate this view in human subjects is not easy. *Cis*-UCA has been shown to suppress human natural killer (NK) cell activity in a dose-dependent manner *in vitro*, while *trans*-UCA has little effect (Gilmour *et al* 1993a). However no correlation was found between the percentage of *cis*-UCA in the skin and the suppression in NK cell activity induced by narrowband UVB therapy of individuals with psoriasis (Guckian *et al* 1995). If human epidermal cells were treated with *cis*-UCA *in vitro* before testing their activity as stimulator cells in a MECLR, a 20% suppression resulted (Hurks *et al* 1997a). The downregulation was increased to 27% by incubation of the epidermal cells with *cis*-UCA for 3-6 days before testing. *Trans*-UCA had no such effect. In addition *cis*-UCA did not alter a mixed lymphocyte reaction (MLR) if present throughout the culture period (Hurks *et al* 1997a). Thus, a partial downregulation in human epidermal antigen presenting activity could be attributed to *cis*-UCA. A further potential *in vivo* mechanism of action of *cis*-UCA includes the stimulation of prostaglandin E_2. This has been reported *in vitro* from human peripheral blood monocytes (Hart *et al* 1993) and keratinocytes (Jaksic *et al* 1995).

Chronic exposure of mice with either broad-band and narrow-band UVB sources resulted in *trans*- to *cis*-UCA conversion. The percentage of *cis*-UCA began to return to normal levels after 4 weeks of chronic narrow-band UVB (3000 J/m^2, 3 times per week), despite continued exposure (El-Ghorr *et al* 1995). CH was suppressed only in mice that had received broad-band UVB, thereby questioning the correlation between *cis*-UCA and CH outcome, after continued irradiation (El-Ghorr *et al* 1995). In contrast, systemic suppression of CH could not be seen in mice genetically deficient in histidase, and therefore skin UCA (<10% normal), upon a single UVB exposure, whereas suppression in wild-type mice was dose-responsive and comparable to that seen previously in BALB/c mice (De Fabo *et al* 1983). Histidase deficient mice exhibited a normal CH response to TNCB.

Incubation of epidermal cells in *cis*-, but not *trans*-UCA, completely inhibited antigen presentation in a spindle-cell tumour antigen system. Neither histamine antagonists nor indomethacin, which inhibit prostaglandins, reversed *cis*-UCA effects. An effect could also be seen on secondary immune responses. Pre-incubation with *cis*-UCA, but not *trans*-UCA, prior to injecting antigen pulsed epidermal cells into the footpads of immune mice, inhibited the DTH response upon challenge (Beissert *et al* 1997). These data indicate that *cis*-UCA may be an important regulator of LC antigen presenting ability in tumour immune responses, and therefore in photocarcinogenesis.

Intraperitoneal injection of *cis*-UCA for 7 consecutive days, starting on the day of transplantation, was able to prolong the survival of allogeneic MHC disparate skin grafts in mice (Gruner *et al* 1992). C3H mice were transplanted with C57Bl/6 skin and *cis*-UCA prevented rejection in a dose-dependent manner. *Trans*-UCA had no protective effect. Furthermore, *cis*-UCA injection delayed the onset of acute graft-versus-host disease and prevented the lethal disease in 30% of animals (Gruner *et al* 1992). *Trans*-UCA did not significantly delay onset of graft-versus-host disease. However, if C3H mice that had previously rejected allografts were given skin from the same donor strain 4 weeks later, and the recipients were treated with *cis*-UCA as

before, grafts were rejected. This indicates that tolerance was not induced by *cis*-UCA in this system

Splenic DC were obtained from mice injected intravenously with 50-200μg *cis*- or *trans*-UCA, and pulsed with DNP_6 Ova. It was found that splenic dendritic cells (DC) from *cis*-UCA injected mice had significantly impaired APC function (assessed as the proliferative response of T cells derived from mice immune to DNP_6 Ova), whereas those from *trans*-UCA injected mice had normal APC ability. This was despite the DC having the same Ia expression (Noonan *et al* 1988). Mixtures of DC from *cis*-UCA treated mice with normal DC did not impair APC function, and direct addition of *cis*-UCA to *in vitro* proliferation assays was without effect. Addition of indomethacin, to inhibit prostaglandin synthesis, did not have any effect (Noonan *et al* 1988). This may indicate that essential mediators of immune suppression are not available once cells are removed from the mice.

Addition of a methyl group to position 2 of the imidazole ring of UCA abrogates the immunosuppressive effects of *cis*-UCA. The side arm may be altered significantly (by reduction, or even replacement) with no loss of activity (Norval *et al* 1989a). Thus, the 5-membered ring is necessary for immunosuppressive activity, perhaps by fitting into a receptor site.

1.4.2. DNA damage

As is the case with *cis*-UCA, rodent models suggest that DNA damage is a critical event in UV-induced immunosuppression (reviewed in Vink *et al* 1996b). Cells of the South American opossum, *Monodelphis domesticus*, contain an enzyme that confers the ability to repair UVR-induced pyridimine dimers in DNA by a process known as photoreactivation. In photoreactivation, cyclobutane dimers between adjacent pyridimines on the same DNA strand are repaired *in situ* by splitting of the dimers. This process is mediated by an enzyme activated by visible (photoreactivating) light in marsupial cells. Exposure of opossums to UVB before contact sensitisation

suppressed CH responses. Extraction of DNA from UV-irradiated skin samples indicated that there was significant induction of pyridimine dimers by immunosuppressive doses of UVB (Applegate *et al* 1989). If UVB was followed by exposure to photoreactivating light, CH responses were restored to levels comparable to those of unirradiated opossums. Analysis of skin samples from opossums which were exposed to photoreactivating light showed that 86% of the pyridimine dimers had been removed.

Vink and colleagues identified cells with cyclobutyl pyridimine dimers within the epidermis, the dermis, and DLN of irradiated sites in mouse skin (Vink *et al* 1996a). When mice were sensitised through irradiated sites with the contact sensitiser FITC, Ia^+ cells bearing FITC and containing dimers could be detected in DLN. Such cells from mice sensitised 3d after UVB exposure exhibited reduced APC function *in vivo*. This was demonstrated by injecting DLNC into the footpads of syngeneic mice, then challenging these recipients with FITC 5d later. DLNC from unirradiated, FITC-sensitised mice were capable of inducing CH in recipients upon challenge. Mice immunised with DLNC from mice which had been sensitised 3d after irradiation had significantly reduced CH responses. Liposomes incorporating T4 endonuclease V (T4N5) accelerate the repair of pyridimine dimers. APC function was completely restored in mice which received T4N5 immediately after irradiation, but only if the liposomes were applied to the exposed site (Vink *et al* 1996a). Further experiments by this group examined the effect of repair of cyclobutyl pyridimine dimers within DLNC from irradiated, contact sensitised mice *in vitro*. When DC enriched from the DLNC population of irradiated mice were injected into syngeneic recipient mice, the CH response was suppressed compared with that elicited in mice which received DC from unirradiated mice. DNA damage was repaired in DC from irradiated mice *in vitro*, by incubating cells with liposomes containing a photolyase, which underwent photoreactivation in visible light. Injection of DC incubated with photolyase restored

CH responses to levels comparable to those achieved by transfer of DC from unirradiated mice (Vink *et al* 1997).

Kremer and colleagues exposed normal human skin to UVB doses *ex vivo*. UVB induced DNA damage could be demonstrated, and damage increased with increasing UVB dose. In contrast to studies in mice, DNA damage within the LC did not lead to a functional impairment to stimulate T cells, as irradiation did not decrease the capacity of LC to induce T cell proliferation on a per cell basis (Kremer *et al* 1997).

Kripke and colleagues demonstrated that injecting supernatants from irradiated PAM-212 cells into mice suppressed the DTH response to alloantigen. Incubation of PAM-212 cells with T4N5 liposomes following UVB irradiation removed the suppressive activity of supernatants (Nishigori *et al* 1996a). Application of T4N5 liposomes to mouse skin immediately after UVB irradiation decreased the number of cyclobutane pyridimine dimers in the epidermis. Additionally, suppression of CH responses by UVB exposure was abrogated in T4N5 treated mice (Kripke *et al* 1992). However, the suppression of CH responses by UVB was not completely reversed by T4N5 liposomes. This group proposed that DNA damage would induce the release of immunoregulatory cytokines (Kripke *et al* 1992). They showed that supernatants from irradiated PAM-212 cells can suppress DTH responses when injected intravenously into mice. Immunoperoxidase staining of irradiated PAM-212 cells revealed induction of IL-10 following irradiation. Incubation of PAM-212 cells with T4N5 reduced the induction of IL-10, thereby supporting their earlier hypothesis. Application of T4N5 liposomes to murine skin *in vivo* reduced the induction of IL-10 within the epidermis and serum following UVB exposure (Nishigori *et al* 1996b). Interestingly, IFN-γ production by T cells stimulated by DC from irradiated mice was impaired. However, if DC from irradiated mice were incubated with photolyase *in vitro* before stimulating T cells, IFN-γ production was restored (Vink *et al* 1997).

Encapsulating the restriction endonuclease *Hind*III in liposomes allows induction of DNA strand breaks *in vivo* and *in vitro*. Topical application of such liposomes to

murine skin impaired the induction of CH responses initiated at either local or distant sites. *Hind*III induced production of TNF-α in the epidermis of mice, and TNF-α and IL-10 production by keratinocytes *in vitro* (O'Connor *et al* 1996). *Hind*III induced DNA strand breaks therefore mimic the effect of UVB irradiation. APC function was impaired in mice treated with *Hind*III liposomes. Interestingly, *Hind*III liposomes suppressed the DTH response to *Candida albicans*, and this was abrogated by antibodies against IL-10 (Nishigori *et al* 1996a).

Evidence to support the role of DNA damage in initiation of immunosuppressive mechanisms is limited in human subjects. Hurks *et al* (1995) showed that the *in vitro* action spectrum for the suppression in the MECLR is associated with UV-induced DNA damage. Recently this study was extended to investigate the action spectra at 4 wavelengths for the induction of thymine dimers, which represent the largest subclass of cyclobutyl pyrimidine dimers, and the suppression in the MECLR following *in situ* irradiation of small pieces of human skin (Hurks *et al* 1997b). The 2 action spectra had close similarities with a high sensitivity at 254, 297 and 302 nm, then a steep decline to 312 nm. However with such large gaps in the wavelengths studied and no information regarding UVA wavelengths, more information is required.

Young and colleagues investigated the depths to which monochromatic light could penetrate and induce DNA damage within human skin. Human volunteers were irradiated *in vivo*, and thymine dimers identified in punch biopsies with a monoclonal antibody. DNA dimer formation was independent of epidermal layer studied, when light ranging from 300 to 360 nm was utilised. There was poor transmission to the basal epidermal layer of wavelengths between 280 and 300 nm. Dimers were detectable within dermal nuclei when wavelengths as short as 300 nm were used (Young *et al* 1998). This contrasts with findings from *ex vivo* studies, and indicates that there is significant transmission of UVB wavebands through the skin.

Repair of pyridimine dimers does not fully restore CH responses in mice (Kripke *et al* 1992), indicating that although DNA damage appears to be important, it is not

wholly responsible for UV-induced immunosuppression. This is supported by the fact that humans have the capacity to rapidly repair cyclobutane pyridimine dimers by a photoreactive enzyme (Sutherland *et al* 1980), but can still be immunosuppressed upon UV irradiation.

1.4.3. Other chromophores

UV can activate the transcriptional factors AP-1 and NF-κB. NF-κB is located in the cytoplasm, in association with the inhibitory molecule, IκB. Upon activation IκB is split from NF-κB, and NF-κB can migrate into the nucleus and initiate gene transcription. It is hard to envisage that UVB can by-pass cytoplasmic NF-κB and only activate it secondary to DNA damage (reviewed in (Schwarz, 1998). Studies by Devary and colleagues involved UVC irradiation of enucleated cells. It was demonstrated that NF-κB could be activated in these cells upon irradiation, indicating that a nuclear signal is not a requirement for NF-κB activation following UV exposure (Devary *et al* 1993; Simon *et al* 1994). UV can activate genes by intracellular signalling pathways, which are triggered by effects on the cell membrane.

Application of α-tocopherol, the most biologically active form of the antioxidant vitamin E, to mouse skin before irradiation with solar-simulated UVR completely reversed UV-induced suppression of CH and migration of LC (Yuen and Halliday, 1997). It was postulated that scavenging of UVR-induced lipid peroxidases and reactive oxygen may have inhibited loss of cell membrane integrity.

When sensory nerves were depleted from mice by injecting with the neurotoxin capsaicin before 4 weeks of age, UV irradiation of the mice at a later time did not lead to suppression of CH responses. When mice were injected with calcitonin gene-related peptide antagonist, CH responses were similarly restored in UV-irradiated mice (Garssen *et al* 1998). This implies that sensory neuropeptides are involved in UV-mediated immune suppression.

PUVA induces monofunctional adducts and cross-links in DNA, as opposed to the cyclobutyl pyridimine dimers induced by UVB irradiation. The cytotoxic action of UVA is dependent upon reactive oxygen species. In human skin, possible endogenous chromophores for generating reactive oxygen species may come from the circulation. Flavins are thought to play a role in photo-induced skin injury, and UVA irradiation of riboflavin produces singlet oxygen, superoxide anions, and triplet-state riboflavin radicals (Sato *et al* 1995). The latter of these mediators are immediately quenched in human skin. If H_2O_2 is produced, damage can occur at sites other than the generation site, due to the long-lived nature of the molecule.

UVB irradiation of PAM-212 cells inhibits induction of interferon regulatory factor-1 (IRF-1) mRNA expression in response to stimulation by IFN-γ (Aragane *et al* 1997). It is also recognised that UV induces clustering and internalisation of the cell surface receptors for TNF-α and IL-1 (Rosette and Karin, 1996). UVB appears to trigger the CD95 (Fas) pathway, by directly activating the CD95 receptor (Aragane *et al* 1998) and thereby resulting in apoptosis of the target cell.

1.5. Effects of UVB on APC

It is apparent that disruption of LC could be a fundamental component of UV-mediated immunosuppression, either by incorrect, inappropriate or inefficient antigen presentation. Alternatively, depletion or apoptosis of LC could lead to antigen presentation by another cell type. Two important signals involved in initiating migration of LC from the epidermis are IL-1β and TNF-α (Cumberbatch *et al* 1997a; Kimber *et al* 1998), and the role of these cytokines in UV irradiated sites is discussed later, in sections 1.9.1 and 1.9.7 respectively.

Several studies have examined LC numbers and morphology within human skin following UV exposure (Yoshikawa *et al* 1990; Skov *et al* 1998b). Photochemotherapy is the combined use of chemicals, such as psoralen, and UV in

treatment of skin ailments. The presence of psoralen in the skin, whether it is applied locally or given systemically, enhances the pigmentary response of the skin to UVR. UVA exposure in combination with psoralen application (PUVA) for skin conditions such as psoriasis altered LC morphology after a single exposure, with loss of fine dendritic processes (Friedmann, 1981). Repeated exposures led to a progressive reduction in numbers, to 10% after 7 treatments. By this time, the remaining cells were elongated with coarse dendrites. LC were detectable in, or under, the basal layer, suggesting migration out of the epidermis, or the preferential survival of cells positioned deeper in the skin. An Australian study showed that UV acts differently on LC of people of different racial origins (Hollis and Scheibner, 1988). Whereas LC were depleted by apoptosis in the darker skin of Aboriginal or Asian Australians, cells in those of Celtic descent died due to membrane disruption and organelle damage (Hollis and Scheibner, 1988). Absolute numbers were less drastically reduced in blacks, and returned to normal levels more quickly.

The effect of low-dose, long-term UVB (300-500 J/m^2 initially, three times weekly for four weeks) on the function of LC was investigated in skin blisters/biopsies from healthy volunteers, and showed a marked suppression of the MECLR (Van Praag *et al* 1994). The reduction in MECLR was not paralleled by changes in LC numbers or in HLA class II expression. Furthermore, the LC appeared ultrastructurally normal (Van Praag *et al* 1994). It is unlikely that low-dose UVB alters these cells at a phenotypic or structural level, but could alter membrane motility (Kobata *et al* 1993), thereby decreasing the efficiency of antigen presentation, or of antigen internalisation and processing.

Further *in vitro* investigations of human LC function showed a UVB dose-dependent decrease of T cell proliferation in the MECLR (Rattis *et al* 1995). Responses by both $CD4^+$ and $CD8^+$ T cells were inhibited by UVB-treated LC. Supernatants from irradiated cells did not inhibit the proliferation induced by unirradiated control LC, suggesting that immunosuppression is not mediated by

soluble factors, as indicated by murine models (Rivas and Ullrich, 1992; Rivas and Ullrich, 1994). Addition of IL-1α, IL-1β or IL-2 did not reverse the defective LC function (Rattis *et al* 1995). Phenotypic analysis indicated that UVB did not alter levels of expression or the percentage of cells displaying HLA-DR. However, cultured LC were less sensitive to UVB-exposure than fresh cells. Cultured LC express higher levels of the costimulatory molecules, ICAM-1 (CD54), B7 and leukocyte function-associated antigen (LFA)-3. UVB may therefore exert an immunosuppressive effect by preventing accessory function development, a phenomenon seen with blood DC (Young *et al* 1993). Again, this could be related to cytoskeletal damage (Zamansky and Chou, 1987), and/or decreased mobility of surface antigens (Kobata *et al* 1993).

Epidermal cells obtained from suction blisters of human skin after exposure *in vivo* to varying doses of UV exhibited altered capacities to stimulate T cells. Epidermal cells were pulsed with purified antigen, either immediately or 24h following UV-treatment, and their ability to induce T cell proliferation assessed. A dose-dependent reduction of activating ability was found (Austad and Braathan, 1985), and could be mimicked by pretreatment of epidermal cells to deplete HLA-DR^+ cells. As LC are the only DR^+ cells resident in normal human epidermis, it is likely that changes in these cells account for these observations. Cell death was limited to DR^- keratinocytes, and was not related to the responses obtained (Austad and Braathan, 1985).

UV also causes changes in intercellular signalling. *In vitro* stimulation by epidermal cells obtained from both UV-exposed and normal control skin induces a greater than 1000-fold increase in mRNA for the T cell growth factor IL-2, in allogeneic $CD4^+$ T cells. However, the mRNA for the IL-2 receptor α-chain (IL-2Rα, CD25) was upregulated only upon stimulation by normal, unirradiated epidermal cells (Stevens *et al* 1995). At the protein level, CD25 expression on T cells was increased within 48h of incubation with normal epidermal cells, but was not altered by

incubation with irradiated epidermal cells. Treatment with antibodies to transforming growth factor (TGF)-β restored the ability of the UV-exposed epidermal cells to upregulate CD25. Thus, the altered immune response after UV-exposure seems to be due in part to TGF-β released by epidermal cells post-UV, initiating an altered early $CD4^+$ T cell gene expression, which is characterised by the deficient IL-2Rα expression (Stevens *et al* 1995). Furthermore, T cells stimulated by UV-exposed epidermal cells have altered growth factor utilisation and cytokine production patterns (Stevens *et al* 1998). T cells cultured with control epidermal cells utilise IL-2, IL-7 and IL-15 for growth, and produce predominantly IFN-γ. Those stimulated with UV-exposed epidermal cells use IL-4 for growth, and more cells produce IL-4 and/or IL-5 compared to controls.

Noonan and collegues showed that short UV wavelengths led to a loss of ATPase staining dendrites and a decrease in total epidermal LC 24h later, in BALB/c mice, in a dose-dependent fashion (Noonan *et al* 1984). Doses of 270 or 290 nm UV used to deplete LC were insufficient to suppress the CH response, although higher doses could. Conversely, a dose of 320 nm UV that caused 50% suppression of CH did not deplete LC, nor did it affect LC morphology (Noonan *et al* 1984). Chronic UVA exposure (10 kJ/m^2, 3 times per week for 4 weeks) reduced $ATPase^+$ and Ia^+ cells in murine epidermis (Aubin *et al* 1991).

When DLNC excised from a contact sensitised mouse were injected into a syngeneic recipient, the recipient mouse was able to elicit a full CH response upon its first exposure to the contact sensitiser. However, if DLNC were tranferred from a donor mouse exposed to UVB 4 days before sensitisation, a specific tolerance was generated in the recipient (Tang *et al* 1992). This observation suggests that UVB affects CH at the level of the APC originating in the skin. Analysis of the DLNC surprisingly revealed that more were FITC (the contact sensitiser)$^+$ in UVB-pretreated animals, and that the fluorescence intensity was no different from sensitised only animals. A higher proportion of the DLNC from the UVB-irradiated mice were

Mac-1$^+$, F4/80$^+$, with few containing Birbeck granules, indicating that UVB changes the APC type.

1.6. UV radiation and hypersensitivity responses

Several studies have indicated that UV irradiation of human subjects generally leads to the downregulation of CH and DTH responses, both of which are considered as important measures of T cell function. Mork and Austad (1992) reported that 70% of patients with allergic chronic dermatitis in their hands could be treated successfully with suberythemal UVB, and the situation was maintained if UVB was given once weekly thereafter. Similarly exposure to suberythemal UVB 4 times weekly over a 3 week period was sufficient to suppress the CH response to nickel in nickel-sensitive individuals (Sjovall and Christensen, 1986). This effect was limited to the UVB wavelengths as UVA did not alter the patch-test scores. In contrast, Tie *et al* (1995) found that subjects, sensitised through normal skin and challenged via UVB-exposed skin, exhibited enhanced CH.

Using carefully graded doses of sensitiser (DNCB), Friedmann *et al* (1989) showed that therapy with both broadband UVB and PUVA (psoralen plus UVA) induced impairment of CH in about 70% of patients with psoriasis. The sensitiser was applied approximately halfway through the therapy and the elicitation occurred 4 weeks after the cessation of therapy. Subjects who were not sensitised successfully during treatment were re-sensitised at a later date, and no evidence for tolerance was obtained in either the UVB or the PUVA group.

Following exposure of a small area of skin to 144 mJ/cm^2 UVB on each of 4 consecutive days, a protocol which reduces LC numbers in the epidermis from 565 to 17 per mm^2, 40% of volunteers demonstrated suppressed CH responses to DNCB applied epicutaneously at the site of exposure, followed by challenge at a distant unirradiated site (Yoshikawa *et al* 1990). In agreement with mouse studies (Streilein

and Bergstresser, 1988), these subjects were designated as UVB-susceptible (UVB-S) and the others, in whom the extent of the CH response was unaltered by the UV exposure, were termed UVB-resistant (UVB-R). Skov *et al* (1998b) reported that 56% of human subjects exhibited suppressed CH responses following 3 MED UVB and sensitisation on the irradiated site. Tolerance was not induced in the unresponsive individuals. Interestingly, tests to assess LC ($CD1a^+HLA\text{-}DR^+$) and macrophage ($CD1a^-HLA\text{-}DR^+$) numbers and the ability of epidermal cells to activate autologous T cells following irradiation revealed no differences between the UVB-susceptible and resistant individuals. Thus susceptibility to UVB cannot be correlated easily with these local changes in epidermal APC populations or functions.

The clear division into UVB-S and UVB-R has not been confirmed in all studies. Cooper *et al* (1992) reported that almost all subjects had reduced ability to respond to DNCB if the sensitiser was applied to the irradiated site. The downregulation was dose dependent to some extent as the lowest exposure used (0.75 MED of UVB daily for 4 days to a small area of skin) induced non-responsiveness in 68% of individuals while the highest (2 MED of UVB daily for 4 days) induced non-responsiveness in 95% of individuals. The most susceptible people exhibited tolerance to DNCB on repeated challenge with the sensitiser. There was a rather modest suppression in CH if the DNCB was applied at a distant unirradiated site after 4 MED UVB was given as a single dose. There may be a wavelength dependence in the local immunosuppressive effect as exposure to UVA did not alter the induction of CH in any subject (Skov *et al* 1997). Another study has demonstrated that solar-simulated UV exposure, corresponding to 3 MED, followed by sensitisation on the irradiated site led to suppressed CH responses in 50% of subjects (Serre *et al* 1997). However, in contrast, Kelly *et al* (1993) found that solar simulated radiation was highly immunosuppressive in everyone tested. A single exposure of a small area of skin to 3 MED was followed by sensitisation with DNCB on the irradiated site and diphenylcyclopropenone on a distant site, and elicitation with both sensitisers occurred

3 weeks later. The CH response was completely suppressed locally in 100% of individuals and systemically in 83% of individuals. A dose of radiation of this magnitude is of biological relevance as it would be experienced within one hour at noon in the summer in the United Kingdom.

It is difficult at this time to draw firm conclusions from these rather disparate observations. However, it is clear that, in all studies, at least a proportion of human subjects exhibit suppressed CH if the sensitiser is applied to the irradiated site. The effect is more marked if small quantities of sensitiser are used. UV-induced tolerance and systemic immunosuppression are not consistently observed, and the contribution of different wavelengths within the UV spectrum is uncertain at present. Studies in rodents provide more consistent data.

Streilein and Bergstresser found that UVB exposure, with 600 J/m^2 on four consecutive days, before contact sensitisation of different mouse strains, led to different outcomes upon elicitation of the CH response (Streilein and Bergstresser, 1988). Some mouse strains exhibited CH responses which were unaltered in comparison with unirradiated counterparts, and were designated as UVB-R. Examples of the UVB-R strains which this group discovered are A/J and BALB/c mice. In other strains, such as C3H/HeN and C57Bl/6 strain mice, the CH response was significantly downregulated by irradiating prior to sensitisation, and these were designated as UVB-S. Experiments conducted in one set of F1 hybrids derived from 2 UVB-S parental strains displayed the UVB-R trait, suggesting gene complementation, and also that more than one gene locus was invovled.

The CH response to DNFB was suppressed in mice irradiated with 720 J/m^2 UVB and contact sensitised throught the same site 6h later. This was to a similar extent as the suppression observed in mice sensitised 72h after irradiation. Partial tolerance could be displayed, as resensitising of mice did not restore the CH response (Hammerberg *et al* 1996). This only occurred when mice were sensitised at the site of UVB exposure, and not if sensitised at a distant site. Mice sensitised 6h after a single

720 J/m^2 UVB exposure exhibited tolerance, and maximum tolerance could be observed in mice sensitised 48h after irradiation.

Garssen and coworkers irradiated mice and then sensitised the mice with either picryl chloride or Ox on the non-UV-exposed abdomen. In mice which were irradiated with UVB before sensitisation, 70-80% suppression of CH responses was observed upon challenge on the UV-protected ears (Garssen *et al* 1999). Both the early and late phases of CH are impaired in mice irradiated before contact sensitisation (Sontag *et al* 1994).

Most experiments have focussed on the effects of UVB before contact sensitisation. In experiments by Polla and colleagues, local exposure of the sites of elicitation (ears) of CH in mice exposed to UVB for 4 days prior to challenge actually enhanced the CH response, in both C3H/HeJ and A/J strain mice. PUVA had no effect on the CH response if given at this stage. The enhancement was due to a local, not systemic, effect of UVB (Polla *et al* 1986).

Like CH responses, DTH responses of mice can be inhibited by UV exposure. In mice which were irradiated with UVB before infection with HSV, the DTH response elicited by injecting viral antigens into the ears later was reduced from the DTH response elicited in animals which were not irradiated (Howie *et al* 1986). UVA1 exposure inhibited the response to HSV antigens, although less efficiently than a broad-band UVB irradiation (El-Ghorr and Norval, 1999). The DTH response to *Mycobacterium bovis* can also be suppressed by exposure of mice to UVB before, during or after infection (Jeevan and Kripke, 1989). Similarly, mice given a single UVB exposure before or after immunisation with *Candida albicans* exhibited a depressed DTH response against this pathogen (Denkins *et al* 1989).

1.7. UV and T cells

While aspects of T cell immunity have been outlined above in the context of hypersensitivity responses (see section 1.5), the effects of UV on T cell numbers are worthy of consideration. Changes in circulating T cell subsets as a result of exposure are controversial and, indeed, it is possible that such modulations are only transient. Thus, for example, Gilmour *et al* (1993b) found no significant changes in the percentages of blood $CD3^+$, $CD4^+$ or $CD8^+$ cells during and after broadband UVB phototherapy or PUVA. In contrast, exposure of people to the midday sun for 1 hour per day for 12 days during the Spring in Sydney, Australia, led to a small but significant decrease in the number of peripheral T cells (Hersey *et al* 1983). Circulating $CD4^+$ T cell numbers declined markedly while $CD8^+$ T cells increased. The reduction in the CD4:CD8 ratio was not completely restored, even 2 weeks post-exposure. The decrease in peripheral $CD4^+$ T cells might be accounted for by migration into irradiated sites.

Limited studies have examined T cell subsets in UV-exposed skin. Biopsies showed an initial decrease, or depletion, of intraepidermal T cells within 2 days of a single erythemal UV dose of solar simulated light (Di Nuzzo *et al* 1996). Dermal T cells increased by 24 hours and were maximal at 48 hours. One week after the irradiation, the migration into the epidermis of $CD3^+$ T cells, expressing CD4 but not CD8, could be detected. These cells were almost exclusively of the memory phenotype ($CD45RO^+$), and lacked expression of the activation markers HLA-DR, VLA-1 and IL-2R (Di Nuzzo *et al* 1998). Thus the cutaneous CD4:CD8 ratio may be increased following UV exposure. The time course of the T cell infiltration into the epidermis and dermis was UV dose-dependent. One explanation for the initial loss of cutaneous T cells may be phototoxicity. It has been shown that T cells are hypersensitive to UV compared with keratinocytes (Teunissen *et al* 1993), monocytes (Kremer *et al* 1995) or B cells (Deeg *et al* 1989).

1.8. UVB-induced immunological tolerance

UV radiation results in an inability to respond at later times to haptens encountered through irradiated skin shortly after exposure. This occurs in UVB-S mice, whereas UVB-R strains can raise a significant CH response upon challenge. Similar traits have been correlated to skin cancers in humans. It has been revealed that 92% of skin cancer patients fall into the UVB-S category (Yoshikawa *et al* 1990), and therefore UVB-induced suppression of CH may act as a risk factor/indicator for skin cancers. However it should be noted that the proportion of blacks with the UVB-S trait is similar to the proportion in Caucasians, despite black populations having a significantly lower incidence of skin cancer (Vermeer *et al* 1991). This may perhaps be attributed to the protective effects of melanin preventing significant DNA damage. Alongside the inability to respond to recall antigens, specific suppressor cells are generated. These can be demonstrated by adoptive transfer experiments, involving transfer of spleen cells from irradiated, immunised mice to naive recipients. Recipients fail to respond to the hapten upon challenge despite never having encountered it themselves.

1.9. UV and NK cells

Natural killer (NK) cells are non MHC-restricted cytotoxic cells which are part of the innate immune system, important in the recognition and lysis of both virally-infected cells and tumour cells (Trinchieri, 1989). There is an inverse correlation between the expression of surface MHC class I molecules on target cells and their susceptibility to NK cell-mediated lysis (Karre, 1992). NK cells also play a role in the directing of immune responses via the release of cytokines such as IFN-γ (Trinchieri *et al* 1984).

UVR causes a dose-dependent inhibition of NK cell activity (Weitzen and Bonavida, 1984; Toda *et al* 1986). *In vitro* exposure demonstrated that NK activity and lymphocyte proliferation, in response to mitogen, were inhibited to the same extent. Both effects were suppressed maximally at 260 nm, consistent with nucleic acid acting as the main chromphore (Schater *et al* 1983), although there is also evidence for the involvement of UCA (Gilmour *et al* 1993a). The dose required for 50% inhibition of NK cell activity at 300 nm was only 3 mJ/cm^2, equivalent to about 12 minutes of midday exposure to the sun in a temperate climate during summer. Although these experiments were conducted *in vitro* which would eliminate the absorptive properties of the skin, it is likely that environmental UV exposure might be sufficient to inhibit NK cell function (Weitzen and Bonavida, 1984; Schater *et al* 1983). As UV penetration through skin layers, assessed by thymine dimer formation, shows a very small decline of 2.5% per cell layer at 300 nm (Chadwick *et al* 1995), at this wavelength the amount of radiation required to inhibit NK cell activity is within that reaching the dermis and capillaries. Also it has been calculated that, when the greater proportion of UVA in solar radiation and its greater penetration into the skin are taken into account, UVA may have an equivalent immunosuppressive effect as that of UVB on NK cell activity (Hersey *et al* 1993).

In vivo studies utilising solarium exposure as a source of UV have concluded that NK cell function is reduced during the course of tanning (Hersey *et al* 1983). This suppression was particularly significant 2 weeks after the last treatment. Circulating NK cell numbers were also decreased, although the correlation between the numbers and function was not absolute. Measurement of NK cell activity after various UV therapies, used for the treatment of psoriasis, showed depression during and 4 weeks after the irradiation course (Gilmour *et al* 1993a). The precise timing and extent of the downregulation varied between the groups (PUVA, broadband UVB, narrowband UVB) and may be related to dose. No change in circulating NK cell numbers was reported (Gilmour *et al* 1993a).

UVR does not prevent NK cells recognising and binding to their target cells but acts at the apoptotic or lytic stage. NK cells, exposed to UVB *in vitro*, were induced to spontaneously release cytotoxic factors, detectable within 30 minutes of exposure (Weitzen and Bonavida, 1984). In agreement with this is the observation that superoxide dismutase (SOD), an antioxidant which scavenges superoxide anions, reversed the UV-induced suppression in NK cell activity (Toda *et al* 1986). IL-2 also reversed the suppression and acted synergistically with SOD. Thus UV may induce NK cells to produce reactive oxygen species, resulting in a less efficient killing of target cells.

In association with a decrease in NK cell activity, a similar decrease in NK-associated cytokines might be expected as a result of UVR. As NK cells are a potent, although not the only source of IFN-γ (Hosoi *et al* 1993), UV could bias the systemic immune system away from cellular immune responses. Induction of suppressive monocytic/macrophagic cells, T cells and their related cytokines, as well as prostaglandin release, could then play a role in exacerbating the downregulation in NK cell activity.

1.10. UVR and its effects on cytokine production

1.10.1. Interleukin (IL)-1

IL-1 is a proinflammatory cytokine synthesised in 2 isoforms (IL-1α and IL-1β) encoded by different genes (D'Eustachio *et al* 1987). IL-1 can stimulate prostaglandin synthesis by many cell types (Pentland and Mahoney, 1990), and is a significant mediator in induction of fever (Long *et al* 1990) and acute inflammatory responses (McIntyre *et al* 1991).

IL-1β plays a vital role in CH responses. Shornick and colleagues engineered an IL-1β deficient mouse, and used these mice to demonstrate the importance of IL-1β in contact sensitisation. Sensitisation of wild-type mice with a range of concentrations of

TNCB led to a dose-dependent swelling of the footpad upon challenge, which was maximal when a sensitising concentration of 0.1% TNCB was used. No footpad swelling could be measured in IL-1β deficient mice upon challenge, following sensitisation with this dose (Shornick *et al* 1996), and a concentration of 1% TNCB was needed to achieve a response equivalent to that induced by 0.1% TNCB wild-type mice. Injection of IL-1β into IL-1β deficient mice immediately before contact sensitisation resulted in a dose-dependent restoration of responsive to challenge. When DLNC from wild-type mice contact sensitised with 0.1% TNCB were incubated with haptenated spleen cells *in vitro*, antigen specific proliferation could be detected. When DLNC from IL-1β deficient mice contact sensitised with 0.1% TNCB were incubated with haptenated spleen cells, there was no antigen specific proliferation (Shornick *et al* 1996).

Cumberbatch and colleagues have revealed that IL-1β is vital for initiating LC migration from the epidermis, and subsequent accumulation of DC within DLN. Intradermal injection with 50ng IL-1β led to LC migration, with a 15% reduction within 2h. DC numbers began to increase in DLN 4h after IL-1β injection (Cumberbatch *et al* 1997c). Intraperitoneal injection of anti-IL-1β antibodies resulted in a 61% inhibition of Ox-induced increases in DC within DLN. Anti-IL-1β could also inhibit LC migration from the epidermis and accumulation of DC induced by TNF-α injection. It was deduced that IL-1β acts in concert with TNF-α, as injection of mice with antibodies against TNF-α prevented the LC migration and accumulation of DC induced by IL-1β injection (Cumberbatch *et al* 1997a).

Enk and Katz identified enhanced expression of TNF-α, IL-10, IL-1α and even IL-1β mRNA, in epidermal cell suspensions prepared 4h after injection of IL-1β *in vivo* (Enk *et al* 1993a). Supernatants of highly enriched cultured LC displayed IL-1 activities. Cell mixing experiments to investigate the role of contaminating keratinocytes showed that IL-1α was predominantly derived from keratinocytes. PCR revealed that IL-1β transcripts were limited to LC (Schreiber *et al* 1992).

Interestingly, induction of apoptosis of mononuclear phagocytes *in vitro* leads to a rapid release of IL-1β (Long *et al* 1990). Apoptosis is a known phenomenon following UVB irradiation of cells (Kitajima *et al* 1996; Aragane *et al* 1998; Schwarz *et al* 1995). IL-1 was shown to be present in the *stratum corneum* of normal human skin, but not in the basal layer. After UVB exposure, IL-1 appeared in the basal cell layer, and was increased in the more exterior regions (Oxholm *et al* 1988). Together with this increase in IL-1, IL-1 receptor antagonist (IL-1ra) was increased markedly following 2-3 MED of UV (Hirao *et al* 1996). IL-1ra is reportedly induced during keratinocyte differentiation, a known effect of UVR or sun-exposure (Matsui *et al* 1996). The ratio of IL-1ra:IL-1α in sun-exposed skin was over 100, an excess sufficient to block IL-1α-induced biological responses (Hirao *et al* 1996). In unexposed skin, the ratio was around 8, which is too low to block activity. Therefore, although UV stimulates an increase in IL-1 levels, with the concomitantly greater rise in IL-1ra, UV may actually inhibit the activities of IL-1α and suppress an inflammatory response. The adhesion molecule VCAM-1 is known to be upregulated on endothelial cells by IL-1, but is unaltered by UV-exposure (Norris *et al* 1991).

1.10.2. Interleukin (IL)-6

IL-1 is a potent inducer of IL-6, a cytokine which is detectable in serum 1-3 hours after 2-4 MED whole-body UV-exposure of human subjects (Kirnbauer *et al* 1989; Urbanski *et al* 1990). IL-6 has a wide range of effects, including fever induction and the synthesis of acute phase proteins. Like IL-1, it is known to be produced by keratinocytes and LC. Although initially regarded as a pro-inflammatory cytokine, recent evidence suggests a significant anti-inflammatory, and even an immunomodulatory, role for IL-6. It can elicit the release of adrenocorticotropic hormone from the central nervous system, increasing synthesis of glucocorticoids in the adrenal gland (Tilg *et al* 1997). In turn, these suppress IL-1 and TNF-α synthesis. It is also suggested that IL-6 induces the soluble form of the TNF receptor, p55 (Tilg

et al 1997). Elevations in IL-6 correlate well with UV-induced fever courses, and precede increases in acute phase proteins, such as C-reactive protein (Urbanski *et al* 1990). IL-6 induced C-reactive protein promotes synthesis of 5-10 fold greater quantities of IL-1ra compared with IL-1β from peripheral blood mononuclear cells (Tilg *et al* 1997). Antibodies to IL-6 inhibit this in a dose-dependent manner. Irradiation of long-term cultured epidermal cells shows that IL-6 production is attributable to UVB wavelengths, with UVA having no effect (Kirnbauer *et al* 1989). Whether IL-6 production is mediated directly by UVB, and/or via IL-1 or other mediators *in vivo* is unresolved.

1.10.3. Interleukin (IL)-10

One of the major immunoregulatory cytokines involved in the balance of an immune response is IL-10. This was first recognised in murine Th2-cell lines, and has been shown to have an inhibitory effect on Th1-like cells (Fiorentino *et al* 1992). By inhibiting the growth and cytokine production of Th1-like cells, IL-10 promotes a Th2-like cytokine profile within localised areas. IL-10 is produced by many cell types, including T lymphocytes, of both helper (Ullrich, 1995) and cytotoxic (Romagnani, 1997) subsets, monocyte/macrophages (Kang *et al* 1994), B cells, mast cells, keratinocytes (Ullrich, 1994) and melanocytes (Teunissen *et al* 1997).

IL-10 in delayed type and contact hypersensitivities

Intraperitoneal injection of IL-10 blocks both phases of DTH. Similarly, the effector phase of CH can be blocked by IL-10, although IL-10 injection does not block the induction phase of CH (Schwarz *et al* 1994). Blocking of the effector phase of CH by IL-10 is not dose-dependent, and only occurs if IL-10 is administered at least 12h before hapten challenge, implying indirect activity of IL-10 on other mediators. Sensitisation with contact allergens induces IL-10 mRNA in murine epidermal cells, detectable at 4h and maximal by 12h (Enk and Katz, 1992b). This quickly diminishes

to 50% by 18h, and background levels at 24h, after painting (Ferguson *et al* 1994). IL-10 injection decreases the inflammatory cell infiltrate, and decreases edema at the dermis compared to mice with a normal CH response (Kondo *et al* 1994). IL-10 application also suppresses the IFN-γ mRNA upregulation normally seen in CH (Kondo *et al* 1994). IL-10 gene targeted mice (IL-10T) mice mount an exaggerated CH response, increased in both magnitude and duration of response (Berg *et al* 1995). Ear swelling was maintained 4 days after challenge in IL-10T mice, and epidermal pustules were larger and more frequent than in WT mice. In many areas these pustules became confluent and formed serocellular crusts on the surface of IL-10T mouse ears. These results clearly implicate IL-10 in control of CH responses within the skin.

Normal mice exhibit suppressed DTH responses after UVB-irradiation, whereas in IL-10T mice, a full DTH response is restored (Teunissen *et al* 1994). Investigation of serum from irradiated wild-type mice shows an IL-10 peak 4 days after a single UVB-exposure, coinciding with optimum immunosuppression. Evidence for the downregulatory role of IL-10 in DTH is provided by intraperitoneal injection of the cytokine prior to either the sensitisation or elicitation phase. Injection leads to blocking of both events (Schwarz *et al* 1994).

The primary source of IL-10 following UVB-irradiation is yet to be determined, although many cell types have been suggested. Potential candidates include keratinocytes and melanocytes, which are located in the epidermis, and infiltrating monocytic/macrophagic cells, which are induced following UVB-exposure. Other candidates may be Th2-like cells, primed in draining lymph nodes by LC migrating from UVB-irradiated skin.

IL-10 effects on antigen presentation

IL-10 does not act directly on Th1-like cells to inhibit their activity (Enk *et al* 1993b; de Waal Malefyt *et al* 1992). Pretreating LC with IL-10 causes loss of antigen presenting function for Th1-like cells, failing to promote proliferation and inducing

clonal anergy (Enk *et al* 1993b). IL-2 and IFN-γ mRNA levels are decreased to around 50% that of untreated cells. Antigen presentation for Th2 cells is unaffected. If cells are cultured for 2-4 days prior to treatment with IL-10, inhibition is reduced. This suggests that IL-10 affects maturation of one or more of the costimulatory molecules expressed on cultured LC, but not on freshly isolated cells (Enk *et al* 1993b). The effect is not on class II MHC expression or cell viability. B7 mRNA levels are normally upregulated on cultured LC, but remain constant after IL-10 pretreatment. Lack of B7 expression is associated with clonal anergy (Gimmi *et al* 1993).

When macrophages are used as APC, the Th1/Th2 balance is similarly affected. However, these effects are mediated by different mechanisms. Upon culture with IL-10, macrophage HLA-DR/DP and HLA-DQ expression is strongly downregulated, and the upregulation usually induced by IFN-γ and/or IL-4 is inhibited. IL-10 alters macrophage appearance in culture, causing rounding-up and decreased adherence. This could prevent further antigen presentation, and possibly act as a migratory trigger *in vivo* (Ferguson *et al* 1994). IL-10 also has an autoregulatory role on macrophages, downregulating its own mRNA expression. Whilst suppressing production of inflammatory cytokines, it enhances production of the anti-inflammatory IL-1 receptor antagonist (IL-1ra).

The antigen-presenting capacity of B cells is upregulated by IL-10, with an increase in class II MHC expresion (Enk *et al* 1993b). Splenic B cells do not inhibit Th1-like cell proliferation (de Waal Malefyt *et al* 1992). IL-10 also acts as a B cell growth factor (Enk *et al* 1993b). The significance of this in relation to UV-induced immunosuppression may be minimal however, as the skin is relatively deficient in B cells. IL-10 can be found in sera after UV exposure, but only with doses over 10 MED (Beissert *et al* 1996; Rivas and Ullrich, 1994).

IL-10 and UVB-induced immunosuppression

With the apparent deviation toward a Th2-like cytokine response following UVB exposure, it is natural to hypothesise a central role for IL-10 in UVB-induced immunosuppression. IL-10 exhibits antagonistic effects towards IFN-γ, inducing downregulation of MHC class II expression, and therefore antigen presentation, on macrophages (Moore *et al* 1993). It suppresses cytokine synthesis when Th1 T cell clones, or NK cells, are activated by IL-2 and accessory cells in an antigen non-specific manner. Furthermore, whilst downregulating inflammatory cytokines (Howard and O'Garra, 1992), IL-10 can upregulate IL-1ra expression and secretion (Cassatella *et al* 1994). IL-10 could thereby play a key role in the production of "split-tolerance".

There is strong evidence for a role for IL-10 mediating immune suppression in the mouse following UVB exposure, especially for the participation of keratinocyte-derived IL-10 (Rivas and Ullrich, 1994). Human studies present a less clear picture, with some groups finding both keratinocyte-derived IL-10 mRNA and secreted protein (Enk and Katz, 1992b; Grewe *et al* 1995), some finding mRNA without protein secretion (Kang *et al* 1994), and others finding neither mRNA (Ried *et al* 1994; Teunissen *et al* 1994) nor protein (Teunissen *et al* 1997; Jackson *et al* 1996).

After exposure of small areas of human skin sites to 3 MED UVB or UVA1, Skov *et al* found that IL-10 protein was slightly increased in suction blister fluid taken 24 hours later. This increase is only significant following UVB irradiation (Skov *et al* 1998a). Kang *et al* (1994) argue that IL-10 within UV-exposed human skin is secreted from infiltrating $CD11b^+$ macrophages rather than resident $CD11b^-$ keratinocytes. LC and UV-induced macrophages respond differently to inflammatory stimuli (Shibaki *et al* 1995). The infiltrating macrophages preferentially activate suppressor $CD4^+$ T cells (Shibaki *et al* 1995), and secrete high levels of IL-10. UV-induced macrophages could thereby favour development of Th2-like suppressor cells. Although keratinocytes synthesize small amounts of IL-10 mRNA, the protein may remain inside the cells.

Teunissen *et al* (1997) performed exhaustive studies on normal human keratinocytes from 40 donors and were unable to detect any IL-10 mRNA or protein following UVB irradiation. Examination of two human cell lines (A341, HaCaT) gave similar results. IL-10 mRNA within melanocytes was detected, without protein secretion. Therefore the positive findings from other groups (Enk and Katz, 1992b; Grewe *et al* 1995) may be due to melanocyte contamination. Teunissen and colleagues (1997) also performed whole body irradiations (1.5 MED), and were unable to detect alterations in serum IL-10 levels outwith the 24 hour biphasic temporal pattern of controls. It is difficult to reconcile this result with a major role for macrophage-derived IL-10 in systemic immunosuppression in human subjects.

Potential sources of IL-10 after UV exposure

Keratinocytes - IL-10 production by human keratinocytes is currently a contentious issue (Jackson *et al* 1996; Grewe *et al* 1996). Ried *et al* (1994) could not detect IL-10 gene expression in human keratinocytes, and could not induce expression with various stimuli (IL-1β, IL-6, IL-8, TNFα, IFN-γ, TPA, retinoic acid, osmotic stress or UV-radiation). However, IL-10 mRNA is detectable in the epidermis in human Th2-like skin diseases, such as Sezary Syndrome, and following tape-stripping (Nickoloff *et al* 1994). IL-10 was predominantly identified throughout all levels of the epidermis, within the cytoplasm of keratinocytes, with accentuation on their membranes in upper level cells. IL-10 cannot be seen in keratinocytes of normal or psoriatic (Th1-like) skin (Nickoloff *et al* 1994).

IL-10 production by murine keratinocytes is generally accepted. Depletion studies have shown keratinocytes to be the main source of IL-10 mRNA after sensitisation with contact allergens (Enk and Katz, 1992b). The murine keratinocyte cell-line, PAM-212, constitutively expresses IL-10 mRNA, with increased protein secretion upon stimulation (Teunissen *et al* 1997). In keratinocyte cultures derived from BALB/c, C57Bl/6, and Swiss mice, IL-10 is detectable by ELISA. Upregulation

of IL-10 mRNA in keratinocytes has also been shown to occur late in the induction phase of murine CH (Enk and Katz, 1995), again suggesting an immunomodulatory role.

Melanocytes - Teunissen *et al* (1997) argue that IL-10 mRNA found within human epidermal cell preparations is due to contamination by melanocytes. Using limiting dilutions to obtain pure cultures of keratinocytes (identified as cytokeratin positive) and melanocytes (NKI-beteb positive), followed by RNA extraction at 24h or 48h after phorbol myristate acid (PMA), IFN-γ or UVB stimulation, IL-10 mRNA was only detectable in melanocyte cultures. This was not translated to detectable levels of protein however.

Monocyte/macrophages - One of the major events in a DTH or CH response is induction of a cellular infiltrate into the responsive area. This infiltration also occurs 24-48h after UVB-exposure. When epidermal cells are separated on the basis of CD11b expression, macrophages ($CD11b^+$), induced to infiltrate by UVB, have reproducibly 200-400 fold higher IL-10 mRNA content relative to keratinocytes ($CD11b^-$) (Kang *et al* 1994). Production and secretion of IL-10 protein is undetectable until 48h after irradiation, and is closely related to macrophage infiltration. Flow cytometric analysis of the macrophage population shows 19% have a greater capacity to retain cell-associated IL-10, and these may be representative of a highly activated subpopulation. Human monocytes are strong producers of IL-10, dependent upon the stimuli (de Waal Malefyt *et al* 1992).

T cells - After UVB-irradiation the ratio of $CD4^+CD8^+$ cells within the skin is increased. This occurs after a delay of around 2 days, at which point migration of $CD4^+$ T cells into the site is detectable. As IL-10 is inhibitory towards APC function for Th1-like cells , but not Th2-like cells (Ferguson *et al* 1994), a positive feedback loop may be established in irradiated skin whereby IL-10 causes an increase in

Th2-like cells, and further generation of IL-10.

1.10.4. Interleukin (IL)-12

IL-12 is implicated in polarisation of immune responses towards a Th1-like cytokine response (Trinchieri, 1994; Openshaw *et al* 1995), by inducing IFN-γ production (Gately *et al* 1991) and by enhancing NK and CTL reponses (Locksley, 1993). IL-12 is formed by the covalent linkage of p35 and p40 subunits to produce a biologically active heterodimeric p70 molecule. The p40 subunit is produced in 10- to 100- fold excess of p35, but evidence for increased secretion is lacking. When recombinant p40 was injected into mice, an antagonist activity was observed (Mattner *et al* 1993), and a recently produced IL-12 p40 transgenic mouse (in which circulating p40 is detectable) has diminished type 1 cytokine responses (Yoshimoto *et al* 1998).

The primary sources of IL-12 are APC such as monocytes, macrophages, neutrophils, DC and B cells (Trinchieri, 1993). IL-12 leads to IFN-γ production, which in turn potentiates APC functions and increases IL-12 production by a positive feedback mechanism. The major biological activity of IL-12 is on NK and T cells (Locksley, 1993), increasing cytokine production, proliferation and cytotoxicity. IL-12 is regarded as the key cytokine in directing cell-mediated immune responses, via generation of Th1 cells (Trinchieri, 1993). However, in p40 transgenic mice, DTH responses were suppressed and there was skewing towards Th2-like cytokine profiles in response to antigen. IFN-γ was decreased, whereas IL-4 and IL-10 were increased, versus control littermates (Yoshimoto *et al* 1998).

IL-12 is essential during the sensitisation and elicitation phases of CH. Injection of anti-IL-12 prior to sensitisation of mice with DNFB led to a suppressed response upon challenge, via generation of a tolerant state (Riemann *et al* 1996). Anti-IL-12 treatment before elicitation of CH to DNFB also leads to a suppressed inflammatory response (Riemann *et al* 1996).

Regulation of IL-12 is central to UV-induced immunosuppression. There is evidence for this in mouse models. Administration of IL-12 4h after a single 15 kJ/m^2 dose of UVB blocked the suppression of DTH and CH observed in UV-irradiated mice, in a dose-dependent manner (Schmitt *et al* 1995). Intraperitoneal injection of IL-12 21h after the last of 4 daily, 1000 J/m^2 UVB exposures also prevented suppression of CH upon elicitation (Schwarz *et al* 1996). Administration of IL-12 prevented the induction of suppressor T cells in both series of experiments. Adoptive transfer of spleen cells from UV-irradiated mice treated with IL-12 had no effect on the immune response of recipient mice (Schmitt *et al* 1995; Schwarz *et al* 1996). Depletion studies identified $CD8^+$, rather than $CD4^+$, T cells, as the cells responsible for transferring suppression to recipient mice (Schwarz *et al* 1998).

IL-12 p35 chain mRNA is expressed constitutively by human keratinocytes, and upon contact sensitisation, p40 mRNA is rapidly expressed (Muller *et al* 1994a). IL-12 protein is detectable in concentrated epidermal cell supernatants, and is readily detectable in keratinocyte cell line supernatants following stimulation with phorbol-12, 13-dibutyrate (Aragane *et al* 1994). Anti-IL-12 treatment of haptenated human epidermal cells causes a 50% reduction in allogeneic T cell proliferation assays (Muller *et al* 1994a). UVB irradiation of human peripheral blood monocytes before using them in APC dependent T cell stimulation assays, resulted in a selective inhibition of antigen presentation to established human Th1 clones (Kremer *et al* 1996). While proliferation and IFN-γ production by Th1 clones was inhibited in a dose-dependent manner, normal proliferative activity and cytokine production were maintained in Th2 clones. Perhaps most interestingly, while the proliferative responses of Th0 clones were normal, there was a preferential suppression of IFN-γ production, skewing cytokine profiles towards those of Th2 clones (Kremer *et al* 1996). These studies indicate the way that resident dermal and/or infiltrating macrophages may behave at irradiated sites.

1.10.5. Interleukin (IL)-15

IL-15 has the ability to stimulate growth of both $CD4^+$ and $CD8^+$ T cell subsets (Grabstein *et al* 1994), to generate lymphokine-activated killer cells (Burton *et al* 1994; Bamford *et al* 1994) and to act as a chemokine for T cells (Wilkinson and Liew, 1995). Conflicting data have been reported regarding IL-15 after UV exposure.

Mohamadzadeh *et al* (1995) found that IL-15 mRNA was constitutively expressed in the human dermis, but was absent in the epidermis. Upon UV-exposure, IL-15 mRNA was enhanced in dermal sheets and induced in the epidermis. Expression was traced to $HLA\text{-}DR^-$ cells, predominantly keratinocytes, but not to $HLA\text{-}DR^+$ cells, probably LC. Immunoblot analysis revealed that IL-15 protein secretion in cultured keratinocytes was enhanced by UVB. In contrast, Blauvelt *et al* (1996) detected constitutive IL-15 mRNA expression in freshly isolated human keratinocytes and LC, and this was downregulated by UVB exposure in a dose- and time-dependent manner.

One important difference between the experimental protocols used by these groups is that Mohamadzadeh *et al* (1995) irradiated intact epidermal and dermal sheets, whereas Blauvelt and colleagues (1996) irradiated after segregation of cell types. Doses of 200 J/m^2 UVB were found to be toxic *in vitro*, but gave optimal IL-15 mRNA expression *in situ*. This may imply that IL-15 expression is under the control of other intercellular UV-inducible factors found within the skin, but which cannot act in culture. It should be noted that IL-15 mRNA appears to be regulated at post-transcriptional levels (Tagaya *et al* 1996), and is not necessarily related to protein secretion.

Macrophages can also produce IL-15 (Carson *et al* 1994), and if recruited locally as a result of UV irradiation, could be another source of this cytokine. IL-15 could play an important role in regulating the local cytokine microenvironment at UV-exposed sites, aiding the outgrowth of suppressor T cells.

1.10.6. Interleukin (IL)-18

IL-18 is one of the most recently discovered cytokines. As its original name, IFN-γ inducing factor (IGIF) suggests, IL-18 is implicated in the bias of immune responses towards the Th1-like arm (Takeda *et al* 1998), promoting cell-mediated immune responses and facilitating CH. IL-18 stimulation results in increased IFN-γ production by antigen-stimulated T cell lines, but has no effect on IL-4 or IL-10 production, in both murine (Kohno *et al* 1997) and human (Micaleff *et al* 1996) systems. NK cell cytotoxicity is also enhanced (Micaleff *et al* 1996; Ushio *et al* 1996). Cellular sources of IL-18 include activated macrophages and Kupffer cells (Ushio *et al* 1996), and murine keratinocytes (Stoll *et al* 1997). IL-18 mRNA is upregulated early in the response to contact allergens (Stoll *et al* 1997), implicating it in directing CH development. The findings that the human IL-18 amino acid sequence includes an IL-1 signature-like sequence (Kohno *et al* 1997), that the IL-1β converting enzyme (ICE) cleaves an IL-18 precursor (Gu *et al* 1997), and that IL-18 signals through IL-1 receptor-associated kinase (IRAK) (Robinson *et al* 1997), suggest that IL-18 is under similar control to IL-1β and may have expression resembling that of IL-1β. Whilst IL-18 enhances T cell proliferation, this can be blocked by treatment with neutralising anti-IL-2 monoclonal antibodies (at least at low IL-18 concentrations (Micaleff *et al* 1996)), so that its activity may be sensitive to changes in both IL-2 and IL-2Rα (CD25) expression. The upregulation of CD25 mRNA in $CD4^+$ T cells is not induced by irradiated epidermal cells (Stevens *et al* 1995).

1.10.7. Tumour necrosis factor (TNF)-α

TNF-α is a trimeric, pro-inflammatory cytokine. The TNF-α gene is polymorphic, containing 5 micro-satellite regions, induction is mostly mediated by a κ3 binding site for NF-κB, and is also regulated at the transcriptional level, predominantly by a UA-rich sequence in the 3'-UTR, controlling mRNA turnover (Rink and Kirchner, 1996). Synthesis of TNF-α can be blocked by phosphodiesterase

inhibitors, corticosteroids, ω-3 polyunsaturated fatty acids and IL-10 (Eigler *et al* 1997).

LPS treatment induces monocytes to produce many pro-inflammatory cytokines, including TNF-α. Monocytes then produce IL-10 8-24h later, and this is dependent on TNF-α (Wanidworanun and Strober, 1993). IFN-γ and/or IL-4 inhibit the TNF-α induced IL-10 increase in monocytes.

Arguably the most important effects of TNF-α are on LC. TNF-α is needed to keep LC alive in culture, whereas without keratinocyte-derived cytokines the cells rapidly involute and die. TNF-α does not have any effects on the maturational state of the cells, and freshly isolated epidermal cells express TNF-α mRNA (Koch *et al* 1990). Cumberbatch and colleagues showed that TNF-α is implicated in migration of LC from the epidermis and accumulation of DC within DLN. Intradermal injection of TNF-α resulted in a decrease in LC numbers in the epidermis (Cumberbatch *et al* 1997a), and in a dose- and time-dependent accumulation of DC (Cumberbatch and Kimber, 1992). Furthermore, treatment of mice with antibodies to neutralise TNF-α inhibited the number of DC in DLN measured 18h after contact sensitisation with Ox or FITC (Cumberbatch and Kimber, 1995). The efficiency of skin sensitisation was similarly reduced, with impaired CH responses in anti-TNF-α treated mice compared to mice treated with control serum. These effects were only detected when TNF-α was inhibited at the time of sensitisation, as injection of anti-TNF-α 18h after contact sensitisation was without effect (Cumberbatch and Kimber, 1995). As mentioned in section 1.9.1, LC require signals from both IL-1β and TNF-α to initiate migration (Cumberbatch *et al* 1997a). Examining the kinetics and interaction of the two signals, it would appear that the TNF-α signal is secondary to IL-1β signalling (Cumberbatch *et al* 1997a; Kimber *et al* 1998). In experiments by Moodycliffe and colleagues, broad-band UVB induced accumulation DC within the DLN of irradiated mice, and suppressed the CH response to Ox. When mice were injected with neutralising

antibodies to TNF-α before irradiation, the accumulation of DC was abrogated and the CH response was restored (Moodycliffe *et al* 1994).

Studies in mouse models suggest that susceptibility to UVB-induced immunosuppression is partly controlled by the Tnf locus (Yoshikawa and Streilein, 1990). Induction of TNF-α could lead to altered, or diminished, antigen presentation, via LC depletion from the epidermis. Injection of anti-TNF-α can block the UV-induced suppression of CH responses, but has no effect on DTH responses to spleen cells (Rivas and Ullrich, 1994).

TNF-α is strongly implicated in the inflammatory response to UVB-exposure. Skov *et al* (1998a) found that irradiating human skin with 3 MED of UVB led to a rapid increase in TNF-α protein in suction blister fluid, which was maximal at 6 hours following exposure. Interestingly, exposure to 3 MED UVA1 (340-400 nm) resulted in a slight decrease in TNF-α at 6 hours (Skov *et al* 1998a).

Conflicting data have been reported on the role of UVB-induced TNF-α in the upregulation of E-selectin. Strickland *et al* (1997) found E-selectin expression on dermal blood vessels proximal to the exposed site by 4 hours, before TNF-α could be detected. Walsh (Walsh, 1995) studied the "sunburn" response, finding that mast cells degranulated upon exposure to 3 MED, releasing stores of intracellular TNF-α, which in turn led to E-selectin and ICAM-1 expression on cutaneous endothelium by 2 hours. Keratinocytes contributed minimally to this effect, which could be blocked with the mast cell inhibitor, disodium cromoglycate (Walsh, 1995). TNF-α is further implicated in sunburn cell formation by *in vitro* studies, as antibodies against TNF-α reduced UV-induced apoptosis in human keratinocytes, although not completely (Schwarz *et al* 1995).

UVB exposure (200 J/m^2) stimulated a similar quantity of TNF-α from cultured human dermal fibroblasts as IL-1α. However, the two treatments synergised to give a 30-40 fold increase in TNF-α, mirrored by mRNA expression (Fujisawa *et al* 1997). As UVB is capable of inducing IL-1α directly, and as incident UVB can penetrate to

the dermis, a TNF-α cascade could be initiated in this site (Fujisawa *et al* 1997). *In vitro* studies using a human keratinocyte cell line showed that blockade of IL-1α activities with monoclonal antibodies also resulted in a partial inhibition of TNF-α release, supporting an autocrine role for IL-1α in TNF-α release (Corsini *et al* 1995). Antioxidants have a similar effect in inducing TNF-α secretion (Corsini *et al* 1995). DNA damage is implicated in TNF-α gene expression following UVB-exposure (Kitibel *et al* 1998). *Cis*-UCA, even above physiological levels, could not augment phorbol 12-myristate 13-acetate (TPA) induced synthesis of either TNF-α mRNA or protein in keratinocytes (Redondo *et al* 1996). Interestingly, UVB mimicks the TNF-α induced modulation of the p55 TNF surface receptor on keratinocytes, and mRNA expression, via a TNF-α mediated autocrine regulatory pathway (Trefzer *et al* 1993).

1.11. UV and skin cancer

Skin cancers are commonly divided into 3 distinct categories; basal cell carcinoma, squamous cell carcinoma and malignant melanoma. Although the first 2 have a low mortality rate, they are important conditions due to the resultant morbidity and cosmetic deformities, and represent 90% of all skin cancers. In addition, a study of non-melanoma skin cancer incidence in the Glasgow area indicated that basal cell carcinomas are under-recorded by 31% and squamous cell carcinomas by 43% (Lucke *et al* 1997). Risk factors for basal cell carcinoma include increasing age, red/blonde hair and fair skin (Lin and Carter, 1986). Outdoor occupations and a family history are also implicated (Hogan *et al* 1989). There is an increased risk of a basal cell carcinoma patient developing squamous cell carcinoma (Karagas, 1994) and malignant melanoma. The link between these associations is thought to be UVR, as UV is regarded as a major etiological factor in these cancers.

UVB appears to be the most important environmental factor in skin cancer pathogenesis (Streilein *et al* 1994a), and approximately 90% of the estimated

900,000 - 1,200,000 new cases of non-melanoma skin cancers (squamous and basal cell carcinoma) in the USA each year are attributed to it (Buckman *et al* 1998). There is a global trend of increasing skin cancer incidence. Currently, each year in Belgium there are 15-20,000 basal cell or spinal cell epitheliomas being diagnosed (Autier, 1998). The incidence of basal cell carcinoma increased 11% between 1985 and 1990 in Australia (Marks, 1995), with similar increases reported in Great Britain (Ko *et al* 1994) and The Netherlands (Coebergh *et al* 1991). Estimates suggest that for every 2.5% decrease in ozone levels, such as that seen over Northern hemisphere mid-latitudes, human skin cancer rates will increase by 10%.

The 1985 Vienna Convention introduced a framework for international restrictions on use of ozone depleting substances. Estimates of the effectiveness of these measures have been calculated. Extrapolating to the year 2100 using a 'no restrictions' scenario or the Montreal Protocol scenario, large increases in skin cancer incidence are predicted (4- and 2-fold respectively). Under the stricter Copenhagen Amendments, in which ozone reaches its minimum levels in 2000, increases in skin cancer incidence are predicted to reach a peak 60 years later of 10% (Slaper *et al* 1996).

The United States Environmental Protection Agency (EPA) report that cutaneous malignant melanoma already has one of the fastest growing incidence rates of all cancers in the USA, with case numbers doubling over the last 20 years (Streilein *et al* 1994a). NASA studies in Puerto Rico have shown that, calculating from their lowest values in 1978 and 1991, the age-adjusted incidence of melanoma has increased 528% and 200% in males and females respectively in 13 years (Matta *et al* 1998). Studies on the Norwegian population show that holidays abroad are now having a detectable effect on melanoma incidence (Bentham and Aase, 1996). In Canada, predictions are that melanoma will become a much larger burden on its health care system in the early part of the 21st century (MacNeill *et al* 1995).

Experiments by Kripke and colleagues in the 1970s demonstrated that

UVB-induced skin tumours are highly immunogenic when transplanted into syngeneic mice (Fisher and Kripke, 1977; Kripke *et al* 1979). When the same tumours were transplanted into UVB-irradiated mice there was no rejection, and this indicated that UVB must induce some form of immunomodulation to prevent tumour rejection.

Daily irradiation with UVR filtered beneath 315 nm (2.5×10^5 J/m^2) induced murine tumours with 50% probability of tumour development (T_{50}) after 68 weeks of irradiation. Tumours included SCC, poorly differentiated (spindle cell) tumours and benign papillomas. Irradiation with UVA/B (FS40, 2.394×10^4 J/m^2) 3 times per week resulted in a T_{50} of 23 weeks. This shows that UVA is carcinogenic in haired mice, although less so than UVB (Strickland, 1986).

As mentioned in section 1.7, when human subjects with a history of skin cancer are assessed for immune function following UVB, the vast majority show depressed CH responses to DNCB, with half of these appearing to be tolerant to rechallenge at unirradiated sites (Yoshikawa *et al* 1990). Therefore, humans can be classified as UVB-susceptible (UVB-S) or UVB-resistant (UVB-R). These putative traits have been linked to the Tnfα and Lps loci in mice (Yoshikawa and Streilein, 1990; Streilein and Bergstresser, 1988). This has recently been narrowed to withina 190kb region on the *Bat5* and H-2D segment of mouse chromosome 17 (Handel-Fernandez *et al* 1999). The UVB-S trait applies to 30-40% of the Caucasian population, but increases to over 90% in skin cancer patients, indicating UVB-S may be a risk factor for skin cancer (Yoshikawa *et al* 1990). Intriguingly, blacks, who have a far lower incidence of skin cancer, also carry the UVB-S trait at a frequency of around 40% (Vermeer *et al* 1991).

1.12. Other human diseases

The eyes are dramatically affected by over-exposure to UV. Ocular damage from UV exposure includes effects on the cornea, lens, iris, and associated epithelial and

conjunctival tissues (Longstreth *et al* 1998). Photokeratosis, also known as snowblindness, is common in outdoor recreationists. There is significant evidence suggesting a correlation between UV exposure and conjunctival pterygium, climatic droplet keratopathy and cataracts (Bergmanson and Sheldon, 1997), and it is likely that ocular melanoma and other cornea/conjunctival condtions (eg. pinguecula) will increase as ozone decreases. Recent quantitative risk assessments developed for cataract idicate that, under the Montreal Adjustments, cataract incidence will peak at just under 3 new cases per 100,000 (Longstreth *et al* 1998).

Many mouse models indicate that UVB can alter immune responses to viruses, bacteria and parasites (reviewed in (Halliday and Norval, 1997). For ethical and pratical reasons, less is known about UV-mediated effects on human diseases.

With the effect of UV on T helper cell subsets, exposure could have important consequences in T cell mediated diseases in humans. UVB could be of particular relevence in human immunodeficiency virus (HIV) patients, where sunlight could accelerate acquired immunodeficiency syndrome (AIDS) progression (Vincek, 1995; Wallace and Lasker, 1992). UV could act by facilitating viral replication and accumulation of stable mRNA (Valerie *et al* 1988), and/or by inducing a Th1 to Th2 switch, a factor implicated in disease exacerbation (Clerici and Shearer, 1993).

In herpes simplex virus (HSV), one of the factors inducing recrudescence and the appearance of cold-sores is exposure to sunlight. HSV-specific lymphoproliferation was markedly reduced (39.8-93.8%) 3 days after whole-body, 1 MED exposure, and remained suppressed for at least a further week (Miura *et al* 1994). This was associated with increased levels of TGF-β (IL-10 was unaltered), secondary to IL-6 induction, in HSV-stimulated peripheral blood leukocyte cultures. The association between UVB-induced immune dysregulation and development of recurrent HSV lesions was incomplete, implying that UVB contributes to, but is not directly responsible for, HSV reactivation.

The immune response to intracellular bacteria could also be altered by UVB. In experiments using the lepromin reaction, a cell-mediated immune response to antigens of *Mycobacterium leprae*, 27 of 29 cases showed a reduced reaction in locally irradiated sites, by at least 20%. Irradiation decreased lymphocyte infiltration, particularly $CD4^+$ T cells, in and around the induced granuloma (Cestari *et al* 1995). As the granulomatous response limits or suppresses infection, it thus follows that UVB could exacerbate the clinical situation.

Damian and colleagues investigated the effect of UV exposure on the DTH response to tuberculin protein purified derivative in Mantoux test positive volunteers. The Mantoux induced erythema was suppressed in skin irradiated with solar simulated radiation, but not in unirradiated sites (Damian *et al* 1998). Interestingly, when individuals were chronically irradiated over 4-5 weeks, the suppression, although significant, was not as large as that achieved by a short course of UV exposure.

Erythemagenic UVR is also toxic in the autoimmune disease systemic lupus erythematosus (SLE) (McGrath *et al* 1994; Dov Golan *et al* 1992). This phenomenon may be due to further suppression of already reduced cell-mediated immunity (Horwitz, 1972). Tissue damage occurs, and may be due to TNF-α production. Suppression of NK cells and perturbed regulation of immunoglobulin production could also exacerbate the disease. Enhanced binding of autoantibodies to the cell membrane of UV-irradiated keratinocytes may perpetuate tissue damage (Dov Golan *et al* 1992).

1.13. Sunscreens

As sunscreens prevent classical sunburn ie. erythema, it would be anticipated that they would also protect against UV-induced immunosuppression. The easiest way to reduce UCA photoisomerisation is to decrease the intensity of incident light, and the rate of *cis*-UCA formation is reduced by topical application of sunscreens (Krien and

Moyal, 1994). This is limited to broad-spectrum (ie. filtering out both UVB and UVA), highly protective sunscreens however. *Cis*-UCA formation is inversely related to the SPF, with protection against isomerisation exceeding the SPF values by a factor of greater than 10. After multiple exposures the protective effect of classical UVB filters is lost, with only those blocking both UVA and UVB retaining activity against photoisomerisation (Krien and Moyal, 1994). Thus, sunscreens which prevent sunburn may protect against at least one mechanism (*cis*-UCA) of immunosuppression, with the degree of protection determined by the nature of the sunscreen.

Attempts to develop systems to assess the immune protection factor of sunscreens, in comparison with their SPF have resulted in much discussion (Davenport *et al* 1998; Gasparro, 1998; Wolf and Kripke, 1998; Chu *et al* 1998). Davenport *et al.*used breast tissue explants as a source of epidermal cells for use in MECLR experiments, finding that the reduction in the elicited response was prevented by application of various sunscreens (Davenport *et al* 1998). The degree of protection is debatable, and may not exceed the SPF (Wolf and Kripke, 1998).

Application of a high protection sunscreen (SPF 29) was found to prevent the UVB-induced suppression of both the primary allergic reaction, and CH elicitation, to DNCB (Whitmore and Morison, 1995). Broad-spectrum sunscreens stopped the immunosuppression of CH to a recall antigen (nickel) when applied before solar-simulated irradiation (suberythemal, once a day for five days). There was only a slight protective effect afforded by a narrow-band sunscreen (Damian *et al* 1997).

Application of sunscreens prevented infiltration of $CD36^+$ macrophages into irradiated sites when an erythemagenic, 4 day UVB exposure protocol was used (Hurks *et al* 1997). The influx of these cells is closely correlated with modulation of MECLR responses. However, when a suberythemagenic, 4 week protocol was employed, suppression of MECLR was not altered by sunscreen application (Hurks *et al* 1997).

Multiple, low-dose exposures to UV in solariums decreased both NK cell function and numbers (Hersey *et al* 1983), a phenomenon which was not prevented by sunscreen application (Hersey *et al* 1987). The sunscreens used in these studies did not block UVA-wavelengths. Other immunologic properties, such as immunoglobulin production, measured in pokeweed mitogen-stimulated cultures of B and T cells, and DTH, remained depressed (Hersey *et al* 1987). A decrease in the circulating CD4:CD8 ratio, with a rise in percentage of $CD8^+$ cells still occurred.

As prevention of UV-induced immunosuppression appears dependent on the nature of the sunscreens, caution regarding their use must exercised. Protection against visible damage such as erythema could encourage longer exposure, unwittingly enhancing damage to the immune system. Behaviour regarding sunscreen application is also vitally important. Physical sunscreens are more difficult to apply than chemical sunscreens, with the result that often less is applied (Diffey and Grice, 1997). Studies show both types of sunscreen are commonly used at volumes below those at which the commercial SPF is calculated (Stenberg and Larko, 1985; Diffey and Grice, 1997).

1.14. Diet

Various food components, including flavonoids (Middleton and Kandaswami, 1992), green tea polyphenols, silymarin, and pyridoxine, can influence the immune system, and when they are either fed or topically applied to irradiated mice, skin cancer incidence (Birt *et al* 1997; Gensler *et al* 1996), pyridimine dimer formation (Chatterjee *et al* 1996), and immune suppression (Reeve *et al* 1995; Katiyar *et al* 1995) are reduced. Pyridoxine-supplemented feed also reduces the suppression of CH after *cis*-UCA painting in mice (Reeve *et al* 1995).

There is more circumstantial evidence for the influence of dietary components in UV-induced immunosuppression in humans. Melanoma patients have decreased levels of selenium in their sera, compared with healthy individuals (Reinhold *et al* 1989).

Selenium is important in inducing free radical scavenging systems, and it is postulated that decreased levels increase susceptibility to various cancers (Clark, 1985). Topical L-selenomethionine application leads to an increased MED (Burke *et al* 1992). Rafferty *et al* (1998) have shown recently that sodium selenite and selenomethionine supplementation protect human keratinocytes and melanocytes *in vitro* from UVB-induced cell death. It has been hypothesised that an antioxidant-rich diet will protect against UV-induced free radical formation within the skin and subsequent cell membrane damage. During the course of a 3-6 months diet including fish-oil supplements, rich in ω-3 fatty acids, the MED rose progressively for each volunteer, but decreased within 10 weeks of termination (Rhodes *et al* 1994). The total fatty acid content within the epidermis rose from 1.8% to 24.2%, and was accompanied by thiobarbituric acid reactive substances (TBARS), indicating lipid peroxidation in irradiated skin. Thus, ω-3 fatty acids decrease UVB-erythemal sensitivity but increase susceptibility of the skin to lipid peroxidation (Rhodes *et al* 1994). In another fish-oil supplementation study, MED was again shown to increase, with a concomitant decrease in PGE_2 levels (Rhodes *et al* 1995). Reduced PGE_2 may be due to ω-3 fatty acid interference at more than one step. The ω-3 fatty acids may compete with the more common ω-6 fatty acids for metabolism of cyclo-oxygenase, or for release from cell membranes by phospholipases. It is also suggested that ω-3 fatty acids can act as an oxidisable buffer, reducing free radical damage to more essential structures.

Another anti-oxidant, vitamin E, offers partial protection against the UVA-induced decrease in the ability of human LC to present antigen (Clement-Lacroix *et al* 1996). These data imply, but do not confirm, a role for oxidative stress in the immunosuppressive effects of UVA.

In one single-diet study, it was found that β-carotene prevented UV-induced suppression of DTH (measured by a Multitest CMI kit, incorporating seven different test antigens), after 10 weeks compared to that in volunteers fed a placebo diet (Fuller *et al* 1992).

The following chapters will detail UV mediated effects on a variety of immune parameters. Firstly, the suppression of CH responses by UVB will be examined. Alterations induced within DLN of irradiated mice during both contact sensitisation and elicitation of CH will be addressed. The effects of UV radiation on LC within the epidermis, and subsequent changes in DC frequency within DLN will be reported. Finally, the molecular signals involved in altering immune paramaters within the skin will be presented. The aims of each approach are summarised at the beginning of each chapter.

Chapter 2

Materials and Methods

2.1. General

2.1.1 - Mice.

Female C3H/HeN (H-2^k) and BALB/c (H-2^b) mice aged 6-10 weeks were obtained from the specific pathogen-free animal breeding facility at the Medical Microbiology Transgenic Unit, University of Edinburgh. Male mice were used for some PCR experiments. Mice were maintained in rooms with UV filtered lighting, and were age matched to within 2 weeks in individual experiments.

2.1.2 - Growth medium and supplements.

RPMI-1640 (Flow Laboratories, Irvine, UK) was supplemented with 100 Iu/ml penicillin, 200 mg/ml streptomycin, 2 mM L-glutamine, 100 μg/ml gentamicin, 10 μg/ml fungizone, 25 mM HEPES buffer (Gibco BRL, Paisley, UK) and 10% heat-inactivated foetal calf serum (FCS; Gibco BRL), designated RPMI-FCS throughout.

2.1.3 - Cells.

PAM-212 (BALB/c strain mice-derived keratinocyte cell line (Yuspa *et al* 1980)) and B16 cells (C57Bl/6 melanoma-derived (Kreider and Schmoyer, 1975)) (both obtained from Dr. R McKenzie, Department of Dermatology, University of Edinburgh) were cultured in RPMI-FCS. Vero cells for HSV assays were also cultured in RPMI-FCS. Cells were harvested using 0.1% trysin/ 0.04% versene solution.

Epidermal cells were prepared from mouse ears. Ears were collected and floated dorsal side down on 0.76% tetrasodium ethylenediamine tetraacetic acid (EDTA; Sigma) in PBS, pH 7.2 for 2 hours, at 37°C. Ears were rinsed by passaging through PBS three times, and epidermal sheets removed with a scalpel blade. Single cell suspensions of were prepared by rubbing epidermal sheets through a steel gauze (J. Staniar and Co., Manchester, UK), before washing by centrifugation at 1000 rpm in RPMI-FCS.

2.1.4 - UV sources and exposures.

Mice were shaved on the back with electric clippers at least 24h before irradiating. Mice were irradiated under two Philips TL-20W/12 bulbs with an output range of 270-350 nm, peak 305 nm, emitting 80 mW/cm^2 (see fig. 2.1; Philips, Eindhoven, The Netherlands). The output of this source was determined using a filtered photodiode meter, calibrated against measurements made with a UV-visible spectroradiometer (model 742, Optronic Laboratories) across the spectral range 250- 400 nm (Dr. N Gibbs, Photobiology Unit, University of Dundee). The tube to target distance was 16 cm. Control mice were shaved and/or anaethetised where appropriate, but not irradiated. One MED for C3H/HeN mice under these conditions was 1500 J/m^2.

For studies examining the effects of narrow-band UVB exposure, a single TL01 lamp (Philips) was used. This lamp emitted predominantly at 311-312 nm, and the total irradiance was 200 $\mu W/cm^2$ (see fig. 2.1). One MED for C3H/HeN mice was 10,000 J/m^2 of TL01.

UVA1 exposure was from a Dr. Hoenle Bluelight lamp, emitting 38.5 W/m^2 (see fig. 2.1). Contaminating UVB was filtered out by shielding mice with a 1cm depth of cation X (provided by Dr. F de Gruijl, Utrecht) in distilled water, at a concentration of 0.1 mg/ml, in a perspex tray. Mice were anaethetised with

Hypnorm/Hypnovel in these experiments due to the length of exposure. One MED for C3H/HeN mice was 500,000 J/m^2 of UVA1.

2.1.5 - UV irradiation of cells.

Cells were monolayered in flat-bottomed 24-well plates (Iwaki, Japan) overnight, then rinsed with PBS. Cells were overlaid with 150 µl phenol-red free PBS and irradiated. PBS was removed and the cells incubated in a 1 ml volume of RPMI-FCS.

2.1.6 - UV irradiation of mice.

Mice were contained in a perspex box, with a maximum of four per irradiation to reduce sheltering by littermates. In experiments where ears were protected from UV-exposure, mice were shaved on the dorsum with electric clippers at least 24h before irradiating, and their heads covered with paper towels during irradiation. Due to the length of exposure needed for 500 kJ/m^2 UVA1, mice were anaethetised by intraperitoneal injection of 150 µl sterile distilled water containing 1.67 mg/ml Hypnorm (Janssen Pharmaceutical, Oxford, UK) and 0.83 mg/ml Hypnovel (Roche, Welwyn Garden City, UK). Control mice for UVA1 experiments were anaethetised, but not irradiated. Mice were carefully spread out in a perspex box during irradiation to reduce sheltering by littermates. Mice were limited to 4 per box for UVB irradiation, but were not anaesthetised.

2.1.7 - Urocanic acid isomers.

Trans-urocanic acid (*trans*-UCA; 4-imidazoleacrylic acid) was purchased from Sigma Chemical Co. (Poole, UK). *Cis*-UCA was prepared by thin layer chromatography after UV irradiation as described previously (Norval *et al* 1989a), by Dr. J Crosby, University of Bristol. Imidazole-4(5)-methylidene malonic acid (IMMA) and methylated imidazole-4(5)-methylidene malonic acid (MeIMMA) were a

gift from Dr. H Morrison, Dept. Chemistry, Purdue University. The chemical structures of these molecules are shown in fig. 4.1. These substances were dissolved in the vehicle DMSO:ethanol (1:9), and were dropped onto mouse ears which had been tapestripped twice 24h previously.

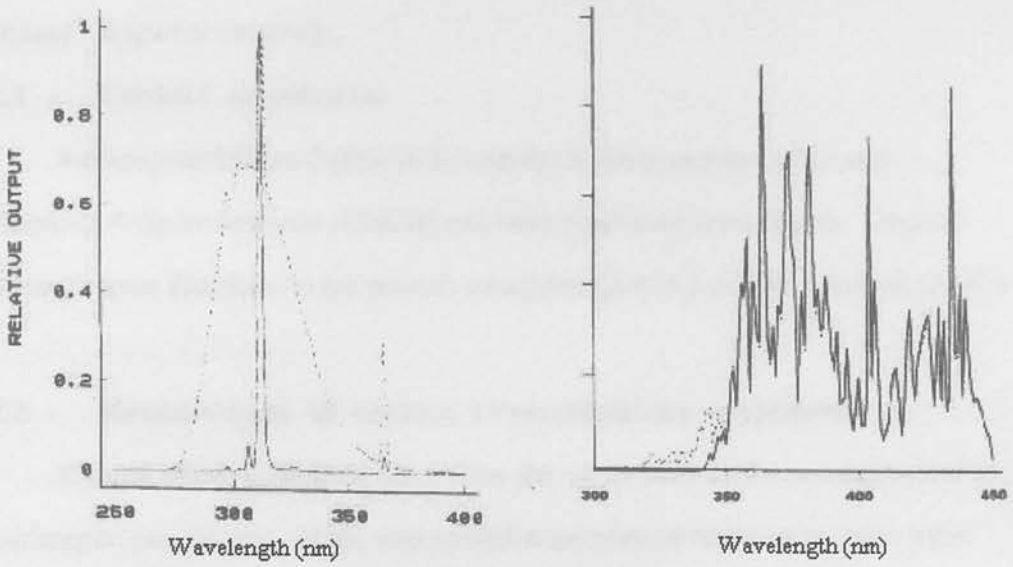


Figure 2.1. The spectral outputs of the UV lamps used during these studies. The outputs of the TL-01 (broad-band) UVB lamp (dotted line), and the TL-12 (narrow-band) UVB lamp (solid line), are shown in the left panel. The right panel shows the output of the Dr. Hoenle Bluelight (UVA1) lamp, with cation X filtration (solid line) or without filtering (dotted line).

2.2. Immune responses.

Contact hypersensitivity.

2.2.1 - Contact sensitisers.

4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone; Ox) and 1-chloro-2, 4-dinitrobenzene (DNCB) and were purchased from Sigma. Contact sensitisers were dissolved to the desired concentration in 4:1 acetone:olive oil (AOO).

2.2.2 - Measurement of contact hypersensitivity responses.

Groups of 6-8 C3H/HeN mice were shaved on their backs two days prior to experimental procedures. At the start of each experimental timecourse, mice were anaethetised, ears covered and the shaved dorsum exposed to doses of UVB as described in individual experiments. On day 0, 50 µl of contact sensitiser in acetone:olive oil (AOO, 4:1) was dropped onto the irradiated area, at sensitising concentrations. Sensitising concentration was 1% Ox, and was titrated for DNCB as described in section 2.3.2. Positive control mice were sensitised but not irradiated, and negative control mice were neither irradiated nor sensitised. Mice were tailmarked 10 days later, their ears measured with an engineer's micrometer (Draper Precision Instruments, Japan) as shown in fig. 2.2, and 20 µl of contact sensitiser diluted in AOO (4:1) to challenge concentration applied to the dorsum of each ear, of all mice. Challenge concentration was 0.25% Ox. A challenge concentration of 1% was used in initial DNCB CH elicitation experiments, and was altered between 0.4%, 0.75% and 1% for further experiments, as described in sections 2.3.4 and 2.3.5. Ears were remeasured 24h later. In some experiments, the challenge was repeated on days 11 and 12, with ear thickness monitored at 24h intervals. All ear measurements were carried out under blinded conditions. The degree of contact hypersensitivity was assessed as the function of the challenge-induced increase in ear thickness, with the mean incremental ear swelling and standard error of the mean (SEM) calculated for each group.

The percentage suppression was calculated according to the formula :-

$$\% \text{ suppression} = 100 - \frac{100 \text{ (increase in experimental- increase in negative control)}}{\text{(increase in positive control - increase in negative control)}}$$

2.2.3 - Proliferative responses of draining lymph node cells following contact sensitisation.

Female C3H/HeN mice were irradiated on the shaved back with 2 MED (2880 J/m^2) UVB (TL-20W/12) on two consecutive days. One day after the last irradiation, mice were separated into groups of 5 animals, and contact sensitised. Two groups had 25 µl of 1% Ox (Sigma) dropped on each ear, whilst another received 25 µl vehicle (AOO). Control groups contained unirradiated mice. Auricular lymph nodes were collected, pooled and single cell suspensions of lymph node cells (LNC) were obtained by rubbing lymph nodes through a steel gauze (J. Staniar and Co.), then washed by centrifugation at 1000 rpm in RPMI-FCS. Cells were resuspended in RPMI-FCS, viable cells counted in trypan blue and 2 x 10^6 viable cells added to wells in a 96-well round-bottom culture plate (Iwaki) in a 200 µl volume. Cells were radioactively pulsed by adding 2 µCi ^{3}H-thymidine to each well over a 24h period. Cells were harvested onto filter mats and the radiation incorporated measured on a scintillation counter (Canberra Packard, Zurich, Switzerland).

2.2.4 - Cytokine production by draining lymph node cells.

C3H/HeN mice were contact sensitised as described in section 2.2.2. Three days following contact sensitisation, draining auricular lymph nodes were removed. Alternatively, mice had CH responses elicited and DLN removed 3 days after the initial challenge (ie. on day 13 after contat sensitisation). Single cell suspensions were prepared by rubbing through a metal gauze (J. Staniar and Co.) and washed by centrifugation at 1000rpm in RPMI-FCS. Cells were seeded at 1 x 10^7 per ml in

24-well culture plates (Iwaki). Supernatants were removed at intervals up to 120h, and frozen at -70°C until assayed for cytokine content (mouse IL-10 and IL-12 ELISA, BioSource International (details in section 2.3.3); mouse IL-10, IFN-γ and IL-12 ELISA, AstraZeneca).

Delayed type hypersensitivity.

2.2.5 - Herpes simplex virus.

Type 1 herpes simplex virus (HSV-1) which was a plaque-purified isolate from a clinical case (Howie *et al* 1986), was passaged *in vitro* in Vero cells at a multiplicity of infection of 0.2 pfu/cell. The titre of stock virus was determined by plaque assay (see section 2.2.6), and the virus stored at -70°C. Killed virus was prepared by irradiating thin films of sonicated viral stocks with approximately 300 mJ/cm^2 UVB.

2.2.6 - Determination of viral titre by plaque assay.

Plaque assays for infectious HSV were performed using monolayers of Vero cells in flat-bottomed 96-well plates (Nunclon, Roskilde, Denmark). 1 x 10^5 cells per well were monlayered overnight. Following sonication of the stock, HSV was added to each well in serial dilutions and allowed to adhere to the cells for 1 hour, before overlaying with 0.25% Seaplaque agarose in the culture medium. After 2 days incubation with HSV at 37°C, cells were fixed in formol saline and stained with 10% Giemsa. Plaques were counted by low power light microscopy, and the titre calculated at 2 x 10^7 pfu/ml.

2.2.7 - Measurement of delayed type hypersensitivity responses.

Groups of 8 C3H/HeN mice were used for delayed type hypersensitivity studies. Mice were irradiated with 1500 J/m^2 UVB 3 days before infection with HSV. Another group of mice were injected subctaneously with 100 μg *cis*-UCA 5h before infection. Mice receiving anti-IL-10 (provided by Dr. SE Ullrich, Dept. Immunology,

University of Texas, Houston; clone. SXC-1) were injected intraperitoneally with 100 μg anti-IL-10 4h after UVB or *cis*-UCA exposure, with control mice injected with 0.1% BSA. In experiments investigating the effect of UCA analogues on DTH responses, analogues were injected subcutaneously 5h before infection.

To infect mice, 5×10^5 plaque forming units of live HSV in 100 μl PBS were injected subcutaneously. Positive control mice were infected but not irradiated, and negative control mice were neither infected nor irradiated. Ten days later, mice were individually tail-marked, their ears measured with an engineer's micrometer (Draper Precision Instruments) as shown in fig. 2.2, and 2×10^5 killed HSV diluted in 10 μl PBS injected into the pinnae of each ear. Mice were killed 24h later, and ears remeasured. All ear measurements were carried out under blinded conditions. The degree of delayed type hypersensitivity was assessed by the challenge-induced increase in ear thickness during this 24h period, with the mean incremental ear swelling and SEM calculated for each group.

The percentage suppression was calculated according to the formula :-

$$\% \text{ suppression} = 100 - \frac{100 \text{ (increase in experimental- increase in negative control)}}{\text{(increase in positive control - increase in negative control)}}$$

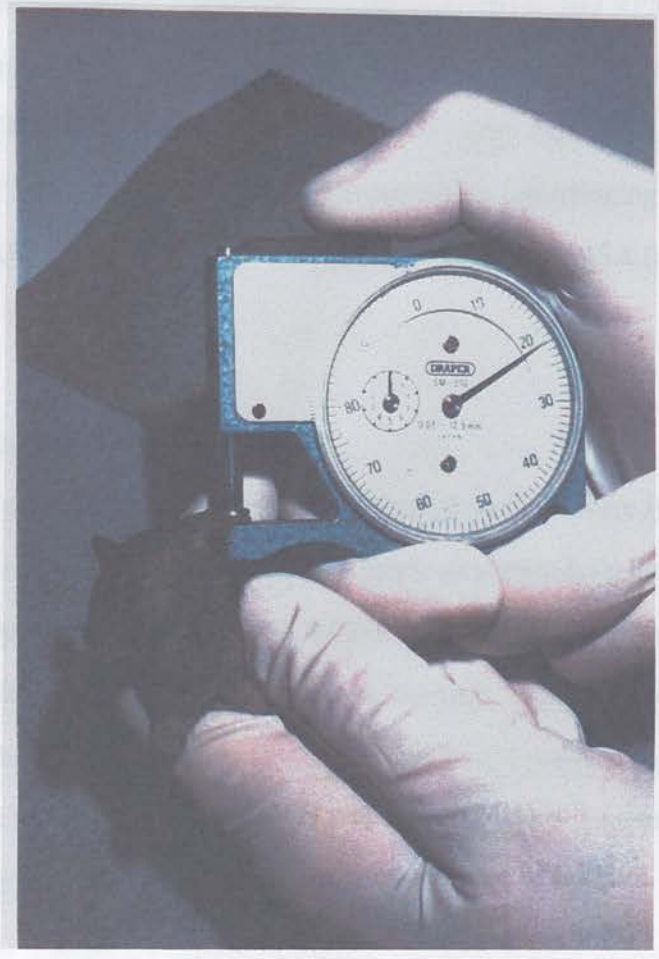


Figure 2.2. Measurement of mouse ear thickness with a micrometer. This was used to assess the inflammation induced by elicitation of CH or DTH responses (reproduced from K Halliday, Ph.D thesis, University of Edinburgh).

2.3. Effects at a cellular level

2.3.1 - Quantitative cytokine analyses in skin samples.

Skin samples (approximately 0.5 cm^2) were cut from the shaved back of C3H mice and weighed. These were finely chopped, removed to 1ml RPMI-FCS and snap frozen. Samples were thawed and homogenised using an Ultra-Turrex T8 hand-held homogeniser (IKA Laboratories, Stafen, Germany), snap frozen again, thawed and sonicated for 10s. Any skin remaining was removed by centrifuging at 3000 rpm for 5 mins, and the supernatant collected for cytokine analysis by ELISA (details in section 2.3.3).

2.3.2 - Preparation of cell lysates.

Cells were treated as described in section 2.1.5. Cells were lysed by adding 2 mls of ice-cold triple-detergent lysis buffer. Triple-detergent lysis buffer comprised 50 mM Tris HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide and 1% NP-40 (all Sigma), with 100 µg/ml phenylmethyl sulfonylfluoride, 1 µg/ml aprotonin and 0.5% sodium deoxycholate (all Sigma) added just before use. Lysates removed 30s after adding the lysis buffer were stored at -70°C until the cytokine content was assayed by ELISA (see section 2.3.3).

2.3.3 - Enzyme Linked ImmunoSorbent Assays (ELISA).

ELISA were conducted for IL-10, IL-12 and TNF-α using Cytoscreen Immunoassay kits (BioSource International, Camarillo, CA), according to the manufacturer's instructions. Briefly, for each assay a standard curve was created by addition of standards to a row of wells (standards ranged from 0-200 pg/ml IL-10, 0-500 pg/ml IL-12 and 0-1250 pg/ml TNF-α). Initial steps involved coating plates with standards and samples. For IL-12 assay, 100 µl standards were added to wells. Samples were diluted 1/2 with diluent buffer, and 100 µl added to wells. A 50 µl volume of biotinylated anti-IL-12 was then added to all wells except a chromagen

blank control. For TNF-α assay, 50 µl of standards and samples were added to each well, followed by 50 µl of biotinylated anti-TNF-α, except a chromagen blank control. Plates were incubated at room temperature for 2h before washing 4 times with wash buffer. For IL-10 assay, 100 µl of standards and samples were added to wells and incubated for 90 mins at 37°C, before washing 4 times with wash buffer. Biotinylated anti-IL-10 was then added to all wells except a chromagen blank for a further 45 mins at 37°C, before washing. As a second step in all assays, streptavidin-HRP was added to all wells except the chromagen blanks, and the plates incubated for a further 30 mins at room temperature (45 mins at 37°C for IL-10). Plates were washed and 100 µl stabilized chromagen added to each well for 15-30 mins in the dark, before addition of 100 µl stop solution. Absorbance at 450 nm of each well was read on a plate reader (MR 700 Plate Reader, Dynatech) blanked against the chromagen blank (consisting of stabilized chromagen and stop solution only). The standard curve was constructed and all sample concentrations calculated from this. Samples reading over the highest standard were diluted with standard diluent and re-analysed, with this additional dilution accounted for in the final calculation. IFN-γ, and some IL-10 and IL-12, assays were conducted using in-house ELISA developed by Dr. RJ Dearman, AstraZeneca Central Toxicology Laboratory.

2.3.4 - Immunoperoxidase staining of skin sections.

Skin sections were frozen in cryomatrix (Life Sciences International, Runcorn, UK) then cut on a cryostat. Sections were placed on microscope slides and fixed in 2% paraformaldehyde. Slides were stored at 4°C until staining. To stain, slides were allowed to heat to room temperature, and covered with methanol including 3% hydrogen peroxide (H_2O_2) for 30 minutes, before washing twice with PBS. Slides were overlaid with 20% normal rabbit serum in PBS for 20 minutes, and washed twice with PBS. Primary antibody (anti-IL-10, clone JES-2A5, Harlan Seralab, Loughborough, UK; anti-CD54 (ICAM-1), clone KAT-1, Serotec, Oxford, UK;

anti-Ia [MHC class II]; clone YE2/36HLK [rat IgG2a], Serotec; anti-IgG2a negative control, clone LO-DNP-16, Serotec) was diluted appropriately in PBS containing 3% bovine serum albumin (BSA), and overlaid overnight at room temperature. Slides were again washed twice with PBS, and overlaid with secondary antibody (biotinylated rabbit anti-rat IgG (H+L), Vector Laboratories, Peterborough, UK) diluted 1/100 in PBS containing 3% BSA, for 30 minutes. Slides were washed twice with PBS, and HRP-avidin (Vector Laboratories) diluted 1/100 in PBS containing 3% BSA added for 30 minutes. Slides were again washed in PBS, and developed by addition of diaminobenzidene (DAB; Sigma) for 5-10 minutes. Finally, slides were washed with PBS then water, allowed to dry, and mounted in DPX under a glass cover slip. Slides were stored at 4°C before examination.

Lymph node cells or splenocytes from untreated mice were used as positive controls. Tissues were collected and single-cell suspensions prepared. Cells were then cytospun and stored at 4^0C until stained.

2.3.5 - Immunoperoxidase staining of cells.

PAM-212 or B16 cells were seeded at 1 x 10^6 cells/chamber on 2-chamber slides, and allowed to adhere overnight. Cells were then irradiated in the presence of 150 µl phenol-red free PBS, which was then replaced with 1 ml RPMI-FCS. At the end of the experimental timecourse, cells were fixed in 2% paraformaldehyde before staining for IL-10 as described in section 2.3.4.

2.3.6 - Expression of ICAM-1 on PAM-212 cells.

PAM-212 cells were suspended in RPMI-FCS, 5 x 10^5 cells per well seeded in 24-well plates (Iwaki), and allowed to adhere overnight. Cells were then cultured in fresh RPMI-FCS, supplemented with varying concentrations of *cis*-UCA, *trans*-UCA or histamine, or combinations thereof, along with varying concentrations of TNF-α

(Genzyme). After 24 hours, cells were harvested by chemical (trypsin/ versene), or mechanical (scraping), removal from the plate, prior to washing by centrifugation in PBS. Cells were incubated with 100 µl of primary monoclonal antibody (1/100 dilution in PBS) (rat anti-mouse ICAM-1, and rat IgG2a anti-mouse negative control; both Serotec) on ice for 45 mins. PAM-212 were washed and incubated with 100 µl secondary detecting antibody (goat anti-rat IgG $F(ab)_2$ FITC-conjugate; Serotec) at a 1/100 dilution, on ice for 45 minutes. Finally, cells were fixed in formol saline and analysed by EPICS (Coulter).

Positive controls included LPS-stimulated peritoneal macrophages and DC from the draining lymph nodes of contact sensitised mice. Peritoneal macrophages were obtained from peritoneal lavage of mice with RPMI-FCS. Cells were washed by centrifugation at 1000 rpm for 5 mins, and viable cells resuspended at 5 x 10^5 cells/ml in RPMI-FCS. Cells were stimulated overnight with 5 µg/ml LPS. DC were identified in lymph node cell single-cell suspensions by their large size and high granularity. Negative controls, demonstrating non-specific staining, were typically set at 1%.

2.4. Effects on Langerhans cells and dendritic cells.

2.4.1 - Preparation of epidermal sheets.

Mice were exposed to UVB or UVA1 as detailed in section 2.1.6, or were painted with *trans*- or *cis*-UCA as detailed in section 2.1.7. In experiments examining the neutralizing effects of anti-TNF-α (20 µg) or anti-IL-1β (20 µg) (both goat anti-mouse antibodies, R&D Systems, Abingdon, UK), the antibodies were injected intraperitoneally in a 100 µl volume 2 hours before irradiation or UCA painting. Control mice were injected with the same volume of normal goat serum as a control. In experiments examining the effect of TNF-α (Genzyme, Cambridge, MA), 1000 U TNF-α was injected intradermally in a 20 µl volume 30 mins before irradiation. Control mice were injected with the same volume of 0.1% BSA as a control. Mouse

ears were collected into PBS and kept on ice prior to splitting with forceps. The ears were floated dorsal side down on 0.76% tetrasodium ethylenediamine tetraacetic acid (EDTA; Sigma) in PBS, pH 7.2 for 2 hours, at 37°C. Ears were rinsed by passaging through PBS three times, and epidermal sheets removed with a scalpel blade.

2.4.2 - ATPase staining of epidermal sheets.

Epidermal sheets were stained for adenine triphosphatase (ATPase) using adenine diphosphate (ADP) as a substrate (Chaker *et al* 1984). Epidermal sheets were fixed by floating in sodium cacodylate/formaldehyde buffer (6.85% sucrose, 1.6% sodium cacodylate, 0.1% formaldehyde in distilled water) for 1 hour at 4°C, before rinsing in PBS. Sheets were stained by adding to ADP-lead [0.12% $MgSO_4$, 5% D-glucose, 0.06% ADP (Sigma) in Tris/mal buffer (3.025% Tris buffer salt, 2.9% maleic acid, 1% NaOH; pH 7.3):distilled water, 2:3] for 70 mins at 37°C, prior to rinsing four times in Tris/mal buffer:distilled water, 2:3. Sheets were developed at room temperature by a 20 minute incubation in 2% ammoniumpolysulphide, and washed with water. Finally, epidermal sheets were mounted in 50% glycerol under a glass cover slip. Slides were stored at 4°C before examination, and the number of $ATPase^+$ cells counted in 10 fields per epidermal sheet (1 field = 0.1 mm^2), with a minimum of 4 sheets per group.

2.4.3 - Ia staining of epidermal sheets.

Epidermal sheets were fixed in prechilled acetone for 20 mins at -20°C. Following fixation, sheets were washed three times in PBS and placed in tissue culture plates for incubation with antibody solutions. Primary antibody (anti-Ia [MHC class II]; clone YE2/36HLK [rat IgG2a], Serotec; anti-IgG2a negative control, clone LO-DNP-16, Serotec) diluted 1/100 in 0.1% bovine serum albumin (BSA)/PBS was added and incubated for 30 mins at room temperature.

For fluorescent development, epidermal sheets were washed in three changes of PBS and secondary antibody (fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-rat IgG, clone STAR69, Serotec) diluted 1/100) in 0.1% BSA/PBS added. Sheets were incubated in the dark for a further 30 mins at room temperature. Antibody solution was discarded and tissue washed in PBS. Epidermal sheets were then mounted on microscope slides in glycerol/saline (9:1), using forceps to tease tissue, and kept in the dark at 4°C before examination by fluorescence microscopy. Concanavilin A (ConA) stimulated splenocytes were used as positive controls.

For immunoperoxidase development, endogenous peroxidase activity was blocked by a 30 min incubation with 3% H_2O_2 in PBS prior to addition of primary antibody. Epidermal sheets were washed in three changes of PBS and secondary antibody (biotinylated anti-rat IgG (H+L), Vector Laboratories, Peterborough, UK) diluted 1/100 in PBS containing 0.1% BSA, for 30 minutes. Slides were washed twice with PBS, and HRP-avidin (Vector Laboratories) diluted 1/100 in PBS containing 0.1% BSA added for 30 minutes. Slides were again washed in PBS, and developed by addition of DAB (Sigma) for 5-10 minutes. Finally, sheets were washed with PBS then water and mounted in DPX under a glass cover slip. Slides were stored at 4°C before examination.

Positive controls for both procedures consisted of either DLNC or spleen cells which were cytospun onto microscope slides. Slides were stained for MHC class II expression in the same manner as epidermal sheets.

2.4.4 - Dendritic cell enrichment.

The method of Macatonia and collegues (Macatonia, 1986) was followed to enrich DC populations from auricular lymph nodes following a variety of treatments. In experiments examining the effect of TNF-α (1000 U) (Genzyme), TNF-α was injected intradermally in a 20μl volume 4h before DLN collection. Control mice (12 per group) were injected with the same volume of 0.1% BSA as a control. In

experiments examining the neutralizing effects of anti-TNF-α (20 μg) or anti-IL-1β (20 μg) (both goat anti-mouse antibodies, R&D Systems, Abingdon, UK), the antibodies were injected intraperitoneally in a 100 μl volume 2 hours before irradiation. Control mice were injected with the same volume of normal goat serum as a control. Single cell suspensions were prepared by rubbing lymph nodes through a 200-mesh steel gauze (J. Staniar and Co.). Cells were washed by centrifugation at 1000 rpm in RPMI-FCS and resuspended in 8 ml RPMI-FCS. The cell suspension was gently underlaid with 2 ml 13.5% w/v metrizamide (Nygaard, Oslo, Norway) diluted in RPMI-FCS, and centrifuged at 600 g for 20 mins at room temperature. A DC enriched population was removed from the resultant interface (shown in fig. 2.3) and washed twice in RPMI-FCS. The pellet was resuspended in a minimal volume of RPMI-FCS, and DC counted by morphologic analysis by light microscopy. The enriched population typically comprised 60-85% DC of total cells counted.

Figure 2.3. The DC interface after centrifugation on a 14.5% Metrizamide gradient. The band contains a DC-enriched population (reproduced from A Moodycliffe, Ph.D thesis, University of Edinburgh).

2.5. Polymerase chain reaction (PCR).

2.5.1 - RNA isolation.

RNA was isolated from tissue samples using the SV total RNA isolation system (Promega, Madison, WI), according to the manufacturer's instructions. Mouse ears or samples of shaved back skin were weighed, removed into 1 ml SV RNA lysis buffer and homogenised. Alternatively, 5 x 10^5 cultured cells were trypsinised and washed, and the cell pellet resuspended in SV RNA lysis buffer. A 175 µl volume of lysate was removed to a microcentrifuge tube and 350 µl SV RNA dilution buffer added. The tube was heated to 70°C for 3 mins, then centrifuged at 12,000 rpm for 10 mins. The lysate was removed to a clean tube and 200 µl 95% ethanol added to it. This mixture was transferred to a spin basket, centrifuged onto a membrane (12,000 rpm, 1 min) and the eluate discarded. SV RNA wash solution was added to the spin basket, which was again centrifuged and the eluate discarded. DNase incubation kit (40 µl yellow core buffer, 5 µl 0.09M $MnCl_2$, 5 µl DNase I per reaction) was added to the spin basket membrane and incubated for 15 mins at room temperature. This reaction was stopped by addition of 200 µl SV DNase stop solution, and the spin basket centrifuged. The membrane was then washed consecutively by centrifugation with 600 µl and 250 µl SV RNA wash solution. Finally, 100µl nuclease-free water was overlaid on the membrane and RNA collected from the membrane by centrifugation into a collection tube. The isolated RNA was stored at -20°C.

2.5.2 - Reverse transcription of RNA isolates.

Purity and concentration of RNA isolates was determined by optical density. RNA was diluted in distilled water to concentration of 2 µg per 9 µl, and denatured by heating to 65°C for 2 mins, before quenching on ice. 11 µl of a reverse transcription (RT) mix was then added to each sample; 1 µl RNA guard (Pharmacia Biotech), 4µl 1st strand buffer, 0.01 M dithiothreitol (DTT; GibcoBRL), 2 µg oligo $dT_{(18)}$, 200 units

Moloney murine leukemia virus reverse transcriptase (MMLV RT; GibcoBRL), 1μl dNTPs (25 mM mix of dATP, dCTP, dGTP, dTTP; Pharmacia Biotech). Reverse transcription was allowed to occur by incubating at 37°C for 1h, and the reaction stopped by heating to 95°C for 5 mins.

Semi-quantitative PCR.

2.5.3 - Primer labelling.

Each primer was γ[^{32}P]-dATP -end labelled in a 30μl reaction mix; 5 μM primer, 30 units T4 polynucleotide kinase (PNK), 1x PNK buffer (both New England Biolab, Hitchin, Herts.) and 0.08 mCi/ml γ[^{32}P]-dATP (ICN Pharmaceuticals). The mixture was incubated at 37°C for 1h, before denaturing at 95°C for 5 mins (Progene, Techne (Cambridge) Ltd, Cambridge). IL-1β and TNF-α primers were kindly supplied by Dr. R McKenzie (Department of Dermatology, University of Edinburgh), β-actin, IFN-γ and IL-10 primers were purchased from Pharmacia Biotech. Primer sequences are detailed in table 2.1.

2.5.4 - PCR amplifications.

PCR was performed on 2 μl RT-reaction in a PCR cocktail containing 0.08 mM dNTP mix (Pharmacia Biotech); 0.5 units thermally stable "Red-hot" DNA polymerase (Advanced Biotechnologies, Leatherhead, Surrey, UK); 1x PCR buffer (Advanced Biotechnologies). For each primer pair the optimum Mg^{2+} ($MgCl_2$; Advanced Biotechnologies) concentration of the final mix was determined to eliminate non-specific signals. To establish the optimum Mg^{2+} concentration, RT products were amplified with unlabelled primers, and resolved on 1.5% agarose gels with ethidium bromide. Products were visualised under UV-light. Reactions were overlaid with mineral oil (Sigma), and 'hot-started' (D'Aquila *et al* 1991), by preheating RT-reactions and PCR cocktail to 85°C. Primers (Pharmacia Biotech) were added in a 1.5 μl mix. Products were amplified at 95°C for 1 min (denaturing), 65°C for 1 min

(annealing) and 72°C for 1 min (polymerisation). The optimum cycle numbers were determined for each primer set by plotting product accumulation against cycle number, following autoradiography (see section 2.5.5).

2.5.5 - SDS-PAGE and autoradiography.

Radioactive $\gamma[^{32}P]$-dATP -labelled PCR products were resolved by 12% SDS-polyacrylamide electrophoresis (SDS-PAGE) at 120 volts. Samples were run against a pUC digest DNA ladder (GibcoBRL). Gels were analysed by capturing with Grab-IT, then calculating raw volume of each band with GelWorks 1D Advanced (both programs from UltraViolet Products, Cambridge).

2.6 - Statistics.

Preliminary statistics were carried out by ANOVA. In examples where ANOVA indicated significant differences, Students *t*-tests were conducted between the relevant groups.

cytokine	primer sequence	product size (bp)	Mg^{2+} (mM)	primer conc^{n} (μM)	cycle no (PAM-212)	cycle no (skin)
β-actin	5' 5' - GTGGGCCGCTCTAGGCACCAA - 3' 3' 5' - CTCTTTGATGTCACGCACGATTTC - 3'	540	3.5	20	38	38
IL-10	5' 5' - ACCTGGTAGAAGTGATGCCCCAGGCA - 3' 3' 5' - CTATGCAGTTGATGAAGATGTCAAA - 3'	237	2	20	33	38
TNF-α	5' 5' - ATGAGCACAGAAAGCATGATCCGC - 3' 3' 5' - CAAAAGTAGACCTGCCCGGACTC - 3'	692	0.5	20	33	33
IL-1β	5' 5' - TCATGGGATGATGATGATGATAACCTGCT - 3' 3' 5' - CCCATACTTTAGGAAGACACGGATT - 3'	502	2.5	5	nd	33
IFN-γ	5' 5' - TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC - 3' 3' 5' - TGGACCTGTGGGTTGTTGACCTCAAACTTGGC - 3'	365	2.5	20	nd	28

Table 2.1. Primer sequences, expected product size and conditions of use for polymerase chain reactions (PCR) (nd = not determined).

Chapter 3

The effect of UVB on cytokine profiles during CH responses in C3H/HeN mice

3.1 Introduction

Many groups have shown an apparent unresponsiveness or immunological tolerance to antigens encountered through UVB irradiated sites (Schwarz *et al* 1996; Hammerberg *et al* 1996; Dai and Streilein, 1997; Niizeki and Streilein, 1997). This tolerance may be mediated by a selective activation of Th2 cells above Th1 cells.

Contact hypersensitivity (CH) is a common and relatively well investigated immune response induced by chemical antigens encountered through the cutaneous microenvironment, as described in section 1.3.6. This chapter investigates UVB-mediated effects on the CH response and alterations in cytokine production by DLNC which may account for UVB-mediated immune suppression. As the induction phase of CH is usually characterised by a Th1-like cytokine profile (Kimber and Dearman, 1997; Dearman *et al* 1996), it is postulated that UVB may alter this by enhancing Th2-like cytokine production, or at least by downregulating Th1-like cytokines. Multiple challenges with contact sensitiser were used to enhance any differences which were made by UVB pretreatment. Repeated antigenic exposure over a period of days is a means of skewing T cell responses to a greater extent, due to the negative cross-regulatory mechanisms both T helper subsets use (described in section 1.3.4). A similar technique has been used previously to investigate cytokine production by DLNC during elicitation of a CH response (Dearman *et al* 1996).

The elicitation phase of CH is also impaired in irradiated mice. As discussed earlier, the ear swelling response of CH occurs by early and late components (see

section 1.3.6). Investigations into the effects of UVB exposure on these components revealed that irradiation impairs both stages (Sontag *et al* 1994).

The aims of this area of research were -

A. to establish a CH protocol for the contact sensitiser DNCB,

B. to examine the effect of local UVB exposure prior to sensitisation on the CH response,

C. to examine cytokine profiles in DLN during the induction and elicitation phases of CH.

It was hypothesised that UVB would reduce the CH response to DNCB if given prior to sensitisation, and that this would possibly be due to a preferential bias towards Th2-like cytokine production by DLNC during at least one phase of the response. The first set of experiments served to establish a suitable CH regimen for study and examine effects of UVB on elicitation of this response, and the second investigates effects that UVB exerts during contact sensitisation.

RESULTS

Establishing a CH protocol for DNCB and investigating cytokine profiles upon the elicitation phase of CH.

3.2 Establishing a CH protocol for DNCB.

A basic protocol giving a suitably large CH response was required. Preliminary experiments were conducted to establish the optimum sensitising dose of DNCB for CH responses in C3H/HeN strain mice. Mice were sensitised by painting 50µl of either 0.1%, 1% or 2.5% DNCB on their shaved back-skin, and were challenged on their ears 10 days later with 20µl 1% DNCB (as described in section 2.2.2). This challenge dose was chosen as it had been used previous studies to investigate cytokine profiles of DLNC (Dearman *et al* 1996). Control groups consisted of mice which were given vehicle (AOO) only at the sensitisation stage. Challenges were repeated on 3 consecutive days with 1% DNCB, and the increase in ear thickness measured. Repeated challenges were used to boost the response and to further polarise cytokine profiles which were to be investigated later. Negative control mice were challenged with AOO only. A sensitisation dose of 0.1% DNCB did not provoke a CH response above that of unsensitised mice which were given only vehicle at the time of sensitisation. There was an increasing irritant response with repeated challenges, as measured by ear swelling in unsensitised mice (vehicle group). The optimal sensitisation dose was found to be 1% DNCB. The CH responses after sensitisation with varying concentrations of DNCB are shown in fig. 3.1.

3.3 Establishing an immunosuppressive UVB regimen for DNCB-induced CH.

The next experiments were designed to investigate whether, and in what regimen, UVB could suppress the CH response to DNCB. UVB was given in 3 different regimens; 1- A single 1440 J/m^2 UVB exposure 3 days before contact sensitisation; 2- 2 x 960 J/m^2 exposures on consecutive days before sensitisation; and

3- 3 x 960 J/m^2 exposures on consecutive days before sensitisation. All regimens suppressed the CH response elicited by a 1% DNCB challenge. Suppression was observed after all challenges, and was calculated at 45.5%, 62.1% and 53.0% for UVB regimens 1, 2 and 3 respectively, at 24h (calculated against the unsensitised, challenged group (veh), as described in section 2.2.2). The effects of the UVB regimens on the CH response can be seen in fig. 3.2. All UVB treatments caused a decreased CH response when compared with the positive control, and this was significant for regimens 2 and 3 at 48h. Therefore, repeated irradiations gave more marked suppression than a single exposure.

3.4 CH responses elicited by repeated challenge with weaker concentrations are more suppressed by UVB.

A protocol for UVB suppression of the CH response to DNCB was designed from the data obtained in sections 3.2 and 3.3. It was decided to use a regimen of 3 daily irradiations with 1440 J/m^2 UVB prior to contact sensitisation with 1% DNCB 1 day after the last irradiation. Mice were challenged with 20µl 0.4% DNCB 10 days later. A challenge dose of 0.4% was used to reduce the inflammatory response in mice due to challenges alone. This is shown in fig. 3.1, where repeated challenge of unsensitised mice with 1% DNCB (vehicle group) resulted in a large ear swelling by the end of the experiment. As can be seen in fig. 3.3, this new regimen suppressed the CH response at all times when repeated challenges with 0.4% DNCB were given, and the degree of suppression was maintained through to the end of the challenge regimen (21.4 and 47.9% suppession at 24h, 74.6 and 77.0% at 48h, and 67.4 and 63.1% at 72h).

3.5 Cytokine profiles of DLNC after CH elicitation by multiple challenges.

It was established above that the CH response to DNCB could be suppressed by UVB irradiation prior to contact sensitisation (section 3.4). To determine if the UVB-mediated suppression could be attributed to an altered cytokine profile, DLN were taken from mice which had been challenged repeatedly with DNCB (the same mice that exhibited depressed CH in fig. 3.3). Single cell suspensions of DLNC were prepared and cultured without any stimulus for up to 5 days, as described in section 2.2.4. Supernatants were collected at various times and then stored for cytokine analysis by ELISA (described in section 2.3.3). As can be seen in fig. 3.4, mice which had been irradiated before contact sensitisation showed similar Th1-like (IL-12 and IFN-γ) cytokine profiles upon elicitation of CH to those of positive control animals. Two positive control groups were conducted to establish the degree of inter-group variation. However, IFN-γ and IL-12 did not increase over the culture period, suggesting that cells may have been optimally activated before DLN collection. The Th2-like cytokine under investigation, IL-10, was not detected in any sample (data not shown).

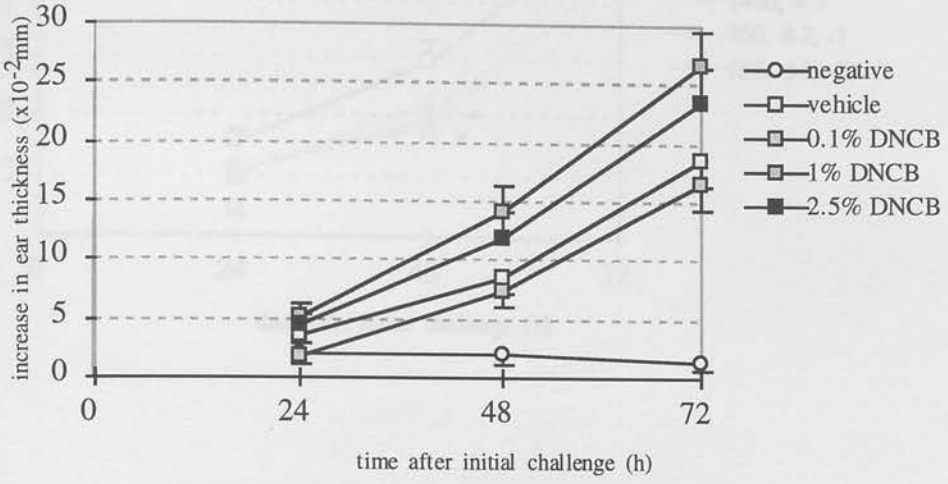


Figure 3.1. The dose response for DNCB sensitisation of C3H/HeN mice. Mice (n= 6) were sensitised on shaved dorsal skin with either 50µl AOO or DNCB, at concentrations stated (w/v). Ten days later mice were challenged by application of 20µl 1% DNCB to the dorsum of each ear (neg was challenged with 20µl AOO). 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. Results are shown expressed as the mean increase in ear thickness for each group, ± SEM. Challenges were repeated at 24 and 48h after the initial challenge.

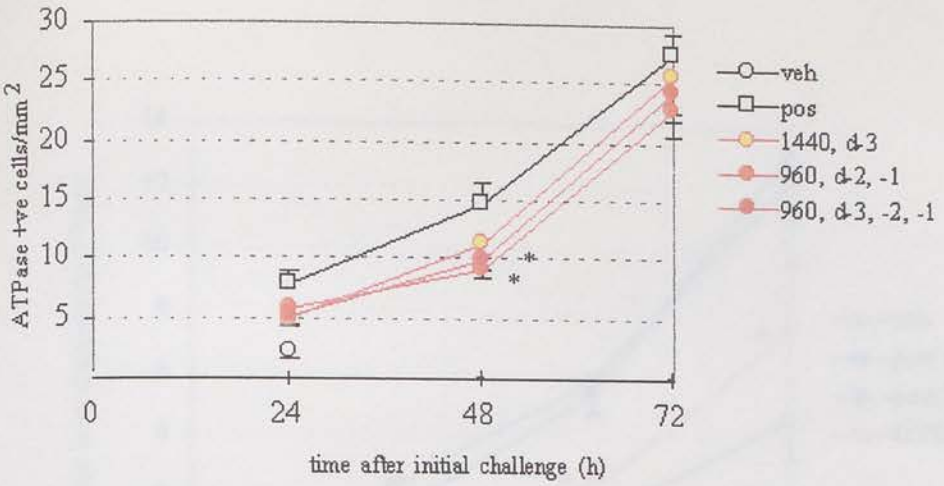


Figure 3.2. Immunosuppression of the CH response to DNCB by various UVB regimens. Mice (n= 6) were shaved on their back and irradiated with either 1440 J/m^2 UVB 3 days before sensitisation, 960 J/m^2 on 3 consecutive days before sensitisation, or 960 J/m^2 on 2 consecutive days before sensitisation. One day later mice were sensitised on shaved area with either 50µl vehicle (AOO) or 50µl 1% DNCB. A further ten days later mice were challenged by application of 20µl 1% DNCB 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. Results are expressed as the mean increase in ear thickness for each group, ± SEM. Challenges were repeated at 24 and 48h after the initial challenge. *= $p < 0.05$, vs. positive control.

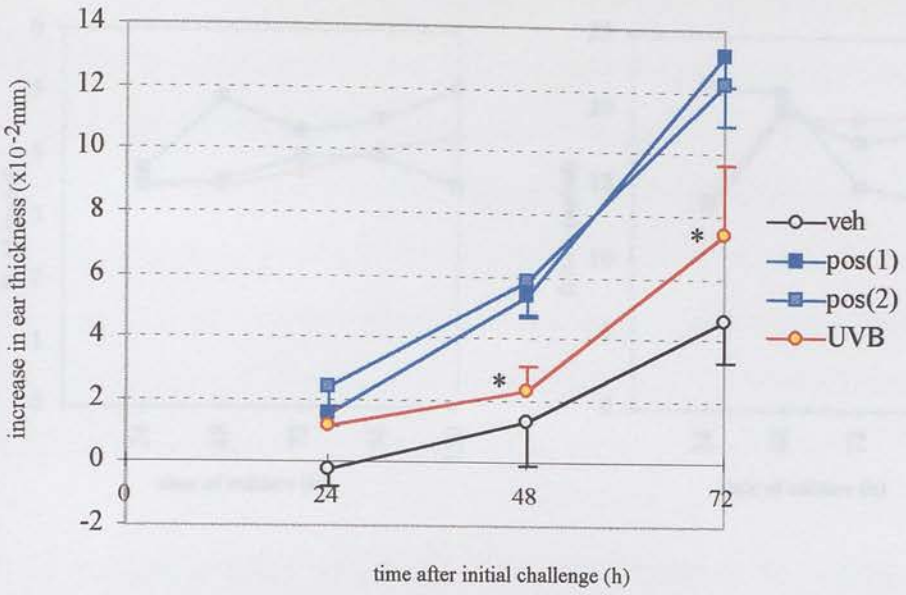


Figure 3.3. UVB can suppress the CH response to DNCB even if several challenges are given. Mice (n= 6) were shaved on their back and irradiated with 1440 mJ/m^2 UVB on three consecutive days. One day later mice were sensitised on shaved area with either 50µl AOO or 50µl 1% DNCB. Ten days later mice were challenged by application of 20µl 0.4% DNCB 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. Challenges were repeated at 24 and 48h after the initial challenge. Significant differences between the positive, unirradiated controls and the irradiated group were determined by Student's *t*-test. *= $p< 0.05$, vs. positive controls.

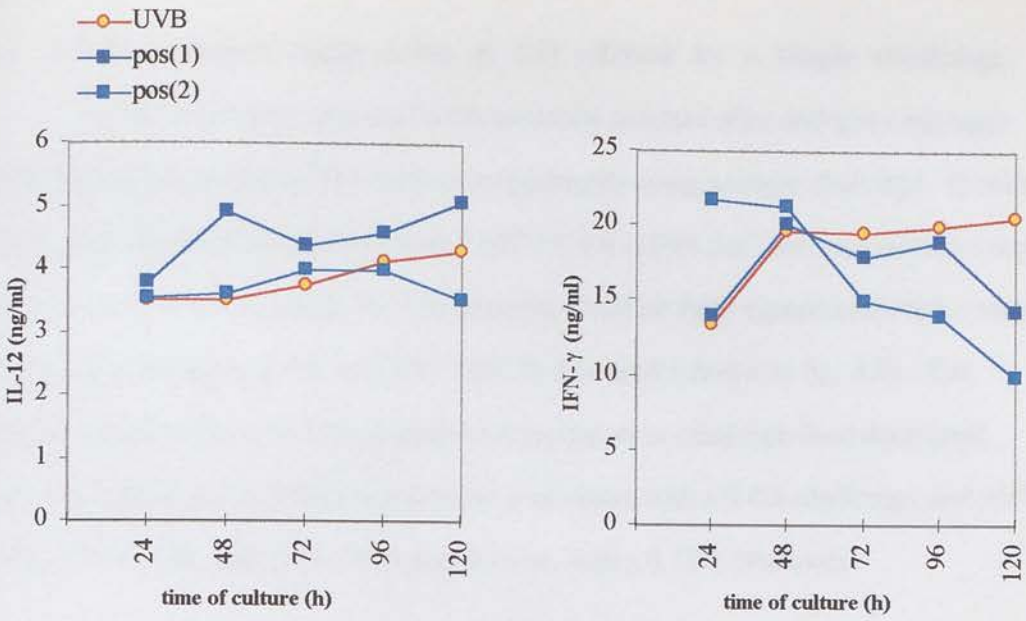


Figure 3.4. Th1-like cytokine production by DLNC following elicitation of CH, with multiple challenges. C3H/HeN mice were irradiated with 1440 J/m^2 UVB on their shaved backs on 3 consecutive days. Mice were sensitised through the irradiated site with either 50µl 1% DNCB or AOO one day after the last irradiation. Similar groups were established with unirradiated control mice, with 2 positive control groups established to counter intra-experimental variation. CH was elicited 10 days later by dropping 20µl 0.4% DNCB onto the ears. This was repeated on day 11 and 12. Auricular lymph nodes were removed on day 13 and single-cell suspensions prepared. Cells were seeded at 1 x 10^7 per ml in 24-well culture plates. Supernatants were removed at intervals up to 120h, and frozen at -70^{0}C until assayed for IL-12 (left) and IFN-γ (right) content (as described in section 2.3.3).

3.6 UVB-mediated suppression of CH elicited by a single challenge.

As Th1 cytokines appeared to be optimally secreted after multiple challenges with DNCB, it was decided to revert to experiments using a single challenge. If mice were given repeated irradiations with 1 MED UVB (1440 J/m^2, for 3 consecutive days) prior to contact sensitisation, the CH response could be fully suppressed when a single challenge with either 0.4% or 0.75% DNCB was used (shown in fig. 3.5). The relative suppression in UVB exposed mice increased as challenge dose decreased. This was calculated at 100% suppression at all times with a 0.4% challenge, and 81% (24h), 69% (48h) and 74% (72h) suppression with a 0.75% challenge.

3.7 Cytokine profiles of DLNC after CH elicitation by a single challenge.

Contact sensitisation was established in groups of unirradiated or UVB irradiated mice. CH reponses were elicited by a single challenge 10 days after sensitisation, and DLN removed 3 days later to examine the effect of cytokine profiles. As shown in fig. 3.6, the CH response was significantly suppressed in the irradiated mice (completely at 48h, and by 56.1 or 51.2% at 72h, in comparison with control groups). Single cell suspensions of DLNC were prepared and cultured without any stimulus for up to 5 days, as described in section 2.2.4. Supernatants were collected at various times and then stored for cytokine analysis by ELISA (described in section 2.3.3). Th1-like cytokines increased throughout the culture period, indicating that the cells were still stimulated 3 days after challenge, and that they had not been driven to optimal secretory patterns. As shown in fig. 3.7, cells from mice which had been irradiated (UVB group) were less able to secrete Th1-like cytokines (IFN-γ and IL-12) than mice which were mounting an ordinary CH response (pos groups). As with the multiple challenge regimen, the Th2-like cytokine under investigation, IL-10, was undetectable at all times (data not shown; IL-10 ELISA limit of detection was 100 pg/ml, in this case).

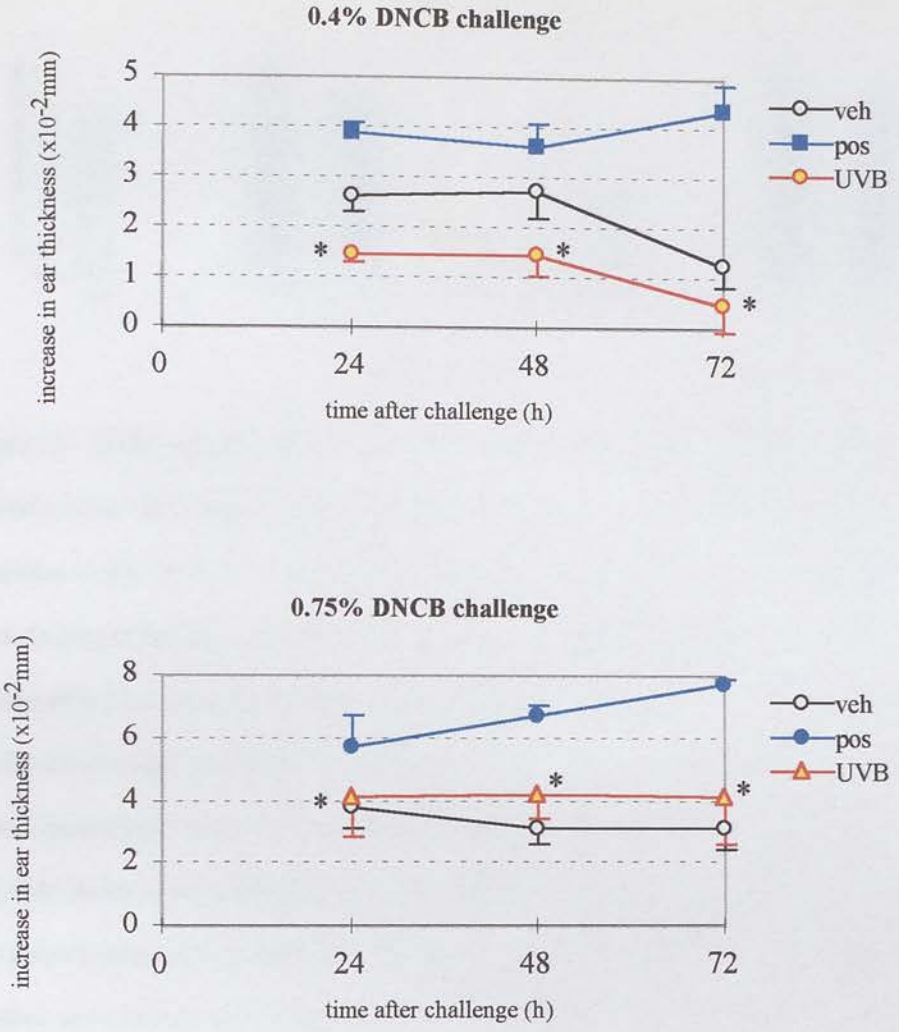


Figure 3.5. UVB exposure induced suppression of the CH response to DNCB, regardless of challenge concentration. Mice (n= 6) were shaved on their back and irradiated with 1440 J/m^2 UVB on three consecutive days. One day later mice were sensitised on the shaved area with either 50µl vehicle (AOO) or 50µl 1% DNCB. Ten days later mice were challenged by application of 20µl 0.4% (top) or 0.75% (bottom) DNCB 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. Measurements were repeated at 24 and 48h after the initial challenge. Significant differences between the positive, unirradiated control groups and the irradiated group were determined by Student's *t*-test. *= $p < 0.05$, vs. positive controls.

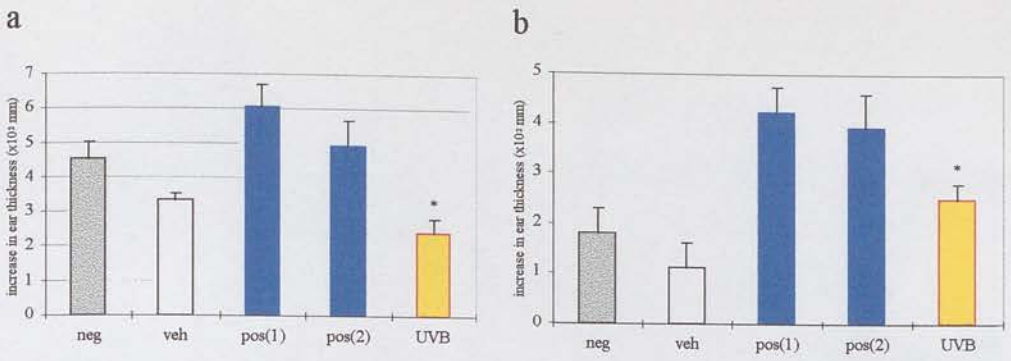


Figure 3.6. UVB suppresses the CH response to DNCB after a single challenge. Mice (n= 8) were shaved on their back and irradiated with 1440 J/m^2 UVB on three consecutive days. Mice were sensitised on shaved area with either 50µl AOO or 50µl 1% DNCB on day 0. Ten days later mice were challenged by application of 20µl 0.4% DNCB to the dorsum of each ear. Negative (neg) control mice received AOO at both phases, whereas vehicle (veh) control mice were not sensitised but were challenged with DNCB. Two positive control groups were established to counter intra-experimental variation. 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. Ear measurements were carried out at 48h (a) and 72h (b). Significant differences between the positive, unirradiated control groups and the irradiated group were determined by Student's *t*-test. *= $p < 0.05$, vs. positive controls.

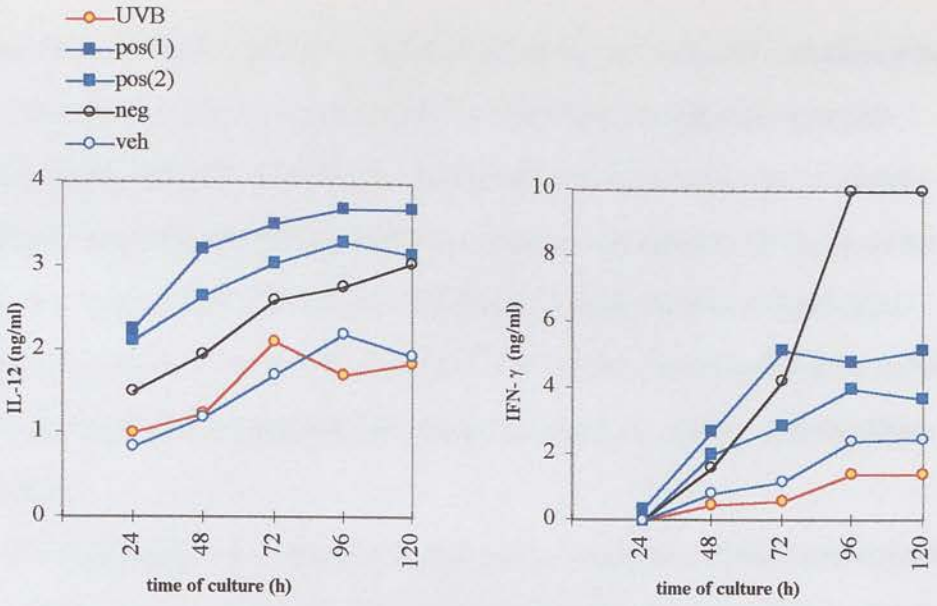


Figure 3.7. Th1-like cytokine production by DLNC following elicitation of CH, after a single challenge. C3H/HeN mice were irradiated with 1440J/m^2 UVB on their shaved backs on 3 consecutive days. Mice were sensitised through the irradiated site with either 50μl 1% DNCB or AOO one day after the last irradiation. Similar groups were established with unirradiated control mice. CH was elicited 10 days later by dropping 20μl 0.4% DNCB onto the ears. Auricular lymph nodes were removed on day 13 and single-cell suspensions prepared. Cells were seeded at 1 x 10^7 per ml in 24-well culture plates. Supernatants were removed at intervals up to 120h, and frozen at -70^{0}C until assayed for IL-12 (left) and IFN-γ (right) content (as described in section 2.3.3).

Investigating cytokine profiles upon induction of contact sensitisation.

There appeared to be a reduction in Th1-like cytokine profiles during the elicitation phase of the CH response to DNCB (when a single challenge was used). The cytokine microenvironment in which an antigen is encountered for the first time is an important factor in determining the subsequent cytokine profile produced upon restimulation of the specific T cells generated. The *in vitro* cytokine profile of cultured DLNC following UVB irradiation, and contact sensitisation, *in vivo*, was therefore investigated.

It was decided to investigate cytokine profiles during the contact sensitisation phase of CH using the contact sensitiser 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone; Ox). Ox is a stronger inducer of TNF-α within the skin than DNCB (Holliday *et al* 1997), and causes a greater lymphoproliferative response and DC accumulation within DLN (Kimber *et al* 1990c). The local lymph node assay, in which proliferation of DLNC following contact sensitisation is measured, can be used as an indicator of the strength of sensitiser (Kimber and Dearman, 1991; Kimber *et al* 1990b). As such, Ox represents a stronger contact sensitiser than DNCB.

A contact sensitisation protocol that had previously been used in our laboratory was adopted. This UVB exposure regimen was 2 daily 1440 J/m^2 exposures before sensitising a day later and had been shown to result in 46% suppression of the elicited CH response (M.B. Lappin, Ph.D thesis, University of Edinburgh). This UVB exposure regimen was adapted to 2 daily exposures of 2880 J/m^2 UVB.

3.8 The effect of UVB on the proliferative response of DLNC after contact sensitisation with Ox.

Mice were irradiated with 2 daily exposures of 2880 J/m^2 UVB and sensitised with 1% Ox one day later. Positive control groups consisted of unirradiated mice which were contact sensitised. Positive control groups were performed in duplicate within individual experiments in order to examine intra-experimental variation. The

cellularity of auricular lymph nodes removed from Ox sensitised mice was greater than those from irradiated, Ox sensitised mice. DNCB induced only a slight increase in DLN weight and cellularity. UVB irradiation resulted in an increase in DLN weight, but not in cellularity (shown in fig. 3.8). The belief that Ox would represent a better contact sensitiser to study the primary (sensitisation) response was reinforced by the findings.

In agreement with DLN cellularity, proliferative responses were reduced slightly by prior UVB irradiation in Ox sensitised mice (shown in fig. 3.9). Ox sensitisation resulted in a high proliferative response of DLNC, which was significantly suppressed by approximately 20% by UVB exposure ($p= 0.012$). In AOO treated (i.e. unsensitised) control mice, DLNC proliferation was slightly, but not significantly, increased following UVB irradiation.

3.9 The effect of UVB on cytokine profiles of DLNC after CH sensitisation.

Cytokine analysis showed that UVB radiation did not significantly alter the early (up to 36h) profiles of IL-12, IFN-γ and IL-10, following the induction phase of contact sensitisation (figs. 3.10a, b and c, respectively). Importantly, the IL-12 ELISA used in these experiments recognises both bioactive IL-12 (p70) and the free p40 subunit. IL-12 production was significantly increased ($p= 0.028$) in cells from UVB irradiated, contact sensitised mice compared with positive control (Ox) mice by 48h. Levels remained greater in UVB irradiated mice compared with the Ox group after this time, but these differences were not significant (shown in fig. 3.10). There was also an increase in IFN-γ by 96 and 120h of culture (shown in fig. 3.10), in supernatants from DLNC from UVB irradiated, contact sensitised mice compared with the Ox group. This increase was not significant. IL-10 was found in approximately 15-20% lower concentrations in cells from UVB, contact sensitised mice from 72h onwards (shown in fig. 3.10). This decrease was not significant at any time.

In UVB irradiated, unsensitised mice there was production of IL-12 from DLNC throughout the study (shown in fig. 3.10). Production was rapid in the first 12h (similar to contact sensitised mice), and this led to detectable IFN-γ by 48h (shown in fig. 3.10). IL-10 was not assayed in irradiated, unsensitised mice.

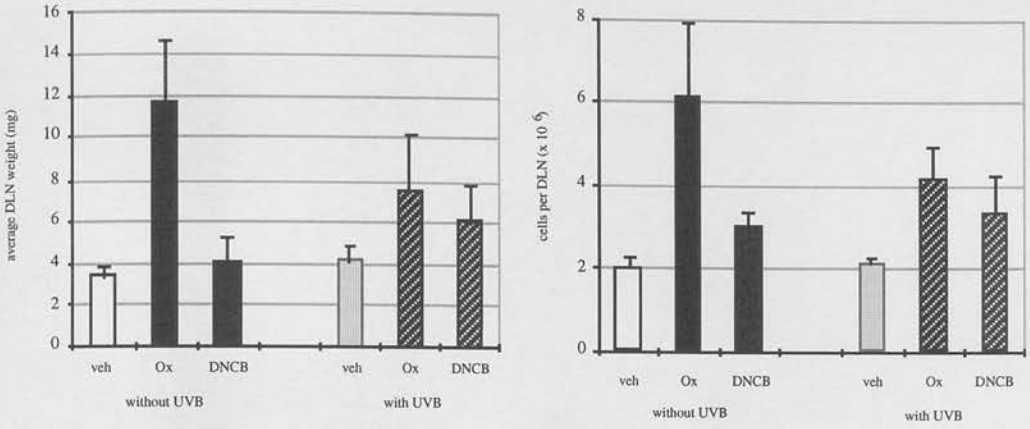


Figure 3.8. UVB irradiation leads to increased numbers of DLNC following induction of contact sensitisation. C3H/HeN mice were irradiated with 2880J/m^2 UVB on 2 consecutive days. One day after the last irradiation, mice were contact sensitised. Mice were sensitised by applying either 25µl 1% Ox, 1% DNCB or AOO to their ears. Similar groups were established with unirradiated mice. Three days following contact sensitisation, auricular lymph nodes were removed and weighed. Single-cell suspensions prepared, cells were counted and total cells per DLN calculated.

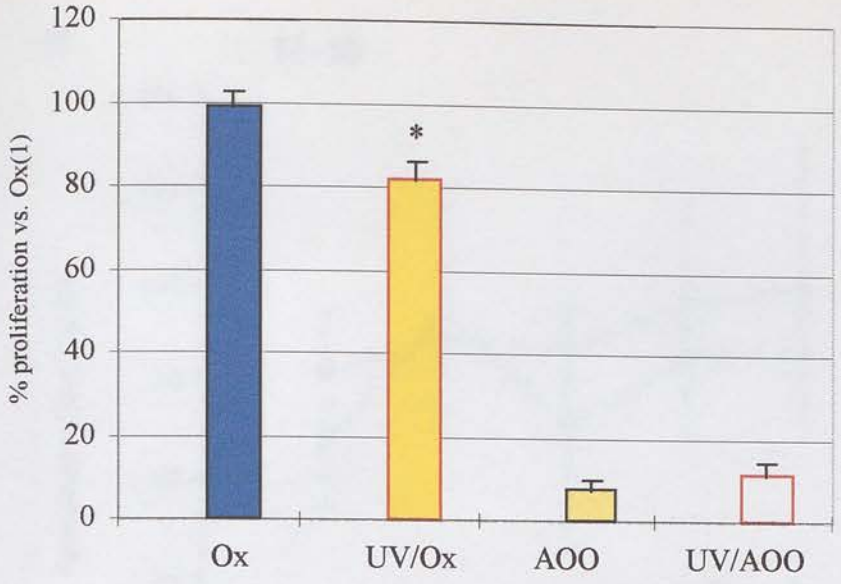


Figure 3.9. Spontaneous proliferation of DLNC following induction of contact sensitisation. C3H/HeN mice were irradiated with 2880J/m^2 UVB on 2 consecutive days. One day after the last irradiation, mice were contact sensitised, by applying either 25µl 1% Ox or AOO to their ears. Similar groups were established with unirradiated control mice. Three days following contact sensitisation, auricular lymph nodes were removed, and single-cell suspensions prepared. Cells were seeded at 2 x 10^6 per 200µl in 96-well round-bottom culture plates, and pulsed with 2µCi tritiated thymidine. Cells were harvested after 24h and the radiation incorporated measured. 3 individual experiments were performed and results are plotted as the average of the mean for individual experiments ± SEM, where 100% represents the proliferation induced by the group Ox (1) for each individual experiment (100% represents ^{3}H-thymidine uptake cpm of 42,190, 70,776 and 80,774 for 3 individual experiments). Values were normalised against these values due to variation in counts between individual experiments. *= $p < 0.05$, vs. unirradiated Ox group.

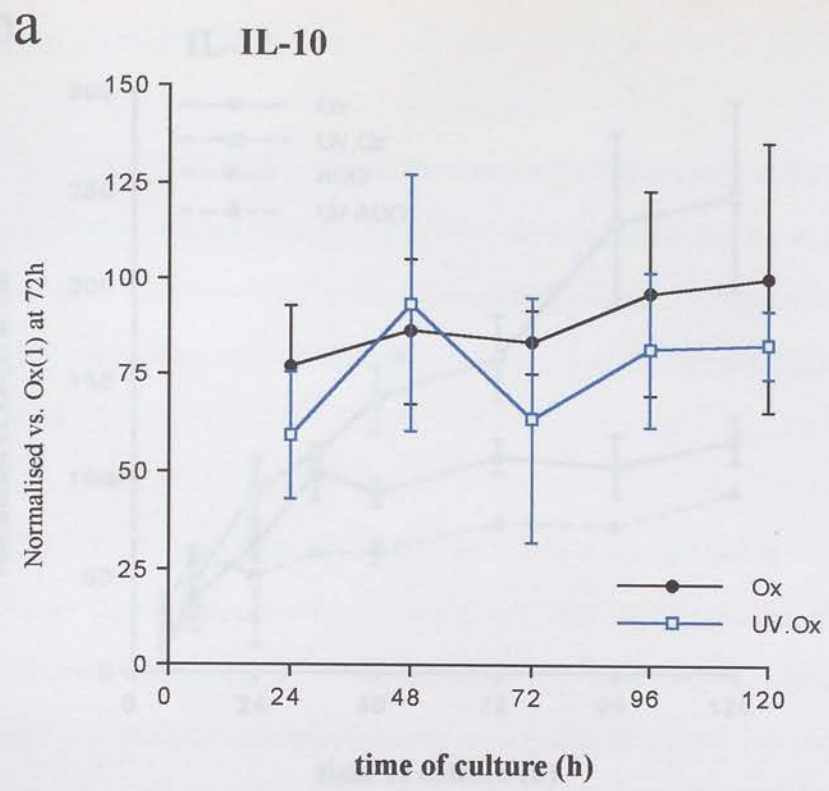
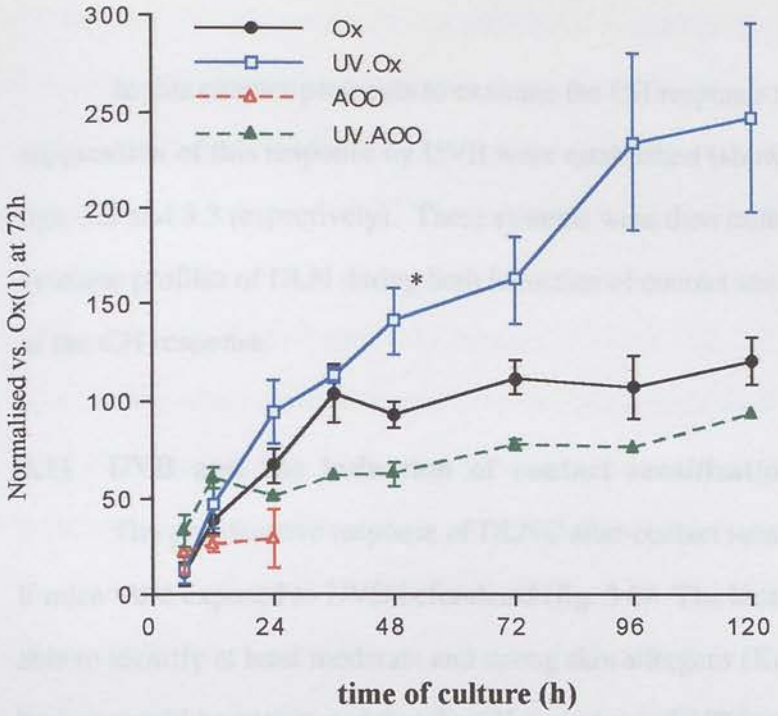
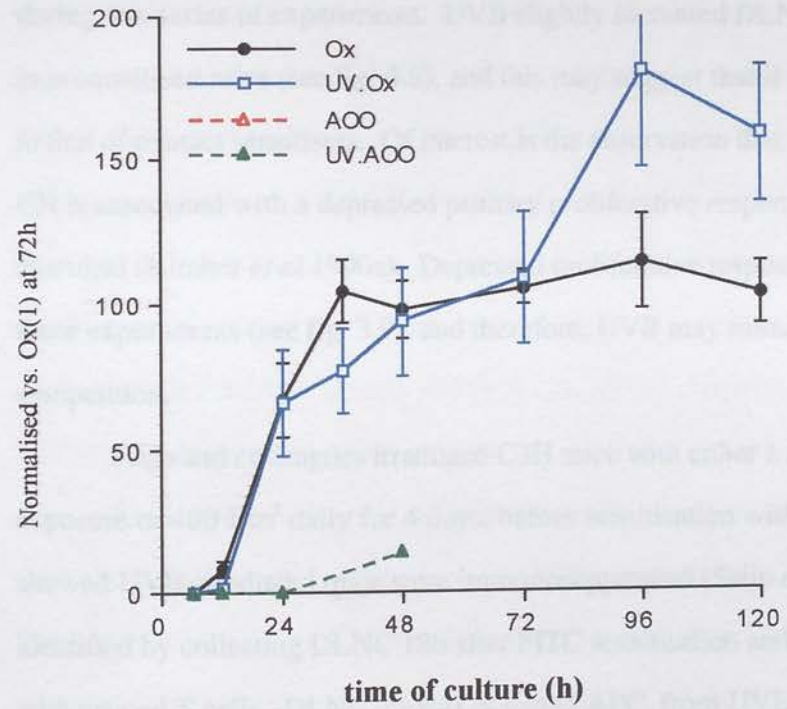


Figure 3.10. Cytokine production by DLNC following induction of contact sensitisation. C3H/HeN mice were irradiated with 2880J/m^2 UVB on 2 consecutive days. One day after the last irradiation, mice were contact sensitised. Mice were sensitised with either 25µl 1% Ox or AOO applied to the ears. Similar groups were established with unirradiated control mice. Three days following contact sensitisation, auricular lymph nodes were removed, and single-cell suspensions prepared. Cells were seeded at 1 x 10^7 per ml in 24-well culture plates. Supernatants were removed at intervals up to 120h, and frozen at -70^{0}C until assayed for cytokine content (as described in Mats and Meths, section 2.3.3). Results from individual experiments were normalised against the 72h value for Ox (1), and are represented as the mean ± SEM for at least 4 experiments (IL-10; 100% = 175.45 pg/ml, range; 168.5 - 182.4 pg/ml, IL-12; 100% = 6.62 pg/ml, range; 1.65 - 15.1 pg/ml, IFN-γ; 100% = 31.76 pg/ml, range; 16.75 - 62.8 pg/ml). * = $p < 0.05$ vs. Ox group.

b


IL-12
Ox
UV.Ox
AOO
UV.AOO
Normalised vs. Ox(1) at 72h
300
250
200
150
100
50
0
*
0
24
48
72
96
120
time of culture (h)

c


IFN-γ
Ox
UV.Ox
AOO
UV.AOO
Normalised vs. Ox(1) at 72h
200
150
100
50
0
0
24
48
72
96
120
time of culture (h)

3.10 DISCUSSION

In this chapter protocols to examine the CH response to DNCB, and suppression of this response by UVB were established (shown in fig. 3.1, and figs. 3.2 and 3.3 respectively). These systems were then utilised to examine the cytokine profiles of DLN during both induction of contact sensitisation and elicitation of the CH response.

3.11 UVB and the induction of contact sensitisation

The proliferative response of DLNC after contact sensitisation was suppressed if mice were exposed to UVB beforehand (fig. 3.6). The local lymph node assay is able to identify at least moderate and strong skin allergens (Kimber, 1992). On this basis it would be anticipated that the CH response in UVB irradiated mice would be reduced from that of unirradiated, contact sensitised mice. This was a regular feature during this series of experiments. UVB slightly increased DLN weight and cellularity in unsensitised mice (see fig. 3.8), and this may suggest that it acts in a manner similar to that of contact sensitisers. Of interest is the observation that antigenic competition in CH is associated with a depressed primary proliferative response to the second chemical (Kimber *et al* 1990a). Depressed proliferative responses are observed in these experiments (see fig. 3.9), and therefore, UVB may mimic antigenic competition.

Saijo and colleagues irradiated C3H mice with either a single 2 kJ/m^2 UVB exposure or 400 J/m^2 daily for 4 days, before sensitisation with FITC. They then showed UVB-irradiated mice were immunosuppressed (Saijo *et al* 1996). This was identified by collecting DLNC 18h after FITC sensitisation and then culturing *in vitro* with primed T cells. DLNC, or FACS-sorted APC, from UVB-irradiated mice induced around 40% less proliferation than those from normal mice.

Chronic activation of both murine and human $CD4^+$ T cells in the presence of IL-10 gives rise to T cells with low proliferative capacity (Groux *et al* 1997). However, UVB irradiation before contact sensitisation did not significantly alter IL-10 or IFN-γ secretion by DLNC compared with DLNC from unirradiated, contact sensitised mice (figs. 3.9 and 3.11 respectively). Exposure resulted in a significant increase in IL-12 production by 48h of culture, as can be seen in fig. 3.10. These data indicate that UVB irradiation may enhance synthesis of IL-12 by DLNC after contact sensitisation whilst having no effect on Th2 cytokine production. They therefore appear to contradict the initial hypothesis which was that, at the very least, Th1-like cytokines would be reduced in mice that had been UVB-irradiated.

In experiments by DiIulio *et al*, the presence of IL-12 during the sensitisation phase of CH increased the magnitude and duration of the response (DiIulio *et al* 1996). Injection of anti-IL-12 before sensitisation resulted in a reduced ear swelling upon challenge (Riemann *et al* 1996). Furthermore, Riemann and colleagues showed that when anti-IL-12 treated mice were rechallenged, the CH response remained minimal, suggesting that tolerance had been induced. Anti-IL-12 treatment after sensitisation but before challenge could also suppress CH (Riemann *et al* 1996). Anti-IL-12 injection before sensitisation inhibited UVB induced suppression of CH (Schwarz *et al* 1996). Injection of IL-12 into UVB treated mice (and sensitised through the same site) before challenge restored normal CH responses (Schwarz *et al* 1996). These observations indicate a role for IL-12 in both stages of CH, and strongly implicate it in UVB mediated immune suppression.

Kremer and colleagues showed that UVB irradiation of monocytes impaired their ability to stimulate proliferation and IFN-γ production of Th1 cells, in a dose-dependent manner, whilst retaining normal APC function to Th2 cells (Kremer *et al* 1996). Th0 cell stimulation was also normal, but exhibited a preferential suppression of IFN-γ production. This was related to decreased production of IL-12 p70 protein.

Recent findings suggest that IL-12 p40 may actually regulate Th1 responses. Mice transgenic for IL-12 p40 (the inducible subunit) have high serum levels of mainly monomers or p40 homodimers. Antigen induced cytokine production is skewed toward a Th2 profile, with decreased production of IFN-γ and increased production of IL-4 and IL-10, suppressed DTH responses and increased susceptiblity to malarial infection (a Th2-mediated disease) (Yoshimoto *et al* 1998).

The IL-12 ELISA used for analysis of DLNC supernatants measured both the IL-12 p40 subunit and the bioactive p70 heterodimer. It is possible that UVB increases IL-12 p40 production by DLNC, and skews immune responses by this antagonistic mechanism. However, IFN-γ began to increase in supernatants from UVB-irradiated DLNC at late timepoints (see fig. 3.11), suggesting that the IL-12 produced may have been biologically active. Interestingly, Schmitt *et al* observed increased IL-12 production by LNC from irradiated mice (Schmitt *et al* 1998). They irradiated mice then stimulated LNC *in vitro* in the presence of LPS. IL-12 production was increased in LNC from UVB-irradiated mice compared with LNC from non-irradiated mice, when measured by ELISA. Conversely, DC-enriched populations from the spleens of irradiated mice produced significantly less biologically active IL-12 than splenic DC from non-irradiated mice. Ullrich argues that secretion of IL-12 p40, which can bind IL-12 receptor, thereby interfering with IL-12 p70 signalling, is a result of UV-induced cytokines acting on DC (Ullrich, 1999). This may explain UV-mediated suppression of Th1 cells. Because IL-4, but not IL-12, is necessary for Th2 cell growth, Th2 cells would be unaffected in UV-irradiated mice.

In experiments conducted by Saijo *et al*, T cells from naive, or FITC-sensitised mice, produced less IFN-γ and IL-2 in response to 24h culture with APC from UV-irradiated mice than in response to APC from unirradiated mice (Saijo *et al* 1996). Th2-like cytokines (IL-4 and IL-10) were not detectable unless supernatants were concentrated, and were not consistently produced. Culture with APC from UV-irradiated mice did not increase Th2-like cytokine synthesis.

In experiments by Simon and colleagues, unirradiated BALB/c epidermal cells, LC, or splenic adherent cells, presented keyhole limpet hemocyanin (KLH) to both antigen specific Th1 and Th2 cell lines. *In vitro* irradiation of epidermal cells and LC with 20 mJ/cm^2 UVB led to loss of their ability to stimulate Th1 cell lines, but the cells retained APC function for Th2 cell lines. Irradiated splenic adherent cells lost APC function for both subsets (Simon *et al* 1990). This selective activation of Th2 cells was due to induction of clonal anergy in Th1 cells. Th1 cells were unable to respond to antigen presented by normal APC at later times (Simon *et al* 1991). This was not reliant on soluble factors, as supernatants from UVB-exposed LC or splenic adherent cells had no effect on Th1 cell proliferation.

IL-10 does not have any effects on anti-CD3 or alloantigen-induced LC-dependent T cell proliferation. Production of IL-2 and IFN-γ (but not IL-6) was reduced in these assays (Enk *et al* 1993b). IL-10 pretreated LC did not alter proliferative responses of Th2-cell clones (D10.G4), but these cells were unable to support proliferation of Th1-cell clones (AE7). Fresh or 1 day cultured LC were inhibitory toward Th1 cells, but by day 2 or 3 of culture, LC were affected to a much lesser extent by IL-10 pretreatment (Enk *et al* 1993b). DC treated with IL-10 were capable of supporting proliferation of, and IFN-γ secretion by, both $CD4^+$ and $CD8^+$ T cells in a mixed leukocyte response. In contrast, IL-10 markedly inhibited DC driven IFN-γ production by purified $CD4^+$ or $CD8^+$ T cells. Under the same conditions, macrophage-dependent proliferation was strongly inhibited. IL-10 inhibited DC induced production of IFN-γ by Th1 clones, and $CD4^+$ and $CD8^+$ T cells from unprimed mice (Macatonia *et al* 1993). These experiments suggest that it would have been beneficial to have purified DLNC into $CD4^+$ and $CD8^+$ T cell subsets, as this would have removed redundant signals between the cell types and may have made any preferential effects of UVB exposure easier to identify.

It has been shown previously that lymphocytes from UVR exposed mice produce less IL-2 and IFN-γ when activated *in vitro* by antigen or anti-CD3 (for

polyclonal T cell activation) (Araneo *et al* 1994). IL-4 secretion was consistently elevated in comparison to normal controls. Isotype analysis showed that specific antibodies to ovalbumin (the immunising antigen) was slightly reduced in all IgG subclasses, accompanied by an enhancement of IgA.

Thomson and colleagues found that IFN-γ was detectable in DLN 3 days after sensitisation with Ox, and this could be amplified by culturing the cells with anti-CD3 antibodies (Thomson *et al* 1993). Treatment of mice with anti-IL-4 before sensitisation did not affect DLNC production of measured cytokines (IFN-γ, IL-3, IL-4, GM-CSF) *in vitro*. Conversely, anti-IFN-γ caused a marked rise in IL-4, a lesser and more variable rise in IL-2, and a decrease in IFN-γ, particularly by $CD4^+$ cells. Despite these effects, anti-IFN-γ did not significantly alter CH reponses to Ox. This suggests that IFN-γ participates in, but is not wholly responsible for, the CH response. Anti-IL-4 did not alter CH outcome either, but it has been postulated that IL-4 may play a role in the effector, rather than the sensitisation, phase of CH (Gautam *et al* 1992). In agreement with this is the finding that IFN-$\gamma^{-/-}$ mice can mount CH responses as well as wild-type (C57Bl/6) mice (Reeve *et al* 1999). UVB and *cis*-UCA suppressed CH responses in both sets of mice (69% and 29% reduction in C57Bl/6, 79% and 27% in IFN-$\gamma^{-/-}$ respectively). Responses could be restored by injection of IFN-γ. This suggests that IFN-γ can antagonise the immunosuppressive action of *cis*-UCA (Reeve *et al* 1999).

Boonstra and colleagues found that IFN-γ production by purified splenic $CD4^+$ T cells was reduced if mice were irradiated before spleen collection and $CD4^+$ cell stimulation *in vitro* with anti-CD3 antibodies (Boonstra and van Oudenaren, 1998). They observed that polarization of both $CD4^+$ T cell compartments was reduced, but that the Th1 compartment was affected more severely than the Th2 compartment.

3.12 UVB and CH elicitation.

Miyauchi and Horio used a similar system to the one used here (described in section 2.2.2) and showed that the degree of CH suppression mediated by UVB was increased as the challenge concentration of contact sensitiser decreased (Miyauchi and Horio, 1995). This finding is corroborated by the results in section 3.4. They also found that suppression was greater if UVB doses were divided i.e. 2 exposures of 500 J/m^2, as opposed to a single 1000 J/m^2 exposure. This was observed in the series of experiments reported here, and is shown in fig. 3.2.

Initial cytokine analysis experiments were conducted using DLNC from mice which had had multiple (3) challenges with DNCB. Multiple challenges were used to enhance any differences which were made by UVB pretreatment, thereby making them easier to identify. Repeated antigenic exposure is a means of skewing T cell responses to a greater extent, with a more defined cytokine profile emerging (Mosmann *et al* 1991). However, it was found that in C3H mice, repeated elicitation of CH fully activated DLNC by 3 days. This was the time after primary elicitation that DLN were removed, and there did not appear to be any further cytokine production during the culture period (see fig. 3.4). Alterations in cytokine profiles were not apparent, and IL-10 could not be detected. A similar technique was used by Dearman and colleagues to investigate the cytokine profile of DLNC following a DNCB elicited CH response, in BALB/c mice (Dearman *et al* 1996). They could not detect IL-10 production in their experiments, but IFN-γ was produced throughout the study. Interestingly, IFN-γ levels detected after multiple challenges in BALB/c mice were similar to those found during investigation of DLNC cytokine profiles after a single challenge (see fig. 3.7). This may indicate that DNCB is a stronger contact sensitiser in C3H/HeN mice than in BALB/c mice.

It has been demonstrated previously that repeated application of the contact sensitiser 2, 4, 6-trinitro-1-chlorobenzene (TNCB) results in a site-restricted shift in

the time course of antigen-specific responses, from a delayed to an early-type response. The cutaneous microenvironment at the time of antigen presentation to T cells during the elicitation phase, rather than the induction phase of CH, is responsible for this shift. This also extends to unrelated third-party antigens. Repeated (chronic) exposure to TNCB led to a situation where high constitutive IL-4 mRNA levels were observed, and IL-10 mRNA was upregulated as early as 1-3h later, in an antigen specific manner. These changes were also observed at the protein level. Acute exposure to TNCB increased mRNA expression of IL-2, IFN-γ, and TGF-β. Chronic exposure elevated the mRNA expression for IL-4 and IL-10, with the immunoregulatory cytokines appearing to differentiate the regimens (Kitagaki *et al* 1997).

Reversion to a single challenge regimen allowed cytokine production to be detected, as shown by increases in IL-12 and IFN-γ during culture (shown in fig. 3.7). IL-12 was measured by an ELISA that recognises both natural IL-12 (p70) and the free p40 subunit. In these analyses, UVB irradiation before contact sensitisation appeared to change the cytokine profile to that of a primary response, as cytokine production by UVB treated DLNC was similar to that of negative control mice (no sensitisation, DNCB challenge). IL-10 was not detectable at any time during culture. These data tentatively support the hypothesis that UVB reduces Th1-like immune responses, but not by upregulation of Th2-like responses (Saijo *et al* 1996).

In a secondary immune response, T cells can encounter and be activated by antigen at the site of application, rather than in DLN as is the case in the primary response. When keratinocytes were used as accessory cells there was a specific defect in T cell IFN-γ production, whereas IL-2 and IL-4 were induced at levels comparable to those seen when professional APC were used as accessories (Goodman *et al* 1994). This was not due to non-productive CD28 engagement, as stimulation of this pathway by monoclonal antibodies still did not induce IFN-γ production in keratinocyte supported cultures. IL-2 was increased 10-fold in these CD28-triggered cultures,

demonstrating a specific deficiency in IFN-γ as opposed to a failure to respond to CD28 signalling. RT-PCR revealed that keratinocytes produced little or no mRNA for IL-12 p40 compared with professional APC, and addition of IL-12 to cultures restored IFN-γ production.

Noonan and colleagues reported that a single UV exposure suppressed the CH response to TNCB and DNFB (Noonan *et al* 1981). BALB/c mice had their ears covered and were irradiated on their shaved backs, then contact sensitised through the same site. A CH response was elicited by challenge on the ears 5-7 days later. The degree of systemic immune suppression was proportional to the $\log_{10}$ dose of irradiation, and exhibited a time delay before detection. Mice sensitised 1 day after UV treatment mounted normal CH responses upon elicitation, but if they were sensitised 3-15 days after irradiation, systemic CH was suppressed by around 70%. When normal mice were immunised with TNP-conjugated splenocytes from normal or UV exposed mice, a normal DTH response could be elicited by a later injection of TNP-conjugated splenocytes. UV-irradiated recipients could not be sensitised with splenocytes from UV-irradiated mice, although a time delay of 3 days was required to establish unresponsiveness (Noonan *et al* 1981). This indicates the blocking of sensitisation in UV-irradiated mice was due to defective antigen presentation. CH to DNFB can be impaired by intradermal injection with *cis*-UCA (200μg) into C3H/HeN, C3H/HeJ, C57Bl/6 and BALB/c mice (Kurimoto and Streilein, 1992).

3.13. Criticisms of experimental design and suggestions for further work

The experiments described herein investigated the effects of UVB exposure on both induction of contact sensitisation and elicitation of CH responses. Whilst IL-10 was detectable during the sensitisation phase, it was not detectable during elicitation. For this reason it would have been useful to have investigated another Th2-like cytokine, such as IL-4. A recent investigation by Garssen and colleagues has

identified IL-4 in culture supernatants following *in vitro* stimulation of DLNC derived from contact sensitised mice. IL-4 production by these cells was unaltered if mice were UVB irradiated before sensitisation (Garssen *et al* 1999).

It would also have been constructive to have investigated the cytokine profiles of purified $CD4^+$ or $CD8^+$ cells. As mentioned above, analysis of cytokine production by a mixed $CD4^+$ or $CD8^+$ cell population suggested that culture with IL-10 treated DC had no effect on cytokine profiles (Macatonia *et al* 1993). However, when cells were purified there was a clear differential effect on Th1- and Th2-like cytokine production. Examination of purified $CD4^+$ cells from spleens of irradiated mice showed a reduction in both Th1 and Th2-like cytokines investigated, but the effect was more severe on the Th1-like compartment (Boonstra and van Oudenaren, 1998). Masking effects may operate in DLN of UVB-irradiated mice, where induction of DC accumulation may lead to macrophage activation within the DLN. Macrophages may phagocytose apoptotic cells, leading to cytokine secretion by these macrophages and further complication of cytokine analysis when looking at the total DLNC population.

Chapter 4

Alterations in LC numbers after UVA1, UVB, or UCA

4.1 Introduction

It is recognised that UVB can suppress cell-mediated immune responses (Fisher and Kripke, 1977; Kripke *et al* 1979; Noonan *et al* 1981). More recent investigations indicate that UVA1 (340-400 nm) may also have such an effect (Clement-Lacroix *et al* 1996; El-Ghorr and Norval, 1999), although this area is controversial (Skov *et al* 1997; Reeve *et al* 1998). It has been shown, for example, that UVA1 at a dose of as small as 1000 J/m^2 can suppress DTH responses in C3H/HeN mice (El-Ghorr and Norval, 1999). In contrast, there is evidence from the hairless mouse (Reeve *et al* 1998), and more recently from C57BL/6 mice (Reeve *et al* 1999), that irradiation with total UVA (320-400 nm) before UVB partially protects mice from UVB-induced immunosuppression of CH. One possible mechanism for this immune suppression is through effects on APC at irradiated sites.

Cis-UCA may represent an important immunoregulatory tool, with potential application in a wide variety of situations. It has several facets that contribute to this. Firstly, *cis*-UCA is a small and naturally occurring molecule, so side-effects with regard to its use should be limited. While UVB phototherapy is used for skin conditions such as psoriasis, its use is limited by its erythemal side-effects. *Cis*-UCA is easily made *in vitro* from *trans*-UCA. *Cis*-UCA can be applied by a non-invasive regimen, in the form of an ointment or cream. Additionally, due to its size, the molecule can readily be manipulated to enhance its efficacy. However, the mechanisms by which *cis*-UCA mediates immune suppression remain unclear. The structures of *cis*- and *trans*-UCA are shown in fig. 4.1.

Imidazole-4(5)-methylidene malonic acid (IMMA) is a molecule which resembles both *trans*- and *cis*-UCA, and as such may be useful in determining immunomodulatory determinants on *cis*-UCA. IMMA has its imidazole and carboxyl functionalities permanently fixed in a configuration analogous to both *trans*- and *cis*-UCA (shown in fig. 4.1). Additionally, photoinactivation of bacteriophage single-stranded DNA is changed by a factor of 0.43 by IMMA, a value which is intermediate between the rate for *trans*-UCA (1.6) and *cis*-UCA (0.014) (Houghtaling *et al* 1996). Methylated IMMA (MeIMMA) is similar to IMMA but has a methyl group added to the 2-position of the imidazole ring (see fig. 4.1).

It has been known for some time that irradiation with UVB results in a decrease of LC, as assessed by CD1a, MHC class II or ATPase expression, from the irradiated site in the epidermis. The following experiments were carried out to investigate whether *cis*-UCA or UVA1 were capable of depleting LC from murine epidermis, as both modalities modulate immune responses which can be initiated by APC at the site of exposure.

The aims of these experiments were-

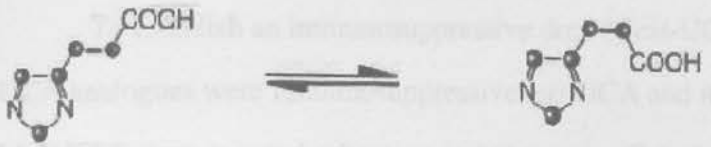
A. To examine the effects of UCA analogues on DTH responses.

B. To examine whether UVA1 could deplete LC when a given in a single, suberythemal dose.

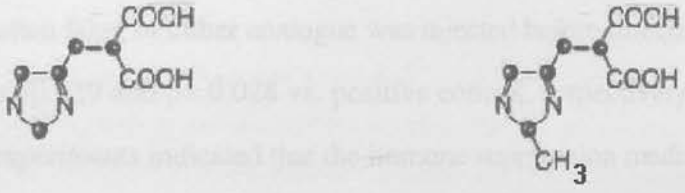
C. To examine wavelength interaction in regard to LC numbers.

D. To examine and establish timecourses for LC depletion mediated by UVB and *cis*-UCA.

E. To examine the role of IL-1β and TNF-α in UV-, and *cis*-UCA, mediated LC depletion.



Trans-UCA *Cis*-UCA



IMMA MeIMMA

Figure 4.1. The molecular structures of both UCA isomers and the UCA analogues, IMMA and MeIMMA.

4.2. UCA analogues can suppress the DTH response to HSV-1.

To establish an immunosuppressive dose of *cis*-UCA and to find out if the UCA analogues were immunosuppressive, *cis*-UCA and its analogues, IMMA and MeIMMA, were injected subcutaneously into mice 5h before infecting mice in the scruff of the neck with HSV-1. A DTH response was elicited 10 days later by injecting inactivated HSV-1 into the pinnae of the ear, and the increase in ear thickness measured 24h later (as described in section 2.2.7). *Cis*-UCA significantly suppressed the DTH response to HSV-1 by 40% when injected at a dose of 50μg ($p= 0.021$ vs. positive control). MeIMMA and IMMA also significantly suppressed the response when 50μg of either analogue was injected before infection (by 69% and 77%; $p= 0.029$ and $p= 0.028$ vs. positive control, respectively) (see fig. 4.2). Further experiments indicated that the immune suppression mediated by *cis*-UCA was dose-dependent, and both IMMA and MeIMMA showed similar tendencies (as shown in fig. 4.3). Immune suppression by *cis*-UCA has previously been shown to be dose-dependent (Norval *et al* 1989a).

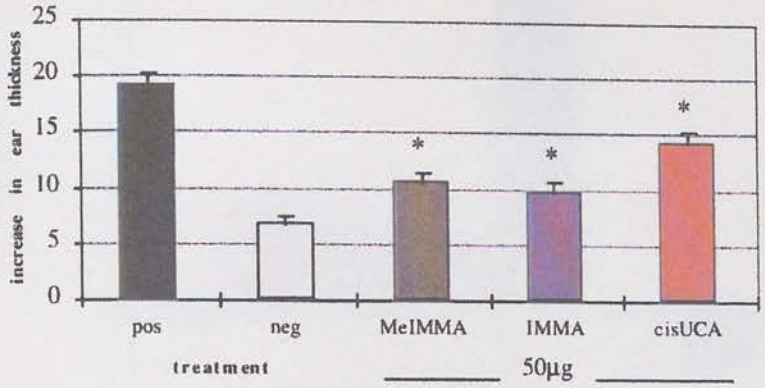


Figure 4.2. UCA analogues can suppress the DTH response to HSV-1. Mice (n=8) were injected subcutaneously with 50µg IMMA, MeIMMA or *cis*-UCA and infected with 5 x 10^5 pfu HSV 5h later. DTH was elicited 10 days later by injecting 2 x 10^4 pfu inactivated HSV into the pinnae of each ear. Increases in ear thickness were measured 24 hours later. The mean increase in ear thickness was used as a measure of the DTH response. Results are expressed mean ± SEM. Positive control mice (pos) were injected with vehicle only before infecting, and negative control mice (neg) were not infected. Significant differences between the positive control (pos) and the analogue treated groups were determined by Student's *t*-test; *= $p<0.05$.

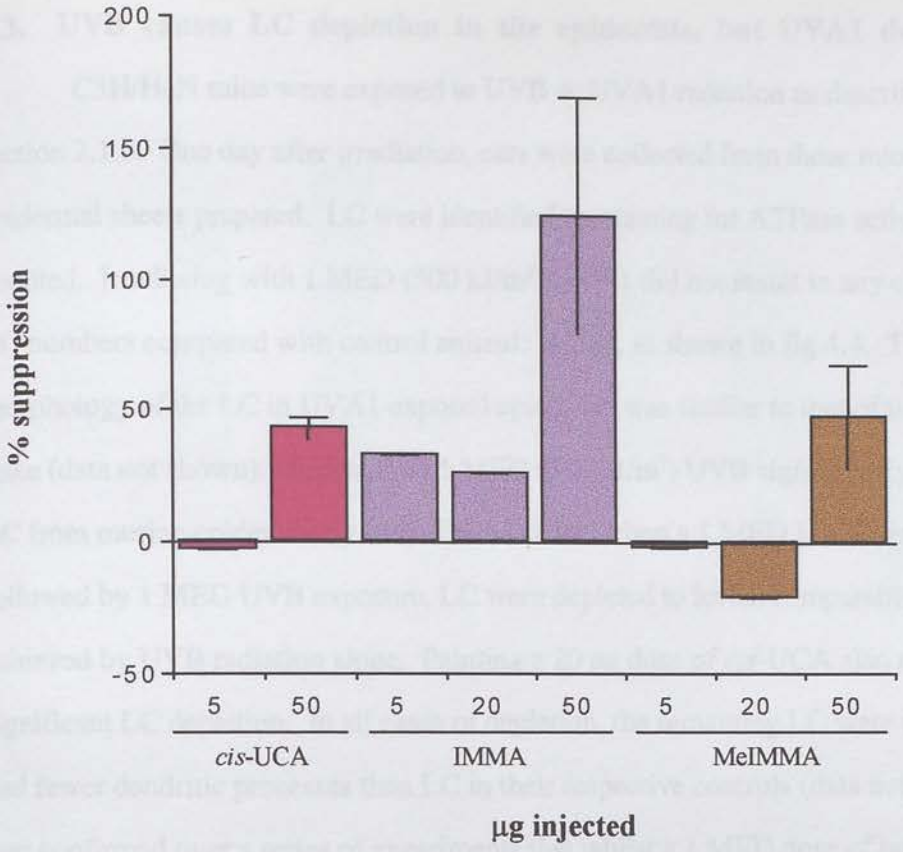


Figure 4.3. UCA analogues can suppress the DTH response to HSV-1. Mice (n=8) were injected subcutaneously with the analogue indicated and infected with 5 x 10^5 pfu HSV 5h later. DTH was elicited 10 days later by injecting 2 x 10^5 pfu inactivated HSV into the pinnae of each ear. Increases in ear thickness were measured 24h later. The mean increase in ear thickness was used as a measure of the DTH response. Results are expressed as percentage suppression for individual experiments, or mean of 2 experiments ± SD.

4.3. UVB causes LC depletion in the epidermis, but UVA1 does not.

C3H/HeN mice were exposed to UVB or UVA1 radiation as described in section 2.1.6. One day after irradiation, ears were collected from these mice and epidermal sheets prepared. LC were identified by staining for ATPase activity, then counted. Irradiating with 1 MED (500 kJ/m^2) UVA1 did not result in any change in LC numbers compared with control animals at 24h, as shown in fig 4.4. The morphology of the LC in UVA1 exposed epidermis was similar to that of unirradiated mice (data not shown). Exposure to 1 MED (1440 J/m^2) UVB significantly depleted LC from murine epidermis by 24h. Furthermore, when a 1 MED UVA1 exposure was followed by 1 MED UVB exposure, LC were depleted to levels comparable to those achieved by UVB radiation alone. Painting a 20 μg dose of *cis*-UCA also resulted in significant LC depletion. In all cases of depletion, the remaining LC were rounded and had fewer dendritic processes than LC in their respective controls (data not shown). It was confirmed over a series of experiments that whilst a 1 MED dose of broad-band UVB depleted LC by 25-30%, a 1 MED UVA1 exposure had no effect on LC frequency (see table 4.1).

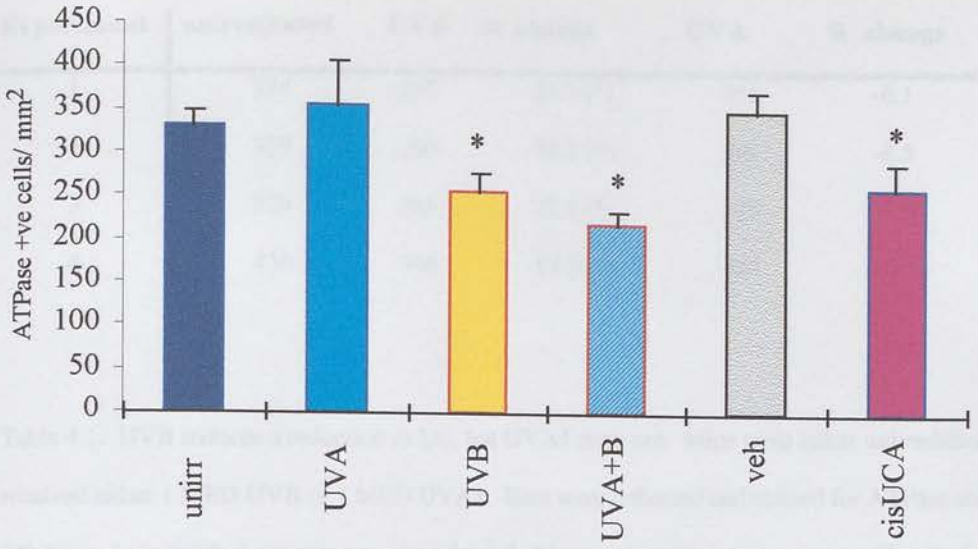


Figure 4.4. UVA1 does not deplete LC numbers and does not confer protection from UVB-induced depletion. Mice were treated with 1 MED UVA1, 1 MED UVB, or 1 MED UVA1 followed immediately by 1 MED UVB. For the veh and *cis*-UCA groups, ears gently were tape-stripped 24h before painting with DMSO/eth or 20μg *cis*-UCA. Ears collected 24h later. For UCA experimental groups, mice had their ears tape-stripped twice 24h before application of veh (DMSO/eth) or *cis*-UCA. Four epidermal sheets from each group were prepared and stained for ATPase activity. Results are shown as the mean ± SEM. Significant differences between the relevant control and the experimental groups were determined by Student's *t*-test. For UV treated groups, *= $p < 0.05$, vs. unirradiated, and for the *cis*-UCA group, *= $p < 0.05$, vs. vehicle.

Experiment	unirradiated	UVB	% change	UVA	% change
1	374	263	-29.7 (*)	351	-6.1
2	329	200	-39.2 (*)	301	-8.5
3	329	256	-22.2 (*)	355	+7.9
4	430	346	-19.5 (*)	432	+0.5

Table 4.1. UVB induces a reduction in LC, but UVA1 does not. Mice were either unirradiated, or received either 1 MED UVB or 1 MED UVA1. Ears were collected and stained for ATPase activity 24h later. In individual experiments, 4 epidermal sheets were prepared each group. Results from 4 individual experiments are shown as mean and percentage change from control values. *= $p < 0.05$, vs. control.

4.4. UVB-mediated LC depletion is dose-dependent.

The next series of experiments investigated the dose of UVB required to deplete LC from irradiated epidermis. As shown in fig 4.5, a dose of as little as 0.5 MED (750 J/m^2) broad-band UVB was enough to alter the morphology and number of LC. The effect was dose-dependent, with increased LC depletion in epidermal sheets irradiated with 1 MED (1500 J/m^2) broad-band UVB. A single MED (10,000 J/m^2) exposure of narrow-band UVB (TL-01) depleted numbers to similar levels compared with the same dose of broad-band UVB, but there was not a significant reduction with a 0.5 MED TL-01 exposure. The effect on morphology was not as marked after narrow-band UVB (TL-01) as with broad-band UVB (TL-12). UVA1 did not significantly deplete numbers, and again, if it preceded a 1 MED broad-band UVB exposure, did not confer protection against LC depletion.

4.5. A timecourse for UVB effects on LC.

Local and systemic immune suppression following UVB exposure exhibit different kinetics. Hammerberg *et al* (1996) examined the time needed between irradiation and contact sensitisation of C57BL/6 mice to establish suppression of CH. Suppression could be observed immediately, and up to 48h, after irradiation, if mice were sensitised through the irradiated site (local suppression). If mice were sensitised through unirradiated sites, a period of 3 days was required before systemic suppression was observable. The timecourse of ATPase$^+$ cell depletion by UVB was therefore examined. Mice were exposed to UVB at various times before collecting their ears and staining all samples for ATPase activity at the same time. There was a highly significant drop in LC within 12h of exposure to 1 MED UVB. 24h after irradiation LC numbers were still depleted, although to a lesser extent. LC frequency exhibited a slow and steady recovery thereafter, which can be seen in

fig 4.6a. Numbers fell by as much as 45% by 12h, with 27% depletion at 24h. Examination at earlier times showed that LC were significantly depleted as early as 6h after UVB exposure (by 32%), but that the peak in reduction was at 12h (see fig. 4.6b).

4.6. An extended examination looking for UVA1 effects on LC.

It was apparent that previous experiments using a standard 24h timepoint to investigate UVA1 effects on LC numbers may have missed the peak in alterations, either by looking after numbers had recovered, or by looking too early for any effects to have been mediated. LC frequency in UVA1 irradiated mouse epidermis was investigated at various times up to 96h after exposure. This can be seen in fig 4.7. A 1 MED UVA1 exposure did reduce LC frequency, but this reduction was not significant at any of the times examined (maximum reduction was from 374 to 318.25 per mm^2 (15%), at 48h; p= 0.17 vs. unirradiated 0h). The morphology of $ATPase^+$ cells present in the epidermis was similar at all times, with long dendritic processes clearly visible (data not shown).

4.7. A timecourse for *cis*-UCA mediated LC depletion from murine epidermis.

Mouse ears were gently tape-stripped twice 24h before they were painted with UCA isomers (as described in section 2.1.7). Tapestripping did not alter LC numbers in the epidermis, as shown in fig. 4.4. Tapestripped and vehicle (DMSO/eth) painted ears had the same LC frequency as untreated, unirradiated ears. Preliminary experiments had established that 20μg *cis*-UCA significantly depleted LC numbers by 24h after painting on to mouse ears (shown in fig. 4.4). Examination of the timecourse for $ATPase^+$ cell depletion following *cis*-UCA painting was then undertaken. UCA isomers were applied at various times before collecting the ears for ATPase staining. As shown in fig 4.8, maximal depletion of LC by *cis*-UCA occured

at 24h, and with a decrease from 374 to 260.75 ATPase$^+$ cells/ mm^2 (30.3%). The timecourse was not extended beyond this time. *Trans*-UCA reduced LC numbers only slightly, and this decrease was not significant at any time (a maximum of 9.3% at 24h). *Cis*-UCA induced LC migration therefore has a delayed timecourse in comparison with UVB irradiation, in which maximal depletion was achieved 12h following exposure.

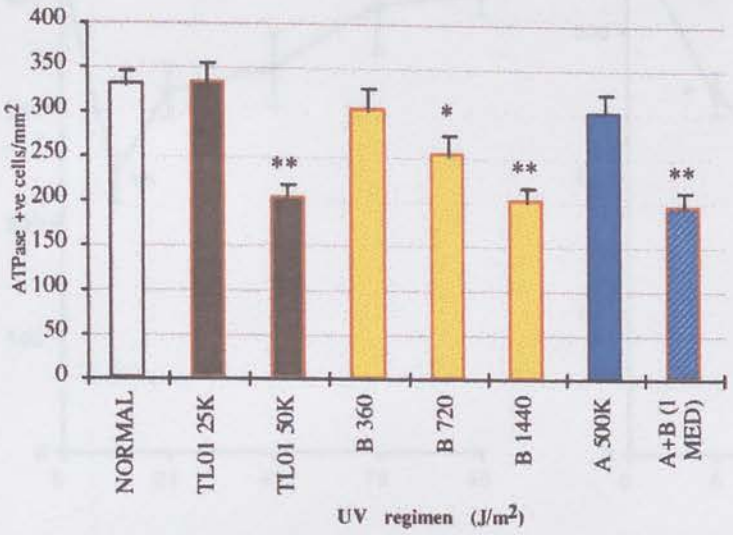


Figure 4.5. UVB-induced alterations in LC numbers are dose-dependent. Mice were exposed to either 25 or 50 kJ/m^2 (1 MED) narrow-band UVB (TL-01), 360, 720 or 1440 J/m^2 (1 MED) broad-band UVB (TL-12), 500 kJ/m^2 (1 MED) UVA1 or 500 kJ/m^2 (1 MED) UVA1 followed immediately by 1440 J/m^2 (1 MED) broad-band UVB (TL-12). Ears collected 24h later and 4 epidermal sheets from each group were prepared and stained for ATPase activity. Results are shown as the mean ± SEM. Significant differences between the normal control and the experimental groups were determined by Student's *t*-test. *= $p< 0.05$, and **= $p< 0.01$, vs. unirradiated.

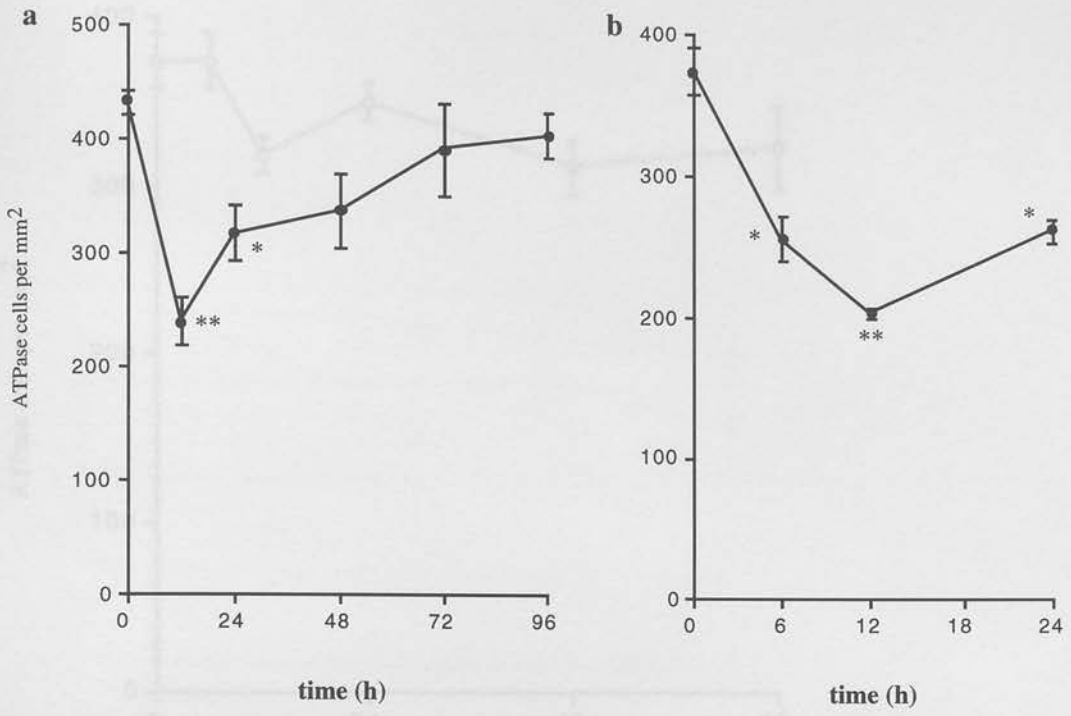


Figure 4.6a and b. The timecourse for ATPase$^+$ cells in murine epidermis following irradiation with 1 MED of UVB (1440 J/m^2). Mice were irradiated and ears collected at various times thereafter. Four epidermal sheets from each group were prepared and stained for ATPase activity. Results from 2 separate experiments are shown, as the mean ± SEM for each timepoint, with 0h representing the number in unirradiated animals. *= $p< 0.05$ and **= $p< 0.01$, vs. unirradiated.

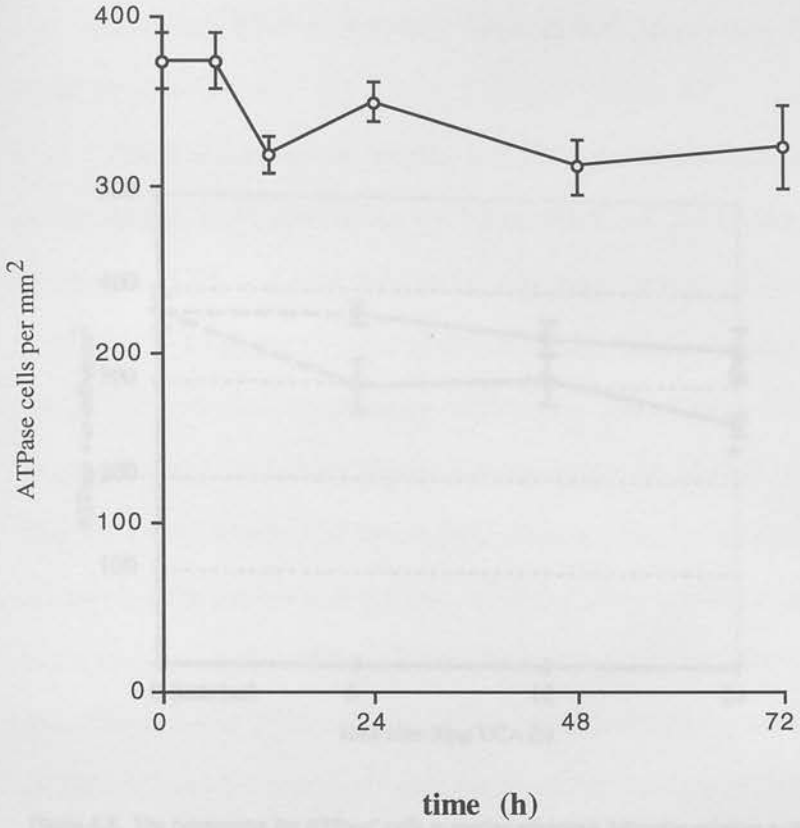


Figure 4.7. The timecourse for ATPase$^+$ cells in murine epidermis following irradiation with 1 MED of UVA1 (500 kJ/m^2). Mice were irradiated as decsribed in section 2.1.6 and ears collected at various times thereafter. Four epidermal sheets from each group were prepared and stained for ATPase activity. Results are shown as the mean ± SEM for each timepoint, with 0h representing unirradiated levels. *= $p< 0.05$ and **= $p< 0.01$, vs. unirradiated.

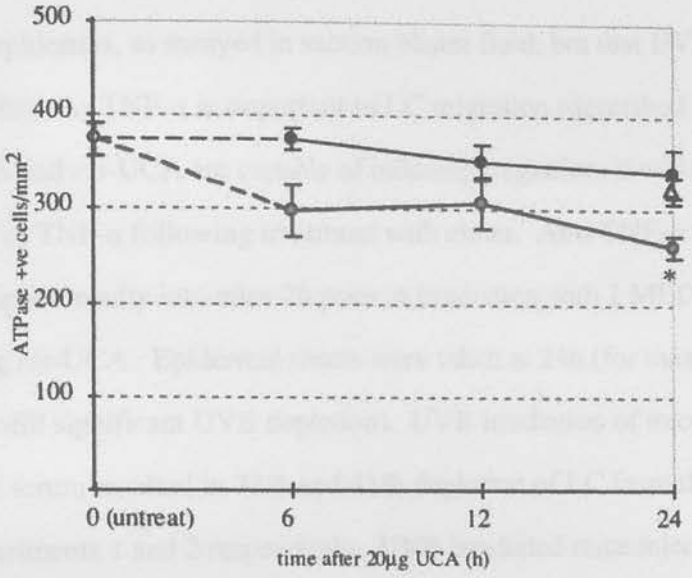


Figure 4.8. The timecourse for ATPase$^+$ cells in murine epidermis following painting with 20µg *cis*- or *trans*-UCA. Mice had their ears tape-stripped twice 24h before application of DMSO/eth or 20µg UCA isomers. Four epidermal sheets from each group were prepared and stained for ATPase activity. Results are shown as the mean ± SEM. Significant differences between the DMSO/eth group and the experimental groups were determined by Student's *t*-test. *= $p < 0.05$, vs. untreated.

4.8. Blocking TNF-α activity inhibits LC migration induced by either UVB or *cis*-UCA.

There is evidence in humans that UVB irradiation induces TNF-α production in the epidermis, as assayed in suction blister fluid, but that UVA1 does not (Skov *et al* 1998a). As TNF-α is important in LC migration (described in section 1.3.1), and both UVB and *cis*-UCA are capable of inducing migration, it was decided to investigate the role of TNF-α following treatment with either. Anti-TNF-α was injected intraperitoneally into mice 2h prior to irradiation with 1 MED UVB or painting with 20μg *cis*-UCA. Epidermal sheets were taken at 24h (for maximal *cis*-UCA depletion and still significant UVB depletion). UVB irradiation of mice injected with normal goat serum resulted in 32% and 41% depletion of LC from the epidermis, for experiments 1 and 2 respectively. UVB irradiated mice injected with anti-TNF-α did not have altered LC numbers, with the frequency being reduced by only 7% and 9% relative to respective controls (shown in fig 4.9a). Fig 4.9b shows that LC retained a normal appearance if animals were injected with anti-TNF-α before UVB irradiation. Similar results were obtained from mice injected with anti-TNF-α before painting their ears with *cis*-UCA (see figs. 4.10a and 4.10b). LC frequency was reduced by 20% and 15% respectively following painting with 20μg *cis*-UCA. If mice were treated with anti-TNF-α 2h before application of *cis*-UCA, reductions in LC frequency were completely abrogated.

4.9. Exogenous TNF-α increases LC depletion mediated by UVB, and provides a signal which can promote LC depletion in UVA1 irradiated mice.

TNF-α was injected into the pinnae of mouse ears 0.5h before collecting the ears for ATPase staining of the epidermis. Injection of TNF-α resulted in LC depletion in the epidermis of mice (a 22.4% decrease from 0.1% BSA injected mice). Mice which were irradiated with UVB exhibited a 19.6% decline in LC within the

epidermis 24h later, whilst UVA1 irradiation had no effect. Mice injected with TNF-α, and irradiated 24h earlier, had LC depletion to a similar degree as TNF-α injection alone. Mice irradiated with UVA1 and injected with TNF-α had a LC frequency of 325.5 per mm^2. UVB irradiated mice and injected later with TNF-α had LC at a frequency of 300 per mm^2 (see fig. 4.11a). UVA1 alone, as expected, had no effect on LC frequency. It is possible that the dose of TNF-α used in these experiments (1000 U) masked any endogenous protein released as a result of irradiation. Importantly, this experiment shows that UVA1 irradiation does not lead to a signal that inhibits the action of TNF-α. Fig. 4.11b shows the morphology of the LC remaining in the epidermis.

4.10. Blocking IL-1β activity inhibits LC migration induced by either UVB or *cis*-UCA.

Another important cytokine involved in LC migration is IL-1β. When the effects of this cytokine were neutralised by injecting anti-IL-1β 2h before UVB-irradiation, LC numbers and morphology were restored to normal. This can be seen in fig. 4.12. In UVB iradiated mice injected with normal goat serum, LC frequency was reduced by 31% and 26% respectively. There was no depletion of LC in mice injected with anti-IL-1β. As with anti-TNF-α injection, injection of anti-IL-1β 2h before *cis*-UCA painting also inhibited depletion of LC from the epidemis, and this can be seen in fig. 4.13. *Cis*-UCA painting resulted in migration of 22% and 29% of LC from the epidermis in experiments 1 and 2 respectively, and this migration was completely prevented by injection of anti-IL-1β 2h earlier.

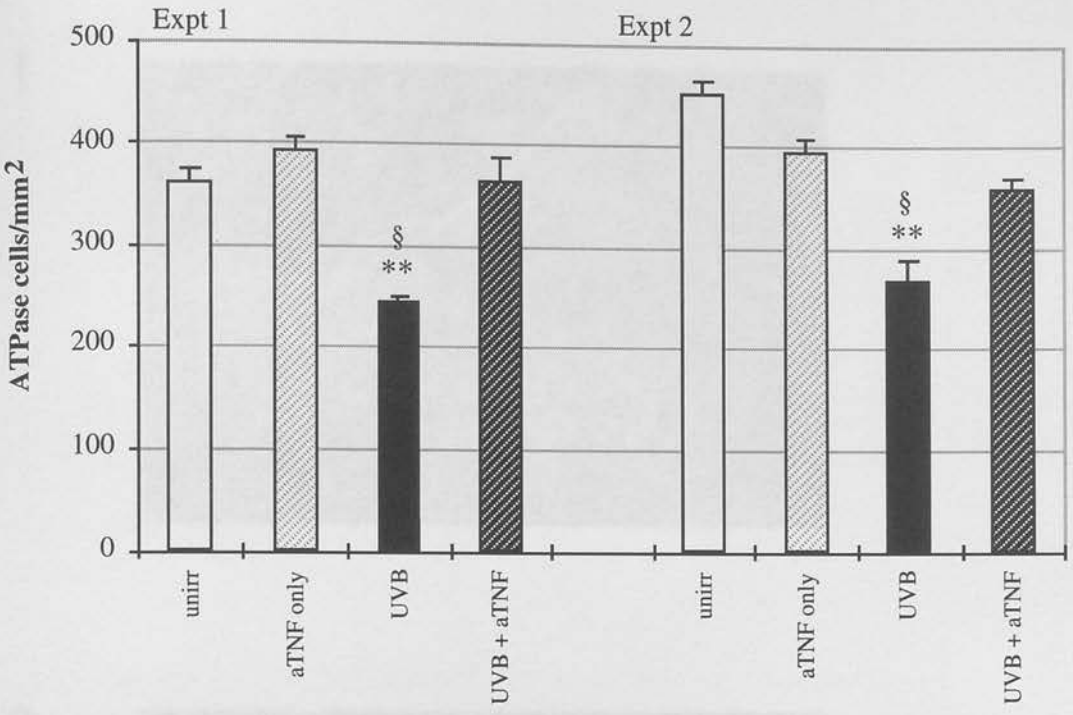
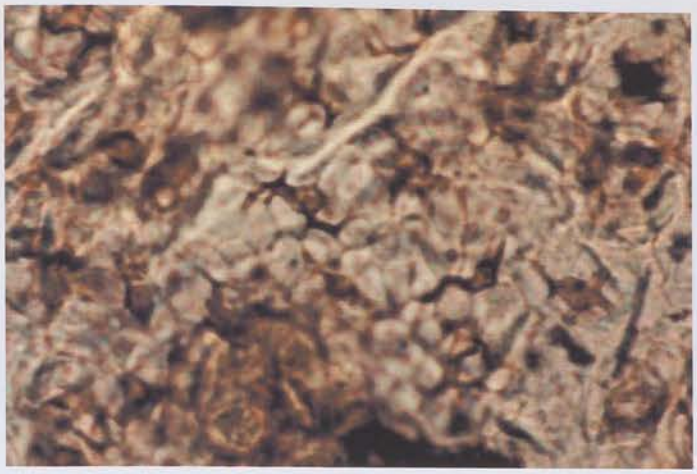


Figure 4.9a. The role of TNF-α in UVB-mediated LC depletion. Mice were injected with normal goat serum or 20μg anti-TNF-α 2h before 1 MED (1440 J/m^2) UVB irradiation. Four epidermal sheets from each group were prepared and stained for ATPase activity. **= $p < 0.01$ vs. control, and §= $p < 0.05$, vs. same treatment without anti-TNF-α antibody.

1



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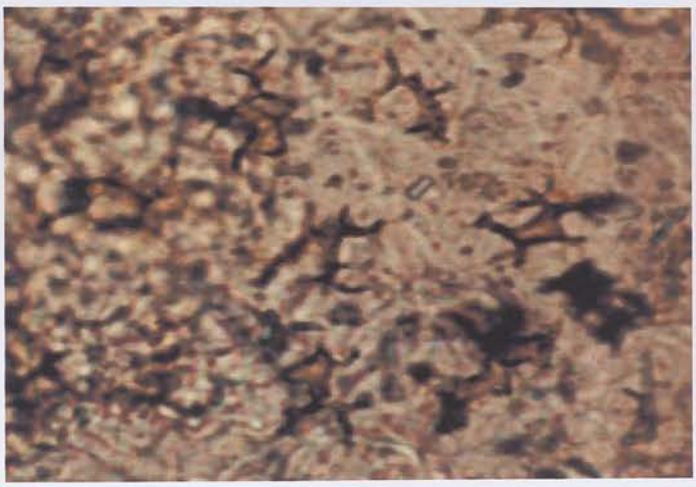


Figure 4.9b. Blocking TNF-α inhibits alterations in LC morphology induced by UVB irradiation. Mice were injected with normal goat serum (1), or anti-TNF-α (2), before irradiating with 1 MED UVB. The morphology of LC remaining within epidermal sheets 24h after irradiation are shown (both magnifications are x 250).

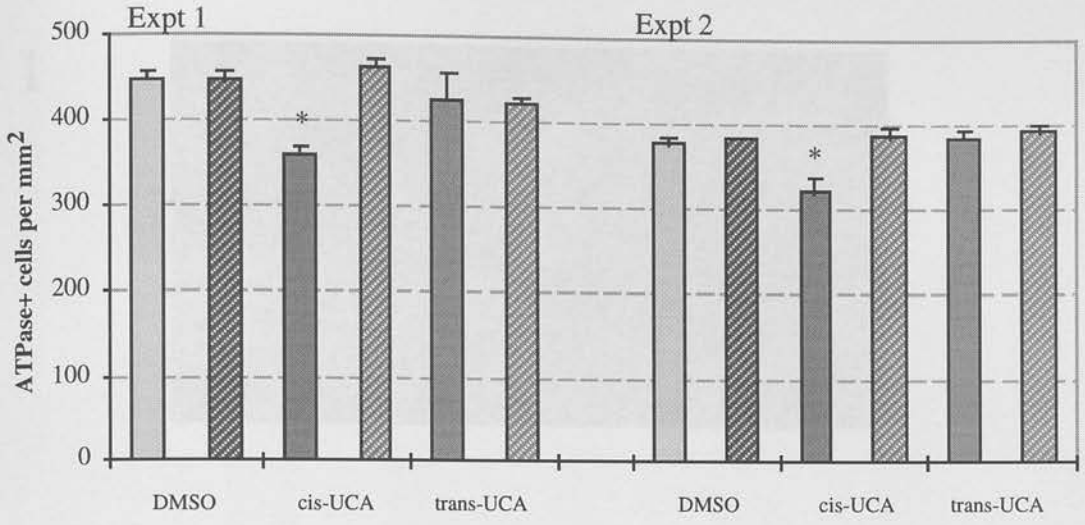
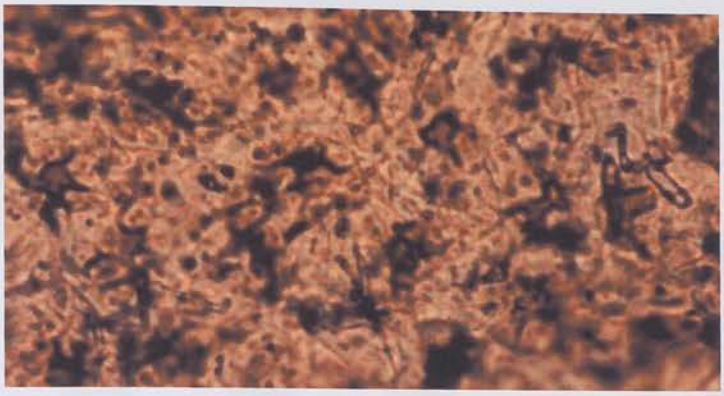


Figure 4.10a. The role of TNF-α in *cis*-UCA-mediated LC depletion. Mice were injected with normal goat serum (solid bars) or 20μg anti-TNF-α (striped bars) 2h before ear painting 20μg of either UCA isomer. Four epidermal sheets from each group were prepared and stained for ATPase activity. Results are shown as the mean ± SEM, *= $p < 0.05$, vs. DMSO.

1



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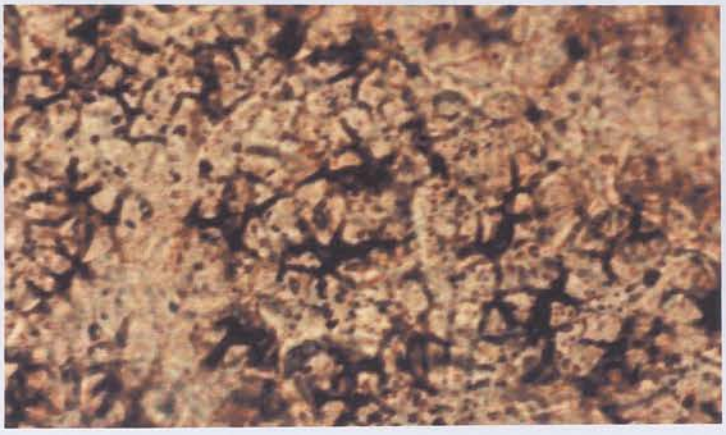


Figure 4.10b. Blocking TNF-α inhibits alterations in LC morphology induced by *cis*-UCA painting. Mice were injected with normal goat serum (1), or anti-TNF-α (2), before painting the ears with 20μg *cis*-UCA. The morphology of LC remaining within epidermal sheets 24h after *cis*-UCA application are shown (both magnifications are x 250).

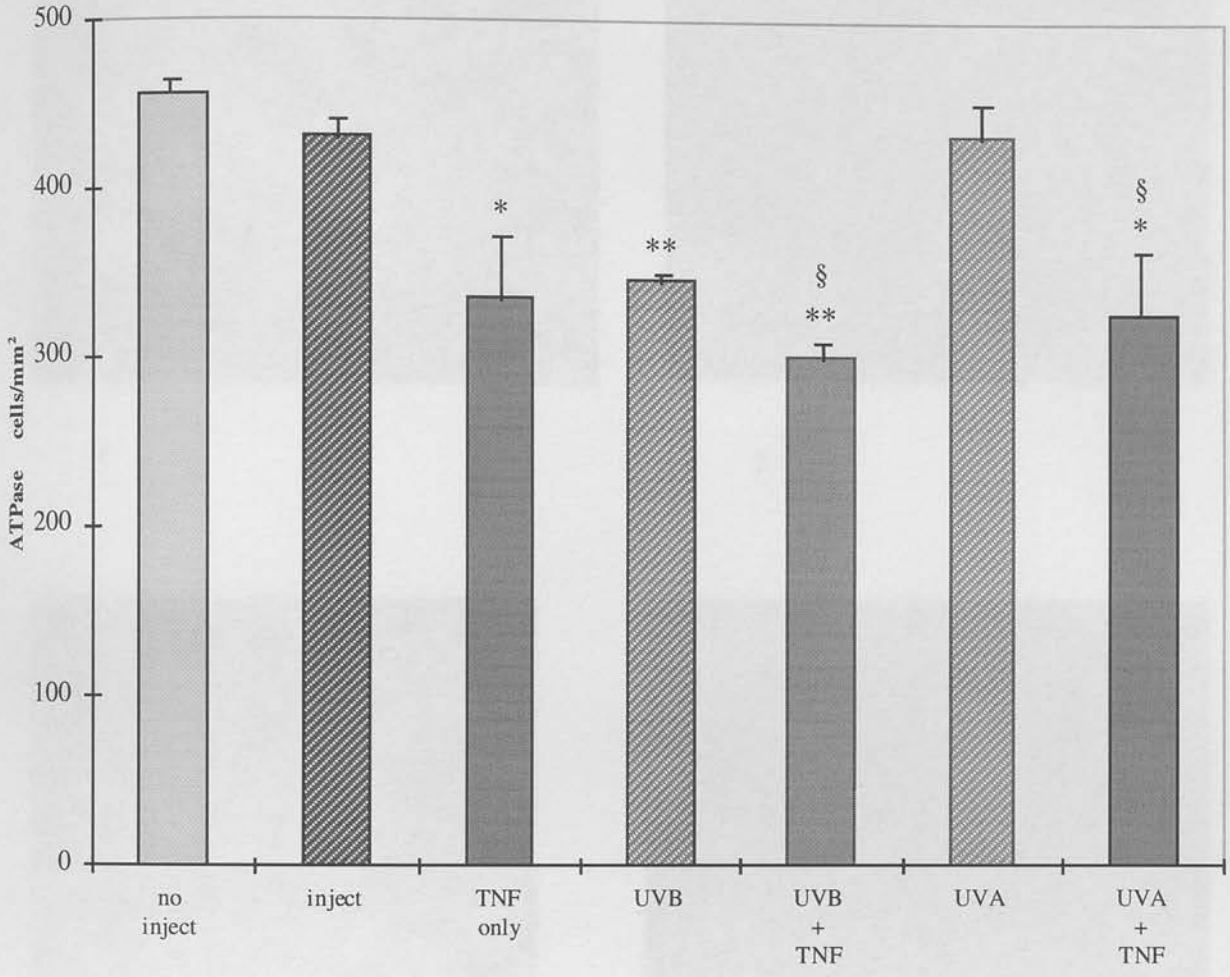
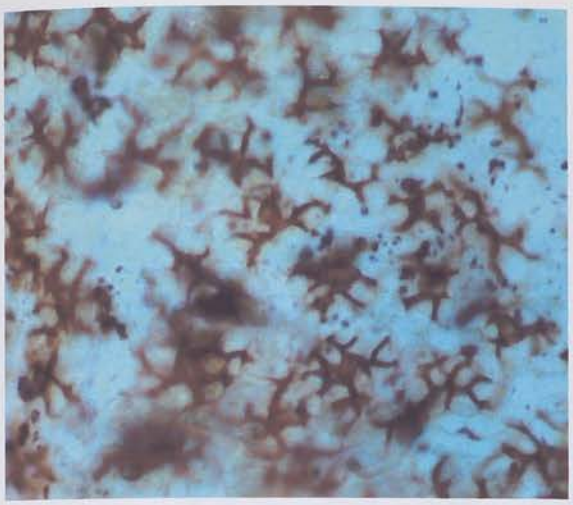


Figure 4.11a. TNF-α induces LC migration in UVA irradiated mice, and enhances UVB-mediated LC depletion. Mice were irradiated with 1 MED (1440 J/m^2) UVB or 1 MED (500 kJ/m^2) UVA1. Mice were injected with 1000 U TNF-α or 0.1% BSA 0.5h before ear collection (24h after UV exposure). Four epidermal sheets from each group were prepared and stained for ATPase activity. *= $p< 0.05$ and **= $p< 0.01$, vs. control, §= $p< 0.05$ vs. same treatment without TNF-α.



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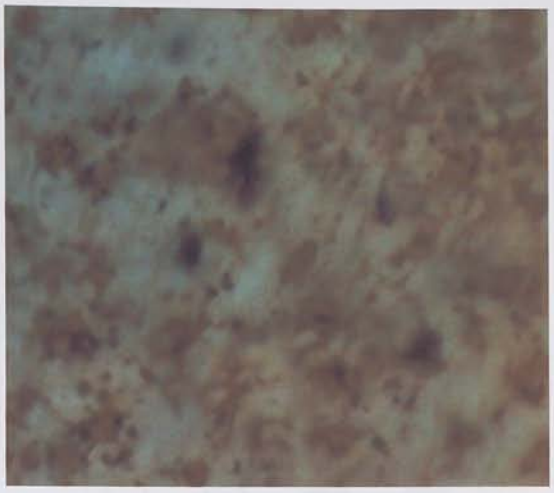

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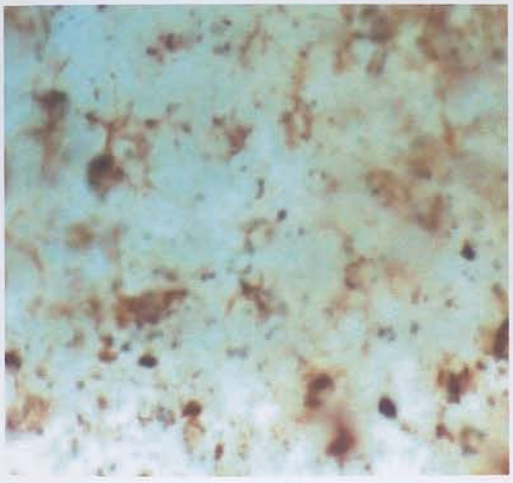

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Figure 4.11b. Morphology of LC remaining in the epidermis following various treatments. Mice were untreated (1), injected with TNF-α (2), irradiated with 1 MED UVB (3) or irradiated with 1 MED UVA1 (4). The morphology of LC remaining within epidermal sheets 24h after irradiation are shown (all magnifications are x 250).

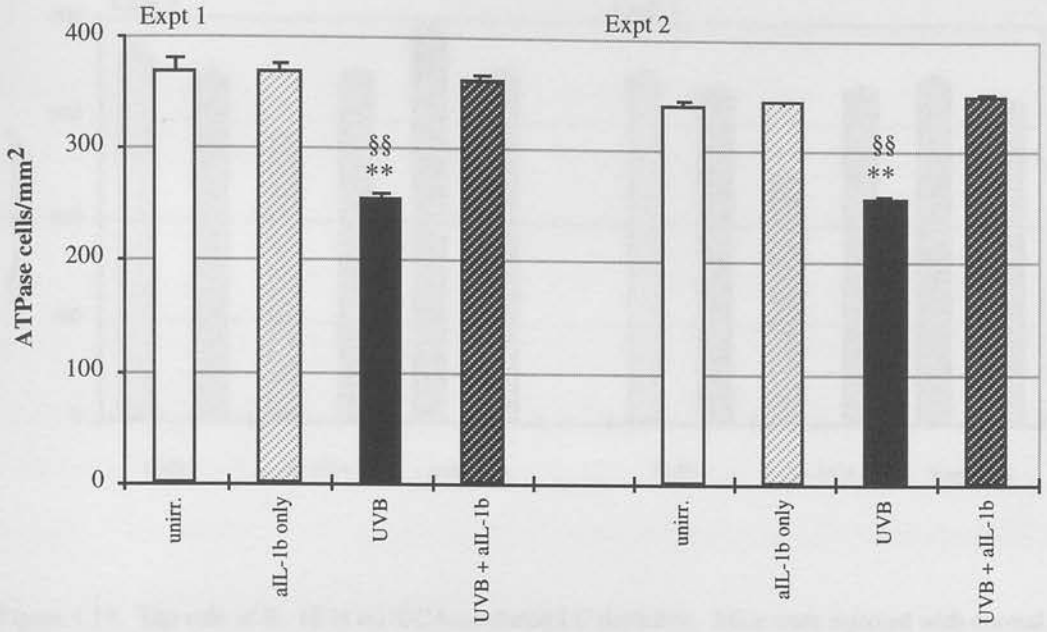


Figure 4.12. The role of IL-1β in UVB-mediated LC depletion. Mice were injected with normal goat serum or 20μg anti-IL-1β 2h before 1 MED (1440 J/m^2) UVB irradiation. Four epidermal sheets from each group were prepared and stained for ATPase activity. **= $p< 0.01$, vs. unirradiated control, and §§= $p< 0.01$, vs. same treatment without anti-IL-1β antibody.

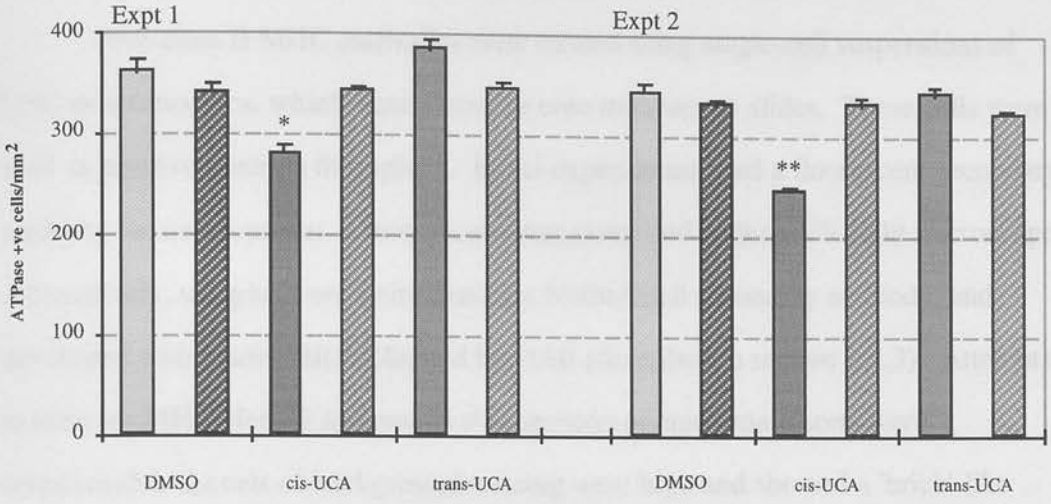


Figure 4.13. The role of IL-1β in *cis*-UCA-mediated LC depletion. Mice were injected with normal goat serum (solid bars) or 20μg anti-IL-1β (striped bars) 2h before ear painting 20μg of either UCA isomer. Four epidermal sheets from each group were prepared and stained for ATPase activity. Results are shown as the mean ± SEM, *= $p< 0.05$, and **= $p< 0.01$, vs. DMSO.

4.11. LC staining on the basis of MHC class II expression.

Anti-class II MHC antibodies were titrated using single-cell suspensions of LNC or splenocytes, which were cytospun onto microscope slides. These cells were used as positive controls throughout. Initial experiments used a fluorescent secondary antibody for development. Fluorescence was visualised under a UV light microscope. Alternatively, samples were stained using a biotinylated secondary antibody, and developed with avidin-HRP followed by DAB (described in section 2.4.3). Attempts to stain for MHC class II antigens in skin sections or epidermal sheets were unsuccessful. Levels of background staining were high and showed a 'brick'-like pattern. This indicates that the keratinocytes bound antibody, and LC were not readily identifiable because of this (data not shown).

4.12. DISCUSSION

IMMA has its imidazole and carboxyl functionalities permanently fixed in a configuration analogous to both *trans*- and *cis*-UCA (Houghtaling *et al* 1996). IMMA was capable of suppressing the DTH response to HSV when it was injected 5h before infection, as described in section 4.2. Like *cis*-UCA, the immunosuppressive effects of IMMA appeared to be dose-dependent.

Norval and collegues showed that addition of a methyl group to position 2 of the imidazole ring of UCA abrogated immunosuppressive effects of *cis*-UCA (Norval *et al* 1989a). The side arm could be altered significantly (by reduction, or even replacement) with no loss of activity. Thus, it appeared that the 5-membered ring was necessary for immunosuppressive activity, perhaps by fitting into a receptor site. MeIMMA resembles IMMA, with the only alteration being addition of a methyl group at the 2-position of the imidazole ring. It was therefore anticipated that this analogue of UCA would have no immunosuppressive properties. However, MeIMMA did suppress DTH responses (figs. 4.1 and 4.2), and this was to a similar extent to *cis*-UCA.

LC possess a Mg^{++}- or Ca^{++}-dependent plasma-membrane ecto-adenosine triphosphatase (ATPase), and this was the marker used to identify LC throughout these experiments. Staining for MHC class II expression was also attempted, but the level of binding to keratinocytes in C3H/HeN mouse ears and skin did not allow LC identification by this method. Regardless, ATPase staining has many advantages over MHC class II staining in UV-irradiated samples. It is well documented that UVB causes an inflammatory response, and one of the predominating cell types in this infiltrate are monocyte/ macrophage cells (Hammerberg *et al* 1996; Meunier *et al* 1995). Although there is a time delay before the inflammatory cell infiltration following UVB irradiation, the infiltrating monocyte/ macrophage cells do express MHC class II, and may therefore complicate identification of LC. Hammerberg and colleagues reported that 24h after a 1.1 MED UVB exposure, infiltrating

monocyte/ macrophage cells represented 1.2% of the total dermal cell population (Hammerberg *et al* 1996). The peak in infiltrating cell numbers occured 48-72h after irradiation. It was anticipated, and observed, that UVB-treatment would result in a loss of dendrites on LC, and this would have made distinction between small, rounded LC and infiltrating macrophages difficult. Additionally, there is evidence that MHC class II expression may also be induced on keratinocytes (Nickoloff and Turka, 1994; Basham *et al* 1985). In contrast, ATPase enzyme protects LC from permeabilization upon exposure to extracellular ATP, whereas keratinocytes and macrophages (J774) are susceptible (Girolomoni *et al* 1992). Loss of ATPase activity correlates with an "activated" state, and application of a second contact sensitiser 24h after the first resulted in tolerance to the second (Hanua *et al* 1989). Therefore, ATPase activity may represent the best, single marker for both LC and their capacity to initiate immune responses.

Noonan *et al.* found that ATPase positive cells were reduced 24h after irradiating mice on the back with narrow-band UVB (270, 290, 320 nm) (Noonan *et al* 1984). Irradiating with 270 or 290 nm UVB led to a loss of dendrites and a decrease in the total epidermal LC, in dose-dependent fashion. A dose of 320 nm UV that caused 50% systemic suppression of CH did not deplete LC. Previous experiments within this laboratory demonstrated that chronic broad-band UVB (TL-12) (1000 J/m^2, 3 times per week) reduced LC by greater than 50% after 2 weeks, but this could not be reduced further with more exposures (El-Ghorr *et al* 1995). Narrow-band UVB (TL-01, 3000 J/m^2, 3 times per week) depleted LC by 20% after 2 weeks. This gradually increased to 40% with further exposures (for up to 6 weeks). In the series of experiments detailed in this chapter it has been consistently shown that a single 1440 J/m^2 broad-band UVB irradiation can reduce LC numbers present in murine epidermis 24h later by around 30%. The reduction in LC frequency mediated by broad-band UVB was dose-dependent, and the peak in LC depletion occurred 12h after irradiation. A single 10,000 J/m^2 exposure to narrow-band UVB (TL-01) resulted in a 38%

reduction 24h later. Local suppression of CH is influenced by the timing of hapten application after UVB exposure. In a previous study, Miyauchi and Horio found the optimal time to induce maximal suppression correlated with the time of minimal ADPase$^+$ cells after 1000 J/m^2 (3 days) (Miyauchi and Horio, 1995). The lamp used for their study had an emission spectrum of 275-375 nm, with peak at 305 nm, and therefore resembles the output of the Philips TL-12 used in these experiments. The timecourse reported by Miyauchi and Horio is in conflict with that described in section 4.5. In the experiments shown here, LC numbers were not significantly depleted, and were returning to normal levels, by 3 days after UVB exposure. One potential reason for this difference may be the source of the epidermal sheets used. Whereas epidermal sheets were derived from ears for all experiments described in this chapter, Miyauchi and Horio used UVB irradiated abdominal skin. Therefore, whereas ears do not have to be treated before experimentation, abdominal skin has to be shaved to remove fur. Additionally, ears are subject to incidental UVR via natural and artificial light sources within animal housing facilities, whilst abdominal skin is protected by its fur and anatomical position. This may provide a reason for the difference in LC numbers found in these experiments, using epidermal sheets from ears, and those of other groups, using abdominal sheets (a mean of 380 against around 800 per mm^2). Abdominal skin is unlikely to have been exposed to UVB except in the earliest stages of a mouse's life, and may therefore be more sensitive to its effects. Ears are commonly exposed to some radiation, and may adapt. As such, the ears would represent a better model for the types of UV exposure that humans receive. Although mice in each individual experiment were age matched, ages ranged from between 7-11 weeks old between experiments. This may account for the variation in mean ATPase$^+$ cells between experiments, with LC frequency increasing with age. Interestingly, Miyauchi and Horio also showed that old mice (26 weeks) are less susceptible to UVB-induced immune suppression than young mice (11 weeks) (Miyauchi and Horio, 1995), and this may suggest adaptive mechanisms over time.

In contrast to the results shown in this chapter, some groups have shown reductions in LC numbers by UVA radiation. However, the protocols of exposure are often different, and many studies are further complicated by the use of psoralen. For instance, Aubin *et al.* used continued UVA exposure (99.5% emitted energy between 320-400 nm, 10 kJ/m^2, 3 times per week for 4 weeks) in conjunction with the psoralen, TMAP (Aubin *et al* 1991). This regimen led to a decrease in ATPase$^+$, Ia$^+$ and Thy-1$^+$ cells in the exposed skin of C3H mice. CH induction at the treated site was reduced, and this was related to impaired APC function in DLN (as shown by adoptive transfer of DLNC from irradiated mice into unirradiated recipients). Chronic UVA exposure alone also reduced ATPase$^+$, Ia$^+$, Thy-1$^+$ cells, but had no effect on immune function (Aubin *et al* 1991). This was also observed in BALB/c mice.

Polychromatic UV light, composed of environmentally relevant wavelengths, was found to disrupt the cytoplasmic microtubule complex in a dose-dependent manner. This did not correlate with the cytotoxicity of the UV light used. UVC did not alter microtubules at doses as high as 100 J/m^2, but microtubules were disrupted after 3000 J/m^2 UVA exposure (Noonan *et al* 1984). No changes could be seen in the actin microfilaments which stretch across the length of control and irradiated cells.

UVA (320-400 nm) isomerises UCA (El-Ghorr and Norval, 1999) but may not lead to immune suppression. A simple additive effect of UVA1, UVA2, and UVA1+2 was seen for *trans*-UCA isomerisation (Webber *et al* 1997). This indicates that failure to produce immune suppression is not due to complex wavelength interaction and/or the presence of an *in vivo* endogenous photosensitiser of UCA photoisomerisation. Webber and colleagues postulated that other factors, such as UVA blocking of signals generated downstream of *cis*-UCA, may be involved.

Trans- to *cis*-UCA conversion can be seen with both broad- and narrow-band UVB lamps (El-Ghorr *et al* 1995). The percentage of *cis*-UCA started to return to normal levels after 4 weeks (3000 J/m^2, 3 times per week) of narrow-band UVB exposure, despite continued exposure. CH was suppressed only in mice that had

received broad-band UVB, suggesting that there was no correlation between *cis*-UCA and the CH outcome (El-Ghorr *et al* 1995).

In the experiments described here, *cis*-UCA was also capable of depleting LC, but took longer to do so than UVB (maximal depletion was 24h after painting). There are many possible reasons for this delayed action. One simple explanation may be that, although mice have the epidermis gently tape-stripped before painting, *cis*-UCA may require time to permeate into the epidermis in this artificial system. A theoretical approach to estimate the distribution of cis-UCA after irradiation indicates that it may diffuse into the deeper layers of the human epidermis at a rate of $10^{-17}m^2/s$ (Krien and Moyal, 1994). *Cis*-UCA is formed immediately in the stratum corneum following UV irradiation, and may be able to mediate effects more rapidly. In experiments by Kurimoto and Streilein, LC were rounded up and possessed fewer dendrites as early as 5h after an intradermal injection of 200μg *cis*-UCA (Kurimoto and Streilein, 1992). *Cis*-UCA (20μg) did not result in as much LC depletion as UVB irradiation (1440 J/m^2) (an average of 20.8%, ranging from 15.1 to 28.7% in 6 experiments, as against around 30% following UVB), even though it was applied at the high-end of physiological dosage (20μg per mouse ear). Untreated murine flank skin contains around $5\mu g/cm^2$ UCA, with 4% in the *cis* isoform ($0.2\mu g/cm^2$) (Norval *et al* 1988). This may indicate that *cis*-UCA acts in concert with other UVB-induced pathways/ mechanisms. It is possible that *cis*-UCA acts in a synergistic manner with UV-induced DNA damage to stimulate LC migration. Blocking, or lack, of either *cis*-UCA or DNA damage have been shown to prevent UVB-induced immune suppression (El-Ghorr and Norval, 1995; De Fabo *et al* 1999; Kripke *et al* 1992; Sutherland *et al* 1980), suggesting that they are not mutually exclusive. This could also explain the disparity in timecourses, as either mechanism (DNA damage or *cis*-UCA) alone could be less effective and take longer to act than UVB irradiation, which would induce both simultaneously.

When *cis*-UCA (200μg) was injected intradermally into C3H/HeN, C3H/HeJ, C57Bl/6 or BALB/c mice, CH to DNFB painted on to the injected site is impaired (by up to 68%). Staining for Ia expression in the epidermis 5h later revealed that LC had lost their dendrites and were rounded up, with frequency reduced in all strains (by 25% compared with positive controls) (Kurimoto and Streilein, 1992). This immunosuppressive effect could be partially restored (CH to 73%, and LC to 90%, of the positive controls) by intraperitoneal injection of anti-TNF-α antibodies 2h before *cis*-UCA treatment (Kurimoto and Streilein, 1992). These experiments indicate that if LC can be retained in the site to be sensitised, CH responses are normal. The experiments described in section 4.8 also show the dependency of *cis*-UCA mediated LC migration upon TNF-α. This dependency is further extended to IL-1β in section 4.10.

Cis-UCA, even above physiological levels, could not augment phorbol 12-myristate 13-acetate (TPA) induced synthesis of either TNF-α mRNA or protein in long-term cultured human keratinocytes (Redondo *et al* 1996). Skov *et al.* (1998a) showed that irradiation of human volunteers with 3 MED UVB led to a rapid increase in TNF-α concentration within suction-blister fluid, which was maximal 6h after exposure. UVA1 caused a slight decrease in TNF-α, although both UV regimens led to a slight increase in *cis*-UCA. Therefore, if UVA1 cannot stimulate TNF-α release or production, it may not provide an essential signal for LC migration. As discussed in section 4.6, and above, LC numbers were not depleted by exposure of mice to 500,000 J/m^2 UVA1. UVB does induce TNF-α secretion, and this and IL-1β are vital in UVB-mediated LC migration. Both these cytokines were shown to be important in *cis*-UCA mediated LC migration also.

Enk and Katz identified a causal relationship between production of IL-1β by epidermal LC and the induction of epicutaneous sensitisation, by injection of IL-1β (Enk and Katz, 1995). Epidermal cell suspensions prepared 4h after IL-1β injection, or application of TNCB, produced almost identical cytokine mRNA profiles. There

was enhanced expression of MHC class II, TNF-α, IL-10, IL-1α and even IL-1β itself. Addition of IL-1β to keratinocytes (derived by washing non-adherent cells from epidermal cell cultures) resulted in increased TNF-α and IL-10, with a less pronounced IL-1α increase (Enk and Katz, 1995). Lymphocytes from UVR exposed mice produced less IL-2 and IFN-γ when activated *in vitro* by antigen or anti-CD3 (polyclonal T cell activation). IL-4 secretion was consistently elevated in comparison to normal controls. A similar change could be induced when mice were treated with LPS or IL-1β. The ability of IL-1β to alter cytokine secretion was not a direct effect, as addition of IL-1β to cultures of antigen-primed T cells did not alter cytokine patterns (Araneo *et al* 1994).

UVB not only alters the frequency of LC in the epidermis, but also has effects on LC function. In experiments by Dittmar and colleagues, UVB irradiation inhibited upregulation of B7 molecules on human LC derived from suction blister roofs after irradiation. These LC could not activate naive resting T cells. In contrast, UVA1 exposed LC did upregulate B7, and were capable of stimulating naive T cells (Dittmar *et al* 1999). Antibodies to CD28 restored the stimulatory capacity of UVB irradiated LC. Experiments by Simon *et al.* showed that unirradiated BALB/c epidermal cells, LC, or splenic adherent cells, but not Ia^- epidermal cells, presented keyhole limpet hemocyanin (KLH) to both antigen specific Th1 and Th2 cell lines. Irradiated epidermal cells and LC lost their ability to stimulate Th1 cell lines, but retain APC function to Th2 cell lines (Simon *et al* 1990). UVB reduced the total number of Ia^+ epidermal cells, but did not reduce intensity of staining on positive cells.

UVA-mediated damage to membranes, proteins, DNA or other cell targets is predominantly related to oxidative stress. Clement-Lacroix and colleagues found that the APC function of cultured LC (measured by MECLR) was dose-dependently reduced by direct UVA exposure, at doses up to 20 J/m^2 (Clement-Lacroix *et al* 1996). Overnight incubation with the anti-oxidant vitamin E partially protected against this immunosuppression, and reduced the number of thiobarbituric acid reactive substances

(TBARS) released into the supernatant (indicating reduced oxidative damage). This suggests that UVA can alter APC functions partly via generation of reactive oxygen species.

As mentioned above and in section 1.3.3, macrophages infiltrate the skin following UVB exposure. Meunier and colleagues identified the macrophages infiltrating human skin after sunburn (3 days after 4 MED FS40) as having the extended phenotype $CD1a^-$ $CD1c^-$ $CD11b^+$ $CD11c^+$ $CD36^+$ $Fc\gamma RII^+$ DR^+) (Meunier *et al* 1995). They observed that after UVR there was expansion of bone-marrow derived DR^+ cells in the perivasculature and sub-basement membrane zone of the papillary dermis. Despite an overall expansion of DR^+ cells, the $CD1a^+$ $CD1c^+$ $CD36^-$ LC-like dendritic APC subset of dermal DR^+ cells was depleted, indicating that LC loss was not accounted for by accumualtion within the dermis. In contrast, there was a selective expansion of the dermal macrophage subset, which is phenotypically identical to the population that appears in the epidermis after UVR injury. These cells derived not only from transcapillary migration, but also from *in situ* proliferation of a dermal precursor (Meunier *et al* 1995). These cells are capable of migration to DLN, as phenotypic experiments investigating hapten carriage after UVB suggest that hapten is presented by macrophage-like cells, rather than LC (Tang *et al* 1992).

This chapter has shown that UVA1, at least at a dose of 500 kJ/m^2 (1 MED), cannot deplete LC from murine epidermis. UVB, both broad- and narrow-band, and *cis*-UCA, can mediate LC depletion. This effect is dose-dependent, and is mediated via the cytokines IL-1β and TNF-α. Blocking of either cytokine abrogated the effects of either UVB exposure or *cis*-UCA painting on LC. LC frequency is significantly reduced as early as 6h after UVB irradiation, but may take longer following *cis*-UCA application.

4.13. Criticisms of experimental design and suggestions for further work

It would have been ideal in all of the experiments detailed above to have corroborated findings by use of another LC marker. This was attempted for MHC class II staining, but did not yield any results (described in section 4.11).

In the experiments described herein, it has been shown that UVB and *cis*-UCA, but not UVA1, mediate migration, or at least depletion, of LC from murine epidermis. The timecourse of LC depletion was established for UVB and potential effects by UVA1 were examined up to 96h after exposure. Depletion following *cis*-UCA painting was found to be maximal in these experiments 24h later, but the timecourse was not extended beyond this point (see fig. 4.8). It cannot be discounted that LC numbers continue to decline after this time, and therefore would have been useful to have extended the timecourse of investigation following *cis*-UCA painting. This was not undertaken as it had been anticipated that the timecourse would have more closely resembled that for UVB (so that LC numbers would have been recovering by 24h), and it has been reported previously that LC were significantly depleted by 5h after *cis*-UCA injection (Kurimoto and Streilein, 1992).

Further investigation of the immune suppression mediated by IMMA and MeIMMA would have been interesting. These were shown to have been equally, if not more, immunosuppressive than *cis*-UCA in the context of the DTH response to HSV-1 (described in section 4.2). This was not examined in the context of CH responses, or LC depletion. A dose-response for effects of *cis*-UCA and its analogues on LC depletion would have provided useful data.

The interaction of UV wavelengths is an area of much current interest. From the data provided in this chapter, it is clear that irradiating C3H/HeN mice with 1 MED UVA1 before 1 MED UVB does not alter the depletion of LC mediated by UVB alone. Reeve and colleagues showed that in the same series of experiments in which this regimen provided photoimmunoprotection (Reeve *et al* 1998), the reverse exposure

protocol, irradiating with 1 MED UVB before 1 MED UVA1, also provided photoimmunoprotection. Examination of the effects of such a protocol on LC numbers within the epidermis would have provided further useful information on wavelength interactions.

Chapter 5

Alterations in the accumulation of DC in DLN following UVA1 and UVB

5.1 Introduction

Dendritic cells (DC) found in DLN are believed to be the mature form of epidermal LC. DC aggregate with T cells within the DLN and form clusters. Freshly isolated LC are not capable of doing this, as the cells need a period of around 3 days to acquire this ability (Inaba *et al* 1986). It has been proposed that following UVB irradiation the ability of DC to present antigen is impaired, due to an inability to form T cell clusters (Muller *et al* 1994b). Vink and colleagues showed that UV-induced DNA damage plays a role in this impairment. When DC from irradiated and contact sensitised mice were injected into syngeneic recipients, there was a failure to induce CH in recipients upon challenge. By incubating DC in the presence of liposomes containing T4 endonuclease V (T4N5) or a photolyase to remove pyridimine dimers, the ability of DC from irradiated mice to induce CH in recipients could be restored (Vink *et al* 1996a; Vink *et al* 1997). Experiments on the murine epidermal-derived DC line, XS52, indicate that UVB irradiation prepares DC to undergo apoptosis upon contact with T cells (Kitajima *et al* 1996). The XS52 cells remained susceptible to T cell-mediated apoptosis 16h after irradiation with even small doses of UVB. The APC capacity of XS52 cells, using $CD4^+$ T cell clones as responders, was inhibited by greater than 50% by irradiation with 5-10 mJ/cm^2 UVB (Caceres-Dittmar *et al* 1995). Pretreatment with catalase prevented the UV-induced inhibition of APC function, whereas exposure to hydrogen peroxide mimicked the effects of UV, thereby indicating a role for reactive oxygen species in UV-induced alteration of DC APC ability.

There exists a correlation between the number of DC arriving in DLN 24h after contact sensitisation and the magnitude of the induced T cell proliferative response (Kimber *et al* 1990c). UVB has been shown previously to induce DC accumulation within DLN. UVB suppresses the antigen presenting ability of DC, as shown by the reduced proliferative responses induced by these cells. This is not due to a reduction in Ia expression (Moodycliffe *et al* 1992), but costimulatory molecules may be altered.

The cytokines IL-1β and TNF-α are important in induction of LC migration from the epidermis, and subsequent DC accumulation in DLN. In previous experiments there was no detectable alteration in ATPase+ cell numbers following UVA1 irradiation (see chapter 4). There is evidence that UVB irradiation induces TNF-α production in the human epidermis, but that UVA1 does not (Skov *et al* 1998a). These experiments were conducted to investigate whether UVA1 and UVB were capable of increasing DC numbers in DLN for the site of exposure. The contribution of both cytokines was investigated in relation to DC increases induced by UVR.

The aims of these experiments were-

A. To examine the effects of UVA1 and UVB on DC accumulation in lymph nodes draining the site of exposure.

B. To examine the dependency of DC accumulation following UVR on TNF-α and IL-1β

5.2. An exposure of 2 MED UVB results in accumulation of DC within DLN.

It has been shown previously that irradiating mice with UVB leads to an increase in DC numbers within DLN (Moodycliffe *et al* 1994). Preliminary experiments were performed to establish the technique of DC enumeration and to corroborate this finding. C3H/HeN mice were exposed to 2 MED UVB (2880 J/m^2). Auricular lymph nodes were removed 48h later, and single-cell suspensions were prepared from pooled samples. DC were enriched by centrifugation on a 14.5% metrizamide gradient, as described in section 2.4.4. There was a substantial increase in total DLNC following UVB exposure, from 8.42 x 10^6 cells per DLN in unirradiated mice to 11.58 x 10^6 cells per DLN in irradiated mice (see fig. 5.1). This increase in total cells was accompanied by an even greater increase in DC per DLN, from 2,330 DC per DLN in unirradiated mice to 13,540 following a 2 MED UVB exposure.

5.3. Both UVA1 and UVB result in an accumulation of DC in DLN of irradiated sites.

It was next examined whether lower doses of UVB were capable of increasing DC numbers, and whether an equivalent dose of UVA1, in terms of MED, had the same effect. Mice were irradiated with either 1 MED UVA1 (500 kJ/m^2) or 1 MED UVB (1440 J/m^2), as described in section 2.1.6. Auricular lymph nodes were collected 24h or 48h later. As shown in table 5.1, DC were increased by both UV regimens. There was an approximate doubling of DC numbers in the DLN by 24h after UVB or UVA1 irradiation, and this value was not substantially increased at 48h after a 1 MED exposure. Total DLN cellularity was increased by 41% after UVA1, and by 86% following UVB, irradiation. It has been reported previously that DC were maximally increased 48h after UVB-irradiation (Moodycliffe *et al* 1992), but this was

not apparent from these experiments.

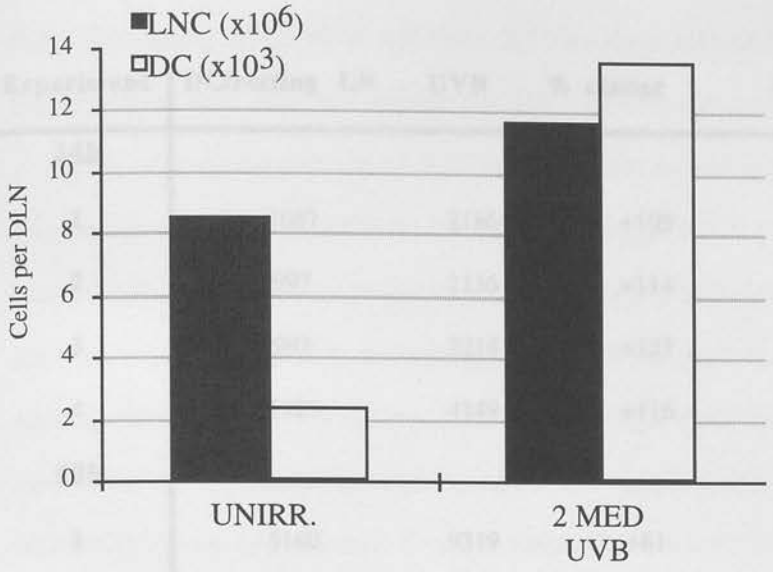


Figure 5.1. UVB induces DC accumulation in DLN. Mice were given a single exposure of 2 MED UVB and auricular lymph nodes collected 48h later. Control mice were unirradiated. Single-cell suspensions were derived from auricular lymph nodes, and total cells counted (solid bars). DC were enriched on a 14.5% metrizamide gradient before counting (clear bars).

Experiment	DC/resting LN	UVB	% change	UVA1	% change
24h					
1	1067	2186	+105	1857	+74
2	997	2136	+114	1929	+93
3	992	2214	+123	3450	+248
4	1923	4149	+116	3606	+88
48h					
1	5160	9319	+81	13320	+158
2	2800	9150	+227	4320	+54
3	4180	6075	+45	4600	+10

Table 5.1. UVA1 and UVB irradiation induce an accumulation of DC in DLN. Mice were given a 1 MED exposure of either UVB or UVA1, and DLN collected 24h or 48h later. Control mice were unirradiated. DC were enriched on a 14.5% metrizamide gradient before counting. Results from several individual experiments are shown as the mean DC per DLN, and the percentage change from their respective control (unirradiated) mice, within individual experiments.

5.4. Blocking of TNF-α inhibits DC accumulation as a result of UVB irradiation, but has no effect on UVA1-induced accumulation.

It was shown previously that intraperitoneal injection of anti-TNF-α could prevent the depletion of ATPase$^+$ cells from the epidermis if given prior to UVB (see section 4.8). It was therefore of interest to examine whether injection of the same antibody could prevent DC accumulation in DLN. It can be seen in fig. 5.2 that in mice given anti-TNF-α, there was significant abrogation of the increase in DC as a result of UVB exposure. In experiment 1, UVB resulted in a 133% increase of DC within DLN over DC numbers found in unirradiated mice (2136 DC per DLN vs. 997). In mice given anti-TNF-α, there was only a 9% increase in DC within DLN from UVB irradiated mice over DC numbers found in unirradiated mice (1257 DC per DLN vs. 1153). In a second experiment, UVB caused a 123% increase in DC within DLN, but this was reduced to a 36% increase in mice treated with anti-TNF-α. There was no significant difference in the numbers of DC per DLN from UVA1 exposed mice and those recovered from UVA1 exposed mice treated with anti-TNF-α. In experiment 1, the increase in DC numbers induced by UVA1 alone was 93% (1929 vs. 997 DC per DLN), and despite being reduced to a 45% increase in mice treated with anti-TNF-α, this was not significant. In a second experiment, UVA1 irradiation caused a 248% increase in DC within DLN, and this was only very slightly, and non-significantly, reduced by anti-TNF-α, to a 236% increase.

5.5. Blocking of IL-1β inhibits DC accumulation as a result of either UVB or UVA1 irradiation.

LC migration from the epidermis following UVB exposure was shown in section 4.10 to be dependent on IL-1β as well as TNF-α. The effect of blocking IL-1β by the use of specific antibodies was therefore examined. When anti-IL-1β was injected 2h prior to either UVA1 or UVB irradiation, subsequent accumulation of DC within the auricular lymph nodes was inhibited. These results are presented in

fig. 5.3. In mice which were injected with normal goat serum and were not irradiated, 1067 DC per DLN were found. Injection with anti-IL-1β reduced this slightly, but not significantly. UVB irradiation of mice injected with normal goat serum increased DC numbers by 103% to 2186 per DLN, with injection of anti-IL-1β preventing this increase (1029 DC per DLN). UVA1 irradiation of mice injected with normal goat serum increased DC numbers by 74% to 1857 per DLN, with injection of anti-IL-1β similarly preventing this increase (929 DC per DLN).

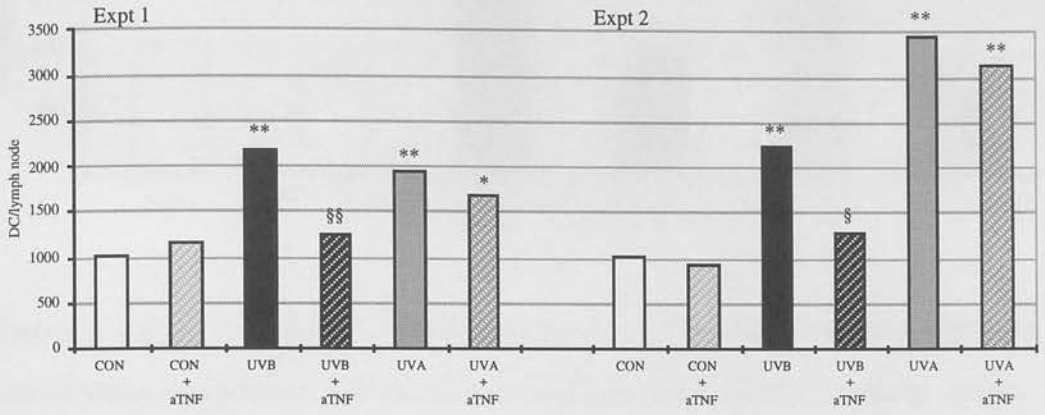


Figure 5.2. Anti-TNF-α reduces DC accumulation following UVB, but not UVA1, irradiation. Mice were injected intraperitoneally with 20μg anti-TNF-α or normal goat serum 2h before irradiating. Mouse ears were given a single exposure of either 1 MED UVB or 1 MED UVA1, and DLN collected 24h later. *= $p< 0.05$ and **= $p< 0.01$, vs. unirradiated control, §= $p< 0.05$ and §§= $p< 0.01$, vs. same irradiation protocol without antibody.

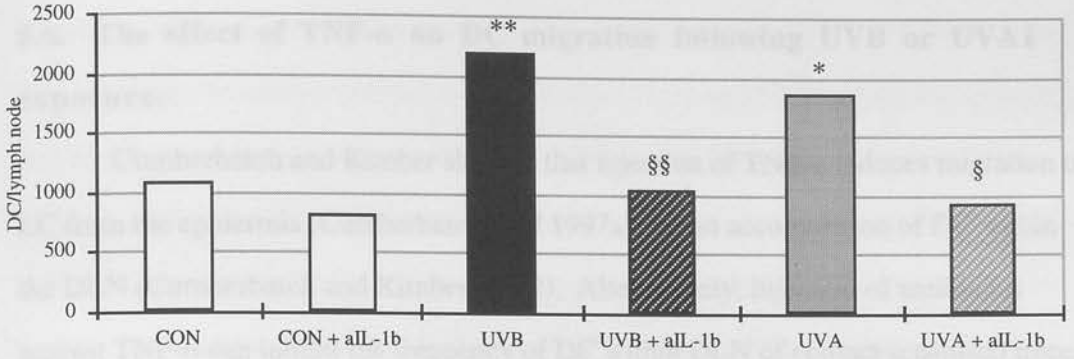


Figure 5.3. Anti-IL-1β reduces DC accumulation following UVB and UVA1 irradiation. Mice were injected intraperitoneally with 20μg anti-IL-1β or normal goat serum 2h before irradiating. Mouse ears were given a single exposure of either 1 MED UVB or 1 MED UVA1, and DLN collected 24h later. *= $p< 0.05$ and **= $p< 0.01$, vs. control, §= $p< 0.05$ and §§= $p< 0.01$, vs. same treatment without antibody.

5.6. The effect of TNF-α on DC migration following UVB or UVA1 exposure.

Cumberbatch and Kimber showed that injection of TNF-α induces migration of LC from the epidermis (Cumberbatch *et al* 1997a) and an accumulation of DC within the DLN (Cumberbatch and Kimber, 1992). Alternatively, injection of antibodies against TNF-α can inhibit the frequency of DC within DLN of contact sensitised mice. Subsequent CH responses were also impaired in these mice (Cumberbatch and Kimber, 1995). Using the same methods, it has been shown that TNF-α is a critical factor in accumulation of DC within DLN of UVB irradiated mice (Moodycliffe *et al* 1994). As it appears that TNF-α is not necessary for accumulation of DC within DLN following UVA1 irradiation (see section 5.4), it was anticipated that injection of TNF-α would enhance DC accumulation above that induced by UVA1 alone. Conversely, TNF-α was found to be critical in UVB-induced accumulation of DC, and it was anticipated that injection of this cytokine would have no marked effect on the number of DC within DLN.

In these experiments, mice were irradiated 24h, and injected with TNF-α 4h, before collecting DLN. The results of this are shown in fig. 5.4. TNF-α injection caused a marked increase of DC numbers, up to 8060 DC per DLN from 1925 in unirradiated mice injected with 0.1% BSA. As expected, DC were increased in mice exposed to 1 MED UVB (by 116% to 4149) and in UVA1 irradiated mice (by 88% to 3606). These levels of accumulation are in agreement with those reported earlier (see section 5.3). TNF-α injection into irradiated mice further increased the number of DC per DLN, to 8685 in UVB irradiated mice and to 9835 in UVA1 irradiated mice. Therefore, UVA1, but not UVB, irradiation, appears to augment the accumulation of DC in DLN of TNF-α injected mice.

Mice exposed to 1 MED UVA1 followed immediately with 1 MED UVB exhibited DC accumulation (an increase of 98% over unirradiated control mice to 3806 DC per DLN) (also shown in fig. 5.4). This increase was therefore comparable to

irradiation with either spectrum alone (UVB = 116% increase; UVA1 = 87% increase). This result can be interpreted in 2 ways. Firstly, UVA1 and UVB act on the same cells and therefore result in similar accumulation of DC, with the accumulation not being additive. Secondly, UVA1 exposure results in accumulation of one population of DC, and inhibits accumulation of another population of DC following UVB exposure. In conjunction with the data found following TNF-α injection, this experiment provides tentative evidence that UVA1 acts on a different DC population than UVB and TNF-α.

An increase in lymph node DC after UVA1 exposure could be detected (section 5.3), but there was no reduction or alteration in ATPase$^+$ cell numbers in the epidermis (section 4.2). Taken together, these data suggest that UVA1 may act on a cutaneous APC population other than LC. To investigate this possibility, it was decided to attempt to examine potential effects on dermal DC. Unfortunately, dermal DC in the mouse comprise a large number of cell types and without defined phenotypic markers. Therefore, macrophages infiltrating the skin after UV irradiation may be misinterpreted as dermal DC, as might LC migrating out of the epidermis. This infiltrate is maximal by 48-72h after UVB irradiation (Meunier *et al* 1995). One technique to evade both these problems is to study cells which have migrated out of the ear during an *ex vivo* culture period, thereby removing the possibility of a macrophagic infiltrate.

The rationale behind this approach was to use spontaneous migration of cells from split ear halves as a starting value, representing APC capacity. DC were identified by morphology under a light microscope. This technique has been used by Larsen and colleagues to obtain migratory cells from the dermis for functional studies (Larsen *et al* 1990). Subsequent treatment would reduce the number of LC within the ears and therefore reduce the number of DC recoverable by floating the ears on RPMI-FCS.

For instance, it has been shown in chapter 4 that UVB irradiation results in migration of LC from the ear *in vivo*. If ears were taken at a time before macrophage infiltration could occur, there would be fewer DC within the ear, and therefore fewer recoverable DC following floating on RPMI-FCS. It was postulated that UVA1 would act on a cell population other than LC, also resulting in migration out of the ear *in vivo*. Therefore, UVA1 would similarly result in fewer cells available for migration into RPMI-FCS upon floating. Finally, if ears were irradiated with both UVB and UVA1, there would be migration of both cell populations *in vivo*. There would be an additive effect and therefore even fewer recoverable DC than with either irradiation protocol alone. This would provide at least circumstantial evidence for UVB and UVA1 acting

on different cell types *in vivo*. A schematic representation of this theory is shown in fig. 5.5. Experiments were conducted to establish the feasibility of such an approach.

5.7. DC migration from ear halves is a quantifiable method and suitable for investigation of UVR effects.

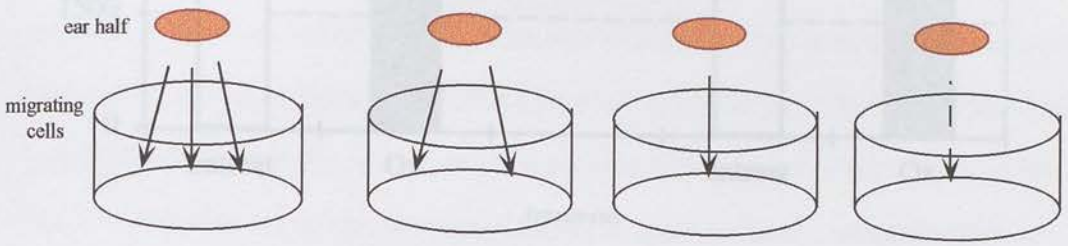
Preliminary experiments investigated whether DC could be recovered from RPMI-FCS. Ears were taken and split into halves, then floated on RPMI-FCS. Supernatants were collected and cells washed by centrifugation. Recovered cells were counted under light microscopy, and DC identified. DC were recoverable after 24h in numbers ranging from 4500 to 6000 cells per ear (data not shown). By 96h of culture RPMI-FCS became contaminated due to deterioration of the ear half and DC could not be recovered.

The next series of experiments investigated whether it was possible to alter the number of recoverable DC. Mice were contact sensitised by applying 20 µl 1% Ox to both ear surfaces, the ears immediately removed and split into halves with forceps. Ear halves were then floated on RPMI-FCS for 24h, allowing for spontaneous migration of DC from ear halves. Medium was then removed, washed by centrifugation and DC counted by light microscopy. The number of cells recovered from the medium are shown in fig. 5.6. DC numbers were not significantly altered by this technique, presumably because cells were not allowed enough time migrate *in vivo*. When ears were left *in vivo* for 1 day following contact sensitisation before removal, DC numbers recovered by floating on tissue culture medium for a further 24h were reduced (see fig 5.6). This indicates that this system may be utilised as a quantitative measure of DC remaining in the ear various times after treatment. Due to time constraints, such experiments involving exposure to various UV regimens were not pursued.

1. *In vivo* treatment =

unirradiated UVB UVA1 UVB, UVA1

2. Time delay -> ears *ex vivo*.



3. Spontaneously migrated cells recovered =

X X - B X - A X - C

4. Cells migrated due to treatment =

B A C

If A = B = C, this would suggest A and B are the same cells.

If C > A or B, this would suggest the cells are from different populations, but if C < A + B, this would suggest some of the cells are the same.

Fig. 5.5. A schematic diagram to explain the theory for investigating DC which spontaneously migrate from ear halves during floating on RPMI-FCS.

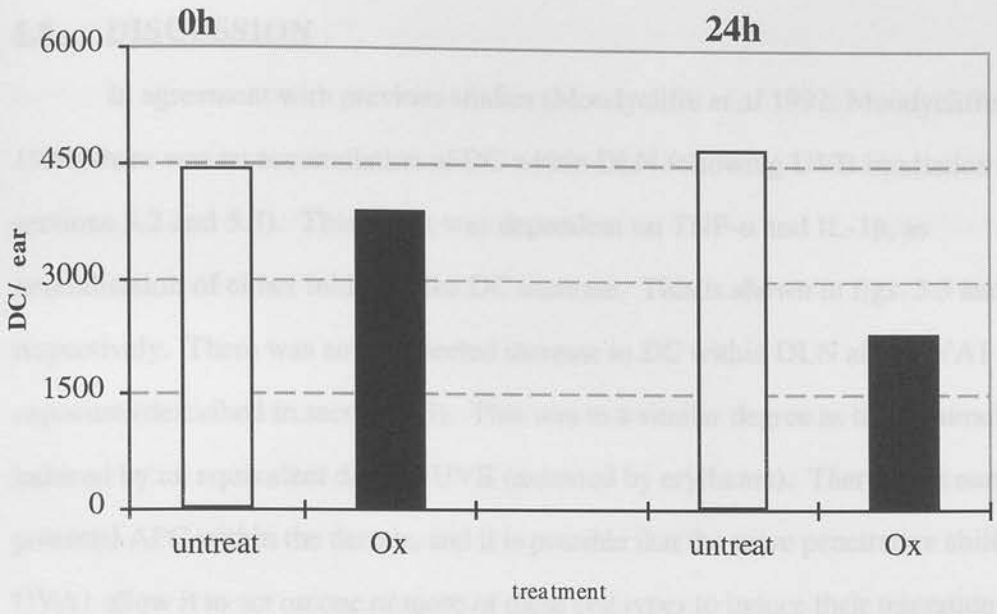


Figure 5.6. There is a detectable reduction of DC recovered from RPMI-FCS if ears are left for 24h after contact sensitisation. Mouse ears were contact sensitised by dropping 25µl 1% Ox or AOO on to both surfaces. Ears were removed either immediately (0h) or 24h later (24h), and split into halves with forceps. Ear halves were then floated on RPMI-FCS and the cells in the medium counted 24h later.

5.8. DISCUSSION

In agreement with previous studies (Moodycliffe *et al* 1992; Moodycliffe *et al* 1994) there was an accumulation of DC within DLN following UVB irradiation (see sections 5.2 and 5.3). This effect was dependent on TNF-α and IL-1β, as neutralisation of either inhibited the DC increase. This is shown in figs. 5.3 and 5.4 respectively. There was an unexpected increase in DC within DLN after UVA1 exposure (described in section 5.3). This was to a similar degree as the accumulation induced by an equivalent dose of UVB (assessed by erythema). There are a number of potential APC within the dermis, and it is possible that the more penetrative abilities of UVA1 allow it to act on one or more of these cell types to induce their migration to DLN. Only antibodies to IL-1β, but not TNF-α, prevented DC accumulation after UVA1 (see sections 5.4 and 5.5). The most likely candidate as an APC to mediate UV-induced immune suppression is dermal DC.

TNF-α was not necessary for accumulation of DC within DLN following UVA1 irradiation, as injection of neutralising antibodies into UVA1 irradiated mice did not prevent DC accumulation (see section 5.4). As such, it was investigated whether injection of TNF-α could augment the accumulation of DC within DLN of irradiated UVA1 alone. It is of interest that TNF-α injection into mice which had been exposed to UVB caused only a small increase in DC per DLN above that induced by TNF-α injection alone. This was from 8060 in unirradiated mice to 8685 in UVB irradiated (an increase of only 625 DC). This increase is probably due to 1 MED UVB not inducing secretion of maximal concentrations of TNF-α, so that injection of exogenous TNF-α may act on cells not affected by UVB to induce their migration and subsequent arrival in the DLN. The increase in UVA1 irradiated, TNF-α injected mice was 1775 above that induced by TNF-α alone (see section 5.6). In comparison with unirradiated mice, UVA1 caused an 87% increase in DC per DLN from 1925 to 3606 (therefore 1681 more DC per DLN). These values (a 1681 increase with UVA1 over control mice, and a 1775 increase over TNF-α injected mice) further strengthen the argument

that UVA1 may promote DC accumulation in DLN independently of TNF-α. Treatment of mice with 1 MED UVA1 followed immediately by 1 MED UVB did not result in DC numbers equivalent to addition of either irradiation protocol alone, but rather equivalent to only UVA1 exposure (see fig. 5.4). This may indicate that UVA1 induces a signal within the skin which inhibits the action of the subsequent UVB exposure.

The efficiency of CH sensitisation is impaired when contact sensitisers are applied to skin naturally deficient, or depleted, of LC. There is evidence that a second pathway of cutaneous antigen presentation exists. Ia^+ DC, present in the perivascular region of the mouse dermis, when hapten-derivatised, are capable of inducing CH in naive mice (Tse and Cooper, 1990). Thus, if UVA1 depleted these cells, it may impair subsequent immune responses.

Alternatively, there also exists a population of Thy-1^+, Ia^- cells, exhibiting a dendritic morphology, within the murine epidermis. It has been suggested that the ratio of these cells to LC influences the intensity of CH (Bigby *et al* 1987). Sullivan and colleagues showed that adoptive transfer of hapten-derivatised Thy-1^+ DC specifically downregulated contact sensitisation (Sullivan *et al* 1986). If UVA1 could initiate migration of these cells to DLN, it may alter the ratio of respective APC and interfere with later immune responses. Chronic UVA (10 kJ/m^2, 3 times per week for 4 weeks) has been shown to reduce Thy-1^+ cells from murine epidermis (Aubin *et al* 1991). This would also provide a possible explanation as to why Reeve *et al.* (Reeve *et al* 1998; Reeve *et al* 1999), when using interacting UV wavelengths, observe immune protection. In their experiments CH responses in mice were not inhibited if the mice were irradiated with UVA before contact sensitisation, but were if mice received UVB before sensitisation. Furthermore, if mice were irradiated with UVA followed immediately by UVB, before contact sensitisation, CH responses elicted later were less suppressed than those in mice given only UVB. It could be that both subsets of cells (Thy-1^+, Ia^-, and LC) would appear in DLN around the same time. Further

studies have indicated that the immunoprotective activity of UVA in this system is due to the induction of heme oxygenase. This molecule protects tissues from oxidative stress. Heme oxygenase progressively increased in UVA irradiated skin, but when expression was blocked, the immunoprotective effect against UVB or *cis*-UCA was abrogated (Reeve and Tyrell, 1999).

An inflammatory infiltrate, consisting of lymphocytes and macrophages, is localised around the vessels of the upper and mid dermis following UVA1 exposure (Lavker and Kaidbey, 1997), indicating that a signal is induced deep in the skin. Young and colleagues identified that irradiation of human skin with monochromatic light of 300 nm results in DNA damage in dermal cells (Young *et al* 1998). Therefore, it seems unlikely that pyridimine dimers are involved in initiation of the signal. This signal is not dependent on TNF-α, as antibodies to neutralise this cytokine did not inhibit DC accumulation following UVA1 exposure. TNF-α is neither chemotactic nor chemokinetic for macrophages (Webb *et al* 1996), so blocking of activity may not alter this infiltrate, but possibly affect subsequent interactions or cytokine release. Blocking IL-1β may prohibit activation of infiltrating macrophages. Another possible reason for migration of these cells as opposed to LC may be differential effects on adhesion molecules which act to retain the cells in the dermis. LC express α6 integrin, whereas DC do not, and antibodies against α6 integrin inhibit Ox and TNF-α induced LC migration from the epidermis (Price *et al* 1997). TNF-α causes LC to round up in the epidermis of mice treated with antibodies against α6 integrin, but does not induce migration. Dermal DC may express different adhesion molecules than LC and may be more susceptible to IL-1β, but not TNF-α, induced migration.

Dermal macrophages are induced to proliferate and infiltrate the epidermis following UVB-induced injury. Meunier and colleagues (1995) recognised that, after exposing humans to a 4 MED UVB, there was an expansion of $CD45^{+}DR^{+}$ (macrophage) cells around the vasculature. This change could be seen as early as 24h, and was maximal between 48 and 72h, after irradiation. Three days following

irradiation, the $CD36^+$ macrophage subset represented 53.3% of dermal DR^+ cells, whereas they represented 18.3% of this population in unexposed skin. Experiments by Tang and colleagues suggest that these cells are capable of migration to DLN, as phenotypic experiments investigating hapten carriage in mice after UVB irradiation indicate that hapten is presented by macrophage-like cells, rather than LC, in DLN of the irradiated mice. Whereas 49% of DLN DC had Birbeck granules in FITC sensitised mice, this fell to 11% in mice which had been irradiated before FITC sensitsation (Tang *et al* 1992).

Moodycliffe *et al.* (1992) showed that UVB irradiation alone induced DC migration to DLN, and UVB prior to contact sensitisation at the same site enhanced migration. The percentage of DC bearing FITC and the quantity of FITC per cell was unaltered by UVB irradiation. Additionally, UVB did not alter Ia expression on FITC-bearing DC. Neither UCA isomer (even above physiological levels, 100-200 μg/mouse) had an effect on DC numbers in lymph nodes of sensitised or unsensitised mice (Moodycliffe *et al* 1992).

UVB not only alters DC numbers, but can change the antigen presenting ability of DC. A UVB dose in excess of 1000 J/m^2 inhibits the stimulation of primary T cell responses by DC from human blood to alloantigen by 60-70% (Young *et al* 1993). The effect was 2- or 3-fold greater on MLR than in polyclonal mitogenesis, using comparable DC numbers and UVB doses. Costimulatory molecules (B7, ICAM-1) were reduced in a dose-dependent manner, although cell viability was unaffected. Sufficient contacts to prime T cell responsiveness to IL-2 could be formed, although subsequent proliferation was severely impaired without supplemental lymphokines. T cell alloreactivity was preserved in the secondary response (Young *et al* 1993).

In experiments by Noonan and colleagues, the APC function of splenic DC was tested after either a single 27 kJ/m^2 UVB exposure or intravenous injection of 50-200μg *cis*- or *trans*-UCA per mouse. Splenic DC (65-90% Ia^+, 8-11% FcR^+, <2% Thy-1.2^+) were obtained by density centrifugation and pulsed with DNP_6 ovalbumin,

then the proliferative response of T cells derived from mice immune to DNP_6 ovalbumin measured. Splenic DC from UVB-irradiated or *cis*-UCA treated mice had significantly impaired APC function, whereas those from *trans*-UCA mice had normal ability (Noonan *et al* 1988). This was despite the DC having the same Ia expression. Mixtures of DC from irradiated or *cis*-UCA treated mice with normal DC did not impair APC function. A time delay for the onset of defective APC function was required, as DC taken 3 days after irradiation had normal activity, whilst those taken 7 days later were suppressive. Direct addition of *cis*-UCA to *in vitro* proliferation assays was without effect, nor did addition of indomethacin, to inhibit prostaglandins, have any effect. This may indicate that essential mediators are not available once cells are removed from UVB-exposed mice.

An experimental protocol to investigate potential effects of UVA1 on cutaneous APC populations other than LC were established. The method of floating ear halves on RPMI-FCS is used to obtain migratory cells from the dermis, traditionally for functional studies. Larsen and colleagues discovered that the majority of migratory leukocytes were Ia^+ LC, with the remainder comprised of Thy-1^+, $CD3^+$, $CD4^-$, $CD8^-$, dendritic epidermal cells and a small population of Ia^-, $CD11a18^+$ macrophages (Larsen *et al* 1990). The number of migratory cells was 8,000-10,000 per ear per day, from C57Bl/6 mouse ears. In the preliminary experiments reported here (section 5.7), cell recovery was 4500-6000 per C3H/HeN mouse ear, within 24h of floating. As cells migrate spontaneously from ear halves upon floating, the rationale behind this experimental protocol was that a reduction in cell numbers recovered *ex vivo*, would indicate an earlier migration of cells *in vivo*. Recoverable cell numbers could be reduced by topical application of 1% Ox 24h before ear collection, but not if ears were removed immediately after contact sensitisation (see fig. 5.6). When assessed with data on LC migration, these observations suggest that contact sensitisation induced migration of cells from the ear within 24h. As the number of migratory cells was reduced at 24h, replenishment of LC or recruitment of other cells would appear not to

have occurred. Thus, the immunostimulatory capacity of the ear as a whole would be reduced. The theory behind tentative identification of different cell populations by this technique is explained in fig. 5.5.

5.9. Criticisms of experimental design and suggestions for further work

The experiments described in this chapter detail the role of IL-1β and TNF-α in UV-induced DC accumulation within DLN. As there appears to be a difference in dependency of these cytokines following UVB irradiation compared with UVA1, it was informative to perform additive experiments. Using both UV exposures, it could be determined whether the number of DC within DLN was increased by the same magnitude as the increase in UVB irradiated mice PLUS the increase in UVA1 irradiated mice. Such an experiment was performed with a 1 MED UVA1 exposure followed immediately by 1 MED UVB. This result showed that DC accumulation was not the equivalent of DC accumulation induced by UVA1 plus DC accumulation by UVB. When assessed with DC accumulation following TNF-α injection, it can be argued that UVA1 may inhibit actions of subsequent UVB irradiation. Due to time constraints and the number of mice involved in such an experiment, the reverse exposure regimen i.e. UVB followed by UVA1, was not performed. It would be anticipated that such an experiment would give DC accumulation suggestive of an additive effect, as any blocking effects mediated by UVA1 would be delivered after UVB.

Functional assays such as MLR would provide information on the immunostimulatory capacity of these apparently different DC populations. Experiments by Dittmar *et al* (1999) indicated that UVB irradiation prevents the upregulation of B7 on LC, but that UVA1 does not. Lack of B7 expression is associated with clonal anergy (Gimmi *et al* 1993). Additionally, phenotypic analysis, by the use of monoclonal antibodies and EPICS, would provide important information on the

immunostimulatory molecules expressed on the DC populations. Ia expression on DC accumulating in DLN following UVB does not appear to differ from that on normal DC, with both sets of cells presenting the same amount of FITC after contact sensitisation (Moodycliffe *et al* 1992).

Previous experiments by Moodycliffe and colleagues (1992) showed that the maximum accumulation of DC within DLN of irradiated mice occured 48h after exposure. Although this was not observed in these experiments, where the increase at 24h was proportionally the same as that observed at 48h, it may be constructive to have investigated UVA1 induced accumulation over time. The hypothesis that UVA1 can induce accumulation of a dermal DC population may be conducive to arrival of these DC in the DLN before epidermal LC.

Chapter 6

Cytokine and ICAM-1 expression in the skin following UVB or UVA1 irradiation

6.1 Introduction

CH and DTH are Th1-like cytokine mediated immune responses, and as such, suppression may be mediated through an altered cytokine balance, leading to Th2 cell growth or activation. This may be in the form of altered antigen presentation, by way of an altered microenvironment, in which specific T cells are activated. The effects of UV exposure on LC and DC have already been discussed (see chapters 4 and 5 respectively). This chapter will detail effects on cytokine and adhesion molecule (ICAM-1) expression in the skin following UV exposure.

Interleukin-10, as discussed in section 1.10.3, is an important cytokine in the development of Th2-like immune responses, promoting humoral immunity. IL-10 can act on APC, rendering LC tolerogenic and downregulating class II MHC expression on macrophages. The importance of IL-10 has been shown in maintenance of tolerance in the gut, as IL-10 deficient mice develop inflammatory bowel disease (Kuhn *et al* 1993).

Induction and expression of IL-10 following high dose UVB was investigated by various techniques; immunoperoxidase staining of mouse skin samples and mouse skin cell lines (PAM-212 and B16), ELISA of skin homogenates, cell line supernatants and lysates, and PCR of skin sample homogenates.

Intercellular adhesion molecule (ICAM)-1 (CD54) can be expressed on keratinocytes. ICAM-1 is the ligand for both leukocyte function associated antigen (LFA)-1 (CD11aCD18) and Mac-1 (CD11bCD18), which are commonly expressed on T cells and cells of the monocyte/ macrophage lineage respectively. Thus, ICAM-1 is

important in attraction, and maintenance, of these cells at sites of expression. It is proposed that UV may modulate immune responses by altering ICAM-1 expression and the subsequent cellular infiltration. It is also possible that UCA, and/or histamine (Mitra *et al* 1993), may affect the level of ICAM-1 expression following irradiation, and this has been investigated.

Finally, TNF-α is implicated in regulation of ICAM-1 expression and in migration of LC from the epidermis (see sections 4.8 and 4.9). IL-1β is also involved in LC migration following UVB exposure. Expression of IL-1β and TNF-α mRNA in irradiated ears was examined by PCR, following either UVA1 or UVB exposure. Samples of irradiated skin, together with supernatants and lysates of cultured cell lines or primary epidermal cells were examined for TNF-α protein.

The aims of these experiments were-

1. To examine the source(s) of cutaneous IL-10, and its role in suppression of DTH, following UVB irradiation of mice.
2. To examine differences in cytokine gene expression in the skinfollowing either UVA1 or UVB exposure of mice.
3. To examine the regulation of ICAM-1 expression on keratinocytes by UCA isomers.

RESULTS

The role and source(s) of IL-10 in UVB-mediated immune suppression.

6.2. The role of IL-10 in UVB and *cis*-UCA mediated suppression of DTH responses.

These experiments were conducted to examine the role of IL-10 in the immunomodulation induced by UVB exposure and *cis*-UCA application on DTH responses. Mice were irradiated with 1500 J/m^2 UVB, 4h before injection with anti-IL-10. Three days later mice were infected with HSV-1. When the DTH response was elicited 10 days later by injecting HSV-1 antigens into the ear pinnae, it was found that the DTH response was significantly suppressed by UVB treatment ($p= 0.001$ and $p= 0.014$, vs. positive controls in individual experiments). Suppression was measured at 61% and 59% within individual experiments. Suppression was enhanced in mice that had been injected with antibodies to IL-10, to 91% and 85% respectively. This enhancement was significant in only one UVB experiment ($p= 0.029$, vs. UVB and control serum in experiment 1). This is shown in fig. 6.1. In parallel experiments, 100μg *cis*-UCA was injected subcutaneously into groups of mice, and mice injected with anti-IL-10 or control serum 4h later. One hour later, HSV-1 infection was established by injecting mice with 5 x 10^5 pfu HSV-1 subcutaneously. The DTH response of these mice is also shown in fig. 6.1. In one of two experiments, *cis*-UCA suppressed the DTH response (by 39%; $p= 0.02$, vs. positive control). Injection of anti-IL-10 4h after *cis*-UCA exposure increased the suppression to 70% and 17%, although this was not significantly different from suppression induced in *cis*-UCA and control serum treated mice. Therefore, in these experiments injection of antibodies to neutralise IL-10 did not reverse UVB or *cis*-UCA mediated suppression of the DTH response to HSV. This suggests that IL-10 does not play a role in UVB-mediated suppression of DTH to HSV.

The antibodies against IL-10 used in this experiments were used in preliminary studies to investigate immunoperoxidase staining of cells for IL-10 expression, and

although not used for later investigations, did result in staining of IL-10. The SXC-1 clone secretes rat anti-mouse IL-10 (Mosmann *et al* 1990), and has previously been shown to have neutralising effects on IL-10 (Rivas and Ullrich, 1992; Rivas and Ullrich, 1994).

6.3. IL-10 in the serum of UVB irradiated mice.

C3H/HeN mice were irradiated with 7 MED (10 kJ/m^2) UVB on the shaved backs and serum collected at daily intervals thereafter. IL-10 content of the sera was measured by ELISA, as described in section 2.3.3. As can be seen in fig. 6.2, IL-10 protein was detectable at all times. IL-10 dropped from levels found constitutively in these mice by 1 day, but then peaked between 3 and 4 days following UVB exposure. IL-10 protein level was at its highest in serum taken 7 days after irradiation. The drop one day following UVB exposure may be due to IL-10 secreting cells being removed from the circulation, such as recruitment of macrophages (Kang *et al* 1994) or T cells (Di Nuzzo *et al* 1998) to irradiated sites. The peak at day 3-4 correlates with the time at which maximal systemic suppression of CH can be induced (Miyauchi and Horio, 1995).

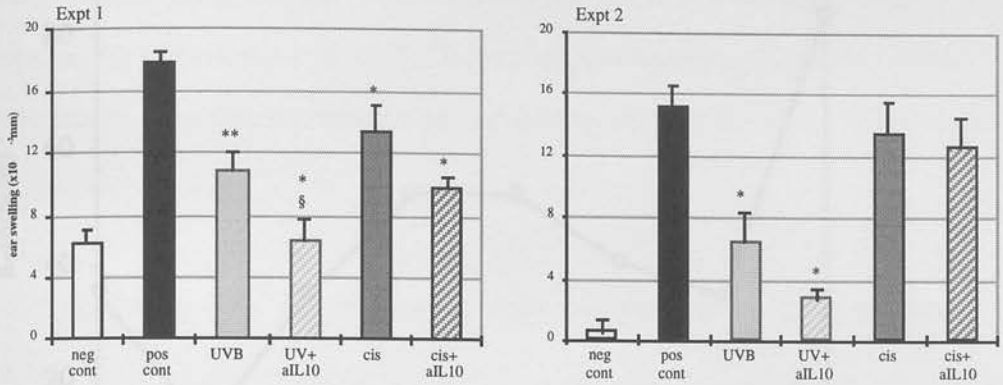


Figure 6.1. UVB or *cis*-UCA suppressed the DTH response to HSV-1, and this was not restored by neutralising IL-10 antibodies. Mice (n=8) were irradiated with 1500 J/m^2 UVB, and injected with anti-IL-10 or control serum (0.1% BSA) 4h later, 3 days before HSV-1 infection. Mice were injected subcutaneously with the 100µg *cis*-UCA 5h before, and injected with anti-IL-10 or control serum 1h before, HSV-1 infection. Mice were infected by subcutaneous injection with 5 x 10^5 HSV pfu. DTH was elicited 10 days later by injecting 2 x 10^5 pfu inactivated HSV into the pinnae of each ear. Increases in ear thickness were measured 24 hours later. Pre- and post-challenge measurements of ear thickness were made and the mean increase in ear thickness used as a measure of the DTH response. Results are expressed mean ± SEM for each group. Significant differences between the positive control and treated groups were determined by Student's *t*-test; *= $p<0.05$ and **= $p< 0.01$, vs. positive control; §= $p<0.05$ vs. same treatment without antibody.

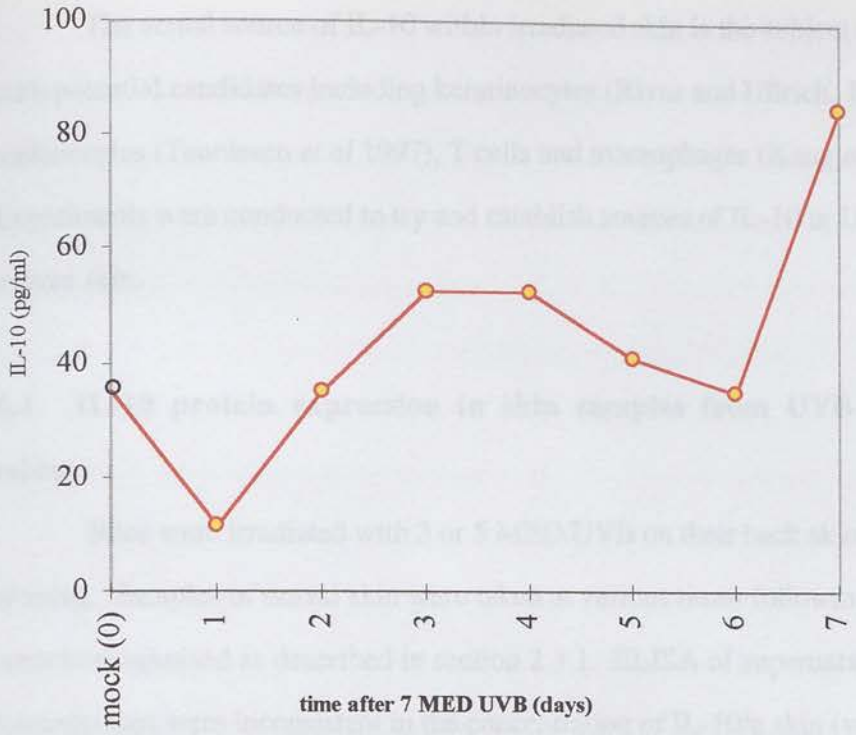


Figure 6.2. IL-10 is present in the serum of UVB irradiated mice. Mice were irradiated with 10 kJ/m^2 and sera collected from 2 mice at various times after exposure. IL-10 content was measured by ELISA, as described in section 2.3.3.

The actual source of IL-10 within irradiated skin is the subject of great debate, with potential candidates including keratinocytes (Rivas and Ullrich, 1992), melanocytes (Teunissen *et al* 1997), T cells and macrophages (Kang *et al* 1994). Experiments were conducted to try and establish sources of IL-10 in UVB-irradiated mouse skin.

6.4. IL-10 protein expression in skin samples from UVB exposed mice.

Mice were irradiated with 3 or 5 MED UVB on their back skin, one day after shaving. Samples of dorsal skin were taken at various times following exposure, and were homogenised as described in section 2.3.1. ELISA of supernatants from skin homogenates were inconsistent in the concentration of IL-10/g skin (wet weight) recovered, but commonly showed increased IL-10 within the first 24h following irradiation. A representative experiment is shown in fig. 6.3. This result suggests that IL-10 is released by cells resident to the skin following UVB irradiation, as the time of increased expression is too early for infiltrating cells to participate. Furthermore, IL-10 protein expression returns to control values after 24h, a time at which macrophages are beginning to appear in the epidermis (Hammerberg *et al* 1996).

6.5. IL-10 protein expression by UVB irradiated cells.

PAM-212 cells stained positively for IL-10 before UVB irradiation, and it was difficult to tell whether the staining became more intense following irradiation with 10 mJ/cm^2 UVB (see fig. 6.4). The staining appeared to become more focused around the nucleus.

B16 cells stained were very intensely, even with isotype control antibody. Attempts to stain B16 cells by using a longer hydrogen peroxide/ methanol incubation, to block endogenous peroxidase, did not significantly reduce the background staining.

Quantitative analysis of IL-10 production by cells were also performed by ELISA. Epidermal cell suspensions were prepared as described in section 2.1.3. Cells were seeded at a density of 1.5 x 10^6 cells/ well and irradiated with scaled doses of UVB (as described in section 2.1.5). Culture supernatants were collected, and cell lysates prepared (as described in section 2.3.2). ELISA of supernatants taken from epidermal cell suspensions 24h after irradiation suggested that IL-10 was secreted by these cells at a concentration of around 200 pg from 1.5 x 10^6 cells. *In vitro* irradiation had no discernible effect on the amount of IL-10 secreted, at doses up to 20 mJ/cm^2.

ELISA of culture supernatants indicated that IL-10 was not secreted by PAM-212 (fig. 6.5) or B16 (fig. 6.6) cells following UVB exposure, at doses up to 20 mJ/cm^2. Concentrations of IL-10 in cell lysates were very inconsistent, but suggested that IL-10 was unaltered within 24h of UVB exposure, by doses up to 20 mJ/cm^2. There was a decline in IL-10 within PAM-212 cells 48h after UVB irradiation, and this appeared to be dose-dependent.

UVB doses up to 20 mJ/cm^2 did not significantly alter viability of either cell line (PAM-212 and B16), so the decline in recoverable intracellular IL-10 could not be attributed to a drop in viable cell numbers.

6.6. IL-10 protein in by cell lines cultured with UCA isomers.

Untreated PAM-212 cells stained positively for IL-10 (see fig. 6.4). IL-10 staining increased in the cytoplasm, and became more focused around the nucleus, after 6h incubation with 10 μg/ml *cis*-UCA (shown in fig. 6.7). Incubation with 10 μg/ml *trans*-UCA had no effect on IL-10. In experiments using 6h incubation with 100 μg/ml UCA, there was no discernible difference between IL-10 staining in *cis*-UCA or *trans*-UCA incubated PAM-212 (see fig. 6.7). When PAM-212 were incubated with 100 μg/ml *cis*-UCA for 24h, there was increased cytoplasmic staining for IL-10 (see fig. 6.7).

Development of immunostaining by a technique other than peroxidase, such as use of fluorescent antibodies, would prevent some of the difficulties encountered during these experiments. The skin produces large quantities of endogenous peroxidase, particularly following UVB exposure. For example, peroxidase is produced during melanogenesis (Okun, 1996).

From the experiments described here, there does not appear to be a clear stereo-specificity with regard to stimulation of IL-10 expression by UCA. *Cis*-UCA appeared to be a slightly better stimulus than *trans*-UCA. It should be noted that despite the use of a cell line (PAM-212), not all cells stained positively for IL-10 expression. A similar pattern was observed following UVB irradiation.

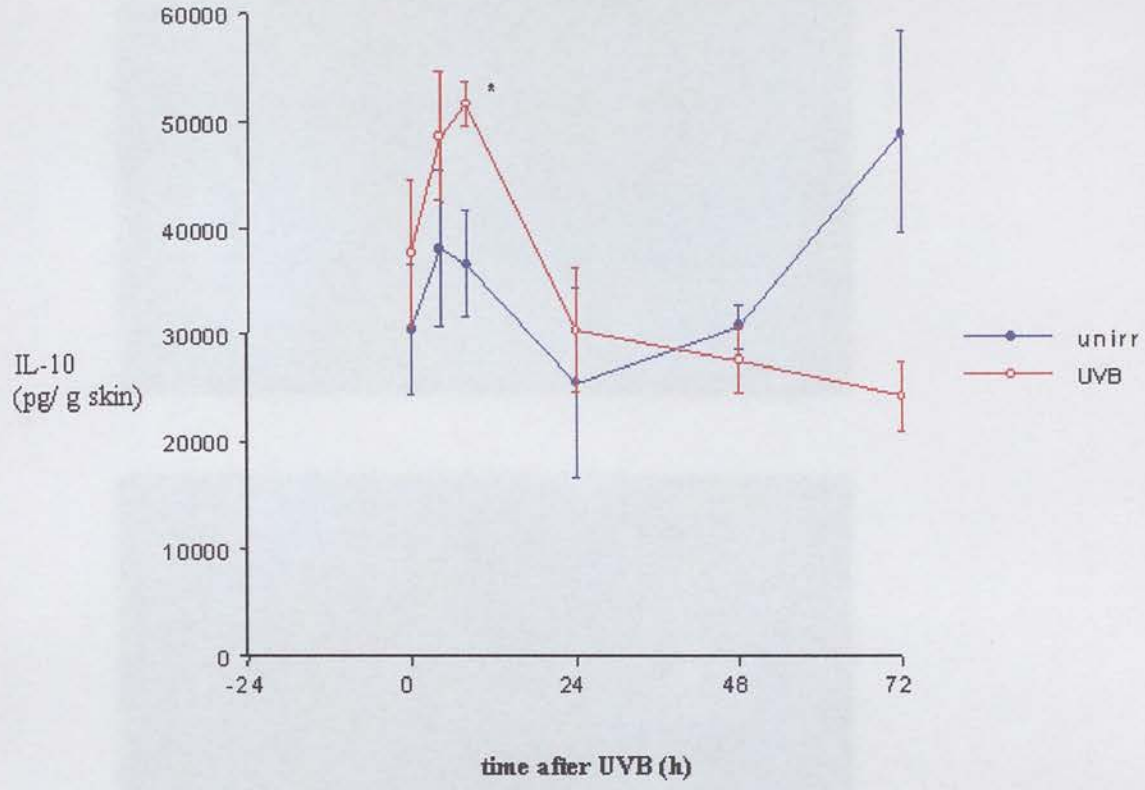
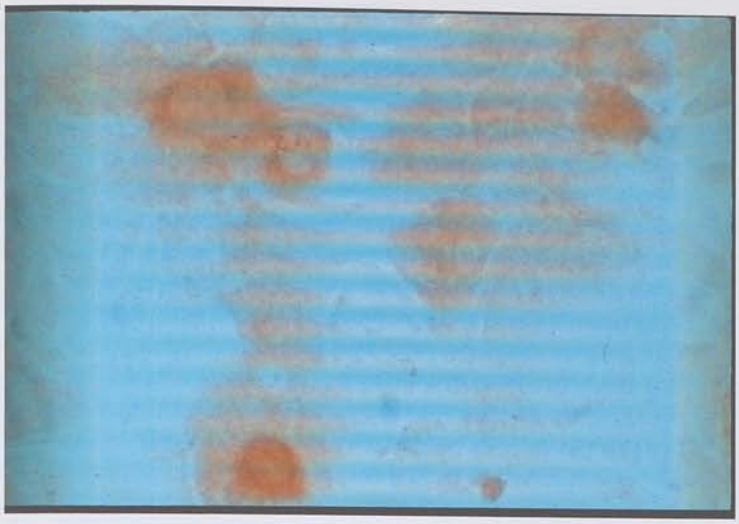


Figure 6.3. IL-10 protein is induced in the skin after 5 MED UVB. Mice (n= 3) were shaved on their dorsal surfaces and irradiated with 5 MED UVB. Skin samples were processed to extract IL-10 as described in section 2.3.1. IL-10 concentrations were determined by ELISA, as described in section 2.3.3. Results are shown as mean ± sem, of 3 samples, for each point. *=$p < 0.05$, vs. unirradiated at the same time.



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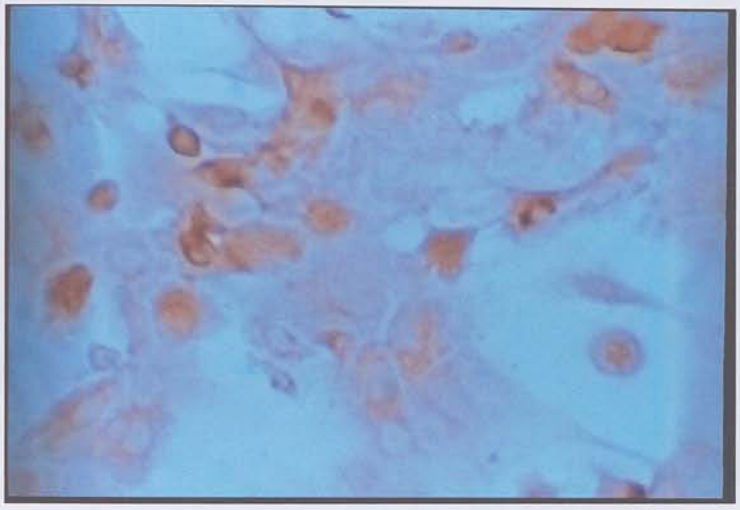

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Figure 6.4. IL-10 staining in PAM-212 cells following UVB irradiation. The top panel shows cells which were not irradiated and the bottom panel shows cells which were stained 24h after irradiation with UVB (both magnifications are x 125; cells incubated with negative control antibody were not stained).

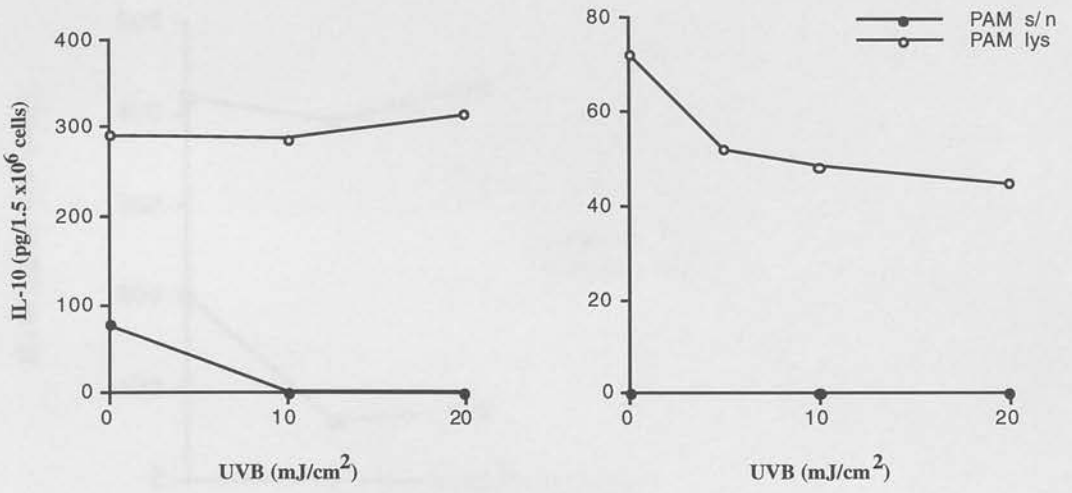

Figure 6.5. IL-10 in lysates or culture supernatants from of PAM-212 cells. 1.5 x 10^6 PAM-212 cells were seeded per well, 24-well plates, and irradiated with scaled doses of UVB. Lysates (open) and supernatants (closed) were collected 24h (left) or 48h (right) after irradiation and IL-10 content measured by ELISA, as described in section 2.3.3.

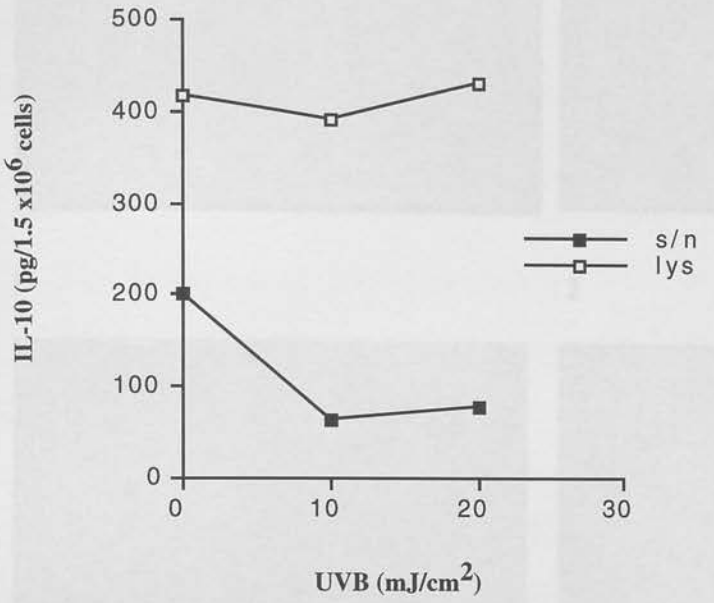

Figure 6.6. IL-10 in lysates or the culture supernatants from B16 cells. 1.5 x 10^6 B16 cells were seeded per well, 24-well plates, and irradiated with scaled doses of UVB. Lysates (open) and supernatants (closed) were collected 24h after irradiation and IL-10 content measured by ELISA, as described in section 2.3.3.

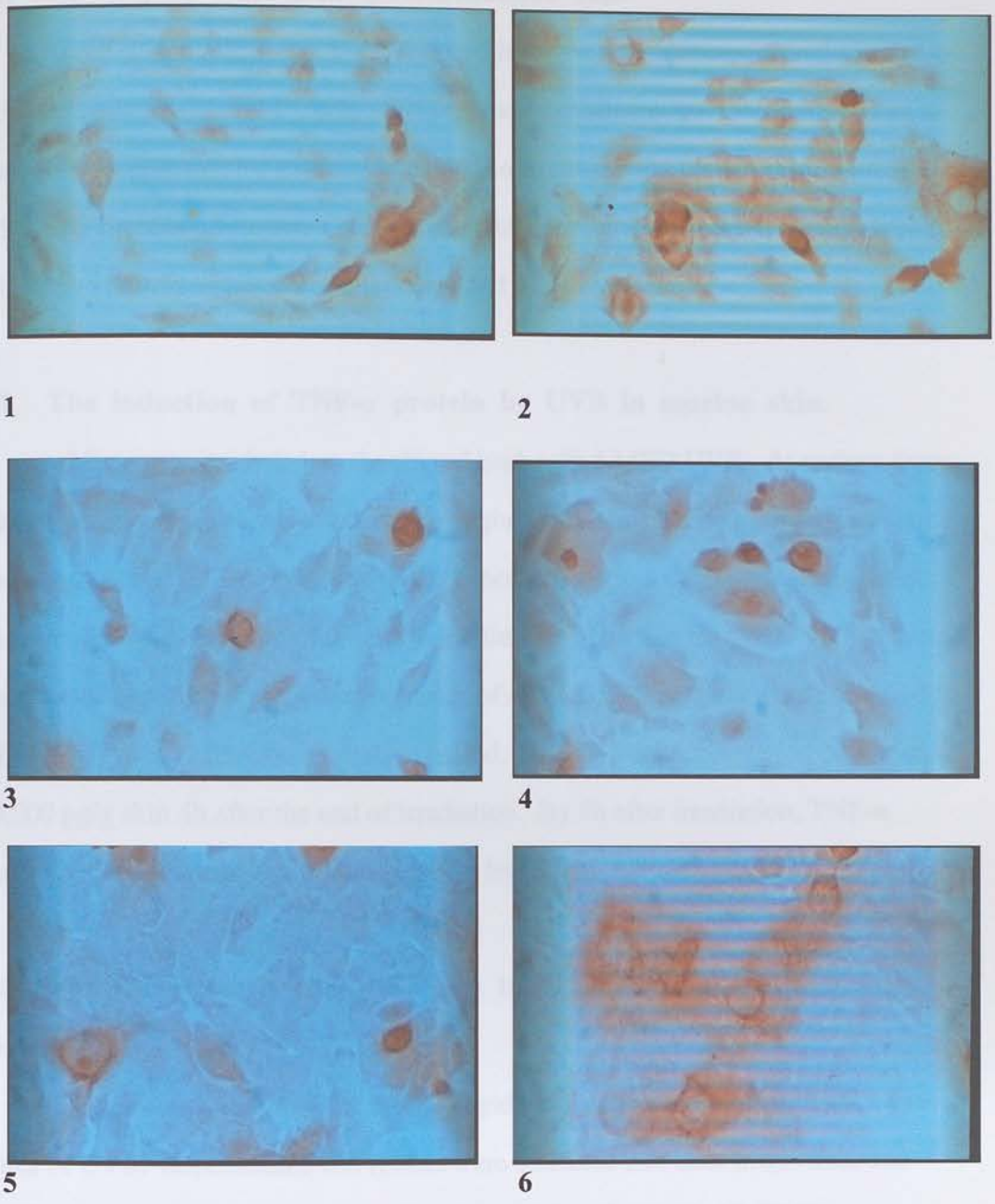

Figure 6.7. Immunostaining for IL-10 in PAM-212 cells incubated with UCA. Cells were incubated with 10 μg/ml (1) or 100 μg/ml (3) *trans*-UCA, and harvested after 6h. Panel 5 shows PAM-212 cells incubated with 100 μg/ml *trans*-UCA and harvested after 24h. The righthand panels show PAM-212 cells which were incubated with *cis*-UCA (10 μg/ml (2) or 100 μg/ml (4) harvested after 6h, and 100 μg/ml (6) harvested after 24h (all magnifications are x 125; cells incubated with negative control antibody were not stained).

The source(s) of TNF-α in UVB-irradiated skin.

TNF-α is implicated in UVB-induced immune suppression, particularly that of CH responses (Rivas and Ullrich, 1994). It has been shown that UV-induced DNA damage is responsible for expression of TNF-α in mouse and human skin (Kitibel *et al* 1998). As mentioned earlier, TNF-α is vital for LC migration and DC accumulation following UVB irradiation (see chapters 4 and 5).

6.7. The induction of TNF-α protein by UVB in murine skin.

Mice were irradiated on the shaved back with 5 MED UVB. At various times thereafter, skin samples were collected, weighed and homogenised. ELISA of skin homogenates for TNF-α revealed that the concentration was increased immediately after irradiation, which took 50 mins to administer (shown in fig. 6.8). TNF-α protein was found constitutively at a concentration of around 6000 pg/g skin, but increased to 15,000 pg/g skin within the irradiation period. The TNF-α concentration peaked at 20,000 pg/g skin 4h after the end of irradiation. By 8h after irradiation, TNF-α concentrations returned to, and remained at, levels comparable to unirradiated mice.

6.8. The induction of TNF-α protein by UVB irradiation of epidermal cells.

Single cell suspensions of primary epidermal cells were irradiated with various doses of UVB. Supernatants and lysates were collected 24h after irradiation, and TNF-α content of both measured by ELISA. As found with IL-10, TNF-α concentrations in supernatants and lysates were not altered significantly by UVB exposure. Concentrations found were 250 ± 30 pg from 1.5 x 10^6 cells.

When cell lines were analysed for TNF-α protein following UVB irradiation, TNF-α could not be found in supernatants from PAM-212 or B16 cultures. Increasing UVB doses reduced TNF-α within cultured cells. TNF-α content of PAM-212 cells was reduced by approximately 50%, 24h following exposure to 5 mJ/cm^2 UVB.

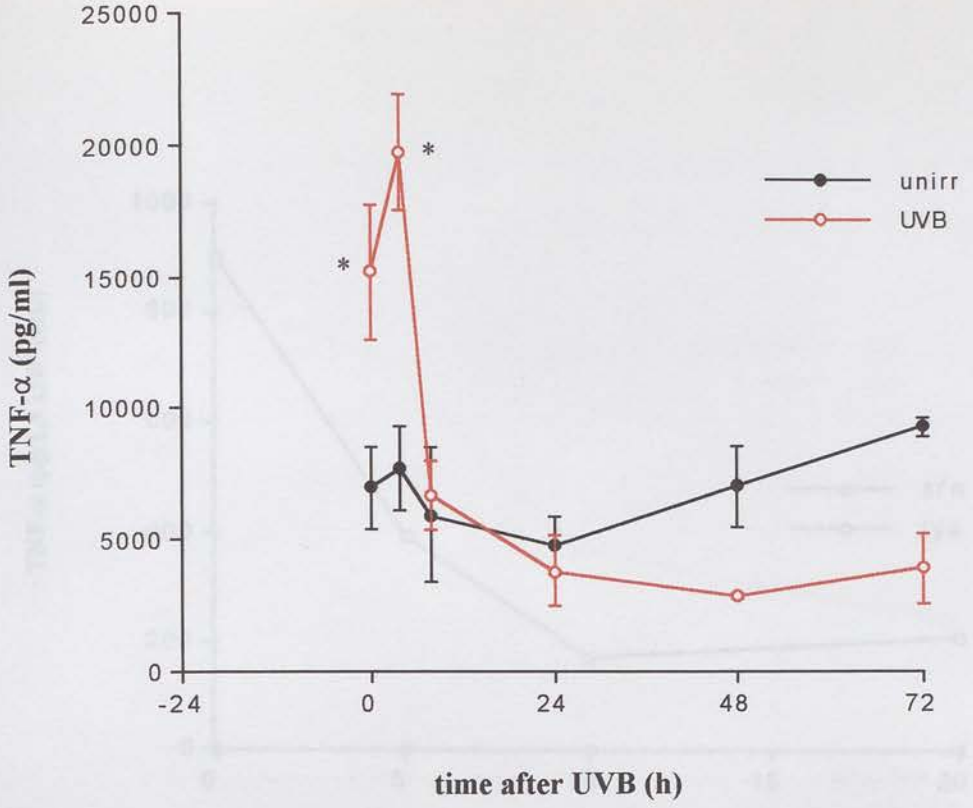

Figure 6.8. TNF-α protein is induced in the skin after 5 MED UVB. Mice (n= 3) were irradiated with 5 MED UVB. Skin samples were taken at various times after irradiation and processed for TNF-α extraction as described in section 2.3.1. TNF-α content was measured by ELISA, as described in section 2.3.3. Results are shown as mean ± sem, *= $p < 0.05$ vs. unirradiated at same time.

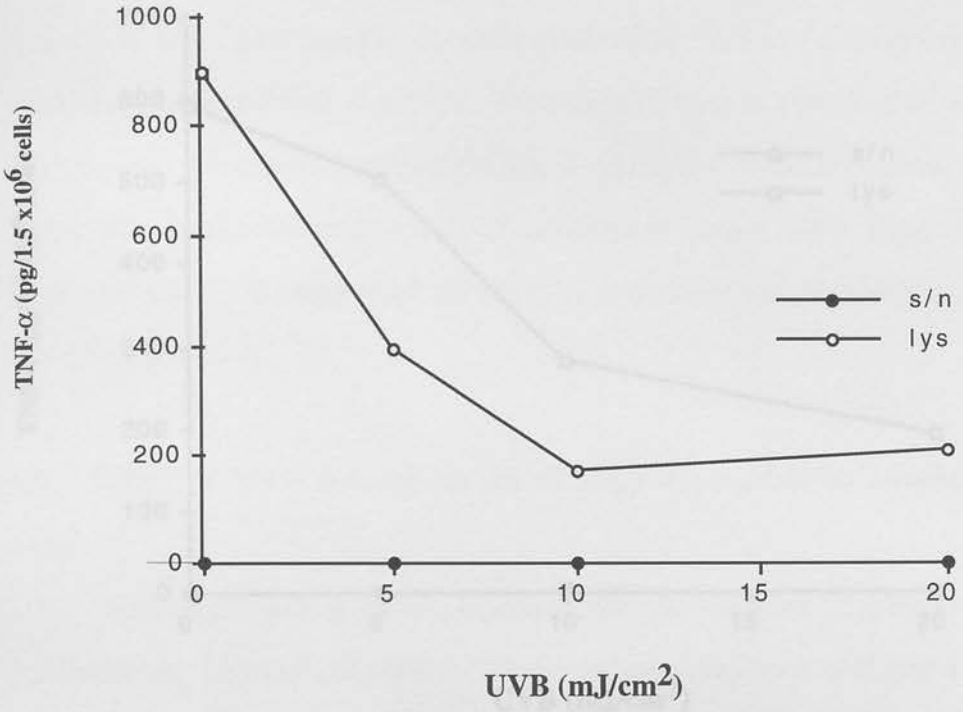

Figure 6.9. TNF-α is not released by keratinocytes (PAM-212), but intracellular stores are reduced by increasing UVB radiation. Cells were seeded at 1.5 x 10^6 per well and allowed to monolayer before irradiating. Culture medium was replaced with PBS during irradiation, and supernatants or cell lysates collected 24h later. TNF-α content of samples was assayed by ELISA, as described in section 2.3.3.

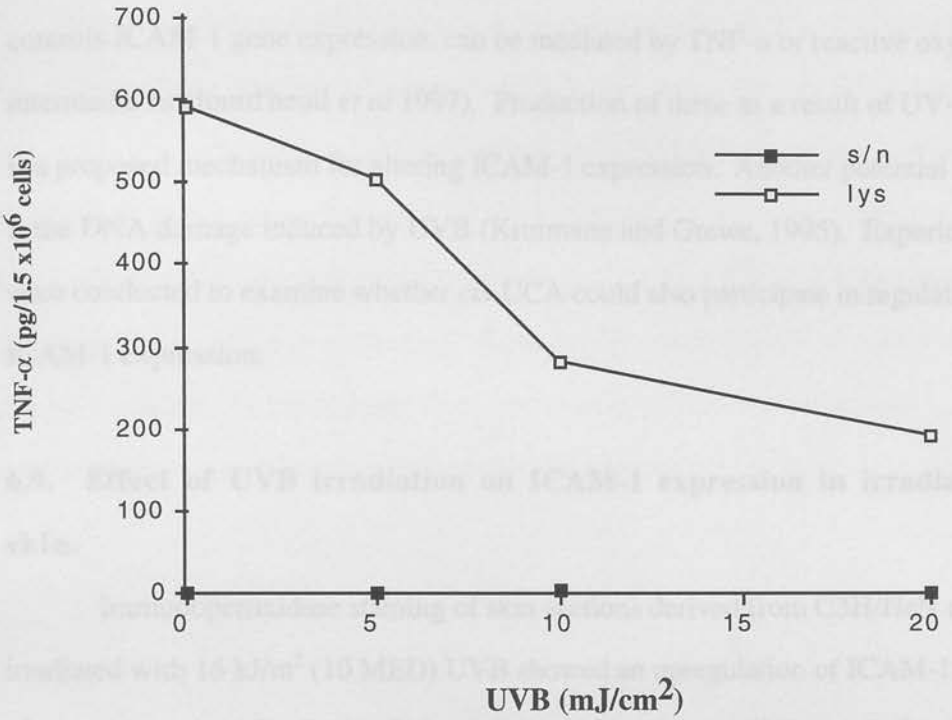

Figure 6.10. TNF-α is not released by melanocytes (B16), but intracellular stores are reduced by increasing UVB radiation. Cells were seeded at 1.5 x 10^6 per well and allowed to monolayer before irradiating. Culture medium was replaced with PBS during irradiation, and supernatants or cell lysates collected 24h later. TNF-α content of samples was assayed by ELISA, as described in section 2.3.3..

ICAM-1 expression following UVB exposure

LFA-1 and ICAM-1 are important for regulating the interaction of leucocytes and keratinocytes in cutaneous immune responses. Induction of NF-κB, which controls ICAM-1 gene expression, can be mediated by TNF-α or reactive oxygen intermediates (Jourd'heuil *et al* 1997). Production of these as a result of UV-radiation is a proposed mechanism for altering ICAM-1 expression. Another potential mediator is the DNA damage induced by UVB (Krutmann and Grewe, 1995). Experiments were conducted to examine whether *cis*-UCA could also participate in regulation of ICAM-1 expression.

6.9. Effect of UVB irradiation on ICAM-1 expression in irradiated skin.

Immunoperoxidase staining of skin sections derived from C3H/HeN mice irradiated with 15 kJ/m^2 (10 MED) UVB showed an upregulation of ICAM-1 at 24h after exposure (see fig. 6.11). This was most apparent on cells beneath the dermis, which lacked ICAM-1 expression before irradiation. There were patches of constitutive staining in the epidermis, and these were also increased by UVB exposure.

6.10. Effect of UVB irradiation on ICAM-1 expression on PAM-212.

ICAM-1 expression on PAM-212 was investigated by flow cytometry. The monoclonal antibody used to detect ICAM-1 expression was titrated against LPS-stimulated peritoneal macrophages, and gave positive staining (43%) at a dilution of 0.01 mg antibody/ml. DC, purified from lymph nodes by centrifugatin on a metrizamide gradient (71% positive, background = 13.3%) and LNC (41% positive, background = 1.6%) also gave positive staining using this protocol.

ICAM-1 expression could not be induced on PAM-212 by TNF-α alone, as background staining with isotype control antibody gave similar flow cytometry profiles to PAM-212 cells stained with anti-ICAM-1. Varying the concentration of *cis*-UCA,

trans-UCA, histamine, or their combinations, also failed to alter ICAM-1 staining above that of isotype control stained cells. This experiment was repeated 16 times and an enhancement in ICAM-1 staining did not occur.

It was possible that the treatment of the cultured cells, such as the method of removal from flasks destroyed the epitope on ICAM-1 which the monoclonal antibody recognised. The cells used as positive controls for ICAM-1 staining were relatively non-adherent, so not normally subjected to trypsinisation. However, when positive control cells were treated with trypsin and then analysed for ICAM-1 expression, the flow cytometry profile was unchanged. Additionally, when PAM-212 cells were removed mechanically from plates, flow cytometry profiles were not altered. Trypsinisation was not therefore responsible for any loss of staining.

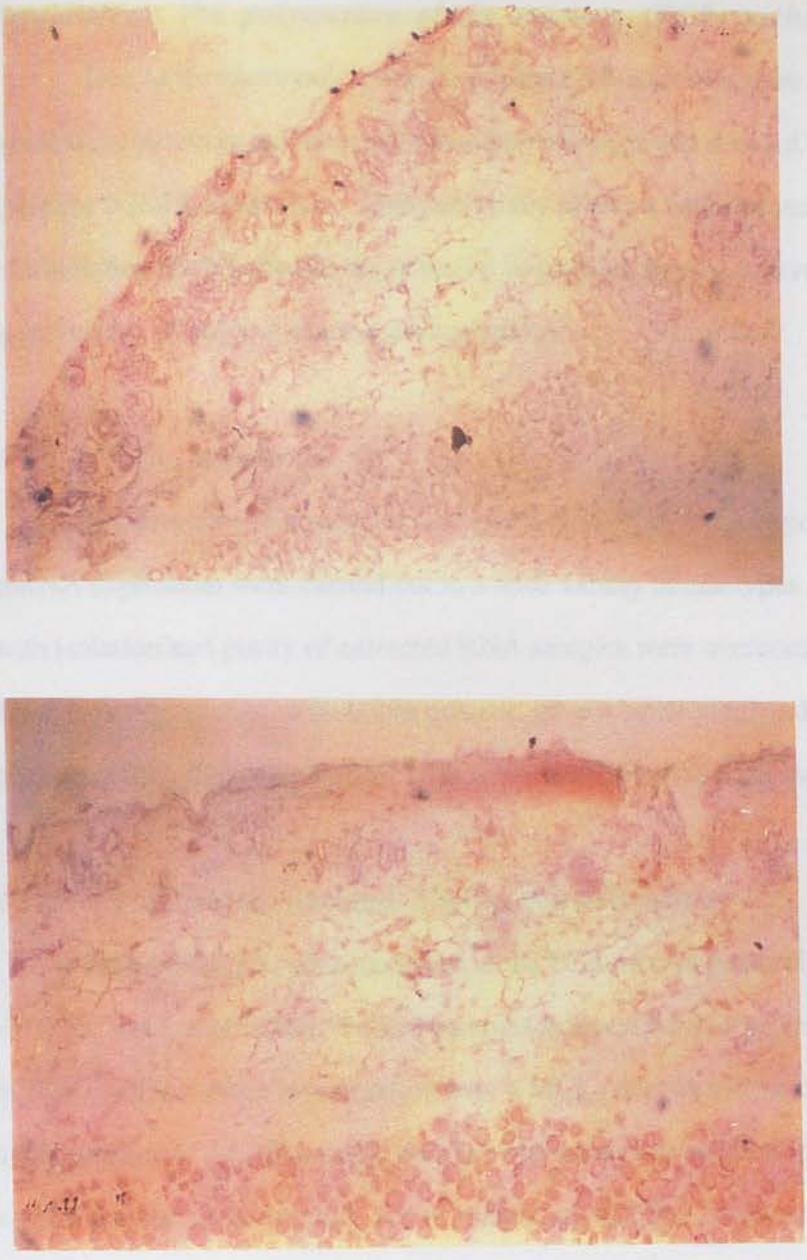

Figure 6.11. Expression of ICAM-1 in mouse skin irradiated with 10 MED UVB. C3H/HeN mice were irradiated with 10 MED UVB (15 kJ/m^2), and skin collected 24h later. Sections were stained with anti-ICAM-1 antibody and developed with DAB as described in section 2.3.4. Areas of ICAM-1 expression are shown in brown. The top panel shows ICAM-1 staining in normal skin and the bottom panel shows ICAM-1 staining in irradiated skin.

Establishing the polymerase chain reaction (PCR) technique.

Due to the observation that IL-10 and TNF-α protein were reduced by UVB irradiation, but were not detectable in supernatants, it was decided to investigate cytokine mRNA expression. This potentially allows a better examination of the effects of irradiation, as it indicates the potential to produce protein, and removes the involvement of any pre-made cytokine stores.

6.11. RNA isolation.

Experiments to investigate the effect of UVR or UCA exposure on cytokine mRNA expression were carried out in a wide variety of cell types. Initial problems with isolation and purity of extracted RNA samples were overcome by adopting an SV RNA isolation system, which was quicker, gave a better yield and proved more consistent than the phenol/chloroform RNA isolation technique used initially.

6.12. Establishing optimal $MgCl_2$ concentration.

Optimal $MgCl_2$ concentrations in the PCR mix for each cell type and each cytokine were established. Pooled samples from each cell type were subjected to PCR for the cytokine under investigation over a MCl_2 concentration range, using a standard cycle number of 40. This cycle number was used as products were likely to plateau using a greater cycle number, due to exhaustion of DNA polymerase and/or other PCR reagents. Products were resolved on 1.5% agarose gels containing ethidium bromide, allowing visualisation under a UV lamp. The $MgCl_2$ concentration which resulted in the brightest product upon development was determined as the optimum for the particular cell type and cytokine under analysis, and this concentration was used for all similar, subsequent PCR reactions (the conditions for PCR are shown in table 2.1).

6.13. Establishing an optimal cycle number for each cytokine.

The results of PCR cycle experiments are shown in fig. 6.12 for RNA derived from cultured cell lines. The optimal cycle number was 33 for TNF-α, 33 for IL-10 and 38 for β-actin.

Positive controls were subjected to PCR reactions over a range of cycle numbers, using radioactively labelled primers. Positive controls consisted of ears from mice which had been contact sensitised with Ox, then CH elicited by ear challenge with 1% Ox. Ears were taken and RNA extracted 24h after challenge. Radioactive products were resolved on 12% polyacrylamide gels, and samples developed by autoradiograph. The raw volume (the arbitrary figure derived by computer analysis of the autoradiograph, see section 2.5.5) for each product was calculated, and the optimal cycle number derived from the linear region of the plot to ensure that the PCR reaction for each cytokine was not saturated. The results of PCR cycle experiments are shown in fig. 6.13 for RNA derived from mouse ears. The optimal cycle number was 33 for TNF-α, 33 for IL-1β, 38 for IL-10, 28 for IFN-γ and 38 for β-actin.

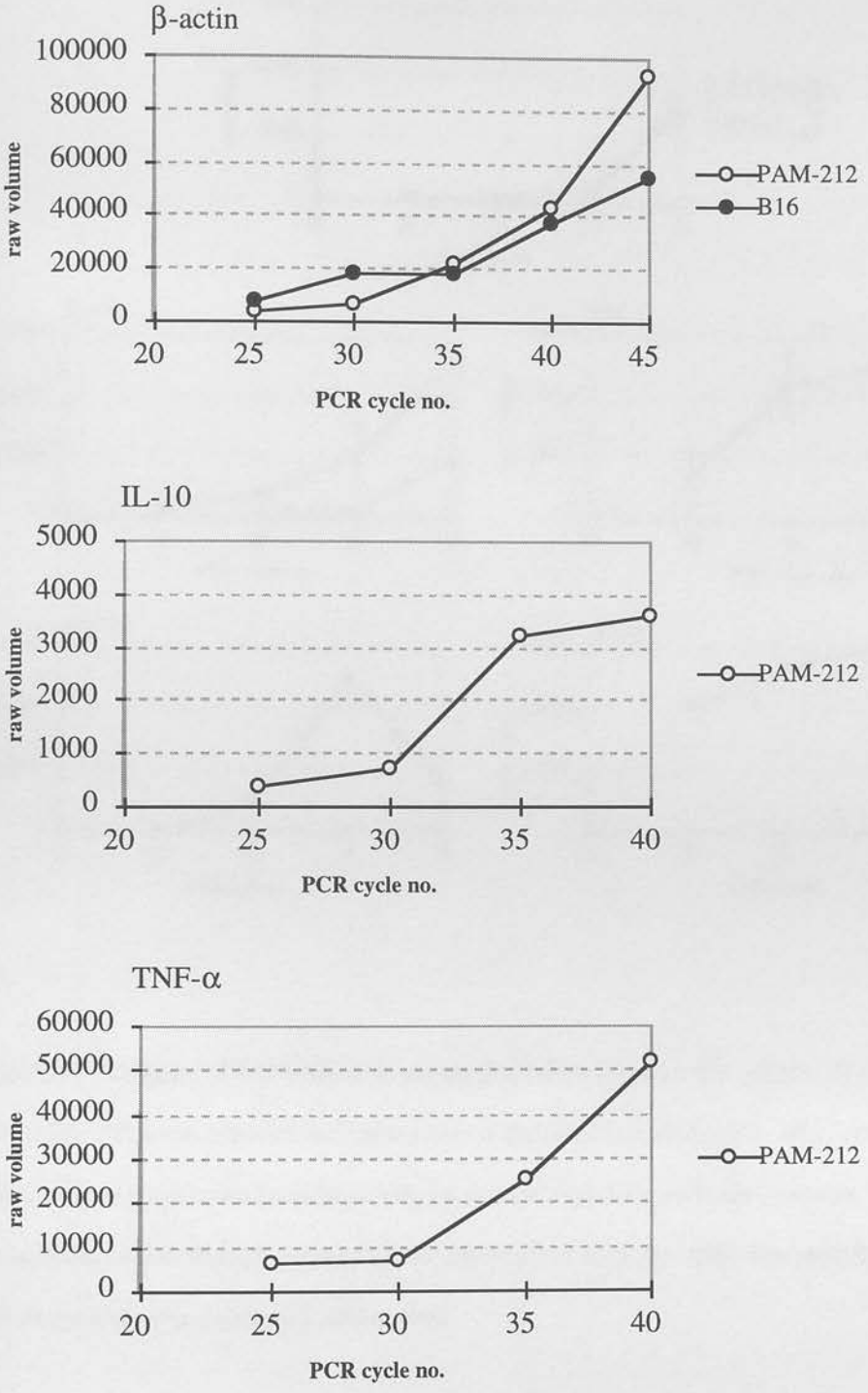

Figure 6.12. Linearity of PCR product accumulation with increasing cycle number, for RNA from cell lines. RNA was extracted from PAM-212 or B16 cells as described in section 2.5.1, and amplified for the relevant cytokine over a range of cycle numbers.

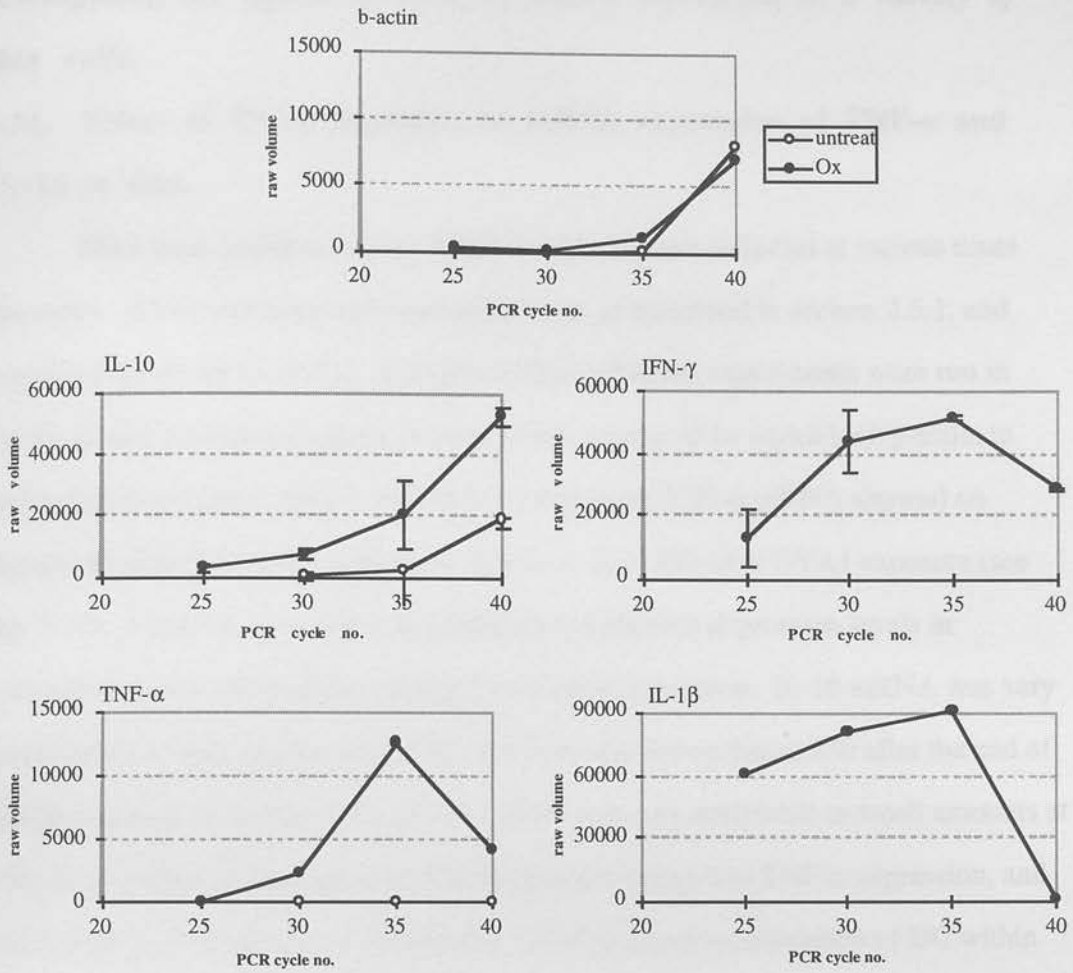

Figure 6.13. Linearity of PCR product accumulation with increasing cycle number, for RNA from mouse ears. RNA was extracted from mouse ears as described in section 2.5.1. Mice were either untreated (untreat), or contact sensitised with Ox and CH elicited by challenge on the ear. Ears were collected 24h later to identify cytokine mRNA expression (Ox group). RNA was amplified for the relevant cytokine over a range of cycle numbers.

6.14. Effect of UVA1 exposure on mRNA expression of TNF-α and IL-1β in skin.

Mice were irradiated with 1 MED UVA1, and ears collected at various times thereafter. RNA was extracted from mouse ears as described in section 2.5.1, and amplified for IL-1β or TNF-α. Samples within individual experiments were run in duplicate and normalised against β-actin, where a value of 1= (cytokine): β-actin, in unirradiated samples. After 1 MED UVA1 exposure, TNF-α mRNA showed no significant alteration from constitutive levels, at up to 48h after UVA1 exposure (see fig. 6.14). Points shown before 0h represent constitutive expression levels in unirradiated ears, which were collected just before irradiation. IL-1β mRNA was very inconsistent in ears exposed to UVA1, but there was upregulation 24h after the end of irradiation (shown in fig. 6.14). IL-10 mRNA was only detectable in small amounts at 96h after irradiation (not shown). UVA1 may not upregulate TNF-α expression, and this would support the earlier finding that UVA1 induced accumulation of DC within DLN is not dependent upon TNF-α (see section 5.4).

6.15. Effect of UVB exposure on mRNA expression of TNF-α and IL-1β in skin.

After 1 MED UVB, TNF-α mRNA was transiently upregulated, peaking 4h after irradiation (a 5-fold increase from unirradiated ears) and remaining increased at 8h (around 3-fold). One day after irradiation, TNF-α mRNA expression had returned to constitutive levels, but increased once more at 48h after UVB exposure. This is shown in fig. 6.15. IL-1β mRNA expression was also upregulated by 5-fold over unirradiated ears 4h after exposure, and returned to constitutive levels by 8h. As with TNF-α mRNA expression, IL-1β mRNA was increased 48h after irradiation (see

fig. 6.15). IL-10 mRNA expression was upregulated within an hour of UVB irradiation, and continued to rise until a peak at 24h. After 24h, expression was slightly reduced but remained elevated from that found in unirradiated ears, even by 96h after UVB exposure (see fig. 6.16). This indicates that UVB has the ability to promote expression of mRNA for the cytokines critical for LC migration. In addition to this, UVB increases the potential of irradiated sites to create a microenvironment favourable for Th2-like cytokine producing cells.

6.16. Effect of UVA1, followed by UVB, exposure on mRNA expression in skin.

Reeve and colleagues have shown that mice irradiated with UVA are partially protected against the immunosuppressive effects of subsequent UVB irradiation (Reeve *et al* 1998), apparently via IFN-γ (Reeve *et al* 1999). As UVA1 did not protect LC from depletion from the epidermis mediated by UVB, the effects of this dual UV regimen were investigated at the mRNA level.

Mice were irradiated with 1 MED UVA1 followed immediately by 1 MED UVB, and ears collected at various times thereafter. RNA was amplified for IL-1β or TNF-α. Samples were run in duplicate and normalised against β-actin, where a value of 1= (cytokine): β-actin, in unirradiated samples. TNF-α mRNA was reduced from constitutive levels immediately after the irradiation period, and remained suppressed throughout the timecourse studied (until 48h after exposure) (see fig. 6.17). IL-1β mRNA expression was transiently upregulated 24h after the end of irradiation, in ears exposed to UVA1 followed by UVB (shown in fig 6.18). These results would suggest that this regimen should prevent LC migration, but this was not observed (see section 4.3).

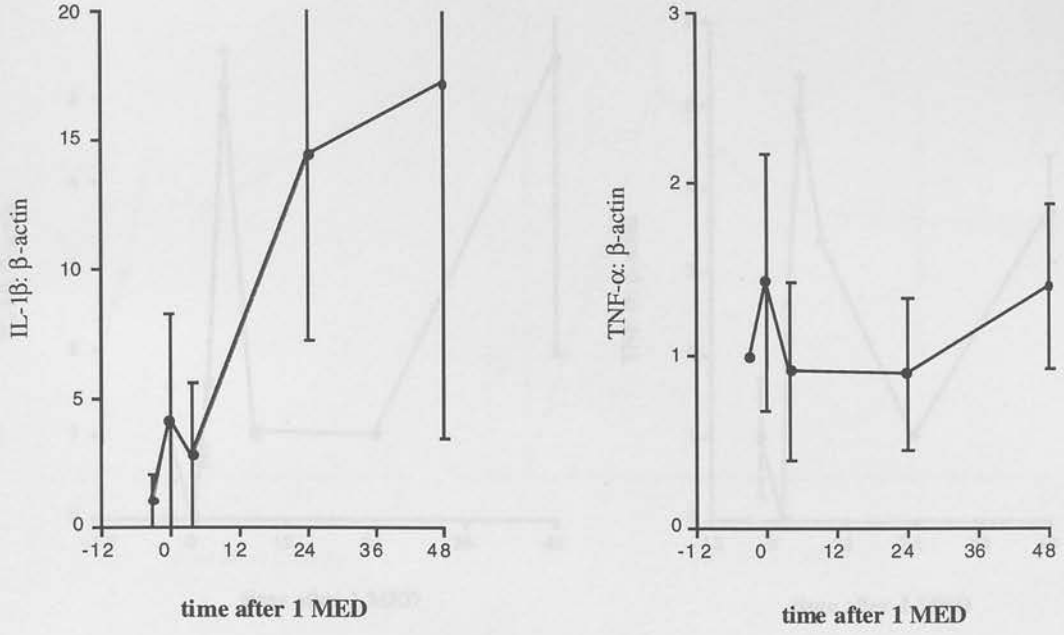

Figure 6.14. A comparison of IL-1β and TNF-α mRNA expression in the ears of UVA1 irradiated mice. Mice were irradiated with 1 MED UVA1, and ears collected at various times thereafter. RNA was extracted from mouse ears as described in section 2.5.1, and amplified for IL-1β (left) or TNF-α (right). Results are represented as the mean ± SEM, for 3 individual experiments. Samples within individual experiments were run in duplicate and normalised against β-actin, where a value of 1= (cytokine): β-actin, in unirradiated samples.

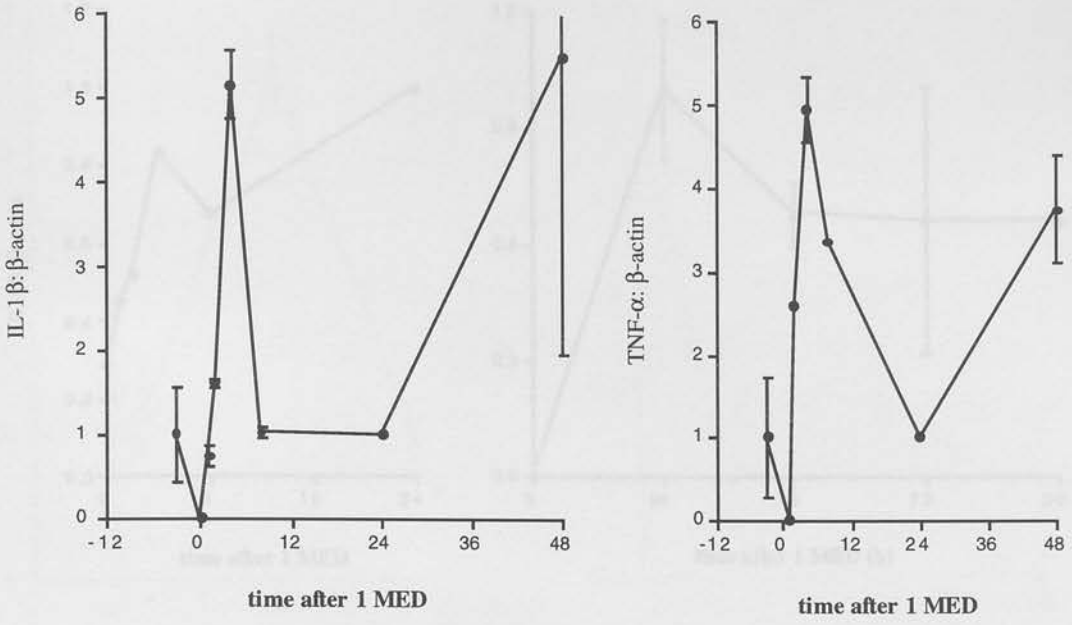

Figure 6.15. A comparison of IL-1β and TNF-α mRNA expression in the ears of UVB irradiated mice. Mice were irradiated with 1 MED UVB, and ears collected at various times thereafter. RNA was extracted from mouse ears as described in section 2.5.1, and amplified for IL-1β (left) or TNF-α (right). Results are represented as the mean ± SEM, for 3 individual experiments. Samples within individual experiments were run in duplicate and normalised against β-actin, where a value of 1= (cytokine): β-actin, in unirradiated samples.

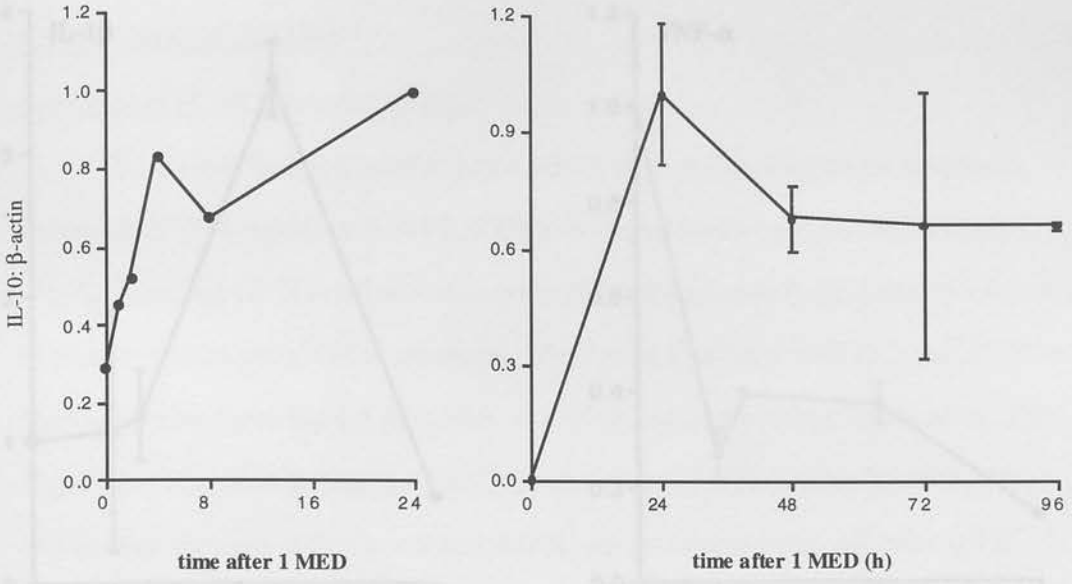

Figure 6.16. IL-10 mRNA expression in mouse ears irradiated with 1 MED UVB. Mice were irradiated with 1 MED UVB, and ears collected at various times thereafter. RNA was extracted from mouse ears as described in section 2.5.1, and amplified for IL-10. Results are represented as the mean ± SD, with samples run in duplicate. IL-10 was not consistently found in unirradiated mouse skin. Therefore, samples were normalised against β-actin, where a value of 1= (cytokine): β-actin, in samples taken 24h after irradiation. Results of 2 individual experiments are shown. Early expression of IL-10 mRNA is shown in the lefthand panel, whilst later expression is shown in the righthand panel.

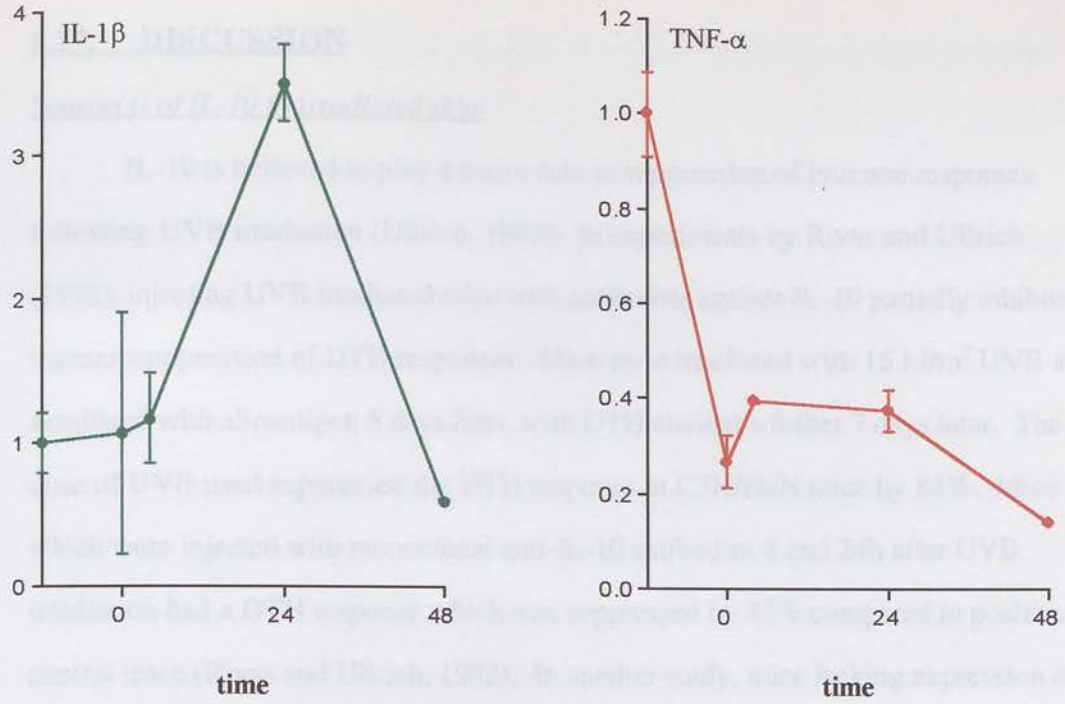

Figure 6.17. Cytokine mRNA expression after 1 MED UVA1 followed immediately by 1 MED UVB. Mice were irradiated with 1 MED UVA1 followed immediately by 1 MED UVB, and ears collected at various times thereafter. RNA was extracted from mouse ears as described in section 2.5.1, and amplified for TNF-α (left) or IL-1β (right). Results are represented as the mean ± SD, with samples run in duplicate. Samples were normalised against β-actin, where a value of 1= (cytokine): β-actin, in unirradiated samples.

6.17. DISCUSSION

Source(s) of IL-10 in irradiated skin

IL-10 is believed to play a major role in suppression of immune responses following UVB irradiation (Ullrich, 1995). In experiments by Rivas and Ullrich (1992), injecting UVB irradiated mice with antibodies against IL-10 partially inhibited immunosuppression of DTH responses. Mice were irradiated with 15 kJ/m^2 UVB and sensitised with alloantigen 5 days later, with DTH elicited a futher 7 days later. The dose of UVB used suppressed the DTH response in C3H/HeN mice by 84%. Mice which were injected with monoclonal anti-IL-10 antibodies 4 and 24h after UVB irradiation had a DTH response which was suppressed by 42% compared to positive control mice (Rivas and Ullrich, 1992). In another study, mice lacking expression of IL-10 (IL-10 targetted; IL-10T mice) were completely resistant to the immunosuppressive effects of UVB on DTH to alloantigens (BALB/c spleen cells) (Beissert *et al* 1996).

In the experiments reported in section 6.2, injection of antibodies against IL-10 did not reverse UVB- or *cis*-UCA mediated suppression of the DTH response to HSV-1. Mice which were injected with anti-IL-10 actually exhibited increased suppression of DTH compared to mice injected with control serum, following UVB irradiation.

It may be that the lack of a second injection, 24h after UVB irradiation or *cis*-UCA application in the experiments in section 6.2 prevented complete neutralisation of IL-10. It is of interest that complete lack of IL-10, such as in IL-10T mice, completely abrogated the immunosuppressive effects of UVB upon DTH responses (Beissert *et al* 1996). In the experiments of Rivas and Ullrich, injection of anti-IL-10 4 and 24h after a highly immunosuppressive UVB dose only halved the degree of immunosuppression observed (84% reduced to 42% immunosuppression of DTH to alloantigen) (Rivas and Ullrich, 1992). It may be that IL-10 has to be neutralised over a longer period following UVB exposure. This idea is further

supported by experiments investigating IL-10 concentrations in sera from irradiated mice.

IL-10 was detectable by ELISA in sera of mice irradiated with 7 MED (10 kJ/m^2) UVB. This peaked at 3-4 days after exposure. This finding is in agreement with the timecourse Beissert and colleagues found for IL-10 protein in the serum of C57BL/6 mice exposed to 30 kJ/m^2 UVB (Beissert *et al* 1996). A quicker, more transient timecourse which peaked at 36h and cleared by 72h, was observed by Rivas and Ullrich, following exposure of C3H/HeN mice to a 15 kJ/m^2 UVB dose (Rivas and Ullrich, 1994). Local and systemic immune suppression following UVB exposure exhibit different kinetics. Local suppression can be observed immediately and up to 48h if C57BL/6 mice are sensitised through the irradiated site. If mice are sensitised through an unirradiated site, a lag period of 3 days is required before systemic suppression is observable (Hammerberg *et al* 1996). In experiments by Horio and Miyauchi, systemic suppression of CH following UVB irradiation was maximal when sensitisation occured 3 days after exposure (Miyauchi and Horio, 1995).

Alterations in cytokine profiles were not apparent during the induction phase of CH (see section 3.9), but there appeared to be a bias towards Th2-like cytokine production at the elicitation phase (see section 3.7). As mentioned previously, the cytokine microenvironment in which an antigen is encountered is important in directing the subsequent immune response. Further experiments investigated the production, and potential sources, of the Th2-like cytokine, IL-10, in irradiated skin.

There are many potential sources of IL-10 within irradiated skin, including keratinocytes (Rivas and Ullrich, 1992), melanocytes (Teunissen *et al* 1997), T cells (Ullrich, 1995; Kitajima *et al* 1996), B cells, mast cells and macrophages (Kang *et al* 1994). IL-10 production by murine keratinocytes is generally accepted. Depletion studies have shown keratinocytes to be the main source of IL-10 mRNA after sensitisation with contact allergens (Enk and Katz, 1992b). Although keratinocytes

synthesize small amounts of IL-10 mRNA, the protein may remain inside the cells (Kang *et al* 1994). Teunissen and colleagues (1997) found that IL-10 mRNA was only detectable in pure melanocyte cultures, but not in pure cultures of primary keratinocytes, following stimulation with phorbol myristate acid (PMA), IFN-γ or UVB. This was not translated to detectable levels of protein however. Thus, the effect of UVB exposure on IL-10 protein in keratinocytes (PAM-212) or melanocytes (B16) was investigated.

PAM-212 cells stained positively for IL-10 before UVB irradiation (described in section 6.4). Staining became more focussed around the nucleus following irradiation. Shreedhar and colleagues interpreted this as a sign of *de novo* synthesis (Shreedhar *et al* 1998), but it could also be a sign of apoptosis, with packaging of cellular material. ELISA of cell lysates indicated that IL-10 content of PAM-212 cells was unaffected 24h after UVB irradiation, and cell viability was unaltered. IL-10 content of PAM-212 cells was reduced in a dose-dependent manner by 48h after irradiation. ELISA of culture supernatants indicated that IL-10 was not secreted by PAM-212 or B16 cells upon UVB exposure (at doses up to 20 mJ/cm^2). Rivas and Ullrich (Rivas and Ullrich, 1992) identified IL-10 in supernatants from PAM-212 cells irradiated with 20 mJ/cm^2 UVB 24h earlier, as there was a band which reacted with anti-IL-10 antibodies on Western blots. In their experiments, IL-10 was not secreted by unirradiated PAM-212 cells. One possible reason for this discrepancy may be the UVB source. Whilst the studies reported here used a Philips TL-12 lamp, Rivas and Ullrich used an FS-40 lamp, which emits 46% of its energy in the UVA waveband (Goèttsch *et al* 1998). Any, or the degree of, contamination by UVA wavelengths may have a critical role in the levels of IL-10 detected. Grewe *et al.* found that UVB or UVA1 caused a time- and dose-dependent induction of IL-10 mRNA expression in, and protein secretion by, long-term cultured human keratinocytes. UVA1 was a stronger inducer than UVB, by a factor of 2 (Grewe *et al* 1995).

B16 cells stained very intensely upon development of immunoperoxidase staining for IL-10, even when isotype control antibody was used. As peroxide is an important factor in melanogenesis, attempts to stain B16 cells in this way required a longer H_2O_2/methanol step to block endogenous peroxide. It may however have been better to have used a different means of development, such as immunofluorescence. Concentrations of IL-10 in B16 cell lysates, measured by ELISA, were very inconsistent between experiments, but again these results suggested that IL-10 was unaltered by doses up to 20 mJ/cm^2.

Goodman and colleagues used keratinocytes as accessory cells in stimulating T cell responses (Goodman *et al* 1994). There was a specific defect in T cell IFN-γ production, whereas IL-2 and IL-4 were induced at levels comparable to those seen when professional APC were used as accessory cells. This was not due to non-productive CD28 engagement, as stimulation of this pathway with monoclonal antibodies did not induce IFN-γ production in keratinocyte supported cultures. IL-2 was increased 10-fold in these cultures, demonstrating a specific deficiency in IFN-γ as opposed to failure to respond to CD28 signalling. RT-PCR revealed that keratinocytes produced little or no mRNA for IL-12 compared with professional APC, and addition of IL-12 to cultures restored IFN-γ production. As IL-10 is directly implicated in reducing cytokine production by Th1-like cells (de Waal Malefyt *et al* 1992; Moore *et al* 1993; Howard and O'Garra, 1992), this is in agreement with a potential role for keratinocyte derived IL-10 in altering immune responses following UVB irradiation.

As mentioned above, Grewe *et al.* examined the effect of UV exposure cultured human keratinocytes. In addition to the effects of UVA or UVB exposure, IL-10 production was increased 2-3 fold when keratinocytes were cultured in high Ca^{2+} medium (Grewe *et al* 1995). Neither addition of TNF-α or IL-1α to unirradiated cells, or addition of neutralising antibodies to TNF-α or IL-1α to UVB irradiated cells, altered the level of IL-10 produced. Similarly, modulation of eicosanoid production by

adding PGE_2, or disturbing cyclo-oxygenase activity by indomethacin, did not affect IL-10 production.

Source(s) of TNF-α in irradiated skin

TNF-α is needed to keep LC alive in culture, whereas without keratinocyte-derived cytokines the cells rapidly involute and die. TNF-α does not have any effects on the maturational state of LC, and freshly isolated epidermal cells express TNF-α mRNA (Koch *et al* 1990).

The concentration of TNF-α protein within samples of mouse skin exposed to 5 MED UVB, was investigated. There was an increase immediately after irradiation (which took 50 mins to administer). Levels were increased 2-fold over those found constitutively, and peaked at 20μg TNF-α per gram of skin, 4h after the end of irradiation. By 8h after UVB exposure, TNF-α protein within the skin had returned to constitutive levels. In a previous experiment (described in section 4.9), TNF-α was injected into the ears at a dose of 1000 U/ ear to induce LC migration. This concentration increased the LC migration induced by 1 MED UVB alone.

Analysis of cultured cells was carried out to determine the source of TNF-α in irradiated skin. In supernatants from PAM-212 and B16 cells, TNF-α was not detectable either constitutively or 24h after irradiation with any dose of UVB up to 20 mJ/cm^2. However, TNF-α was detectable in lysates of unirradiated cells. Analysis of lysates 24h after irradiation revealed that TNF-α was lost from both PAM-212 and B16 cells in a dose-dependent manner. This reduction in TNF-α was more marked for PAM-212 cells than B16 cells (see figs. 6.11 and 6.12).

Walsh investigated the human sunburn response, both *in vitro* and *in vivo* (Walsh, 1995). UVB resulted in rapid degranulation of mast cells, with a consequential release of TNF-α. This was accompanied by expression of the TNF-inducible adhesion molecules, ELAM-1 and ICAM-1, on the cutaneous epithelium 2h later, which peaked between 4 and 6h later. This effect could be blocked

by the mast cell inhibitor, disodium cromoglycate. Keratinocytes contributed minimally to the TNF-α released. Twice as much TNF-α was released when dermal explants were irradiated, indicating that removal of the epidermis, and thereby protective granules, augmented the effect. In the experiment described in section 6.9, ICAM-1 expression was detected in small patches in the *stratum corneum* of unirradiated mice. Expression was upregulated in these areas following irradiation of C3H/HeN mice with 10 MED UVB. There was no expression of ICAM-1 expression in the dermis of unirradiated skin, but ICAM-1 was induced following a 10 MED UVB exposure.

LFA-1 and ICAM-1 are important for regulating the interaction of leucocytes and keratinocytes in cutaneous immune responses. ICAM-1 gene expression is controlled by nuclear factor (NF)-κB, which is inducible by TNF-α. Induction of NF-κB by reactive oxygen intermediates (Jourd'heuil *et al* 1997), produced as a result of UV-radiation, is a proposed mechanism for altering ICAM-1 expression. Another potential mediator is the DNA damage induced by UVB (Krutmann and Grewe, 1995). TNF-α is implicated in DNA damage following UVB exposure, as injection of antibodies against TNF-α reduced DNA fragmentation, and caused a significant, but incomplete, reduction in sunburn cell formation (Schwarz *et al* 1995). Experiments were conducted to examine whether *cis*-UCA could also participate in regulation of ICAM-1 expression.

Experiments by Mitra and colleagues (1993) indicated that UCA, and/or histamine, may play a role in determining the level of ICAM-1 expression following irradiation. Incubation of primary human keratinocytes with TNF-α induced ICAM-1 expression by 48h of culture. Addition of irradiated UCA resulted in ICAM-1 expression increased from that induced by TNF-α alone. *Cis*-UCA, even above physiological levels, could not augment phorbol 12-myristate 13-acetate (TPA) induced synthesis of either TNF-α mRNA expression in, or protein secretion from, keratinocytes (Redondo *et al* 1996).

In *in vitro* experiments, PAM-212 cells did not appear to express ICAM-1 on their surface, despite incubation with TNF-α (see section 6.10). One difference in the treatment of positive control cells (LPS-stimulated peritoneal macrophages, DC or LNC) and the PAM-212 cells to be studied was in harvesting of the cells. PAM-212 form a monolayer during culture, making it necessary to remove them enzymatically. A detrimental effect of trypsin/ versene harvesting on ICAM-1 expression was discounted by removing cells from the monolayer by a mechanical method. Analysis of cells harvested by this technique yielded similar results. Corver *et al.* investigated the effect of monolayer dissociation with trypsin/EDTA on 3 ovarian carcinoma cell lines, and found one of these to have decreased, but still detectable, ICAM-1 expression as a result (Corver *et al* 1995). In another study, tryptic dissociation of monolayered cells caused loss of CD44 expression, but not ICAM-1, using 13 ovarian carcinoma cell lines and 2 mesothelial cell lines (Gardner *et al* 1995). Therefore, it is unlikely that dissociation of the PAM-212 monolayer by trypsin depletes ICAM-1 from the cell surface.

Interestingly, Kashihara-Sawami *et al.* found that ICAM-1 was increased on keratinocytes grown in low calcium medium, which correlated with basal cell keratin K5 expression (Kashihara-Sawami and Norris, 1992). This is supported by the observation that increased TNF-α was released upon irradiation of dermal explants (Walsh, 1995). Immunostaining of skin sections from irradiated mice suggested that ICAM-1 was induced under the dermis (see section 6.9). Thus, the differentiation/ developmental status of the cell may play a role in keratinocyte ICAM-1 expression, and this could explain why ICAM-1 is undetectable on PAM-212. Additionally, ICAM-1 expression on basal keratinocytes was induced by intracutaneous injection of tuberculin-purified protein derivative (PPD) to elicit DTH in humans, but was not significantly induced by UVB treatment (Norris *et al* 1991).

The timing of investigation of ICAM-1 expression following UV irradiation on keratinocytes may also be critical. Norris and colleagues (1990) found that UVR had a

biphasic effect on ICAM-1 expression on human keratinocytes, with a downregulation preceding an upregulation. In experiments by Krutmann and colleagues (1990), *in vitro* irradiation of human keratinocytes prior to stimulation with TNF-α inhibited ICAM-1 upregulation in a dose-dependent manner. UVB irradiation did not affect the low constitutive ICAM-1 expression of KB cells (epidermoid carcinoma cells) within a 24h incubation period. Further studies by the same group revealed that UVB exposure of keratinocytes inhibited ICAM-1 upregulation in response to either TNF-α or IFN-γ. Restoration of responsiveness to TNF-α required at least a 24h recovery period (Krutmann *et al* 1992a). There was a significant increase in ICAM-1 expression 48h after irradiation. In experiments by Norris *et al* (1990), UVR inhibited keratinocyte ICAM-1 expression induced by IFN-γ, 24h after irradiation. However, UVR induced expression to greater levels than IFN-γ alone by 48, 72 and 96h of culture, and enhanced the upregulation induced by IFN-γ.

In an ICAM-1 transgenic mouse model, with gene expression under control of the basal keratin K14 (Williams and Kupper, 1994), CH responses were not potentiated as expected, and $CD45^+$ leukocyte infiltration was not increased at ICAM-1 expression sites. These points argue that ICAM-1 expression on keratinocytes may not play a key role in UV-induced immunomodulation in murine models, or at the very least, cannot act on its own.

An attempt was made to compare expression of IL-1β and TNF-α mRNA in the ears of mice irradiated with either 1 MED UVA1 or 1 MED UVB. These cytokines are central to LC migration, and contrasting effects of UVA1 and UVB on LC migration have been shown in chapter 4. A true comparison of timings was very difficult due to the different power outputs of the respective lamps. The dose used throughout the experiments was assessed physiologically, and represented 1 MED for either lamp. Whilst it took only 10 mins to administer 1 MED broad-band UVB (1440 J/m^2), it took approximately 3h to administer 1 MED UVA1 (500 kJ/m^2). Attempts to reduce the administration time for 1 MED UVA1 involved reducing the tube to target distance.

However, this caused overheating and proved detrimental to the health of the irradiated mice. The overheating problem was reduced by placing a fan in the exposure room, but temperature was only controllable by placing the lamp further away from the mice. As such, any timings are taken from the end-point of 1 MED UVA1 irradiation. If the irradiation time had been cut short, mice would have received a dose of less than 1 MED.

The timecourse for TNF-α mRNA induction by UVB radiation (section 6.15) correlated to that found for protein expresion in irradiated skin (see fig. 6.8). Both showed an early (4h) and transient peak in expression, before returning to constitutive levels. TNF-α mRNA expression was increased 5-fold, whilst protein was increased 2-fold. IL-1β mRNA expression was similarly upregulated by approximately 5-fold over expression in unirradiated skin samples, by 4h after UVB irradiation. Expression quickly reverted to constitutive levels before rising again at 48h after exposure.

IL-10 mRNA expression was upregulated within an hour of UVB irradiation, and continued to rise until a peak at 24h (see fig. 6.16). IL-10 protein in irradiated skin peaked at 8h after UVB exposure, and had returned to constitutive levels by 24h (see fig. 6.3).

TNF-α expression was not altered from constitutive levels at any time after exposure to 1 MED UVA1 (see fig. 6.14). IL-1β mRNA expression was inconsistent in UVA1 irradiated skin, but showed an increase by 24h after the end of exposure. Therefore, UVA1 does not induce expression of both cytokines shown to be critical for LC migration (Kimber *et al* 1998).

In mice which were exposed to both UVA1 and UVB, TNF-α mRNA expression was downregulated. IL-1β was increased at 24h, and had returned to constitutive levels at 48h, after exposure (see fig. 6.17). This dual exposure regimen appears to prevent (TNF-α) or delay (IL-1β) the signals necessary for LC migration, and this may explain the photoprotective effect seen by others (Reeve *et al* 1999).

However, it should be noted that LC migration and accumulation of DC do occur in these mice (see figs. 4.4, 4.5 and 5.4 respectively).

TNF-α mRNA was minimally increased by 8h after UVB exposure of human skin (2.5 MED), and reached a maximum by 24h. Timecourses were not taken beyond the 24h timepoint. No alteration was seen in ICAM-1 or VCAM-1 expression, although E-selectin was increased from 4h onwards, reaching maximal levels at 24h (Strickland *et al* 1997).

In agreement with the results in sections 6.14 and 6.15, there is evidence that UVB irradiation induces TNF-α production in the human epidermis, but that UVA1 irradiation does not (Skov *et al* 1998a). 3 MED UVB led to a rapid increase in TNF-α concentration in suction-blister fluid, which was maximal 6h after exposure. UVA1 caused a slight decrease. Both UV regimens led to a slight increase in IL-10 and *cis*-UCA, although this was larger with UVB (Skov *et al* 1998a). As mentioned in section 1.9.2, IL-6 expression can be induced by IL-1. Kirnbauer *et al.* reported that UVB, but not UVA, induced IL-6 production from human epidermal cells (Kirnbauer *et al* 1989). They therefore supply circumstantial data that UVA does not alter IL-1 expression to a significant degree.

In experiments by Enk and Katz, there was enhanced expression of TNF-α, IL-10, IL-1α and even IL-1β mRNA, in epidermal cell suspensions prepared 4h after injection of IL-1β *in* vivo. Addition of IL-1β to keratinocytes (derived by washing non-adherent cells from epidermal cell cultures) resulted in increased TNF-α and IL-10, with a less pronounced increase of IL-1α (Enk and Katz, 1995).

Supernatants of highly enriched cultured LC display IL-1 and TNF-α, but not IL-4 or IFN-γ activities. Cell mixing experiments to investigate the role of contaminating keratinocytes showed that IL-1 and TNF-α were predominantly derived from these keratinocytes. PCR revealed that IL-1β transcripts were limited to LC (Schreiber *et al* 1992).

Throughout the UVB timecourse there was a gradual increase in IL-1β mRNA above constitutive levels, and this may correlate with replenishment of LC. As IL-1β and TNF-α are involved in LC migration, a quick upregulation following UVB exposure may have been anticipated. Both cytokine mRNAs were transiently upregulated 4h, and TNF-α protein was upregulated 8h, following UVB exposure. UVA1 did not induce an upregulation of TNF-α mRNA expression within irradiated skin, and UVA1 did not result in LC depletion. These results therefore suggest a mechanism for this.

	UVB	UVA1
IL-10 - mRNA	upregulated at 1h peak at 24h above constitutive until 96h	upregulated at 96h
IL-10 - protein	peak at 8h constitutive at 24h	nd
TNF-α - mRNA	peak at 4h constitutive at 24h upregulated at 48h	? small peak at 4h
TNF-α - protein	peak at 4h constitutive at 24h	nd

Table 6.1. The effects of UVB and UVA1 on IL-10 and TNF-α at the mRNA and protein levels. nd= not done.

6.19. Criticisms of experimental design and suggestions for further work

The UV doses used in the experiments described herein were equivalent on the basis of erythemal activity, and have been shown to be immunosuppressive (El-Ghorr and Norval, 1999). However, UVA1 is less efficient at UCA photoisomerisation than UVB. A 1 MED UVA1 exposure results in 17%, whereas 1 MED UVB results in over 40%, *cis*-UCA formation. The LC depletion observed with UVB was dose-dependent, and at 360 J/m^2 UVB no significant reduction in LC numbers could be detected. *Cis*-UCA formation is around 20% at this dose (El-Ghorr and Norval, 1999). Therefore, there may be a threshold percentage *cis*-UCA at which LC depletion is mediated.

Now that the optimal times for cytokine upregulation following irradiation have been identified, it would be interesting to investigate the effect of varying UV doses upon expression. Additionally, the effect of topical apllication of UCA isomers can now be examined. It would be of interest to establish if there is a maximum threshold for inducing cytokine expression, particularly with reference to the cytokines involved in inducing LC migration.

The PCR technique can also be applied to investigate cytokine mRNA expression within the skin at various points of the CH response, in mice irradiated before sensitisation or not. This would appear to be critical, as UVB did not alter cytokine secretion from DLNC of mice following contact sensitisation, but did so after elicitation of the CH response (see chapter 3). Experiments by Garssen and coworkers (1999) indicate that mRNA expression for IFN-γ is downregulated in spleen cell populations following secondary stimulation. It would be of interest to examine if selection of T helper cell profiles occurs within sites of elicitation which have previously been irradiated, and if so, at what time. This could have major implications upon the potential of an irradiated site to elicit a CH response comparable to responses in unirradiated animals.

Development of immunostaining by a technique other than peroxidase, such as use of fluorescent antibodies, has to be undertaken. Alternatively, use of a melanocyte cell line which does not produce as much endogenous peroxidase as B16 may facilitate immunoperoxidase staining. Peroxidase is produced during melanogenesis, so in cell lines producing less, peroxidase may be easier to block.

Chapter 7

General discussion

The preceding chapters have detailed UV mediated effects on a variety of immune parameters. These included suppression of immune responses by UVB, via effects on LC within the epidermis and changes in DC frequency within DLN, and the subsequent cytokine profiles of DLN during both contact sensitisation and elicitation of CH. The molecular signals involved in altering these immune parameters within the skin were examined. Some of the findings of this study are summarised in table 7.1.

7.1. The effects of UVR on cytokines involved in LC migration.

Kimber and colleagues have postulated that topical application of a contact sensitiser leads to quick release of IL-1β within the cutaneous site, which in turn induces TNF-α secretion. The two cytokines then induce LC migration to the DLN (Kimber *et al* 1998). Supernatants of highly enriched cultured LC display IL-1 and TNF-α. Cell mixing experiments to investigate the role of contaminating keratinocytes showed that TNF-α was predominantly derived from this cell type. PCR revealed that IL-1β transcripts were limited to LC (Schreiber *et al* 1992). Injection of anti-IL-1β into the skin prior to TNCB treatment completely prevented sensitisation to this chemical (Enk *et al* 1993a). IL-1β deficient mice require greater concentrations of contact sensitiser than their wild-type counterparts in order to mount CH responses upon challenge (Shornick *et al* 1996). When DLNC from wild-type mice contact sensitised with 0.1% TNCB were incubated with haptenated spleen cells *in vitro*, antigen specific proliferation could be detected. When DLNC from IL-1β deficient mice contact sensitised with 0.1% TNCB were incubated with haptenated spleen cells, there was no antigen specific proliferation (Shornick *et al* 1996). Intradermal injection of TNF-α

results in a decrease of LC numbers in the epidermis (Cumberbatch *et al* 1997a), and in a dose- and time-dependent accumulation of DC (Cumberbatch and Kimber, 1992). The efficiency of skin sensitisation is similarly reduced, with impaired CH responses in anti-TNF-α treatred mice compared to mice treated with control serum. These effects were only detected when TNF-α was inhibited at the time of sensitisation, as injection of anti-TNF-α 18h after contact sensitisation was without effect (Cumberbatch and Kimber, 1995). Mice lacking the p75 TNF-α receptor have reduced LC migration and CH responses (Wang *et al* 1997). Therefore, if UVR can initiate transcription of the IL-1β and/or TNF-α gene(s), it may result in migration of LC from the epidermis. Presentation of antigen subsequently encountered through the irradiated site may thereby be altered, or diminished, via the absence of LC within the epidermis.

Contrasting effects of UVA1 and UVB on LC migration have been shown in chapter 4. IL-1β mRNA expression was upregulated by 4h following UVB exposure, quickly reverted to around constitutive levels before rising again 48h after exposure. Induction of TNF-α mRNA expression by UVB radiation (section 6.15) correlated to increased protein expresion in skin irradiated with 5 MED (see fig. 6.8).

Following UVA1 exposure, IL-1β mRNA expression showed an increase not earlier than 24h, and TNF-α mRNA expression was not altered form constitutive expression levels.

In summary, UVB irradiation results in early induction (within 4h) of IL-1β and TNF-α mRNA expression in mouse skin. UVA1 does not cause upregulation of IL-1β until 24h after exposure, and has does not consistently alter TNF-α mRNA expression.

In experiments by Enk and Katz, there was enhanced expression of TNF-α, IL-10 and even IL-1β mRNA, in epidermal cell suspensions prepared 4h after injection of IL-1β *in* vivo. Addition of IL-1β to cultured keratinocytes resulted in increased TNF-α and IL-10 (Enk and Katz, 1995). Infiltrating monocyte/macrophage cells

could be influenced by the altered microenvironment. Monocyte production of IL-10 occurs 8-24h after LPS treatment, and this is dependent on TNF-α (Wanidworanun and Strober, 1993).

In experiments by Hirao and colleagues, together with an increase in IL-1, IL-1ra was increased markedly following 2-3 MED of UVR. Following sun-exposure the ratio of IL-1ra:IL-1α in skin increased to over 100, an excess sufficient to block IL-1α-induced biological responses (Hirao *et al* 1996). Therefore, although UV stimulates an increase in IL-1 levels, with the concomitantly greater rise in IL-1ra, UV may inhibit the activities of IL-1α and suppress inflammatory responses (Norris *et al* 1991).

7.2. LC migration following UVR.

LC possess a plasma-membrane ATPase, an enzyme which protects LC from permeabilization upon exposure to extracellular ATP, whereas keratinocytes and macrophages (J774) are susceptible (Girolomoni *et al* 1992). Loss of ATPase activity correlates with an "activated" state, and application of a second contact sensitiser 24h after the first resulted in tolerance to the second (Hanua *et al* 1989).

Noonan *et al.* found that ATPase positive cells were reduced 24h after irradiating mice on the back with monochromatic UVB (270, 290, 320 nm) (Noonan *et al* 1984). Irradiating with 270 or 290 nm UVB led to a loss of dendrites and a decrease in the total epidermal LC. A dose of 320 nm UV that caused 50% systemic suppression of CH did not deplete LC.

In the experiments described in chapter 4, UVB depleted LC from the epidermis in a dose-dependent fashion. This depletion in LC numbers was dependent on IL-1β and TNF-α, as blocking of either cytokine prevented any alterations normally induced by UVB. Following irradiation with 1 MED UVB, LC numbers within the epidermis were reduced by 6h, and maximally reduced by 12h. Interestingly, in experiments by Cumberbatch and colleagues, LC numbers began to recover 12-17h

after intradermal injection with TNF-α, although numbers were still significantly reduced from control values (Cumberbatch *et al* 1997c). In section 4.9, injection of TNF-α into the ears of irradiated mice did not increase LC migration above that in mice injected with TNF-α alone.

The steady-state turnover rate for LC is 7 days (Ghaznawie *et al* 1999). Therefore, a 2 day timespan for repopulation of LC from the 30% reduction induced by a 1 MED UVB irradiation is in agreement with this. Recent studies indicate that repopulation of LC can occur by cells residing in the hair follicles. Irradiation of human skin with 4 MED UVB showed that the hair follicle is a critical reservoir of LC (Gilliam *et al* 1998). Cells deep within the follicle are reasonably protected from UVB, which could only penetrate, and induce thymine dimers in, the superficial hair follicle opening. LC migrated out of the follicle and into the epidermis 48h, and maximally 72h, after exposure. These LC exhibited a deficiency in the T cell costimulatory molecules, B7.1 and B7.2.

In the studies of Aubin and colleagues, a continued PUVA regimen led to a decrease in $ATPase^+$, Ia^+ and $Thy\text{-}1^+$ cells in the exposed skin of C3H or BALB/c mice. Chronic UVA exposure alone also reduced $ATPase^+$, Ia^+, $Thy\text{-}1^+$ cells, but had no effect on immune function (Aubin *et al* 1991).

As mentioned above, a single, 1 MED UVA1 exposure could not stimulate a marked increase in TNF-α expression, and therefore, it may not provide an essential signal for LC migration. Similarly, IL-1β was not upregulated at early timepoints following UVA1 irradiation. In agreement, UVA1 did not mediate LC depletion from the epidermis at up to 96h after exposure.

UVA-mediated damage to membranes, proteins, DNA or other cell targets is predominantly related to oxidative stress. Clement-Lacroix and colleagues found that the APC function of cultured LC (measured by MECLR) was dose-dependently reduced by direct UVA exposure, at doses up to 20 J/m^2 (Clement-Lacroix *et al* 1996). APC function was restored by preventing oxidative damage by overnight incubation of

LC with the anti-oxidant vitamin E. UVA1, but not UVB or UVC, induced various non-nuclear changes in mammalian cells, including damage to cytoplasmic organelles, increased membrane permeability and cell lysis (Beer *et al* 1993). Polychromatic UV light, composed of environmentally relevant wavelengths, was found to disrupt the cytoplasmic microtubule complex in a dose-dependent manner. Microtubules were disrupted after 3000 J/m^2 UVA exposure, but not following UVC irradiation (Noonan *et al* 1984). No changes could be seen in the actin microfilaments which stretch across the length of control and irradiated cells.

Therefore, UVA1 may not induce LC migration from the epidermis, but may prevent irradiated LC from functioning properly. The cells may become immobilised, and not be able to function properly. However, treatment with either UVB or TNF-α after UVA1 irradiation did result in migration of LC, and unlike LC after UVB exposure, UVA1 exposed LC do upregulate B7, and are capable of stimulating naive T cells (Dittmar *et al* 1999).

Cis-UCA did deplete LC, but took longer than UVB, with maximal depletion 24h after topical application. In experiments by Kurimoto and Streilein, LC were rounded up and possessed fewer dendrites as early as 5h after an intradermal injection of 200μg *cis*-UCA (Kurimoto and Streilein, 1992). Therefore the discrepancy in the time of maximum LC depletion following either UVB irradiation against *cis*-UCA application, may simply be due to the time needed for UCA to absorb and penetrate to the target LC within the epidermis. LC depletion following *cis*-UCA painting was similar to UVB in its dependence upon IL-1β and TNF-α (see sections 4.8 and 4.10). Kurimoto and Streilein showed that loss of LC dendrites and suppression of CH could be partially restored by intraperitoneal injection of anti-TNF-α antibodies 2h before *cis*-UCA treatment (Kurimoto and Streilein, 1992). These experiments indicate that if LC can be retained in the site to be sensitised, CH responses are normal.

Caution regarding extrapolation of these results across species barriers is required. Goettsch and colleagues showed that changes in the morphology of LC

within mouse or rat skin were detectable after 3-9 kJ/m^2 UVB, but not in human LC. UVB suppressed the function of epidermal cells in the MECLR, with cells from mice most susceptible and humans more resistant, with an interspecies effect variation of 5.5 (Goettsch *et al* 1998).

In summary, UVB and *cis*-UCA deplete LC from the epidermis in mice, and this is dependent on IL-1β and TNF-α. UVA1 does not reduce LC numbers within the epidermis.

7.3. UVR and its effects DC accumulation within DLN

In agreement with UVB depleting LC from the epidermis, there was an accumulation of DC within DLN following UVB irradiation (see sections 5.2 and 5.3). As with LC migration, this effect was dependent on TNF-α and IL-1β (see figs. 5.3 and 5.4 respectively). There was an unexpected increase in DC within DLN after UVA1 exposure (described in section 5.3). Only antibodies to IL-1β, but not TNF-α, prevented an accumulation of DC within DLN following UVA1 (see sections 5.4 and 5.5).

There are a number of potential APC within the dermis other than LC, and it is possible that UVA1 acts on one or more of these cell types to induce their migration to DLN. There is evidence that a second pathway of cutaneous antigen presentation exists. Ia$^+$ DC, present in the perivascular region of the mouse dermis, when hapten-derivatised, are capable of inducing CH in naive mice (Tse and Cooper, 1990). Thus, if UVA1 depleted these cells, it may impair subsequent immune responses. Alternatively, there also exists a population of Thy-1$^+$, Ia$^-$ cells, exhibiting a dendritic morphology, within the murine epidermis. It has been suggested that the ratio of these cells to LC influences the intensity of CH (Bigby *et al* 1987; Sullivan *et al* 1986). Migration of these cells mediated by UVA1 may interfere with immune responses due to alterations in the ratio of respective APC within DLN.

Moodycliffe *et al.* (1992) showed that UVB irradiation alone induced DC migration to DLN. Neither UCA isomer had an effect on DC numbers within DLN (Moodycliffe *et al* 1992). An antibody to *cis*-UCA does not prevent the accumulation of DC following UVB irradiation (El-Ghorr and Norval, 1995).

UVB not only alters DC numbers, but can change the antigen presenting ability of DC (Young *et al* 1993; Noonan *et al* 1988). A time delay for the onset of defective APC function was required, as DC taken 3 days after irradiation had normal activity, whilst those taken 7 days later were suppressive. DC treated with IL-10 were capable of supporting proliferation of, and IFN-γ secretion by, both $CD4^+$ and $CD8^+$ T cells in an MLR. In contrast, IL-10 markedly inhibited DC driven IFN-γ production by purified $CD4^+$ or $CD8^+$ T cells. Under the same conditions, macrophage-dependent proliferation was strongly inhibited. IL-10 inhibited DC induced production of IFN-γ by Th1 clones, and by $CD4^+$ and $CD8^+$ T cells from unprimed mice in a primary allogeneic MLR (Macatonia *et al* 1993).

In summary, UVB irradiation results in an accumulation of DC within DLN, and this is dependent on IL-1β and TNF-α. UVA1 also causes an IL-1β dependent accumulation of DC, but this is not dependent on TNF-α.

7.4. UVR and its effects on contact sensitisation

The strength of the proliferative response to the sensitiser correlates with the level of sensitisation and the severity of elicitation (Kimber *et al* 1990a). There similarly exists a correlation between the number of DC arriving in DLN 24h after sensitisation and the magnitude of the induced proliferation (Kimber *et al* 1990b; Kimber *et al* 1990c). The proliferative response of DLNC after contact sensitisation was suppressed if mice were exposed to UVB before sensitisation (fig. 3.6). The local lymph node assay is able to identify at least moderate and strong skin allergens (Kimber, 1992). Thus, the depressed proliferative responses observed in these

experiments (see fig. 3.9), would indicate that UVB reduces the relative strength of the sensitiser.

In vitro irradiation of epidermal cells and LC with 20 mJ/cm^2 UVB led to loss of their ability to stimulate Th1 cell lines, but the cells retained APC function for Th2 cell lines. Irradiated splenic adherent cells lost APC function for both subsets (Simon *et al* 1990). UVB irradiation of monocytes impaired their ability to stimulate proliferation and IFN-γ production of Th1 cells, whilst retaining normal APC function to Th2 cells (Kremer *et al* 1996). Th0 cell stimulation was also normal, but exhibited a preferential suppression of IFN-γ production. This was related to decreased production of IL-12 p70 protein. IL-10 did not have any effects on anti-CD3 or alloantigen-induced LC-dependent T cell proliferation, but production of IL-2 and IFN-γ. IL-10 pretreated LC did not alter proliferative responses of Th2-cell clones (D10.G4), but were unable to support proliferation of Th1-cell clones (AE7) (Enk *et al* 1993b). Chronic activation of both murine $CD4^+$ T cells in the presence of IL-10 gives rise to T cells with low proliferative capacity (Groux *et al* 1997). However, UVB irradiation before contact sensitisation did not significantly alter IL-10 or IFN-γ secretion by DLNC compared with DLNC from unirradiated, contact sensitised mice (figs. 3.9 and 3.11 respectively).

Irradiation of mice led no increase in IFN-γ production from DLNC towards the end of the culture period (see fig 3.10). Anti-IFN-γ did not significantly alter CH responses to Ox. This suggests that IFN-γ participates in, but is not wholly responsible for, the CH response (Thomson *et al* 1993). IFN-$\gamma^{-/-}$ mice can mount CH responses as well as WT mice (C57Bl/6). UVB and *cis*-UCA can suppress CH responses in both wild-type (C57Bl/6) and IFN-$\gamma^{-/-}$ mice (Reeve *et al* 1999).

T cells from naive, or FITC-sensitised mice, produced less IFN-γ and IL-2 in response to culture with APC from UV-irradiated mice than in response to APC from unirradiated mice (Saijo *et al* 1996). IL-4 and IL-10 were not detectable unless culture

supernatants were concentrated, and were not consistently produced. Culture with APC from UV-irradiated mice did not increase Th2-like cytokine synthesis.

Injection of anti-IL-12 before sensitisation resulted in a reduced ear swelling upon challenge (Riemann *et al* 1996). Furthermore, Riemann and colleagues showed that when anti-IL-12 treated mice were rechallenged, the CH response remained minimal, suggesting that tolerance had been induced (Riemann *et al* 1996). Anti-IL-12 injection before sensitisation inhibited UVB induced suppression of CH (Schwarz *et al* 1996). However, UVB irradiation before sensitisation resulted in increased IL-12 production by 48h of culture (see fig. 3.10). Schmitt *et al* observed increased IL-12 production by LNC from irradiated mice (Schmitt *et al* 1998). IL-12 production was increased in LNC from UVB-irradiated mice compared with LNC from non-irradiated mice, as measured by ELISA. DC-enriched populations from the spleens of irradiated mice produced significantly less biologically active IL-12 than splenic DC from unirradiated mice.

Antigen induced cytokine production in mice transgenic for IL-12 p40 is skewed toward a Th2 profile, with decreased production of IFN-γ and increased production of IL-4 and IL-10, suppressed DTH responses (Yoshimoto *et al* 1998). The IL-12 ELISA used for analysis of DLNC culture supernatants measured both the inducible p40 subunit and the bioactive p70 heterodimer. UVB may increase IL-12 p40 production by DLNC. Secretion of IL-12 p40, which can bind the IL-12 receptor, may interfere with signalling via IL-12 p70 and skew immune responses by this antagonistic mechanism.

There is evidence that exposure to sub-optimal concentrations of skin sensitisers may tip the balance between promoting or inhibitory signals in CH (Sy *et al* 1977). Accordingly, it was observed that UVB-mediated immune suppression was enhanced by using lower challenge concentrations (see section 3.4). LC depletion may be a mechanism to reduce the antigenic dose presented to T cells, and may therefore cause suppression of T cell responses.

In summary, UVB decreases the proliferative response of DLNC following contact sensitisation. UVB also appears to increase Th1-like cytokine production, but this may be mis-interpreted due to IL-12 p40 induction. IL-10 production by DLNC of contact sensitised mice is unaltered by UVB irradiation.

7.5. UVB and its effects on elicitation of CH responses.

In these analyses, UVB irradiation before contact sensitisation appeared to change the cytokine profile upon CH elicitation. IFN-γ and IL-12 were produced in culture supernatants of DLNC from mice undergoing a CH response, but were reduced in culture supernatants from DLNC of mice which were irradiated before contact sensitisation. The profiles were changed to that of a primary response, as cytokine production by UVB treated DLNC was similar to that of negative control mice (see section 3.7). IL-10 was not detectable at any time during culture. These data support the hypothesis that UVB reduces Th1-like immune responses, but not by upregulation of Th2-like responses (Saijo *et al* 1996).

Garssen and colleagues have used a similar system to that used in this study, with pre-exposure to suberythemal UVB on 4 days prior to sensitisation of BALB/c mice with Ox 4 days after the last irradiation. They could not detect IFN-γ or IL-4 in supernatants taken from DLNC after 24h of culture (Garssen *et al* 1999). When DLNC were stimulated *in vitro* with conA or Ox coupled to BSA, there was decreased production of IFN-γ by cells from irradiated animals. Similar results were found by PCR, indicating that these effects also occur at the mRNA level. Further investigation using picryl chloride (PCl) as the contact sensitiser showed that IFN-γ and IL-12 were reduced in *in vitro* stimulated culture supernatants from irradiated mice, but IL-4 production was unaltered. IL-10 was not detectable in these assays.

In a secondary immune response, T cells can encounter and be activated by antigen at the site of application, as well as in DLN. The cytokine microenvironment in which an antigen is encountered is important in directing the subsequent immune

response. In experiments by Goodman and colleagues, when keratinocytes were used as accessory cells there was a specific defect in T cell IFN-γ production, whereas IL-2 and IL-4 were induced at levels comparable to those seen when professional APC were used as accessories (Goodman *et al* 1994). RT-PCR revealed that keratinocytes produced little or no mRNA for IL-12 p40 compared with professional APC, and addition of IL-12 to cultures could restore IFN-γ production. IL-10 application can suppress the upregulation of IFN-γ mRNA normally seen in challenged skin (Kondo *et al* 1994).

In summary, UVB irradiation suppressed CH responses in mice. Production of Th1-like cytokines by DLNC was decreased in mice which were irradiated before contact sensitisation.

7.6. UVB effects on the cutaneous microenvironment.

Injection of IL-10 (1-2μg) into the ears of mice 8h before application of TNCB, then challenging on the contra-lateral ear 5d later, led to significant inhibition of CH (Enk *et al* 1994). If sensitised to another chemical at a later time, IL-10 treated mice were able to respond, indicating that suppression was hapten-specific. Hapten-specific anergy could also be observed in the DLNC of IL-10 treated mice. Sensitisation with contact allergens induces IL-10 mRNA in murine epidermal cells, detectable at 4h and maximal by 12h (Enk and Katz, 1992b). The effector phase of CH can also be blocked by IL-10 (Schwarz *et al* 1994). IL-10 injection decreases the inflammatory cell infiltrate, and decreases edema at the dermis compared to mice with a normal CH response (Kondo *et al* 1994). Evidence for the downregulatory role of IL-10 in DTH is provided by intraperitoneal injection of the cytokine prior to either the sensitisation or elicitation phase. IL-10 injection leads to blocking of both events (Schwarz *et al* 1994). IL-10 injection therefore results in similar changes in immune responsiveness to UVB irradiation.

Mice lacking expression of IL-10 (IL-10T mice) were completely resistant to the immunosuppressive effects of UVB on DTH to alloantigens (BALB/c spleen cells) (Beissert *et al* 1996). Mice which were injected with monoclonal anti-IL-10 antibodies 4 and 24h after UVB irradiation had a DTH response which was suppressed by half as much as mice not treated with antibody (Rivas and Ullrich, 1992). In these studies, a single injection of antibodies against IL-10 did not reverse UVB- or *cis*-UCA mediated suppression of the DTH response to HSV-1 (see section 6.2). It may be that IL-10 has to be neutralised for a longer time following irradiation to prevent its activity. This would also indicate that it is likely that there are many factors involved in IL-10 production following UVB exposure, and potentially, many sources.

Local suppression can be observed immediately and up to 48h if C57BL/6 mice are sensitised through the irradiated site. IL-10 mRNA expression was upregulated within an hour of UVB irradiation, and continued to rise until a peak at 24h (see fig. 6.16). Expression remained elevated above constitutive levels 96h after exposure. IL-10 protein in irradiated skin peaked at 8h after UVB exposure, and had returned to constitutive levels by 24h (see fig. 6.3). IL-10 was detectable by ELISA in sera of mice irradiated with 7 MED UVB, and peaking 3-4 days after exposure. This finding is in agreement with a previous timecourse found for IL-10 protein in the serum of C57BL/6 mice exposed to UVB (Beissert *et al* 1996). If mice are sensitised through an unirradiated site, a lag period of 3 days until contact sensitisation is required before maximal systemic suppression of CH can be observed (Miyauchi and Horio, 1995; Hammerberg *et al* 1996).

ELISA of cell lysates indicated that IL-10 content of PAM-212 cells was unaffected 24h after UVB irradiation. IL-10 content of PAM-212 cells was reduced in a dose-dependent manner by 48h after irradiation, and this was not due to decreased cell viability. ELISA of culture supernatants indicated that IL-10 was not secreted by PAM-212 or B16 cells upon UVB exposure (at doses up to 20 mJ/cm^2). Rivas and Ullrich (Rivas and Ullrich, 1992) identified IL-10 in supernatants from PAM-212 cells

irradiated with 20 mJ/cm^2 UVB 24h earlier, but not in supernatants from unirradiated PAM-212 cells.

UVB may alter the lymphocytes populations within irradiated sites. This may be facilitataed by effects on adhesion or costimulatory molecules. CD44 expression on LC is important in lymphocyte homing and T cell activation. TNF-α can upregulate CD44 expression, whereas IL-10 downregulates expression, both in a concentration-dependent manner. When added simultaneously to cultures of LC, the two cytokines mutually neutralised each other's activity (Osada *et al* 1995). As discussed above, both TNF-α and IL-10 are present in UVB-irradiated skin samples shortly after exposure (see figs. 6.8 and 6.3 respectively). In the experiment described in section 6.9, ICAM-1 expression was induced in the subdermal compartment following a 10 MED UVB exposure. ICAM-1 gene expression is controlled by nuclear factor (NF)-κB. Induction of NF-κB by reactive oxygen intermediates (Jourd'heuil *et al* 1997), or by TNF-α, is a proposed mechanism for altering ICAM-1 expression. TNF-α is also implicated in DNA damage following UVB exposure, as injection of antibodies against TNF-α reduced DNA fragmentation (Schwarz *et al* 1995). DNA damage induced by UVB is another potential mediator (Krutmann and Grewe, 1995). *Cis*-UCA, even above physiological levels, could not augment phorbol 12-myristate 13-acetate (TPA) induced synthesis of either TNF-α mRNA expression in, or protein secretion from, keratinocytes (Redondo *et al* 1996).

Norris and colleagues (1990) found that UVR had a biphasic effect on ICAM-1 expression on human keratinocytes, with a downregulatory effect preceding an upregulation. In experiments by Krutmann and colleagues (1990), *in vitro* irradiation of human keratinocytes prior to stimulation with TNF-α inhibited ICAM-1 upregulation in a dose-dependent manner. Further studies by the same group revealed that UVB exposure of keratinocytes inhibited ICAM-1 upregulation in response to either TNF-α or IFN-γ. Restoration of responsiveness to TNF-α required at least a

24h recovery period (Krutmann *et al* 1992a). There was a significant increase in ICAM-1 expression 48h after irradiation.

In summary, UVB causes an increase in TNF-α and IL-10 protein within irradiated sites. These may play a role in altering the cytokine microenvironment for subsequent immune responses.

	UVB	UVA1	*cis*-UCA
immune suppression	CH	CH (reported in 1)	CH (reported in 2)
	DTH	DTH (reported in 1)	DTH
LC depletion	YES	NO	YES
from epidermis	(max. at 12h)		(max. at 24h)
	TNF-α dependent,		TNF-α dependent,
	IL-1β dependent		IL-1β dependent
DC accumulation	TNF-α dependent,	TNF-α dependent,	NONE
in DLN	IL-1β dependent	IL-1β dependent	(reported in 3)
cytokine mRNA	TNF-α (peak at 4h)	NO TNF-α	
in skin	IL-1β (peak at 4h)	IL-1β (upreg at 24h)	
	IL-10 (continued rise)		

Table 7.1. The effects of UVB, UVA1 and *cis*-UCA on the immune parameters investigated.

(1 = El-Ghorr and Norval, 1999, 2 = Kondo *et al*, 1995, 3 = Moodycliffe *et al*, 1992).

7.7 Summary

In any response to antigen, there is a chain of events leading to the final immunological decision and outcome, which can be clearance, ignorance or exacerbation of the disease state. UVB plays a role in this decision making by biasing the system and having knock-on effects throughout the subsequent response.

There is evidence that UVB irradiation induces TNF-α production in the human epidermis, but that UVA1 does not (Skov *et al* 1998a). It is therefore rational to expect LC migration following UVB exposure, and this has been identified in this study. UVA1 did not cause a decrease in LC frequency within the epidermis. A predominant role for TNF-α in human monocyte IL-10 synthesis has been shown (Wanidworanun and Strober, 1993). Lymphocytes from UVR exposed mice produce less IL-2 and IFN-γ, but more IL-4, when activated *in vitro* by antigen. A similar change could be induced when mice were treated with LPS or IL-1β. The ability of IL-1β to alter cytokine secretion is not a direct effect, as addition of IL-1β to cultures of antigen-primed T cells does not alter cytokine patterns (Araneo *et al* 1994). Leukocytes produce TNF-α and IL-1β in response to stimulation by LPS. LPS is also a potent stimuli for IL-1ra mRNA expression and release, and upregulation of IL-1ra has been reported in irradiated skin (Hirao *et al* 1996). Release of IL-1ra can be potentiated in the presence of IL-10. IL-10 alone does not increase IL-1ra release, but manages to stabilise IL-1ra mRNA. IL-10 inhibits production of TNF-α, IL-1β, and IL-8 under the same conditions (Cassatella *et al* 1994). Similarly, injection of IL-10 inhibits the mRNA signals for IL-1α, IL-1β and TNF-α (Enk *et al* 1994).

Contact sensitisation after irradiation resulted in an unresponsiveness to the sensitiser upon challenge. A low zone tolerance effect can be mediated by cells other than LC (Steinbrink *et al* 1996). Use of radiolabelled allergen showed that chemicals can leave the site of application via local blood vessels, whereas a small fraction of allergen remains in the site for days (Macher and Chase, 1969). Timed excision of sensitised sites showed that the chemical does not have to become cell associated, and

that the escaping chemical established tolerance rather than sensitisation. Loss of LC from the irradiated epidermis robs the cutaneous immune system of its primary APC, increasing the likelihood of antigen presentation by (an)other cell type(s) and facilitating leakage of contact sensitiser into the systemic environment. Additionally, any sensitiser encountered at the site will do so in an environment more favourable toward Th2-like immune responses. Interestingly, a recent study has found that IL-4 inhibits LC migration induced by TNF-α, via downregulation of TNFR2 expression (Takayama *et al* 1999).

Irradiated epidermal cells and LC lose their ability to stimulate KLH-specific Th1 cell lines, but retain APC function to KLH-specific Th2 cell lines (Simon *et al* 1990). When keratinocytes are used as accessory cells there is a specific defect in T cell IFN-γ production. This is because keratinocytes produce little or no mRNA for IL-12 compared with professional APC (Goodman *et al* 1994). Incubation of epidermal cells in *cis*-, but not *trans*-UCA completely inhibited antigen presentation in a spindle-cell tumour antigen system. Secondary immune responses were also affected, as incubation with *cis*-UCA prior to injecting antigen-pulsed epidermal cells into the footpads of immune mice inhibited the DTH response (Beissert *et al* 1997).

A proposed mechanism of action leading to immune modulation following UVB irradiation is shown in fig. 7.1, based upon the results of this study, and related to the findings of other groups. UVB photoisomerises UCA. *Cis*-UCA, in conjunction with another UV-induced 'factor' induces secretion of IL-1β. IL-1β in turn initiates in TNF-α production and release, with both cytokines inducing LC migration from the epidermis (with subsequent accumulation of DC within the DLN). IL-1β and TNF-α also contribute to IL-10 synthesis and secretion, either from resident cells or infiltrating macrophages, thereby altering the cutaneous microenvironment. IL-10, aided by IL-1ra, then downregulates IL-1β and TNF-α expression, making it more difficult for presentation of antigens encountered through the site shortly after UVB

irradiation. T cells within DLN of irradiated mice appear to be primed in the normal fashion however, exhibiting the same cytokine profiles as unirradiated mice, although with a lower proliferative response. Upon homing to the site of sensitisation, the cells are not fully activated due to the Th2-like cytokine microenvironment.

Effects on disaease states

Clearance

Ever since Finsen's first recognition that UVR could heal the skin lesions of skin tuberculosis (*Lupus vulgaris*) (Finsen, 1899), phototherapy has operated on a largely empirical basis. Narrow-band UVB (311-312 nm) is as effective as PUVA in treatment of psoriasis (Van Weelden *et al* 1990). The epidermis of psoriasis patients contains about 3 times as much UCA as that of normal subjects, and the antigen-presenting ability of epidermal cells is reduced during UVB therapy (Gilmour *et al* 1993).

UVB suppressed human ACD both locally and systemically following Ni allergy elicitation, whilst UVA exposure had no effect (Sjovall and Christensen, 1986). In a study by Simons and colleagues, UVB was slightly better at treating chronic hand dermatitis than PUVA, although both therapies relieved the condition (Simons *et al* 1997). However, the side-effects, such as erythema and pigmentation, were greater following PUVA, and the group recommended that patients be treated with UVB initially, then PUVA if this failed. Conversely, UVA1 is a better treatment for atopic eczema than UVB (Krutmann *et al* 1992b; Krutmann *et al* 1998).

Graft survival can be prolonged by treating the recipient with UVB or PUVA, whereas UVA alone has no effect (Gruner *et al* 1992). Injection of *cis*-UCA starting on the day of transplantation was able to prolong the survival of allogeneic MHC disparate skin grafts in mice. Furthermore, *cis*-UCA injection delayed the onset of acute graft versus- host disease and prevented the lethal disease in 30% of animals

(Gruner *et al* 1992). Acute graft versus- host disease is regarded as a Th1 cell mediated disease, whereas the milder chronic form is chararcterised by activation of $CD4^+$ T cells secreting Th2-like cytokines (Rus *et al* 1995; Corsini *et al* 1995).

Ignorance

As has been shown in this study, UVB can prevent sensitisation to contact sensitisers. It has also been shown that UVB, and *cis*-UCA, can prevent DTH responses. It is likely that UVB and *cis*-UCA can also prevent sensitisation to, and subsequent recognition of, tumour neoantigens (Streilein *et al* 1994b). This appears to be as a biological side-effect, which has evolved to prevent the immune system rejecting sunburned skin containing altered, self-antigens.

UVB caused a reduction in immunisation rates to DPCP in human volunteers, when given 3 days before sensitisation and CH was elicited 3 weeks later. UVA was without effect (Skov *et al* 1997).

Exacerbation

Erythemagenic UVR is toxic in the autoimmune disease systemic lupus erythematosus (SLE) (McGrath *et al* 1994; Dov Golan *et al* 1992). This phenomenon may be due to further suppression of already reduced cell-mediated immunity (Horwitz, 1972). Tissue damage occurs, and may be due to TNFα production. Suppression of NK cells and perturbed regulation of immunoglobulin production could also exacerbate the disease. Enhanced binding of autoantibodies to the cell membrane of UV-irradiated keratinocytes may perpetuate tissue damage (Dov Golan *et al* 1992).

UVB could be of particular relevence in HIV patients, where sunlight could induce AIDS progression (Vincek, 1995; Wallace and Lasker, 1992). Irradiation of a chronically HIV-infected promonocytic cell line (U1) induced elevated reverse transcriptase activity and p24 antigen levels in culture supernatants (Stanley *et al*

1989). There was also a time-dependent upregulation in viral antigen after UVB exposure. A switch from Th1 to Th2-like cytokine profiles is implicated in disease exacerbation (Clerici and Shearer, 1993). However, most published data indicate few, if any, adverse affects on HIV-positive individuals following phototherapy (reviewed in Adams *et al* 1996).

Immune response to intracellular bacteria could also be altered by UVB. The lepromin reaction, a cell-mediated immune response to antigens of *Mycobacterium leprae*, is reduced in locally irradiated sites. Irradiation decreases lymphocyte infiltration, particularly $CD4^+$ T cells, in and around the induced granuloma (Cestari *et al* 1995). As the granulomatous response limits or suppresses infection, it thus follows that UVB could exacerbate the clinical situation.

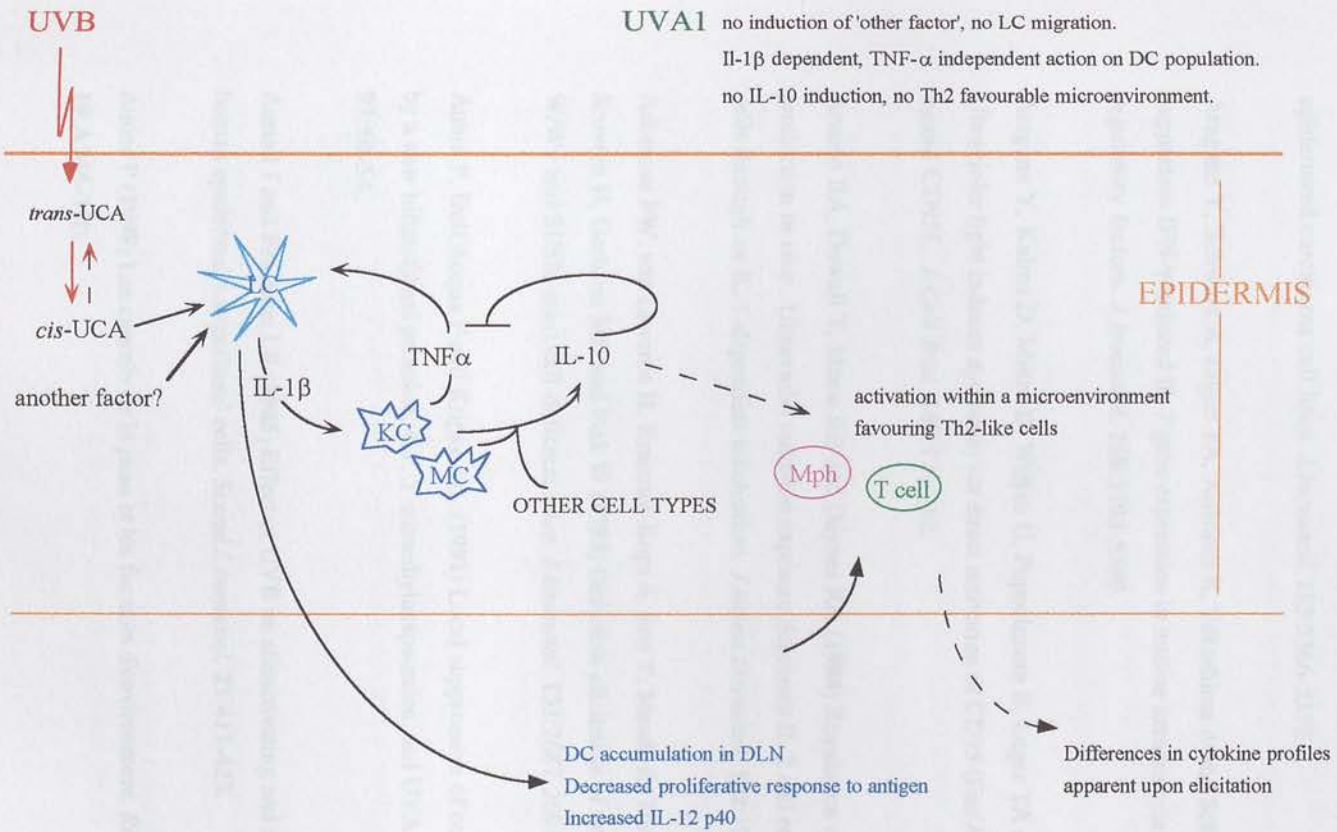

Figure 7.1. A proposed mechanism of action leading to immune suppression following UVB irradiation

(KC = keratinocyte, MC = melanocyte, Mph = macrophage).

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Presentations

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OP166 ULTRAVIOLET-B IRRADIATION OF MICE ENHANCES IL-12 p40 PRODUCTION IN LYMPH NODE CELLS

MS Duthie, RJ Dearman[1], I Kimber[1] and M Norval
Medical Microbiology, University of Edinburgh Medical School, Edinburgh EH8 9AG, and [1]Zeneca Central Toxicology Laboratory, Macclesfield SK10 4TJ.

Ultraviolet (UV)-B irradiation suppresses cell-mediated immune responses to antigens encountered subsequently through the exposed site. It has been suggested that UV-B mediates these effects by blocking the induction of Th1-like cytokine responses. In the present study C3H/HeN mice were irradiated with 2 minimum erythemal doses of UV-B prior to contact sensitisation on their ears with 1% oxazolone; conditions which resulted in approximately 50% suppression of contact hypersensitivity. The auricular lymph nodes draining the irradiated sensitised site were excised and the lymph node cells cultured *in vitro* for 120 h with measurement of cytokine production by ELISA. UV-B irradiation caused no change in IL-10 or IFN-γ secretion compared with the unirradiated group. However exposure resulted in a significant increase in IL-12 p40 production by 48 h. These data indicate that UV-B irradiation enhances synthesis of IL-12 by lymph node cells while not affecting Th2 cytokine production. How these changes relate to the immunosuppression induced by UV-B exposure remains uncertain as yet.

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DIFFERENTIAL EFFECTS OF UVA AND UVB ON LANGERHANS CELL MIGRATION.

MS Duthie, RJ Dearman[1], I Kimber[1], and M Norval
Dept. of Medical Microbiology, University of Edinburgh Medical School, Edinburgh, UK. [1]Astra-Zeneca Central Toxicology Laboratory, Macclesfield, UK.

Time-courses for the reduction of Langerhans cells numbers in the epidermis of mice following exposure to UVA or UVB irradiation, or skin painting with *cis*-urocanic acid (UCA), have been established. Using doses which suppressed delayed hypersensitivity in all cases, both UVB and *cis*-UCA resulted in a reduction of Langerhans cell numbers within 24h of exposure, whereas UVA had no effect (at up to 72h). Furthermore, UVA did not exert any protection against the UVB-mediated depletion in Langerhans cell numbers. The reduction in Langerhans cells following both UVB and *cis*-UCA exposure could be blocked by pre-treatment of mice with neutralising antibodies to either IL-1β or TNF-α. UVB irradiation has been shown to cause an increase in dendritic cell numbers in lymph nodes draining irradiated sites. UVA irradiation also caused an increase. However, pre-treatment with anti-TNF-α blocked this effect following UVB exposure, but did not alter the UVA-mediated increase. These results indicate that UVB induces Langerhans cell migration from the epidermis, possibly via UCA isomerisation and subsequent secretion of TNF-α and IL-1β. UVA may act through a different mechanism and may target another cutaneous antigen presenting cell type, possibly dermal dendritic cells.

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DIFFERENTIAL EFFECTS OF UVA1 AND UVB RADIATION ON LANGERHANS CELL MIGRATION IN MICE

Malcolm S. Duthie, Ian Kimber*, Rebecca J. Dearman* and Mary Norval§

Department of Medical Microbiology

University of Edinburgh Medical School

Teviot Place, Edinburgh, UK.

*AstraZeneca Central Toxicology Laboratory

Alderley Park,

Macclesfield, Cheshire, UK.

§Corresponding author:

Dr Mary Norval

Department of Medical Microbiology

University of Edinburgh Medical School

Teviot Place, Edinburgh EH8 9AG, Scotland.

Tel: +44 131 650 3167 Fax: +44 131 650 6531 Email: M.Norval@ed.ac.uk

Running title: UVA1 and UVB-induced LC migration

Key words: dendritic cells, tumor necrosis factor-α, interleukin 1β

Abbreviations: CH, contact hypersensitivity; DTH, delayed type hypersensitivity; DC,

dendritic cells; DLN, draining lymph nodes; IL, interleukin; LC, Langerhans cells; MED, minimal erythema dose; TNF, tumor necrosis factor

ABSTRACT

The UVB- and UVA1 (340-400 nm)-induced migration of Langerhans cells (LC) from the epidermis and accumulation of dendritic cells (DC) in the lymph nodes draining the exposed skin site of C3H/HeN mice have been investigated. One minimum erythemal dose (MED) of UVB (1.5 kJ/m^2) and of UVA1 (500 kJ/m^2) were chosen, amounts which had been shown previously to suppress delayed hypersensitivity (DTH). UVB irradiation resulted in a reduction in epidermal LC numbers which was most apparent 12 h after exposure, but, in contrast, UVA1 had no significant effect up till 72 h after exposure. UVA1 did not exert any protection against the UVB-mediated depletion in LC numbers. The reduction in LC following UVB exposure could be prevented by treatment of the mice with neutralising antibodies to either tumor necrosis factor (TNF)-α or interleukin (IL)-β 2 h before irradiation. It was known already that exposure to UVB caused an increase in the number of dendritic cells (DC) in the lymph nodes draining the irradiated skin site. In the present study we have shown that UVA1 had a similar effect. Pre-treatment of the mice with neutralising antibodies to IL-1β substantially inhibited the increase after both UV regimens. However TNF-α antibodies only affected the UVB-induced increase and did not alter the increase in DC numbers following UVA1 exposure. These results indicate that UVB causes the migration of LC from the epidermis and an accumulation of DC in the draining lymph nodes by a mechanism that involves both TNF-α and IL-1β. UVA1, on the other hand, does not cause LC migration from the epidermis and the UVA1-induced accumulation of DC in the draining lymph nodes requires IL-1β, but not TNF-α. Therefore it is likely that UVA1 may act through a different mechanism from UVB and may target a cutaneous antigen presenting cell other than LC, such as the dermal DC.

INTRODUCTION

In the course of a normal cutaneous immune response, Langerhans cells (LC), the major antigen presenting cells of the epidermis, internalise and process antigen, and, in response to changes in the local cytokine milieu, they migrate to the draining lymph node. During the migration in the afferent lymphatics, LC mature, as revealed by the altered expression of various adhesions and co-stimulatory molecules. Once in the paracortical region of the lymph node, where they are known as dendritic cells (DC), they present antigen peptides to specific T cells, which are then stimulated to proliferate and to produce a particular cytokine profile. The key migratory signals are believed to be provided in an autocrine fashion by interleukin (IL)-1β, and in a paracrine fashion by tumor necrosis factor (TNF)-α (Kimber *et al*, 1998; Wang *et al*, 1999). LC express only the type 2 (p75) TNF-α receptor, and mice deficient in expression of this receptor demonstrate significantly impaired LC migration (Wang *et al*, 1997). Blocking of TNF-α activity by neutralising antibodies revealed that TNF-α acts early in the response, as injection of the antibodies prior to contact sensitisation, but not 18 h after, inhibited the sensitisation phase and the subsequent contact hypersensitivity (CH) on challenge (Cumberbatch and Kimber, 1995). IL-1β is produced solely by LC in the normal mouse epidermis, and its synthesis is enhanced by topical exposure to chemical allergens (Enk and Katz, 1992). Intradermal injection of IL-1β stimulates LC migration from the epidermis, but at a slower rate than that induced by injection of TNF-α (Cumberbatch *et al*, 1997a). Therefore it is believed that IL-1β acts as the initial signal to upregulate TNF-α production and release from keratinocytes, and delivers an autocrine signal for LC migration simultaneously (Kimber *et al*, 1998).

UV radiation is divided into three wavebands: UVC (200-280 nm), UVB (280-315 nm) and UVA (315-400 nm), further divided into UVA1 (340-400 nm) and UVA2 (315-340 nm). It is recognised that UVB exposure can suppress cell-mediated immune responses to a variety of antigens, including contact sensitisers

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The effects of ultraviolet radiation on the human immune system

M.S.DUTHIE, I.KIMBER* AND M.NORVAL

Department of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.
**Central Toxicology Laboratory, Zeneca plc, Alderley Park, Macclesfield, Cheshire SK1 4TJ, U.K.*

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Summary The adverse outcome of increased ultraviolet (UV) irradiation on human health is currently of concern. While many experiments have been carried out in rodent models, fewer have been designed to test the effects of UV exposure in human subjects. This review concentrates on the modulations induced in the human immune system by UV, and outlines changes in antigen presentation by Langerhans cells and macrophages, in the activities of natural killer cells and T cells, and in cytokine regulation. Precautionary measures which might be taken to help protect people against the immunosuppressive action of UV irradiation are considered.

Key words: cytokine regulation, immune system, immunosuppression, Langerhans cells, macrophages, natural killer cells, T cells, ultraviolet irradiation

As well as links with skin cancer, ultraviolet (UV) irradiation causes alterations in immune functions. While the mechanisms of these effects have been widely investigated in inbred mouse strains, and to some extent in rats, ethical and practical considerations have meant that relatively few human studies have been conducted.

UV radiation can be divided into three categories, dependent on wavelength: UVC, 200–280 nm; UVB, 280–315 nm; UVA, 315–400 nm. Stratospheric ozone effectively blocks wavelengths shorter than 290 nm, together with 70–90% of UVB. Between 1970 and 1987, a substantial decrease in the protective ozone layer occurred,[1,2] with a resultant increase in the amount of UVB reaching the Earth's surface, which was particularly apparent over Antarctica. In spite of this change, it is difficult to determine if there is a global trend towards increasing UVB levels over populated regions of the world, due to the relatively short timespan of the measurements. However, life-styles have altered in recent decades, leading to increased personal exposure to UV in many cases. Such factors include more leisure time, 'sunshine' holidays, the use of sunbeds for cosmetic tanning, the wearing of minimal clothing outdoors and the insufficient use of sunscreens. In addition, phototherapy is used more frequently for the treatment of a number of medical conditions such as psoriasis and acute graft-versus-host disease. Finally, some industrial UV exposure can occur, as is the case with welding arcs or tungsten halogen lamps.

Most of the UV which irradiates human skin is absorbed by the epidermis, with transmission of the longer wavelengths only into the dermis. Most experiments thus far have concentrated on the UVB waveband which is regarded as the most important in mediating immunosuppression. However, there is growing interest in the effects of UVA[3] and in solar-simulated radiation. It is believed that UV acts by initiating a cascade of events in the skin starting with absorption by a chromophore or chromophores locally and ending in immunomodulation. Among the first changes, membrane damage, induction of cytoplasmic transcription factors, DNA damage and isomerization of urocanic acid (UCA)[4,5] appear to play important parts. Alterations in antigen-presenting cell populations occur, so that antigens encountered through UV-exposed skin are presented differently, or not at all, from those encountered through normal skin. Changes at the molecular level are induced so that intercellular and intracellular signalling mechanisms are modified, and the local microenvironment becomes more favourable to the development of T-helper (Th) 2-like immune responses. These effects can be demonstrated not only at a local level but, at least in rodent models, at distant sites also, resulting in antigen-specific systemic immunosuppression.

Correspondence: Dr Mary Norval.

Cellular aspects of ultraviolet radiation

Several studies have indicated that UV irradiation of human subjects generally leads to the downregulation of delayed hypersensitivity, considered as an important measure of T-cell function. The first two of these involve suppression of already established responses. Mork and Austad[6] reported that 70% of their patients with allergic chronic dermatitis were treated successfully with suberythemal UVB, and the situation was maintained if UVB was given once weekly thereafter. Similarly, exposure to suberythemal UVB four times weekly over a 3-week period was sufficient to suppress the contact hypersensitivity (CH) response to nickel in nickel-sensitive individuals.[7] This effect was limited to the UVB wavelengths, as UVA did not alter the patch-test scores. In contrast, Tie *et al.*[8] found that subjects sensitized through normal skin and challenged via UVB-exposed skin, exhibited enhanced CH.

Using carefully graded doses of sensitizer (1-chloro-2,4-dinitrobenzene, DNCB), Friedmann *et al.*[9] showed that therapy with both broadband UVB and psoralen plus UVA (PUVA) induced impairment of CH in about 70% of patients with psoriasis. The sensitizer was applied approximately half-way through the therapy and the elicitation occurred 4 weeks after the cessation of therapy. Subjects who were not sensitized successfully during treatment were resensitized at a later date, and no evidence for tolerance was obtained in either the UVB or the PUVA group.

Following exposure of a small area of skin to 144 mJ/cm^2 UVB on each of 4 consecutive days, a protocol which reduces Langerhans cell numbers in the epidermis from 565 to 17/mm^2, 40% of volunteers demonstrated suppressed CH responses to DNCB applied epicutaneously at the site of exposure, followed by challenge at a distant unirradiated site.[10] In parallel with mouse studies, these subjects were designated as UVB susceptible and the others, in whom the extent of the CH response was unaltered by the UV exposure, were termed UVB resistant. It was also found that 92% of skin cancer patients fall into the UVB-susceptible category,[10] and therefore UVB-induced suppression of CH may act as a risk factor/indicator for skin cancers. However, it should be noted that the proportion of black people with the UVB-susceptible trait is similar to that in Caucasians, despite the significantly lower incidence of skin cancer in this population,[11] although this could be attributed to the protective effects of melanin preventing significant DNA damage. Skov *et al.*[12] also reported that 56% of human subjects exhibited suppressed CH responses following 3 minimal erythema doses (MEDs) of UVB and sensitization on the irradiated site. Tolerance was not induced in the unresponsive individuals. Interestingly, tests to assess Langerhans cell (CD1a+ HLA-DR+) and macrophage (CD1a− HLA-DR+) numbers and the ability of epidermal cells to activate autologous T cells following irradiation revealed no differences between the UVB-susceptible and UVB-resistant individuals. Thus, susceptibility to UVB cannot be correlated easily with these local changes in epidermal antigen-presenting cell populations or functions.

The clear division into UVB susceptible and UVB resistant has not been confirmed in all studies. Cooper *et al.*[13] reported that almost all subjects had a reduced ability to respond to DNCB if the sensitizer was applied to the irradiated site. The downregulation was dose dependent to some extent as the lowest exposure used (0·75 MED of UVB daily for 4 days to a small area of skin) induced non-responsiveness in 68% of individuals while the highest (2 MED of UVB daily for 4 days) induced non-responsiveness in 95% of individuals. The most susceptible people exhibited tolerance to DNCB on repeated challenge with the sensitizer. There was a modest suppression in CH if the DNCB was applied at a distant unirradiated site after 4 MED UVB was given as a single dose. There may be a wavelength dependence in the local immunosuppressive effect, as exposure to UVA did not alter the induction of CH in any subject.[14] Another study has demonstrated that solar-simulated UV exposure, corresponding to 3 MED, followed by sensitization on the irradiated site, led to suppressed CH responses in 50% of subjects.[15] However, in contrast, Kelly *et al.*[16] found that solar-simulated radiation was highly immunosuppressive in all subjects tested. A single exposure of a small area of skin to 3 MED was followed by sensitization with DNCB on the irradiated site and diphenylcyclopropenone on a distant site, and elicitation with both sensitizers occurred 3 weeks later. The CH response was completely suppressed locally in 100% of individuals and systemically in 83% of individuals. A dose of radiation of this magnitude is of biological relevance as it would be experienced within 1 h at noon in the summer in the U.K.

It is difficult at this time to draw definite conclusions from these rather disparate observations. However, it is clear that, in all studies, at least a proportion of human subjects exhibit suppressed CH if the sensitizer is applied to the irradiated site. The effect is more marked if small quantities of sensitizer are used. UV-induced tolerance and systemic immunosuppression are not consistently

observed, and the contribution of different wavelengths within the UV spectrum is uncertain at present.

Antigen-presenting function

The primary antigen-presenting cells in the epidermis are the Langerhans cells, which form a network of dendritic cells. Following contact with antigen, they internalize and process it, with changes in local cytokine production leading to their migration to the draining lymph node. During migration via the afferent lymph, Langerhans cells mature, as revealed by the altered expression of various adhesion and costimulatory molecules. Once in the paracortical region of the lymph node, they present processed antigenic peptides to specific T cells, which are stimulated to proliferate and produce a particular cytokine profile. It is apparent that disruption of Langerhans cells could be a fundamental component of UV-mediated immunosuppression, either by incorrect, inappropriate or inefficient antigen presentation. Alternatively, depletion or apoptosis of Langerhans cells could lead to antigen presentation by another cell type.

Several studies have examined Langerhans cell numbers and morphology within human skin following UV exposure, two of which have been outlined above.[10,12] Photochemotherapy (PUVA) for skin conditions such as psoriasis altered Langerhans cell morphology after a single exposure, with loss of fine dendritic processes.[17] Repeated exposures led to a progressive reduction in numbers, to 10% after seven treatments. By this time, the remaining cells were elongated, with coarse dendrites. Langerhans cells were detectable in, or under, the basal layer, suggesting migration out of the epidermis, or the preferential survival of cells positioned deeper in the skin. An Australian study showed that UV acts differently on the Langerhans cells of people of different racial origins.[18] Whereas Langerhans cells were depleted by apoptosis in the darker skin of Aboriginal or Asian Australians, cells in those of Celtic descent died due to membrane disruption and organelle damage.[18] Absolute numbers were less drastically reduced in black people, and returned to normal levels more quickly.

The effect of low-dose, long-term UVB (300–500 J/m^2 initially, three times weekly for 4 weeks) on the function of Langerhans cells was investigated in skin blisters/biopsies from healthy volunteers, and showed a marked suppression of the mixed epidermal cell lymphocyte reaction (MECLR).[19] MECLR is used as a measure of immune responsiveness; epidermal cells act as stimulators to induce proliferation of allogeneic peripheral blood responder cells. The reduction in MECLR was not paralleled by changes in Langerhans cell numbers or in HLA class II expression. Furthermore, the Langerhans cells appeared ultrastructurally normal.[19] It is unlikely that low-dose UVB alters these cells at a phenotypic or structural level, but it could alter membrane motility,[20] thereby decreasing the efficiency of antigen presentation, or of antigen internalization and processing.

Further *in vitro* investigations of human Langerhans cell function showed a UVB dose-dependent decrease of T-cell proliferation in the MECLR.[21] Responses by both CD4 + and CD8 + T cells were inhibited by UVB-treated Langerhans cells. Supernatants from irradiated cells did not inhibit the proliferation induced by unirradiated control Langerhans cells, suggesting that immunosuppression is not mediated by soluble factors, as indicated by murine models.[22,23] Addition of interleukin (IL)-1α, IL-1β or IL-2 did not reverse the defective Langerhans cell function.[21] Phenotypic analysis indicated that UVB did not alter levels of expression or the percentage of cells displaying HLA-DR. However, cultured Langerhans cells were less sensitive to UVB exposure than fresh cells. Cultured Langerhans cells express higher levels of the costimulatory molecules intercellular adhesion molecule (ICAM)-1 (CD54), B7 and leucocyte function-associated antigen 3. UVB may therefore exert an immunosuppressive effect by preventing accessory function development, a phenomenon seen with blood dendritic cells.[24] Again, this could be related to cytoskeletal damage[25] and/or decreased mobility of surface antigens.[20]

Epidermal cells obtained from suction blisters of human skin after exposure *in vivo* to varying doses of UV exhibited altered capacities to stimulate T cells. Epidermal cells were pulsed with purified antigen, either immediately or 24 h following UV treatment, and their ability to induce T-cell proliferation assessed. A dose-dependent reduction of activating ability was found,[26] and could be mimicked by pretreatment of epidermal cells to deplete HLA-DR + cells. As Langerhans cells are the only DR + cells resident in normal human epidermis, it is likely that changes in these cells account for these observations. Cell death was limited to DR – keratinocytes, and was not related to the responses obtained.[26]

UV also causes changes in intercellular signalling. *In vitro* stimulation by epidermal cells obtained from both UV-exposed and normal control skin induced a greater than 1000-fold increase in mRNA for the T-cell growth factor, IL-2, in allogeneic CD4 + T cells. However, the

mRNA for the IL-2 receptor α-chain (IL-2Rα, CD25) was upregulated only upon stimulation by normal, unirradiated epidermal cells.[27] At the protein level, CD25 expression on T cells was increased within 48 h of incubation with normal epidermal cells, but was not altered by incubation with irradiated epidermal cells. Treatment with antibodies to transforming growth factor (TGF)-β restored the ability of the UV-exposed epidermal cells to upregulate CD25. Thus, the altered immune response after UV exposure seems to be due in part to TGF-β released by epidermal cells post-UV, initiating an altered early CD4 + T-cell gene expression, which is characterized by the deficient IL-2Rα expression.[27] Furthermore, T cells stimulated by UV-exposed epidermal cells had altered growth factor utilization and cytokine production patterns.[28] T cells cultured with control epidermal cells utilized IL-2, IL-7 and IL-15 for growth, and produced predominantly interferon (IFN)-γ. Those stimulated with UV-exposed epidermal cells used IL-4 for growth, and more cells produced IL-4 and/or IL-5 compared with controls.

Monocytes leave the circulation by binding to the endothelial leucocyte adhesion molecule (ELAM)-1. This is induced on the vascular endothelium in the proximity of a UV-irradiated site, or after injection with purified protein derivative (PPD), which elicits delayed-type hypersensitivity (DTH).[29] ELAM-1 was first detectable at 6 h, and maximally at 24 h, in both cases. Infiltration by macrophages into the treated site had a similar time course. By 3 days, however, ELAM-1 was more strongly expressed in DTH sites than in UV exposed skin. This difference may be due to local cytokine activity. Thus, following UV, ELAM-1 could act by recruiting antigen-presenting macrophages, temporarily replacing the Langerhans cells which had migrated from the skin. Other adhesion molecules induced by intradermal injection of PPD to elicit DTH, such as vascular cell adhesion molecule (VCAM)-1 and ICAM-1, were unaltered in UV-exposed skin.[29]

Investigation of epidermal cell suspensions, derived from skin which had been subjected to erythemogenic UVB each day for 4 days, showed a doubling in numbers of CD36 + macrophages compared with unirradiated controls.[30] This increase correlated with the recovery, and enhancement, of MECLR responses.[30,31] *In situ* immunohistochemistry and flow cytometry of cell suspensions revealed that UV radiation induced not only infiltration of CD36 + monocyte/macrophage cells into the epidermis, but also expansion of the dermal macrophage subset, which is phenotypically identical.[32]

T cells

While aspects of T-cell immunity have been outlined above in the context of hypersensitivity responses, the effects of UV on T-cell numbers are worthy of consideration. Changes in circulating T-cell subsets as a result of exposure are controversial and, indeed, it is possible that such modulations are only transient. Thus, for example, Gilmour *et al.*[33] found no significant changes in the percentages of blood CD3 +, CD4 + or CD8 + cells during and after broadband UVB phototherapy or PUVA. In contrast, exposure of people to the mid-day sun for 1 h/day for 12 days during the spring in Sydney, Australia, led to a small but significant decrease in the number of peripheral T cells.[34] Circulating CD4 + cell numbers declined markedly, while CD8 + cells increased. The reduction in the CD4/CD8 ratio was not completely restored even 2 weeks postexposure. The decrease in peripheral CD4 + T cells might be accounted for by migration into irradiated sites.

Limited studies have examined T-cell subsets in UV-exposed skin. Biopsies showed an initial decrease, or depletion, of intraepidermal T cells within 2 days of a single erythemal UV dose of solar-simulated light.[35] Dermal T cells increased by 24 h and were maximal at 48 h. One week after the irradiation, the migration into the epidermis of CD3 + T cells, expressing CD4 but not CD8, could be detected. These cells were almost exclusively of the memory phenotype (CD45RO +), and lacked expression of the activation markers HLA-DR, VLA-1 and IL-2R.[36] Thus, the cutaneous CD4/CD8 ratio may be increased following UV exposure. The time course of the T-cell infiltration into the epidermis and dermis was UV dose dependent. One explanation for the initial loss of cutaneous T cells may be phototoxicity. It has been shown that T cells are hypersensitive to UV compared with keratinocytes,[37] monocytes[38] and B cells.[39]

Natural killer cells

Natural killer (NK) cells are major histocompatibility complex (MHC)-unrestricted cells which are part of the innate immune system, important in the recognition and lysis of both virally infected cells and tumour cells. They also play a part in the development of Th1-like immune responses via the release of IFN-γ.

UV radiation causes a dose-dependent inhibition of NK cell activity.[40,41] *In vitro* exposure demonstrated that NK activity and lymphocyte proliferation, in response to mitogen, were inhibited to the same extent. Both effects were suppressed maximally at 260 nm, consistent with

nucleic acid acting as the main chromophore,[42] although there is also evidence for the involvement of UCA (see section below). The dose required for 50% inhibition of NK cell activity at 300 nm was only 3 mJ/cm^2, equivalent to about 12 min of mid-day exposure to the sun in a temperate climate during summer. Although these experiments were conducted *in vitro*, which would eliminate the absorptive properties of the skin, it is likely that environmental UV exposure might be sufficient to inhibit NK cell function.[40,42] As UV penetration through skin layers, assessed by thymine dimer formation, shows a very small decline of 2·5% per cell layer at 300 nm,[43] at this wavelength the amount of radiation required to inhibit NK cell activity is within that reaching the dermis and capillaries. In addition, it has been calculated that when the greater proportion of UVA in solar radiation and its greater penetration into the skin are taken into account, UVA may have an equivalent immunosuppressive effect to that of UVB on NK cell activity.[44]

In vivo studies utilizing solarium exposure as a source of UV have concluded that NK cell function is reduced during the course of tanning.[34] This suppression was particularly significant 2 weeks after the last treatment. Circulating NK cell numbers were also decreased, although the correlation between the numbers and function was not absolute. Measurement of NK cell activity after various UV therapies, used for the treatment of psoriasis, showed depression during and 4 weeks after the irradiation course.[45] The precise timing and extent of the downregulation varied between the groups (PUVA, broadband UVB, narrowband UVB) and may be related to dose. No change in circulating NK cell numbers was reported.[45]

UV radiation does not prevent NK cells recognizing and binding to their target cells but acts at the apoptotic or lysis stage. NK cells, exposed to UV *in vitro*, were induced spontaneously to release cytotoxic factors, detectable within 30 min of exposure.[40] In agreement with this is the observation that superoxide dismutase (SOD), an antioxidant which scavenges superoxide anions, reversed the UV-induced suppression in NK cell activity.[45] IL-2 also reversed the suppression and acted synergistically with SOD. Thus, UV may induce NK cells to produce reactive oxygen species, resulting in a less efficient killing of target cells.

In association with a decrease in NK cell activity, a similar decrease in NK-associated cytokines might be expected as a result of UV radiation. As NK cells are a potent, although not the only source of IFN-γ,[46] UV could bias the systemic immune system away from cellular immune responses. Induction of suppressive monocyte/macrophage cells, T cells and their related cytokines, as well as prostaglandin release, could then play a part in exacerbating the downregulation in NK cell activity.

Molecular aspects of ultraviolet radiation

Urocanic acid

UCA is formed by the enzyme histidine ammonia-lyase during keratinization. It is found naturally as the *trans*-isomer within the stratum corneum and is a major absorber of UV in the skin. On UV irradiation, *trans*-UCA isomerizes to *cis*-UCA in a dose-dependent fashion until the stationary state is reached when approximately equal quantities of the two isomers are present. It takes approximately 2 weeks for the *cis*-UCA to return to a baseline level. The isomerization is efficient between 305 and 341 nm (i.e. in both the UVB and UVA wavebands) in Caucasian skin[47] and has a maximum at 290–310 nm.[48] The percentage of *cis*-UCA in suction blister fluid 24 h after 3 MED of either UVB or UVA1 (340–400 nm) is increased over that found in fluid from unirradiated skin.[49] The elevated percentage of *cis*-UCA in suction blister fluid seems to be maintained for several weeks following irradiation.[33] There is a large interindividual variation in UCA concentration but little difference between body sites within one person.[50,51] There is no correlation between the total UCA concentration and skin type, degree of pigmentation, MED or stratum corneum thickness.[50,51] A study of seasonal variation in UCA isomers in human skin showed that the total UCA content was lower in the summer months in all six body sites tested, of which some were normally exposed and others not exposed to sun.[52] The percentage of *cis*-UCA was close to the maximum obtainable during the summer months, except in the buttocks, and it decreased to below 7% in the winter months in all body sites apart from the forehead. A recent study of patients who had been treated for basal cell carcinoma and malignant melanoma concluded that there was no difference in the absolute content of UCA isomers between the skin of the two cancer groups and normal controls.[53] Irradiating these subjects with a single test UV dose resulted in a relatively higher production of *cis*-UCA in both cancer groups compared with the controls. The significance of this change, if any, is not known at present.

UCA was regarded initially as a potential sunscreen but tests to evaluate its sun protection factor (SPF) have

shown that this is not the case. Addition of *trans*-UCA to human skin in amounts about 200 times greater than those occurring naturally afforded a SPF of only 1·58.[54] Rodent experiments have suggested that *cis*-UCA can initiate at least some of the events leading to UV-induced immunosuppression (see reviews[55,56]), and the pioneering work of De Fabo and Noonan showed that the wavelength dependency of suppression of CH by UV has a similar action spectrum to the absorption spectrum of UCA.[4,5] Evidence to corroborate this view in human subjects is obviously not easy. *cis*-UCA has been shown to suppress human NK cell activity in a dose-dependent manner *in vitro*, while *trans*-UCA had little effect.[45] However, no correlation was found between the percentage of *cis*-UCA in the skin and the suppression in NK cell activity induced by narrowband UVB therapy in individuals with psoriasis.[57] If human epidermal cells were treated with *cis*-UCA *in vitro* before testing their activity as stimulator cells in a MECLR, a 20% suppression resulted.[58] The downregulation was increased to 27% by incubation of the epidermal cells with *cis*-UCA for 3–6 days before testing. *trans*-UCA had no such effect. In addition, *cis*-UCA did not alter a mixed lymphocyte reaction if present throughout the culture period.[58] Thus, a partial downregulation in human epidermal antigen-presenting activity could be attributed to *cis*-UCA. A further potential *in vivo* mechanism of action of *cis*-UCA includes the stimulation of prostaglandin E_2 (PGE_2) production, which has been reported *in vitro* from human peripheral blood monocytes[59] and keratinocytes.[60]

DNA damage

As is the case with *cis*-UCA, rodent models suggest that DNA damage is a critical event in UV-induced immunosuppression (see review[61]). Evidence to support this view is limited in human subjects. Hurks *et al.*[62] showed that the *in vitro* action spectrum for the suppression in the MECLR is associated with UV-induced DNA damage. This study was recently extended to investigate the action spectra at four wavelengths for the induction of thymine dimers, which represent the largest subclass of cyclobutyl pyrimidine dimers, and the suppression in the MECLR following *in situ* irradiation of small pieces of human skin.[63] The two action spectra had close similarities with a high sensitivity at 254, 297 and 302 nm, then a steep decline to 312 nm. However, with such large gaps in the wavelengths studied and no information regarding UVA wavelengths, more information is required.

Cytokine induction

UV induces changes in epidermal cytokine profiles, involving both pro-inflammatory and anti-inflammatory cytokines, from a wide range of cell types.

Interleukin-1. Keratinocytes are known to be a rich source of IL-1, which stimulates prostaglandin synthesis by many cell types.[64] IL-1 was shown to be present in the stratum corneum of normal human skin, but not in the basal layer. After UVB exposure, IL-1 appeared in the basal cell layer, and was increased in the more exterior regions.[65] Together with this increase in IL-1, IL-1 receptor antagonist (IL-1ra) was increased markedly following 2–3 MED of UV.[66] IL-1ra is reportedly induced during keratinocyte differentiation, a known effect of UV radiation or sun exposure.[67] The ratio of IL-1ra: IL-1α in sun-exposed skin was over 100, an excess sufficient to block IL-1α-induced biological responses.[66] In un-exposed skin, the ratio was about 8, which was too low to block activity. Therefore, although UV stimulates an increase in IL-1 levels, with the concomitantly greater rise in IL-1ra, UV may actually inhibit the activities of IL-1α and suppress an inflammatory response. The adhesion molecule VCAM-1 is known to be upregulated on endothelial cells by IL-1, but is unaltered by UV exposure.[29]

Tumour necrosis factor-α. Studies in mouse models suggest that susceptibility to UVB-induced immunosuppression is partly controlled by the *Tnf* locus.[68] Tumour necrosis factor (TNF)-α is involved in Langerhans cell migration from the skin,[69] and induction of this cytokine could lead to altered, or diminished, antigen presentation, via Langerhans cell depletion. TNF-α is strongly implicated in the inflammatory response to UVB exposure. Skov *et al.*[49] found that irradiating human skin with 3 MED of UVB led to a rapid increase in TNF-α protein in suction blister fluid, which was maximal at 6 h following exposure. Interestingly, exposure to 3 MED UVA1 resulted in a slight decrease in TNF-α at 6 h.[49]

Conflicting data are reported on the role of UVB-induced TNF-α in the upregulation of E-selectin. Strickland *et al.*[70] found E-selectin expression on dermal blood vessels proximal to the exposed site by 4 h, before TNF-α could be detected. Walsh[71] studied the 'sunburn' response, finding that mast cells degranulated upon exposure to 3 MED, releasing stores of intracellular TNF-α, which in turn led to E-selectin and ICAM-1

expression on cutaneous endothelium by 2 h. Keratinocytes contributed minimally to this effect, which could be blocked with the mast cell inhibitor disodium cromoglycate.[71] TNF-α is further implicated in sunburn cell formation by *in vitro* studies, as antibodies to TNF-α reduce UV-induced apoptosis in human keratinocytes, although not completely.[72]

UVB exposure (200 J/m^2) stimulates a similar quantity of TNF-α from cultured human dermal fibroblasts as does IL-1α. However, the two treatments synergize to give a 30–40-fold increase in TNF-α, mirrored by mRNA expression.[73] As UVB is capable of inducing IL-1α directly, and as incident UVB can penetrate to the dermis, a TNF-α cascade could be initiated in this site.[73] *In vitro* studies using a human keratinocyte cell line showed that blockade of IL-1α activities with monoclonal antibodies also resulted in a partial inhi-bition of TNF-α release, supporting an autocrine role for IL-1α in TNF-α release.[74] Antioxidants have a similar effect in inducing TNF-α secretion.[74] DNA damage is implicated in TNF-α gene expression following UVB exposure.[75] *cis*-UCA, even above physiological levels, could not augment phorbol 12-myristate 13-acetate-induced synthesis of either TNF-α mRNA or protein in keratinocytes.[76] Interestingly, UVB mimics the TNF-α-induced modulation of the p55 TNF surface receptor on keratinocytes, and mRNA expression via a TNF-α-mediated autocrine regulatory pathway.[77]

Interleukin 6. IL-1 is a potent inducer of IL-6, a cytokine which is detectable in serum 1–3 h after 2–4 MED whole-body UV exposure of human subjects.[78,79] IL-6 has a wide range of effects, including fever induction and the synthesis of acute phase proteins. Like IL-1, it is known to be produced by keratinocytes and Langerhans cells. Although initially regarded as a proinflammatory cytokine, recent evidence suggests a significant anti-inflammatory, and even an immunomodulatory, role for IL-6. It can elicit the release of adrenocorticotrophic hormone from the central nervous system, increasing synthesis of glucocorticoids in the adrenal gland.[80] In turn, these suppress IL-1 and TNF-α synthesis. It is also suggested that IL-6 induces the soluble form of the TNF receptor, p55.[80] Elevations in IL-6 correlate well with UV-induced fever courses, and precede increases in acute phase proteins such as C-reactive protein.[79] C-reactive protein induced by IL-6 promotes synthesis of five- to 10-fold greater quantities of IL-1ra compared with IL-1β from peripheral blood mononuclear cells.[80] Antibodies to IL-6 inhibit this in a dose-dependent manner. Irradiation of long-term cultured epidermal cells shows that IL-6 production is attributable to UVB wavelengths, with UVA having no effect.[78] Whether IL-6 production is mediated directly by UVB, and/or via IL-1 or other mediators *in vivo* is unresolved.

Interleukin 10. With the apparent deviation toward a Th2-like cytokine response following UVB exposure, it is natural to hypothesize a central role for IL-10 in UVB-induced immunosuppression. IL-10 exhibits antagonistic effects towards IFN-γ, inducing downregulation of MHC class II expression, and therefore antigen presentation, on macrophages.[81] It suppresses cytokine synthesis when Th1 T-cell clones, or NK cells, are activated by IL-2 and accessory cells in an antigen non-specific manner. Furthermore, while down-regulating inflammatory cytokines,[82] IL-10 can upregulate IL-1ra expression and secretion.[83] IL-10 could thereby play a key part in the production of 'split-tolerance'.

There is strong evidence for this in the mouse following UVB exposure, especially for the participation of keratinocyte-derived IL-10.[23] Human studies present a less clear picture, with some groups finding both keratinocyte-derived IL-10 mRNA and secreted protein,[84,85] some finding mRNA without protein secretion,[86] and others finding neither mRNA[87,88] nor protein.[89,90]

After exposure of small areas of human skin sites to 3 MED UVB or UVA1, Skov *et al.* found that IL-10 protein was slightly increased in suction blister fluid taken 24 h later. This increase was only significant following UVB irradiation.[49] Kang *et al.*[86] argue that IL-10 within UV-exposed human skin is secreted from infiltrating CD11b+ macrophages rather than resident CD11b− keratinocytes. Langerhans cells and UV-induced macrophages respond differently to inflammatory stimuli.[91] The infiltrating macrophages preferentially activate suppressor CD4+ T cells,[91] and secrete high levels of IL-10. UV-induced macrophages could thereby favour development of Th2-like suppressor cells. Although keratinocytes synthesize small amounts of IL-10 mRNA, the protein may remain inside the cells. Teunissen *et al.*[89] performed exhaustive studies on normal human keratinocytes from 40 donors and were unable to detect any IL-10 mRNA or protein following UVB irradiation. Examination of two human cell lines (A341, HaCaT) gave similar results. IL-10 mRNA within melanocytes was detected, without protein secretion. Therefore, the positive findings from other groups[84,85] may be due to melanocyte contamination. Teunissen *et al.*[89] also performed whole body

irradiations (1·5 MED), and were unable to detect alterations in serum IL-10 levels outwith the 24-h biphasic temporal pattern of controls. It is difficult to reconcile this result with a major role for macrophage-derived IL-10 in systemic immunosuppression in human subjects.

Interleukin 12. As IL-12 is regarded as the key cytokine in directing cell-mediated immune responses, via generation of Th1 cells[92,93] and enhancement of NK cell activity,[94] regulation of this cytokine is also central to UV-induced immunosuppression. There is evidence for this in various mouse models.[95,96] IL-12 p35 chain mRNA is expressed constitutively by human keratinocytes, and upon contact sensitization, p40 mRNA is rapidly expressed.[97] IL-12 protein is detectable in concentrated epidermal cell supernatants, and is readily detectable in keratinocyte cell line supernatants following stimulation with phorbol-12,13-dibutyrate.[98] Anti-IL-12 treatment of haptenated human epidermal cells causes a 50% reduction in allogeneic T-cell proliferation assays.[97] UVB irradiation of human peripheral blood monocytes, before using them in antigen-presenting cell-dependent T-cell stimulation assays, resulted in a selective inhibition of antigen presentation to established human Th1 clones.[99] While proliferation and IFN-γ production by Th1 clones was inhibited in a dose-dependent manner, normal proliferative activity and cytokine production were maintained in Th2 clones. Perhaps most interestingly, while the proliferative responses of Th0 clones were normal, there was a preferential suppression of IFN-γ production, skewing cytokine profiles towards those of Th2 clones.[99] These studies indicate the way that resident dermal and/or infiltrating macrophages may behave at irradiated sites.

Interleukin 15. IL-15 has the ability to stimulate growth of both CD4 + and CD8 + T-cell subsets,[100] to generate lymphokine-activated killer cells[101,102] and to act as a chemokine for T cells.[103] Conflicting data have been reported regarding IL-15 after UV exposure. Mohamadzadeh *et al.*[104] found that IL-15 mRNA was constitutively expressed in the human dermis, but was absent in the epidermis. Upon UV exposure, IL-15 mRNA was enhanced in dermal sheets and induced in the epidermis. Expression was traced to HLA-DR – cells, predominantly keratinocytes, but not to HLA-DR + cells, probably Langerhans cells. Immunoblot analysis revealed that IL-15 protein secretion in cultured keratinocytes was enhanced by UVB. In contrast, Blauvelt *et al.*[105] detected constitutive IL-15 mRNA expression in freshly isolated human keratinocytes and Langerhans cells, and this was downregulated by UVB exposure in a dose- and time-dependent manner.

One important difference between the experimental protocols used by these groups is that Mohamadzadeh *et al.*[104] irradiated intact epidermal and dermal sheets, whereas Blauvelt *et al.*[105] irradiated after segregation of cell types. Doses of 200 J/m^2 UVB were found to be toxic *in vitro*, but gave optimal IL-15 mRNA expression *in situ*. This may imply that IL-15 expression is under the control of other intercellular UV-inducible factors found within the skin, but which cannot act in culture. IL-15 mRNA appears to be regulated at post-transcriptional levels,[106] and is not necessarily related to protein secretion. Macrophages can also produce IL-15,[107] and if recruited locally as a result of UV irradiation, could be another source of this cytokine. IL-15 could play an important part in regulating the local cytokine microenvironment at UV-exposed sites, aiding the outgrowth of suppressor T cells.

The sources and various interactions of some of these cytokines following UV irradiation are represented in Figure 1.

Precautionary measures

Sunscreens

As sunscreens prevent classical sunburn, i.e. erythema, it would be anticipated that they would also protect against UV-induced immunosuppression. The easiest way to reduce UCA photoisomerization is to decrease the intensity of incident light, and the rate of *cis*-UCA formation is reduced by topical application of sunscreens.[108] This is limited to broad-spectrum (i.e. filtering out both UVB and UVA), highly protective sunscreens however. *cis*-UCA formation is inversely related to the SPF, with protection against isomerization exceeding the SPF values by a factor of greater than 10. After multiple exposures, the protective effect of classical UVB filters is lost, with only those blocking both UVA and UVB retaining activity against photoisomerization.[108] Thus, sunscreens which prevent sunburn may protect against at least one mechanism (*cis*-UCA) of immunosuppression, with the degree of protection determined by the nature of the sunscreen.

Attempts to develop systems to assess the immune protection factor of sunscreens, in comparison with their SPFs, have resulted in much discussion.[109–112] Davenport *et al.* used breast tissue explants as a source

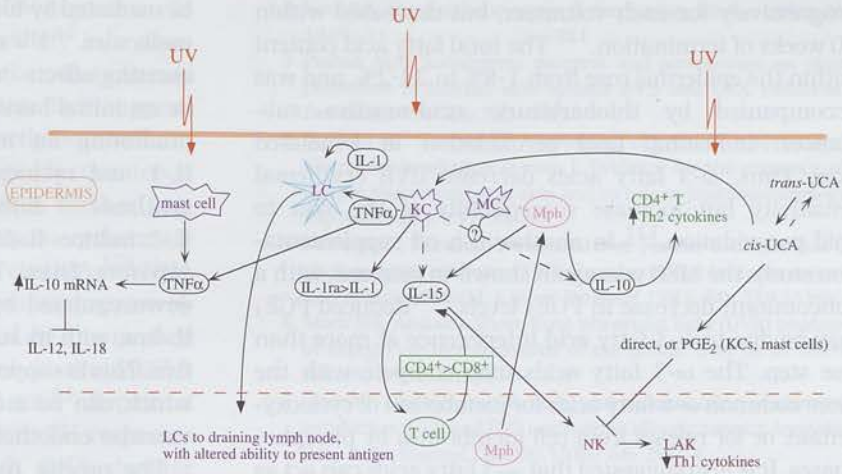

Figure 1. A potential mechanism by which ultraviolet (UV) radiation may alter the cellular and cytokine components of the human epidermis, resulting in immunosuppression. IL, interleukin; TNFα, tumour necrosis factor-α; UCA, urocanic acid; PGE_2, prostaglandin E_2; KC, keratinocyte; LAK, lymphokine-activated cell; LC, Langerhans cell; MC, melanocyte; Mph, monocyte/macrophage; NK, natural killer cell; →, released by/acting upon (– –→, weakly); ⟶, migration; ⊥, blocking; ↑/↓, increased/decreased.

of epidermal cells for MECLR experiments, finding that the reduction in the elicited response was prevented by application of various sunscreens.[109] The degree of protection is debatable, and may not exceed the SPF.[111] Application of a high protection sunscreen (SPF 29) was found to prevent the UVB-induced suppression of both the primary allergic reaction, and CH elicitation, to DNCB.[113] Broad-spectrum sunscreens stopped the immunosuppression of CH to a recall antigen (nickel) when applied before solar-simulated irradiation (suberythemal, once a day for 5 days). There was only a slight protective effect afforded by a narrowband sunscreen.[114]

Application of sunscreens prevented infiltration of CD36+ macrophages into irradiated sites when an erythemogenic, 4-day UVB exposure protocol was used.[30] The influx of these cells is closely correlated with the modulation of MECLR responses. However, when a suberythemogenic, 4-week protocol was employed, suppression of MECLR was not altered by sunscreen application.[30]

Multiple, low-dose exposures to UV in solaria decreased both NK cell function and numbers,[34] a phenomenon which was not prevented by sunscreen application.[115] The sunscreens used in these studies did not block UVA wavelengths. Other immunological properties, such as immunoglobulin production, measured in pokeweed mitogen-stimulated cultures of B and T cells, and DTH, remained depressed.[115] A decrease in the circulating CD4:CD8 ratio, with a rise in the percentage of CD8+ cells, still occurred. As prevention of UV-induced immunosuppression appears dependent on the nature of the sunscreens, caution regarding their use must be exercised. Protection against visible damage such as erythema could encourage longer exposure, unwittingly enhancing damage to the immune system. Behaviour regarding sunscreen application is also vitally important, as studies show they are commonly used in insufficient amounts[116] and volumes below those at which the commercial SPF is calculated.

Diet

Various food components, including flavonoids,[117] green tea polyphenols, silymarin and pyridoxine, can influence the immune system, and when they are either fed or topically applied to irradiated mice, skin cancer incidence,[118,119] pyrimidine dimer formation[120] and immune suppression[121,122] are reduced. Pyridoxine-supplemented feed also reduces the suppression of CH after *cis*-UCA painting in mice.[121]

There is more circumstantial evidence for the influence of dietary components in UV-induced immunosuppression in humans. Melanoma patients have decreased levels of selenium in their sera, compared with healthy individuals.[123] Selenium is important in inducing free radical scavenging systems, and it is postulated that decreased levels increase susceptibility to various cancers.[124] Topical L-selenomethionine application leads to an increased MED.[125] Rafferty *et al.*[126] have shown recently that sodium selenite and selenomethionine supplementation protects human keratinocytes and melanocytes *in vitro* from UVB-induced cell death. It has been hypothesized that an antioxidant-rich diet will protect against UV-induced free radical formation within the skin and subsequent cell membrane damage. During the course of a 3–6 month diet including fish oil supplements, rich in ω-3 fatty acids, the MED rose

progressively for each volunteer, but decreased within 10 weeks of termination.[127] The total fatty acid content within the epidermis rose from 1·8% to 24·2%, and was accompanied by thiobarbituric acid-reactive substances, indicating lipid peroxidation in irradiated skin. Thus, ω-3 fatty acids decrease UVB erythemal sensitivity but increase susceptibility of the skin to lipid peroxidation.[127] In another fish oil supplementation study, the MED was again shown to increase, with a concomitant decrease in PGE_2 levels.[128] Reduced PGE_2 may be due to ω-3 fatty acid interference at more than one step. The ω-3 fatty acids may compete with the more common ω-6 fatty acids for metabolism of cyclooxygenase, or for release from cell membranes by phospholipases. It is also suggested that ω-3 fatty acids can act as an oxidizable buffer, reducing free radical damage to more essential structures.

Another antioxidant, vitamin E, offers partial protection against the UVA-induced decrease in the ability of human Langerhans cells to present antigen.[129] These data imply, but do not confirm, a role for oxidative stress in the immunosuppressive effects of UVA. In one single-diet study, it was found that β-carotene prevented UV-induced suppression of DTH (measured by a Multitest CMI kit, incorporating seven different test antigens) after 10 weeks, compared with that in volunteers fed a placebo diet.[130]

Discussion

UV appears to be able to influence immune responses at multiple levels. By altering the appearance, function and numbers of Langerhans cells, UV can change the cell type by which antigen is presented. ELAM-1 is induced on vascular endothelium after UV,[29] facilitating a monocytic infiltration at the exposed site. Macrophages could subsequently present antigen in a less efficient manner compared with Langerhans cells. In races with darker skin, Langerhans cells are not depleted as markedly, and return to basal levels more quickly,[18] than in Caucasians. Therefore, such individuals may be less immunosuppressed, and this may contribute to the lower incidence of skin cancer in black people. One difficulty with this theory is the role of IL-10. If Teunissen *et al.* are correct in their belief that melanocytes are the main source of IL-10,[89] greater immunosuppressive effects in more melanized skin might be expected.

Long-term, low-dose UVB does not deplete Langerhans cell numbers, but may act at a cytoskeletal level[25] to prevent efficient antigen presentation, by altering processing, phagocytosis or migration. This appears to be mediated by blocking the expression of costimulatory molecules.[24] UV may alter the local cytokine milieu, by exerting effects on various cell types. There appears to be an initial burst of bioactive IL-1 from keratinocytes, promoting an inflammatory response. UVB-induced IL-1 and cyclooxygenase may initiate prostaglandin synthesis.[131] Either UVB directly, or via the action of IL-1, induces IL-6 release and the characteristic post-UV exposure fever. The inflammatory response may be downregulated by UV- or IL-6-induced production of IL-1ra, with its subsequent release rendering IL-1 inactive. This is supported by the observation that VCAM-1, which can be induced by IL-1, is not upregulated on vascular endothelium after UV exposure.[29]

The precise role, and source, of IL-10 in an UV-irradiated site remain to be elucidated. It is conceivable that melanocytes release small amounts upon exposure, and this could bias the site towards a Th2-like response in the event of an immunological challenge. This could also be achieved by prevention of IL-12 upregulation, and could explain the suppression of CH responses. Alternatively, a similar mechanism involving the recently discovered cytokine IL-18[132,133] may have a role. This effect could be enhanced by damage to Langerhans cells. Membrane perturbation of Langerhans cells in pale skin may be an important step in macrophage activation, resulting in further IL-10 secretion. In black skins, apoptosis of Langerhans cells may prevent this enhancement, and could explain why black people are not more immunosuppressed than white people. Also, TNF-α is an inducer of IL-10 mRNA expression,[134] and may be of relevance to infiltrating monocyte/macrophage cells.

Although IL-15 release after UV exposure is another controversial area, it is perhaps more conceivable that IL-15 is induced,[104] rather than downregulated,[105] after UV. IL-15 could then play an important part in attracting T cells into the exposed site, a phenomenon observed over time. Initial decreases in T-cell numbers may be due to phototoxic effects. The delay in recruitment could also be accounted for if macrophages were the major source of IL-15. T-cell recruitment would then be determined by macrophage infiltration kinetics. IL-15 may also be important in the inhibition of NK cells, by promoting their development into lymphokine-activated killer cells. These may quickly become exhausted, with decreased cytotoxic properties. Alternatively, prostaglandin generated by membrane damage may suppress NK cell activity.[135,136] Loss of NK cells would also deprive the immune system of a key source of cytokines which promote cell-mediated immunity.

Isomerization of *trans*-UCA to *cis*-UCA has also been

shown to be an important result of UV exposure. *cis*-UCA can directly suppress NK cell activity in a dose-dependent manner,[45] as well as delay the onset of acute graft-versus-host disease.[137] This latter fact suggests that *cis*-UCA can act directly or indirectly on a cellular source to promote Th2-like cytokine production (or at least inhibit Th1-like cytokines). This is further supported by exacerbation of systemic lupus erythematosus, an antibody-mediated disease, by *cis*-UCA.[138] DNA damage may contribute more to immunosuppression by shorter UVB wavelengths, as indicated by the marked fall in thymine dimer formation, and *in situ* MECLR suppression, between 302 and 312 nm.[63] Repair of pyrimidine dimers does not fully restore CH responses in mice,[139] indicating that although DNA damage appears to be important, it is not wholly responsible for UV-induced immunosuppression. This is supported by the fact that humans have the capacity for rapidly repairing cyclobutyl pyrimidine dimers by a photoreactive enzyme,[140] but can still be immunosuppressed upon UV irradiation. Various studies have shown that blocking of *cis*-UCA or DNA damage prevent UV-induced immunosuppression, and it may be that the two do not act by mutually exclusive mechanisms.

The effects of UV radiation on the human immune system are extremely complicated and highlight the complexity involved in mounting an immune response. There seems to be no single definitive, primary action, but rather a complex series of events initiated simultaneously, or in quick succession, after irradiation. Many of the processes may be redundant, making dissection of events extremely difficult. Attempts to compare mouse and human data are being made, and give some indication of the relative risks of UV radiation.[141,142]

Photoimmunology is a recent addition to the field of immunology, with its relevance to disease and infectious states being poorly understood. While reduction of stratospheric ozone has been relatively well documented and is now being remedied, its potential effects on health are only beginning to be appreciated.[143] Further study is essential to provide guidance regarding 'safe' levels of UV exposure, and to develop more effective protective measures. In addition, innovative vaccination regimens suggest that transcutaneous vaccinations may be possible in the near future,[144,145] making investigations into skin immunology even more important.

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(reviewed in Beissert and Schwarz, 1999). One mechanism may be through effects on LC at the irradiated sites. It is known that UVB irradiation leads to a reduction in LC numbers at the exposed site, with a loss of dendritic processes in the ones which remain (Toews *et al*, 1980; Aberer *et al* , 1981; Lynch *et al*, 1981).

We have demonstrated that UVB irradiation results in the accumulation of DC in the lymph nodes draining the irradiated sites (Moodycliffe *et al*, 1992). TNF-α was found to be a key cytokine in this response as treatment of mice with TNF-α antibodies before irradiation blocked the UVB-induced increase (Moodycliffe *et al*, 1994). A similar accumulation has been reported following contact sensitisation, with inhibition by TNF-α antibodies (Cumberbatch and Kimber, 1995; Cumberbatch *et al*,1997b).

In previous studies we have shown that irradiation of C3H/HeN mice with either UVB or UVA1 can suppress both the CH response, using oxazolone as the skin sensitiser, and the delayed type hypersensitivity (DTH) response, using an infectious model with herpes simplex virus (El-Ghorr and Norval, 1999). However, the doses required to exert such immunomodulatory effects were found to differ markedly. For CH, significant downregulation occurred with a dose of 500 kJ/m^2 UVA1 (equivalent to 1 minimal erythemal dose, MED) or higher, and a dose of 5 kJ/m^2 UVB (equivalent to 3.3 MED) or higher. For DTH, significant downregulation occurred with a dose of 1 kJ/m^2 UVA1 or higher, and a dose of 0.1 kJ/m^2 UVB or higher. These results indicate that different mediators and pathways may be involved in the immunomodulatory cascade, initiated by UV absorbance in the skin, which depend on the waveband of the irradiation as well as on the immune parameter involved. In addition some recent evidence demonstrates that waveband interactions may occurs. For example irradiation with total UVA before UVB partially protected mice from the suppression of CH responses induced by UVB (Reeve *et al*, 1998, Reeve *et al*, 1999).

The aim of the present study was to investigate whether UVA1 irradiation induces a loss of LC from the epidermis of C3H/HeN mice and an accumulation of DC in the lymph nodes draining the exposed skin sites, as has been reported for UVB. Doses of UVA1 and UVB were chosen which are equivalent in terms of the induction of erythema at 1 MED each. The roles of TNF-α and IL-1β in these processes were identified by neutralising experiments using antibodies to each cytokine, injected prior to the UVA1 and UVB irradiation.

MATERIALS AND METHODS

Mice

Female C3H/HeN mice, aged 6-8 weeks, were obtained from the specific pathogen-free animal breeding facility in the Medical Microbiology Transgenic Unit, University of Edinburgh. Mice were age-matched to within one week in individual experiments.

UV sources and irradiation of mice

Two Philips TL-12 lamps with an output range of 270-350 nm, peak 305 nm, emitting 80 mW/cm^2 were used as the broadband UVB source. One MED for the C3H/HeN mice was 5 kJ/m^2. The UVA1 exposure was from a Dr Hoenle Bluelight lamp, emitting 38.58 W/m^2. Wavelengths below 340 nm were filtered out by passage through a 1 cm depth of cation X (provided by Dr F. de Gruijl, University of Utrecht) in distilled water at a concentration of 0.1 mg/ml, contained in a perspex tray. One MED for the C3H/HeN mice was 500 kJ/m^2. The output of both sources was determined using a filtered photodiode meter, calibrated against measurements made with a UV-visible apectroradiometer (model 742, Optronic Laboratories) across the spectral range 250-450 nm, with a tube to target distance of 16 cm. The spectroradiometer was calibrated against tungsten halogen and deuterium standard

sources which are calibrated by the UK National Physics Laboratory (Teddington, UK).

Mice were UVB-irradiated in a perspex box with a maximum of 4 per box. Due to the length of exposure needed for the UVA1, mice were first anaesthesised by intraperitoneal injection of 150 µl sterile distilled water containing 1.67 mg/ml Hyponorm (Janssen Pharmaceutical, Oxford, UK) and 0.83 mg/ml Hyponovel (Roche, Welwyn Garden City, UK). They were then spread out in perspex boxes for irradiation to reduce shielding by littermates. Control mice for the UVA1 experiments were anaesthesised but not irradiated.

Epidermal LC numbers

Mice were exposed to UVB or UVA1 or not irradiated, as detailed above. In experiments examining the neutralising effects of TNF-α and IL-1β antibodies, 20 µg of each antibody (both goat immunoglobulins prepared after immunisation with mouse recombinant proteins, R&D Systems, Abingdon, UK) was injected intraperitoneally in a 100 µl volume 2 h before irradiation. Control mice, both irradiated and unirradiated, were injected with the same volume of normal goat serum at the same time. Mouse ears were collected into phosphate buffered saline at various times after irradiation and split with foceps. Epidermal sheets from the dorsal surface only were prepared and stained for ATPase, using ADP as substrate, as a marker for LC (Chaker *et al*, 1984). Sheets were mounted in 50% glyerol under a glass cover slip and the number of $ATPase^+$ cells counted in 10 fields per sheet (1 field = 0.1 mm^2), with a minimum of 4 sheets per group. Results are shown as the mean ± SEM. Significant differences between the relevant control and the experimental groups were calculated by Student's *t*-test.

DC enrichment and counting

The method outlined in Moodycliffe *et al* (1993) was used. In brief, the auricular lymph nodes were collected from groups of 8-12 mice 24 h after irradiation. In some experiments the mice were pretreated with TNF-α or IL-1β antibodies 2 h before the exposure, as detailed above. The lymph nodes from each group were pooled and single cell suspension prepared by rubbing through a 200-mesh steel gauze (J. Staniar and Co., Manchester, UK). The cells were washed and suspended in 8 ml RPMI-1640 containing 100 I.U./ml penicillin, 200 mg/ml streptomycin, 2 mM L-glutamine, 100 μg/ml gentamicin, 10 μg/ml fungizone, 25 mM HEPES buffer and 10% heat-inactivated foetal calf serum (all Flow Laboratories, Irvine, UK) [RPMI-FCS]. The cell suspension was gently underlaid with 2 ml 14.5% w/v metrizamide (Nygaard, Oslo, Norway) and centrifuged at 600 g for 20 min at room temperature. The DC-enriched population was removed from the interface and washed twice in RPMI-FCS. The pellet was resuspended in RPMI-FCS and the DC counted by morphological analysis in the light microscope. DC were counted in three separate fields and the results shown are the means of these counts.

RESULTS

Irradiation with 1 MED UVA1 does not deplete epidermal LC, whereas UVB causes a dose-dependent depletion

The frequency of epidermal cells was assessed as a function of $ATPase^+$ cells in epidermal sheets prepared from the dorsal ear skin of C3H/HeN mice. Data pooled from 17 independent experiments revealed that the number of LC in untreated (naive) skin was 384 ± 11.5 per mm^2 (mean ± SEM). Twenty-four h after exposure to 1 MED UVA1, there was no change in the number or the morphology of the LC. In contrast, irradiation with 1 MED UVB reduced the LC numbers significantly. The results of 4 individual experiments are shown in Table 1, and demonstrate a mean drop in epidermal LC numbers of approximately 28%. The LC remaining in the epidermis after UVB exposure were rounded and had fewer dendritic processes than in the unirradiated controls. Two smaller doses of UVB were used in comparison with the 1 MED dose. When the LC were counted 24 h later, the numbers fell by 39.4% after 1 MED ($p<0.01$ v. unirradiated control), by 23 % after 0.5 MED ($p<0.05$ v. unirradiated control) and by 7.6% after 0.25 MED (no significant change), demonstrating that LC migration induced by UVB exposure is dose-dependent.

It has been reported that irradiating mice with UVA1 before UVB led to a partial reversal of the immunosuppressive effects of UVB on CH (Reeve *et al*, 1999). To determine whether such a combination altered LC numbers in the epidermis, mice were irradiated with 1 MED UVA1 followed by 1 MED UVB. At 24 h the LC numbers were depleted to levels comparable with those achieved by UVB alone (UVA+UVB 192.5 per mm^2 ± 19.8, UVB 200 ± 15.9, unirradiated control 329 ± 17.6 in one experiment; and UVA+UVB 219 ± 16.5, UVB 256 ± 23.6, unirradiated control 329 ± 18.8 in a second independent experiment).

The timecourse for LC depletion following UVB irradiation

The numbers of LC in the epidermis at various times after exposure to 1 MED UVB were examined and the results are shown in Figure 1a. There was maximal depletion at 12 h, followed by a steady recovery back to the resting levels. The LC were rounded and had fewer dendrites at 12 h, but the morphology recovered to normal as the numbers of LC increased. In a second experiment, a 6 h time point was included and a significant reduction in LC numbers was demonstrated. The fall was 32% at 6 h ($p<0.05$ v. unirradiated) and 45.3% ($p<0.01$ v. unirradiated control) at 12 h.

LC were also monitored between 6 and 72 h after exposure to 1 MED UVA1 but there was no significant reduction at any time (Figure 1b). The morphology of the LC was unaltered throughout with long dendritic processes clearly visible.

Neutralisation of TNF-α and IL-1β prevents LC depletion after UVB irradiation

The available evidence indicates important roles for TNF-α and IL-1β in the induction of LC migration from the epidermis following contact sensitisation (Kimber *et al*, 1998). Therefore experiments were performed to investigate the contribution of these cytokines to the UVB-induced reduction in LC numbers. Neutralising goat immunoglobulins against either murine TNF-α or IL-1β were injected intraperitoneally into mice 2 h prior to UVB irradiation. Control animals were injected with normal goat serum before exposure. The results of 2 independent experiments are shown in Figure 2a for anti-TNF-α, and in Figure 2b for anti-IL-1β. First, the antibodies themselves did not alter LC numbers significantly in unirradiated animals compared with those injected with normal goat serum. Second, the UVB-irradiated group injected with the normal goat serum showed a significant reduction in LC numbers compared with the unirradiated group also injected with the normal goat serum (a decrease of 32% and 41% for the TNF-α experiments in Figure

2a, and 31% and 26% for the IL-1β experiments in Figure 2b). Third, there was no significant reduction in LC numbers in the mice pretreated with the cytokine antibodies before irradiation compared with unirradiated animals pretreated with the same antibody (7.3% and 9% for the TNF-α experiments in Figure 2a, and 2% and 0% for the IL-1β experiments in Figure 2b), and the morphology of the LC was no different from that in the unirradiated animals. On the basis of these results, it is concluded that TNF-α and IL-1β are both critical cytokines in the migration of LC from the epidermis following UVB exposure.

UVA1 and UVB exposure lead to the accumulation of DC in the lymph nodes draining the irradiated sites

We have shown previously that UVB irradiation causes the accumulation of DC in lymph nodes draining the exposed skin site (Moodycliffe *et al*, 1992). Similar experiments were conducted to corroborate this finding and to investigate whether UVA1 also induces such an increase in DC in the draining lymph nodes (DLN). Mice were exposed to 1 MED UVB or UVA1, and the auricular lymph nodes draining the ears collected 24 h later. Control mice were not irradiated. The DC in the lymph nodes were counted following enrichment on a metrizamide gradient and the results of 4 independent experiments are shown in Table 2. The DC numbers per lymph node were increased by both UV regimens by approximately two-fold.

Neutralisation of TNF-α prevents the UVB-induced, but not the UVA1-induced, accumulation of DC in DLN

An important role for TNF-α in DC accumulation induced by UVB exposure had been revealed in a previous study (Moodycliffe *et al*, 1994). The results in Figure 3a for one representative experiment substantiate this finding. There was a 123% increase in DC numbers in UVB-irradiated mice compared with unirradiated

controls, and this was reduced to 36% in animals pretreated with TNF-α antibodies compared with unirradiated controls given the same antibodies.

However, for UVA1 the finding were quite different and are also shown in Figure 3a. There was a 248% increase in DC numbers in UVA1-irradiated mice, and a 236% increase in animals pretreated with TNF-α antibodies compared with unirradiated controls, also given the TNF-α antibodies. Thus, as the TNF-α antibodies did not affect substantially the increase in DC numbers following exposure, a major role for this cytokine in UVA1-induced DC accumulation is not indicated.

Neutralisation of IL-1β prevents both the UVB and the UVA1-induced accumulation of DC in DLN

As Il-1β was shown to have a significant effect on the migration of LC from the skin, a similar approach to that just described for TNF-α was employed but using IL-1β antibodies. The results of such an experiment are shown in Figure 3b. It was found that the IL-1β antibodies, injected 2 h prior to either UVB or UVA1 exposure, substantially inhibited the accumulation of DC in the DLN. The antibody itself reduced the number of DC slightly compared with mice injected with normal goat serum. UVB exposure induced a 103% increase in DC numbers, and UVA1 a 74% increase. In both cases, the IL-1β antibodies prevented this accumulation almost completely and to a similar extent.

DISCUSSION

Under normal circumstances, LC represent the major antigen presenting cells of the epidermis and their numbers and morphology are assessed most frequently by Ia or ATPase staining of epidermal sheets. Both methods give the same results using the C3H/HeN strain of mouse (Aubin *et al*, 1991). One mechanism whereby UV

radiation results in a modulation of immune responses is thought to be by trafficking of a proportion of LC from the epidermis. The ones that remain show abnormal morphology and have a reduced capacity for antigen presentation. Most studies in this area have used broadband UVB sources or solar simulated irradiation, and the contribution of the UVA waveband to this process is not well defined. Limited information indicates that the effect on LC may be wavelength dependent. Noonan *et al* (1984) reported that irradiation at 270 and 290 nm resulted in a decrease in epidermal LC numbers with associated changes in dendrites, but this did not happen at 320 nm. Similarly, using monochromatic light, Obata and Tagmani (1985) found that UVA from 320-400 nm was almost ineffective in reducing LC numbers: wavelengths shorter than 300 nm were the most efficient. Previously we reported that a single dose of 40 kJ/m^2 UVA did not result in a significant reduction in LC numbers 24 h later (El-Ghorr *et al*, 1994). However Aubin *et al* (1991) demonstrated that chronic UVA exposure (10 kJ/m^2, 3 times weekly for 4 weeks) reduced the number of ATPase$^+$ cells in the epidermis of C3H/HeN mice by approximately 45% compared with unirradiated controls.

In the present study UVB was found to reduce epidermal LC numbers, while UVA1 did not (Table 1 and Figure 1). The doses from the two lamps were equivalent in terms of erythema. This parameter was chosen as it has been suggested that the action spectrum for LC depletion from the epidermis is similar to that of the induction of erythema (Obata and Tagmani, 1985). We had shown previously that DTH responses could be suppressed by either of the UV doses used, and CH responses by the UVA1 dose but a 3.3-fold higher dose of UVB was required (El-Ghorr and Norval, 1999). Therefore no correlation is apparent between the migration of LC from the epidermis and the UV-induced modulation of DTH or CH. This has also been indicated elsewhere. For example, Noonan *et al* (1984) found no loss in epidermal LC following exposure at 320 nm, even at doses which caused 50% suppression of CH, and the chronic UVA irradiation protocol used by Aubin *et al*

(1991) which resulted in a considerable loss of LC did not alter CH responses. It is interesting to note that low zone tolerance to contact sensitisers can be mediated without LC migration (Steinbrink *et al*, 1996).

Previously the importance of TNF-α in inducing LC migration from the epidermis was demonstrated (Moodycliffe *et al*, 1994). This result was corroborated in the present study (Figure 2) and, by pretreatment of mice with IL-1β antibodies prior to UVB exposure which restored the LC numbers to the level found in unirradiated controls (Figure 2), a role for IL-1β was also established. These two cytokines are critical in LC movement during the early phase of contact sensitisation (Kimber *et al*, 1998), together with the downregulation of E-cadherin, expressed on the LC surface (Schwarzenberger and Udey, 1996).

The induction of TNF-α may be wavelength dependent. Skov *et al* (1998) found that irradiating human skin with 3 MED UVB led to a rapid increae in TNF-α protein in suction blister fluid, maximal at 6 h after exposure. However UVA1 resulted in a slight decrease at 6 h. Furthermore in mice carrying a chloramphenicol acetyl transferase reporter transgene bearing the entire TNF-α promoter, UVA1 did not induce the expression of TNF-α in skin, in contrast to UVB (de Kossodo *et al*, 1995). DNA damage, occurring at predominantly dipyrimidine sites and including the formation of cyclobutane pyrimidine dimers, may be the initiating factor for TNF-α gene expression following UVB irradiation (Kitibel *et al*, 1998). Other types of DNA damage, mainly oxidative forms, are found at longer wavelengths which may not be appropriate for the induction of TNF-α. Such a situation has been described recently for another UV-induced cytokine, IL-6. The wavelength dependence for IL-6 expression in keratinocytes was found to match the spectra for both DNA absorption and cyclobutane pyrimidine dimer formation (Petit-Frere *et al*, 1998). For example at 365 nm, approximately one-thousandth of the quantity of IL-6 was produced compared with the same dose but at 302 nm.

Although IL-1β is known to be essential for contact sensitisation in the mouse and to cause LC depletion from the epidermis (Cumberbatch *et al*, 1997a), little information is available regarding the wavelength and the timing of its induction by UV. In one study of human subjects, IL-1β was detected at elevated concentrations in suction blister exudates at 15 h after 3 MED solar simulated radiation, an increase which was sustained for at least the next 72 h (Barr *et al*, 1999).

In agreement with previous studies (Moodycliffe *et al*, 1992 and 1994), we detected an accumulation of DC in the lymph nodes draining the irradiated skin site following UVB exposure (Table 2). This was dependent on TNF-α and IL-1β as neutralisation of either cytokine inhibited the increase in numbers (Figure 3). Perhaps surprisingly, given the lack of effect of UVA1 on the epidermal LC numbers, exposure to this waveband also resulted in an accumulation of DC in the DLN (Table 2). A similar role for IL-1β in UVA1- as in UVB-induced accumulation was indicated. In contrast to the situation with UVB, TNF-α did not appear to be involved after UVA1 exposure as TNF-α antibodies failed to alter substantially the increase in DC (Figure 3).

Therefore UVA1 irradiation may not lead to a significant upregulation in TNF-α expression in the epidermis, with the lack of LC migration as a consequence. However, as DC still accumulate in the DLN after UVA1, the source of DC must be other than the LC. It is possible that they originate in the dermis. DC are found in the papillary dermis of normal skin and are considered to be migrating LC, either precursors on their way to the epidermis or mature cells on their way to the DLN. In addition the dermis contains other subsets of DC, with one in the mouse showing a LC-like phenotype (Duraiswamy *et al*, 1994). An inflammatory infiltrate consisting of lymphocytes and macrophages is found around the vessels of the upper and mid-dermis following UVA1 irradiation (Lavker and Kaidbey, 1997), indicating that cellular changes are induced by this waveband in the dermis. TNF-α is neither chemotactic nor chemokinetic for macrophages (Webb *et al*, 1996), so blocking of its

activity is unlikely to alter this response. The same may be true for the dermal DC moving from the skin to the DLN, in response to UVA1. In addition DC from different sites may express different adhesion molecules and the ones on dermal DC may render the cells susceptible to IL-1β-induced, but not to TNF-α-induced migration.

In summary, we have shown that 1 MED UVB irradiation causes the migration of epidermal LC and the accumulation of DC in lymph nodes draining the exposed skin site. TNF-α and IL-1β are mediators at both of these stages. One MED UVA1 does not cause the migration of epidermal LC but DC still accumulate in the DLN, an effect which involves IL-1β but not TNF-α.

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LEGENDS FOR FIGURES

Figure 1. The timecourse for ATPase$^+$ cells in murine epidermis following irradiation with 1 MED UVB (a) or 1 MED UVA1 (b). Four epidermal sheets from the ears of each group were prepared and stained for ATPase. Ten fields were counted per individual sheet. Results are shown as the mean ± SEM for each timepoint, with 0 h representing the number in unirradiated animals. *=$p<0.05$ and **=$p<0.01$ v. unirradiated.

Figure 2. The roles of TNF-α and IL-1β in UVB-mediated LC depletion from the epidermis. Mice were injected with normal goat serum (NGS) or TNF-α antibodies (a) or IL-1β antibodies (b) 2 h before 1 MED UVB irradiation. Four epidermal sheet from the ears of each group were prepared and stained for ATPase activity. **=$p<0.01$ v. unirradiated, §=$p<0.05$ and §§=$p<0.01$ v. the same treatment with the cytokine antibody.

Figure 3. The roles of TNF-α (a) and IL-1β (b) in DC accumulation in lymph nodes following 1 MED UVB or UVA1 irradiation. Mice were injected with normal goat serum (NGS) or TNF-α antibodies (a) or IL-1β antibodies (b) 2 h before irradiating. Auricular lymph nodes were collected 24 h later, and the DC counted following enrichment.

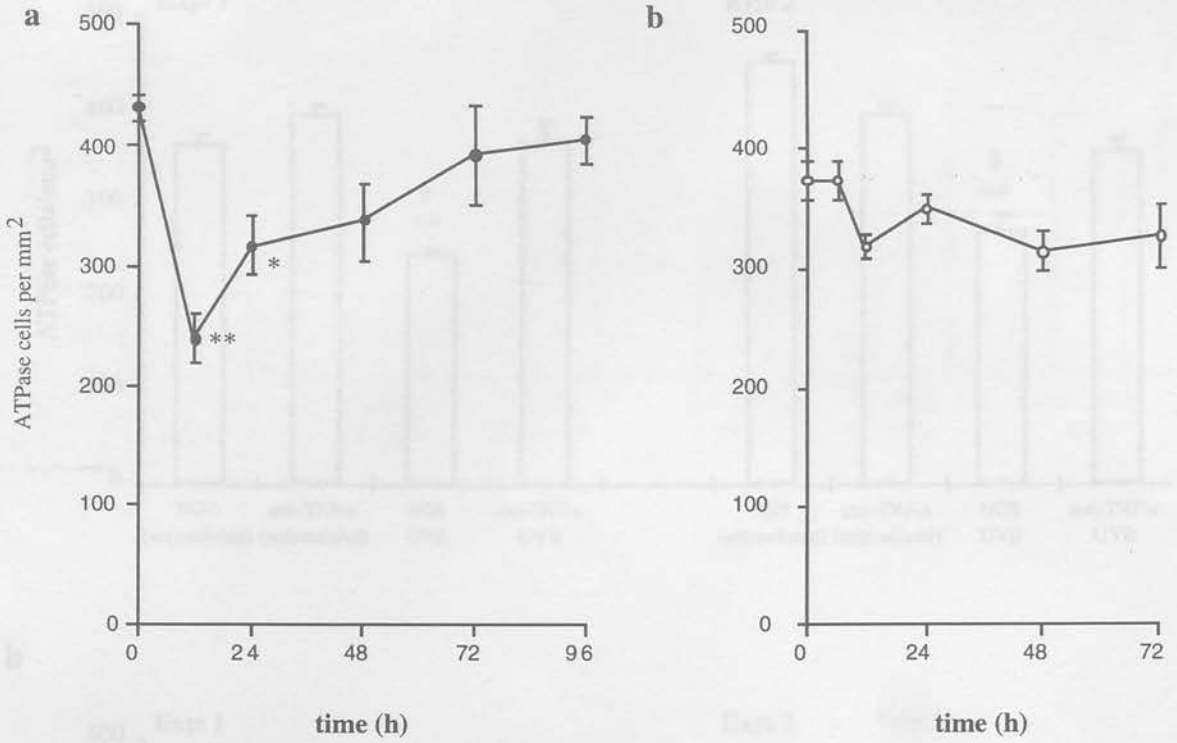
Experiment	unirradiated	UVB	% change	UVA	% change
1	374 ± 16.6	263 ± 8.7	-29.7 (*)	351 ± 11.6	-6.1
2	329 ± 17.6	200 ± 15.9	-39.2 (*)	301 ± 21.4	-8.5
3	329 ± 18.8	256 ± 23.6	-22.2 (*)	355 ± 53.3	+7.9
4	430 ± 9.9	346 ± 4.3	-19.5 (*)	432 ± 19.5	+0.5

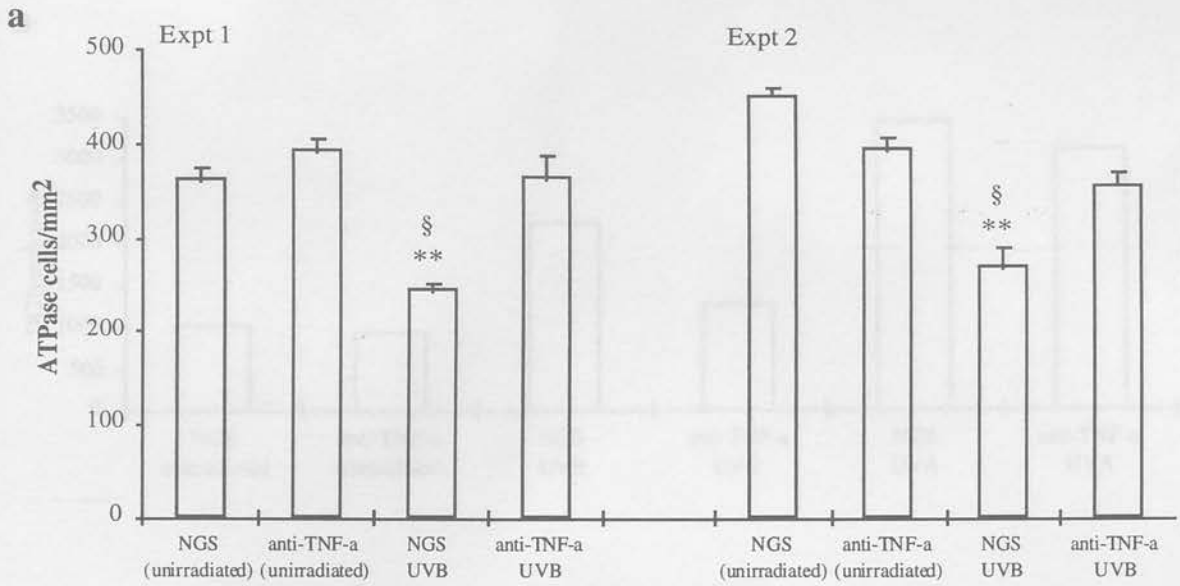
Table 1. UVB induces a reduction in LC, but UVA1 does not. Mice were either unirradiated, or received either 1 MED UVB or 1 MED UVA1. Ears were collected and stained for ATPase activity 24h later. In individual experiments, 4 epidermal sheets were prepared each group. Ten fields were counted per individual sheet. Results from 4 individual experiments are shown as mean ± sem, and percentage change from control values.

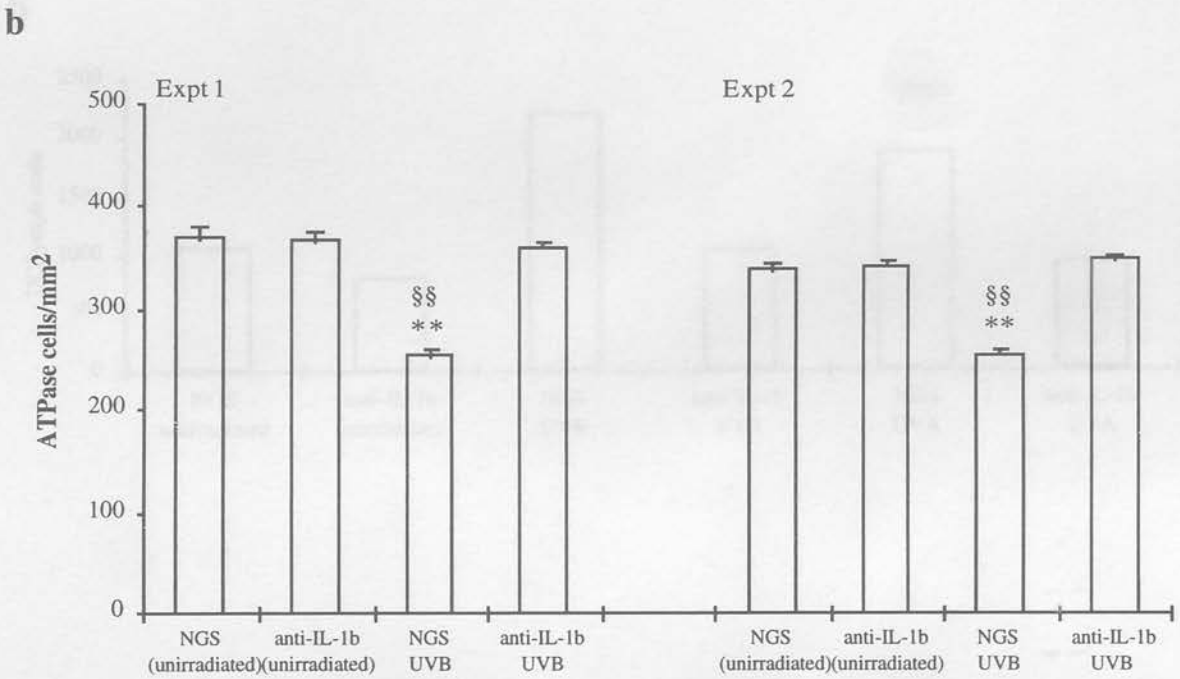
*= $p < 0.05$, vs. control.

Experiment	DC/resting LN	UVB	% change	UVA1	% change
1	1067	2186	+105	1857	+74
2	997	2136	+114	1929	+93
3	992	2214	+123	3450	+248
4	1923	4149	+116	3606	+88

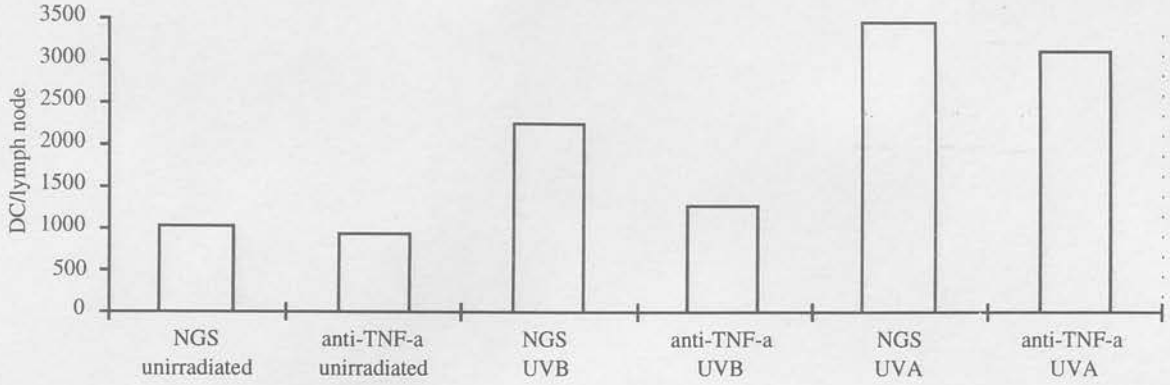
Table 2. UVA1 and UVB irradiation induce an accumulation of DC in DLN. Mice were given a 1 MED exposure of either UVB or UVA1, and DLN collected 24h later. Control mice were unirradiated. DC were enriched on a 14.5% metrizamide gradient before counting. Results from several individual experiments are shown as the number of DC per DLN, and the percentage change from their respective control (unirradiated) mice, within individual experiments.


a
b
500
400
300
200
100
0
ATPase cells per mm 2
**
*
0
24
48
72
96
time (h)

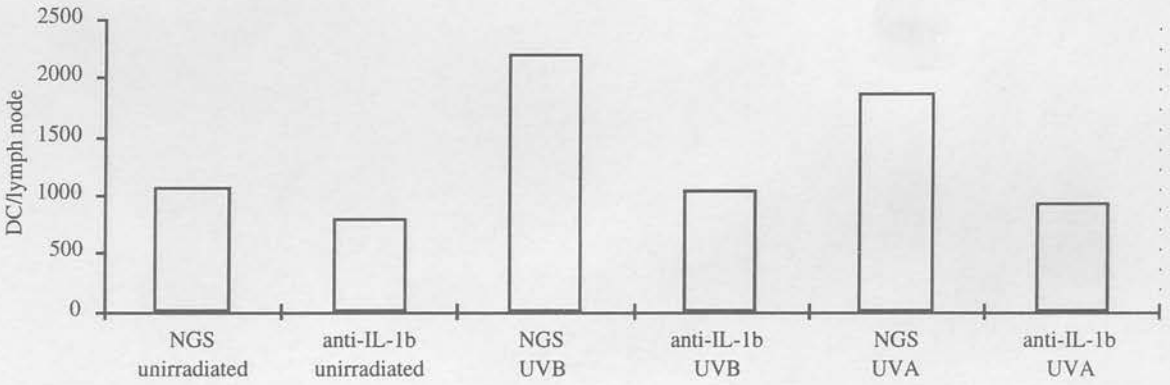

a
Expt 1
Expt 2
ATPase cells/mm2
500
400
300
200
100
0
§
**
§
**
NGS (unirradiated)
anti-TNF-a (unirradiated)
NGS UVB
anti-TNF-a UVB
NGS (unirradiated)
anti-TNF-a (unirradiated)
NGS UVB
anti-TNF-a UVB


b
Expt 1
Expt 2
ATPase cells/mm2
500
400
300
200
100
0
§§
**
§§
**
NGS (unirradiated)
anti-IL-1b (unirradiated)
NGS UVB
anti-IL-1b UVB
NGS (unirradiated)
anti-IL-1b (unirradiated)
NGS UVB
anti-IL-1b UVB

a


DC/lymph node
3500
3000
2500
2000
1500
1000
500
0
NGS unirradiated
anti-TNF-a unirradiated
NGS UVB
anti-TNF-a UVB
NGS UVA
anti-TNF-a UVA

b


DC/lymph node
2500
2000
1500
1000
500
0
NGS unirradiated
anti-IL-1b unirradiated
NGS UVB
anti-IL-1b UVB
NGS UVA
anti-IL-1b UVA