MECHANISM OF IMMUNOSUPPRESSION INDUCED BY ULTRAVIOLET - B LIGHT IRRADIATION

Thesis submitted for the Degree of Doctor of Philosophy

by

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This thesis is dedicated :

To the memory of a beautiful piscean who drowned in the sea of life trying to save a soul, exstinguishing the flame that burn't so brightly, forever

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To two very special friends, James and Margaret, whose constant support and encouragement made the completion of this thesis possible.

An opening speech by Louis Pasteur as professor at Lille on December 7, 1854 invoking the spirit of Benjamin Franklin:

Without theory, practice is but routine born of habit. Theory alone can bring forth and develop the spirit of invention. It is you especially who are obliged not to share the opinion of those narrow minds who reject everything in Science which has no immediate application. You know Franklin's charming saying? He was witnessing the first application of a purely scientific discovery and people around him asked, " But what is its use?" Franklin answered them, "what is the use of a newborn babe?" Yes, gentlemen, what is the use of a newborn child? And yet, perhaps, at that tender age, germs already existed in you of the talents which distinguish you. Among your baby boys, fragile things that they are, there are incipient magistrates, scientists, heroes as valient as those who are now covering themselves with glory under the walls of Sebastopol. And thus, gentlemen, a theoretical discovery has but the merits of its existence: it awakens hope and that is all. But let it be cultivated, let it grow and you will see what it will become.

DECLARATION

I declare that the studies presented in this Thesis are the result of my own independent investigation, with the exception of the fluorescent flow cytometric analysis and HPLC analysis which were carried out with the assistance of Mr W Neill and Mr T Reid.

This work has not been, and is not being currently submitted for candidature for any other degree.

Angus M Moodycliffe

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ABSTRACT

Irradiation with ultraviolet-B light (UV-B) suppresses some cell-mediated immune responses to a variety of antigens, including contact sensitizers. Following UV irradiation there is a modulation of Langerhans cell markers, and keratinocytes are induced to synthesize and secrete tumour necrosis factor-alpha (TNF-α). It has been postulated that there is a photoreceptor in the skin which mediates the effects of UV-B radiation on the immune system. One candidate is urocanic acid (UCA) found naturally in the stratum corneum of the epidermis as the trans-isomer, which converts to the cis-isomer on irradiation. Cis-UCA has been demonstrated to suppress immune responses in several experimental systems.

The mechanism of UV-B induced suppression of contact hypersensitivity (CH) responses and the role of cis-UCA were examined using a murine model of CH. UV-B irradiation was demonstrated to suppress the induction of CH responses whilst cis-UCA had little, if any, effect. Next, the migration of dendritic cells (DC) to draining lymph nodes (DLN) following UV-B irradiation or epicutaneous application of UCA isomers was examined in unsensitized mice and mice sensitized with FITC. It was found that UV-B irradiation alone induced DC migration to DLN with a maximum number of DC being present in DLN 48hr following irradiation. In addition UV-B irradiation followed by skin sensitization at the same site enhanced DC migration. In sensitized mice, the percentage of DC bearing FITC and the quantity of FITC per DC were unaltered by prior UV exposure. Further, the percentage of DC expressing Ia or ICAM-1 molecules and the amount of Ia or ICAM-1 expressed per DC was unaffected by UV-B irradiation. In contrast to these results with UV-B irradiation, neither isomer of UCA had any significant effect on DC numbers in DLN in sensitized or unsensitized mice.

The UV-B source used in the above studies emitted light over the range 270-350 nm. Another source was tried which emitted a narrow band only (311-

312 nm) and isomerized trans to cis-UCA efficiently. It was revealed that narrow band irradiation had no effect on DC accumulation in DLN or in suppressing the induction of the CH response. This confirms that UV-B induced DC migration does not occur as a result of the local production of cis-UCA and that cis-UCA is not sufficient by itself to induce suppression of the CH response. Administration of dexamethasone (a transcriptional inhibitor of TNFα) or TNFα antibodies to mice prior to UV-B irradiation decreased DC accumulation in DLN suggesting that UV-B induced DC migration to DLN is stimulated by TNFα release. If mice were treated with TNFα antibodies prior to UV-B exposure, UV-B induced suppression of the CH response to oxazolone was significantly inhibited. Thus UV-B induced suppression of CH is mediated by intracutaneous release of TNFα.

High performance liquid chromatography (HPLC) proved successful in quantifying UCA isomers in normal and UV-B irradiated skin extracts of several mouse strains. The analysis of other mouse tissues by HPLC was not possible because of the complexity of substances present and the fact that UCA represents a major UV absorbing constituent only in the skin. Subsequently, a murine monoclonal antibody to cis-UCA was prepared and tested by ELISA using UCA isomers conjugated to protein as antigens. The interaction of the antibody with structural analogues of UCA was assessed by competitive inhibition ELISA which indicated that the antibody had a high specificity for cis-UCA. Screening of mouse sera at various times after UV-B irradiation of mice by competitive inhibition ELISA using the monoclonal antibody showed that cis-UCA was present, probably in an unbound form, for at least 2 days after the exposure. Thus, cis-UCA produced in the epidermis following UV-B irradiation reached the serum a few hours later where it may gain access to other areas of the body such as DLN, spleen or unirradiated skin.

ABBREVIATIONS USED IN TEXT

SALT	Skin Associated Lymphoid Tissue
SIS	Skin Immune System
TNFα	Tumour Necrosis Factor Alpha
TNFβ	Tumour Necrosis Factor Beta
UCA	Urocanic Acid
UV-A	Ultraviolet A Light
UV-B	Ultraviolet B Light
UV-C	Ultraviolet C Light
CH	Contact Hypersensitivity
DC	Dendritic Cell
DLN	Draining Lymph Node
HPLC	High Performance Liquid Chromatography
MHC	Major Histocompatibility Complex
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon Gamma
DEC	Dendritic Epidermal Cell
LFA-1	Lymphocyte Function-Associated Antigen 1
LFA-3	Lymphocyte Function-Associated Antigen 3
IL	Interleukin
CSF	Colony Stimulating Factor
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
LC	Langerhans Cell
DNCB	Dinitrochlorobenzene
DNFB	Dinitrofluorobenzene
TNCB	Picryl chloride
FITC	Fluorescein isothiocyanate

oxazolone	4-ethoxymethylene-2-phenyloxazol-5-one
HSV	herpes simplex virus
DTH	delayed type hypersensitivity
TNP	trinitrophenol
DNP_6OVA	dinitrophenylated-ovalbumin
s.c.	subcutaneous
i.p.	intraperitoneal
i.v.	intravenous
i.d.	intradermal
m.wt.	molecular weight
CSIF	cytokine synthesis inhibitory factor
PBS	phosphate buffered saline
h r	hours
BSA	bovine serum albumin
KLH	keyhole limpet haemocyanin

CHAPTER 1

INTRODUCTION

1.1 The Skin as a Lymphoid Organ

The skin is the main interface between the internal milieu of the organism and the environment. Consequently, it is subject to a constant barrage of antigenic challenges from pathogenic micro-organisms and environmental chemicals, that represent a broad spectrum of antigenic specificities. The skin and the immune system collaborate to provide a cutaneous defence mechanism against this continuous onslaught of antigens. The principal physical function of the skin is that of a relatively impenetrable physicochemical barrier over the entire surface of the body, keeping most, but not all, invading pathogens and noxious agents at bay. As a second line of defence, the immune system recognizes and responds in a highly specific manner to pathogens and chemical agents that penetrate this barrier.

It has been well documented that the skin has the capacity to function as an appropriate microenvironment for the efferent arm of the immune response. Indeed, many of the classical demonstrations of both cell and antibody mediated immune reactions have used the skin as an immunological substrate. Studies suggest that the skin is more than just an immunologically permissive microenvironment, but can be considered a lymphoid organ in its own right, containing a characteristic architecture of lymphoid and nonlymphoid elements capable of complex interactions. This lymphoid organ, termed the skin-associated lymphoid tissue (SALT) [Streilein, 1983 and 1990], is a unique organization comprising of 1) keratinocytes which create a microenvironment that is favourable to antigen uptake and its recognition by lymphocytes; 2) distinctive populations or recirculating T lymphocytes that display a natural proclivity for migrating to skin; 3) a specialised set of antigen-presenting

cells within the epidermis called Langerhans cells (LC) which are responsible for processing and presenting antigens to immunocompetent lymphocytes in situ or in the draining lymph nodes (DLN); and 4) a set of peripheral DLN, integrating this multicellular system, that contain, along with the dermis, blood vessels with endothelial cells whose surfaces capture lymphocytes passing through the blood. The complementary functional properties of each of these cell types and their cooperative interactions provide the skin with immune protection.

Over the past eight years it has become apparent, at least in mice, that another constituent of SALT is a relatively sessile, dendritic epidermal cell that bears Thy-1 alloantigen (Thy-1+ DEC) but is Ia negative. The potential role that these cells might play in SALT is a subject of intense interest and experimentation, and at present a single specific in vivo function for these cells has not been agreed upon.

Since several other immunologically relevant cells populate epidermal and dermal tissues: mast cells, tissue macrophages (histiocytes), granulocytes and indeterminant cells [Bos and Kapsenberg, 1986; Kapsenberg et al, 1990; Van Loveren et al, 1990], Bos and Kapsenberg (1986) proposed that all these cells including the cells comprising SALT, but excluding skin-draining lymph nodes, form an intricate and complex system called the "skin immune system" (SIS) (Figure 1). With the introduction of this term, all elements i.e. both innate and adaptive (aquired) subsystems, contributing to immunological defence within the skin, are described under one heading.

1.1.1 Keratinocytes

Keratinocytes which constitute the growing epidermal component of the skin are enclosed in an aqueous microenvironment bounded most distally by the stratum corneum and proximally by the basement membrane of the dermal epidermal junction. Although, keratinocytes are the major cell types within this

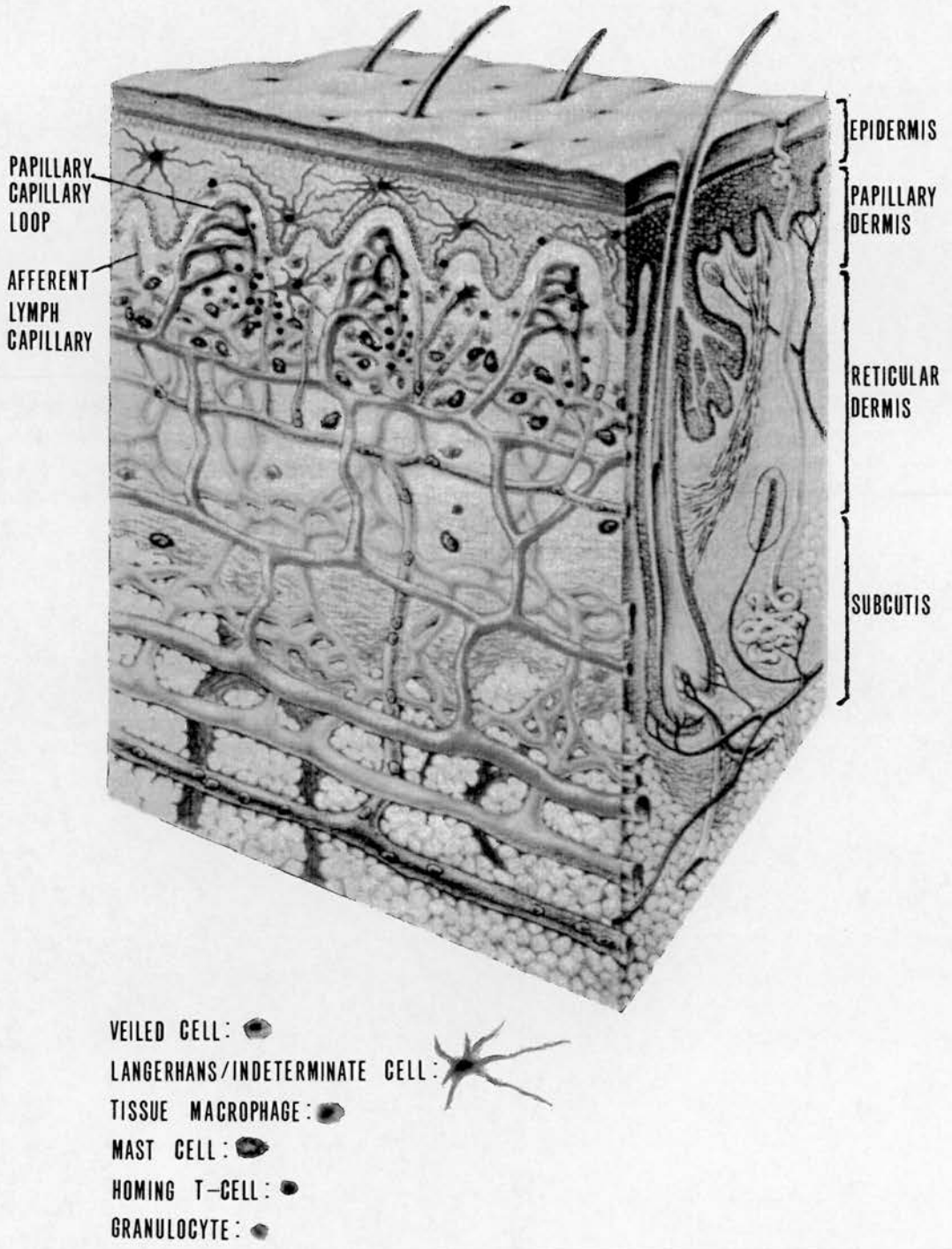

Figure 1 Schematic representation of type and distribution of skin immune system cells and their blood and lymphatic drainage systems depicted in the frontal projection; cells and structures not primarily involved in skin immune responses are drawn in the lateral projection.

space, several different bone-marrow derived cells also occupy it: LC, lymphocytes and in mouse skin a population of Thy-1+ DEC. A number of important findings indicate that keratinocytes may have a significant and extensive influence on the contents and properties of this fluid microenvironment. The first was that keratinocytes could be induced to express major histocompatibility complex (MHC) class II molecules and molecules important in facilitating T lymphocyte/keratinocyte interactions: intercellular adhesion molecule 1 (ICAM-1) and OKM5 antigen. This observation has generated a great deal of interest in the possible role of the keratinocyte as an accessory cell in antigen-specific T cell responses. Another major finding was that keratinocytes, under appropriate circumstances secrete a diverse array of cytokines that could augment this possible accessory cell function as well as influencing intraepidermal immune responses.

1.1.1A Expression of MHC Class II and Adhesion Molecules

Since the first report by Lampert et al (1981) that keratinocytes in human graft-vs-host disease expressed MHC class II molecules, further examination of other skin diseases revealed that this expression was related to the presence of a lymphoid infiltrate within the skin [Lampert, 1984]. It is well known that gamma interferon (IFN-γ), a product of activated T cells can induce the expression of MHC class II molecules on cells that do not normally express this antigen such as endothelial cells and fibroblasts [Geppert and Lipsky, 1985], implying that this cytokine may be the agent responsible for the expression of MHC class II molecules by keratinocytes. Indeed, it has now been demonstrated that IFN-γ is able to induce the expression of MHC class II molecules by human and murine keratinocytes [Basham et al, 1985; Aiba and Tagami, 1987].

In addition to inducing the expression of MHC class II molecules on keratinocytes, IFN-γ has been shown to induce two other antigens on the surface of keratinocytes: OKM5 and ICAM-1. The OKM5 antigen is expressed on a number of

other cells including endothelial cells, monocytes, platelets and accessory cells [Bamwell et al, 1985; Cooper et al, 1986], where it is involved in cell adhesion. The expression of this antigen by keratinocytes may suggest a role for these cells in trapping T cells or other leukocytes within the epidermis. Normal keratinocytes express no or very low levels of ICAM-1, but following stimulation with inflammatory cytokines such as IFN-γ and tumour necrosis factor alpha (TNFα) expression is increased to easily recognized levels [Dustin et al, 1988]. The raised level of expression was found to be associated with an increase in the adhesion of T lymphoblasts to monolayers of cultured keratinocytes, presumably due to binding of lymphocyte function-associated antigen (LFA-1) on the lymphocyte to its ligand ICAM-1 on the keratinocyte. Further, Krutmann and his colleagues (1990) have shown recently that tumour necrosis factor beta (TNFβ) can up-regulate ICAM-1 cell surface expression on keratinocytes [Krutmann et al, 1990]. It is suggested that the expression of ICAM-1 on keratinocytes by inflammatory cytokines such as IFN-γ may play an important role in the trafficking of T cells through the epidermis during inflammatory responses [Nickoloff, 1989; Lewis et al, 1989].

1.1.1B Cytokine Production by Keratinocytes

Keratinocytes exhibit the capacity to secrete a large number of cytokines: interleukin (IL)-1α, IL-6, IL-8, IL-10 colony stimulating factors (CSF) such as granulocyte/macrophage CSF (GM-CSF), TNFα and platelet-derived growth factor [Luger and Schwarz, 1990; Enk and Katz, 1992]. The multifunctional nature of these cytokines enables them on their own, or in concert, to carry out numerous functions, which initiate, up-regulate and amplify intraepidermal immune and inflammatory responses. These functions include inducing and increasing the expression of MHC class I and II molecules and ICAM-1 molecules on various cell types (e.g. endothelial cells); acting as a chemoattractant for T lymphocytes, neutrophils, monocytes and macrophages; activating T lymphocytes, neutrophils and

macrophages; inducing haematopoiesis; inducing the production of other cytokines from a variety of cells and enhancing the proliferation of antigen-specific T cells [McKenzie and Sauder, 1990; Luger and Schwarz, 1990]. As well as initiating, up-regulating and enhancing the immune response by providing immunostimulatory mediators, keratinocytes can also down-regulate ongoing inflammatory or immunological reactions by secreting suppressive mediators which include: prostaglandins [Rola-Pleszczynski, 1985], urocanic acid [De Fabo and Noonan, 1983], contra IL-1 [Schwarz et al, 1987] and a number of, as yet, structurally undefined inhibitory molecules. The conditions under which suppressive factors are released by keratinocytes and their possible role in suppressing certain immune responses are discussed in detail later in this chapter.

The dermal-epidermal junction is a semipermeable membrane which permits the bidirectional translocation of molecules into dermis and epidermis. Subsequently, factors secreted by keratinocytes can cross the dermal-epidermal junction and influence inflammatory or immunological reactions taking place locally within the epidermis and/or dermis or systemically at other sites in the body.

1.1.1C Functional Activities of Keratinocytes

Keratinocytes constitutively secrete IL-1 [Luger and Schwarz, 1990] and can be induced to express MHC class II molecules as well as molecules that facilitate T cell/keratinocyte interactions: ICAM-1, OKM5 antigen. Thus, these cells possess three of the major signals necessary for accessory cell function in presenting antigen to T cells. However, despite attempts to establish whether or not keratinocytes can present antigen [Breathnach et al, 1986; Tjernlund and Scheynius, 1987; Cunningham and Noble, 1989], it is not clear if these cells can perform this function, mainly because unpurified epidermal cell populations were used in the studies.

It is known that keratinocytes are phagocytic [Luger et al, 1983], more so than epidermal LC. Whether the effect of this property of keratinocytes would be to increase or decrease the possibility that antigens within this compartment would be successfully presented to T cells is unknown at present. However, based on the findings that LC phagocytose very poorly, the concept that keratinocytes may function to take up particulate antigen, modify/partially degrade it and release it into the epidermal microenvironment enabling LC to then take up the fragments and process them for presentation is an attractive one.

1.1.2 Skin-Seeking T Lymphocytes

Normal human [Bos et al, 1987] and mouse [Barker and Billingham, 1972] skin harbour lymphocytes, an overwhelming majority, if not exclusively of T cell type only. Studies examining the immunophenotypes of T cells in normal skin [Bos et al, 1987] revealed that the majority of intracutaneous T cells are located within the dermis where they are clustered around post-capillary venules and are approximately evenly distributed over CD4+, CDW29+ (4B4+) memory and CD8+ suppressor/cytotoxic T cell subsets. On the other hand, CD4+, CD45R+ (2H4+) naive T cells were found to be relatively rare in the skin, although these cells form approximately 50% of peripheral blood CD4+ T cells. Intraepidermal lymphocytes were mostly of the CD8+ suppressor-cytotoxic T cell subset, accounting for less than 2% of the total number of lymphocytes present in normal skin. Studies examining the immunophenotype of T cells in diseased skin such as contact dermatitis [Bos et al, 1989] also revealed that intra-cutaneous T cells of the CD4+ subset were almost exclusively of the memory T cell subpopulation; CD4+, CD45R+ (2H4+) naive T cells were rare. Further, intraepidermal cells in diseased skin were found to be mostly of the CD8+ suppressor-cytotoxic T cell subset. These results indicate that T cells in normal and diseased skin, as far as the CD4+ T cell subset is concerned, have already met antigen in association with MHC class II molecules, as

they have mainly a memory immunophenotype. It is not known whether these T cells have immigrated into the skin in an activated state or whether they have been locally activated or reactivated.

The concept that the T cell component of the skin represents a distinct subpopulation of skin-seeking lymphocytes is supported by studies suggesting that the skin may be a distinct regional lymphocyte homing specificity. For example several investigators have shown experimentally preferential lymphoid migration to skin [Issekutz et al, 1980; Issekutz et al, 1986] and certain skin-localizing T cell malignancies have been demonstrated clinically in patients [Abel, 1985; Miller et al, 1980]. More direct evidence for the existence of a skin-specific lymphocyte phenotype has recently been provided by Picker and his colleagues [Picker et al, 1990]. These investigators reported that cutaneous T cells differ from their counterparts in noncutaneous sites by the expression of an epitope recognized by the monoclonal antibody HECA-452. Interestingly, the existence of a distinct compartmentalized gut associated lymphoid tissue (GALT) is supported by the identification of cell-surface antigens in both humans and rats that are preferentially expressed on intestinal lymphocytes [Cerf-Bensussan et al, 1987; Cerf-Bensussan et al, 1986] and by studies showing that certain gut-derived lymphoid populations are GALT specific in their homing/recirculation properties [Butcher et al, 1980; Jalkanen et al, 1989; Schmitz et al, 1988]. Thus, the report by Picker and co-workers [Picker et al, 1990] of the existence of a skin-specific lymphocyte phenotype provides significant new evidence supporting the hypothesis that SALT is an immunologically unique lymphoid organ.

1.1.3 Langerhans Cells: Dendritic Cells of the Skin

1.1.3A Dendritic Cells

Dendritic cells (DC) were originally described by Steinman and Cohn (1973), as 'a novel cell type in peripheral lymphoid organs of mice', with a

morphology that resemble macrophages but with a characteristic dendritic appearance. Like macrophages, DC are bone marrow derived, but may be regarded as a separate cell line, reacting with DC-specific antibodies [Nussenzweig et al, 1982; Kraal et al, 1986]. Further, DC constitutively express MHC class II molecules [Steinman and Nussenzweig, 1980] in contrast to macrophages where this is an inducible trait [Unanue and Allan, 1987] and are specialized to initiate primary T cell responses [Steinman and Inaba, 1989]. Also, unlike macrophages, DC are only weakly phagocytic [Steinman and Nussenzweig, 1980].

Since their original description [Steinman and Cohn, 1973] DC have been isolated from spleens and lymph nodes of mice, rats and humans [Tew et al, 1982; Steinman et al, 1979; Klinkert et al, 1982; Van Voorhis et al, 1982]. They have also been observed in the medullary area of the thymus [Van Ewijk, 1984]. Apart from their presence within primary and secondary lymphoid organs, DC are present in various other sites in the body. They can be isolated from peripheral blood (circulating DC) [Van Voorhis et al, 1982; Knight et al, 1986] and from afferent lymph (veiled cells) [Knight, 1984]. DC in low numbers have been identified in the interstitial connective tissues of all organs, including the portal area of the liver, kidney, heart, gut and respiratory tract but not the brain [Hart and Fabre, 1981]. Most strikingly, DC are abundantly present in stratified squamous epithelia as LC.

1.1.3B Langerhans Cells

Large numbers of LC form a regular and almost closed network of dendrites within the basal and suprabasal layers of the epidermis. LC are bone marrow-derived [Katz et al, 1979; Frelinger et al, 1979], have a low buoyant density [Teunissen et al, 1988] and a low phagocytic activity [Streilein and Bergstresser, 1984]. It has long been regarded that LC form a reticulo-epithelial trap for antigen encountered at skin surfaces, and that they transport it, via afferent lymphatics to DLN [Shelly and Juhlin, 1976; Silberberg-Sinakin et al, 1976].

Evidence supporting and confirming this hypothesis will be discussed later in this chapter. It is proposed that LC that leave the skin are replaced from the blood by circulating LC precursors (the identity of which remains unknown) [Bos and Kapsenberg, 1986]. Alternatively or in addition, epidermal LC that have left the skin may be replaced by division from an intra-epidermal LC pool [Miyauchi and Hashimoto, 1987; Czerneielewski and Demarchez, 1987]. An array of molecules are displayed on the surface of LC, some of which help to establish the cell's identity and some of which have defined functional properties: class II MHC molecules, Fc and C3b receptors, CD1 (T6), T200 (Ly5), ICAM-1 (very low levels), LFA-3, CD4 (on human LC), IL-2 receptor (on murine LC), F4/80 and membrane ATPase activity [Bos and Kapsenberg, 1986; Teunissen et al, 1990; Cumberbatch et al, 1992]. Apart from the membrane molecules which are associated with antigen presenting function, the function of the other molecules is less clear; the role of CD1 (T6), and CD4 and IL-2 receptor expressed on human and murine LC, respectively remains particularly enigmatic. It has recently been suggested, based on the structural relationships between CD1 antigens and MHC class I molecules, that CD1 antigens are important in the transport and release, at the LC surface, of soluble self peptides which may interact with Thy-1+ DEC [Hanau et al, 1990]. LC contain within their cytoplasm organelles called Birbeck granules. Although the precise function of these organelles is unknown, available evidence suggests that they are morphological expressions of endocytosis [Hanau et al, 1987A and B] and are critical for antigen processing or presentation [Stossel et al, 1990; Hanau et al,1987B; Bucana et al, 1992].

LC are considered the principal antigen-presenting cells of the skin [Stingl et al, 1989]. Recently, investigators have shown LC to undergo important phenotypic and functional changes in culture, transforming them into potent immunostimulatory DC [Schuler and Steinman, 1985; Romani et al, 1989A; Romani et al, 1990; Romani and Schuler, 1989; Shimada et al, 1987]. It appears that this

functional maturation is effected by GM-CSF and IL-1 [Witmer-Pack et al, 1987; Heufler et al, 1988], products of keratinocytes [Luger and Schwarz, 1990]. Freshly isolated LC can process native protein antigens efficiently for presentation to antigen specific T cell clones but are weak in stimulating resting T cells. Conversely, LC cultured for a total of 48-72hr lose the capacity to process antigen but acquire potent stimulatory capacity for resting T cells [Romani et al, 1989B; Streilein and Grammer, 1989; Witmer-Pack et al, 1987; Schuler and Steinman, 1985]. Based on these observations it is viewed that fresh and cultured LC are in vitro representatives of their in vivo counterparts: intraepidermal LC and LC that have migrated to DLN, respectively. With this view in mind it is suggested that intraepidermal LC are especially programmed for efficient antigen processing and can readily present antigen to primed or memory T cells but during migration to the DLN this facility is 'exchanged' for potent immunostimulatory activity (achieved in part, from the acquisition of accessory molecules) and the ability to present antigen to unprimed T cells [Romani et al, 1989B; Streilein and Grammer, 1989; Cumberbatch et al, 1991A and B; Cumberbatch et al, 1992].

1.1.4 Specialized Endothelial Cells Responsible for Directing Lymphocyte Migration into Extravascular Tissue

The vascular endothelium which consists of a monolayer of squamous cells lining the whole circulatory system forms an anatomical barrier between the blood and extravascular tissues of the body. Consequently, a mechanism must exist that permits skin-seeking lymphocytes to selectively escape from the vascular tree into the dermis/epidermis (and DLN). Lymphocytes have been seen to cluster around the post-capillary venules that occupy the papillary and reticular dermis in normal skin and (in amplified numbers) in inflammed skin [Bos and Kapsenberg, 1986]. It is believed that the cellular and molecular mechanism(s) of lymphocyte emigration from the blood into the skin is promoted by the endothelial cells that line these small

vessels [Streilein, 1990]. A hypothetical model for the mechanism of selective lymphocyte emigration from blood into extravascular tissue has been proposed [Butcher et al, 1982]. This model suggests that lymphocytes possess on their surface specific adhesion molecules (referred to as homing receptors) which recognize and interact with appropriate ligand molecules expressed on the surface of endothelial cells that line post-capillary venules. A brief summary of the experimental data available describing these cell surface molecules and implicating their role in the selective migration of lymphocytes from the vascular compartment into extravascular tissue is given below.

A number of monoclonal antibodies have been produced which can block lymphocyte binding to peripheral lymph node post-capillary venules in vitro and lymphocyte homing to peripheral lymph nodes in vivo, by recognizing a glycoprotein antigen of between 80 to 90 KDaltons expressed on the surface of these cells [Gallatin et al, 1983; Rasmussen et al, 1985; Jalkanen et al, 1986]. Another set of antibodies has also been identified which block lymphocyte binding to Peyer's patch post-capillary venules, but not peripheral lymph node post-capillary venules [Jalkanen et al, 1986; Chin et al, 1986]. An additional cell surface molecule on lymphocytes which has been shown to be important in binding to endothelial cells of the lymph node and skin is LFA-1 [Hamann et al, 1988; Haskard et al, 1987]. It has been reported that if cultured human endothelial cells either from umbilical cords or the microvasculature of the skin are pretreated with a variety of inflammatory mediators such as IFN-γ, IL-1 and/or TNF, leukocyte adherence was greatly increased [Yu et al, 1985; Bevilacqua et al, 1985; Gamble et al, 1985]. Pre-incubating lymphocytes with anti-LFA-1, however, resulted in a substantial (but not total) inhibition of lymphocyte binding to both non-treated or inflammatory mediator-treated cultured endothelial cells [Mentzer et al, 1986; Haskard et al, 1986; Haskard et al, 1987]. The observation that LFA-1 is important in the

binding of lymphocytes to endothelial cells in lymph nodes and skin, suggest that this molecule is not tissue specific.

Endothelial cells are functionally very diverse [Pasyk and Cherry, 1990] being able to respond to a variety of inflammatory mediators by expressing a multitude of cell surface molecules believed to be important in lymphocyte trafficking. Before focussing on the cell surface molecules on endothelial cells, it is worth mentioning that some of the endothelial cell surface antigens represent specific receptor ligand-like molecules for known lymphocyte molecules, whereas other endothelial cell antigens are less well characterized with respect to the identification of the complementary recognition molecule on the lymphocyte. ICAM-1 which is expressed on vascular endothelial cells, macrophages, lymphocytes and thymus and mucosal epithelial cells [Dustin et al, 1986] is a ligand for LFA-1 [Marlin and Springer, 1987]; so lymphocytes expressing LFA-1 are able to bind to endothelial cells via the ICAM-1 molecule. In an analogous fashion to the previously discussed results in which anti-LFA-1 antibodies on lymphocytes block binding to endothelial cells, antibody to ICAM-1 on endothelial cells blocks this adherence [Nickoloff, 1990]. Further, it has been demonstrated that IFN-γ and TNF enhance the expression of ICAM-1 on cultured endothelial cells from normal skin after 24hr [Griffiths et al, 1989]. Also, markedly enhanced vascular endothelial cell expression of ICAM-1 in several skin diseases has been reported [Griffiths et al, 1989] substantiating the in vitro data and supporting the notion that the expression of ICAM-1 molecules by endothelial cells is important in the trafficking of lymphocytes into skin. In contrast to the expression of ICAM-1 molecules on the surface of vascular endothelial cells, a number of tissue-specific endothelial cell molecules which are involved in lymphocyte homing have been identified [Butcher et al, 1980; Streeter et al, 1988].

1.1.5 Thy-1+ Dendritic Epidermal Cells

It has been recognized for ten years that the epidermis of all strains of normal mice is populated by a resident, bone-marrow-derived population of DC phenotypically distinct from epidermal LC [Bergstresser et al, 1983; Tschachler et al, 1983; Romani et al, 1985]. These cells express Thy-1, CD3 in association with a gamma/delta T cell receptor, asialo GM-1, but not CD4, CD8 or class II MHC molecules, and are referred to as Thy-1+ dendritic epidermal cells (DEC) [Tigelaar et al, 1990; Streilein, 1990; Koning et al, 1987]. Whether the T cell receptor on Thy-1 + DEC is used for recognizing antigen by itself or in association with MHC molecules is unknown. However, preliminary evidence suggests that at least some of the Thy-1+ DEC appear to recognize class I MHC molecules [Janeway et al, 1988]. It has been reported that the human epidermis harbours a very low number of a similar subset of gamma/delta T cells [Groh et al, 1988].

In an attempt to explain the biological function of Thy-1 + DEC it has been proposed that these cells are dedicated to providing immunosurveillance for the "epithelia" [Tigelaar et al, 1990; Janeway et al, 1988; Streilein, 1990] which may be accomplished by cytotoxic destruction of foreign/altered target cells [Nixon-Fulton et al, 1988]. On the other hand, the demonstration that administration of hapten-derivatized Thy-1 + DEC to naive mice induces a state of specific immunological unresponsiveness [Sullivan et al, 1986] suggests that Thy-1 + DEC may have an important role to play in regulating immune responses (which will be discussed in more detail later in this chapter). Despite these suggestions not a single, specific in vivo function for Thy-1 + DEC has been agreed upon.

1.2 Immunological Mechanism in Contact Hypersensitivity

J. Jadassohn is reported to be the first investigator to describe the phenomenon of skin sensitization [cited by Polak, 1980]. He observed in 1895 that certain inflammations caused by contact of the skin with chemicals were not due to the irritating properties of these substances but to a specific increase in skin sensitivity, acquired by repeated contact with the same substance. Since that time a number of important advances have been made in our understanding of the biology of contact hypersensitivity (CH), a cutaneous delayed hypersensitivity reaction. Progress has been facilitated by the development of suitable animal models of CH and by a greater comprehension of the molecular and cellular events which initiate and regulate immune responses in general.

CH is a biphasic phenomenon comprising an induction or afferent phase during which sensitization is initiated, and an elicitation or efferent phase when, following subsequent exposure to the same chemical, the sensitized animal exhibits a cutaneous hypersensitivity reaction. A variety of immunoregulatory mechanisms serve to modulate or contain both the induction and elicitation phases of the CH response.

1.2.1 The Induction Phase

The inductive phase may be further subdivided into five stages:

a) the preparatory phase (formation of the conjugate)

b) the antigen uptake phase

c) the antigen recognition phase

d) the proliferation and differentiation phase

e) The propagation phase consisting of the release of effector and memory cells into the circulation and the spread of sensitivity over the entire body.

1.2.1A The Preparatory Phase

When applied to the skin, haptens, such as dinitrochlorobenzene (DNCB), picryl chloride (TNCB) and oxazolone penetrate quickly through the stratum corneum (the outermost layer of the epidermis) entering the epidermal compartment that contains extracellular and cell-surface epidermal proteins [Nishioka, 1985; Streilein, 1990]. Haptens that result in CH commonly exhibit three features: chemical reactivity, lipid solubility and low molecular weight [Bergstresser, 1984]. Chemical reactivity is required because most haptens covalently bind to larger molecules, most importantly to structural and cell surface proteins creating many novel hapten-protein conjugates [Bergstresser, 1989]. This chemical binding of haptens to larger molecules has been given a variety of interchangeable terms, such as derivatization, haptenation and conjugation, which describe the process by which relatively small reactive haptens bind to formerly non-immunogenic structures, converting them to immunogenic ones. Protein-hapten conjugates produced in this way become the materials against which the resulting hypersentivity response is directed. This process is referred to as the preparatory phase.

1.2.1B Uptake of Antigen by Epidermal Antigen Presenting Cells

A distinctive feature of the CH response is that the processing and presentation of antigen (hapten-derivatized epidermal protein) in association with class II MHC molecules is believed to be performed by LC. A considerable body of evidence has been gathered to support the hypothesis that LC are important antigen-presenting cells for the induction of CH responses. Numerous investigators have reported that the efficiency of sensitization is impaired, or actively suppressed, when hapten is applied to areas of skin naturally poor in, or depleted of, LC [Toews et al, 1980; Streilein et al, 1980B; Semma and Sagami, 1981; Rheins and Nordlund, 1986; Halliday and Muller, 1987]. However, there is evidence that LC are not an

absolute prerequisite for the initiation of CH. Studies in the guinea pig [Baker et al, 1985] and mouse [Streilein, 1989] suggest that contact sensitization may proceed in the absence of epidermal LC, implying that a second pathway of cutaneous antigen presentation may exist. In support of this view is the recent report by Tse and Cooper (1990) that Ia^+ DC, located in the perivascular region of the mouse dermis, when derivatized with hapten can induce contact sensitivity in naive mice.

1.2.1C Antigen Recognition Step (Transmission of Antigenic Information to T-Lymphocytes)

It is now very clear that contact sensitization is dependent upon the activation and clonal expansion of hapten-reactive T lymphocytes [Parrott and De Sousa, 1966; Davies et al, 1969; De Sousa and Parrott, 1969; Pritchard and Micklem, 1972]. However, a great deal of controversy has centred on the actual site of primary stimulation (sensitization) of T cells i.e. the site of transmission of antigenic information to T-lymphocytes. Although a historical hypothesis has proposed "peripheral sensitization" [Medawar, 1965], the majority of evidence suggests that these events are initiated in the lymph nodes draining the site of sensitization [Frey and Wenk, 1957; Parker and Turk, 1970; Asherson and Mayhew, 1976; McFarlin and Balfour, 1973]. However, for the activation and expansion of reactive T lymphocytes, antigen must reach the DLN in an appropriate form. It has been thought for some time that the transport of antigen by LC from the skin to the DLN represents the major route of cutaneous sensitization [Silberberg-Sinakin et al, 1976]. In support of this is the observation that topical exposure of mice to DNFB caused a temporary but marked, depletion of identifiable LC from the epidermis [Bergstresser et al, 1980]. In recent years it has become clear that within hours of contact sensitization there is an accumulation of DC in the lymph nodes draining the site of application [Knight et al, 1985A; Macatonia et al, 1986; Knight et al, 1985B; Macatonia et al, 1987; Kinnaird et al, 1989] and that a

significant proportion of these cells bear high levels of antigen [Macatonia et al, 1986; Macatonia et al, 1987; Kinnaird et al, 1989]. That these antigen-bearing DC in DLN derive from epidermal LC which travel from the skin via afferent lymphatics has been corroborated by studies of athymic mice bearing skin allografts [Kripke et al, 1990]. It was found that the Ia^+ antigen-bearing DC which accumulated in the DLN following contact sensitization at the site of the allograft were of graft donor origin. There is now strong evidence suggesting that the stimulus for the migration of LC to DLN is TNFα [Cumberbatch and Kimber, 1992].

1.2.1D Proliferation and Differentiation Phase

Considerable evidence suggests that the antigen-bearing DC which accumulate in DLN play an important or decisive role in the induction of contact sensitization. They have been demonstrated to be potent stimulators of both primary and secondary T lymphocyte proliferative responses in vitro [Jones et al, 1989; Robinson, 1989; Knight et al, 1985A; Macatonia et al, 1986; Macatonia et al, 1987] and small numbers will efficiently induce contact sensitization in naive animals [Knight et al, 1985A; Knight et al, 1985B; Kinnaird et al, 1989; Macatonia and Knight, 1989]. Further, it has been reported that a correlation exists between the number of DC which arrive in the DLN within 24 hr of skin sensitization and the vigour of the primary lymphocyte proliferative response [Kimber et al, 1990A].

It is thought that the antigen-laden DC migrating into DLN come to rest in the paracortical area of the lymph node where they are referred to as interdigitating cells [Teunissen et al, 1990]. Within the paracortical area, those T cells that specifically bind to the antigen being presented (in association with MHC class II molecules) by interdigitating DC, are activated. It is thought that these initiator T lymphocytes upon activation, proliferate and release interleukins which recruit another subset of T cells (T_{DH}) to the lymph node, which upon presentation of

antigen become activated and proliferate and differentiate into effector T cells of CH [Nishioka, 1985].

In order for DC to activate T cells, a direct interaction between these two cell types is mandatory [Inaba et al, 1989]. It has been proposed that DC have a reversible "antigen-independent" mechanism for surveying T cells [Inaba and Steinman, 1987; Austyn and Morris, 1988; Austyn et al, 1988; Inaba et al, 1989]. This provides time for antigen-MHC class II complexes on the surface of the DC to align with specific receptors on the T cell. If complementarity occurs the T cell is retained for subsequent activation but if not, the T cell leaves and new clones have an opportunity to bind.

1.2.1E Propagation Phase

This step involves systemic sensitization. The effector T cells of CH after being produced in the lymph nodes, re-enter the circulation (via the efferent lymphatics) and join the circulating T cell pool. As well as recirculating through the lymphoid organs, these recently activated T_{DH} cells enter peripheral tissues, particularly the skin [Andersen et al, 1987]. The mechanism by which these cells enter the skin has been previously discussed in section 1.1.4.

It is worth mentioning that keratinocytes may have an important role to play in the induction phase of CH. It has previously been mentioned (Section 1.1.1C) that keratinocytes are phagocytic, much more so than LC. Thus, any antigenic particles that gain access to the epidermal compartment will be taken up by keratinocytes and perhaps degraded. Consequently, the possibility arises that the uptake of antigenic particles by keratinocytes might actually interfere with, rather than promote, processing and presentation of antigen by LC.

1.2.2 The Elicitation Phase

The elicitation phase of CH occurs following challenge of previously sensitized animals, with a peak response approximately 24hr after exposure and is characterized by an infiltration of mononuclear cells and in some instances basophils [Dvorak and Mihm, 1972]. The sequence of events thought to take place are as follows. Upon challenge, haptens penetrate into the epidermis to form the same hapten-carrier protein complexes that are produced in the induction phase. LC present in the epidermis take up these antigen complexes and present them to effector T_{DH} cells of CH which escape into the dermis from the blood vasculature (as described in section 1.1.4) and migrate into the epidermis. Upon presentation these T cells are activated and release a variety of lymphokines inducing an amplified cellular infiltrate and local inflammation. These lymphokines which include IL-2, chemotactic factor and IFN-γ, have a variety of stimulatory effects on other lymphocytes, mononuclear phagocytes and vasculature. These effects include causing activated T lymphocytes to proliferate and release mediators; increasing the expression of MHC class II molecules on the surface of LC; augmentation of the cytotoxic activity of T cells; recruitment of mononuclear phagocytes to the site; induction of mitosis of lymphocytes to increase their numbers; reduction of the migratory activity of macrophages to increase their metabolic activity to process antigens and toxic substances; dilation of blood vessels and increase in their permeability [Goh, 1988]. The T lymphocytes contained within the infiltrate are CD4+ and CD8+, usually with the former predominating [Wood et al, 1986] and comprise both specific and non-specific elements [Scheper et al, 1985].

The local response is amplified further as a consequence of the capacity of IFN-γ to activate keratinocytes in three important ways: 1) to initiate the expression of MHC class II molecules; 2) by inducing keratinocytes to release several cytokines that modulate T cell activity; and 3) by inducing keratinocytes to express cell adhesion molecules such as ICAM-1. It is thought that the expression of

MHC class II molecules on keratinocytes may amplify the in situ potential for antigen presentation, thereby activating additional T cells. T cell activation at the local site could be further accelerated as a result of the release of certain cytokines by keratinocytes and the expression of adhesion molecules may play a role in directing T cell migration from dermis into epidermis [Streilein, 1990].

There is considerable interest in the role of mast cells and vasoactive amines in the elicitation phase of CH. It is postulated that an early event takes place following challenge involving the degranulation of mast cells and the release of vasoactive amines such as histamine and serotonin, which induce vasodilation and the creation of gaps between adjacent endothelial cells, facilitating the entry of effector T lymphocytes which mediate the classical delayed hypersensitivity reaction [Van Loveren and Askenase, 1984]. This view is supported by the observation that challenge reactions in mice comprise both an early (1-2hr) and a late (24-48hr) phase of tissue swelling [MacKenzie et al, 1981; Van Loveren et al, 1983] and that the elicitation of contact sensitivity may be more effective in areas of skin rich in mast cells [Gershon et al, 1975]. Further, reserpine, an agent that depletes mast cell serotonin, markedly impairs the elicitation of sensitization [Askenase et al, 1980; Back and Groth, 1983]. It has been suggested that for mast cells to participate in challenge reactions, the sequential action of two independent T lymphocyte populations is required [Van Loveren and Askenase, 1984; Van Loveren et al, 1984]. There is evidence that the early component of the elicitation reaction is mediated by a T lymphocyte-derived antigen-binding factor that 'sensitizes' tissue mast cells and possibly other vasoactive amine-containing cells [Meade et al, 1988; Herzog et al, 1989A]. Following challenge it is thought that antigen binds to such sensitized cells, initiating degranulation and allowing subsequent infiltration of the second population of T lymphocytes which cause the late-phase reaction [Meade et al, 1988; Van Loveren et al, 1986]. However, the role of mast cells and vasoactive amines in delayed-type hypersensitivity remains controversial. For instance, mice

genetically deficient in mast cells display 24hr challenge reactions comparable with normal animals [Galli and Hammel, 1984; Mekori et al, 1987]. Also, reserpine has been demonstrated to virtually abolish the expression of contact reactions in mast cell-deficient mice [Galli and Hammel, 1984; Askenase et al, 1983]. It is thought that this is as a consequence of a direct effect of reserpine on T lymphocyte function [Mekori et al, 1987; Mekori et al, 1985]. Thus, the role of mast cells and vasoactive amines in the elicitation phase of CH remains unresolved.

1.2.3 Immunoregulation

A great deal of work has been done to investigate the immunoregulation of CH [Asherson et al, 1980; Claman et al, 1980A; Claman et al, 1980B], much of which has involved deliberately perturbing the immune system to provoke the appearance of immunoregulatory mechanisms [Dieli et al, 1987; Taborski et al, 1986; Miller et al, 1977; Miller et al, 1978; Asherson et al, 1977]. Such studies have revealed that active regulation of CH can be achieved by suppressor cells which influence the induction stage of sensitization (afferent-acting suppressor cells) [Moorhead, 1976; Thomas et al, 1979; Dieli et al, 1987], by a complex network of interacting suppressor cells (and molecules) which inhibit the elicitation reaction (efferent-acting suppressor cells) [Miller et al, 1978; Asherson et al, 1984] or by clonal inhibition [Miller et al, 1977]. In recent years, the description of contrasuppressor cells which modify suppression in an antigen-specific manner distinct from T cell help [Ptak et al, 1984; Green and Ptak, 1986] has added further to the complexity of cellular interactions in induced immunoregulatory processes.

Although studies which have involved inducing immunoregulatory mechanisms have advanced our understanding of the various cellular and molecular mechanisms which may serve to modulate the immune system, they have provided little information about the regulatory events which actually influence the development and expression of CH following topical sensitization. On the other hand, indirect evidence for the appearance of regulatory mechanisms following

epicutaneous exposure derives from experiments in which cyclophosphamide, given prior to sensitization, has been shown to augment CH reactions, presumably through selective impairment or elimination of suppressor cells or their precursors [Maguire and Ettore, 1967; Polak and Rinck, 1977; Parker and Turk, 1982]. These studies suggest that, following topical sensitization, there may be both inducing or promoting signals and inhibitory (suppressive) effects with the balance between them influencing the severity and duration of reactions and longevity of sensitization. There is some evidence that this balance may be tipped in favour of the appearance of cyclophosphamide-sensitive immunosuppression depending on the concentration of skin-sensitizing chemical administered [Sy et al, 1977; Asherson et al, 1979]. Polak (1980) suggested that contact sensitizers which appear to be relatively weak sensitizers are such because of their ability to induce more active suppression and not because of any lack of inherent immunogenicity.

1.3 Ultraviolet Radiation

1.3.1 Natural Source

Ultraviolet (UV) radiation which is emitted by the sun comprises of electromagnetic radiation of wavelengths ranging from 200-400nm. Like other forms of electromagnetic radiation, UV is delivered in photons (wave/particle 'packets of energy'); the energy of a photon depends on the wavelength of radiation, shorter wavelengths being more energetic. UV radiation is divided into three subregions termed UV-A, UV-B and UV-C. The amount of UV radiation in sunlight that reaches the earth's surface is directly dependent on its wavelength. Of the three UV bands, UV-C (200-280nm) is most efficient in the induction of erythema, but the penetration of its short wavelengths to the earth's surface is blocked by nitrous oxide in the thin upper atmosphere and by oxygen and ozone at lower altitudes of the stratosphere (15-30km). On the other hand, solar radiation in the UV-B (280-320nm) and UV-A (320-400nm) region are present in terrestrial sunlight. UV-B radiation is often referred to as the sunburn spectrum of solar radiation because it is many more times efficient in inducing erythema than UV-A.

It is now established that there is a seasonal hole in the stratospheric ozone layer over the Antarctic [Farman et al, 1985] and that ozone depletion may be occuring outside this geographic boundary [Proffitt et al, 1989] as well as within the Arctic circle [Hofmann et al, 1989]. Further, Lubin et al (1989) have provided the first direct ground-based measurements of increased levels of UV-B radiation during an ozone depletion episode in Antarctica. These findings are confirming what has long been predicted - a decrease in the stratospheric ozone concentration and a subsequent increase in the amount of solar UV-B radiation reaching the earth's surface will occur as a direct result of man-made perturbations to the atmosphere. Since wavelengths in the middle UV region of the spectrum (UV-B 280-320nm) are readily absorbed by living organisms and are responsible for much of the biological

activity of solar UV radiation, increased environmental exposure to UV-B radiation would have profound effects on human health and animal and plant life.

1.3.2 Artificial UV Sources

Various types of lamps exist which emit across the UV region. For example, a lamp used to investigate the influence of broad band UV-B (280-320nm) radiation on experimental animals is the Philips TL20W/12 fluorescent lamp. However, although this type of UV source is convenient, there are disadvantages. As can be seen from the relative spectral energy distribution of this UV-B sunlamp (Figure 2), radiation on both sides of the UV-B band i.e. in the UV-A and UV-C, is also being emitted. Thus, mice exposed to this lamp are exposed simultaneously to other wavelengths of radiation, in addition to UV-B [Van Weelden et al, 1988].

A new UV-B fluorescent sunlamp, the Philips TL-01 lamp has recently been developed. In contrast to the TL20W/12 lamp, the spectrum of this lamp is dominated by a strong and narrow peak (band width 2.5nm) around 311-312nm, with a second peak around 305nm and has much smaller output at wavelengths of 300nm and below than the broad band UV-B sources (Figure 3) [Flindt-Hansen et al, 1991; Van Weelden et al, 1988 and 1990].

1.3.3 The Biological Effects of UV Radiation

UV-B radiation is among the most ubiquitous agents encountered in the environment. Consequently, in mammalian systems the skin is the major substrate upon which this nonionizing radiation directly interacts with an organism; such an interaction determining the biological effects of UV radiation. Also, it should be noted that UV-B penetrates very little, if at all, beyond the skin [Agin et al, 1981], partly because of scattering of the radiation and partly because there are many efficient absorbers of UV-B radiation in the skin. UV-A, in contrast, penetrates considerably further [Agin et al, 1981].

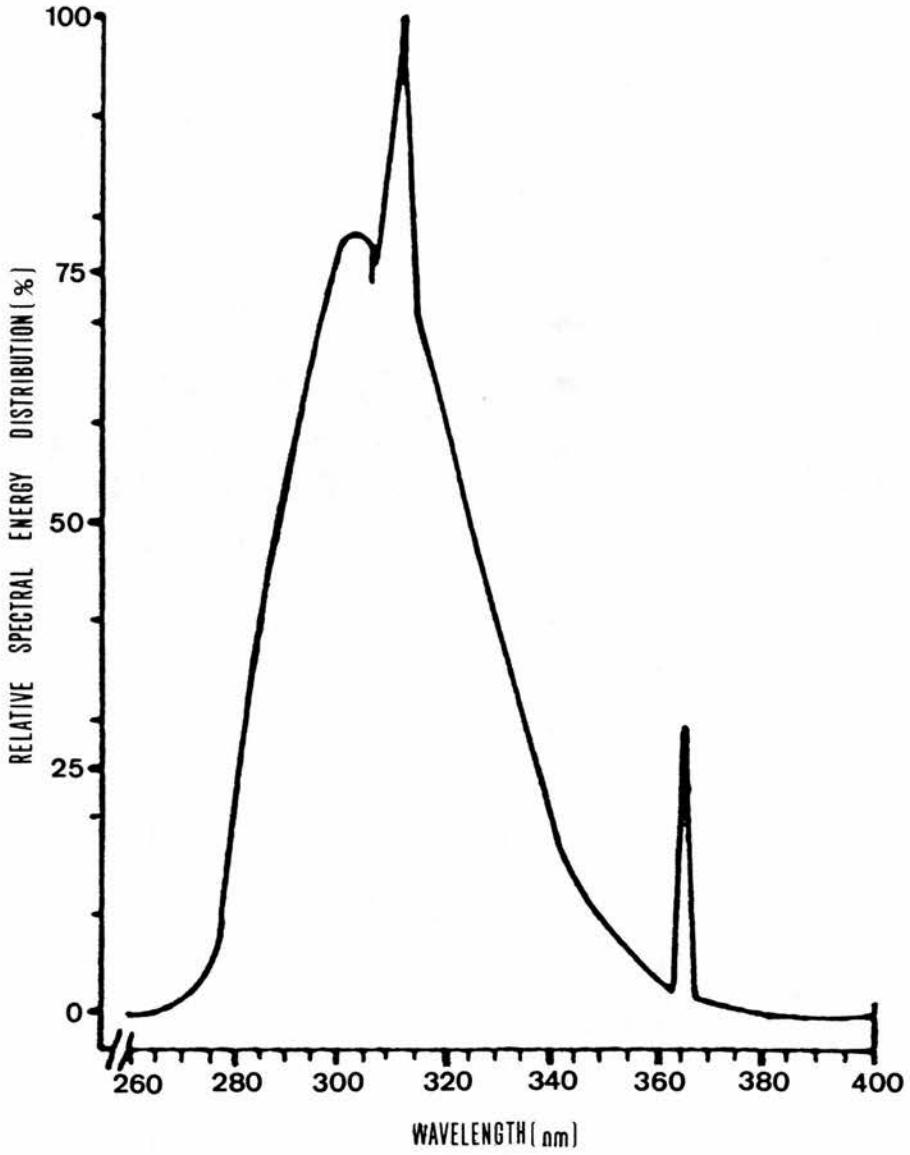

Figure 2 Relative spectral energy distribution of a Philips TL-20W/12 UV-B lamp.

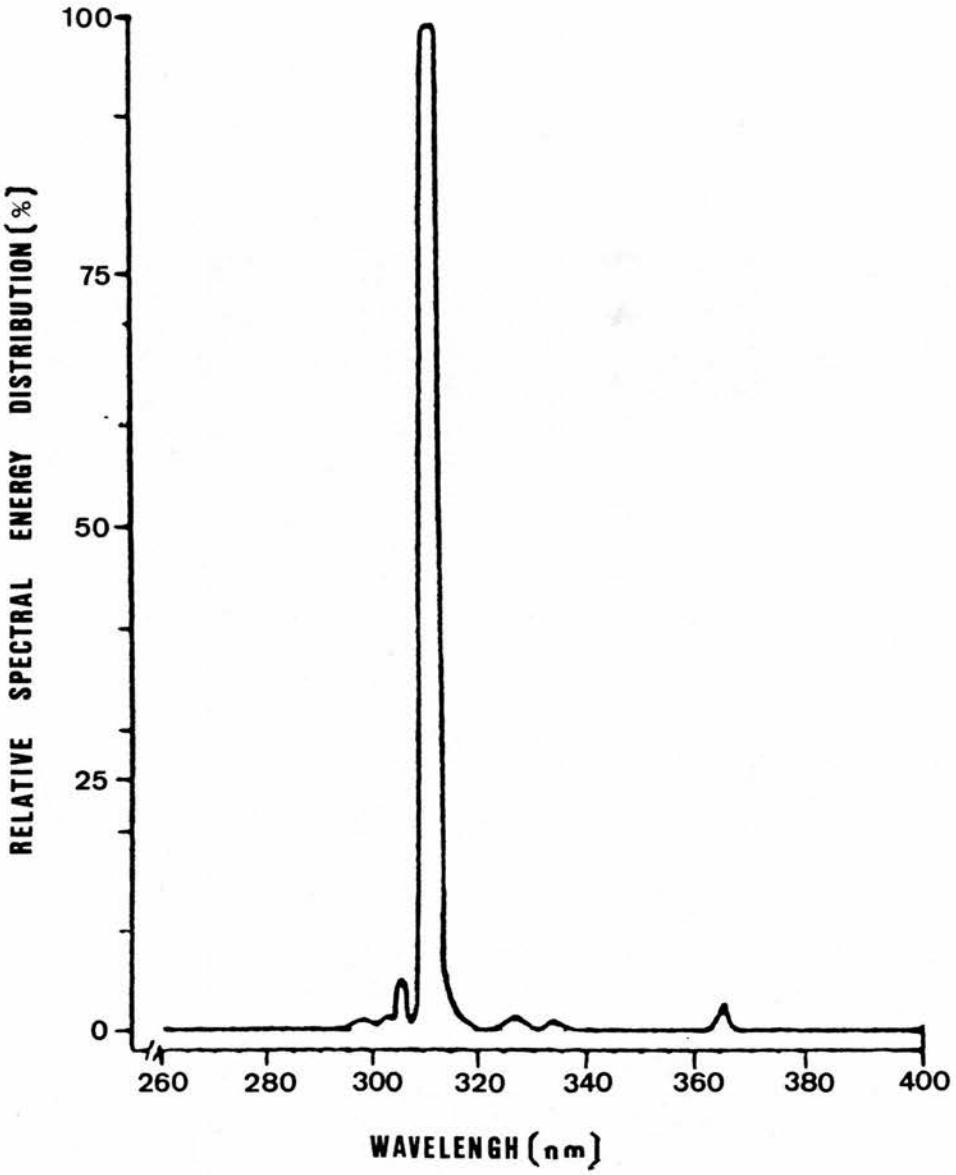

Figure 3 Relative spectral energy distribution of the experimental fluorescent lamp, Philips TL-01.

A major critical biological molecule which absorbs UV-B radiation is DNA with the result that it is damaged. Damage may be direct such as the formation of pyrimidine dimers by absorption of UV photons or indirect via a route involving "excited-state" oxygen produced by the splitting of water molecules. In addition to pyrimidine dimers other damage can occur in UV irradiated DNA including DNA-protein cross-links and DNA strand breaks [Setlow and Carrier, 1966; Elkind and Han, 1978; Peak et al, 1985; Miguell and Tyrrell, 1983].

The most serious biological consequences of UV radiation on proteins are due to absorption by a few amino acids - tryptophan, tyrosine, phenylalanine, cystine, and cysteine and the peptide bond. Since cystine with its chain-linking disulphide bond is critical in tertiary structure formation, this is probably the most important target in the inactivation of proteins. Changes which occur following UV absorption of proteins include increased sensitivity to heat, enzyme inactivation and antigenic changes due to altered amino acid residues [Smith and Hanawalt, 1969].

An important function of UV-B radiation is that it converts skin stores of 7-dehydrocholesterol (provitamin D_3) to previtamin D_3 which then slowly converts to vitamin D_3. Vitamin D is essential for calcium and bone metabolism [Holick, 1985]. This appears to be the only well documented beneficial effect of UV-B radiation, although exposure to UV-B radiation is very effective in the treatment of a large number of skin diseases.

Despite many dark coloured cultures considering light coloured skin very beautiful, many caucasians through vanity and sociological pressures constantly seek a tanned skin. During the process of tanning a number of biological events arise. UV radiation upon penetrating the skin destroys and damages skin cells (i.e. Keratinocytes and LC) [Obata and Tagami, 1985] and at the same time triggers melanocytes (pigment cells) present in the epidermis to produce melanin which acts as a shield to absorb damaging UV radiation. It is the UV-B induced production of melanin which produces the tan. Further, during the process of tanning UV-B

radiation induces erythema which is a local effect caused by the dilation of blood vessels near the skin's surface and increased blood flow to the skin, making the skin hot, swollen and red - sunburnt. Sunburn frequently causes the upper layers of the epidermis to be discarded so that the skin peels and is replaced by new skin.

Depending on a person's predisposition, the effects of sunburn may cause permanent damage which is only manifested after a number of years. Sunburn may lead to degeneration of the connective tissue, increased susceptibility to infection and skin carcinomas. There are three forms of skin cancer: basal-cell carcinoma, squamous-cell carcinoma and malignant melanoma, all of epidermal origin. Although non-melanoma skin cancers are the most common of all tumours in the caucasian population, with a low mortality rate of 1% or less [Pollock Shea, 1988], the incidence of malignant melanoma skin cancer is increasing at an alarming rate, particularly in Northern hemispheres [Mackie et al, 1992]. The mortality rate of malignant melanoma skin cancer is much higher than non-melanoma skin tumours. There is little question of the causal link between UV radiation and non-melanoma skin cancer. These cancers correlate with latitude, and by implication UV levels. The closer to the equator a white-skinned person lives, the more likely he or she is to get basal-cell or squamous-cell carcinoma. Moreover, these carcinomas occur on parts of the body we expose the most: face, hands, arms and neck. The formation of these cancers appears to be dependent on the cumulative effect of exposure to sunlight. Mice chronically exposed to UV radiation develop non-melanoma skin cancer [Blum, 1959]. People who work outdoors are particularly prone to non-melanoma skin cancers. De Gruijl and van der Leun (1991) reported that the action spectrum of non-melanoma skin cancers is similar to that for erythema i.e. UV-B wavelengths are more efficient than UV-A. Although the link between UV exposure and malignant melanoma is not as clear cut as with non-melanoma skin cancer, it seems to be associated with acute burning experiences since it occurs more frequently in fair-skinned individuals with a history of blistering sunburn in their

childhood. However, it is puzzling that malignant melanomas are not restricted to UV-exposed areas of the body and vary in their distribution between men and women.

Other biological effects of UV-B radiation include photoageing, arising as a consequence of frequent and prolonged exposure to UV radiation over a substantial period and manifesting itself in the form of dryness and wrinkling [Kligman, 1989]; the formation of certain types of ocular cataracts [Taylor et al, 1988; Pitts et al, 1986] and an alteration in immunological function (discussed in detail in section 1.4).

1.4 Immunosuppression by Ultraviolet B Radiation

Irradiation with UV-B in vivo suppresses some cell-mediated immune responses to a variety of antigens. For example CH responses to chemical haptens [Greene et al, 1979; Toews et al, 1980; Noonan et al, 1981A & C; Lynch et al, 1981, 1983; De Fabo and Noonan, 1983; Elmets et al, 1983; Streilein and Bergstresser, 1988; Vermeer et al, 1991] and delayed-type hypersensitivity responses to herpes simplex virus (HSV) [Howie et al, 1986A; Yasumoto et al, 1987] and alloantigens [Ullrich, 1986; Mottram et al, 1988] are suppressed. Other examples of UV-induced immunosuppression include suppression of highly antigenic UV-induced tumours [Fisher and Kripke, 1977, 1978; De Fabo and Kripke, 1979] and suppression of Leishmania parasite-induced skin lesions [Giannini, 1986].

A model system used by many investigators to investigate the mechanism of this effect is the UVB-induced suppression of the CH response to contact sensitizers such as TNCB or dinitrofluorobenzene (DNFB).

1.4.1 Local and Systemic Immunosuppression

Irradiation of mice with UV-B suppresses CH either "locally" i.e. when the contact sensitizer is applied to the UV irradiated site [Toews et al, 1980; Lynch et al, 1981; Streilein and Bergstresser, 1988], or "systemically" i.e. when the sensitizer is applied to a site distant from the site of UV-B exposure [Noonan et al, 1981A, C; De Fabo and Noonan, 1983]. Investigators using protocols that produce local suppression of immune responsiveness have achieved their effect by exposing the skin to relatively low doses of UV-B (20-480mJ/cm^2) radiation. Conversely, investigators using protocols that induce systemic suppression have only been able to achieve suppression using high doses of UV-B (1500-9000mJ/cm^2). Since local suppression requires lower doses of UV-B radiation than does systemic suppression it has been suggested that different mechanisms are therefore responsible for

initiating local and systemic suppression [Freeman et al, 1982; Elmets et al, 1983; Morison, 1984; Bergstresser, 1986; Kripke, 1986; Krutmann and Elmets, 1988; Streilein and Bergstresser, 1988]. Consequently, throughout the literature the terms "high dose" and "low dose" suppression have been used extensively to designate the differences between systemic and local suppression, respectively. In an attempt to clarify the issue of high dose versus low dose suppression, and systemic versus local suppression, Noonan and De Fabo (1990) recently carried out an analysis of the UV dose-responses and kinetics of generation of UV-induced local and systemic suppression of CH using two different mouse strains, Balb/c and C57BL/6, which had been used previously in other laboratories for studies of systemic [Lynch et al, 1981; Noonan et al, 1981A, C; De Fabo and Noonan, 1983] and local [Toews et al, 1980; Streilein and Bergstresser, 1988] suppression, respectively. These investigators reported for the first time that for a given strain of mouse, the UV dose-response for local and for systemic suppression was identical, indicating that the use of the terms "low dose" and "high dose" to refer respectively to local and systemic suppression by UV irradiation are incorrect. They were able to demonstrate that the differences between the dose-response characteristics of local and systemic suppression found previously, were due to differences in the time-course of generation of local and systemic suppression after UV irradiation and differing sensitivities to UV suppression between mouse strains. They found that local suppression was initiated if the sensitizer was applied immediately, or between 1 and 3 days after a single dose of UV whilst systemic suppression was initiated only if the sensitizer was applied between 1 and 3 days after UV irradiation and that C57BL/6 mice were approximately 6-fold more sensitive to local or systemic suppression than Balb/c mice.

These findings confirmed and extended the studies of Lynch et al (1983) and Applegate et al (1989), who reported that for a given strain of mouse local suppression could be detected by sensitization immediately or 3 days after UV

irradiation and systemic suppression could only be detected if sensitization occurred 3 days after UV exposure. Further, Noonan and De Fabo's (1990) findings support the view that differences in susceptibility to UV suppression exist [Streilein and Bergstresser, 1988]. Taken together these results suggest that a common mechanism may initiate UV-induced local and systemic suppression of CH by the immediate formation, at the site of UV irradiation, of an immunosuppressive signal which takes several days to act systemically.

Throughout the literature investigators studying UV-induced local immunosuppression have focused on the direct effects of UV-B radiation on immunocompetent cells at the irradiation site. Since UV-B radiation does not penetrate beyond the skin, investigators studying UV-induced systemic alterations in immune reactivity have focused on the indirect effects of UV-B radiation perhaps occurring as a result of the release of soluble mediators which can act systemically. In light of the report by Noonan and De Fabo (1990) that a common mechanism, perhaps mediated by a UV-B induced soluble mediator, is responsible for initiating local and systemic suppression, it seems reasonable to postulate that all or some of the effects on immunocompetent cells at the irradiation site may arise as a consequence of a UV-B induced mediator acting locally within the skin. Alternatively, the direct and indirect effects of UV-B irradiation may both operate in suppressing the induction of CH suggesting that more than one mechanism may be involved in UV-B induced immunosuppression. Evidence that this is the case was provided by Chung et al (1986A) who demonstrated that the direct effects of UV-B radiation play a role in local UV-B induced immunosuppression whereas the indirect effects of UV-B play a role both in local and systemic UV-B mediated immunosuppression. They found that the capacity of systemically immunosuppressed mice to mount a CH response to DNFB could be completely restored by treatment with indomethacin (an inhibitor of prostaglandin activity). In contrast, immunological responsiveness of locally suppressed mice could not be

restored by indomethacin pretreatment, where DNFB application was confined to the UV-B irradiation site. When a portion of the UV-B exposure site was shielded and DNFB applied to the area of skin that was covered, an immunosuppressed response was also observed. In this latter case, treatment with indomethacin was able to reverse the suppression.

Both local [Elmets et al, 1983; Glass et al, 1990] and systemic suppression [Ullrich, 1987; Morison and Kripke, 1984; Yee et al, 1989B] are associated with the appearance of antigen-specific suppressor T lymphocytes in the animals' spleens and lymph nodes which prevent the induction of CH upon transfer to a normal recipient. Subsequently, it has been postulated that the generation of these cells is essential for the production of unresponsiveness to cutaneously applied haptens [Elmets et al, 1983; Ullrich, 1987]. Thus there has been considerable interest in identifying the cell(s) responsible for activating suppressor T-cell circuits following UV-B exposure; possible candidates will be discussed in later sections.

1.4.2 Direct Effects of UV-B Irradiation in Suppressing the Induction of Contact Hypersensitivity

1.4.2A The role of Langerhans Cells in UV-B Induced Immunosuppression

The major target of the direct effects of UV-B irradiation appears to be the LC of the skin. To investigate whether a direct relationship exists between the surface density of LC and the capacity of a cutaneous surface to support the induction of CH, Streilein et al (1980B) and Toews et al (1980) developed a mouse model system in which acute, low-dose exposure of body wall skin to UV-B was shown to deplete surface expression of ATPase activity on epidermal LC. When contact sensitizers, such as DNFB, were painted on cutaneous areas depleted of epidermal LC expressing ATPase activity by UV-B irradiation, CH was not induced [Toews et al, 1980; Streilein et al, 1980B]. Mice treated in this way also proved to be

specifically unresponsive to the same hapten when they were skin painted subsequently on a normal site [Toews et al, 1980]. This hapten-specific unresponsiveness was mediated by hapten-specific T suppressor cells [Elmets et al, 1983]. It was concluded that relatively low doses of UV-B radiation deplete the irradiated skin of normally functioning LC, and thus prevent the induction of CH to haptens by removing the critical antigen-presenting cells that are required to initiate the immune process. Further data to substantiate this hypothesis, were provided by Streilein et al (1980A) and Streilein and Bergstresser (1981) who demonstrated that cutaneous surfaces physiologically deficient in LC (mouse tail skin, hamster cheek pouch) also failed to support the induction of CH. However, the validity of this postulate was questioned by Sauder and Katz (1983), who examined the capacity of haptens to induce CH through tail skin of various inbred strains of mice. They discovered that strains C57BL/6, C57BL/10 and AB.Y mice failed to develop CH (and instead tolerance is induced) whilst Balb/c, A/J and CBA mice displayed vigorous CH. No significant strain differences in the density of ATPase positive cells in tail skin was found and thus they concluded that the ability to become sensitive or tolerant is not related to LC density (as detected by ATPase staining).

Work by Lynch et al (1981) and Aberer et al (1981) served to emphasize that the presence (or absence) of a phenotypic characteristic of LC (such as ATPase activity) cannot be used as a reliable indicator of either the presence (or absence) or of the functional capabilities of these cells. Thus, more direct evidence for the role of LC in UV-B induced suppression of CH was needed other than attempting to elucidate whether or not alterations in the number of LC present in the skin following UV-B irradiation were responsible for immunosuppression. This was provided by a number of investigators [Stingl et al, 1981; Sauder et al, 1981; Austaad and Braathen, 1985; Cruz et al, 1989; and Cruz et al, 1990]. Stingl et al (1981) observed that exposure to a single low dose of UV-B radiation resulted in an inhibition of the ability of epidermal cells to serve in vitro as antigen-presenting

cells for dinitrophenylated-ovalbumin (DNP_6 OVA) and PPD-induced proliferation of immune T cells. In a related experiment using human epidermal cells Austaad and Braathen (1985) reported that low-dose irradiation similarly reduced the ability of these cells to mediate PPD - and alloantigen-induced T cell proliferation. Although, non-purified epidermal cells were utilized by both groups of investigators, these experiments suggested that LC were the targets of UV-B radiation based on previous circumstantial evidence that LC stood alone among epidermal cells in their capacity to present antigen effectively to sensitized T lymphocytes. In a different set of experiments also utilizing non-purified epidermal cells, Sauder et al (1981), who had demonstrated that trinitrophenol (TNP)-derivatized epidermal cells injected subcutaneously (s.c.) into mice could induce CH, found that exposure of these cells to a single low dose of UV-B radiation, prior to hapten-derivatization, robbed them of this capacity. Instead, the induction of a state of specific immunological tolerance was observed which could be adoptively transferred via spleen cells from mice that had previously received the irradiated epidermal cells.

More recently Cruz et al (1989) have provided direct evidence that LC are the relevant immunological targets of low-dose UV-B irradiation. These investigators utilized intravenous (i.v.) immunization with flow cytometry-purified epidermal cell subpopulations for the induction of CH as a tool to characterize the effects of low-dose UV-B irradiation on the immunogenic properties of these cells. They found that not only does UV-B irradiation abrogate the capacity of Ia^+ epidermal cells (presumably LC) to sensitize for CH, but such treatment results in down-regulation of CH responses indicating that LC are indeed the relevant immunological targets of low dose UV-B irradiation. It was also found that the down-regulation induced by the UV-B irradiated Ia^+ epidermal cells was hapten-specific. Thus, it appears from these results that low-dose UV-B irradiated LC may be capable of activating suppressor mechanisms. The studies by Cruz et al (1989) however, addressed neither the fate of the transferred cells nor the relevance of this technique

to CH, where sensitization is ordinarily achieved epicutaneously. This issue was clarified by Cruz et al (1990) who examined the migration of epidermal cells after i.v. infusion, the immunogenic properties of LC that return to skin and the effect of low dose UV-B radiation on these processes. LC (Ia^+ epidermal cells) were found to home preferentially to skin and were competent to induce CH. Moreover, low-dose UV-B irradiation confered upon these "migrant" LC the capacity to mediate immunological unresponsiveness and down-regulation of subsequent CH responses, confirming that LC are the relevant immunological targets of low-dose UV-B radiation.

It has been demonstrated that some strains of mice (C57BL/6, C57BL/10 and AB.Y) fail to develop CH whilst other strains (Balb/c, A/J and CBA) display vigorous CH [Sauder and Katz, 1983]. Streilein and Bergstresser (1988) found that a similar genetic effect was operative in the low-dose UV-B irradiation model. They found that when DNFB was applied to UV-B irradiation sites, unresponsiveness developed in some strains of mice (e.g. C57BL/6, C57BL/10, C3H/HeN) but vigorous CH was induced in others (e.g. Balb/c, A/J). However, these investigators found that all the strains tested were equally susceptible to the effects of UV-B radiation on LC (as measured by ATPase staining) at UV-B treated sites, implying that there is a dissociation between the effects of UV-B radiation on epidermal LC and the capacity of a cutaneous surface to support the induction of CH. The observations of Sauder and Katz (1983) and Streilein and Bergstresser (1988) called into question whether LC are always required for the induction of CH and tended to imply that in the so called UV-B resistant strains of mice, alternative (non-LC dependent) mechanisms allow the induction of CH.

In an effort to explain these findings Streilein (1989) proposed that two antigen-presentation pathways for the induction of CH may exist in the skin. He suggested that one pathway which is LC dependent, is present in all mice, but the other pathway, which is LC independent, may be functional only in mice that are

designated UV-B resistant. Streilein (1989) speculated that MHC class II$^+$ DC/macrophages of the dermis may be responsible for the LC independent pathway. Interestingly, recently Tse and Cooper (1990) reported that dermal cells can initiate a CH reaction in mouse skin. These investigators found that, like dendritic LC of the epidermis, perivascular dendritic Ia$^+$ cells of the dermis were capable of initiating T-cell mediated CH in vivo. Streilein (1989) tested the hypothesis that UV-B resistant mice utilize extraepidermally located antigen-presenting cells for CH induction by tape stripping the epidermis of UV-B susceptible and resistant strains. Since tape stripping of mouse epidermis had previously been shown to completely (albeit transiently) remove LC from the epidermis [Streilein et al, 1982], he speculated that tape stripping would resemble UV-B irradiation in the ability to rob the skin of UV-B susceptible animals of its capacity to support the induction of CH. He demonstrated that tape stripped skin of UV-B resistant Balb/c mice did support the induction of hapten-induced CH in the absence of epidermal LC. However, he found that tape stripped skin of UV-B susceptible mice also supported the induction of CH. These results were consistent with the hypothesis that cells (presumably in the dermis) other than LC can be important in the induction of CH [Tse and Cooper, 1990] but failed to illuminate the reason why some strains of mice are susceptible to UV-B radiation and others not. Based on the observations of these investigators [Sauder and Katz, 1983; Streilein and Bergstresser, 1988; Streilein, 1989] it was necessary to re-examine the epidermis for another UV-B induced perturbation, other than a direct effect on LC.

1.4.2B The role of I-J+ and Thy-1+ Epidermal Cells in UV-B Induced Immunosuppression

Other than UV-B irradiated LC, two other cell types present in the epidermis of mice have been identified that are capable of initiating down-regulatory signals to topically applied haptens. These are I-J+ cells and the Thy-1+ DEC.

Granstein et al (1984) first described the epidermal I-J+ cell when it was observed that hapten-coupled, UV-B irradiated epidermal cells were capable of generating afferent acting, antigen-specific, transferable suppressor T cells. Treatment of the UV-B-irradiated epidermal cell population with an antibody directed against the I-J+ determinant abrogated its capacity to activate the suppressor T cell pathway. Further studies have shown that this I-J+ cell is a high density, cyclophosphamide sensitive, adherent cell that is Ia^+, but not Thy-1+ [Granstein, 1985]. I-J+ epidermal cells were found to exhibit resistance to UV-B irradiation [Granstein et al, 1984]. However, since its original description there have been no other reports confirming that such a cell exists.

Another epidermal cell, distinct from LC, which is thought to preferentially deliver down-regulatory signals for cell-mediated immune reactions is the Thy-1+ DEC. It has been shown that low-dose UV-B irradiation does not seem to affect Thy-1+ DEC [Aberer et al, 1986] suggesting that this cell population may also represent a UV-resistant cell population capable of inducing suppression of CH. When purified Thy-1+ DEC are conjugated with the hapten TNCB and then injected i.v. into mice they mediated down-regulation of CH [Sullivan et al, 1986]. However, Sullivan et al (1986) found that s.c. injection of these TNCB conjugated cells produced no effect. Further, Cruz et al (1989) have demonstrated that when purified populations of haptenated unirradiated and irradiated Thy-1+ DEC were injected i.v. both delivered down-regulatory signals. It is unclear why haptenated Thy-1+ DEC do not initiate a down-regulatory signal when injected by the s.c. route. Sullivan et al (1986) proposed that derivatized Thy-1+ DEC may only be perceived as down-regulating when they are allowed access to the systemic (i.v.) compartment, which they suggest may be accomplished either by the migration of Thy-1+ DEC from the epidermis or by a change in cellular traffic to the epidermis. Interestingly, studies by Okamoto and Kripke (1987) have demonstrated that the DLN of mice painted with fluorescein isothiocyanate (FITC) on skin irradiated with

low-dose UV-B contain a population of fluoresceinated Ia⁻/Thy-1+ cells. These investigators showed that injection of DLN cell suspensions from these mice into naive recipients produced inhibition of CH which was associated with the presence of suppressor T lymphocytes. They further demonstrated that the DLN Thy-1+ cells were essential for the production of these suppressor T lymphocytes since depleting the Thy-1+ cells from the DLN population abrogated the ability of this population to induce suppressor T lymphocytes. If it is possible to demonstrate that the Thy-1+ cells that initiate suppression in this system are part of the same population of Thy-1+ DEC that reside within the epidermis, this would provide evidence for the ability of Thy-1+ DEC to induce suppressor cell activity. In support of the notion that these DLN Thy-1+ cells originate from the skin is the recent report by Cruz et al (1990) which showed that i.v. injected Thy-1+ DEC traffic to skin and lymphoid tissues.

So, the possibility exists that a UV-B resistant population of cutaneous cells (either Thy-1+ DEC or perhaps the cell described functionally by Granstein et al (1984)) governs the induction of signals leading to suppression of CH, and that these putative cells may serve as the medium through which the genetic basis of UV-B susceptibility is expressed. Interestingly, Sauder and Katz (1983) and Streilein and Bergstresser (1988) both showed that there were no significant strain differences in the density of ATPase positive cells or in the depletion of epidermal LC (as detected by ATPase staining) by low-dose UV-B irradiation respectively, supporting the notion that the genetic basis for strain differences may reside on differences in the number of Thy-1+ DEC or I-J+ epidermal cells between strains. It has been hypothesized [Sullivan et al, 1986] that the outcome of an epicutaneous application of contact sensitizer in the murine system may depend on the balance between the activation signals from LC and down-regulatory signals provided by Thy-1+ DEC or I-J+ epidermal cells. Thus, it can be speculated that UV-B susceptible and resistant mouse strains differ in the ratio of their LC to Thy-1+ DEC or I-J+ epidermal cell. Evidence to support the theory that UV-B susceptible and

resistant mouse strains may differ in the ratio of their LC to Thy-1+ DEC was provided by Bigby et al (1987) who demonstrated that the ratio of LC to Thy-1+ DEC varies markedly in different strains of mice. More importantly they showed that the ratio of LC to Thy-1+ DEC appears to influence the intensity of CH in different strains of mice. Although, these investigators did not alter the number of LC or Thy-1+ DEC with pharmacological or physical modalities such as topical corticosteroids or UV-B radiation, their results extended the findings of Streilein and Bergstresser (1988) who demonstrated that when a chemical hapten was applied to UV-B irradiated sites, unresponsiveness developed in some strains of mice (C57BL/10 and 6) but vigorous CH was induced in others (Balb/c and A/J). Bigby et al (1987) showed that C57BL/10 and 6 mice developed very small CH responses to chemical haptens and also had very low ratios of LC to Thy-1+ DEC whilst Balb/c and A/J mouse strains developed very vigorous CH responses and had a significantly high ratio of LC to Thy-1+ DEC.

It has been proposed [Sullivan et al, 1986] that normally, the cutaneous system is poised for immunity and LC are a dominant signal for a positive immunogenic response. The down-regulating influence of Thy-1+ DEC and/or I-J+ epidermal cells may provide one mechanism by which the immune system regulates the expression of the CH response, thus establishing a functional balance between the effects of the three epidermal populations. This could be important physiologically in limiting the extent of tissue damage in the host. If there is a breakdown in the balance and functioning LC are not apparent, this helpful down-regulatory mechanism may be perceived as a primary suppressive mechanism that may occur on its own or in concert with other suppressive circuits. Thus, it can be hypothesized that these UV-B resistant epidermal cell populations (distinct from LC) which are responsible for down-regulatory signals, could mediate the local, low-dose UV-B mediated suppression of CH responses.

It should be emphasized that although the suppression-inducing cells (Thy-1+ DEC and I-J+ epidermal cells) have been thought of as being antigen-presenting cells [Granstein, 1985; Sullivan et al, 1986], it is equally possible that they represent precursors of suppressor T lymphocytes. If this is the case then this would imply that the suppressor circuit may be activated by direct contact with the antigen, rather than by interaction with antigen bound to an antigen-presenting cell. This possiblity is interesting in view of the finding by Siliciano et al (1985) that certain FITC-specific human T lymphocyte clones can bind FITC directly without the participation of an antigen-presenting cell.

1.4.2C The Role of Suppressor T Lymphocytes in UV-B Induced Immunosuppression

It has been demonstrated that low-dose UV-B induced suppression of CH in mice is associated with antigen-specific, afferent suppressor T cells that selectively act on the induction phase of sensitization [Elmets et al, 1983]. The finding that UV-B irradiation impairs the induction of CH in some strains of mice and has no apparent effect on CH in others suggested that UV-B susceptible strains of mice generate hapten-specific suppressor T cells, whereas their UV-B resistant counterparts may not. However, this possibility was excluded by examining the capacity of UV-B radiation and hapten to generate suppressor T cells in several different inbred strains of mice [Glass et al, 1990]. Glass and his colleagues (1990) reported that UV-B irradiation generates immune suppressor cells in resistant strains as well as in susceptible ones.

1.4.3 Indirect Effects of UV-B Irradiation in Suppressing the Induction of Contact Hypersensitivity

1.4.3A The role of a Specific Skin Photoreceptor in Initiating UV-B Induced Immunosuppression

1.4.3A1 *Urocanic Acid*

Irradiation with UV-B initiates systemic immunosuppression of CH [Noonan et al, 1981A, C; De Fabo and Noonan, 1983]. Since the wavelengths responsible do not penetrate beyond the skin, the sequence of events leading to systemic immune suppression must be initiated by the absorption of UV radiation by some moiety in the skin. It has been demonstrated that this UV-induced systemic suppression of CH is not dependent on a UV-induced loss of LC. Both Noonan et al (1984) and Morison et al (1984) observed that with the use of different wavelengths of UV radiation, the UV effects on LC numbers and morphology were seen to be separable from UV-induced systemic suppression of CH, suggesting that systemic suppression is not generated by a UV-induced loss of LC from the UV-irradiated site or by an alteration in LC morphology at the UV-irradiated site. These investigators also showed that a UV-induced systemic effect on LC numbers at the site of sensitization is not responsible for the UV-induced generation of systemic suppression of CH. Further, UV-induced systemic suppression of CH is not dependent on gross skin damage or erythema [De Fabo and Noonan, 1983; Noonan et al, 1981B; Noonan et al, 1981C] and is observed in pigmented and nonpigmented mice and humans [Vermeer et al, 1991; Noonan and De Fabo, 1990].

It has been proposed by De Fabo and Noonan (1983) that the initial photo event that triggers systemic suppression is the interaction of UV-B radiation with urocanic acid (UCA). This hypothesis is based on the location of UCA in the stratum corneum and on similarities between its absorption spectrum and the action spectrum of UV-B induced systemic suppression of CH. De Fabo and Noonan (1983)

also found that if the stratum corneum was removed by tape stripping then suppression of the CH response did not occur.

UCA is a major UV-absorbing component of the stratum corneum and represents one of the major chemical components of the epidermis comprising 0.5% of its dry weight [Tabachnick, 1959]. It was originally proposed that UCA may have an important function as a natural "sun-screen" to protect against sunburn [Zenisek et al, 1955] and secondly that it may function as a natural photoprotecting agent for DNA as it absorbs strongly at 277nm and because of its location [reviewed by Morrison, 1985]. UCA is synthesized from histidine in the stratum corneum by the enzyme histidine ammonia-lyase (histidase). Histidine ammonia-lyase is itself produced in the granular and spinous layers of the epidermis where it is inactive. As the granular cells move upwards into the stratum corneum, they become enucleated, the pH drops, various non-structural proteins including the keratohyalin granule are degraded and the enzyme is activated. Upon activation, UCA is formed and accumulates as the cells become highly impermeable on keratinization [Baden and Pathak, 1967; Scott, 1981; Taylor et al, 1991]. On absorption of UV-B radiation either in vitro or in mammalian skin, including human skin [Baden and Pathak, 1967; Norval et al, 1989A; Morrison et al, 1980; Pasanen et al, 1990], UCA undergoes a trans to cis-isomerization in a dose-dependent manner until a photostationary state is reached [Anglin et al, 1961; Baden and Pathak, 1967; Morrison, 1985] (Figure 4).

De Fabo and Noonan (1983) proposed that UV-B radiation exerts its immuno-modulatory properties via cis-UCA and that trans-UCA is the photoreceptor for the UV-B induced systemic suppression of CH. To test this hypothesis further, De Fabo et al (1983) investigated UV-B induced systemic suppression of CH in mice genetically deficient in the enzyme histidine ammonia-lyase and thus in skin UCA. These animals had greatly impaired UV-induced immune suppression. Recently, Reilly and De Fabo (1991) carried out a reciprocal experiment with mice that had

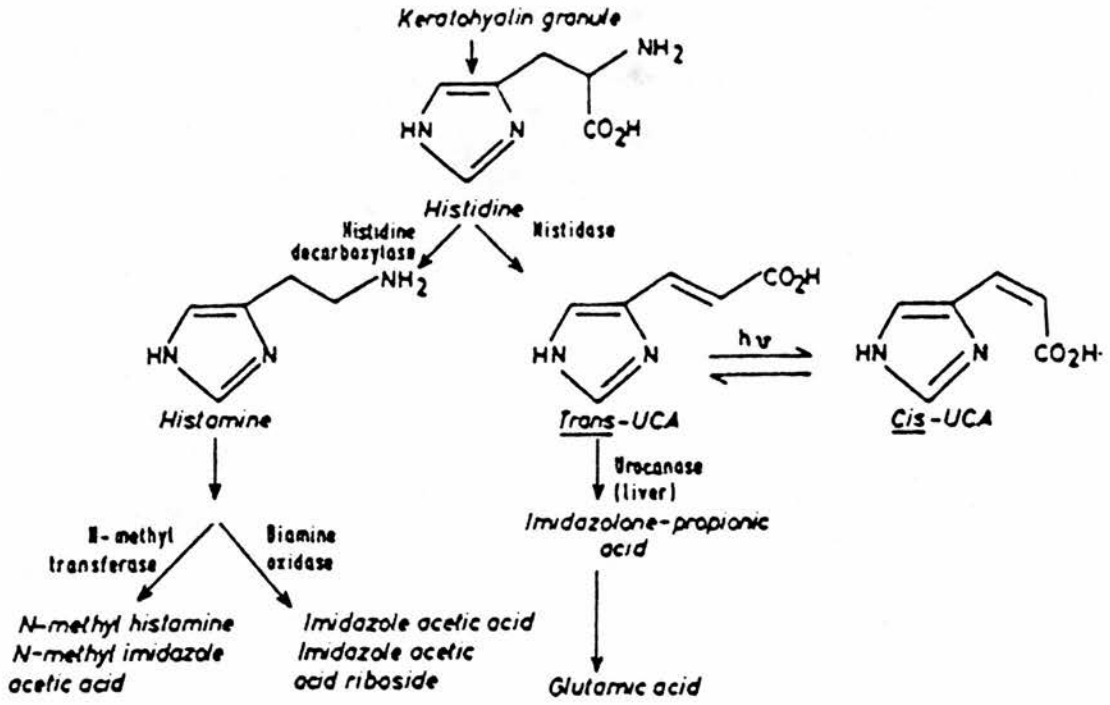

Figure 4 Metabolic pathways of histidine, histamine and urocanic acid.

increased skin levels of UCA when fed with a diet containing a high level of histidine, the metabolic precursor of UCA. They showed significantly greater UV-induced immune suppression of CH than mice fed a normal diet.

Direct evidence implicating cis-UCA as a mediator of the indirect effects of UV-B radiation on UV-B induced local and systemic suppression of CH has been provided by several investigators [Harriott-Smith and Halliday, 1988B; Kurimoto and Streilein, 1992; Reeve et al, 1989 and 1993]. Harriott-Smith and Halliday (1988B) injected cis-UCA i.v. 3 days prior to sensitization with TNCB and measured the CH response developed, compared with mice which had received trans-UCA by the same route. They found that i.v. injection of cis-UCA suppressed the induction of CH to TNCB whereas trans-UCA had no significant effect. In experiments carried out by Reeve et al (1989 and 1993) UV-irradiated UCA was shown to suppress both local and systemic CH reactions when it was topically applied to the shaved dorsum of mice 3hr before sensitization on either the dorsum or ventrum. Further, Kurimoto and Streilein (1992) demonstrated that cis-UCA can impair the induction of CH whether injected intradermally (i.d.) or applied epicutaneously to skin immediately or 5hr prior to hapten painting on the same site.

Evidence that cis-UCA acts as a mediator of UV-induced immuno-suppression in other experimental systems is also available. For example, cis-UCA has been shown to suppress delayed-type hypersensitivity (DTH) responses in a murine model of HSV infection [Ross et al, 1986], to delay rejection of transplant allografts [Oesterwitz et al, 1990; Williams et al, 1990; Gruner et al, 1992; Guymer and Mandel, 1990], prevent and delay the onset of acute lethal graft-versus-host disease [Gruner et al, 1992] and to enhance UV-induced tumour yield and malignancy in the hairless mouse [Reeve et al, 1989].

Using a variety of UCA analogues including histamine Norval et al (1989B) provided evidence that the 5-membered imidazole ring of UCA is necessary for suppressive activity, while the side arm may be modified considerably without

affecting immune function. Since histamine was found to suppress the DTH response and is structurally similar to cis-UCA i.e. it possesses a 5-membered imidazole ring, these investigators speculated that cis-UCA may act via histamine-like receptors present in the epidermis. Support for this notion was provided by the demonstration that H_1 and H_2 histamine receptor antagonists negated the effects of cis-UCA in reducing the number of ATPase positive cells in murine epidermis and suppressing the DTH response to HSV [Norval et al, 1990]. New evidence indicating that cis-UCA interacts with a target cell in the skin via a histamine receptor has recently been provided. Palaszynski et al (1992) reported that trans-UCA and histamine are biologically active in vitro in human dermal fibroblasts, inducing adenyl cyclase as measured by cyclic AMP formation while cis-UCA can actively down-regulate the production of this molecule. Further, they demonstrated that the trans-UCA induction of cyclic AMP can be down-regulated with an H_2 histamine receptor antagonist (cimetidine), thus confirming and extending the findings of Norval et al (1989B and 1990). The down-regulation of the production of an important second messenger such as cyclic AMP may ultimately allow cascading signals to occur leading to immune suppression.

1.4.3A2 *DNA*

A second candidate for the target molecule, suggested by the action spectrum of UV-induced immunosuppression [De Fabo and Noonan, 1983] is DNA. However, it is difficult to imagine that DNA itself would serve as a chemical mediator of immunosuppression. It is more likely that UV-induced damage to the DNA of a particular cell present in the skin triggers the release of a chemical mediator that, in turn, initiates the immunological effects. Alternatively, it is possible that repair mechanisms which are error prone may lead to increased mutations resulting in the synthesis of suppressive mediators. An indirect approach to testing whether UV-B induced damage to DNA of a particular skin-target cell might be the initiating event

in the subsequent suppression of CH was carried out by Kripke et al (1983). This group selected another agent i.e. the chemical photosensitizer 8-methoxypsoralen (8-MOP) in combination with UV-A radiation (PUVA), which like UV-B radiation also induces DNA damage in the skin [Pathak et al, 1974] and tested it to ascertain whether it produced similar immunological alterations. Indeed, they found that this treatment as with UV-B irradiation systemically suppressed the CH response. More direct evidence that DNA is the primary target of UV-B radiation and that local and systemic suppression of CH is initiated as a consequence of DNA damage was provided by Applegate et al (1989). These investigators used the Opossum as an animal model because the cells of these marsupials possess an enzyme that is activated by visible light (photoreactivating enzyme) and repairs UV radiation induced pyrimidine dimers in DNA. They reported that exposure of these animals to a single low dose of UV-B radiation prevented them from developing a CH response to DNFB applied either at the site of irradiation or an unirradiated site. By treating the animals with wavelengths that activate the repair enzyme immediately following UV-B irradiation, Applegate et al (1989) were able to demonstrate that the UV-B induced local and systemic suppression generated could be inhibited almost completely. A different approach to further test the hypothesis that UV-induced lesions in DNA are responsible for the immunosuppressive effects of UV-B irradiation, was carried out by Kripke et al (1991). Based on the observation that epicutaneous application of liposomes containing T4 endonuclease V (T4N5 liposomes) to irradiated murine skin, immediately following exposure, increases repair of UV-induced pyrimidine dimers in the skin, these investigators found using this approach that increasing the repair of UV-induced lesions in DNA abrogates the UV-induced systemic suppression of CH and DTH responses.

1.4.3B The Role of UV-B Induced Soluble Mediators in UV-B Induced Immunosuppression

A number of UV-B induced soluble factors have been identified which are capable of suppressing the CH response in mice and thus could be mediators of the indirect effects of UV-B irradiation. These include prostaglandins, IL-1, TNFα and a number of as yet unidentified mediators transiently present in the serum of irradiated mice or induced in epidermal cell cultures following UV-B irradiation. There is also evidence that suppressor T cells induced as a consequence of UV-B exposure produce and release mediators important in suppressing CH.

1.4.3B1 *Prostaglandins*

Numerous studies have shown that indomethacin is able to block the indirect effects of UV-B radiation on immune function and consequently prostaglandins have been implicated as mediators of these activities [Rheins et al, 1987; Chung et al, 1986A; Robertson et al, 1987; Jun et al, 1988]. More direct evidence for the involvement of prostaglandins was provided by Rheins et al (1987) who reported that topical application of various prostaglandins prior to sensitization with DNFB at the same site suppressed the induction of CH.

1.4.3B2 *IL-1*

IL-1 (a cytokine produced by keratinocytes and LC) may be a mediator of the indirect UV-B induced immunosuppressive effects on the CH response. In vivo studies in mice [Gahring et al, 1984] have demonstrated that IL-1 activity can be detected in the serum of subjects 3 days after exposure to high doses of UV-B radiation. Robertson et al (1987) have shown that i.v. administration of pyrogenic doses of IL-1 to normal mice depressed a subsequent immunological response to DNFB. In addition the capacity of IL-1 to depress CH responses in normal mice was due to an indomethacin sensitive process, suggesting that the suppression of the CH

response to DNFB observed, was in fact due to the IL-1 induced generation and action of prostaglandins.

1.4.3B3 *TNFα*

As discussed in section 1.4.2A the phenomenon by which UV-B irradiation mediates the inhibition of the induction phase of CH appears to be determined genetically. Impaired CH following UV-B exposure occurs in some strains of mice (UV-susceptible), but not in others (UV-resistant) [Streilein and Bergstresser, 1988]. Recently, it has been demonstrated that susceptibility to UV-B irradiation is dictated by alleles at the *Lps* and *TNFα* loci which influence the amount of intracutaneous TNFα produced in response to UV-B irradiation [Yoshikawa and Streilein, 1990]. There is now evidence that keratinocytes synthesize and release TNFα following UV-B irradiation [Kock et al, 1990A; Oxholm et al, 1988]. Moreover, TNFα has been shown to act as an important mediator of the suppressive effects of UV-B irradiation on the induction of CH [Yoshikawa and Streilein, 1990].

1.4.3B4 *Mediators Transiently Present in the Serum of Mice*

Swartz (1984) described a factor in serum 2-6hr following UV-B irradiation of mice which suppressed CH. Interestingly, a specific IL-1 inhibitor (serum-contra-IL-1) exhibiting a molecular weight (m.wt.) of 40KDaltons and found at a maximum concentration in sera 24hr following whole-body UV-B exposure of mice was reported by Schwarz et al (1988). Also, a soluble suppressor factor (mwt > 15000 Daltons) was found in serum 3-5 days after UV-B irradiation which was neither antigen-specific or genetically restricted [Harriott-Smith and Halliday, 1988A]. Harriott-Smith and Halliday (1988A) could not detect significant amounts of IL-1 in serum containing suppressive activity, 3 days after irradiation. By increasing the dose of UV-B irradiation to levels used by Gahring et al (1984) who did observe significant amounts of IL-1 in serum 3 days following

UV-B irradiation, they were still unable to detect any significant amounts of IL-1 in the serum.

Harriott-Smith and Halliday (1988A) obtained evidence that the UV-induced serum factor was produced by suppressor T cells. They found that the suppressive effect of UV-B irradiation was abrogated by cyclophosphamide, but that this restored reactivity was still inhibited by serum from irradiated donors, suggesting that the serum factor requires suppressor T cells for its production but not for its action. Yee et al (1990) have provided further evidence that suppressor T cell factors released by UV-induced suppressor T cells play a role in the suppression of CH responses. They showed that a suppressor T cell factor-specific monoclonal antibody inhibited the suppression of CH by inactivating a 45-60K Dalton suppressor T cell factor released by UV-induced suppressor T cells. It was demonstrated previously by Yee et al (1989A and B) that multiple suppressor factors, both hapten-specific and non-specific may be involved in the suppression of CH responses exhibited in UV-B irradiated, sensitized mice.

It is worth noting that each of the investigators mentioned, used different irradiation protocols when attempting to identify their particular serum soluble suppressor factor - some used high doses of UV-B radiation (e.g. Swartz, 1984 used doses of 8600mJ/cm^2) and others used comparatively lower doses of UV-B radiation (e.g. Harriott-Smith and Halliday, 1988A used doses of 780mJ/cm^2). This is likely to account for the variety of suppressor factors identified, at various time intervals after UV irradiation.

1.4.3B5 *Mediators Induced in Epidermal Cell Cultures*

It has been observed that a factor in supernatants from normal as well as transformed keratinocytes, UV-B irradiated in vitro, suppresses the induction but not elicitation of CH after i.v. injection [Schwarz et al, 1986]. Due to the high m.wt of this factor (15-50K Dalton) and the fact that treatment with indomethacin before

UV-B irradiation did not abolish the suppression of CH Schwarz et al (1986) suggest that it was not a prostaglandin. The specificity of the suppression induced by this factor was not demonstrated. In addition, under identical conditions the same cells and cell lines produce a closely related, if not identical factor, exhibiting a m.wt. of 40KDaltons which blocks the biological activity of IL-1 specifically [Schwarz et al, 1987]. Interestingly, Kim et al (1990) have reported that UV irradiated keratinocytes release multiple suppressor factors each having different immunosuppressive properties and each being produced by different wavelengths of UV radiation. They found that i.v. injection of supernatants into mice obtained from epidermal cell cultures or transformed keratinocytes (Pam212 cells) exposed to UV-B (280-320nm) radiation suppressed the induction of DTH to alloantigens and TNP-modified self-antigens in syngeneic and allogeneic mice, while having no effect on the induction of CH to TNCB. On the other hand, treatment of the cell cultures with UV-A (320-420nm) radiation induced the release of a factor which suppressed CH but not DTH. Further analysis of the suppressive factor released by epidermal cells exposed to UV-B radiation revealed that it is a glycoprotein, perhaps 68KDaltons in m.wt. and its suppressive activity is not H-2 restricted [Ullrich et al, 1990]. It has also been reported that PUVA-treated keratinocytes release suppressive factors that suppress both CH and DTH responses, depending on the dose of UV-A radiation used [Aubin et al, 1991]. If keratinocytes are treated with 8-MOP followed by $50mJcm^{-2}$ UV-A radiation the release of suppressive factors that depressed both DTH and CH was induced, whereas treatment of the keratinocytes with 8-MOP plus $5mJcm^{-2}$ UV-A radiation induced a suppressive factor that only inhibited CH.

1.4.3C Possible Targets and Mechanism of Action of those Soluble Factors that Mediate the Indirect Effects of UV-B Irradiation

Each of the UV-B induced soluble suppressor factors identified are believed to be involved in both local and systemic suppression of CH. Further, they

may function alone or in combination with other factors to carry out their role in UV-B induced immunosuppression of CH. It is also possible that they are all part of one or more pathways and thus each rely on the production of another factor before they themselves can be formed.

The immunological target activity which is imparied/altered by those factors that mediate the indirect effects of UV-B irradiation is uncertain. There is evidence in the literature to suggest that these factors may act to: 1) inhibit or alter the function of antigen-presenting cells [Noonan et al, 1988; Araneo et al, 1989; Ullrich et al, 1990; Ross et al, 1987, 1988 and 1987/88; Robertson et al, 1987]; 2) alter the normal trafficking pattern of T lymphocytes, with the result of diverting cells away from sites of active immunological reactions [Spangrude et al, 1983; Chung et al, 1986B]; 3) stimulate other cells to release soluble suppressor factors [Harriott-Smith and Halliday, 1988B; Kurimoto and Streilein, 1992] or 4) influence the migration of epidermal DC [Cumberbatch and Kimber, 1992]. It is likely that some or all of these effects are expressed and may act together or independently in suppressing the system.

1.4.3C1 *Mediators that Interfere with the Function of Antigen-Presenting Cells*

Associated with the systemic suppression of CH, alterations in the function of antigen-presenting cells present in the spleen have been observed [Greene et al, 1979; Noonan et al, 1981A]. This observed antigen-presenting cell defect was associated with the formation of antigen-specific suppressor T lymphocytes. De Fabo and Noonan (1983) postulated that this antigen-presenting cell defect and the resultant formation of antigen-specific suppressor T cells is initiated by the UV-B induced formation of the cis isomer of UCA. Support for this notion was later provided by Noonan and her colleagues (1988) who demonstrated that i.v. administration of the UV-B induced isomer of UCA, cis-UCA, like in vivo

UV-B irradiation, depresses the antigen-presenting function of splenic DC. Trans-UCA did not have this effect.

More direct evidence that the UV-B induced production of cis-UCA is important in inducing the formation of suppressor T lymphocytes is available. Ross et al (1987 and 1988) demonstrated that cis-UCA-induced local and systemic suppression of the DTH response to HSV was antigen-specific and mediated by suppressor T cells of two phenotypes, namely $L3T4^{+}Ly2^{-}$ and $L3T4^{-}Ly2^{+}$. An experiment involving the transfer of epidermal cells, from mice skin-painted 24hr previously with cis-UCA, to naive syngeneic recipient mice at the same time and site of infection with HSV revealed that only one T subset of phenotype $L3T4^{+}Ly2^{-}$ was generated by modified epidermal cell transfer [Ross et al, 1987/88]. This suggested that two signals may be generated by cis-UCA, one local inducing the $L3T4^{+}Ly2^{-}$ subset possibly through a unique antigen presenting event in skin and the other systemic,interpreted by antigen-presenting cells possibly in the lymph node or spleen and inducing either $L3T4^{-}Ly2^{+}$ alone or both $L3T4^{+}Ly2^{-}$ and $L3T4^{-}Ly2^{+}$. These findings exactly mimicked the immunosuppressive effect of UV-B irradiation on the DTH response to HSV [Howie et al, 1986A and B; Howie et al, 1987].

There is evidence from a number of studies which indicate that other soluble suppressor factors may act on antigen-presenting cells, modifying their function to induce the formation of antigen-specific suppressor T cells. Associated with the suppression of the CH response induced by injecting recombinant IL-1 into mice was the formation of suppressor T cells in the spleen of these mice [Robertson et al, 1987]. The findings of Simon et al (1990 and 1991) show that UV-B radiation abrogates the capacity of LC to present antigen to TH1 cells (effectors of DTH responses) while not affecting their ability to stimulate the proliferation of TH2 cells. In the context of these studies Araneo et al (1989) provided evidence which suggests that this altered antigen presenting function of LC may be mediated through an IL-1 dependent mechanism. Further, Ullrich et al (1990) found that a

factor released from UV-B irradiated keratinocytes which suppressed the DTH response to alloantigens induced the formation of an antigen-specific CD4+ suppressor T cell in the spleen.

Although, in systemic suppression of CH, there is evidence that UV-B radiation may mediate its indirect effect through the release of mediators which alter the function of antigen-presenting cells in the spleen, such that upon encountering antigen they induce the formation of suppressor T cells, investigators have not been able to demonstrate alterations in the function of antigen-presenting cells at the site of antigen application [Noonan et al, 1984; Lynch et al, 1983] or in the DLN [Kripke and McClendon, 1986; Alcalay and Kripke, 1991; Chung et al, 1986A].

It is not clear how suppressor T cells are generated in the spleen, after epicutaneous introduction of haptens through unirradiated or irradiated skin. One possibility is that antigen-binding cells in the DLN represent only one of several antigen-presenting cell populations capable of inducing CH. A second pathway, not involving cells in the DLN may be altered by the direct and/or indirect effects of UV-B radiation, and, upon encountering antigen, transport it to the spleen where suppressor T cells are induced. However, the existence of such alternative pathways for contact sensitization has not been demonstrated. A second possibility is that soluble mediators released from UV-B irradiated skin alter the vascular permeability of both exposed and unexposed skin, causing an influx of the hapten into the bloodstream which could then gain access into the spleen. The uptake of antigen by splenic antigen-presenting cells following exposure of these cells to UV-B induced soluble mediators could then generate suppressor T cells.

1.4.3C2 *Alteration in the Normal Trafficking Pattern of T lymphocytes by Soluble Mediators*

Studies by Spangrude et al (1983) have demonstrated that exposure of mice to UV radiation causes alterations in normal lymphocyte recirculation, with an

enhancement of the localization potential of normal lymphocytes for the DLN. However, an increase in the ability of circulating lymphocytes to localize into peripheral lymph nodes of UV-irradiated animals may not be the only explanation for the increase in the size and lymphocyte content of the lymph nodes which drain the exposed skin. It is feasible that a decreased rate of lymphocyte egress might also be taking place. This type of delay in lymphocyte migration through lymph nodes has been termed "efferent blockade", and is associated with a reduction in the numbers of lymphoid cells found in the efferent lymphatic drainage of antigen-stimulated nodes [Chung et al, 1986B]. Chung et al (1986B) showed that exposing mice to UV radiation induces an efferent blockade of lymphocyte egress from the peripheral lymph nodes which drain the irradiated skin, resulting in a marked retention of lymphocytes. More importantly, they established that prostaglandins may be involved in the generation of an efferent blockade. Although, the exact site of their action within the lymph node still remains to be defined, this observation suggests another mechanism by which mediators of the indirect effects of UV-B radiation could cause systemic and local suppression of CH.

1.4.3C3 *Stimulation of Other Cells to Release Soluble Suppressor Factors*

Palaszynski et al (1992) reported that cis-UCA can actively down-regulate the production of cyclic AMP within dermal fibroblasts in vitro and proposed that this may stimulate these cells to produce and release systemic factors responsible for immunosuppression. Further support for the notion that cis-UCA may act on skin cells to produce soluble mediators of suppression was provided by Kurimoto and Streilein (1992). These investigators were able to demonstrate that the reduced capacity of skin, injected i.d. with cis-UCA, to support the induction of CH to DNFB painted subsequently on to the injected skin site could be reversed by intraperitoneal (i.p.) injection of anti-TNFα antibodies, suggesting that cis-UCA achieves its inhibitory effects, al least in part, through TNFα release. Kurimoto and

Streilein (1992) suggested that cis-UCA may achieve its effect by binding to a receptor on an appropriate target skin cell (such as a keratinocyte) and inducing this cell to activate its TNFα genes (which are transcriptionally silent in normal epidermis). Thus, TNFα released intraepidermally could then act to suppress the induction of the CH response.

Harriott-Smith and Halliday (1988B) reported that production of the suppressive factor they identified was dependent on the UV-induced conversion of trans-UCA in the skin to the more soluble cis-form. Harriott-smith and Halliday (1988B) proposed that cis-UCA interacts directly with suppressor T cells which subsequently appear in the spleen and stimulates them to produce the circulating suppressor factor(s) that inhibit induction of CH.

1.4.3C4 *Mediators that Influence the Migration of Epidermal Dendritic Cells*

Cumberbatch and Kimber (1992) demonstrated that i.d. injection of murine recombinant TNFα causes a rapid and concentration-dependent accumulation of DC in lymph nodes draining the site of application. Since keratinocytes have the potential to synthesize and secrete TNFα following UV-B irradiation [Kock et al, 1990A; Oxholm et al, 1988] it is conceivable that the production and/or release of this cytokine after UV exposure may have a profound local and/or systemic effect on the migration of LC to DLN prior to sensitization, thus influencing the number of available LC to pick up antigen and carry it to the lymph node. Further there is some evidence that cis-UCA may influence epidermal LC migration. Treatment with cis-UCA resulted in a reduction in the number of Ia^+ cells at the site of application while trans-UCA had no effect [Ross et al, 1987/88]. Also, Norval et al (1990) found that cis-UCA reduced the numbers of ATPase positive cells in the epidermis at the site of treatment.

1.5 The Aims of this Investigation

The main aim of the first part of this research project was to elucidate the mechanism of UV-B induced immunosuppression and to establish whether the effects of UV-B radiation are mediated by cis-UCA, using a murine model of CH.

As previously discussed in the Introduction, the available evidence suggests that following epicutaneous application of a contact sensitizer, LC at the site pick up antigen and migrate via the afferent lymphatics to the DLN, a process which appears to be essential for the induction of CH responses. Thus any alteration or inhibition in this migratory behaviour would have profound implications for the impairment of the CH response. Indeed there is evidence that UV-B irradiation or topical application of cis-UCA decrease epidermal LC numbers at the site of treatment suggesting that the UV-B induced formation of cis-UCA and subsequent stimulation of epidermal LC migration away from the skin prior to sensitization on the exposed site may be one or part of the mechanism of UV-B induced immunosuppression. However, since it has been revealed that the presence or absence of a particular cell surface marker is not necessarily evidence of the presence or absence of the particular cell, there is some controversy as to the validity of these findings. Hence, in the first part of the study the influence of UV-B irradiation and UCA isomers on DC accumulation in DLN of unsensitized and sensitized mice was examined. Also, since it has been demonstrated that LC from irradiated skin have an impaired ability to present antigen, another aim was to investigate the influence of UV-B radiation and UCA isomers on the antigen-presenting function of lymph node DC.

Cis-UCA has been demonstrated to be a mediator of the suppressive effects of UV-B radiation in a number of systems including CH. How cis-UCA alters immune function is not known or, indeed if it is confined solely to the epidermis following UV-B irradiation. Establishing if this molecule is able to leave the skin

and thus act systemically would provide some insight into its possible mode of action. Thus, the aim of the second part of the project was to investigate the localization of cis-UCA following UV-B irradiation of mice. The first approach involved an analysis of various tissues from mice exposed to UV-B radiation using high performance liquid chromatography (HPLC). The second approach involved the production of a monoclonal antibody with specificity for cis-UCA which could be used in subsequent experiments to detect cis-UCA in serum and other tissues following UV-B irradiation of mice.

CHAPTER 2

MATERIALS AND METHODS

2.1.1 Mice

Female and male Balb/c, C3HBu/Kam and C57BL/6 mice were bred, maintained and supplied by the Department of Medical Microbiology Animal House, University of Edinburgh. Female C3HBu/Kam mice were used throughout unless stated otherwise. The mice were aged 6-8 weeks at the start of each experiment and did not differ in age by more than 2 weeks within each experiment. They were housed in a room where ambient light was regulated on a 12hr light/dark cycle.

2.1.2 Growth Media

2.1.2A RPMI-FCS Medium

This medium consisted of RPMI-1640 growth medium (Flow Laboratories Ltd., Irvine, Ayrshire) supplemented with 5 x 10^{-5}M 2-mercaptoethanol, 100i.u./ml penicillin, 200µg/ml streptomycin, 2mM L-glutamine, 100µg/ml gentamicin, 20µg/ml fungizone and 10% heat-inactivated foetal calf serum.

2.1.2B R10 Medium

This medium consisted of RPMI-1640 growth medium supplemented with 50µg/ml penicillin, 50µg/ml streptomycin, 2mM L-glutamine, 2.5µg/ml fungizone, 100µg/ml kanamycin, 5 x 10^{-5}M 2-mercaptoethanol, 2mM HEPES, 1mM sodium pyruvate and 10% heat-inactivated foetal calf serum.

2.1.2C R20 Medium

This medium was identical to the growth medium described in section 2.1.2B except that it was supplemented with 20% heat-inactivated foetal calf serum.

2.1.2D HAT Medium

This medium consisted of 500ml of R20 medium and 10ml of hypoxanthine (5×10^{-3}M), thymidine (8×10^{-4}M) and aminopterin (2×10^{-5}M) (HAT) solution (x50) (Sigma).

2.1.2E HT Medium

This medium consisted of 500ml of R20 medium and 10ml of hypoxanthine (5×10^{-3}M) and thymidine (8×10^{-4}M) (HT) solution (x50) (Sigma).

2.2 UV Irradiation

Mice were exposed to one of two different types of UV radiation. They were irradiated under two Philips TL-20W/12 bulbs with an output of 80μW/cm^2 in the UVB broad band range between 270-350nm (Figure 2) or under a Philips TL-01 lamp with an output of 200μW/cm^2 in the UVB narrow band range of 311-312nm (Figure 3).

The backs of mice were shaved with an electric clipper and both or none of their ears were protected with autoclave tape during irradiation. Unshaven mice had one or none of their ears protected. The mice were irradiated for various times in separate compartments of a high-sided perspex box to prevent shielding by cage mates.

2.3 Extraction and Quantification of UCA Isomers

The backs of fifteen female mice of each strain of C3HBu/Kam, Balb/c and C57BL/6 mice were shaven and their ears left unprotected. Five female mice from

each of these strains were either exposed to a single dose of 96mJcm^{-2} UV-B radiation, were sensitized on the dorsum of both their ears with 25µl of 2.5% oxazolone (as described in section 2.5.1) or left untreated. The backs of five male mice from each of these strains were also shaved and their ears left unprotected. Mice irradiated with UV-B or sensitized were killed 48 and 24hr later, respectively. Untreated male and female mice were killed at the same time. After killing by anaesthetisation their ears were removed. Lymph nodes, spleens and kidneys from untreated and irradiated female C3HBu/Kam mice were also removed. Prior to killing urine was collected from these mice. Tissues and urine were stored in the dark at -20°C.

2.3.1 Extraction of UCA Isomers

The method outlined by Norval et al (1988) was used. In brief after measuring the wet weight of a single ear or organ from the appropriate strain of mouse, it was then placed in a small glass tube with 100mg fine sand and 100µl 0.1M NaOH, and homogenized by grinding by hand with a glass rod, while cooling in ice, until no visible particles of tissue remained. Nine hundred µl 0.1M NaOH was used to rinse the glass rod and walls of the tube. After allowing the contents of the tube to settle, the supernatant was centrifuged at 2000g for 5 min. The supernatant was filtered through a 1.2µm Gelman Metricel filter. The volume of the resulting clear filtrate was measured and one-tenth of the volume of 0.5M phosphoric acid added to give a solution of pH 7. Urine was analysed directly by HPLC.

2.3.2 Quantification of UCA Isomers

HPLC analysis of urine and tissue extracts from individual mice was performed by Mr. T. Reid at Bristol University using the method of Norval et al (1988). Analysis was carried out on a system consisting of a Waters 6000A solvent delivery system, a Rheodyne 7125 sample injection valve with a 20µl loop and a

Waters M440 UV-detector set at 254nm combined with a plotting integrator (Spectra-Physics Autolab Minigrator). The column was a 15 cm x 4.6 mm ID cartridge column (Capital HPLC, Bathgate, Scotland) packed with 5µm APS Hypersil. The mobile phase was 10mM acetic acid adjusted to pH 5 with 2M NaOH. The flow rate was 1.0 ml/min. UCA isomers in the samples were quantified by using standard solutions of cis-UCA and trans-UCA (see below) and comparing the peak areas. Typical elution peaks of a standard solution of cis and trans-UCA is shown in Figure 5 .

2.4 Preparation of UCA Isomers and Treatment of Mice

Trans-UCA (Sigma Chemical Co., Poole, Dorset) or cis-UCA (prepared by Professor T. Simpson's Laboratory in Bristol University using preparative thin layer chromatography of UVB irradiated trans-UCA [Norval et al,1989B]) was dissolved at a concentration of 40mg/ml in dimethylsulfoxide (DMSO) at 30°C for 5 min. The solution was then diluted 10, 20, 40, 200 or 2000-fold in acetone and 50µl applied topically to the shaved backs of mice. Alternatively, two lots of 25µl or a single application of 25µl was similarly applied to the dorsum of one or both ears. The solution was also diluted 20 or 40-fold in phosphate buffered saline (PBS) and 100µl injected i.p. or s.c. into the flank. An equal volume of the appropriate vehicle was applied in the same way to control mice. The mice were treated at various times with the appropriate dilution of the solution.

2.5 Contact Hypersensitivity Response

2.5.1 Induction and Expression of Contact Hypersensitivity

Fluorescein isothiocyanate (FITC, Sigma) was used at various concentrations in 1:1 acetone:dibutylphthalate [Kimber et al, 1990A]. 4-

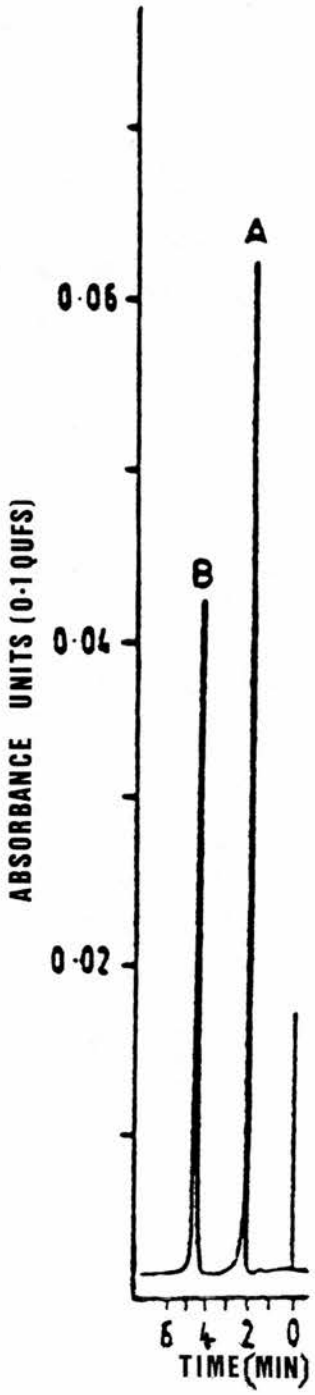

Figure 5 Chromatogram of urocanic acid standard solution containing 5µg/ml of each isomer. Peak A corresponds to cis-isomer and peak B to trans-isomer (taken from Norval et al, 1988).

ethoxymethylene-2-phenyloxazol-5-one (oxazolone; Aldrich Chemical Co.) was used at various concentrations in 4:1 acetone:olive oil [Kimber et al, 1990A]. Fifty µl of oxazolone or FITC or an equal volume of the appropriate vehicle was applied topically to the shaved backs of mice on day 0. CH was elicited on day 5 or 6 by challenging the dorsum of both ears with 25µl of oxazolone or FITC (as appropriate) or with an equal volume of the appropriate vehicle alone. Alternatively, mice received 25µl of oxazolone or FITC or an equal volume of the appropriate vehicle on the dorsum of their left ear on day 0 and were challenged with 25µl of oxazolone or FITC (as appropriate) or an equal volume of the appropriate vehicle on the dorsum of their right ear on day 5 or 6.

Ear thickness was measured using a spring loaded caliper (Figure 6) 24hr following challenge and compared with ear thickness prior to challenge. The increment in ear swelling was used as a measure of the development of CH. Mean values for incremental ear swelling and standard errors of the mean were calculated for each group. A minimum of 5 mice were used per group.

2.5.2 Suppression of Contact Hypersensitivity

Shaved mice were UV irradiated with both their ears protected whilst unshaven mice had their right ear protected during irradiation. UCA isomers were applied topically to either the shaved backs of mice or to the left ear of unshaven mice. Twenty-four hr after the last UV irradiation or UCA treatment, mice were sensitized with the appropriate contact sensitizer at the same site as treatment . The percentage suppression of CH was calculated according to the following formula:

$$\% \text{ Suppression} = 1 - \frac{[(\text{experimental} - \text{negative control})]}{[(\text{positive control} - \text{negative control})]} \times 100$$

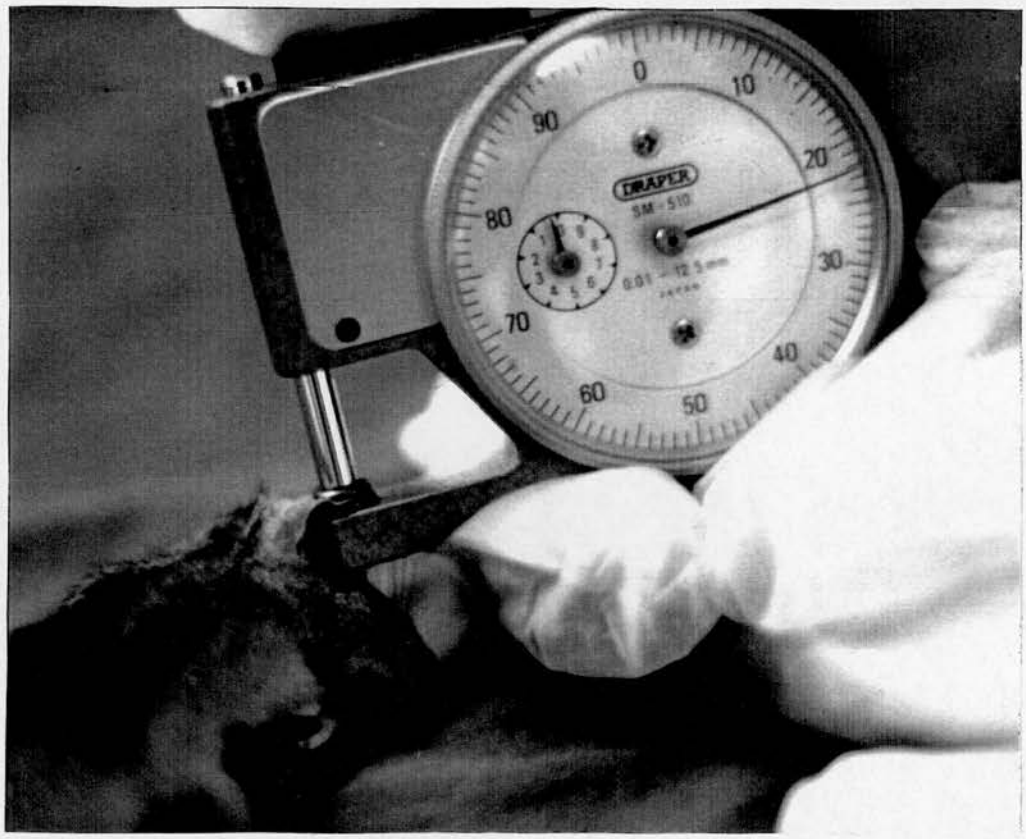

Figure 6 Measuring the thickness of an untreated mouse ear using a spring loaded caliper.

Experimental represents the mean increment in ear swelling of mice treated with UV radiation or UCA isomers prior to sensitization. The positive control represents the mean increment in ear swelling of untreated mice, sensitized and challenged with contact sensitizer. The negative control represents the mean increment in ear swelling of untreated mice sensitized with vehicle and challenged with contact sensitizer [Cruz et al, 1989].

2.6 Influence of UV Irradiation and UCA Isomers on Dendritic Cell Migration and on Migration Induced by Sensitization

2.6.1 Treatment of Mice with and without Contact Sensitization

Unshaven mice whose ears were unprotected, were exposed for various times to UV radiation (at the appropriate dose) or to UCA isomers (at the appropriate concentration) applied topically to the dorsum of both ears. Twenty-four hr after the last UV irradiation or UCA treatment, mice received 25µl of a 1% FITC solution on the dorsum of both ears, and were killed 18hr later. Control mice received an equal volume of the appropriate vehicle on the dorsum of both ears 18hr before killing. Mice not exposed to contact sensitization were killed 12, 24, 36, 42, 48, 60 and 72 hr after the last UV irradiation or UCA treatment.

2.6.2 Isolation, Identification and Enumeration of Lymph Node Dendritic Cells

Draining auricular lymph nodes were excised, pooled for each experimental group which consisted of a minimum of 6 mice and a single cell suspension of lymph node cells was prepared by mechanical disaggregation through a 200-mesh stainless steel gauze (John Stanier and Co., Manchester). Viable cell counts were performed using 0.5% trypan blue and the cell concentration adjusted to 5×10^6 cells/ml RPMI-FCS (Section 2.1.2A). DC-enriched populations were

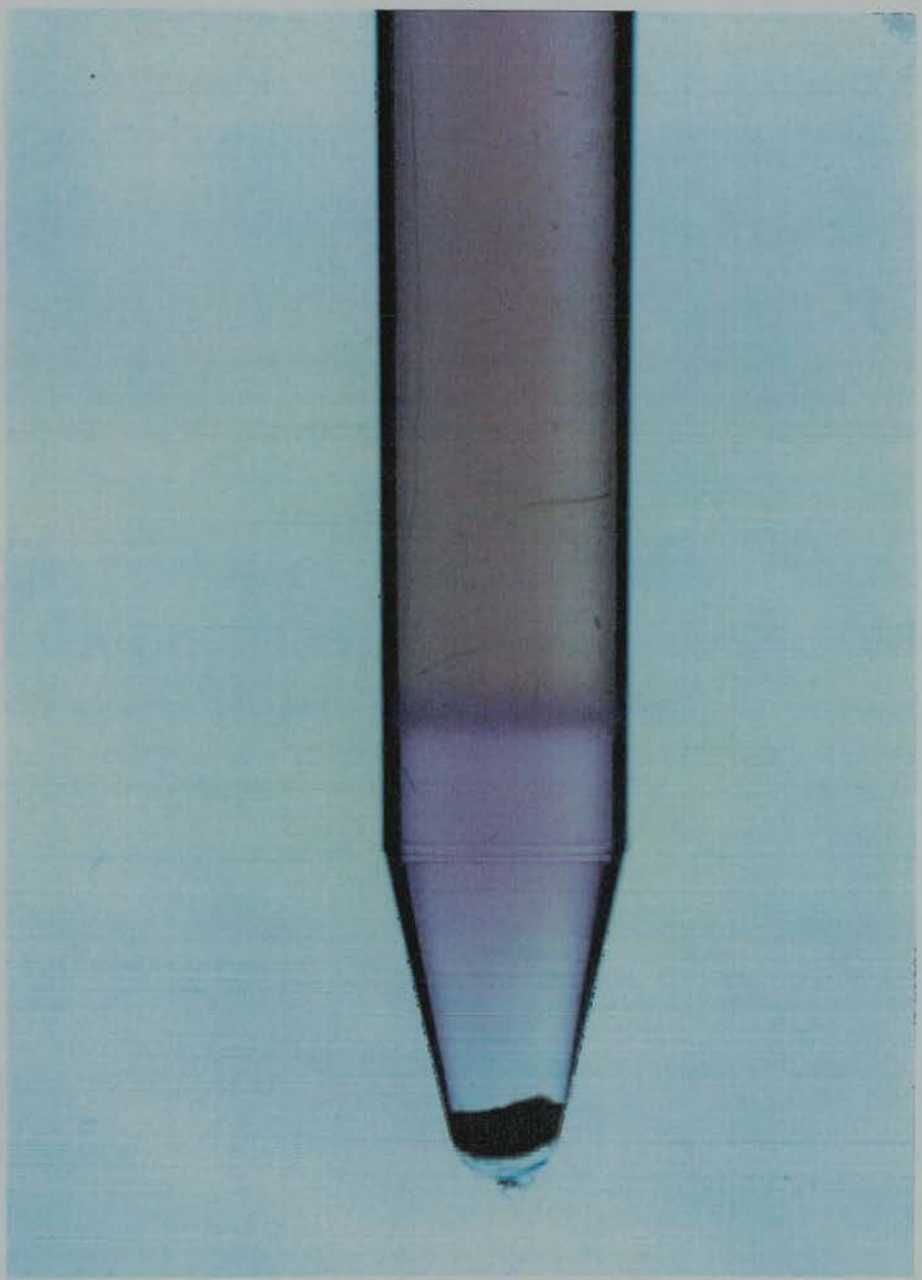

Figure 7 Photograph showing the band (containing a dendritic cell-enriched population) obtained after density gradient centrifugation.

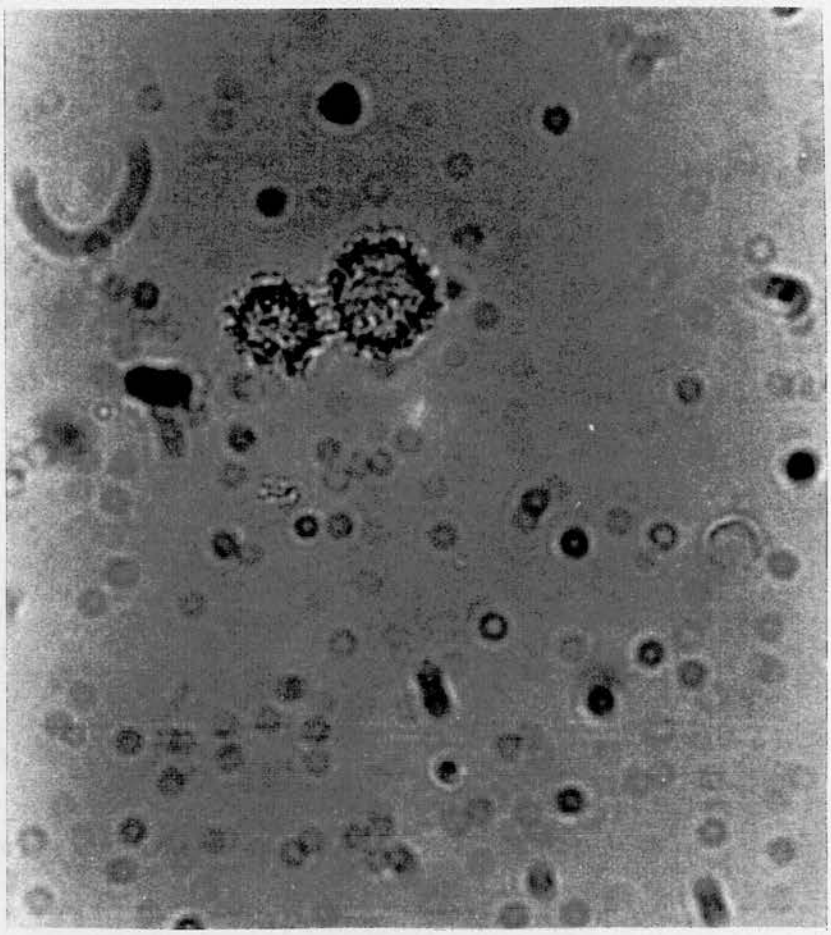

Figure 8 Photograph showing 2 dendritic cells in close contact, under a light microscope (magnification = x400). These DC were derived from the low buoyant density fraction obtained after density gradient centrifugation.

prepared by density gradient centrifugation as described previously [Kinnaird et al, 1989]. Briefly, 8ml of the cell suspension was added to 10ml conical-bottomed test tubes (Sterilin) and was gently underlayed with 2ml of 14.5% metrizamide [Nygaard, Oslo, Norway] in RPMI-FCS. The tubes were then centrifuged for 15 min (600g) at room temperature. The DC-enriched population accumulating at the interface (Figure 7) was collected, washed once and resuspended in RPMI-FCS. The number of DC within the low-buoyant density fraction was assessed routinely by direct morphological examination using light microscopy (Figure 8). For each experimental group, five counts were made and the mean number of DC present within a single lymph node was calculated.

2.6.3 Analysis of FITC-bearing Dendritic Cells

DC-enriched preparations derived from draining auricular lymph nodes of mice whose ears had been exposed to UV irradiation or painted with UCA isomers prior to sensitization with FITC, were analysed by Mr. W Neill on an EPICS "C" flow cytometer (Coulter Electronics) equipped with a 5 Watt (W) argon laser tuned to 488nm wavelength and operating at 100mW. A total of 5000 cells from each sample was analysed at a flow rate of 200 cells per second. DC were identified on a 2-parameter histogram measuring size and side-scatter and then green fluorescence analysed from a bit map onto a 252 channel histogram using log amplification. The percentage of $FITC^+$ cells within this population was measured and also antigen density per cell (fluorescence intensity) by mean channel analysis.

2.6.4 Ia Antigen Staining of Dendritic Cells and FITC-Bearing Dendritic Cells

The ears of mice were either sensitized with FITC or left unsensitized following UV irradiation or the topical application of UCA isomers. DC-enriched preparations derived from draining auricular lymph nodes, were washed and 5 x

10^4 cells stained with anti-mouse Ia monoclonal antibody (clone 11.5.2.1.9.-ECACC, Porton Down, Salisbury) at a dilution of 1:100 for 1hr on ice. The cells were then washed and stained with either a sheep anti-mouse IgG ($F(ab')_2$ fragment) labelled with FITC (Sigma) at a dilution of 1:40 or a sheep anti-mouse IgG labelled with phycoerythrin (Sigma) at a dilution of 1:40 for 40 min on ice. Simultaneously, 5 x 10^4 cells from DC-enriched preparations were incubated with an irrelevant antibody followed by FITC or phycoerythrin-labelled secondary antibody to act as background controls in the EPICS analysis. The cells were analysed in an EPICS "C" flow cytometer by Mr. W Neill. To measure the percentage of DC expressing Ia, the cells were labelled with anti-Ia and FITC secondary antibody before analysis as outlined in the section above. To measure Ia expression on DC after sensitization with FITC, the cells were labelled with anti-Ia and phycoerythrin secondary antibody and a double colour analysis performed in the following manner. Dichroic mirrors were used to separate light into the 90° scatter detector (500 long pass) and into both red (560 short pass followed by a 575/25 band pass filter) and green (530/30 band pass) fluorescence detectors. Any residual spectral overlap of green fluorescence into the red detector was removed by electronic compensation.

2.6.5 ICAM-1 Antigen Staining of Dendritic Cells

Mice were killed 42hr after their last UV radiation treatment. DC-enriched preparations derived from draining auricular lymph nodes were washed and 5 x 10^4 cells were incubated on ice with rat anti-mouse ICAM-1 monoclonal antibody (YN-11.7.4) [Cumberbatch et al, 1992] diluted 1:50 in RPMI-FCS for 45 min. Following incubation the cells were washed in 2ml of cold RPMI-FCS, centrifuged for 5 min (300g) at 2°- 8°C and the supernatant discarded. The cells were incubated on ice for 45 min with a FITC-labelled $F(ab')_2$ rabbit anti-rat IgG antibody (Serotec Ltd) diluted 1:100 with RPMI-FCS. The cells were washed and resuspended in 0.3ml of cold RPMI-FCS and retained on ice until analysis in an

EPICS "C" flow cytometer by Mr. W Neill. Control experiments were performed using an isotype-matched (IgG2b) rat anti-human HLA Class 1 antibody (Clone YTH 862.2; Serotec), in place of anti-ICAM-1. DC were identified as described in section 2.6.3. The percentage of cells expressing ICAM-1 within this population was measured and also the amount of ICAM-1 expression per cell (fluorescence intensity) by mean channel analysis.

2.6.6 Dexamethasone Treatment of Mice

Dexamethasone (Sigma) was dissolved at a concentration of 2mg/ml in sterile PBS. The solution was then diluted 67-fold in sterile PBS and 250µl of this dilution was administered i.p. to mice 5hr and 1hr before UVB irradiation.

2.6.7 Anti-TNFα Antibody Treatment of Mice

Rabbit anti-mouse TNFα antiserum (Genzyme) which had a neutralizing activity of approximately 10^6 neutralizing units/ml was diluted 1:5 with sterile PBS. Two hr prior to each UVB treatment, 100µl of this solution was injected i.p. into each mouse [Yoshikawa and Streilein, 1990].

2.7 Spontaneous Lymphoproliferation Assay

The method outlined by Kimber and Weisenberger (1989) was used. Each experimental group consisted of 3 mice. Unshaven mice whose ears were unprotected, were exposed at various times to the appropriate dose of UVB radiation. Alternatively, at various times, the appropriate amount of UCA isomers was either applied topically to the dorsum of both ears or injected i.p. or s.c. into the flank. Twenty-four hr after the last treatment, the dorsum of both ears of each mouse was then sensitized with 25µl of either 0.25% oxazolone or 0.5% FITC or an equal volume of the appropriate vehicle. Mice that had been sensitized with oxazolone or FITC were killed after 3 and 4 days, respectively and their draining auricular lymph

nodes excised. The lymph nodes were pooled for each experimental group and a single cell suspension of lymph node cells was prepared by mechanical disaggregation through a sterile 200-mesh stainless steel gauze (John Stanier and Co., Manchester). Lymphocyte suspensions were then centrifuged for 10 min (600g) in PBS at room temperature and resuspended in RPMI-FCS growth medium (section 2.1.2A). Viable cell counts were performed using 0.5% trypan blue and the cell concentration adjusted to 7.5 x 10^6 cells/ml. Lymphocyte suspensions were seeded into round bottomed 96-well microtitre plates (Sterilin) at a concentration of 1.5 x 10^6 cells/well (10 wells per group). The wells were then pulsed with 0.75µCi of ^{3}H-thymidine (Amersham) and cultured for 24hr in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were then harvested onto glass fibre discs using an automatic harvester. When dry the discs were counted in a toluene-based liquid scintillator for 1 min. The percentage suppression of spontaneous lymphoproliferation was calculated according to the following formula :

$$\% \text{ Suppression} = 1 - \left[\frac{(\text{experimental} - \text{negative control})}{(\text{positive control} - \text{negative control})}\right] \times 100$$

Experimental represents the mean lymphoproliferative response of lymph node cells from mice treated with UV radiation or UCA isomers prior to sensitization. The positive control represents the mean lymphoproliferative response of lymph node cells from mice sensitized only. The negative control represents the mean lymphoproliferative response of lymph node cells from mice sensitized with vehicle only.

2.8 Secondary Lymphoproliferative Response Assay

2.8.1 Preparation of Stimulator Cells

Mice that had received 25µl of either 5% FITC or 2.5% oxazolone on the dorsum of both ears were killed 18 and 21hr later, respectively. Their draining auricular lymph nodes were excised and DC-enriched populations prepared and counted as described previously (section 2.6.2), with the only difference being that sterile conical-bottom test tubes (Alpha Laboratories Ltd.) and sterile 200-mesh stainless steel gauze was used. The concentration of DC was adjusted so that it was the same for each experimental group.

2.8.2 Preparation of Responder Cells

Mice that had received 25µl of 5% FITC or 0.5% oxazolone on the dorsum of both ears were killed 7 days later. Their draining auricular lymph nodes were removed and a single cell suspension was prepared by mechanical disaggregation through a sterile 200-mesh stainless steel gauze. The cells were washed and resuspended in RPMI-FCS. A viable cell count was made using 0.5% trypan blue and the cell concentration adjusted to 2.5×10^6 cells/ml.

2.8.3 Lymphocyte Proliferation Assay

The method outlined by Jones et al (1989) was used. Briefly, lymph node cells were cultured alone or in the presence of a variable number of stimulator cells in 96-well microtitre plates (Sterilin) at a concentration of 5×10^5 cells/well (5 wells per group) for 48hr at 37°C in a humidified atmosphere of 5% CO_2 in air. Sixteen hr prior to culture termination the cells were pulsed with 0.75µCi of ^{3}H-thymidine (Amersham). Culture was terminated by automatic cell harvesting and ^{3}H-thymidine incorporation determined by β-Scintillation counting. In all experiments the capacity of stimulator populations to incorporate ^{3}H-thymidine when cultured alone was assessed also.

2.9 Production of Anti-Cis-UCA Monoclonal Antibody

2.9.1 Immunization

The KLH-cis UCA conjugate (which consisted of 8 molecules of cis UCA per KLH molecule) used for immunizing the mice was produced and provided by Professor T. Simpson's Laboratory in Bristol University. Three female Balb/c mice were immunized by s.c. injection of 200µg KLH-cis-UCA conjugate in 0.1ml of Freund's incomplete adjuvant per mouse. A first booster injection (200µg of KLH-cis UCA in 0.2ml sterile PBS i.p.) was performed 36 days after the first injection followed by a second and a third boost on day 61 and day 166, respectively. Mice were bled 7 days after the second booster and screened for anti-cis-UCA activity by ELISA.

2.9.2 Somatic Cell Fusion and Cloning

Approximately 10^5 NS-O mouse myeloma cells (a gift from Dr M. McCann) were resuspended in 2ml R10 medium (section 2.1.2B) containing ciprofloxacin (Bayer UK Limited, Berks) at 10µg/ml. The cells were then transferred to a tissue culture flask (Falcon) and cultured in an atmosphere of 5% CO_2 in air at 37°C. The cells were subcultured every few days over a period of 3 weeks in R10 growth medium supplemented with ciprofloxacin (10µg/ml), maintaining maximum cell viability. After 3 weeks the cells were then subcultured 3-4 times in ciprofloxacin-free R10 medium, once again maintaining maximum cell viability. The mouse producing the highest titre of anti-cis-UCA antibodies in polyclonal antisera was killed 3 days after the third booster immunization. Splenocytes (10^8) were collected and fused with 10^7 NS-O cells using a standard fusion technique. Cells obtained after fusion were suspended in HAT medium (section 2.1.2D) containing mixed thymocyte medium (MTM) (prepared as described by Reading, 1982) diluted 1:10 and seeded in 96-well round bottomed microtitre

plates (Sterilin). The wells were then viewed on days 2, 5, 7, and 10 under an inverted light microscope for the presence of HAT-resistant hybridomas. The culture fluids of HAT-resistant hybridomas were tested for anti-cis UCA activity by ELISA at 14 days after fusion. The single positive culture obtained was cloned by limiting dilution (0.5, 1 and 10 cells/well) on feeder layers of MTM diluted 1:3 with HT medium (section 2.1.2E) in 96-well round bottomed microtitre plates (Sterilin). After 4 days the HT medium was replaced with R20 medium (section 2.1.2C) containing MTM diluted 1:3. Following selection of the wells, in which cell growth was detected visually, the culture supernatants were screened by ELISA. A second subcloning procedure was performed on positive microwells. The positive cultures were then expanded, first into flat-bottomed 24-well culture plates (Falcon) and then into culture flasks (Falcon).

2.9.3 Screening by ELISA

An ELISA was used for the detection of anti-cis-UCA antibodies in polyclonal antisera and in culture supernatants of hybridoma cell lines. ELISA plates (Gibco) were coated overnight at 4°C with cis or trans-UCA-BSA conjugate (which consisted of 3.7 molecules of cis-UCA or 4.5 molecules of trans-UCA per BSA molecule and was produced by Professor T. Simpson's laboratory, Bristol University) diluted in 0.1M carbonate-bicarbonate coating buffer, pH 9.6 (5µg in 100µg/well). The plates were then washed extensively with PBS-Tween pH 7.2. Antisera or culture supernatants were left undiluted or diluted from 1:100-1:12800 in PBS-Tween with 1% Bovine serum albumin (BSA; Sigma), and 100µl added to each well in triplicate. The plates were then incubated for 3hr at room temperature. The plates were washed again and incubated with a 100µl of anti-mouse IgG alkaline phosphatase (Sigma Chemical Co.) conjugate (diluted 1:1000 in PBS-Tween with 1% BSA) for 3hr at room temperature. The plates were washed once again and the reaction product was then developed by adding 100µl of alkaline

phosphate substrate buffer pH 9.8 (9.5ml diethanolamine, 80ml distilled H_20, 5ml 1M HCl and 1mg/ml p-nitrophenyl phosphate) to each well. The reaction was stopped after 20 min with 3M NaOH (50µl per well). Absorbance was read at 405nm using an ELISA plate reader.

The negative control used for antiserum was serum drawn from the mouse prior to immunization and in the case of hybridoma cell supernatants, the supernatant from the non-secreting NS-O myeloma cell line used for fusion. The positive control used for antiserum was a rabbit anti-UCA polyclonal antisera (diluted 1:100), derived from a rabbit immunized s.c. on 2 sites with 4500µg KLH-cis-UCA (1:8) in 1ml Freund's incomplete adjuvant on day 0, 14 and 33, and bled on day 47. The rabbit antisera had previously been demonstrated in an ELISA to react with cis or trans-UCA-BSA conjugate. An anti-rabbit IgG alkaline phosphatase (Sigma Chemical Co.) conjugate (diluted 1:1000) was used as the secondary antibody in this case. Mouse antiserum was used as the positive control for hybridoma cell supernatants. The test was considered positive if the mean absorbance was above the mean absorbance of negative control wells plus 3 times the standard deviation.

In other assays histamine-BSA conjugate (produced and provided by Professor T. Simpson's laboratory, Bristol University) key hole limpet haemocyanin (KLH; Sigma) and BSA (Sigma) were used as antigen, all at 5µg in 100µl/well.

2.9.4 Characterization of Anti-Cis-UCA Monoclonal Antibody

2.9.4A Typing of Anti-Cis-UCA Monoclonal Antibody

The immunoglobulin subclass of the monoclonal antibody generated was determined by an isotyping kit according to the manufacturer's recommendations (Amersham).

2.9.4B Analogues Used

Various analogues [Norval et al, 1989B] of UCA (all produced and provided by Professor T.Simpson's laboratory, Bristol University) (Figure 9) including cis and trans-UCA isomers conjugated with KLH (where there are 62 molecules of trans-UCA and 29 molecules of cis-UCA per KLH molecule), histamine-BSA conjugate, histamine (Sigma Chemical Co.) and histidine (Sigma Chemical Co.), were used in competitive inhibition ELISA assays, in order to ascertain the specificity of the monoclonal antibody generated.

2.9.4C Competitive Inhibition ELISA

ELISA plates (Gibco) were coated overnight at 4°C with cis-UCA-BSA conjugate diluted as appropriate in 0.1M carbonate-bicarbonate coating buffer, pH 9.6. The plates were then washed extensively with PBS-Tween, pH 7.2. The appropriate inhibitor was dissolved at a concentration of either 20, 5 or 1 mg/ml in DMSO at 37°C for 5 min and then diluted to the required concentration in PBS-Tween with 1% BSA. Fifty µl of the inhibitor solution was added to each well in triplicate or more followed by 50µl of monoclonal antibody culture supernatant (diluted as appropriate in PBS-Tween with 1% BSA). The plates were then incubated for 3hr at room temperature after which time the plates were washed and the procedure carried out as previously described in section 2.9.3.

The background control used was 100µl of PBS-Tween with 1% BSA instead of 50µl of inhibitor and 50µl of monoclonal antibody. The appropriate positive control used depended on the solution the inhibitor had been dissolved in.. The first consisted of 50µl of DMSO dissolved as appropriate in PBS-Tween with 1% BSA and 50µl of monoclonal antibody cell supernatant. The second consisted of 50µl of PBS-Tween containing 1% BSA and 50µl of monoclonal antibody cell supernatant.

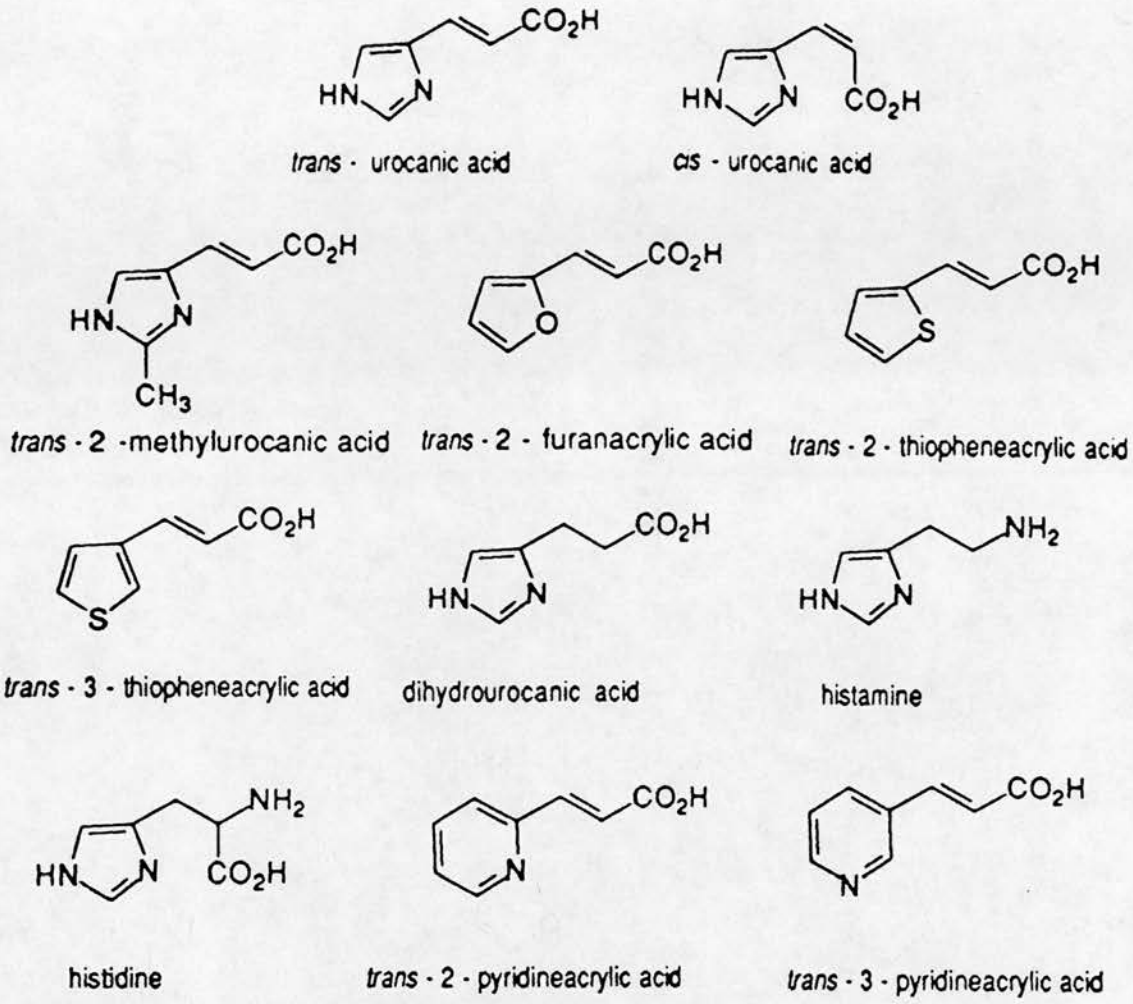

Figure 9 Structure of urocanic acid isomers and analogues.

Inhibition of the monoclonal antibody was calculated as follows:

$$\% \text{ Inhibition} = 1 - \frac{[(\text{mean absorbance test} - \text{mean absorbance background})]}{[(\text{mean absorbance max} - \text{mean absorbance background})]} \times 100$$

Mean absorbance max represents maximal binding of the monoclonal antibody without inhibitor, whereas mean absorbance test represents binding in the presence of an inhibitor. Mean absorbance background is the average background. The standard deviation of the mean absorbance max, test and background was less than 10%.

2.9.5 Purification of Anti-Cis-UCA Monoclonal Antibody by Protein G Affinity Purification

This was carried out as outlined by Hudson and Hay (1989). In brief, 1ml of Protein G-Agarose (Calbiochem Co.) was added to a 1ml syringe with a nylon wool plug at its base. The syringe was packed until the beads settled at the 1ml mark. The syringe column was equilibrated by washing the column with 3ml of PBS. Monoclonal antibody supernatant was diluted 1:2 in PBS and then 10ml was applied to the column. Unbound proteins were washed through with 3ml of PBS. Bound IgG monoclonal antibody was then eluted with 3ml glycine-HCl buffer (0.1M pH 2.8). The pH of the purified IgG solution was titrated to near neutrality with Tris buffer pH 7.48 and then dialysed against PBS. The column was regenerated by washing with 3ml of PBS.

The eluted IgG fraction, the fraction obtained during the loading of the column with the monoclonal antibody supernatant and the fraction collected during the wash were all screened for anti-cis-UCA monoclonal antibody activity by ELISA. ELISA plates were coated overnight at 4°C with cis-UCA-BSA conjugate diluted in 0.1M carbonate-bicarbonate coating buffer, pH 9.6 (0.078µg in 100µl/well) and the procedure carried out as described previously in section 2.9.3.

2.9.6 Protein Estimation

The protein concentration of affinity purified and unpurified monoclonal antibody cell culture supernatant was determined by a protein assay kit according to the manufacturer's recommendations [Biorad].

2.9.7 Anti-Cis-UCA Monoclonal Antibody Treatment of Mice

Affinity purified anti-cis-UCA monoclonal antibody (0.05mg/ml) was injected (100µl) i.p. 2hr prior to each UVB treatment.

2.9.8 Screening of Mouse Serum for Cis-UCA Using Competitive Inhibition ELISA

2.9.8A Treatment of Mice

Mice were bled via the orbital vein and 5hr later shaved mice were exposed once to 216mJ/cm^2 of UVB radiation or were exposed twice to 144mJ/cm^2 of UVB radiation (with an interval of 24hr before the second exposure). At various times thereafter two mice out of a group of ten or fourteen were bled from the vena cava until death and the serum from each mouse pooled.

2.9.8B Competitive Inhibition ELISA

2.9.8.B1 *Titration of Inhibitory Effect of Cis and Trans-UCA in Normal Mouse Serum*

Cis or trans-UCA was dissolved at a concentration of 2mg/ml in distilled water at 37°C for 10 min. This solution was then diluted 1:2, 1:2.7, 1:4, 1:8 and 1:20 in PBS-Tween containing 1% BSA and then each dilution further diluted 1:25 in normal mouse serum (diluted 1:3 in PBS-Tween with 1% BSA) in order to ensure that the mouse serum was diluted to the same extent regardless of the concentration of UCA. Fifty µl of each dilution was then added to each of 6 wells of an ELISA plate

coated with cis-UCA-BSA conjugate (at 0.078µg/well) and washed as described in section 2.9.3 followed by 50µl of monoclonal antibody culture supernatant (diluted as appropriate in PBS-Tween with 1% BSA). The plates were then incubated for 3hr at room temperature after which time the plates were washed extensively and the procedure carried out as previously described in section 2.9.3.

The negative control used was 50µl of normal mouse serum diluted 1:3 in PBS-Tween with 1% BSA and 50µl of PBS-Tween with 1% BSA. The positive control consisted of normal mouse serum diluted 1:3 in PBS-Tween with 1% BSA and 50µl of monoclonal antibody culture supernatant. The percentage inhibition was calculated as described in section 2.9.4C.

2.9.8B2 *Screening of Serum for Presence of Cis -UCA*

ELISA plates were coated with cis-UCA-BSA conjugate (at 0.078µl/well) and washed as described in section 2.9.3. Prebleed serum and serum from mice exposed to UVB radiation was diluted 1:3 in PBS-Tween with 1% BSA. Fifty µl of the diluted serum was added to each of 6 wells or more followed by 50µl of monoclonal antibody culture supernatant (diluted as appropriate in PBS-Tween with 1% BSA). The plates were then incubated for 3hr at room temperature after which time the plates were washed extensively and the procedure carried out as described previously in section 2.9.3.

The negative control used was 50µl of prebleed serum or serum from mice exposed to UVB radiation diluted 1:3 in PBS-Tween with 1% BSA and 50µl of PBS-Tween with 1% BSA. This value was subtracted from the test value. A further control incorporated in this assay involved adding 50µl of cis or trans-UCA which was prepared and diluted appropriately as described in section 2.9.8B1, to each of 4 wells followed by 50µl of monoclonal antibody culture supernatant. The percentage inhibition was calculated as described in section 2.9.4C.

2.9.8B3 *Screening of Dialysed Serum for Presence of Cis-UCA*

One ml of prebleed serum and 1ml of serum taken from a group of ten mice 25hr after one exposure to 216mJ/cm^2 of UVB radiation, was dialysed using Visking tubing with a m.wt. cut off in the range 12,000 to 14,000 for 14hr against PBS at 4°C. The volume of the dialysed serum was measured and diluted (in PBS-Tween with 1% BSA) such that it was diluted 1:3 overall. Dialysed and undialysed serum were then compared using a competitive inhibition ELISA as described in section 2.9.8B2. The percentage inhibition was calculated as described in section 2.9.4C.

CHAPTER 3

RESULTS

3.1 Quantification of UCA Isomers in Murine Tissue Before and After UV-B Irradiation or Contact Sensitization

3.1.1 Introduction

It has been known for several years that UV-B irradiation of mammalian skin, suppresses some cell-mediated immune responses to a variety of antigens, including contact sensitizers [Noonan et al, 1981A and C; Fisher & Kripke, 1978; Howie et al, 1986A; Giannini, 1986; Mottram et al, 1988;]. It has been postulated that there is a photoreceptor in the skin which mediates the indirect effects of UV irradiation on immune function. One candidate, first proposed by De Fabo and Noonan (1983), is UCA found naturally in the stratum corneum of the epidermis as the trans isomer, which converts to the cis-isomer on UV irradiation. There is now considerable evidence available from a variety of experimental systems that cis-UCA acts as a mediator of UV-induced immunosuppression [Noonan et al, 1988; Harriott-Smith and Halliday, 1988B; Ross et al, 1986; Williams et al, 1990; Guymer and Mandel, 1990; Reeve et al, 1989; Gruner et al, 1992].

3.1.2 Analysis of UCA Isomers and Total UCA Content in Ears from Male and Female Mice of Different Strains, Before and After UV-B Irradiation or Contact Sensitization

UCA isomers have been measured successfully in skin extracts by HPLC [Norval et al, 1988; Juhlin et al, 1986; Jansen et al, 1991]. It has been demonstrated that the ears of female C3HBu/Kam mice aged 2 weeks or more have one-third more UCA than neonatal mice and that 4% of this is in the cis-form

[Norval et al, 1988]. These investigators showed that after UV-B irradiation, 31.1% of UCA within the ears was present as the cis-isomer. It has been established that in vivo and in vitro, a photostationary state is reached where no more isomerization of UCA from the trans to the cis form occurs despite continued UV-irradiation [Morrison et al, 1980; Baden and Pathak, 1967; Anglin et al, 1961]. However, it seems reasonable to speculate that if the UCA content of skin varies between sexes or different strains of mice then although UV-B irradiation may result in the same percentage of isomerization of UCA, ultimately, more cis-UCA will be formed in skin containing a higher concentration of UCA. Since there is a correlation between the proportion of cis-UCA formed in the epidermis after UV-B irradiation and the degree of immunosuppression induced [Norval et al, 1988] such a speculation would imply that different sexes or strains of mice would vary in the level of suppression induced after UV-B irradiation. Interestingly, it has been found that the capacity of UV-B radiation to impair the induction of CH when the hapten is painted directly on the irradiated site varies between different strains of mice [Streilein and Bergstresser, 1988]. This speculation, however, would only be valid if one was to assume that differences in skin pigmentation, which does arise between different sexes and strains of mice, had no effect on the capacity of UV-irradiation to isomerize trans to cis-UCA.

In an attempt to explore whether the UCA content of skin varies between sexes or different strains of mice and to establish whether or not a relationship exists between skin pigmentation and the amount of trans to cis-UCA isomerization, five ears from three strains of unirradiated male and female and irradiated ($96 mJcm^{-2}$ UV-B) female mice were analysed by HPLC for the presence of UCA isomers. It was not known what effect (if any) contact sensitization might have on the total UCA content of skin or on the isomerization of trans to cis-UCA. In order to investigate this, five ears from female C3HBu/Kam, C57BL/6 and Balb/c mice each

Table 1 Quantification of Urocanic Acid Isomers in C3HBu/Kam Murine Ears Before and After UV-B Irradiation or Sensitization

Treatment	Wet Weight of Ear(mg)	UCA (ng / mg wet weight) Cis UCA	Trans UCA	Cis UCA as a % of Total UCA	Mean ± Standard Error Mean Cis UCA as a % of Total UCA	Mean Total UCA	Statistical Significance (by t-test)¥
Female Untreated	32	50	390	11.4	5.2 ± 1.8*	371 ± 24	
	23	30	380	7.0			
	29	10	300	3.2			
	35	5	330	1.5			
	29	10	350	2.7			
Male Untreated	18	20	460	4.2	4.0 ± 0.6	470 ± 38	
	22	20	420	4.5			
	20	20	330	5.7			
	21	20	560	3.4			
	20	10	490	2.0			
Female UV-B($96mJcm^{-2}$)	27	120	330	26.7	24.0 ± 1.1	434 ± 12	$p < 0.001$
	31	110	350	23.9			
	32	90	300	23.1			
	40	90	350	20.5			
	22	110	320	25.6			
Female OX 2.5%	55	2	150	1.3	1.0 ± 0.1	152 ± 15	NSπ
	54	2	190	1.0			
	54	1	140	0.7			
	42	1	120	0.8			

The mean total UCA content of male C3H ears was significantly different from the mean total UCA content of male Balb/c and C57B/6 ears ($p < 0.01$; $p < 0.001$, respectively).
The mean total UCA content of female C3H ears was significantly different from the mean total UCA content of female C57B/6 ears ($p < 0.001$) but not significantly from the mean total UCA content of female Balb/c ears ($p > 0.5$).

¥ Significance of difference of mean cis-UCA (as a % of total UCA) from control group*

π Not significantly different from control group*

Table 2 Quantification of Urocanic Acid Isomers in Balb/c Murine Ears Before and After UV-B Irradiation or Sensitization

Treatment	Wet Weight of Ear(mg)	UCA(ng/mg wet weight) Cis UCA	Trans UCA	Cis UCA as a % of Total UCA	Mean ± Standard Error Mean Cis UCA as a % of Total UCA	Mean Total UCA	Statistical Significance (by t-test)¥
Female Untreated	32	4	330	1.2	1.4 ± 0.2*	396 ± 62	
	27	7	450	1.5			
Male Untreated	43	20	250	7.4			
	33	5	220	2.2			
	34	6	240	2.4	3.3 ± 1.0	267 ±17	
	37	6	260	2.3			
	32	8	320	2.4			
Female UV-B(96mJcm^{-2})	17	150	310	32.6			
	12	350	590	37.2			
	21	190	280	40.4	37.0 ± 1.3	566 ± 95	p < 0.001
	24	160	270	37.2			
	22	200	330	37.7			
Female OX 2.5%	41	5	260	1.9			
	39	5	250	2.0			
	57	4	160	2.4	2.3 ± 0.2	194 ± 42	NSπ
	51	1	40	2.4			
	43	7	240	2.8			

The mean total UCA content of male Balb/c ears was significantly different from the mean total UCA content of male C57B/6 ears ($p < 0.001$).
The mean total UCA content of female Balb/c ears was significantly different from the mean total UCA content of female C57B/6 ears ($p < 0.02$).
¥Significance of difference of mean cis-UCA (as a % of total UCA) from control group*
πNot significantly different from control group*

Table 3 Quantification of Urocanic Acid Isomers in C57BL/6 Murine Ears Before and After UV-B Irradiation or Sensitization

Treatment	Wet Weight of Ear(mg)	UCA (ng/mg wet weight) Cis UCA	Trans UCA	Cis UCA as a % of Total UCA	Mean ± Stanard Error Mean Cis UCA as a % of Total UCA	Mean Total UCA	Statistical Significance (by t-test)¥
Female Untreated	45	1	190	0.5	2.5 ± 0.6*	182 ± 27	
	40	6	190	3.1			
	35	4	100	3.8			
	29	3	150	2.0			
	17	8	260	3.0			
Male Untreated	38	2	100	2.0	1.5 ± 0.3	102 ± 12	
	39	1	80	1.2			
	32	0.7	70	1.0			
	32	3	120	2.4			
	27	1	130	0.8			
Female UV-B(96mJcm^{-2})	13	90	220	29.0	30.1 ± 1.0	264 ± 49	p < 0.001
	14	120	310	27.9			
	22	60	150	28.6			
	22	50	100	33.3			
	23	70	150	31.8			
Female OX 2.5%	58	7	160	4.2	2.7 ± 0.4	191 ± 35	NSπ
	43	4	150	2.6			
	38	6	310	1.9			
	83	2	110	1.8			
	51	6	200	2.9			

¥Significance of difference of mean cis-UCA (as a % of total UCA) from control group*
πNot significantly different from control group*

exposed to 25µl of 2.5% oxazolone were analysed 24hr later by HPLC for the quantity of UCA isomers.

The UCA content in terms of ng/mg wet weight and percentage of cis-isomer in all the samples for each strain is given in Table 1, 2 and 3. From the results it was difficult to reach any conclusion as to whether there is a definitive difference between the UCA content of male and female ears. However, it was possible to conclude that a difference in the UCA content of male and female ears between strains exists. The ears of male and female C57BL/6 mice had a significantly lower UCA content compared with male and female ears of the other two strains. Also, the UCA content of male ears differed significantly between all three strains of mice. As expected the percentage of cis-isomer in the ears of all three strains of mice increased significantly after UV-B irradiation. This increase was accompanied by an increase in the UCA content of ears of C3HBu/Kam, Balb/c and C57BL/6 mice exposed to UV-B radiation. However, the percentage of cis-isomer produced in the ears of Balb/c mice after irradiation was higher than in the other two strains. Although there was no obvious difference between the percentage of cis-isomer present in unsensitized and sensitized ears of Balb/c and C57BL/6 mice, the percentage of cis-isomer in the ears of C3HBu/Kam mice was reduced following sensitization. Also, the wet weight of sensitized C3HBu/Kam, C57BL/6 and Balb/c ears compared with untreated ears increased. This increase in wet weight correlated with a 50% reduction in the total UCA content of ears of Balb/c and C3HBu/Kam mice.

3.1.3 Analysis of Kidneys, Spleens, Lymph Nodes and Urine for the Presence of UCA Isomers

UCA was first described in the urine of dogs [Jaffe, 1874]. It is present in human sweat [Zenisek, 1953] and in the epidermis of a variety of species

including man [Everett et al, 1961], guinea pig [Tabachnick, 1957], mice and rats although not in snakes [Baden and Pathak, 1967]. UCA is located predominantly in the stratum corneum (the outermost layer of the skin) and the liver. However, it is not known at present, despite attempts such as by Reeve et al, (1991), whether cis-UCA formed from the trans-isomer on UV-B irradiation stays within the epidermis exerting its immunosuppressive effect entirely locally and/or systemically or whether it leaves the skin via the lymphatic or blood system where it may gain access to other areas within the body such as the draining lymph node, spleen or unirradiated skin. To investigate whether or not UCA isomers are present in tissues other than the skin including urine, the lymph nodes, spleens, kidneys and urine from unirradiated and irradiated female C3HBu/Kam mice were analysed by HPLC.

HPLC analysis of lymph nodes, spleens, kidneys and urine from unirradiated and irradiated mice for the presence of UCA isomers proved difficult and it was not possible to conclude whether any UCA isomers were present (results not shown). It was difficult to analyse the tissue and urine samples easily by HPLC due to the complexity of substances present. Also, in contrast to the skin where UCA represents a major UV absorbing constituent, any UCA isomers that may be present in other tissues may be present in trace amounts, requiring the detection system to be highly sensitive, thus further contributing to the difficulties in quantifying UCA isomers in other tissues. Subsequently, a monoclonal antibody specific for the cis-isomer of UCA was produced for use in detecting the presence of cis-UCA in serum and other tissues, following UV-B irradiation. The production, characterization and use of this monoclonal antibody in detecting cis-UCA in serum after UV-B irradiation of mice is discussed in section 3.6.

3.2 Influence of UCA Isomers and UV-B Irradiation on the Induction of Contact Hypersensitivity Responses

3.2.1 Introduction

Exposure of body wall skin of some (but not all) mice [Toews et al, 1980; Streilein and Bergstresser, 1988], hamsters [Streilein and Bergstresser, 1981], and human beings [Yoshikawa et al, 1990] to low doses of UV-B radiation impairs the induction of CH responses to haptens, painted directly on the irradiated surface. A number of investigators using different mouse strains and protocols have demonstrated local UV-B induced suppression of CH responses to a variety of haptens including DNFB (Gurish et al, 1983; Yoshikawa and Streilein, 1990], oxazolone [Satoh et al, 1990], FITC [Okamoto and Kripke, 1987] and picryl chloride (PCl) [Satoh et al, 1990].

Evidence from a wavelength dependence study of UV-B induced suppression of CH responses suggests that suppression is initiated by an interaction between UV-B radiation and a specific signal-transducing photoreceptor molecule in mouse skin [De Fabo and Noonan, 1983]. As mentioned previously (in section 3.1.1) it is hypothesized that the photoreceptor molecule is the trans-isomer of UCA, a major UV-absorbing component of the stratum corneum [De Fabo and Noonan, 1983], which isomerizes to cis-UCA on absorption of UV-B radiation. Noonan et al [1988] have demonstrated that cis-UCA but not trans-UCA administered systemically in vivo, in the absence of UV-B radiation, initiates an antigen-presenting cell defect in splenic DC that is indistinguishable from the one caused by UV-B irradiation. In similar and relevant studies, Howie et al (1986A) demonstrated that UV-B irradiation of mice before infection with HSV resulted in suppression of a virus-specific DTH response and Ross et al (1986) found that s.c. injection of cis-UCA into mice before infection with HSV also impaired the development of the DTH response. Cis-UCA has also been shown to delay rejection of

transplantation allografts [Williams et al, 1990; Guymer and Mandel, 1990] and to enhance UV-induced tumour yield and malignancy in hairless mice [Reeve et al, 1989]. Thus, there is considerable evidence from several experimental systems, that cis-UCA is involved in the suppressive effects of UV-B irradiation on cutaneous immunity.

In order to elucidate the mechanism of local UV-B induced suppression of CH responses it was first necessary to set up a murine model of CH to demonstrate UV-B induced suppression and to explore under what conditions (if any) the isomerized form (cis-UCA) of the proposed UV-B photoreceptor molecule (trans-UCA) can suppress the CH response.

3.2.2 Titration to Establish Optimal Sensitization Concentration of oxazolone and FITC for Measurements of Suppression of Contact Hypersensitivity

Contact sensitization responses are induced by processing of the allergen by epidermal LC, which transport the processed antigenic determinant (hapten) to draining lymph nodes. Therein, antigen-specific helper T cells recognize LC-bound antigen (in conjunction with class II MHC antigen) and are triggered to proliferate and promote the differentiation and dissemination of effector and memory T cells that are able to elicit a cutaneous response upon their subsequent encounter with the inducing antigen [Nishioka, 1985; Katz, 1985; Bos and Kapsenberg, 1986]. It has been demonstrated that challenge-induced increases in ear thickness convey the skin-sensitizing activity of contact sensitizers [Asherson and Ptak, 1968; Maisey and Miller, 1986]. The skin-sensitizing potential of different contact sensitizers varies - some are strong (e.g. oxazolone) while others are comparitively weak (e.g. FITC) [Gad et al, 1986; Maisey and Miller, 1986].

Two different model systems for investigating the influence of UV-B radiation and UCA isomers on the induction of CH responses were used throughout.

One involved UV-B irradiating or applying UCA isomers to the shaved dorsal surface of mice whose ears were protected during irradiation in contrast to irradiating the shaved abdominal skin as carried out by other investigators [Yoshikawa and Streilein, 1990; Streilein and Bergstresser, 1988; Okamoto and Kripke, 1987; Streilein et al, 1980A], applying the contact sensitizer to the same site 24hr after the last UV or UCA treatment and then eliciting the CH response by challenging the dorsum of both ears 5 or 6 days after sensitization. The other model system involved UV-B irradiating or applying UCA isomers to the dorsum of the left ear of unshaven mice whose right ear was protected during irradiation, applying the contact sensitizer to the dorsum of the left ear 24hr after the last UV or UCA treatment and then eliciting the CH response by challenging the right ear 5 or 6 days after sensitization. UCA isomers were also administered i.p. and s.c. at sites distant from the site of sensitization for both of these models in order to assess the systemic effect of these isomers on the CH response.

Titration experiments were performed to establish the optimal sensitization concentration of oxazolone when applied to the shaved dorsal surface (Table 4) or to the left ear (Table 5) which would induce a measurable ear swelling response such that a significant suppression of the CH response could be measured. Mice were challenged on day 5 regardless of the site of sensitization as carried out by Satoh et al, (1990). Although the skin-sensitizing potential of different contact sensitizers varies [Gad et al, 1986; Maisey and Miller, 1986] it is not known whether this can influence directly the degree of suppression induced by UV-B irradiation. Assuming that the skin-sensitizing potential of contact sensitizers does affect the ability of UV-B radiation to induce suppression it seemed reasonable to speculate that a weaker contact sensitizer may shift the balance in favour of suppression. Subsequently, FITC (a weaker contact sensitizer than oxazolone) was also used with the aim of increasing the possibility of significant UV-B and cis-UCA induced suppression. A titration experiment was performed to find the optimal

Table 4 Titration to Establish Optimal Sensitization Concentration of oxazolone for Measurements of Inhibition of CH

Sensitization*	Challenge$^{\pi}$	Mean Ear Swelling ± SE (mm x10^{-2})$^{\Delta}$
Vehicle	Vehicle	0.3 ± 0.3
Vehicle	25µl 0.25% OX	0.5 ± 0.3
50µl 2.5% OX	" " "	9.0 ± 1.7
50µl 1.0% OX	" " "	11.4 ± 2.3
50µl 0.1% OX	" " "	12.1 ± 2.3
50µl 0.025% OX	" " "	2.6 ± 0.7

* The backs of mice were shaved and 50µl of oxazolone or vehicle applied topically to the shaved surface

$^{\pi}$ Both ears of the mice (n=6) were challenged with 25µl of oxazolone or vehicle 5 days later

$^{\Delta}$ Ear swelling responses were measured at 24hr. Data are presented as mean ear swelling response ± standard error of mean (SE)

Table 5 Titration to Establish Optimal Sensitization Concentration of oxazolone for Measurements of Inhibition of CH

Sensitization*	Challenge¥	Mean Ear Swelling ± SE (mm x 10^{-2})Δ
Vehicle	Vehicle	0.8 ± 0.3
Vehicle	25µl 0.25% OX	1.5 ± 0.3
25µl 2.5% OX	" " "	13.7 ± 1.5
25µl 0.25% OX	" " "	7.3 ± 0.8
25µl 0.1% OX	" " "	5.6 ± 0.9

* The left ear of each mouse was sensitized with 25µl of oxazolone or vehicle

¥ The right ear of each mouse (n=6) was challenged with 25µl of oxazolone or vehicle 5 days later

Δ Ear swelling responses were measured at 24hr. Data are presented as mean ear swelling response ± standard error of mean (SE).

Table6 Titration to Establish Optimal Sensitization Concentration of FITC for Measurements of Inhibition of CH

Sensitization*	Challenge¥	Mean Ear Swelling ± SE (mm x 10^{-2})Δ
Vehicle	Vehicle	0.6 ± 0.3
Vehicle	25µl 0.5% FITC	0.8 ± 0.3
50µl 2.5% FITC	" " "	12.1 ± 1.9
50µl 1.0% FITC	" " "	12.5 ± 0.9
50µl 0.5% FITC	" " "	10.0 ± 1.6
50µl 0.25% FITC	" " "	9.1 ± 1.3

* The backs of mice were shaved and 50µl of FITC or vehicle applied topically to the shaved surface

¥ Both ears of the mice (n=6) were challenged with 25µl of FITC or vehicle 6 days later

Δ Ear swelling responses were measured at 24hr. Data are presented as mean ear swelling response ± standard error of mean (SE)

sensitization concentration of FITC when applied to the shaved dorsal surface (Table 6) which would induce a measurable ear swelling response such that a significant suppression of the CH response could be measured. However, a titration experiment was not performed for mice sensitized with FITC on the dorsum of their left ear due to limited availability of mice. The chosen sensitizing dose (0.5%) and challenging dose (0.5%) did, however, give a reasonable ear swelling response of approximately 8mm x 10^{-2} such that a significant suppression of the CH response could be measured. Mice exposed to FITC either on the shaved dorsal surface or the dorsum of their left ear were challenged on day 6 as carried out by Okamoto and Kripke, (1987).

Since it is not known whether or not the sensitizing dose of a contact sensitizer can affect the degree of suppression induced by UV-B irradiation, doses of contact sensitizers that were likely to induce a moderate (7mm x 10^{-2}) to good (12mm x 10^{-2}) ear swelling response based on the results of the titration experiments shown in Table 4, 5 and 6 were chosen.

3.2.3 Influence of UV-B Irradiation on the Induction of Contact Hypersensitivity Responses to oxazolone and FITC

It can be seen from Table 7 and 8 that mice exposed to UV-B radiation daily for three consecutive days (96mJcm^{-2}/day) prior to sensitization with oxazolone or FITC at the same site significantly suppressed the induction of CH responses to these chemical antigens. A single exposure of mice to 96mJcm^{-2} of UV-B radiation 3 days prior to sensitization did not, however, suppress the induction of the CH response to oxazolone (Table 7).

Table 7 Influence of UCA Isomers and UV-B Irradiation on the Induction of the CH Response to oxazolone

Treatment (Days/hr) Prior to Sensitization	Sensitization	Challenge	% Suppression	Statistical[¥] Significance(by t-test)
Trans UCA 5hr (100µg) s.c.Flank[†]	50µl 0.5% Ox[ø]	25µl 0.25% Ox[∂]	7	NS
Cis UCA 5hr (100µg) s.c.Flank[†]	" " "	" " "	0	-
Trans UCA 5hr (100µg)[π]	" " "	" " "	0	-
Cis UCA 5hr (100µg)[π]	" " "	" " "	6	NS
Cis UCA 7D (100µg) i.p.[†]	" " "	" " "	14	NS
" " 3D (100µg) i.p.[†]	" " "	" " "	16	NS
" " 1D (100µg) i.p.[†]	" " "	" " "	15	NS
UV-B 3D (96mJcm^{-2})[ß]	" 2.5% "	" " "	20	NS
Cis UCA 1D (200µg) s.c.Flank[†]	" " "	" " "	5	NS
" " 1D (200µg)[π]	" " "	" " "	15	NS
" " 4D (200µg) s.c.Flank[†]	" " "	" " "	15	NS
" " 4D (200µg)[π]	" " "	" " "	0	-
UV-B 3D (96mJcm^{-2})[ß]	" " "	" " "	4	NS
Cis UCA 1D (200µg) s.c.Flank[†]	" " "	" " "	40	$p < 0.01$
" " 1D (200µg)[π]	" " "	" " "	37	$p < 0.01$
" " 4D (200µg) s.c.Flank[†]	" " "	" " "	35	NS
" " 4D (200µg)[π]	" " "	" " "	32	$p < 0.05$
UV-B 3D (96mJcm^{-2})[ß]	" 0.1% "	" " "	0	-
Cis UCA 1D (200µg) s.c.Flank[†]	" " "	" " "	12	NS
" " 1D (200µg)[π]	" " "	" " "	3	NS
" " 4D (200µg) s.c.Flank[†]	" " "	" " "	11	NS
" " 4D (200µg)[π]	" " "	" " "	3	NS
UV-B 3D (96mJcm^{-2})[ß]	" " "	" " "	0	-
Cis UCA 1D (200µg) s.c.Flank[†]	" " "	" " "	34	NS
" " 1D (200µg)[π]	" " "	" " "	20	NS
" " 4D (200µg) s.c.Flank[†]	" " "	" " "	31	NS
" " 4D (200µg)[π]	" " "	" " "	0	-
UV-B 3,2,1D (288mJcm^{-2})[&]	25µl 0.25% Ox[Δ]	25µl 0.25% Ox[*]	34	NS
Cis UCA 3,2,1D (200µg)[π]	" " "	" " "	18	NS
Trans UCA 3,2,1D (200µg)[π]	" " "	" " "	0	-
Cis UCA 3,2,1D (200µg)s.c.Flank[†]	" " "	" " "	0	-
UV-B 3,2,1D (288mJcm^{-2})[&]	" " "	" " "	67	$p < 0.05$
Cis UCA 3,2,1D (200µg)[π]	" " "	" " "	0	-
Trans UCA 3,2,1D (200µg)[π]	" " "	" " "	0	-
Cis UCA 3,2,1D (200µg)s.c.Flank[†]	" " "	" " "	24	NS
UV-B 3,2,1D (288mJcm^{-2})[&]	" " "	" " "	75	$p < 0.001$
Cis UCA 3,2,1D (200µg)[π]	" " "	" " "	11.4	NS

NS = Not significantly different from positive control group

[¥] Significance of difference from positive control group
[†] Mice were treated at a site distant from the site of sensitization
[ø] 50µl of 0.5% Ox was topically applied to the shaven backs of mice
[∂] 25µl of 0.25% Ox was topically applied to both ears 5 days later
[π] Mice were topically treated at the same site as sensitization
[ß] The shaven backs of mice were exposed to a single dose (96mJcm^{-2}) of UV-B radiation
[&] The left ear of each mouse was exposed to 96mJcm^{-2} of UV-B radiation each day
[Δ] 25µl of 0.25% Ox was topically applied to the dorsum of the left ear
[*] 25µl of 0.25% Ox was topically applied to the dorsum of the right ear 5 days later

Table 8 Influence of UCA Isomers and UV-B Irradiation on the Induction of the CH Response to FITC

Treatment (Days) Prior to Sensitization*	Sensitization	Challenge	% Suppression	Statistical¥ Significance(by t-test)
Cis UCA 3,2,1D(200µg)†	25µl 0.5% FITC&	25µl 0.5% FITCβ	33	NS
Trans UCA3,2,1D(200µg)†	" " "	" " "	29	NS
UV-B3,2,1D(288mJcm-2)ç	" " "	" " "	94	p < 0.001
Cis UCA 3,2,1D(200µg)†	" " "	" " "	34	NS
UV-B3,2,1D(288mJcm-2)ç	" " "	" " "	90	p < 0.001
Cis UCA 3,2,1D(200µg)†	" " "	" " "	59	p < 0.02
UV-B3,2,1D(288mJcm-2)ç	" " "	" " "	94	p < 0.01
Cis UCA 3,2,1D(200µg)†	" " "	" " "	45	NS
Trans UCA3,2,1D(200µg)†	" " "	" " "	39	NS
Cis UCA 3,2,1D(200µg)ø	50µl 1.0% FITC#	25µl 0.5% FITCΔ	45	NS
Cis UCA 3,2,1D(200µg)ø	" " "	" " "	34	NS
Cis UCA 3,2,1D(200µg)ø	" " "	" " "	35	NS
Trans UCA3,2,1D(200µg)ø	" " "	" " "	5	NS
Cis UCA 3D(200µg)ø	" " "	" " "	18	NS
Trans UCA 3D(200µg)ø	" " "	" " "	29	NS
Cis UCA 2D(200µg)ø	" " "	" " "	0	-
Trans UCA 2D(200µg)ø	" " "	" " "	54	p < 0.001
Cis UCA 1D(200µg)ø	" " "	" " "	25	NS
Trans 1D(200µg)ø	" " "	" " "	43	p < 0.02
Cis UCA 2,1D(200µg)ø	" " "	" " "	55	p < 0.02
Trans UCA 2,1D(200µg)ø	" " "	" " "	39	NS
Cis UCA 2,1D(200µg)ø	" " "	" " "	26	NS
Trans UCA 2,1D(200µg)ø	" " "	" " "	6	NS
Cis UCA 2,1D(100µg)ø	" " "	" " "	28	NS
Trans UCA 2,1D(100µg)ø	" " "	" " "	21	NS
Cis UCA 2,1D (50µg)ø	" " "	" " "	37	NS
Trans UCA 2,1D (50µg)ø	" " "	" " "	3	NS
Cis UCA 2,1D (10µg)ø	" " "	" " "	17	NS
Trans UCA 2,1D (10µg)ø	" " "	" " "	23	NS
Cis UCA 2,1D(200µg)ø	" " "	" " "	0	-
Trans UCA 2,1D(200µg)ø	" " "	" " "	25	NS
Cis UCA 2,1D (50µg)ø	" " "	" " "	34	NS
Trans UCA 2,1D (50µg)ø	" " "	" " "	0	-
Cis UCA 2,1D (10µg)ø	" " "	" " "	1	NS
Trans UCA 2,1D (10µg)ø	" " "	" " "	37	NS

NS = Not significantly different from control group

*Mice were treated at the same site as sensitization
¥ Significance of difference from positive control group
† UCA isomers were topically applied to the left ear of mice
& 25µl of 0.5% FITC was topically applied to the dorsum of the left ear
β 25µl of 0.5% FITC was topically applied to the dorsum of the right ear 6 days later
ç The left ear of mice was exposed to 96mJcm-2 of UV-B radiation each day
ø UCA isomers were topically applied to the shaven backs of mice
50µl of 1.0% FITC was topically applied to the shaven backs of mice
Δ 25µl of 0.5% FITC was topically applied to the dorsum of both ears 6 days later

3.2.4 Influence of UCA Isomers on the Induction of Contact Hypersensitivity Responses to oxazolone and FITC

A single topical application of 100μg of cis or trans-UCA to the shaved dorsum of mice 5hr prior to sensitization with oxazolone at the same site had no effect on the CH response (Table 7). Likewise, a single s.c. injection of 100μg of cis or trans-UCA into the flank 5hr prior to sensitization with oxazolone on the shaved dorsal surface had no systemic effect. It was also not possible to suppress systemically the CH response to oxazolone by administering 100μg of cis-UCA i.p. 7, 3 or 1 day prior to sensitization. However, significant local and systemic suppression was demonstrated when 200μg of cis-UCA was topically applied to the shaved dorsal surface 1 or 4 days prior to sensitization or injected s.c. into the flank 1 day prior to sensitization, respectively (Table 7). It could not be concluded whether this suppression of the CH response occurred as a result of administering a higher concentration of cis-UCA since the time of application prior to sensitization was different from the above mentioned experiments and the sensitizing dose of oxazolone was higher (2.5%). Unfortunately it was not possible to reproduce this result even if the sensitizing dose was decreased to 0.1%. Using a different model system, it was found that topical application of 200μg of cis or trans-UCA to the dorsum of the left ear 3, 2 and 1 day prior to sensitization with oxazolone at the same site failed to induce suppression. Also, administration of 200μg cis or trans-UCA s.c. into the flank 3, 2 and 1 day prior to sensitization on the dorsum of the left ear also failed to induce suppression of the CH response. It was concluded that cis or trans-UCA do not significantly induce local or systemic suppression of the CH response to oxazolone regardless of its concentration, the sensitizing dose or the time of application prior to sensitization (Table 7).

It was possible that oxazolone which is a strong contact sensitizer (as mentioned previously) was having an influence on the ability of UCA isomers to suppress the CH response. Subsequently, a number of experiments were carried out

in an attempt to explore whether cis or trans-UCA when administered by topical application to the shaved dorsum or left ear of mice, was capable of locally suppressing the induction of the CH response to FITC (a weaker contact sensitizer than oxazolone) (Table 8). It was found that 200μg of cis-UCA induced significant local suppression of the CH response to FITC when topically applied to the shaved dorsum of mice 2 and 1 day prior to sensitization. It was observed also that 200μg of trans-UCA induced suppression when applied in the same way 2 and 1 day prior to sensitization. This suggested that the concentration of cis-UCA being administered was too high. Subsequently, dose-response experiments were conducted in order to separate out the suppressive effect of cis and trans-UCA on the CH response to FITC when applied to the shaved dorsal surface 2 and 1 day prior to sensitization. However, it can be seen from Table 8 that it was both not possible to reproduce the significant suppression of the CH response observed when 200μg of cis-UCA was applied 2 and 1 day prior to sensitization or to separate out the effect of cis and trans-UCA by decreasing their concentration. Using a different model system it was found that topical application of 200μg of cis-UCA to the dorsum of the left ear 3, 2 and 1 day prior to sensitization with FITC at the same site did induce significant suppression of the CH response. Once again, however, this result was not reproducible under the same experimental conditions. It was observed also that application of trans-UCA under the same conditions in different experiments induced similar levels of suppression as the cis-UCA isomer.

It was reasonable to conclude from the results shown in Table 7 and 8 that cis-UCA had little, if any, consistent effect on the induction of CH responses to oxazolone or FITC.

3.3 Influence of UV-B Irradiation and UCA Isomers on Dendritic Cell Migration

3.3.1 Introduction

The mechanism by which UV-B irradiation impairs the induction of the CH response is unknown. There is a great deal of evidence that indicates that antigen-bearing DC in DLN are derived from epidermal LC which travel to the lymph nodes in the afferent lymph [Kinnaird et al, 1989; Macatonia, et al, 1987; Silberberg-Sinakin et al, 1976; Kripke et al, 1990; Larsen et al, 1990] and that these antigen-bearing DC are responsible for inducing contact sensitization [Macatonia and Knight, 1989; Kinnaird et al, 1989]. Subsequently, it has been postulated that immobilization or migration of LC away from the skin at UV-B irradiated sites may contribute to the failure of mice to develop CH responses following the painting of hapten on these sites. However, the evidence implicating either one of these possible effects of UV-B irradiation on LC at the exposed site as a possible mechanism for local suppression, is based on controversial interpretations of whether LC have in fact left the skin.

In an attempt to explore whether UV-B irradiation affects epidermal DC migration and whether such effects are mediated via the postulated UCA photoreceptor molecule, the influence of UV-B irradiation and UCA isomers on the migration of DC to DLN was examined in unsensitized and sensitized mice. In addition the influence of UV-B irradiation on the carriage of antigen and on the expression of Ia and ICAM-1 antigens by DC was measured.

3.3.2 Effect of UV-B Irradiation and Cis or Trans-UCA on Dendritic Cell Accumulation in DLN of Unsensitized Mice

Forty-two hr after irradiating mice or painting their ears with cis or trans-UCA, the mice were killed, their auricular lymph nodes excised and the number of DC present in each lymph node was estimated. Table 9 which shows the

results of four experiments, demonstrates that UV-B radiation induces significant and reproducible accumulation of DC in DLN and that neither isomer of UCA has a consistent effect on DC migration to DLN. This suggests that UV-B induced DC migration is not attributable to the local production of cis-UCA.

Assuming that the DC accumulating within the DLN as a consequence of UV-B irradiation are derived from the skin, it was important to generate a clear picture of the kinetics/tempo of this migration after exposure to UV-B radiation. Thus, the number of DC present within the DLN of mice at various times following a single dose of UV-B irradiation ($144mJcm^{-2}$) was calculated. Figure 10 illustrates that DC migration to DLN starts to take place between 12 and 24hr and reaches a maximum at 48hr before decreasing. To clarify further that cis-UCA does not induce DC to migrate to the DLN, the number of DC accumulating in the DLN at various times following the cutaneous application of 100μg of cis-UCA to the dorsum of each ear was also estimated. It can be seen from Figure 10 that irrespective of the time after painting with cis-UCA, the numbers of DC in draining nodes were unaltered.

It was important to establish whether or not the DC migrating to the DLN as a consequence of UV-B irradiation had the potential to present antigen and so the number of DC expressing Ia antigen in the DLN at various times after UV-B irradiation was examined. It can be seen from the results shown in Table 10 that UV-B irradiation had no effect on either the percentage of DC in the DLN expressing Ia or on the intensity of expression of Ia per cell. A further investigation of the influence of UV-B irradiation on the antigen-presenting cell function of the DC accumulating within the DLN was carried out by examining the number of DC expressing ICAM-1 and the level of expression per cell (Table 11). Neither the percentage of DC expressing ICAM-1 nor the intensity of expression per cell was influenced by UV-B irradiation regardless of the total dose received or the time of exposure.

Table 9 Effect of UV-B Irradiation and Cis or Trans-UCA on DC Migration to DLN

	DC count / lymph node				
	Experiment				
Treatment at 66 and 42hr prior to analysis	1*	2*	3Δ	4Δ	Mean ± SD
Vehicle	2850	2860	2079	1950	2435 ± 488
Cis-UCA	5162	3050	2550	2494	
Trans-UCA	7040	4446	2050	2925	
UV-B (144mJcm^{-2})†	10005	9344	9720	9643	9678 ± 272¥

* 200μg of UCA was painted onto each ear
Δ 100μg of UCA was painted onto each ear
† Both ears were exposed to UV-B radiation
¥ Significantly different from vehicle group ($p < 0.001$ by t - test)

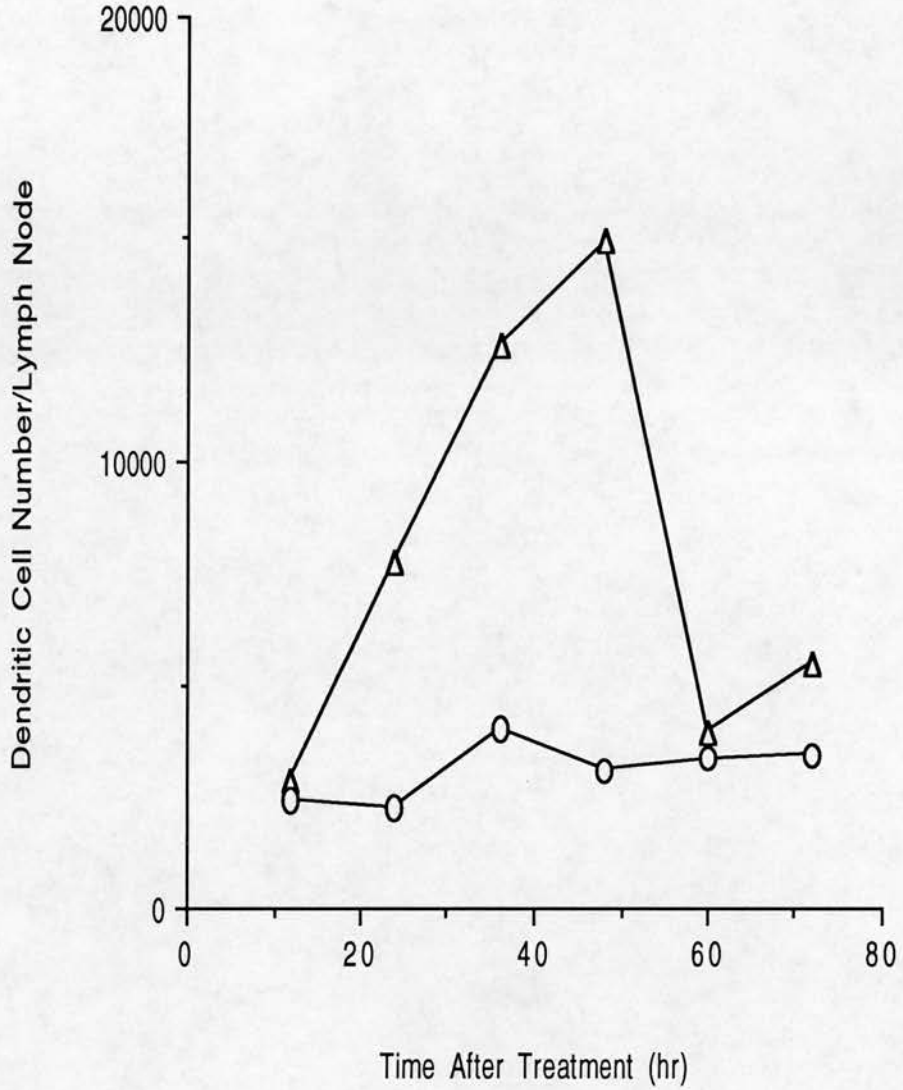

Figure 10 The effect of UV-B irradiation and cis-UCA on the kinetics of DC migration. The ears of unshaven mice were exposed to a single dose (144mJcm^{-2}) of UV-B radiation (Δ) or painted with 100μg of cis-UCA (o) at various times prior to killing. The DC count in naive mice used as a background control for UV-B irradiation was 2925 DC/lymph node. The DC count in naive mice (treated at 48hr with 25μl DMSO on each ear) used as a background control for cis-UCA treatment was 3524 DC/lymph node.

Table 10 The Effect of UV-Irradiation on Ia Expression in DC from DLN

Time After UV Irradiation (hr)	% of Cells Within Gated Population Expressing Ia	Peak Channel Number
Untreated DC	93.8	162
12	83.6	150
24	92.0	154
36	94.7	150
48	91.8	157
60	86.2	155
72	91.5	157

The ears of unshaven mice were exposed to a single dose ($144mJcm^{-2}$) of UV-B radiation. At various times following exposure the mice were killed, their auricular lymph nodes removed and the enriched DC were then stained for Ia using an anti-mouse Ia monoclonal antibody, followed by staining with a sheep anti-mouse IgG FITC - labelled secondary antibody. To measure the percentage Ia expression, 5×10^4 cells from each sample was analysed in a EPICS "C" flow cytometer. DC were identified on a 2 - parameter histogram measuring size and side - scatter and then green fluorescence analysed from a bit map onto a 252 channel histogram using log amplification. The percentage of Ia positive cells within this population was measured and also antigen density per cell (fluorescence intensity) by mean channel analysis.

Table11 Influence of UV-B Irradiation on the percentage of DC Expressing ICAM-1 and on the Level of Expression of ICAM-1

Treatment ¥	% of cells within gated population expressing ICAM-1	Peak Channel Number
-	81.2	167
UV-B 66 and 42hr (288mJcm^{-2})$^{\pi}$	93.7	163
UV-B 66 and 42hr (432mJcm^{-2})*	94.3	167
UV-B 90, 66 and 42hr (432mJcm^{-2})^{ø}	94.5	169

Forty - two hr after the last exposure, the mice were killed, their auricular lymph nodes removed and the enriched DC were then stained for ICAM-1 using an anti - mouse ICAM-1 monoclonal antibody followed by staining with a FITC - labelled rabbit anti - rat IgG antibody. To measure the percentage ICAM-1 expression, 5 x 10^4 cells from each sample was analysed in a EPICS "C" flow cytometer. DC were identified on a 2 - parameter histogram measuring size and side scatter and then green fluorescence anaysed from a bit map onto a 252 channel histogram using log amplification. The percentage of ICAM-1 positive cells within this population was measured and also antigen density per cell (fluorescence intensity) by mean channel analysis.

¥ The ears of unshaven mice were exposed to a single dose of UV-B radiation at different times prior to killing
$^{\pi}$ 144mJcm^{-2} at each time point
* 216mJcm^{-2} at each time point
^{ø} 144mJcm^{-2} at each time point

3.3.3 Influence of UV-B Irradiation and Cis or Trans-UCA on Dendritic Cell Migration Induced by FITC

Irradiated mice or mice painted with cis or trans-UCA were sensitized on their ears with FITC 18hr before killing. Auricular lymph nodes were removed and the number of DC present in each node measured. It can be seen from Table 12, which summarises the results of four independent experiments, that whilst the UCA isomers have no effect on DC migration induced by FITC, UV-B irradiation significantly increases DC migration to the DLN.

There is now a great deal of evidence that DC play a major role during the induction of skin sensitization. Within hours of topical exposure of mice to contact sensitizers there is an accumulation of DC in the lymph nodes draining the site of application [Knight et al, 1985A; Gerberick et al, 1991]. It has been demonstrated from studies in which skin-sensitizing fluorochromes, such as FITC, have been used, that a proportion of the DC which arrive in the DLN bear significant levels of antigen [Macatonia et al, 1986; Kinnaird et al, 1989; Macatonia et al, 1987; Cumberbatch and Kimber, 1990] and that, initially at least, all antigen-bearing cells found within the DLN express Ia antigen on their surface [Cumberbatch and Kimber, 1990]. These antigen-bearing DC in the DLN are highly immunocompetent. They are potent stimulators of both primary and secondary T lymphocyte proliferative responses in vitro [Macatonia et al, 1986; Macatonia et al, 1987; Jones et al, 1989] and efficiently induce contact sensitization in naive animals [Kinnaird et al, 1989; Macatonia and Knight, 1989]. A correlation has been found between the number of DC which are present in DLN 24hr after skin sensitization and the extent of the primary lymphocyte proliferative response [Kimber et al, 1990A].

Thus, it was of considerable importance to establish whether UV-B irradiation influenced the number of DC carrying antigen or the expression of Ia molecules by these antigen-bearing DC in the DLN. The percentage of DC bearing

Table 12 Influence of UV-B Irradiation and Cis or Trans-UCA on DC Migration to DLN Induced by FITC

Treatment at 66 and 42hr	FITC at 18hr	DC Count / lymph node Experiment 1	2	3	4	Mean ± SD	Statistical Significance (by t - test)[¥]
Vehicle	-	6750	8775	4788	8125	7110 ± 1763	
Vehicle*	+	12222	13754	10196	13294	12367 ± 1583	
100μg Cis-UCA	+	15238	16406	9111	11984	13185 ± 3298	NS[Δ]
100μg Trans-UCA	+	14175	19511	11500	15640	15207 ± 3343	NS[Δ]
UV-B (144mJcm^{-2})	+	23490	20174	16422	19600	19922 ± 2895	$p < 0.01$

Both ears of unshaven mice were irradiated or received 100μg of cis or trans-UCA. The mice were sensitized with 25μl of 1% FITC or an equal volume of the appropriate vehicle on the dorsum of both ears 18hr before killing

¥ = Significance of difference from vehicle group*
Δ = Not significantly different from vehicle group*

Table 13 Influence of UV-B Irradiation on the Percentage of DC Carrying FITC and Expressing Ia

Treatment at 66 and 42hr	FITC at 18hr	DC Count/lymph node	% of cells within gated population bearing FITC	Peak Channel	% of cells within gated population bearing FITC#	Peak Channel#	% of cells within gated population expressing Ia$^{\Delta}$
_	-	8125	-	-	-		91 ± 5
_	+	13294	40	165	38 ± 8	150 ± 9	85 ± 7
UV-B (144mJcm^{-2})	+	19600	34	172	38 ± 5	152 ± 16	87 ± 9

The ears of unshaven mice were exposed to a single dose of UV-B radiation and 18hr before killing, 25µl of 1% FITC or an equal volume of the appropriate vehicle was topically applied to the dorsum of both ears. Following killing, their auricular lymph nodes were excised and enriched DC stained for Ia (using an anti - mouse Ia monoclonal antibody followed by staining with a sheep anti - mouse IgG phycoerythrin -labelled secondary antibody) or left alone were analysed in a EPICS "C" flow cytometer to measure the percentage of Ia and FITC positive cells respectively and the density of FITC antigen expressed on the surface of each cell (fluorescence intensity). DC were identified on a 2 - parameter histogram measuring size and side scatter and then green fluorescence analysed from a bit map onto a 252 channel histogram using log amplification. Flourescence intensity was measured by mean channel anaysis.

= Expressed as arithmetic mean of 4 experiments ± 1SD
$^{\Delta}$ = Expressed as arithmetic mean of 2 experiments ± 1SD

FITC from the draining auricular lymph nodes of mice whose ears had been exposed to UV-B radiation prior to sensitization with FITC, was examined (Table 13). UV-B irradiation which increased DC migration induced by FITC did not influence the percentage of DC carrying FITC. Also, the mean intensity of staining per cell of FITC-bearing DC was found to be unaltered by UV-B irradiation. It was therefore concluded that UV-B irradiation prior to sensitization increases the number of FITC-bearing DC accumulating in the DLN. It can be seen from Table 13 that UV-B irradiation had no effect on the expression of Ia molecules by DC accumulating in the DLN as a result of FITC induction. In addition, by using double colour fluorescence, it was possible to demonstrate that UV-B irradiation did not alter Ia expression on FITC-bearing DC (data not shown).

3.4 The Role of TNFα and Cis-UCA in UV-B Induced Dendritic Cell Migration and on Suppression of Contact Hypersensitivity Responses

3.4.1 Introduction

The mechanism by which UV-B irradiation of mouse skin impairs the induction of CH responses if hapten is painted directly on the irradiated site [Toews et al, 1980; Lynch et al, 1981; Yoshikawa and Streilein, 1990] appears to be genetically determined. It has been reported that UV-B irradiation impairs the induction of CH in some (C57BL/6, C3H/HeN) but not other (Balb/c, C3H/HeJ, A/J) genetically defined strains of mice which are referred to as UV-B susceptible and UV-B resistant, respectively [Streilein and Bergstresser, 1988]. Susceptibility to UV-B irradiation is dictated by alleles at the Lps and TNFα loci which influence the amount of intracutaneous TNFα produced in response to UV-B [Yoshikawa and Streilein, 1990]. Interestingly, there is now evidence that keratinocytes synthesize and release TNFα after UV-B irradiation [Kock et al, 1990A]. In addition, Oxholm et al (1988) have reported that TNFα can be detected in the epidermis of UV-B exposed human skin and their photomicrographs suggest that TNFα is in keratinocytes. Recently, TNFα has been shown to act as an important mediator of the suppressive effects of UV-B irradiation on the induction of CH [Yoshikawa and Streilein, 1990]. Based on this observation and the demonstration that UV-B irradiation or i.d. TNFα altered the morphology of epidermal LC, Vermeer and Streilein concluded that TNFα prevents effective sensitization following UV-B irradiation by immobilizing LC within the epidermis [Vermeer and Streilein, 1990]. Further, it has been reported that no active hapten-bearing DC could be found within DLN, if the skin on which the hapten was painted had been exposed previously to UV-B radiation [Bigby et al, 1989].

3.4.2 Influence of TNFα on UV-B Induced Dendritic Cell Accumulation in DLN

It has recently been demonstrated that TNFα induces DC migration to DLN [Cumberbatch and Kimber, 1992] and that both i.d. TNFα and UV-B irradiation reduce the density of Ia^+ cells in the epidermis [Vermeer and Streilein, 1990]. To determine whether UV-B induced DC migration was stimulated by TNFα release, 5 and 1hr prior to exposing the ears of two panels of unshaven mice to a single dose of UV-B ($144mJcm^{-2}$) radiation one of these panels received i.p. injections of dexamethasone (a transcriptional inhibitor of TNFα) whilst the other received i.p. injections of sterile PBS. Two other panels of mice were treated in the same way at the same time but were not exposed to UV-B radiation. All four panels of mice were then killed 48hr following UV-B exposure, their draining auricular lymph nodes excised and the number of DC present in each lymph node calculated. The result shown in Figure 11 demonstrates that dexamethasone treatment inhibits UV-B induced DC migration to the DLN.

However, the action of dexamethasone is not restricted to the inhibition of TNFα production and has a number of other biological effects. Dexamethasone has been shown to alter the phenotype and function of peripheral blood lymphocytes from patients with multiple schlerosis [Salmaggi et al, 1991] and induce changes in protein formation in thyroidectomized rats [Brtko et al, 1990]. Subsequently in order to further investigate the effect of TNFα on UV-B induced DC migration, 2hr prior to irradiating the ears of two panels of unshaven mice with a single dose of UV-B ($144mJcm^{-2}$) one of these panels received i.p. injections of neutralizing rabbit anti-murine TNFα antibodies whilst the other received i.p. injections of normal rabbit serum (NRS). Two other panels of mice not exposed to UV-B radiation were treated in the same way and at the same time. Forty-eight hr following UV-B irradiation, all four panels of mice were then killed, their draining auricular lymph nodes excised and the number of DC present in each lymph node measured. As

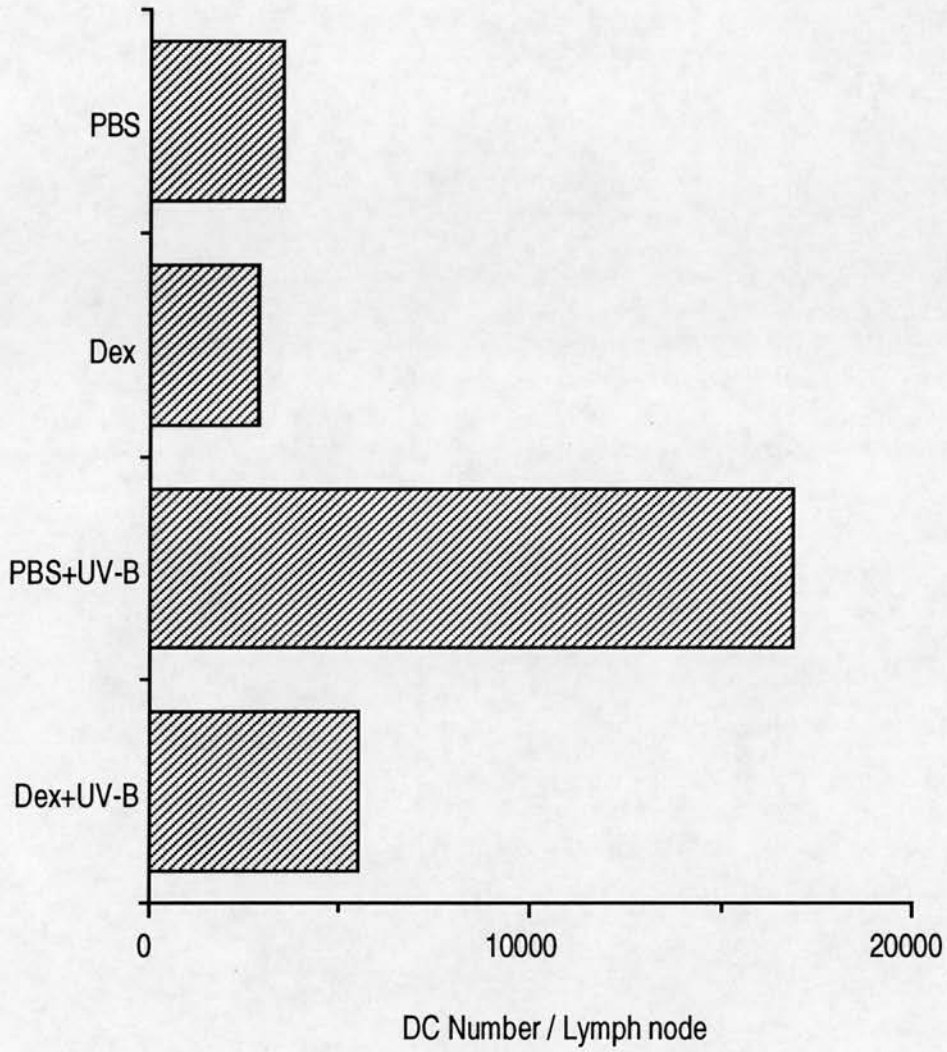

Figure 11 Effect of dexamethasone on DC accumulation in DLN 48hr after UV-B irradiation. Groups of unshaven mice (n=10) were injected i.p. with 250µl of dexamethasone (0.03mg/ml) (Dex+ UV-B) or sterile PBS (PBS+UV-B) 5 and 1 hr prior to UV-B irradiation (144mJcm^{-2}). Negative control mice (n=10) (Dex or PBS) were treated in the same way but were not exposed to UV-B radiation. The number of DC in DLN 48hr following UV-B exposure was measured.

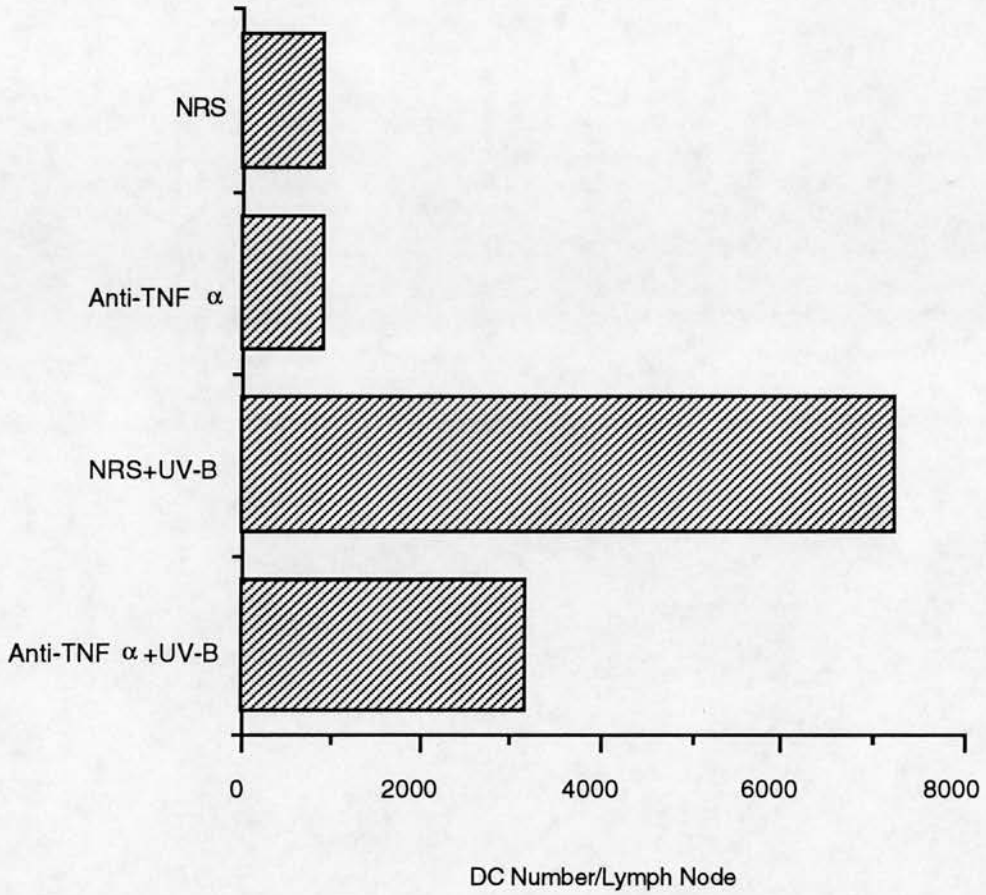

Figure 12 Effect of rabbit anti-murine TNFα antiserum on DC accumulation in DLN 48hr after UV-B irradiation. Groups of unshaven mice (n=10) were injected i.p. with 100µl of rabbit anti-murine TNFα antiserum (anti-TNFα+UV-B) or normal rabbit serum (NRS+UV-B) 2 hr prior to UV-B irradiation (144mJcm^{-2}). Negative control mice (n=10) (anti-TNFα or NRS) were treated in the same way but not exposed to UV-B radiation. The number of DC in DLN 48hr following UV-B irradiation was calculated.

illustrated in Figure 12, treatment of mice with anti-TNFα antibodies 2hr prior to UV-B irradiation decreased DC accumulation in DLN 48hr after UV-B treatment, suggesting that UV-B induced migration to DLN is stimulated by TNFα release.

3.4.3 Effect of TNFα on UV-B Induced Suppression of Contact Hypersensitivity Responses

Having demonstrated that TNFα release as a result of exposure to UV-B radiation induces DC accumulation in DLN it was important to explore the relationship between TNFα release and UV-B induced suppression of the CH response. Accordingly, 2hr prior to exposing the shaved dorsal surface of three panels of mice whose ears were protected to a single dose of UV-B (144mJcm^{-2}) radiation on day 0 and 1, one panel received i.p. injections of rabbit anti-murine TNFα antibodies, another received i.p. injections of NRS whilst the third panel was left untreated. Two other panels of mice received i.p. injections of rabbit anti-murine TNFα antiserum or NRS at the same time but were not exposed to UV-B radiation. These five panels of mice were then sensitized on their shaved dorsal skin with 50μl 1% oxazolone (in acetone:oil) on day 2. Six days later the dorsum of both ears of all these mice were challenged with 25μl 0.25% oxazolone (in acetone:oil) and the ear swelling response measured 24hr later. Positive and negative control mice were sensitized with oxazolone or vehicle, respectively without prior treatment or exposure to UV-B radiation. The ears of the positive control and one negative control were challenged with oxazolone, whilst the ears of another negative control were challenged with vehicle. The results of one experiment presented in Figure 13, demonstrated that administration of anti-TNFα antibodies prior to UV-B irradiation significantly inhibited UV-B induced suppression of the CH response to oxazolone supporting the view that UV-B susceptibility is mediated by intracutaneous production or release of TNFα.

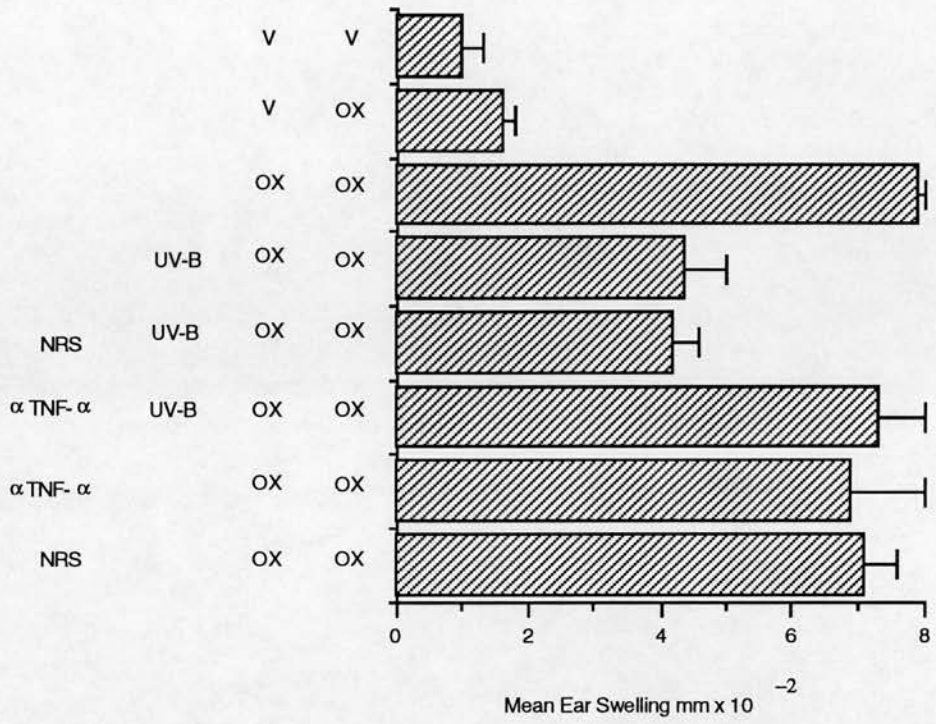

Figure 13 Influence of rabbit anti-murine TNFα antiserum on UV-B induced suppression of the CH response. Panels of 10 mice were injected i.p. with 100µl of rabbit anti-murine TNFα antiserum or or normal rabbit serum (NRS) 2 hr prior to exposing their shaved dorsal skin to a single dose of UV-B ($144mJcm^{-2}$) radiation on day 0 and 1. Control mice were either treated in the same way but not exposed to UV-B radiation or their shaved dorsal skin exposed to UV-B ($144mJcm^{-2}$) radiation on day 0 and 1 without any prior treatment. On day 2, 50µl of 1% oxazolone was topically applied to the shaved dorsal skin of all these mice. Six days later, the dorsum of both ears of these mice was challenged with 25µl 0.25% oxazolone. Bars represent mean (± SE of the mean) ear swelling response 24hr after challenge expressed as mm x 10^{-2}. The decreased ear swelling response of mice injected with normal rabbit serum prior to UV-B irradiation was significantly different from the positive control ($p<0.001$). The ear swelling response of mice injected with anti-TNFα antibodies prior to UV-B exposure was not significantly different from the positive control ($p>0.1$).

3.4.4 Effect of Narrow Band UV-B Irradiation on Dendritic Cell Accumulation in DLN

In an attempt to dissect out the effects of broad band UV-B irradiation on DC migration to DLN and clarify the role of cis-UCA in UV-B induced DC migration, a UV lamp which emits a narrow band of longer UV-B wavelengths around 311-312nm and isomerizes trans to cis-UCA very efficiently in vivo [Gibbs et al, 1993] was used. Accordingly, the ears of three panels of unshaven mice were exposed to a single dose of narrow band UV-B radiation at 144 or 864mJcm^{-2} or to a single dose of broad band UV-B radiation at 144mJcm^{-2}. Control mice were not exposed to UV radiation. Forty-eight hr following exposure all the mice were killed, their draining auricular lymph nodes excised and the number of DC in each lymph node calculated. It can be seen from Figure 14 that narrow band UV-B radiation which efficiently induces the formation of cis-UCA in the skin had no effect on DC accumulation in DLN regardless of the dose administered, further implying that UV-B induced migration does not occur as a result of the local production of cis-UCA. It is also fair to speculate from this result that the UV-B wavelengths emitted by the TL-01 lamp did not induce the production or release of TNFα since no DC migration was observed.

3.4.5 Effect of Narrow Band UV-B Irradiation on the Induction of Contact Hypersensitivity

Since the TL-01 lamp is known to emit wavelengths of UV-B which isomerize trans to cis-UCA efficiently and does not appear to stimulate TNFα release it was possible to explore the role of cis-UCA in UV-B induced suppression of CH responses. Subsequently, the shaved dorsal skin of three panels of mice whose ears were protected were exposed to a single dose of narrow band UV-B radiation at 144 or 432mJcm^{-2} or to a single dose of broad band UV-B radiation at 144mJcm^{-2} on day 0 and 1. On day 2 these three panels of mice were then sensitized on their shaved

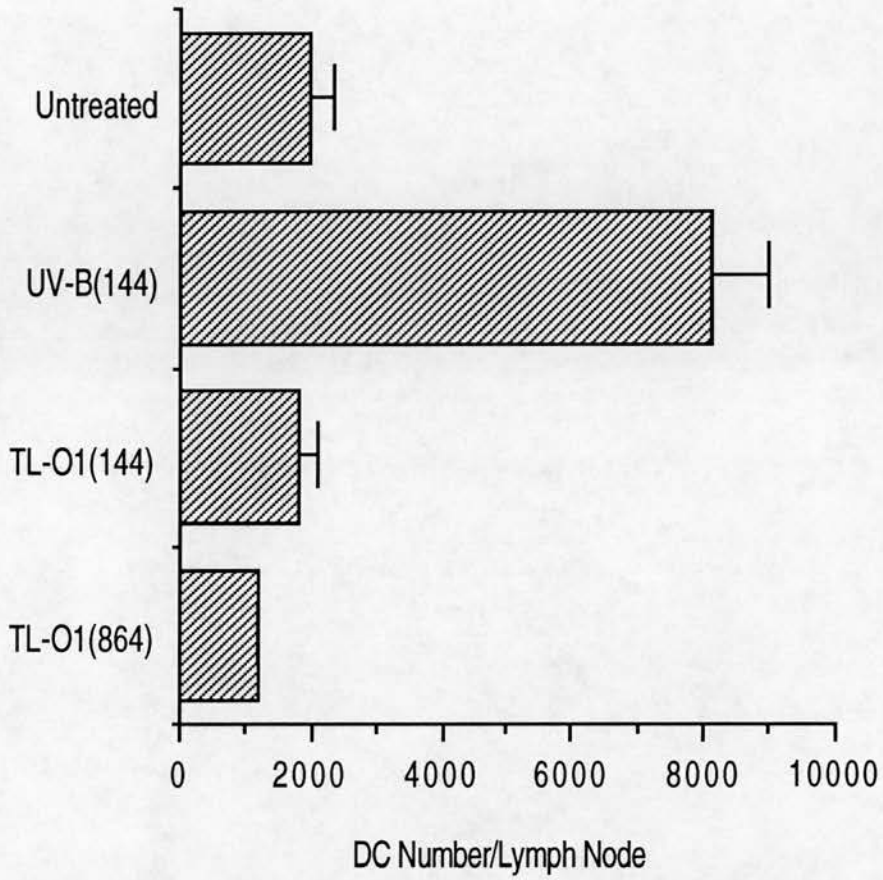

Figure 14 The effect of narrow and broad band UV-B irradiation on DC accumulation in DLN 48hr later. Three panels (n=10) of unshaven mice were exposed to a single dose of narrow band UV-B radiation of 144 (TL-01-144) or 864mJcm^{-2} (TL-01-864) or to a single dose of broad band UV-B radiation at 144mJcm^{-2} (UV-B-144). Negative control mice (n=10) were untreated. The number of DC in DLN 48hr following exposure was measured. The data illustrated represent the results of a single experiment (TL-01-864) or the mean ± standard error of three experiments.

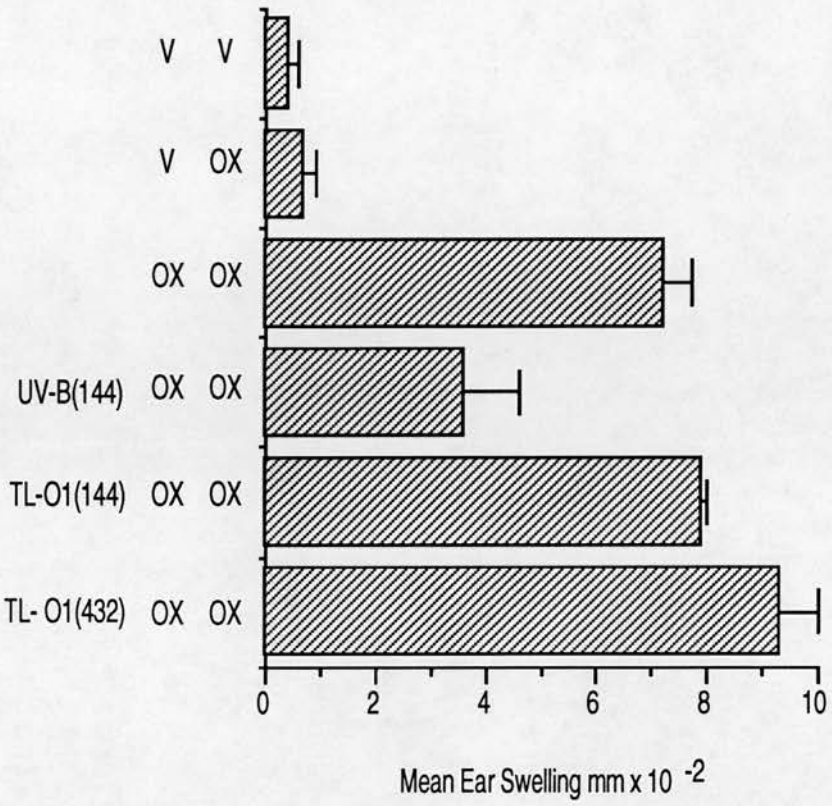

Figure 15 The effect of narrow and broad band UV-B irradiation on the induction of the CH response to oxazolone. The shaved dorsal skin of three panels (n=10) of mice were exposed to a single dose of narrow band UV-B radiation of 144 (TL-01-144) or 432mJcm^{-2} (TL-01-432) or to a single dose of broad band UV-B radiation at 144mJcm^{-2} (UV-B-144) on day 0 and 1. All of these mice were then sensitized on their shaved dorsal skin with 50µl 1% oxazolone on day 2. Six days later, the dorsum of both ears of these mice was challenged with 25µl 0.25% oxazolone. Positive and negative control mice (n=10) were sensitized with oxazolone or vehicle on their shaved dorsal skin, respectively. Bars represent mean (± SE of the mean) ear swelling response 24hr after challenge expressed as mm x 10^{-2} of two independent experiments. The decreased ear swelling response of mice exposed to broad band UV-B radiation was significantly different from the positive control ($p<0.01$) for each experiment. The ear swelling response of mice exposed to either dose of narrow band UV-B radiation was not significantly different from the positive control ($p>0.1$) for each experiment.

dorsal skin with 50µl 1% oxazolone (in acetone:oil). Six days later the dorsum of both ears of all these mice were challenged with 25µl 0.25% oxazolone (in acetone:oil) and the ear swelling response measured 24hr later. Positive and negative control mice were sensitized with oxazolone or vehicle on their shaved dorsal surface, respectively. The ears of the positive control mice and one negative control panel were challenged with oxazolone whilst the ears of a second panel of negative control mice were challenged with vehicle. As illustrated in Figure 15, which shows the results of two representative experiments, narrow band UV-B irradiation, irrespective of the dose administered, did not induce suppression suggesting that trans to cis-isomerization of UCA in response to UV-B irradiation is not sufficient by itself to induce local suppression of the CH response.

3.4.6 Influence of Anti-Cis-UCA Monoclonal Antibody on UV-B Induced Suppression of the Contact Hypersensitivity Response

In an attempt to further explore the role of cis-UCA in UV-B induced suppression of CH responses, 2hr prior to exposing the shaved dorsal skin of three panels of mice to a single dose of broad band UV-B radiation (144mJcm^{-2}) on day 0 and 1, one panel was injected i.p. with 100µl of affinity purified anti-cis-UCA monoclonal antibody (0.05mg/ml), another received i.p. injections of 100µl of sterile PBS whilst the third panel was left untreated. Another panel of mice was injected i.p. with 100µl of affinity purified anti-cis-UCA monoclonal antibody (0.05mg/ml) (see section 3.6) at the same time but was not exposed to UV-B irradiation. These mice were then sensitized on their shaved dorsal skin with 50µl 1% oxazolone (in acetone:oil) on day 2. Six days later the dorsum of both ears of all these mice was challenged with 25µl 0.25% oxazolone (in acetone:oil) and the ear swelling response measured 24hr later. Positive and negative control mice were sensitized with oxazolone or vehicle on their shaved dorsal skin, respectively without prior treatment or UV-B irradiation and their ears challenged with

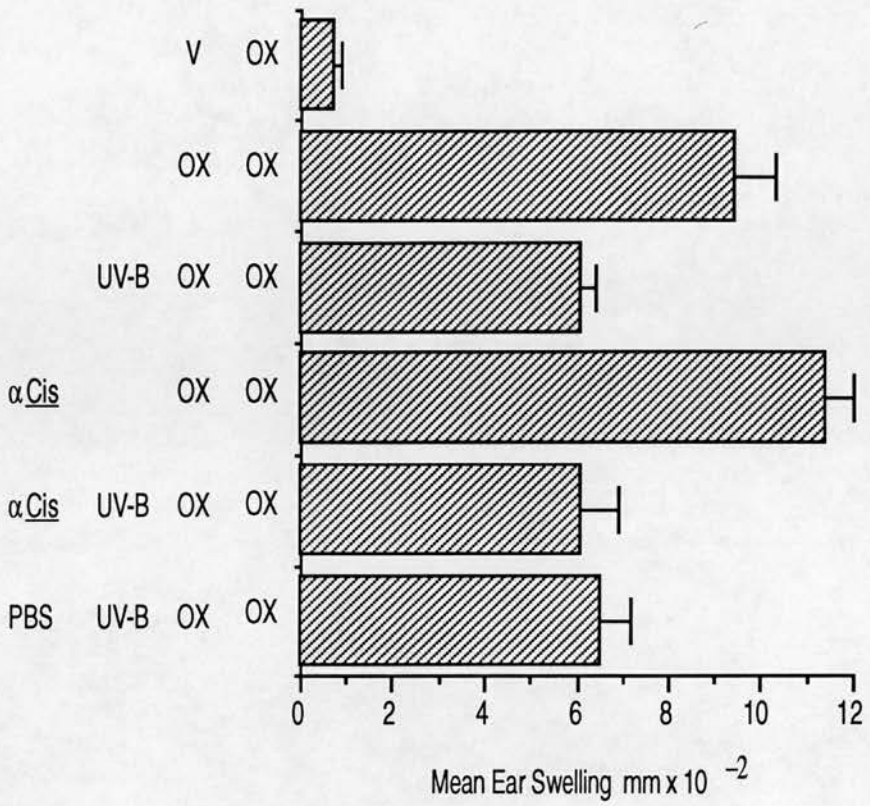

Figure 16 Influence of anti-cis-UCA monoclonal antibody on UV-B induced suppression of the CH response. Two hr prior to irradiating the shaved dorsal skin of three panels of mice (n=8) with a single dose of broad band UV-B radiation ($144mJcm^{-2}$) on day 0 and 1, one panel was injected i.p. with anti-cis-UCA monoclonal antibody, another received i.p. injections of sterile PBS while the third was left untreated. Another panel (n=8) was injected i.p. with anti-cis-UCA monoclonal antibody at the same time but was not irradiated. The mice were then sensitized on their shaved backs with 1% oxazolone on day 2 and 6 days later both their ears were challenged with 0.25% oxazolone. Bars represent mean (± SE of the mean) ear swelling response 24hr after challenge expressed as mm x 10^{-2}. The decreased ear swelling response of mice injected with PBS prior to UV-B irradiation was significantly different from the positive control ($p < 0.05$). The ear swelling response of mice injected with anti-cis-UCA monoclonal antibody prior to UV-B exposure was significantly different from the positive control ($p < 0.02$).

oxazolone 6 days later. It can be seen from Figure 16 which shows the results of one experiment that administration of anti-cis-UCA monoclonal antibody prior to UV-B irradiation did not inhibit UV-B induced suppression of the CH response suggesting that trans to cis-isomerization of UCA in response to UV-B irradiation is not important in inducing local suppression of the CH response.

3.5 Influence of UV-B Irradiation and UCA Isomers on Primary and Secondary Lymphocyte Proliferation Responses

3.5.1 Introduction

There is compelling evidence that DC which are considered to be of central importance in the presentation of antigen to T-lymphocytes [Inaba and Steinman, 1984] play a major role during the induction of skin sensitization. Following epicutaneous exposure of mice to contact sensitizers, there is a rapid accumulation in DLN of DC [Knight et al, 1985A and B; Macatonia et al, 1986; Macatonia et al, 1987; Kinnaird et al, 1989; Kimber et al, 1990A], a significant proportion of which bear high levels of antigen [Macatonia et al, 1986; Cumberbatch and Kimber, 1990]. There is considerable evidence which suggests that these antigen-bearing DC are derived from epidermal LC which are stimulated to migrate, via the afferent lymphatics to the draining nodes [Shelley and Juhlin, 1976; Silberberg-Sinakin et al, 1976; Larsen et al, 1990; Steinman, 1991; Kripke et al, 1990].

The antigen-bearing DC which arrive in DLN following skin-sensitization and which are derived from epidermal LC are highly immunogenic. It has been demonstrated that DC isolated from draining nodes of skin-sensitized mice are efficient inducers of both primary and secondary T-lymphocyte proliferative responses in vitro and small numbers transfer contact sensitivity to naive recipients [Knight et al, 1985A; Macatonia et al, 1986; Kinnaird et al, 1989; Macatonia et al, 1987; Jones et al, 1989; Macatonia and Knight, 1989]. Indeed, there exists a correlation between lymphocyte proliferative responses and DC accumulation in local lymph nodes following skin painting with contact sensitizers [Kimber et al, 1990A].

A number of studies in mice using a variety of assays to assess cell-mediated immunity indicated that UV-B irradiation in vivo or in vitro inhibited the function of splenic antigen-presenting cells [Letvin et al, 1980B; Gurish et al,

1982; Letvin et al, 1980A]. More recently, Bigby and his co-workers produced a series of hapten specific and Ia restricted T cell hybridomas and found that prior treatment of mouse skin with low dose UV-B irradiation before epicutaneous application of a contact sensitizer significantly reduced the capacity of lymph node hapten-bearing DC to stimulate the hybridomas [Bigby et al, 1989]. UV-B radiation has also been shown to have an inhibitory effect on the antigen-presenting function of murine epidermal cells [Austaad and Braathen, 1985; Stingl et al, 1981; Lynch et al, 1983; Gurish et al, 1983; Tang and Udey, 1991]. Cooper and his co-workers have demonstrated that following UV-B exposure in humans, epidermal LC are impaired in their antigen-presenting capacity. These investigators found that in vitro exposure of these cells to UV-B radiation rendered them ineffective as accessory cells in vitro assays assessing T cell dependent responses to alloantigens [Cooper et al, 1985]. As previously mentioned it is hypothesized that UV-B radiation might exert its immunomodulatary properties via cis-UCA [De Fabo and Noonan, 1983]. Interestingly, the administration, in vivo of cis-UCA, but not trans-UCA, in the absence of UV-B irradiation, has been demonstrated to inhibit antigen- presenting cell function of splenic DC and peritoneal adherent cells [Noonan et al, 1988].

It was demonstrated (Section 3.3.3) that following UV-B irradiation, there is a greater number but the same proportion of antigen-bearing DC entering the DLN than would occur with skin sensitization alone. It was shown also that Ia and ICAM-1 expression by DC accumulating in the DLN as a consequence of UV-B irradiation was apparently unaffected suggesting that antigen-presenting cell function may not be lost at least due to changes in expression of these molecules. In an attempt to investigate further the antigen-presenting cell potential of these antigen-bearing DC accumulating in the DLN following UV-B irradiation an examination of the ability of these cells to stimulate primary and secondary lymphocyte proliferative responses was carried out. An investigation of the

influence of UCA isomers on the antigen-presenting cell function of antigen-bearing DC induced to migrate to the DLN by skin sensitization was also carried out in the same way.

3.5.2 Influence of UV-B Irradiation and UCA Isomers on Primary Lymphocyte Proliferative Responses

At various times the ears of mice were exposed to a single dose of 96 or 148mJcm^{-2} of UV-B radiation or 200µg of cis or trans-UCA was applied topically to the dorsum of both ears or injected i.p. or s.c. into the flank. Twenty-four hr after the last treatment, the dorsum of both ears of each mouse was then sensitized with either 25µl of 0.25% oxazolone or 0.5% FITC. Control mice received an equal volume of the appropriate vehicle on the dorsum of both their ears at the same time. Mice sensitized with oxazolone or FITC were killed on day 3 and 4, respectively, their draining auricular lymph nodes excised and pooled for each experimental group. Spontaneous proliferation of single cell suspensions of lymph node cells was then measured (Table 14 and 15).

As shown in Table 14, proliferative responses of lymph node cells from mice exposed to a single dose of 96 or 148mJcm^{-2} UV-B radiation daily for 3 and 2 consecutive days respectively, prior to sensitization with oxazolone at the same site, were significantly and reproducibly suppressed. Although it was not possible to reproduce, a decreased proliferative response of lymph node cells from mice exposed to a single dose of 96mJcm^{-2} UV-B radiation daily for 3 consecutive days, prior to sensitization with FITC was also observed (Table 15). The proliferative response of lymph node cells from mice that had received 200µg of cis-UCA either topically or s.c. each day over 3 days prior to sensitization with oxazolone were reduced but once again it was not possible to reproduce these results (Table 14). On the other hand, the observed decreased proliferative response of lymph node cells from mice whose

Table 14 Influence of UCA Isomers and UV-B Irradiation on Spontaneous Lymphocyte Proliferative Responses Following Sensitization with oxazolone

Treatment (Days) Prior to/after Sensitization	Sensitization Δ	cpm ± SD$^{\sigma}$	% Decrease in lymphopro-liferation	Statistical Significance (by t-test)$^{¥}$
-	Vehicle	4569 ± 731		
-	0.25% Ox*	55763 ± 4320		
UV-B 2,1D(296mJcm^{-2})	" "	32542 ± 875	45	$p < 0.001$
Cis UCA 1D(200µg)$^{\pi}$	" "	55247 ± 5455	1	NS$^{\partial}$
Trans UCA 1D(200µg)$^{\pi}$	" "	47172 ± 7633	17	$p < 0.001$
-	Vehicle	2159 ± 282		
-	0.25% Ox*	61304 ± 5679		
UV-B 2,1D(296mJcm^{-2})	" "	46074 ± 2811	26	$p < 0.001$
Cis UCA 1D(200µg) i.p.†	" "	59232 ± 6678	3.5	NS$^{\partial}$
Cis UCA 1D(200µg) s.c.Flank†	" "	70956 ± 9899	0	-
Cis UCA 2D After(200µg)$^{\pi}$	" "	62820 ± 10290	0	-
Cis UCA 3,2,1D(200µg)$^{\pi}$	" "	42356 ± 9169	32	$p < 0.001$
UV-B 1D(96mJcm^{-2})	" "	40740 ± 5978	35	$p < 0.001$
UV-B 2D After(96mJcm^{-2})	" "	78738 ± 6074	0	-
-	Vehicle	4162 ± 733		
-	0.25% Ox*	72201 ± 5285		
UV-B 3,2,1D(288mJcm^{-2})	" "	52491 ± 2353	29	$p < 0.001$
Cis UCA 3,2,1D(200µg)$^{\pi}$	" "	63954 ± 12425	12	NS$^{\partial}$
CisUCA3,2,1D(200µg)s.c.Flank†	" "	83648 ± 8374	0	-
Trans UCA 3,2,1D(200µg)$^{\pi}$	" "	113617 ± 7306	0	-
-	Vehicle	3742 ± 183		
-	0.25% Ox*	128716 ± 3429		
UV-B 3,2,1D(288mJcm^{-2})	" "	97201 ± 5994	25	$p < 0.001$
Cis UCA 3,2,1D(200µg)$^{\pi}$	" "	145719 ± 4464	0	
Trans UCA 3,2,1D(200µg)$^{\pi}$	" "	99438 ± 5630	23	$p < 0.001$
CisUCA3,2,1D(200µg)s.c.Flank†	" "	111146 ± 5116	14	$p < 0.002$
-	Vehicle	2668 ± 262		
-	0.25% Ox*	82843 ± 3753		
UV-B 3,2,1D(288mJcm^{-2})	" "	59099 ± 4359	30	$p < 0.001$
Cis UCA 3, 2,1D(200µg)$^{\pi}$	" "	79854 ± 6612	4	NS$^{\partial}$

Mice were killed 3 days following sensitization, their draining auricular lymph nodes excised and pooled for each group. Spontaneous proliferation of single cell suspensions of lymph node cells was then measured.

Δ The dorsum of both ears of each mouse was sensitized with 25µl of 0.25% oxazolone or an equal volume of the appropriate vehicle, 24hr after the last UV or UCA treatment

$^{\sigma}$ Counts per minute ± standard deviation (SD)

$^{¥}$ Significance of difference from positive control group*

$^{\pi}$ UCA isomers were topically applied to the dorsum of both ears

† Mice were treated at a site distant from the site of sensitization

$^{\partial}$ Not significantly different from control group*

Table 15 Influence of UCA Isomers and UV-B Irradiation on Spontaneous Lymphocyte Proliferative Responses Following Sensitization with FITC

Treatment (Days) Prior to Sensitization	Sensitization Δ	cpm ± SDØ	% Decrease in lymphopro-liferation	Statistical Significance (by t-test)¥
-	Vehicle	4776 ± 1575		
-	0.5% FITC*	56464 ± 3554		
UV-B 3,2,1D(288mJcm^{-2})	" "	45085 ± 3303	22	$p < 0.001$
Cis UCA 3,2,1D(200µg)π	" "	51157 ± 5111	10	$p < 0.01$
-	Vehicle	4300 ± 385		
-	0.5% FITC*	45502 ± 16348		
UV-B 3,2,1D(288mJcm^{-2})	" "	66905 ± 7207	0	
Cis UCA 3,2,1D(200µg)π	" "	46007 ± 4318	0	
-	Vehicle	1552 ± 186		
-	0.5% FITC*	55094 ± 3699		
UV-B 3,2,1D(288mJcm^{-2})	" "	51538 ± 4313	7	$p < 0.05$
Cis UCA 3,2,1D(200µg)π	" "	56026 ± 2997	0	
Trans UCA 3,2,1D(200µg)π	" "	50621 ± 2757	8	$p < 0.01$
-	Vehicle	4570 ± 1040		
-	0.5% FITC*	22506 ± 2508		
Cis UCA 3,2,1D(200µg)π	" "	19693 ± 1430	16	$p < 0.01$
Trans UCA 3,2,1D(200µg)π	" "	24350 ± 9249	0	
Cis UCA 3,2,1D(200µg)π	Vehicle	6698 ± 596		
Trans UCA 3,2,1D(200µg)π	"	4186 ± 700		

Mice were killed 4 days following sensitization, their draining auricular lymph nodes excised and pooled for each group. Spontaneous proliferation of single cell suspensions of lymph node cells was then measured.

Δ The dorsum of both ears of each mouse was sensitized with 25µl of 0.5% FITC or an equal volume of the appropriate vehicle, 24hr after the last UV or UCA treatment

Ø Counts per minute ± standard deviation

¥ Significance of difference from positive control group*

π UCA isomers were topically applied to the dorsum of both ears

ears had been painted with 200µg of cis-UCA daily for 3 consecutive days prior to sensitization with FITC was reproducible (Table 15).

3.5.3 Influence of UV-B Irradiation and UCA Isomers on Secondary Lymphocyte Proliferative Responses

Before assessing the immunostimulatory properties of DC from mice exposed to UV-B radiation or UCA isomers prior to sensitization, as a function of their ability to initiate proliferative responses by sensitized lymph node cells in vitro, it was first necessary to establish the optimal concentration of DC from sensitized mice which would stimulate a significant proliferative response. Subsequently, the ears of two panels of mice were each sensitized with 25µl of 5% FITC or 2.5% oxazolone and killed 18 and 21hr later, respectively. Their draining auricular lymph nodes were excised and DC-enriched stimulator populations prepared. Responder lymph node cells from mice which had been sensitized 7 days previously on the dorsum of both ears with 25µl of 5% FITC or 0.5% oxazolone were then cultured alone or in the presence of different numbers of stimulator cells from mice sensitized with the homologous contact sensitizer. As a control for non-specific stimulation, responder lymph node cells from mice sensitized 7 days previously, with 5% FITC or 0.5% oxazolone were cultured with an appropriate number of stimulator cells from mice sensitized with oxazolone and FITC respectively. Table 16 and 17 both document the results obtained from one experiment in which various responder : stimulator ratios were employed. It was found that DC-enriched populations prepared from DLN 18 and 21hr following exposure to 5% FITC and 2.5% oxazolone, respectively caused a significant stimulation of proliferation by lymphocytes sensitized to the appropriate homologous contact sensitizer, at a ratio of 17:1. It was observed also that DC fractions isolated from mice exposed to oxazolone which induced a marked proliferative response by lymphocytes sensitized to the same contact sensitizer, induced a significantly smaller response by lymphocytes

Table 16 Stimulation of Antigen (FITC) - Primed Lymph Node Cell Proliferation by DC Isolated from the DLN 18hr Following Skin Sensitization With 5% FITC

Stimulator Cells¥	Responder Cells	Responder : Stimulator Ratio	Lymphocyte Proliferation ^{3}H-Thymidine Incorporation (cpm ± SE)
None	5×10^5	-	1196 ± 102
3×10^4	"	17 : 1	8651 ± 563*
10^4	"	50 : 1	4903 ± 390
6×10^3	"	83 : 1	3121 ± 133
3×10^3	"	167 : 1	2765 ± 271
1.5×10^3	"	333 : 1	1822 ± 100
6×10^3	$5 \times 10^{5}\pi$	83 : 1	3681 ± 286

¥ Stimulator cells exhibited no significant incorporation of ^{3}H-thymidine compared with background controls
* Significantly elevated compared with primed LNC cultured in the absence of stimulator cells ($p < 0.001$)
π oxazolone-primed LNC

Table 17 Stimulation of Antigen (oxazolone) - Primed Lymph Node Cell Proliferation by DC Isolated from the DLN 21hr Following Skin Sensitization With 2.5% oxazolone

Stimulator Cells¥	Responder Cells	Responder : Stimulator Ratio	Lymphocyte Proliferation ^{3}H-Thymidine Incorporation (cpm ± SE)
None	5×10^5	-	3551 ± 247
3×10^4	"	17 : 1	8880 ± 307*
10^4	"	50 : 1	5903 ± 278
5×10^3	"	100 : 1	5624 ± 507
2.5×10^3	"	200 : 1	4214 ± 370
10^4	$5 \times 10^{5\pi}$	50 : 1	2481 ± 267

¥ Stimulator cells exhibited no significant incorporation of ^{3}H-thymidine compared with background controls
* Significantly elevated compared with primed LNC cultured in the absence of stimulator cells ($p < 0.001$)
π FITC-primed LNC

primed to an unrelated contact sensitizer at the same responder : stimulator ratio, suggesting that the influence of the DC fraction on lymphocyte proliferation was largely, but not wholly antigen-specific in nature (Table 17). It was not possible, however, to reach such a conclusion with DC-enriched populations prepared from mice exposed to FITC (Table 16).

Having established that DC which accumulate within the DLN of mice exposed topically to a contact sensitizer initiate proliferative responses by appropriately sensitized lymph node cells in vitro, an investigation of the influence of UV radiation and UCA isomers on the ability of these DC to stimulate secondary proliferative responses was carried out. The ears of shaved mice were exposed to a single dose of $96mJcm^{-2}$ UV-B radiation daily for 3 consecutive days or 200µg of cis or trans-UCA was topically applied to the dorsum of each ear over the same period. Positive control mice were treated in the same way except they received two lots of 25µl of 20% DMSO in acetone on the dorsal surface of each ear. Twenty-four hr following their last treatment all the mice were sensitized on the dorsal surface of each ear with 25µl of 5% FITC. They were then killed 18hr following sensitization, their draining auricular lymph nodes excised and DC-enriched populations prepared. Responder lymph node cells from mice which had been sensitized 7 days previously on the dorsum of both ears with 25µl of 5% FITC were cultured alone or in the presence of different numbers of these stimulator cells. As illustrated in Table 18 regardless of the ratio of responder : stimulator cells UV-B radiation and topical exposure to UCA isomers significantly inhibited the antigen-presenting cell function of DC isolated from DLN 18hr following skin sensitization with 5% FITC. However, it was not possible to reproduce the data shown in Table 18 which represents a single experiment, because DC from mice sensitized with contact sensitizer only were unable to initiate consistent and significant proliferative responses of responder cells thereafter.

Table 18 Influence of UV-B Irradiation and UCA Isomers on the Functional Activity of DC Isolated from the DLN 18hr Following Skin Sensitization with 5% FITC, to Stimulate FITC - Primed LNC Proliferation

In Vivo Treatment of Stimulator cells (DC)	Stimulator Cells (DC)	2^0 Lymphoproliferation (cpm ± SE)	% Suppression	Statistical Significance (by t-test)
-ve Control	-	4312 ± 581		
+ve Control $^{\#\,\pi}$	3×10^4	55144 ± 2797*		
	10^4	36789 ± 1395∂		
UV-B 3,2,1D(288mJcm^{-2})$^{\dagger\pi}$	3×10^4	31817 ± 644	46	p < 0.001¥
	10^4	10841 ± 1726	80	p < 0.001ø
Cis UCA 3,2,1D$^{\Delta\,\pi}$	3×10^4	38493 ± 2488	33	p < 0.01¥
	10^4	31115 ± 2388	17	NSß
Trans UCA 3,2,1D$^{\Delta\,\pi}$	3×10^4	22134 ± 1634	72	p < 0.001¥
	10^4	12212 ± 1653	76	p < 0.001ø

The dorsum of both ears were painted with 25µl of 20% DMSO
π Twenty-four hr following their last treatment the mice were sensitized on the dorsal surface of each ear with 25µl of 5% FITC
† Both ears of shaved mice were exposed to a single dose (96mJcm^{-2}) of UV-B radiation daily
¥ Significance of difference from control group*
ø Significance of difference from control group∂
Δ The dorsum of both ears were painted with 200µg of UCA daily
ß Not significant

The mice were killed 18hr after sensitization, their draining auricular lymph nodes removed and DC-enriched populations prepared. Responder lymph node cells from mice sensitized 7 days previously with 5% FITC were cultured alone or in the presence of different numbers of stimulator cells

3.6 Characterization of a Monoclonal Antibody to Cis-UCA and its Use in Detecting Cis-UCA in Serum Following UV-B Irradiation of Mice

3.6.1 Introduction

The mechanism by which cis-UCA alters immune function is not known. Indeed, it has not been demonstrated whether cis-UCA formed from the trans-isomer on UV-B irradiation stays within the epidermis, exerting its immunosuppressive effect entirely locally and/or systemically or whether it leaves the skin via the lymphatic or blood system where it may gain access to other areas of the body.

In an attempt to elucidate whether or not cis-UCA formed from the trans-isomer on UV-B irradiation leaves the skin, a monoclonal antibody with specificity for cis-UCA was produced and used to detect cis-UCA in serum following UV-B irradiation of mice.

3.6.2 Preparation of a Monoclonal Antibody Against Cis-UCA

Mice were immunized by s.c. injection of 200μg cis-UCA-KLH conjugate in 0.1ml Freund's incomplete adjuvant per mouse. A first booster injection given i.p., consisting of 200μg cis-UCA-KLH conjugate in 0.2ml sterile PBS, was administered after 36 days, followed by a second after 61 days and a third after 166 days. Mice were bled 7 days after the second booster and sera screened for anti-cis-UCA antibody by ELISA. Mice showing antibody titres to cis-UCA-BSA conjugate were killed 3 days after the third booster and spleen cells fused with NS-O mouse myeloma cells. One hybridoma was found which produced an antibody recognizing cis-UCA-BSA conjugate on ELISA. After cloning the single positive culture by limiting dilution twice, a titration of the hybridoma culture supernatant was made by ELISA to establish the end point titre and the antibody dilution giving 50% binding activity of this antibody with 5μg cis-UCA-BSA conjugate per well as antigen (Figure 17). The end point titre of the hybridoma culture supernatant was 256000

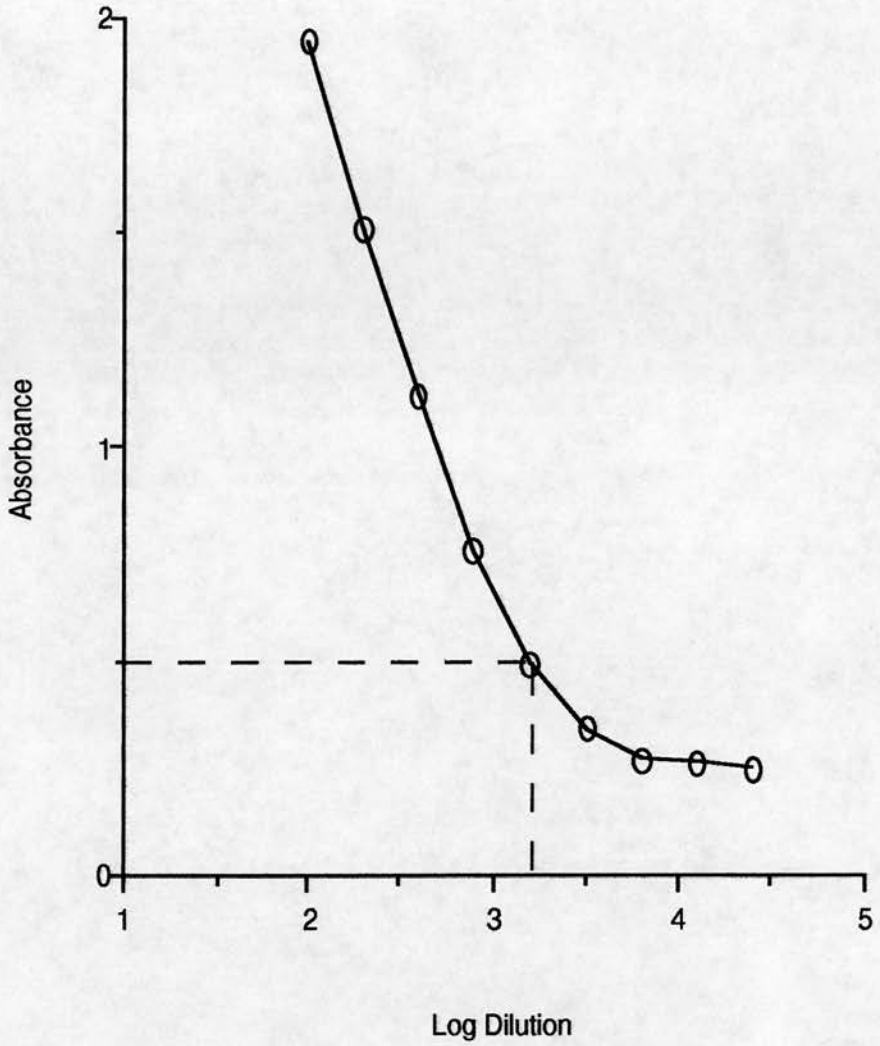

Figure 17 Titration of hybridoma cell supernatant. The antibody dilution giving 50% binding activity was 1 : 1600 (log 3.2)

i.e. the highest dilution which gave a mean absorbance more than the mean of an equivalent dilution of culture medium plus 3 x standard deviation of the mean. The antibody dilution giving 50% binding activity was 1:1600. Using an isotyping kit, the monoclonal antibody was found to be IgG_1 Kappa.

This culture supernatant was tested for activity against other conjugates and proteins as antigens as shown in Table 19. Trans-UCA-BSA, histamine-BSA, BSA or KLH were not recognized.

3.6.3 Characterization of the Monoclonal Antibody

A competitive inhibition ELISA was used to determine the fine specificity of the monoclonal antibody recognizing cis-UCA-BSA conjugate. The sensitivity of this assay increases if the quantity of antigen coated on the plate is as low as possible, since at low concentrations a variation in the amount of competing antigen has a larger impact on the interaction with the monoclonal antibody being tested [Tijssen, 1987]. Subsequently, a checkerboard titration was performed with doubling dilutions of both cis-UCA-BSA conjugate starting at 1.25µg per well, and the monoclonal antibody starting at 1:100 (Figure 18). The lowest concentration of cis-UCA-BSA conjugate (0.08µg per well) which gave a reasonable O.D. (0.3) with monoclonal antibody diluted at 1:400, was chosen for the competitive inhibition ELISAs carried out to demonstrate the specificity of the monoclonal antibody. In these tests, the results of which are shown in Table 20 , UCA isomers and analogues or conjugates were added to the well together with the monoclonal antibody and the inhibition of binding of the monoclonal antibody to cis-UCA-BSA calculated. It was found that while cis-UCA inhibited the binding of the monoclonal antibody to the antigen down to a level of 0.1µg per well, trans-UCA had little effect in comparison; inhibition was apparent only if trans-UCA was present at 5µg per well. Cis-2-methyl-UCA was the only UCA analogue to show any inhibition in binding but only at the high concentration of 5µg per well. Trans-2-methyl-UCA had no effect. Cis and

Table 19 Binding of monoclonal antibody (1 : 1600 dilution) to various antigens (5μg per well) measured by ELISA

Antigens	Absorbance ± SD
Cis-UCA-BSA	0.408 ± 0.015
Trans-UCA-BSA	0.044 ± 0.005
Histamine-BSA	0.049 ± 0.011
BSA	0.037 ± 0.005
KLH	0.060 ± 0.005

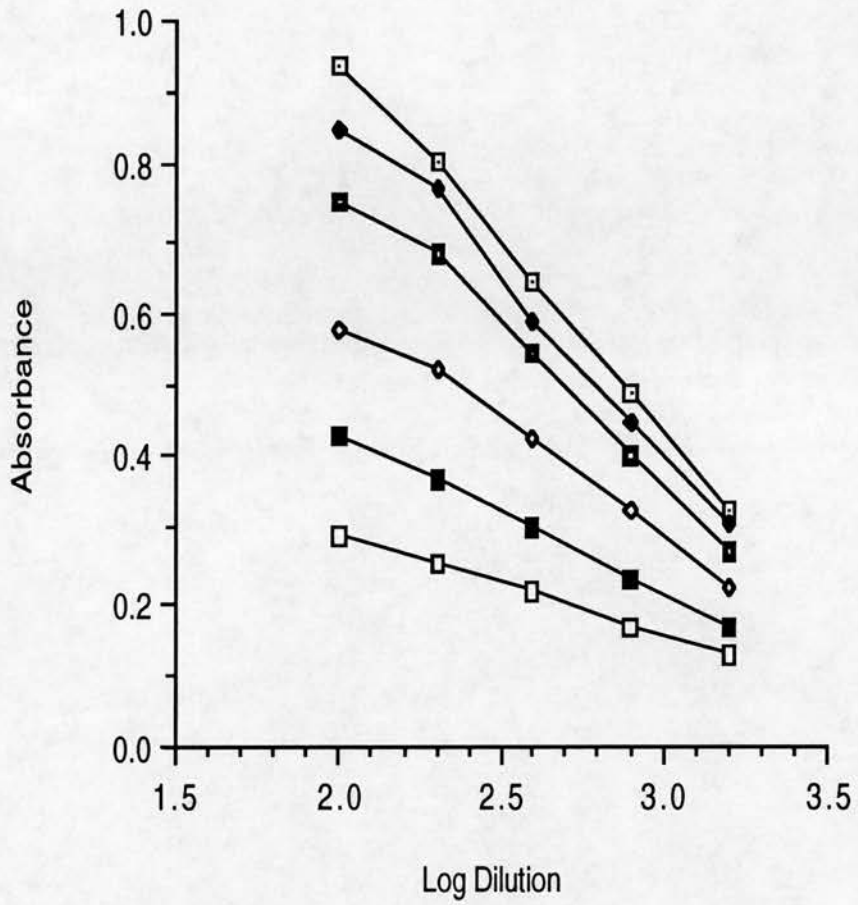

Figure 18 Binding of monoclonal antibody to cis-UCA-BSA conjugate at concentrations of 1.26 (⊡), 0.63 (◆), 0.32 (◘), 0.16 (◇), 0.08 (■), and 0.04 (□) µg per well by ELISA.

Table 20 Competitive inhibition ELISA to test effects of UCA analogues and conjugates on binding of monoclonal antibody (1 : 400 dilution) to cis-UCA-BSA conjugate (0.08μg / well)

	% Inhibition of Binding			
μg analogue or conjugate per well	5	1	0.1	0.03
Cis-UCA	100	80	16	0
Trans-UCA	27	0	0	0
Histamine	ND	0	0	0
Histamine-BSA	ND	0	0	ND
Cis-UCA-KLH	ND	96	60	21
Trans-UCA-KLH	ND	83	6	2
Cis-2-methyl-UCA	59	6	0	0
Trans-2-methyl-UCA	0	0	0	0

Cis and trans - pyridine - 2 - acrylic acid, cis and trans - pyridine - 3 - acrylic acid, cis and trans - 2 - furanacrylic acid, cis and trans - 2 - thiophenoacrylic acid, cis and trans - 3 - thiopheneacrylic acid, dihydrourocanic acid and histidine (all at 1 μg and 0.1 μg per well) did not inhibit the binding of the monoclonal antibody.

ND = not done

trans-UCA-KLH conjugate were found to be both inhibitory down to 0.03μg per well, although the monoclonal antibody had a higher functional affinity for the cis-UCA-KLH conjugate compared with trans-UCA-KLH.

3.6.4 Screening of Murine Sera for Cis-UCA

It was first necessary to explore how sensitive the competitive inhibition ELISA system would be for detecting quantitatively various concentrations of cis-UCA in serum. Thus, various amounts of cis and trans-UCA were added to murine serum to obtain a standard curve. The result of one such experiment is shown in Figure 19. It appears that amounts of cis-UCA between 0.75 and 0.1μg may be detected accurately by this method, while trans-UCA is not detected at all.

The assay was then applied to the detection and measurement of cis-UCA in serum at various times after UV-B irradiation of mice (Table 21). In the first experiment the ears and shaved dorsal skin of ten mice were exposed to a single dose of UV-B radiation ($216mJcm^{-2}$) and 14, 25, 39, 48 and 114hr after irradiation serum from two mice was collected by bleeding via the vena cava. All of these mice were eyebled 5hr prior to irradiation for control serum. As illustrated in Table 21 which shows the result of a representative experiment, after a single exposure of UV-B radiation the significant inhibition in binding of the monoclonal antibody observed indicated a maximum amount of cis-UCA in the serum 25hr after irradiation, equivalent to 0.1μg cis-UCA in each well of the assay i.e. about 6μg cis-UCA per ml serum. In a second experiment the ears and shaved dorsal skin of fourteen mice were exposed twice to $144mJcm^{-2}$ of UV-B radiation with 24hr between each exposure and 1, 5, 11, 24, 29, 47 and 114hr after the second exposure, serum from two mice was collected by bleeding via the vena cava. Five hr prior to irradiation all of these mice were eyebled for control serum. It can be seen from Table 21 which shows the results of a representative experiment that after two exposures of UV-B radiation, cis-UCA was detected in serum 1hr after the

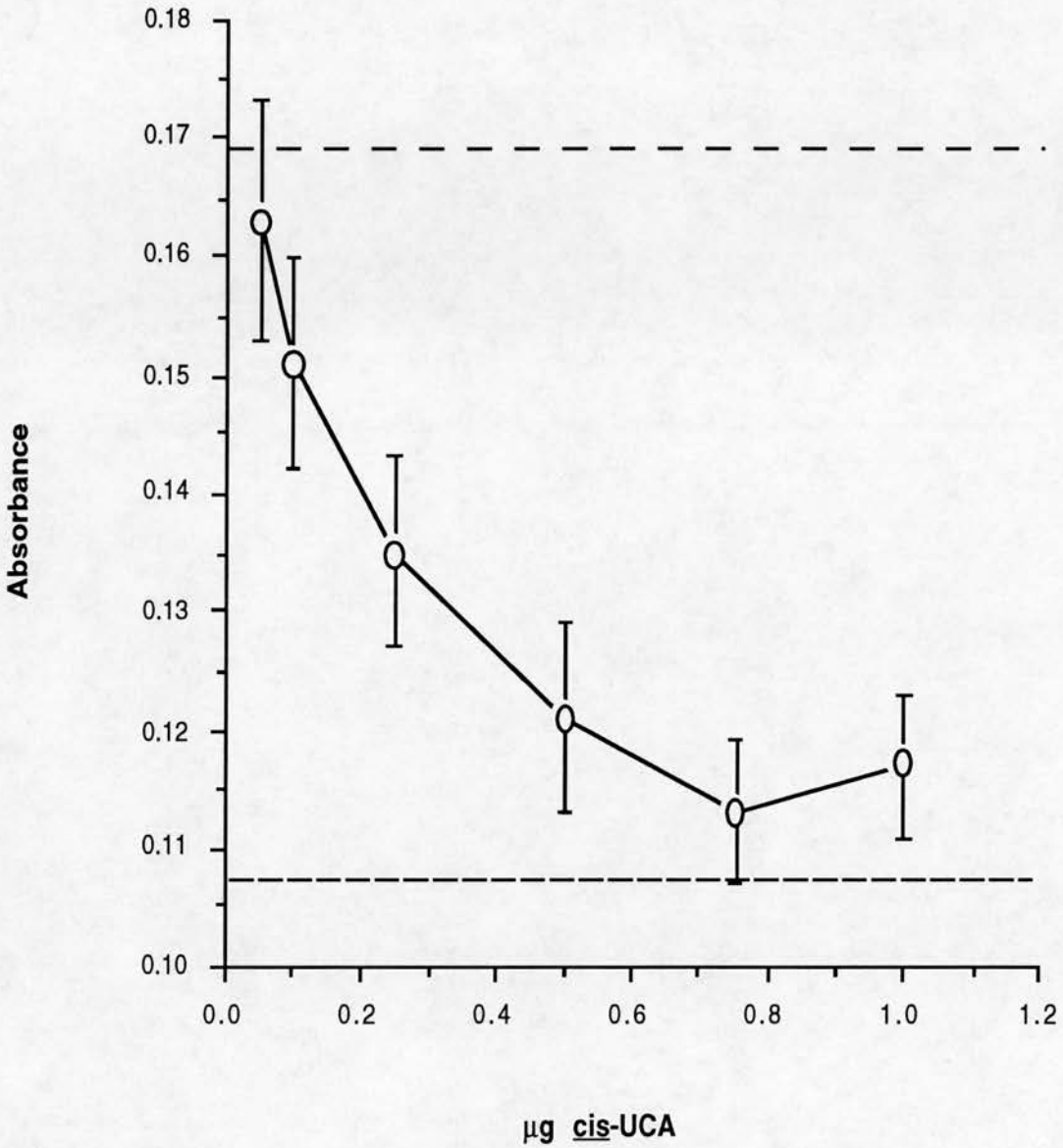

Figure 19 Standard curve showing the effect of varying concentrations of cis-UCA in the competitive inhibition ELISA. The monoclonal antibody was used at a dilution of 1 : 400 and the cis-UCA-BSA conjugate at 0.08µg per well. The mean absorbance of the positive control (— — —) was 0.169 and the mean absorbance of the negative control (———·) was 0.107. Trans-UCA added at 1µg and 0.5µg per well, showed absorbances of 0.176 and 0.188 respectively.

Table 21 Competitive inhibition ELISA to assay cis-UCA in serum at various times after irradiation of mice.

Experiment 1				Experiment 2			
Time after irradiation (hr)[π]	Absorbance	Significance*	% Inhibition of binding	Time after second irradiation (hr)[¥]	Absorbance	Significance*	% Inhibition of binding
Prebleed serum	0.157			Prebleed serum	0.355		
14	0.124	NS[ø]	21	1	0.294	$p < 0.01$	17
25	0.116	$p < 0.001$	26	5	0.225	$p < 0.01$	35
39	0.139	NS[ø]	11	11	0.256	$p < 0.001$	28
48	0.142	$p < 0.01$	10	24	0.291	$p < 0.01$	18
114	0.161	NS[ø]	0	29	0.283	$p < 0.01$	20
				47	0.341	NS[ø]	4
				114	0.338	NS[ø]	5
Prebleed+0.5μg Cis-UCA	0.050	$p < 0.001$	68	Prebleed+0.5μg Cis-UCA	0.224	$p < 0.001$	37
Prebleed+0.5μg Trans-UCA	0.163	NS[ø]	0	Prebleed+0.5μg Trans-UCA	0.340	NS[ø]	4
Prebleed+0.1μg Cis-UCA	0.112	$p < 0.001$	29	Prebleed+0.1μg Cis-UCA	0.291	$p < 0.001$	18
Prebleed+0.1μg Trans-UCA	0.171	NS[ø]	0	Prebleed+0.1μg Trans-UCA	0.335	NS[ø]	6

The monoclonal antibody was used at a dilution of 1: 400 and the cis-UCA-BSA conjugate at 0.08μg per well

[π] One exposure of 216mJcm^{-2} UV-B radiation
* Significance of difference from prebleed serum (Student's t-test)
[¥] Two exposures of 144mJcm^{-2} UV-B radiation with 24hr between
[ø] Not significant

Table 22 Competitive inhibition ELISA to assay cis-UCA in dialysed and undialysed serum 25hr after irradiation of mice with 216mJcm^{-2} of UV-B radiation

Serum sample	Absorbance	% Inhibition of binding
Prebleed : undialysed	0.149*	
Prebleed : dialysed	0.109ø	
Post-irradiation : undialysed	0.085π	43
Post-irradiation : dialysed	0.108¥	0
Prebleed : undialysed + 1.0μg Cis-UCA	0.039	74
" " + 0.5μg Cis-UCA	0.063	58
" " + 0.1μg Cis-UCA	0.096	36

The monoclonal antibody was used at a dilution of 1 : 400 and the cis-UCA-BSA conjugate at 0.08μg per well

π Significantly different from* ($p < 0.001$; Student's t-test)
¥ Not significantly different fromø ($p > 0.01$; Student's t-test)

second irradiation reaching a maximum at 5hr of approximately 30μg per ml serum, and decreasing to approximately 6μg per ml serum by 29hr.

It was not known whether the cis-UCA in the serum was present in a free form or conjugated to another molecule. To test this, 25hr after the ears and shaved dorsal skin of ten mice had been exposed to a single dose of UV-B radiation ($216mJcm^{-2}$), serum was collected from these mice (as described previously) and dialysed. The dialysed serum was then compared with undialysed serum collected from the same mice in a competitive inhibition ELISA (Table 22). It can be seen that there was a significant inhibition in binding of the monoclonal antibody to the antigen in the presence of serum removed from mice 25hr after irradiation and that this inhibition was removed by dialysis of the serum. Therefore cis-UCA in the serum of irradiated animals was in an unbound form or conjugated to a molecule with a m.wt. below 12000.

CHAPTER 4

DISCUSSION

4.1 Mechanism of UV-B Induced Immunosuppression

It is well established that exposure of mice to UV-B radiation impairs the immune response to contact-sensitizing haptens applied to the irradiated skin [Toews et al, 1980; Streilein and Bergstresser, 1988; Yoshikawa et al, 1990]. Further, under these conditions, hapten-specific suppressor T lymphocytes are generated in the lymphoid organs of mice [Elmets et al, 1983]. At present there is controversy as to whether the decreased immune response observed following UV-B irradiation is mediated by these suppressor T cells [Elmets et al, 1983; Glass et al, 1990]. Moreover, the mechanism of this UV-B induced immunosuppression is unknown. However, since it is now more or less accepted that the migration of antigen-bearing epidermal LC from the skin to DLN is necessary for the induction of CH responses [Okamoto and Kripke, 1987; Macatonia et al, 1987; Kripke et al, 1990], a UV-B induced perturbation in the migration of these cells would have profound implications both for the impairment of the induction of CH and the generation of antigen-specific suppressor T lymphocytes. Evidence that UV-B irradiation may influence the migration of LC was first provided by Toews and his colleagues [Toews et al, 1980]. These investigators reported that LC density was decreased in UV-B exposed skin (as detected by ATPase staining) and that there was a clear correlation between this decrease and the ability of irradiated skin to support the induction of CH. However, since these observations it has become apparent that UV-B irradiation modulates surface markers expressed by LC and consequently these may not provide a reliable indicator of the presence or absence of LC in the skin [Lynch et al, 1981; Aberer et al, 1981; Hanau et al, 1985]. Thus, it is uncertain

whether UV-B irradiation influences LC migration. In an attempt to explore further the possibility that UV-B irradiation may influence LC migration, in the first part of this investigation the effect of UV-B irradiation on the number of DC accumulating in the DLN of unsensitized and sensitized mice was examined. The results reported here demonstrate that UV-B irradiation alone (Table 9), or prior to skin sensitization with FITC at the same site (Table 12), induced or enhanced DC migration to DLN, respectively. Further, the data reveal that the percentage of DC bearing FITC in the DLN of unirradiated and irradiated mice was identical (Table 13), implying that UV-B irradiation prior to sensitization increased the number of antigen-bearing DC migrating to DLN. In addition, it was demonstrated that UV-B exposure had no influence on the amount of antigen being carried by these cells, as they migrate to DLN (Table 13).

In view of these results it is not possible to conclude that the immunosuppressive effect of UV-B radiation is mediated through the simple inhibition of antigen transport by LC to the DLN as postulated by several investigators [Bigby et al, 1989; Vermeer and Streilein, 1990; Streilein et al, 1990]. Equally, it appears unlikely that impaired sensitization following local UV-B treatment would be a consequence of the prior migration of functionally mature LC from the epidermis as suggested by the results of Toews et al (1980). However, in contrast to the data reported here, Bigby et al (1989) found that after low dose UV-B treatment of skin prior to hapten painting, they were unable to demonstrate the presence of hapten-bearing antigen-presenting cells in DLN, implying that LC from the hapten-painted UV-B treated site had failed to migrate. Despite this report, the results presented here have been corroborated by the findings of Okamoto and Kripke (1987) and more recently by Tang et al (1992). Both Okamoto and Kripke (1987) and Tang et al (1992), using flow cytometry and fluorescence microscopy respectively, reported that there was no difference in the percentage of $FITC^+$ cells in the DLN of unirradiated and irradiated mice. Tang et al (1992) extended these

findings by demonstrating that the number of $FITC^+$ cells in the DLN of UV-B irradiated mice was greater than that in the DLN of non-irradiated mice and that there was no difference in the fluorescence intensity of the cells from the two groups.

In an attempt to elucidate further the mechanism of UV-B induced immunosuppression it was necessary to explore the obvious question raised by the findings presented here which was, whether the antigen-bearing DC in the DLN of UV-B irradiated mice are capable of functioning as normal antigen-presenting cells in stimulating the induction of CH responses. This was carried out by investigating the ability of antigen-bearing DC accumulating in DLN following UV-B irradiation, to stimulate primary and secondary lymphocyte proliferative responses.

Circumstantial evidence is available in the literature which suggests that the extent to which an animal becomes sensitized following topical exposure to a contact allergen is determined by the magnitude of the primary T lymphocyte proliferative response in the DLN [Kimber et al, 1989; Kimber et al, 1990B; Moorhead, 1976; Dieli et al, 1987; Kimber and Dearman, 1991]. Moreover, Kimber et al (1990A) have provided strong evidence that this primary response is in turn associated with the number of antigen-bearing DC accumulating in DLN following skin painting. In light of this evidence it is interesting that despite a UV-B induced increase in the migration of FITC-bearing DC to DLN found here, the lymphocyte proliferative response of cells isolated from these lymph nodes was decreased (Table 15). Although, this result was not reproducible, it is clear from the data recorded in the present study that UV-B irradiation was able to induce a reproducible and significant reduction in the spontaneous lymphocyte proliferative response of DLN cells from mice sensitized with oxazolone (Table 14). Thus, assuming that oxazolone is carried by DC to the DLN following UV-B exposure, the results suggest that, as a consequence of UV-B irradiation, the antigen-presenting function of antigen-bearing DC in DLN is decreased. The spontaneous

lymphoproliferation assay is, however, a relatively crude measure of the influence of UV-B irradiation on the ability of antigen-bearing DC to stimulate a primary T lymphocyte response. It does not permit discrimination between alterations in the ability of antigen-presenting cells to activate appropriate antigen-specific lymphocytes from other alterations in immune function that may occur within the lymph node, as the result of exposure of the skin to UV irradiation.

However, despite this, further support for the notion that UV-B irradiation exerts its immunomodulatory effect at the level of the antigen-presenting cell was provided by the demonstration that the proliferative response of FITC-primed lymph node cells from the DLN of unirradiated mice is reduced when these cells are incubated in vitro with DC-enriched populations from the DLN of mice sensitized epicutaneously with FITC following UV-B exposure (Table 18). It is important to point out that this finding does not indicate whether, as a consequence of UV-B irradiation, the antigen-presenting function of this DC-enriched population is diminished or merely altered. Two subsets of CD4+ T cells have been identified in mice [Mosmann et al, 1986A; Mosmann and Coffman, 1987; Powrie and Mason, 1988; Bottomly, 1988]. These subsets can be distinguished on the basis of cytokine production and function. Th1, but not Th2 cells produce IL-2, IFN-γ, and lymphotoxin (TNFβ), whereas Th2 cells express IL-4, IL-5, IL-6, and IL-10. Th1 cells appear to be the effectors of DTH reactions [Cher and Mosmann, 1987; Mosmann and Coffman, 1989A; Mosmann and Coffman, 1989B] whereas Th2 cells are more efficient at promoting B cell responses to soluble antigen [Bottomly, 1988; Mosmann et al, 1986A], stimulating IgE production [Lebman and Coffman, 1988], and enhancing the growth and differentiation of mast cells and eosinophils [Mosmann et al, 1986B]. There is considerable evidence that Th1 and Th2 cells differ in terms of their requirements for antigen presentation [Mosmann and Coffman, 1989A; Gajewski et al, 1989; Weaver et al, 1988; Weaver and Unanue, 1990; Gajewski et al, 1990]. Thus, it seems reasonable to speculate that if UV-B irradiation could

induce an alteration in the signalling machinery of lymph node DC (presumably derived from LC) to favour Th2 cell activation, these cells would be unable to activate antigen-primed Th1 cells from the DLN of unirradiated mice. Indeed, the reports by Araneo et al (1989) and Simon et al (1990) both support this notion. Araneo and her colleagues demonstrated that irradiation of mice with high doses of UV-B changed the pattern of lymphokine secretion by activated T cells from a Th1 to a Th2 pattern. More direct evidence was provided by Simon et al (1990) who showed that low dose UV-B irradiation inhibited the capacity of LC to induce proliferation of Th1 cells while not perturbing their ability to stimulate Th2 cells.

Evidence that the FITC-bearing DC, which accumulate in the DLN following UV-B irradiation, have a decreased or altered antigen-presenting cell function has been demonstrated in vivo by a number of investigators [Chung et al, 1986A; Okamoto and Kripke, 1987; Alcalay and Kripke, 1991]. Cells were collected from the DLN of UV-B irradiated or unirradiated mice, epicutaneously sensitized with FITC or DNFB. In contrast to the CH response exhibited by normal mice injected with the DLN cells from unirradiated, sensitized donors, the ability of these recipient mice injected with DLN cells from UV-B irradiated, sensitized donors to induce a CH response was abrogated. Furthermore, the injection of DLN cells from UV-B treated, sensitized donors was demonstrated to induce the formation of hapten-specific suppressor T lymphocytes [Chung et al, 1986A; Okamoto and Kripke, 1987].

In light of the results presented here and the evidence available which has been discussed above, it is important to consider the possible nature of this antigen-presenting cell defect. It is well established that in order for an antigen to be recognised by T lymphocytes, it must be expressed on the surface of an antigen-presenting cell in association with MHC class I or II molecules. Subsequently, any reduction in the number of antigen-bearing DC expressing Ia molecules, that accumulate in the DLN following UV-B irradiation, would impair the induction of the

CH response. Most studies examining the influence of UV-B radiation on MHC class II expression have used epidermal LC as targets. Using immunofluorescence, no alteration in the constitutive expression of these molecules has been observed following exposure of human LC to UV-B doses that are sufficient to impair their antigen-presenting function [Cooper et al, 1985]. Similar findings have been obtained with murine LC [Aberer et al, 1986]. These immunofluorescence studies have also been confirmed by immunochemical studies [Aberer and Leibl, 1987]. Assuming that at least the majority of antigen-bearing DC in the DLN of UV-B irradiated mice are derived from epidermal LC, then the findings by these investigators correlate closely with the data reported here. It was shown that UV-B irradiation had no influence on the proportion of DC expressing Ia antigen in the DLN of mice sensitized with FITC (Table 13). Further, it was found that all the FITC-bearing DC in the DLN of unirradiated and irradiated mice expressed Ia antigen on their surface. These observations are consistent with the findings of Tang et al (1992) who reported that the same percentage of FITC/Ia$^+$ cells are present in the DLN of UV-B irradiated and unirradiated mice. There is now strong in vitro [Schuler and Steinman, 1985; Shimada et al, 1987; Picut et al, 1988] and in vivo [Cumberbatch et al, 1991B] evidence which suggests that as LC migrate to DLN they are subject to a phenotypic maturation involving an elevation in the expression of Ia antigens on their surface, consistent with the acquisition of active antigen-presenting cell function. Interestingly, although investigators have been unable to observe a UV-B induced alteration in the constitutive expression of these molecules on the surface of LC, Shimada et al (1987) demonstrated that treatment of LC with UV-B prior to culture inhibited both the augmentation in class II antigen expression and the increase in antigen-presenting capacity. The results presented here on the other hand demonstrate that there is no difference in the intensity of Ia expression on DC from the DLN of irradiated and unirradiated mice (Table 10).

It is generally accepted that adhesion-molecule pairs LFA-1/ICAM-1 and CD2/LFA-3 play an essential role during initiation of physical contact between antigen-presenting cells and T cells [Springer, 1990; Bierer and Burakoff, 1989; Makgoba et al, 1989]. This interaction helps to overcome the mutual repulsion of antigen-presenting cells and T cells and establishes an intimate antigen-independent contact, which enables an antigen/MHC molecule complex to come into contact with the T cell receptor, providing a signal for T-cell activation. Furthermore, these adhesion molecules act as signal transducers, delivering stimulatory signals into the T cell after binding to their respective ligands. It has been demonstrated that short-term culture of human [Teunissen et al, 1990; Romani et al, 1989A] and murine [Tang and Udey, 1991] LC results in a marked elevation in ICAM-1 expression. Also, Cumberbatch et al (1992) were able to demonstrate that LC migration to DLN is associated with a marked increase in ICAM-1 expression, consistent with the development of the ability to cluster T lymphocytes and the acquisition of potent immuno-stimulatory and antigen-presenting potential [Cumberbatch et al, 1991A]. Thus, it is reasonable to speculate that a failure of antigen-bearing DC to express the increased levels of ICAM-1 normally associated with LC migration or a decrease in the number of antigen-bearing DC expressing this molecule, may, at least in part, explain why the DC which accumulate in DLN following UV-B irradiation exhibit normal increases in Ia antigen but a functional deficit. However, although this notion is supported by the demonstration that exposure of epidermal cells to low dose UV-B radiation causes a decrease in the ability of LC to express increased amounts of ICAM-1 in vitro, without affecting surface levels of class II MHC molecules [Tang and Udey, 1991], the results recorded here (Table 11) fail to indicate that this is the case.

Although, the exact nature of this antigen-presenting cell defect cannot be resolved from the data reported here, it is important to consider other possible forms in which this defect may be manifested. Using MHC class II and/or LFA-3

expressing or MHC class II and/or ICAM-1 expressing L- cell transfectants, it has been shown that the presence of LFA-3 as well as ICAM-1 is essential for successful T-cell stimulation [Altmann et al, 1989; Bierer et al, 1988]. This has recently been confirmed by Teunissen (1992) who observed that the antigen-specific T cell response induced by cultured human LC was suppressed in a dose-dependent fashion when anti-LFA-3 or anti-ICAM-1 antibodies were continuously present in the co-cultures and that simultaneous addition of both antibodies resulted in an even more pronounced reduction of T-cell proliferation. Thus, assuming that the observed elevation in LFA-3 expression on LC during culture [Teunissen et al, 1990] is an in vitro representative of the in vivo counterpart i.e. phenotypic maturation of epidermal LC as they migrate to DLN, a UV-B induced selective impairment or modulation in the expression of this accessory molecule would impair sensitization.

Alternatively, UV-B radiation may perturb other mechanisms required for efficient T cell activation. For example, UV-B radiation may inhibit or induce LC to produce certain co-stimulatory factors necessary or inhibitory for T-cell stimulation, respectively. It is also feasible that a UV-B induced down-regulation of antigen processing capacity may occur in LC that migrate to DLN following contact sensitization. Although the precise localization of antigen processing (i.e. cleavage of exogenous antigens and binding to class II molecules) for class II-restricted presentation in LC is not clear, it has been postulated that the Birbeck granule and Birbeck granule-like structures may be involved in antigen processing or presentation [Hanau et al, 1985; Takigawa et al, 1985; Hanau et al, 1987A]. This hypothesis has been strengthened considerably by the observation that internalization of Ia molecules into these organelles takes place in LC obtained from normal skin [Hanau et al, 1987B] and in DC from the DLN of contact sensitized mice [Bucana et al, 1992]. Assuming that these granules are important components of the antigen processing or presentation machinery, it would be predicted that a decrease or abrogation in the formation of these organelles in LC, following UV-B exposure

prior to hapten painting on the irradiated site, would reduce or inhibit the ability of these cells to successfully present antigen to unsensitized T cells in the DLN. Interestingly, such a UV-B induced perturbation has been observed in epidermal LC [Hanau et al, 1985] and in DC from the DLN of sensitized mice [Tang et al, 1992].

In the context of attempting to establish the nature of the antigen-presenting cell defect it is relevant to consider whether the antigen-bearing DC present in the DLN of UV-B irradiated mice are derived from epidermal LC. Although there is considerable evidence that the antigen-bearing DC in the DLN of unirradiated mice are derived from epidermal LC [Kinnaird et al, 1989; Macatonia et al, 1987; Silberberg-Sinakin et al, 1976; Kripke et al, 1990; Larsen et al, 1990], it is not possible to conclude from the data recorded here whether the $FITC^+$ DC present in the DLN of irradiated mice are derived from these cells. It is conceivable that rather than a direct effect on epidermal LC that carry antigen to the DLN the suppressive effect of UV-B exposure is mediated through the induction of a unique population of cells (distinct from LC) which infiltrate the skin, pick up antigen and transport it to the DLN where they induce a down-regulatory signal. In the model of UV-B induced suppression of CH reported here, the contact sensitizer was applied to the irradiated skin of mice 24hr following UV-B treatment on 2 or 3 consecutive days, providing sufficient time for the influx of another population of cells into the UV-irradiated site. This interpretation is supported by Tang et al (1992) who have reported recently that a higher proportion of the $FITC^+$ DLN cells from UV-irradiated mice were Mac-1^+ and F4/80^+ compared with those from unirradiated mice, implying that the $FITC^+$ cell population in the DLN of UV-irradiated mice may be composed predominantly of inflammatory cells (e.g. macrophages), rather than LC. Alternatively, UV-irradiation may induce more immature LC to migrate to the DLN or change or induce expression of these molecules by LC.

Although, it is well established that irradiation of murine body wall skin with low doses of UV-B radiation impairs the induction of CH responses to haptens

painted directly on the irradiated site, it is relevant to compare the findings reported here with those of other investigators. It is clear from the data presented here that exposure of mice to a single low dose of 96 or 144 $mJcm^{-2}$ of UV-B radiation on 3 (Table 7 and 8) or 2 (Figure 13, 15 and 16) consecutive days, respectively prior to sensitization 24hr after the last exposure, locally suppresses the induction of the CH response to oxazolone or FITC. These findings are more or less consistent with those of other investigators who demonstrated that mice exposed to a single low dose (8-70$mJcm^{-2}$) of UV-B radiation on 4 consecutive days with sensitization taking place immediately following the last treatment and/or 24hr later, were locally suppressed [Toews et al, 1980; Okamoto and Kripke, 1987; Elmets et al, 1983; Yoshikawa and Streilein, 1990]. Although exposure of mice to a single low dose of UV-B radiation on 2, 3 or 4 consecutive days prior to sensitization induces significant suppression of the CH response, it is not possible to conclude from the studies presented here and elsewhere whether this is related to the total dose of UV-B radiation received by the mice over a period of time or to the time of exposure prior to sensitization. However, the findings of Noonan and De Fabo (1990) may help clarify this issue. They demonstrated that similar levels of local suppression (>55%) to the levels recorded here and elsewhere can be induced following a single exposure of 250$mJcm^{-2}$ UV-B radiation immediately or 3 days prior to sensitization. This suggests that while the timing of exposure is not relevant to the induction of local suppression, the total dose of UV-B administered is. In support of this notion is the observation here that a single exposure of 96$mJcm^{-2}$ UV-B radiation 3 days prior to sensitization failed to induce any suppression (Table 7).

An important aim of this investigation was to explore whether the suppressive effect of UV-B radiation is mediated through the UV-B induced production of a soluble factor. Since a large body of evidence is available supporting the notion that the suppressive effect of UV-B irradiation on immune function is mediated via cis-UCA (the isomerized form of the proposed UV-B photoreceptor

molecule) an investigation of the influence of this molecule on the induction of the CH response was carried out. From the data recorded here, regardless of the concentration, timing or route of administration (whether applied topically or injected i.p. or s.c.), cis-UCA failed to have any consistent significant local or systemic suppressive effect on the induction of the CH response to oxazolone or FITC (Table 7 and 8). These results, however, contrast with studies by other investigators who were able to demonstrate that administration of cis-UCA locally or systemically suppresses the induction of the CH response. Local suppression was seen following epicutaneous application of cis-UCA immediately or 3 or 5hr prior to hapten painting on the treated site [Reeve et al, 1989; Kurimoto and Streilein, 1992], and systemic suppression when cis-UCA was injected i.v. 3 days prior to sensitization [Harriott-Smith and Halliday, 1988B] or applied topically to the dorsum 3hr prior to sensitization on the ventrum [Reeve et al, 1989]. It is interesting that in all the cases reported, where cis-UCA was demonstrated to locally suppress the induction of the CH response, this molecule was applied immediately or several hours prior to sensitization. In contrast, the time period between cis-UCA treatment and sensitization for all the experiments recorded here except one, was 24hr or more. Since the amount of cis-UCA (100-200μg per mouse), used in other investigations was no different from the range of concentrations examined here, it is conceivable that cis-UCA is capable of inducing local immunosuppression of the CH response only when administered immediately or within a few hours before contact sensitization. On the other hand, it is difficult to provide a satisfactory explanation from the data reported here as to why cis-UCA was unable to suppress the CH response systemically. Based on these studies, the implication is that cis-UCA is not a mediator of the suppressive effects of UV-B radiation on CH responses. Evidence supporting this notion is provided by two separate findings. The first was that exposure of mice on 2 consecutive days to narrow band UV-B radiation (which efficiently isomerizes trans-UCA to cis-UCA in vivo) failed to induce local

suppression of the CH response regardless of the dose, when the contact sensitizer was applied 24hr following the last exposure; however, exposure to broad band UV-B radiation under the same conditions significantly suppressed the response (Figure 15). The second finding was that mice injected with anti-cis-UCA monoclonal antibodies (see later in section 4.2) prior to UV-B exposure on 2 consecutive days failed to inhibit suppression of the CH response to haptens applied to the irradiated site 24hr after the last exposure (Figure 16). It is conceivable, however, that the concentration of anti-cis-UCA monoclonal antibodies administered was not high enough to influence a suppressive effect resulting from the local formation of cis-UCA following UV-B irradiation.

An investigation of the influence of cis-UCA on epidermal DC migration to DLN in unsensitized and sensitized mice revealed that epicutaneous application of this molecule alone (Table 9 and Figure 10) or prior to skin sensitization at the same site (Table 12) had no effect. In support of this finding was the demonstration that exposure of mice to narrow band UV-B radiation did not induce DC accumulation in DLN (Figure 14), further implying that UV-B induced DC migration to DLN is not mediated through the local formation of cis-UCA. Although, cis-UCA was not administered immediately or several hours prior to hapten painting, these findings suggest that the mechanism by which cis-UCA induces suppression of the CH response as reported by several investigators, is not through the induction of epidermal DC migration to DLN. These findings contrast with the report that skin-painting with UV-irradiated UCA resulted in a decrease in the number of cells expressing Ia antigens in the epidermis 1 day later [Ross et al, 1987/88]. Further, Kurimoto and Streilein (1992) reported recently that cis-UCA administered i.d. resulted in a significant reduction of Ia^+ epidermal cells. However, Noonan et al (1985) demonstrated that incubation of mouse epidermal sheets in the presence of cis-UCA decreased the number of Ia^+ epidermal cells by 25-35% without affecting ATPase positive counts suggesting that cis-UCA merely modulates the expression of

Ia molecules on the surface of epidermal cells without inducing them to migrate away from the skin.

Noonan et al (1988) found that i.v. injection of cis-UCA (50-200μg per mouse) depressed the antigen-presenting cell function of purified splenic DC, assessed by the proliferative response of purified T cells from mice immune to DNP_6OVA to DC pulsed with this antigen. These data imply that cis-UCA is capable of inducing an antigen-presenting cell defect in vivo. Similar findings suggesting that cis-UCA is capable of inducing such a defect in vivo are reported here. It was found that enriched-DC from the DLN of mice painted with cis-UCA (400μg) daily for 3 consecutive days prior to sensitization had a significantly impaired/altered antigen-presenting cell function, assessed by the ability of these cells to stimulate secondary lymphoproliferative responses of lymph node cells from mice sensitized with the same hapten (Table 18). Also, administration of cis-UCA topically (400μg) or s.c. (200μg) to mice under the same conditions prior to sensitization resulted in decreased spontaneous lymphoproliferative responses of DLN cells (Table 14 and 15). However, the doses of cis-UCA (1200 or 600μg/mouse) administered to mice were not within the biological range : that is, they did not reflect the amounts of cis-UCA that are generated in mouse skin in vivo (100-200μg) by immunosuppressive doses of UV-B radiation. Therefore, it is possible that the cis-UCA induced decrease/alteration in antigen- presenting cell function of lymph node DC observed here, does not reflect the situation in vivo following UV-B irradiation. The demonstration that enriched-DC from the DLN of mice treated with identical doses of trans-UCA inhibited the secondary lymphoproliferative response of lymph node cells twice as much as that observed with cis-UCA suggests that this may be the case. It is, however, conceivable that the reduced antigen-presenting cell ability of lymph node DC observed when higher than supra - physiological levels of cis-UCA are administered to mice is directly responsible for the inconsistent but significant impaired CH responses reported here.

Streilein and Bergstresser (1988) reported that the capacity of UV-B irradiation to impair the induction of CH in mice depends upon the genotype of the host. Specifically, certain strains of mice (C57BL/6, C3H/HeN) are susceptible i.e. they fail to develop CH if the contact sensitizer is painted on UV-B irradiated skin while other strains (Balb/c, A/J, C3H/HeJ) are UV-B resistant, exhibiting vigorous CH responses when the hapten is painted on the UV-B treated site. Since a correlation between the proportion of cis-UCA formed in the epidermis following UV-B irradiation and the degree of suppression of DTH appears to exist [Norval et al,1988], it seemed reasonable to postulate that a variation in the UCA content of different mouse strains may account for the genetic differences observed, assuming that skin pigmentation does not influence the amount of trans to cis-isomerization that takes place on UV irradiation. Indeed, the investigation presented here (Table 1, 2 and 3) suggests that the UCA content of different strains of mice does vary. However, since a reciprocal relationship was observed between the amount of UCA present in the skin of C57BL/6 and Balb/c mice and the potential of each of these strains to exhibit a suppressed CH response after UV-B irradiation, this implies that susceptibility or resistance to the effects of UV-B exposure is not related to the total amount of UCA present in the skin. In view of the observation that black mice (C57BL/6) are more readily suppressed than albino animals (Balb/c) and that isomerization of UCA takes place immediately on UV-B irradiation in the stratum corneum above the pigment layer, it is unlikely that skin colour has any influence on the degree of isomerization. In support of this notion is the finding reported here which indicates that there is no obvious difference in the percentage of cis-UCA formed in the skin of C57BL/6 mice compared with Balb/c mice following UV-B irradiation. Further, Yoshikawa et al (1990) detected no relationship between UV-B susceptibility and skin colour type in Caucasian subjects and Vermeer et al (1991) found that black individuals with the darkest skin colour may be UV-B susceptible.

From the evidence discussed previously it appears that irradiation of mice with broad band UV-B on 2 or 3 consecutive days prior to sensitization 24hr after the last exposure induces local suppression of the CH response via a mechanism independent of the local formation of cis-UCA in the skin. This is not necessarily unexpected in view of the finding that different wavelengths of UV radiation are able to induce epidermal cells to release a variety of mediators capable of suppressing the induction of CH or DTH responses [Kim et al, 1990]. One mediator of the suppressive effect of UV-B irradiation on the induction of CH whose production in response to UV-B irradiation appears to be genetically linked with UV-B susceptibility, is TNFα [Yoshikawa and Streilein, 1990; Vermeer and Streilein, 1990]. Interestingly, from the data presented here (Figure 13) it was revealed using polyclonal anti-TNFα antibodies, that the UV-B induced suppression observed when the contact sensitizer was applied to the irradiation site 24hr following the last exposure, is mediated by TNFα, supporting the view that this molecule is an important and possibly critical molecular mediator of the down-regulatory effect of UV-B exposure on the induction of CH.

Further, the studies described in this report demonstrate that the induction of DC migration to DLN by UV-B irradiation is mediated by TNFα release (Figure 11 and 12). These data confirm the report by Cumberbatch and Kimber (1992) that TNFα induces DC migration to DLN. Having provided evidence that TNFα is produced and/or released following exposure of murine skin to broad band UV-B radiation it is important to question the source of TNFα and its possible mode of interaction with LC.

A number of cutaneous cells could serve as the source of TNFα. Macrophages are a possible source especially since LPS causes excessive release of this cytokine from LPS^n macrophages [Beutler et al, 1985; Mahoney et al, 1985]. Assuming these cells are the only possible source, then one would predict that the UV-B effect is mediated by macrophages in the dermis, the compartment of skin

containing the largest number of these types of cells. However, since most of the energy of UV-B is absorbed as the light passes through the epidermis, with little UV-B reaching the dermis [Agin et al, 1981], this possibility seems unlikely. Epidermal LC could serve as the source of TNFα. In active psoriatic lesions, high levels of TNFα mRNA have been detected in LC, and TNFα mRNA has even been detected in LC within normal human epidermis [Nickoloff et al, 1990]. Furthermore, activated LC have been demonstrated to release TNFα [Larrick et al, 1989]. Evidence from photomicrographs of UV-B exposed human skin suggest that TNFα is present in keratinocytes [Oxholm et al,1988]. This observation is supported by the finding that UV-B irradiation promotes the synthesis and secretion of TNFα by human keratinocyte cell lines [Kock et al, 1990A]. Since keratinocytes comprise the great majority of the epidermis and are known to absorb UV-B radiation these cells are the most probable source of TNFα. It seems reasonable therefore, to speculate that exposure of murine skin to UV-B radiation stimulates the production and/or release of TNFα by keratinocytes and that this cytokine stimulates LC to leave the epidermis and migrate to the DLN via the afferent lymphatics.

In light of the studies presented here and elsewhere which suggest that TNFα acts as a signal for the migration of LC from the skin, it is necessary to consider the possible mode of interaction of TNFα with these cells. It is conceivable that TNFα may induce or up-regulate the release of another mediator that directly interacts with LC inducing their migration to DLN. For example it has been shown that TNFα can induce the production of IL-1 [Dinarello et al,1986] which shares many biological properties with TNFα [Dinarello, 1989; Le and Vilcek, 1987] and that IL-1 is able to cause a reduction in the density of Ia^+ epidermal LC [Lundqvist and Back, 1990]. It has recently been found that mice possess two receptors for TNFα, designated mTNF-R1 and mTNF-R2, which differ with respect to both extracellular nucleotide sequence and species specificity [Lewis et al, 1991].

Further, mTNF-R1 was found to bind human and mouse TNFα with equivalent affinity while mTNF-R2 exhibited strong species specificity for the mouse cytokine [Lewis et al, 1991]. Although it is not known whether both or one or the other of these receptors is expressed constitutively on the surface of LC, based on the observations of Koch et al (1990B) and Cumberbatch and Kimber (1992), it seems reasonable to speculate that the interaction of TNFα with mTNF-R2 expressed on the the surface of LC is critical in influencing the migratory behaviour of these cells. Koch et al (1990B) observed that under conditions where murine TNFα maintained the viability of mouse LC in culture, the same concentrations of human TNFα were without effect. Also, more recently, Cumberbatch and Kimber (1992) found that in contrast to the increased accumulation of DC in DLN of mice following i.d. injection of murine recombinant TNFα, administration of human recombinant TNFα of comparable specific activity into mice failed to have any influence on DC migration.

Since the density of LC present in the epidermis is approximately 770 cells/mm^2 [Toews et al, 1980] it appears from the results reported here and elsewhere [Kimber et al, 1990A; Macatonia et al, 1986; Knight et al, 1985A and B; Macatonia et al,1987; Kinnaird et al, 1989] that only a proportion of epidermal DC (approximately 18-24000 DC/Lymph node, varying with time, and concentration and type of hapten) are induced to migrate to DLN following contact sensitization. It is possible to explain this if the skin is viewed as a primary lymphoid organ i.e. a site continuously infiltrated by immature LC from the blood which provides the necessary microenvironment for the development of these cells prior to migration to secondary lymphoid organs such as the DLN. Thus, epidermal LC would represent a heterogeneous population of cells varying in age and subsequently exhibiting different levels of phenotypic and functional maturation. It is plausible that only cells that have reached a certain level of maturity are able to leave the skin and migrate to DLN following epicutaneous application of a contact sensitizer or following

UV-B irradiation. Epidermal LC that perhaps express a threshold level of mTNF-R2 on their surface are the only cells that can leave the skin.

It is interesting that the cis-UCA induced local suppression of CH reported by Kurimoto and Streilein (1992) was demonstrated to be reversed by prior treatment of mice with anti-TNFα antibodies implying that cis-UCA achieves its inhibitory effects, at least in part, through TNFα. Although it was not possible to demonstrate suppression of CH by epicutaneous application of cis-UCA, it seems highly unlikely based on the data presented here that cis-UCA mediates its effects through stimulating cells to produce and/or release TNFα. If this was the case, exposure of mice to narrow band UV-B radiation or to topical application of cis-UCA would be predicted to induce DC migration to DLN.

Based on their findings that UV-B irradiation or i.d. TNFα altered the morphology of epidermal LC, Vermeer and Streilein (1990) concluded that TNFα prevents effective sensitization following UV-B irradiation by immobilizing LC within the epidermis. This interpretation contrasts very much with the data presented here which indicate that the local production of TNFα following UV-B irradiation is responsible for increasing the number of LC-bearing antigen that would normally migrate to DLN with sensitization alone. Also, Vermeer and Streilein (1990) demonstrated that both i.d. TNFα and UV-B irradiation reduce the density of Ia^+ cells in the epidermis, supporting the notion that UV-B exposure may induce LC migration through the local release of TNFα. Thus, in view of the data presented here it is relevant to consider how TNFα may achieve inhibition of the induction of CH. In vivo evidence suggests that LC stimulated to migrate to DLN as a result of sensitization undergo a phenotypic and functional maturation [Cumberbatch et al, 1991B; Cumberbatch et al, 1992], similar to that observed in vitro [Shimada et al, 1987; Picut et al, 1988]. The changes effected during short term culture of freshly isolated LC are mediated by GM-CSF [Witmer-Pack et al, 1987] and may be enhanced further by IL-1 [Heufler et al, 1988], both products of UV-B irradiated

or mitogen activated keratinocytes [Kupper et al, 1988; Ansel et al, 1988]. These changes, however, do not appear to be effected by TNFα [Kock et al, 1990B]. The process by which cells receive and translate messages/signals delivered by cytokines is finely tuned. In other words any slight deviation in the timing at which a particular chemical mediator delivers its signal compared with another or in the amount of signal delivered will be interpreted differently by the cell. It is tempting to speculate that upon application of skin allergens, keratinocytes are induced to release cytokines such as IL-1, GM-CSF and TNFα which are then responsible for initiating the migration and functional maturation of LC which carry antigen to DLN, so that once they arrive in the DLN they have matured into potent immunostimulatory cells able to initiate a primary immune response. However, the UV-B induced production of excessive local (cutaneous) amounts of TNFα prior to sensitization may stimulate the migration of LC to DLN which are phenotypically and/or functionally immature but are able to pick up antigen. These cells may subsequently be unable to activate and stimulate the proliferation of hapten-specific virgin T cells, resulting in an impaired response and/or may activate and induce the proliferation of T-cells capable of down-regulating the CH response.

In summary, it would appear that the suppressive effects of UV-B radiation on CH are mediated by TNFα and not cis-UCA. These findings contrast very much with findings from studies examining the effect of UV-B irradiation and cis-UCA on the DTH response to HSV. Norval et al (1988) demonstrated that a correlation exists between the amount of cis-UCA present in the epidermis at various times following UV-B irradiation and the degree of immunosuppression of the DTH response to HSV. They showed that immediately following a single exposure of C3H murine skin to 96mJcm^{-2} of UV-B radiation, there is a conversion of UCA in the skin to over 30% as the cis-isomer, from a background level of 4.7% and that this level is maintained for at least 16hr, declining slowly over the next 7 days. Interestingly, these findings are extended by data recorded here which suggests that

48hr following UV-B exposure of C3H skin, the level of cis-UCA formed decreases by 25% (Table 1). Further, Norval et al (1988) demonstrated that local suppression of the DTH response to HSV by a single exposure of 96mJcm^{-2} of UV-B radiation was generated if the interval between irradiation and sensitization was more than 5hr or less than 14 days; no suppression was observed immediately following UV-B irradiation and maximum suppression was generated with a delay of between 2 and 3 days but declined thereafter. These findings suggest that the proportion of cis-UCA in the epidermis correlates with the degree of immunosuppression of the DTH response except immediately following irradiation. Direct evidence implicating cis-UCA as the mediator of UV-B induced suppression of the DTH response to HSV was provided by Ross et al (1986). These investigators showed that if irradiated UCA (70% cis-isomer) at concentrations ranging from 3-100ug was painted on to the shaved skin or if it was injected s.c. followed by infection with HSV 5hr or 3 days later, then a dose dependent suppression of the DTH response to the virus ensued. It was found later by Norval et al (1989B) that doses of as little as 1μg cis-UCA per mouse could suppress the DTH response to HSV.

Thus, it would appear that in UV-B induced immunosuppression of CH and DTH responses two different suppressive mechanisms are operative, one dependent on the UV-B induced production of TNFα and the other on cis-UCA. Further, It has recently been reported that neutralising anti-IL-10 antibody inhibits the depression of DTH responses by UV-B exposure but will not reverse UV-B induced suppression of CH (personal communication by Dr. S. Ullrich), supporting the view that different suppressive mechanisms operate in CH and DTH responses. Also, this report implies that a variety of suppressive mediators acting independently or relying on each other for their production, may act to suppress DTH responses. Although DTH and CH are two closely related T cell-mediated immune responses requiring the presentation of antigen in two distinct phases termed the sensitization and effector phases, these findings suggest that the two responses may be regulated

by separate mechanisms. Indeed, there is strong evidence available which indicates that this is the case.

It has been found that 24-48hr CH and DTH skin swelling reactions that result from local antigen challenge of actively sensitized mice are preceded by an early skin swelling reaction which is maximal 2hr after challenge [Matsushima and Stohlman, 1991; Van Loveren et al, 1984]. Two different cells mediate the early and late components of CH and DTH responses; a helper cell responsible for both the early reaction and the activation of the effector cell which mediates the late phase reaction. However, although the antigen-specific, MHC-restricted effector cell which mediates the late phase reaction of CH and DTH is the same (i.e Thy-1+, Lyt-1+, CD4+, CD8-, CD3+, I-A$^-$), the helper cells responsible for the early response of CH and DTH are different. The initiating/helper cell which is activated in CH is a Thy-1+, Lyt-1+, CD4-, CD8-, CD3-, IL-3R+, B220+ cell and acts in an antigen-specific but MHC-unrestricted fashion [Herzog et al, 1989B]. On the other hand the initiating/helper cell which is activated in DTH is a Thy-1+, Lyt-1+, CD4-, CD8-, CD3-, IL-2R+, B220-, I-A$^-$ cell which also functions in an MHC-unrestricted manner but provides help in an antigen-nonspecific manner [Matsushima and Stohlman, 1991].

Furthermore, due to the availability of two strains of mice with specific defects in related immunological effector mechanisms, it is now clear that different populations of antigen-presenting cells are involved in inducing CH and DTH responses. In C57BL/Ler-vit/vit mice which lack the CH response due to the absence of LC in the skin [Amornsiripanitch et al, 1988], the closely related DTH response and all other immunological effector mechanisms that require the participation of antigen-presenting cells appear normal. An age-dependent macrophage defect in SJL mice that results in DTH unresponsiveness in young adult ($\leq$ 8 week old) SJL mice has been reported [Stohlman et al, 1985; Matsushima and Stohlman, 1988]. Unlike 16 other mouse strains, in which the adult level of DTH

responsiveness was observed by 6 weeks of age [Matsushima and Stohlman, 1989], young adult SJL mice did not exhibit a DTH response after immunization with a variety of soluble and particulate antigens. By contrast, mature adult SJL mice (≥ 10 weeks) did respond to these antigens, indicating that the regulation of the DTH response in SJL mice is under unique maturational control [Stohlman et al, 1985; Matsushima and Stohlman, 1988; Matsushima and Stohlman, 1989]. Importantly, the CH response and other antigen-presenting cell-dependent immunolgical effector mechanisms were normal in these DTH-unresponsive mice [Matsushima and Stohlman, 1989]. Recently, Matsushima and Stohlman (1991) identified a unique subset of antigen-presenting cells in SJL mice that function as the sole antigen-presenting cell required for the induction of CD4+ DTH effector T cells. This cell is adherent, Mac-1^+, Mac-2^-, Mac-3^+ and I-A^+ and does not participate in the induction of CH responses [Matsushima and Stohlman, 1989]. These investigators have also identified an adherent, Mac-1^+, Mac-2^-, Mac-3^-, I-A^- accessory cell which appears to be crucial in the activation of the CD4+ DTH helper cell, although the interaction between these two cells is neither antigen-specific nor MHC-restricted.

Based on these studies it appears that the DTH and CH response require distinct cell-cell interactions for generating immune responsiveness, implying that they are regulated by different mechanisms. In view of the fact that DTH antigens are given s.c. and contact sensitizers are applied topically, the demonstration that LC and a unique subset of macrophages are critical antigen-presenting cells in the induction of CH and DTH responses, respectively, is not all that surprising. However, these findings do provide a greater insight into the possible mode of action of cis-UCA and TNFα in UV-B induced immunosuppression and suggest that the influence of these UV-B induced mediators is dependent on the site into which antigen is administered. Thus, it seems reasonable to speculate that the suppressive influence of TNFα is mediated at the level of the LC, affecting the number of responsive cells that migrate

to DLN to initiate CH responses, while cis-UCA excerts its influence on a subset of macrophages important in processing and presenting antigen to induce CD4+ DTH effector cells. Since an accessory cell recently described by Matsushima and Stohlman (1991) appears to be important in the activation of the CD4+ DTH helper cell which is critical for the activation of the effector cell, it is also feasible that cis-UCA could act on this cell. Interestingly, Rasanen et al (1989) reported that cis- but not trans-UCA was able to reduce monocyte IL-1 production in a dose-dependent fashion and suppress HLA-DR antigen expression, in vitro.

It is well established that local impairment of the CH response following UV-B irradiation is accompanied with the induction of specific immunological tolerance to haptens applied to the unirradiated site at a later date, which is believed to be mediated by antigen-specific afferent acting suppressor Ly-1+CD8- (presumably CD4+) T cells. In an attempt to clarify whether the induction of afferent acting suppressor T cells is responsible for the down-regulation of CH responses and long lasting unresponsiveness following UV-B exposure, Glass et al (1990) explored whether UV-B resistant strains of mice are capable of generating these cells. Unexpectedly, Glass and his colleagues found that an apparent universal consequence of treatment of murine skin with UV-B radiation and hapten is the induction of antigen-specific suppressor T cells that act on the induction phase of CH, irrespective of whether the recipient displays normal CH or not, suggesting that future attempts to elucidate the mechanism of UV-B induced local immunosuppression should be focussed on the ability of UV-B radiation to interrupt induction of effector mechanisms.

The finding that there are two subsets of CD4+ T cells termed Th1 and Th2 which differ in their cytokine production and function raises the possibility that regulation of the immune system may be a direct product of the preferential activation of only one of these CD4+ T cell subsets in response to antigen. Thus, a shift in the ratio of Th1 and Th2 cells activated following UV-B irradiation would

ultimately result in a complete alteration in the secretion of distinct cytokines following T cell activation and in a depression in the function of certain types of cellular immune response requiring cytokines no longer being produced. Indeed, the demonstration that UV-B irradiation is able to alter the pattern of cytokine secretion by activated T cell from a Th1 to a Th2 profile [Araneo et al, 1989] and alter the antigen-presenting function of LC for Th1 cells from one that is immunogenic to one that is tolerogenic (via functional inactivation), while not affecting their ability to activate Th2 cells, support this notion [Simon et al, 1990 and 1991].

During the course of a normal cell-mediated immune response to a contact sensitizer, it is conceivable that Th1 and Th2 cells are generated; the ratio between these subsets within various cellular compartments such as the DLN and spleen, perhaps varying considerably. It seems reasonable to speculate that Th2 cells (effectors important in promoting B cell responses) may predominate in the spleen since this is an important site of antibody production while Th1 cells (effectors of DTH reactions) predominate in DLN, important sites for sensitization. Assuming that the behaviour/function of cells is restricted by the anatomical site in which they are activated, it is feasible that the preferential activation of Th2 cells within DLN may transform them into cells capable of actively suppressing Th1 responses. In support of this notion is the demonstration that Th2 cells are able to secrete a factor that can suppress cytokine production and proliferation of Th1 cells [Fiorentino et al, 1989; Mosmann and Moore, 1991; Mosmann, 1991; MacNeil et al, 1990]. This factor, originally known as cytokine synthesis inhibitory factor (CSIF), is IL-10. Interleukin-10 acts to inhibit synthesis of most or all cytokines by Th1 cells but not Th2 cells. It is thought that this may be related to the kinetics of action; IL-10 shows little or no inhibition of cytokine production before 8hr and then inhibits synthesis very effectively from this time onward [Mosmann, 1991]. Based on this delay in onset of inhibitory activity, and on the demonstration that IL-10 acts only when antigen-presenting cells are present it is suggested that IL-10

acts on antigen-presenting cells inhibiting the production of a costimulatory signal required for the activation of Th1 cells [Mosmann,1991]. Taking into account these findings and the studies of Simon et al (1990 and 1991) this would imply that as a consequence of the UV-B induced alteration in the antigen-presenting cell function of LC, two down-regulatory signals may be initiated: 1) a long-lasting unresponsiveness in Th1 cells; and 2) active suppression of the induction of Th1 responses mediated by Th2 cells.

In view of these findings it is relevant to consider an alternative interpretation of Glass et al (1990) observations. It is conceivable that UV-B induced suppression of the CH response is ultimately dependent on the balance between the number of activated Th1 and Th2 cells. In other words, activated Th1 and "suppressor-like" Th2 cells may coexist within the DLN and only if a threshold level in the number of activated Th2 cells relative to activated Th1 cells is reached will down-regulation of the CH response be expressed. Thus, in UV-B resistant mice this threshold level may not be attainable due to the LC of these mice being less susceptible to the modifying influence of UV-B irradiation. However, it is conceivable that the original constraints on the function of activated Th2 cells in the DLN of UV-B resistant mice will be removed following the introduction of these cells into naive recipient mice prior to sensitization as carried out by Glass and his colleagues, thus enabling them to suppress the induction phase of the CH response.

4.2 Development of a Monoclonal Antibody Specific for the Cis-Isomer of UCA and its Use in detecting Cis-UCA in the Serum of Irradiated Mice

It is not understood exactly how cis-UCA alters immune function. In order to elucidate its mechanism of action it is necessary to identify its site of action and cellular target. At present it is not known if cis-UCA is confined solely to the epidermis following irradiation or is able to leave this site and act systemically. In this investigation, a monoclonal antibody with specificity for cis-UCA was produced and applied to the detection of UCA in serum of irradiated mice.

Although a minimum number of haptens per carrier protein of 15-30 is recommended for the induction of antibodies [Bauminger and Wilchek, 1980], a good antibody response was elicited in mice immunized with cis-UCA-KLH conjugate with an epitope density of 8 cis-UCA molecules per KLH molecule. This is perhaps not all that surprising as it has been reported that as few as 5 epitopes per carrier molecule give a good IgG response [Klaus and Cross, 1974]. Since UCA is amphoteric with a basic imidazole ring and an acidic carboxylic acid group, the carrier proteins (KLH and BSA) could couple through either group, creating two different structures (Figure 20). It is not known which of these forms was synthesized and subsequently which structure was recognized by the monoclonal antibody.

Using an ELISA it was established that the monoclonal antibody was specific for the cis-isomer of UCA when conjugated to BSA. This was further corroborated using a competitive inhibition ELISA where cis-UCA competed, even at low concentrations, with cis-UCA-BSA antigen bound to the well for binding of the antibody; trans-UCA only competing when it was present at high concentration. Out of 15 UCA analogues tested, only cis-2-methyl UCA displayed inhibitory activity, although it was not so active as cis-UCA itself. When cis-UCA-KLH and trans-UCA-KLH conjugates were used as competitors, they both demonstrated inhibitory activity

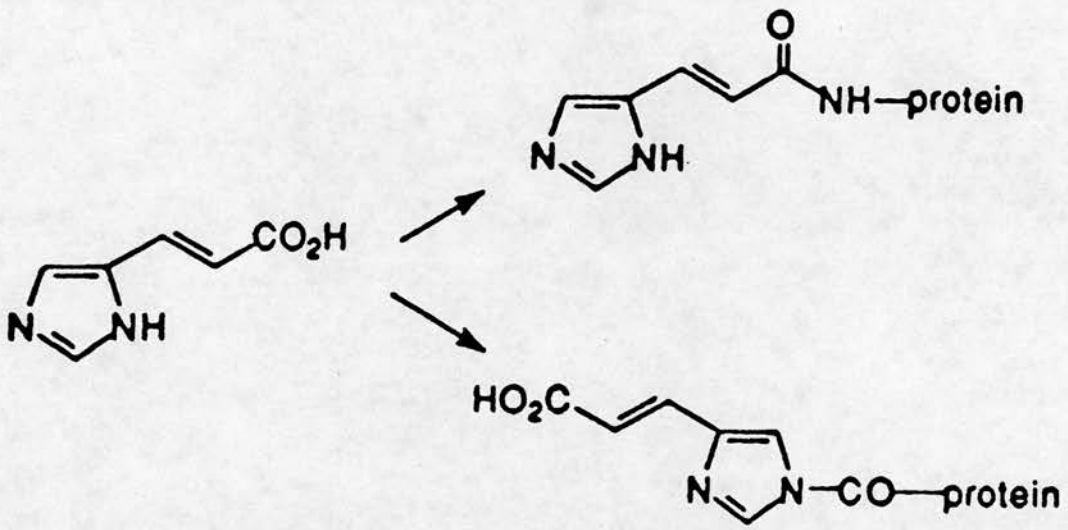

Figure 20 Two different structures that could be generated following coupling of urocanic acid to the carrier protein.

roughly 3-fold better by weight (equivalent to around 1000-fold better in molar terms) than free UCA. This suggests that the paratope of the monoclonal antibody is highly specific for an epitope formed from the combined structure of UCA and a region on the carrier to which it is bound. In support of this notion was the finding that the monoclonal antibody was unable to interact with KLH. It would appear that when the monoclonal antibody is exposed to cis or trans-UCA-KLH it is less able to distinguish between these isomers unless they are present at low concentrations (0.1 - 0.3μg/well). However, the isomers of UCA alone or conjugated to another protein were distinguished and thus it can be concluded that the monoclonal antibody is operationally specific for cis-UCA unless trans-UCA is present in large quantities.

Use of the monoclonal antibody enabled cis-UCA in serum of irradiated mice to be assayed by competitive inhibition ELISA. The sensitivity of detection was approximately 40μM. However, despite the low sensitivity in comparison with HPLC where the sensitivity of detection in homogenised ears was about 0.14μM, representing 1ng/mg wet weight tissue [Norval et al, 1988], cis-UCA was detected in serum soon after irradiation, its concentration diminishing over the next 2 days. Dialysis of the serum revealed that cis-UCA was in a dialysable form and therefore unbound or conjugated to a small molecule with a m.wt. below 12000, and unlikely to be conjugated to serum proteins or membranes. In support of these findings is the report [Reeve et al, 1991] that following painting of mice with ^{14}C-UCA synthesized from [2-^{14}C]-malonic acid, low but significant levels of ^{14}C were detected in serum. The counts were highest in mice exposed to UV-B radiation after painting, and were present 2hr after irradiation and not 6 days later. Electrophoresis of serum proteins suggested that ^{14}C was associated with specific proteins.

Thus there is evidence that a proportion of cis-UCA formed in the epidermis on UV irradiation reaches the serum either through capillaries or lymph, enabling it to exert its influence on immune function systemically, in lymph nodes

or spleen. At present the cellular target for cis-UCA is unknown although there is some evidence that it may be in the skin [Palaszynski et al, 1992]. The findings presented here imply on the other hand that cis-UCA has the potential to act on a cellular target present at a site other than the skin.

4.3 Further Extensions

It is important to understand the cellular mechanisms involved in UV-B induced immunosuppression for several reasons. First, there is an association between UV-B induced immunosuppression and the development of primary skin cancers in mice [Fisher and Kripke, 1982] and humans [Yoshikawa et al, 1990]. Thus, an insight into the cellular mechanisms by which UV suppresses the immune response using animal models, may be useful in providing new approaches for the treatment and prevention of skin cancers. Second, the local and systemic immunological alterations caused by UV radiation, especially the suppression of DTH responses, may be a predisposing factor for an increased incidence or severity of infectious disease. Third, in addition to the immunosuppressive effect of UV-B radiation being antigen-specific, it is highly selective. Despite CH and DTH responses, and the rejection of UV-induced tumours being suppressed following exposure to various regimens of UV radiation, most other immune responses such as antibody production and the generation of cytolytic T cells are normal [Spellman et al, 1977; Kripke et al, 1977]. Thus, studies attempting to elucidate the mechanism(s) of UV-induced immunosuppression may in the process yield new insights into the differential immunoregulation of different compartments of the immune system.

There is strong evidence that a common mechanism initiates local and systemic UV-B induced suppression of CH responses, based on the demonstration that the dose responses of UV-B induced local and systemic suppression are identical. However, based on the data recorded here and elsewhere it appears that more than one mechanism may be involved, acting independently of the other to induce local and/or systemic immunosuppression. Evidence in the literature that the antigen-presenting cell function of LC and DC present at the UV irradiated site or in lymph nodes draining this site, respectively, is decreased, whereas the antigen-presenting cell function of these cells at sites distant from the site of UV exposure is unaltered except in the spleen, further suggests that other mechanisms

are at play. The majority of studies carried out to ascertain whether local or systemic suppression is associated with the generation of antigen-specific T suppressor cells, involved adoptively transferring lymph node and spleen cells to naive syngeneic recipient mice. Thus in light of the evidence that UV-B irradiation appears to have disparate effects on the function of different populations of antigen-presenting cells, depending on whether suppression is being induced locally or systemically, it is imperative to establish what role if any, each of the lymphoid compartments has in the induction of local and systemic immunosuppression. This could perhaps be achieved by splenectomising mice, thus enabling an assessment of the importance of this organ and DLN in the generation of local and systemic suppression of CH responses to be made.

Investigators examining whether local suppression of CH is mediated via the reduced/altered antigen-presenting cell activity of DLN cells demonstrated that the ability of these cells to induce CH when transferred to normal mice was abrogated and associated with the induction of antigen-specific T suppressor cells. Although these findings question the role of suppressor T cells formed in the spleen during local suppression, they suggest that local immunosuppression of CH occurs as a result of UV radiation exerting its immunomodulatory effect at the level of the lymph node dendritic antigen-presenting cell. More direct evidence that this is indeed the case could be achieved by adoptively transferring antigen-bearing DC-enriched cells from the DLN of mice exposed to UV-B radiation to syngeneic recipient mice. Further, the role of lymph node and spleen DC from UV irradiated mice in the induction of local and systemic immunosuppression could be elucidated by this method.

It is reported here that the ability of antigen-bearing DC from the DLN of UV-B irradiated mice to stimulate secondary lymphoproliferative responses <u>in vitro</u> was diminished/altered. Although it was not possible to reproduce these findings, optimization of this particular assay system would enable a variety of studies to be carried out. For example the influence of various wavelengths and

doses of UV radiation and suppressive mediators such as TNFα, IL-10 and cis-UCA on the antigen-presenting cell function of lymph node DC in vivo could be assessed. Also, using hapten-specific immune Th1 and Th2 clones, the effect of UV radiation and suppressive mediators in altering the antigen-presenting cell function of lymph node DC could be examined.

There is evidence that freshly isolated DC from the spleen resemble freshly isolated LC from the epidermis and that both cells undergo parallel phenotypic and functional changes during short term culture [Girolomoni et al, 1990]. The parallel modifications that LC and spleen DC exhibit after culture imply that both cells respond similarly to factors in the microenvironment. An obvious question raised by these findings is what is the stimulus for the phenotypic and functional maturation of spleen DC? There is a consensus that the introduction of an antigen into the epidermal compartment stimulates epidermal cells to release a variety of cytokines important in inducing the migration of antigen-bearing LC to DLN and their functional and phenotypic maturation as they do so. It is equally conceivable that such a process occurs with spleen DC and that these cells upon encounter with antigen are induced to migrate away from the spleen perhaps to peripheral lymph nodes. Thus, an examination of whether DC numbers in the spleen fluctuate following sensitization and a comparison of the events observed in this case with those occuring when sensitization takes place after UV-B exposure would yield some interesting results.

The inference of the results reported here is that the UV-B induced local production of TNFα and subsequent enhanced migration of epithelial DC bearing antigen to DLN is an important component in the expression of local immunosuppression. Since it is not known at present whether the increased number of antigen-carrying DC accumulating in the DLN as a consequence of the UV-B induced formation of TNFα represents a functionally and/or phenotypically immature DC derived from epidermal LC or a completely different cell population it is important that a phenotypic analysis be carried out on these cells. Furthermore,

it would be interesting to compare the phenotype of the antigen-bearing DC present in the DLN of mice exposed to UV-B radiation with antigen-bearing DC in the DLN of mice treated with anti-TNFα antibodies prior to UV-B exposure.

Although several investigators have reported that systemic suppression of CH responses is not related to a systemic effect of UV radiation on the number and morphology of LC at the unirradiated site of contact sensitization as detected by ATPase staining and electron microscopy, it would be interesting to confirm these observations by investigating the influence of UV-B irradiation on the induction of DC migration to DLN by hapten painting at an unirradiated site.

The studies presented here failed to indicate that local UV-B induced suppression of CH is dependent on the formation of cis-UCA following UV-B irradiation. These observations contrast very much with studies examining the influence of cis-UCA on DTH responses to HSV, implying that the mechanisms of UV-B induced suppression of CH and DTH responses are different. This is perhaps not surprising considering that the sensitizing antigens used to induce CH and DTH responses are different: one being a live organism and the other a chemical hapten. Thus, it would be interesting to investigate whether the dose response and kinetics of generation of UV-B and cis-UCA induced suppression of DTH responses alter when sensitizing doses of inactivated HSV are administered topically or s.c. to mice.

The recent availability of an anti-cis-UCA monoclonal antibody generated during the course of these studies provides an excellent opportunity to clarify the role and mode of action of cis-UCA in UV-B induced suppression of CH and DTH responses. The influence of cis-UCA in suppressing DTH and CH responses could be examined by administering sufficient concentrations of this monoclonal antibody locally or systemically prior to UV-B irradiation. Whether the direct or indirect effects of cis-UCA on DTH or CH responses is mediated through an alteration/decrease in the antigen-presenting cell function of epidermal LC, spleen DC or lymph node DC, could be assessed by injecting anti-cis-UCA monoclonal antibodies locally or systemically prior to UV-B exposure of mice and then

examining the ability of these antigen-presenting cells to stimulate secondary lymphoproliferative responses in vitro. A novel finding reported here is that following UV-B irradiation, cis-UCA can be detected in the circulation, implying that this molecule has the potential to act systemically i.e. at sites within the body, distant from the site of production. Thus, establishing if this molecule can reach local lymph nodes and/or the spleen would provide valuable information about its possible mode of action. This could be achieved by mechanical disaggregation of lymphoid organs from UV irradiated mice through a stainless steel wire mesh and testing the cell-free supernatant, following washing of the cell suspension, using the inhibition ELISA system described in these studies. It is of considerable interest to elucidate whether cis-UCA exerts its immunosuppressive effect by interacting with a receptor expressed on the surface of a specific population of cells present in the epidermis, dermis, lymph node or spleen. The availability of a monoclonal antibody specific for the cis-form of UCA provides an ideal opportunity to explore this possibility.

There is no doubt that susceptibility to the local and systemic suppressive effects of UV-B radiation is genetically determined in the mouse. Thus, carrying out the afore mentioned investigations, while at the same time examining differences between UV-B resistant and UV-B susceptible strains of mice, would provide a greater understanding of the mechanism(s) of UV-B induced immunosuppression.

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APPENDIX

Publications Arising from the Work Contained in this Thesis

Publications

The publications listed below were derived from the work carried out in this study. Copies of the manuscripts are found below.

Moodycliffe A.M., Kimber I. and Norval M. (1992) The effect of ultraviolet B irradiation and urocanic acid isomers on dendritic cell migration. Immunology. 77, 394

Moodycliffe A.M., Norval M., Kimber I. and Simpson T.J. (1993) Characterization of a monoclonal antibody to cis-urocanic acid: detection of cis-urocanic acid in the serum of irradiated mice by immunoassay. (In Press)

Moodycliffe A.M., Norval M. and Kimber I. (1993) The role of cis-urocanic acid and tumour necrosis factor-α in UV-B induced dendritic cell accumulation in draining lymph nodes and on suppression of contact hypersensitivity responses. (Submitted)

logy 1992 77 394–399

The effect of ultraviolet B irradiation and urocanic acid isomers on dendritic cell migration

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SUMMARY

Irradiation with ultraviolet-B light (UV-B) suppresses some cell-mediated immune responses to a variety of antigens, including contact sensitizers. Following UV irradiation there is modulation of Langerhans' cells' markers and keratinocytes are induced to synthesize and secrete tumour necrosis factor-α (TNF-α). *Cis*-urocanic acid (*cis*-UCA) has been suggested as a photoreceptor for UV and has been demonstrated to suppress immune responses in several experimental systems. UCA is found naturally in the stratum corneum as the *trans*-isomer and converts to the *cis*-isomer on irradiation. In the present study the migration of dendritic cells (DC) to lymph nodes following UV-B irradiation or epicutaneous application of UCA isomers was examined in unsensitized mice and mice sensitized with fluorescein isothiocyanate (FITC). It was found that UV-B irradiation alone induced DC migration to draining lymph nodes (DLN) and that UV-B irradiation prior to skin sensitization at the same site enhanced DC migration. A maximum number of DC was present in DLN 48 hr following irradiation. In sensitized mice, the percentage of DC bearing FITC and the quantity of FITC per DC was unaltered by prior UV exposure. In contrast, neither isomer of UCA had any significant effect on DC numbers in sensitized or unsensitized mice. It was concluded that UV-B irradiation induced the migration of DC from the epidermis to draining lymph nodes, an effect possibly mediated by TNF-α release, while UCA may act by a different mechanism, perhaps via histamine-like receptors in the epidermis.

INTRODUCTION

ng epicutaneous exposure of mice to skin-sensitizing ls there is a rapid accumulation of dendritic cells (DC) in ıing lymph nodes (DLN).[1-3] A significant proportion of found within DLN bear antigen suggesting that they -ginate from the epidermis and may have an antigen- ng function.[3-5] Topical exposure of mice to 2,4-dinitro- nzene (DNFB) causes a temporary but marked deple- -angerhans' cells (LC) from the epidermis.[6] In addition ;ome DC which accumulate in DLN following contact tion contain Birbeck granules, characteristic of LC.[5] ıd other studies indicate that the antigen-bearing DC in ɜ derived from epidermal LC which travel to the lymph nodes as veiled cells in the afferent lymph. Recently Kripke *et al.* have corroborated this hypothesis by showing that cells from DLN of athymic nude mice, grafted with allogeneic skin and contact sensitized through the graft, contain antigen-bearing DC of graft donor origin.[7] However, it is possible that, while LC initiate contact sensitization under normal circumstances, skin cells other than LC may be capable of inducing the sensitization particularly when the potential of LC to present antigen has been abrogated.[8,9]

Antigen-bearing DC in DLN are potent stimulators of both primary and secondary T-lymphocyte proliferative responses *in vitro*[2,10] and efficiently induce contact sensitization in naive animals.[3,11] A correlation is found between the number of DC which are present in DLN 24 hr after skin sensitization and the extent of the primary lymphocyte proliferative response.[12]

Irradiation with ultraviolet-B light (UV-B) is known to suppress some cell-mediated immune responses to a variety of antigens including contact sensitizers (reviewed in ref. 13). It has been speculated that a UV-induced loss of LC is responsible for suppression as epidermal LC numbers in UV-irradiated skin decrease in parallel with a decrease in contact hypersensitivity (CH) responses.[14] However, a number of other studies have not shown such a correlation[15,16] although there is evidence for the modulation of LC markers after UV irradiation.[17]

:viations: CH, contact hypersensitivity; DC, dendritic cells; yed hypersensitivity; DLN, draining lymph node; DMSO, sulphoxide; DNFB, 2,4-dinitrofluorobenzene; FITC, fluores- ıiocyanate; ICAM-I, intercellular adhesion molecule-1; LC, ns' cells; RPMI-FCS, supplemented RPMI-1640 medium plus al calf serum; TNF, tumour necrosis factor; UCA, urocanic B, ultraviolet-B light.

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been reported that UV-B irradiation impairs the
of CH in some (UV susceptible), but not other (UV
genetically defined strains of mice.[18] Susceptibility to
ictated by alleles at the Lps and Tnfa loci which
he amount of tumour necrosis factor-α (TNF-α)
n response to UV-B.[19] Recently, TNF-α has been
ct as an important mediator of the suppressive effects
n the induction of CH.[19] Interestingly, there is now
iat keratinocytes synthesize and release TNF-α after
liation.[20]
kely that there is a photoreceptor in skin which
ie effects of UV irradiation on the immune system.
date, first proposed by De Fabo and Noonan,[21] is
cid (UCA), found naturally in the stratum corneum
ns-isomer, which converts to the *cis*-isomer on
. There is considerable evidence from several experi-
tems that *cis*-UCA plays an important role in UV-
mmunosuppression. For example, *cis*-UCA has been
nodify antigen-presenting cell function *in vivo*,[22] to
elayed hypersensitivity (DH) responses in a murine
erpes simplex virus infection,[23,24] to delay rejection of
ition allografts[25,26] and to enhance UV-induced
ld and malignancy in the hairless mouse.[27]
present study the effects of UV irradiation and UCA
the migration of DC to DLN have been examined in
d and sensitized mice. In addition the carriage of
d expression of Ia antigens by the DC have been

MATERIALS AND METHODS

am ($H\text{-}2^k$) female mice, aged 6–8 weeks were used
t. The mice were bred and maintained in the Dept. of
Microbiology Animal House, University of Edin-
K.

liation
irradiated for 30 min under two Philips TL20/12
h gave a dose of 144 mJ/cm^2 in the range of 270–350
were irradiated in separate compartments of a high-
ex box to prevent shielding by cage mates. They were
nd their ears unprotected.

ment
A (Sigma Chemical Co., Poole, U.K.) or *cis*-UCA
by preparative thin layer chromatography of UV-B-
rans-UCA)[28] was dissolved at a concentration of 40
dimethylsulphoxide (DMSO) at 37° for 5 min. The
as then diluted 10-fold in acetone and 25 μl applied
o the dorsal surface of both ears. An equal volume of
riate vehicle was applied in the same way to control

nsitization
n isothiocyanate (FITC; Sigma) was used as a 1%
1:1 acetone:dibutylphthalate. Mice received 25 μl of
n equal volume of the appropriate vehicle on the
both ears 18 hr before killing.

Isolation, identification and enumeration of lymph nodes DC
Draining auricular lymph nodes were excised, pooled for each experimental group and a single-cell suspension of lymph node cells was prepared by mechanical disaggregation through a 200-mesh stainless steel gauze. Viable cell counts were performed using 0·5% trypan blue and the cell concentration adjusted to 5×10^6 cells/ml in RPMI-1640 growth medium (Flow Laboratories, Irvine, U.K.) supplemented with 5×10^{-5} M 2-mercaptoethanol, 100 IU/ml penicillin, 2 mM L-glutamine, 100 μg/ml gentamicin, 20 μg/ml fungizone and 10% heat-inactivated foetal calf serum (RPMI-FCS). DC-enriched populations were prepared by density gradient centrifugation as described previously.[3] Briefly, 8 ml of the cell suspension was added to 10 ml conical-bottom test tubes and was gently underlayed with 2 ml of 14·5% metrizamide (Nygaard, Oslo, Norway) in RPMI-FCS. The tubes were then centrifuged for 15 min (600 ***g***) at room temperature. The DC-enriched population accumulating at the interface was collected, washed once and resuspended in RPMI-FCS. The number of DC within the low-buoyant density fraction was assessed routinely by direct morphological examination using light microscopy. For each experimental group, five counts were made and the mean number of DC present within a single lymph node was calculated.

Analysis of FITC-bearing DC
DC-enriched preparations derived from draining auricular lymph nodes of mice whose ears had been exposed to UV-B irradiation or painted with UCA isomers prior to sensitization with FITC, were analysed on an EPICS 'C' flow cytometer (Coulter Electronics, Luton, U.K.) equipped with a 100 mW argon laser tuned to 488 nm wavelength. A total of 5000 cells from each sample was analysed at a flow rate of 200 cells/second. DC were identified on a two-parameter histogram measuring size and side-scatter and then green fluorescence analysed from a bit map onto a 252 channel histogram using log amplification. The percentage of $FITC^+$ cells within this population was measured and also antigen density/cell (fluorescence intensity) by mean channel analysis.

Ia antigen staining of DC and FITC-bearing DC
DC-enriched preparations derived from draining auricular lymph nodes, were washed and 5×10^4 cells stained with anti-mouse Ia monoclonal antibody (clone 11.5.2.1.9; ECACC, Porton Down, Salisbury, U.K.) at a dilution of 1:100 for 1 hr on ice. The cells were then washed and stained with either a sheep anti-mouse IgG [$F(ab')_2$fragment] labelled with FITC (Sigma) at a dilution of 1:40 or a sheep anti-mouse IgG labelled with phycoerythrin (Sigma) at a dilution of 1:40 for 40 min on ice. Simultaneously, 5×10^4 cells from DC-enriched preparations were incubated with an irrelevant antibody followed by FITC or phycoerythrin-labelled secondary antibody to act as background controls in the EPICS analysis. The cells were analysed in an EPICS 'C' flow cytometer (Coulter Electronics) equipped with 5 W argon laser operating at 488 nm wavelength. To measure the percentage of DC expressing Ia, the cells were labelled with anti-Ia and FITC secondary antibody before analysis as outlined in the section above. To measure Ia expression on DC after sensitization with FITC, the cells were labelled with anti-Ia and phycoerythrin secondary antibody and a double-colour analysis carried out in the following manner. Dichroic mirrors were used to separate light into the 90° light

scatter detector (500 long pass) and into both red (560 short pass followed by a 575/25-band pass filter) and green (530/30 band pass) fluorescence detectors. Any residual spectral overlap of green fluorescence into the red detector was removed by electronic compensation.

RESULTS

Effect of UV-B irradiation and *cis* or *trans*-UCA on DC accumulation in DLN of unsensitized mice

After irradiating mice or painting their ears with *cis* or *trans*-UCA, the mice were then killed, their auricular lymph nodes excised and the number of DC present in each lymph node was estimated. Table 1, which shows the results of four experiments, demonstrates that neither isomer of UCA has a consistent effect on inducing DC migration to DLN, whereas UV-B irradiation increases DC numbers within the DLN.

Time–course of the effect of UV-B irradiation and *cis*-UCA on DC migration and Ia expression

The number of DC present within the DLN of mice at various times following a single dose of UV-B irradiation of 144 mJ/cm^2 was calculated. Figure 1 illustrates that DC migration to DLN starts to take place between 12 and 24 hr, and reaches a maximum at 48 hr before decreasing. On the other hand, at various times after the cutaneous application of *cis*-UCA, the numbers of DC were unaltered (Fig. 1).

The number of DC expressing Ia antigens in the DLN at various times after UV-B irradiation was examined; the results are shown in Table 2. The same mean intensity of staining per cell was found throughout the experiment (data not shown).

Influence of UV-B irradiation and *cis* or *trans*-UCA on DC migration induced by FITC

Irradiated mice or mice painted with UCA isomers were sensitized on their ears with FITC 18 hr before killing. Their auricular lymph nodes were removed and the number of DC present in each lymph node was measured. It can be seen from Table 3, which shows the results of four independent experiments, that whilst the isomers have no effect on DC migration induced by FITC, UV-B irradiation increases DC m the DLN considerably.

Influence of UV-B irradiation on the percentage of D FITC and Ia expression in the DLN

The draining auricular lymph nodes of mice, whos been exposed to UV-B radiation prior to sensitiz FITC, were examined for the percentage of DC bea (Table 4). UV-B irradiation which increased DC induced by FITC did not influence the percenta carrying FITC. The mean intensity of staining/cel bearing DC was found to be unaltered by UV-B irra can therefore be concluded that UV-B irradiatio sensitization increases the number of FITC-beari cumulating in the DLN.

It can be seen from Table 4 that UV-B irradiat effect on the expression of Ia molecules by DC accu

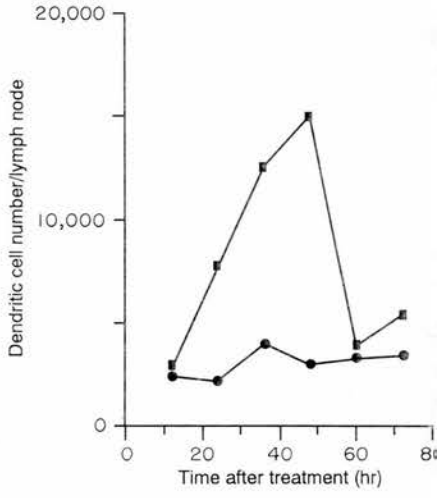

Figure 1. Effect of UV-B irradiation (■) and *cis*-UCA (●) or of DC migration. The DC count/lymph node in naive mi background control for UV-B irradiation was 2925. The lymph node in mice (treated at 48 hr with 25 μl DMSO on ea as background control for *cis*-UCA treatment was 3524.

Table 1. Effect of UV-B irradiation and *cis* or *trans*-UCA on DC migration to DLN

Treatment at 66 and 42 hr prior to analysis	DC count/lymph node experiment				
	1*	2*	3†	4†	Mean ± SD
Vehicle	2850	2860	2079	1950	2435 ± 488
cis-UCA	5162	3050	2550	2494	
trans-UCA	7040	4446	2050	2925	
UV-B (144 mJ/cm^2)	10,005	9344	9720	9643	9678 ± 272‡

* 200 μg of UCA was painted onto each ear.

† 100 μg of UCA was painted onto each ear.

‡ Significantly different from control group ($P < 0{\cdot}001$ by Student's *t*-test).

Table 2. The effect of UV-B irradiation on expression in DC from DLN

Time after UV irradiation (hr)	% of cells within the gat population expressing
Untreated DC	93·8
12	83·6
24	92·0
36	94·7
48	91·8
60	86·2
72	91·5

At various times after UV irradiation (single do of 144 mJ/cm^2) the mice were killed, their auricu lymph nodes removed and the enriched DC we then stained for Ia.

Table 3. Influence of UV-B irradiation and *cis* or *trans*-UCA on DC migration to DLN induced by FITC

Treatment at 66 and 42 hr	FITC at 18 hr	DC count/lymph node experiment 1	2	3	4	Mean ± SD	Statistical† significance (by Student's *t*-test)
Vehicle	–	6750	8775	4788	8125	7110 ± 1763	
Vehicle*	+	12,222	13,754	10,196	13,294	12,367 ± 1583	
100 μg *cis*-UCA	+	15,238	16,406	9111	11,984	13,185 ± 3298	NS
100μg *trans*-UCA	+	14,175	19,511	11,500	15,640	15,207 ± 3343	NS
UV-B (144 mJ/cm^2)	+	23,490	20,174	16,422	19,600	19,922 ± 2895	$P < 0{\cdot}01$

† Significance of difference from vehicle group*.
NS, not significantly different from vehicle group.

Table 4. Influence of UV-B irradiation on the percentage of DC carrying FITC and expressing Ia

Treatment at 66 and 42 hr	FITC at 18 hr	DC count/ lymph node	% of cells within gated population bearing FITC	% of cells within* gated population bearing FITC	% of cells within† gated population expressing Ia
Vehicle	–	8125	—	—	91 ± 5
Vehicle	+	13,294	40	38 ± 8	85 ± 7
UV-B (144 mJ/cm^2)	+	19,600	34	38 ± 5	87 ± 9

* Expressed as arithmetic mean of four experiments ± 1 SD.
† Expressed as arithmetic mean of two experiments ± 1 SD.

s a results of FITC induction. In addition, by using
our fluorescence, UV-B irradiation did not alter Ia
on FITC-bearing DC (data not shown).

DISCUSSION

reported here demonstrate that UV-B irradiation
ior to skin sensitization at the same site, induces or
C migration to DLN. In addition our data show that
unable to induce similar changes, indicating that the
of its action may be different from irradiation.

C are the major antigen-presenting cells of the skin
rucial role in the induction of CH responses, they are
ndidates to mediate the immunosuppressive effects
adiation. It has been suggested that the immobiliz-
C at UV-B-irradiated sites may contribute to the
ice to develop CH when hapten is painted subse-
these sites.[29,30] Bigby *et al.*[29] reported that there were
earing antigen-presenting cells in DLN, if the skin
e hapten was painted had been exposed previously to
s known that UV-B irradiation stimulates the
d secretion of TNF-α by keratinocytes.[20] Vermeer
n found that TNF-α inhibited the induction of CH to
sed on this observation and the demonstration that
radermal TNF-α altered the morphology of epider-
rmeer and Streilein concluded that TNF-α prevents
sitization following UV-B irradiation by immobili-
hin the epidermis.[30] In contrast to these findings,
sented here indicate that UV-B irradiation increases
of migrating DC induced by FITC, while the
percentage of DC bearing antigen in DLN is unaltered. Also, we have found that treatment of mice with dexamethasone, a transcriptional inhibitor of TNF-α, prior to UV irradiation inhibits DC migration to DLN (A. M. Moodycliffe, I. Kimber and M. Norval, unpublished data). The recent demonstration by Cumberbatch and Kimber[31] that TNF-α induces DC migration to DLN, supports the concept that one of the effects of UV-B exposure is to induce DC accumulation in DLN by stimulating TNF-α release. Further support is provided by Vermeer and Streilein who have shown that both intradermal TNF-α and UV-B irradiation reduce the density of Ia$^+$ cells in the epidermis,[30] suggesting that UV-B exposure may induce LC migration through the local release of TNF-α.

It is not possible to conclude from our results whether or not the induction of DC migration by UV-B radiation to DLN, either by itself or in conjunction with some other important components(s), is responsible for UV-B induced suppression of CH responses. However, we have shown that, following UV-B irradiation, there was a greater number of antigen-bearing DC entering the DLN than would occur with skin sensitization alone. Ia expression was unaffected suggesting that antigen-presenting cell function may not be lost, although it could be altered. There is evidence, that during migration from the skin to lymphoid tissue, LC are subject to phenotypic and functional maturation.[32] The functional maturation of LC *in vitro* is stimulated by granulocyte–macrophage colony-stimulating factor,[33] a product of keratinocytes.[34] It is possible that, following UV irradiation, the migrating DC do not have time to mature into fully functional antigen-presenting cells. T-cell interactions with DC may then be affected, for example, by altered ability to

synthesize and secrete accessory molecules, such as interleukin-1 (IL-1) and IL-6, or by altered expression of appropriate adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and LFA-1. Indeed it has been shown recently that membrane ICAM-1 is expressed on lymph node DC, while it is present in only very low amounts on epidermal LC; increased ICAM-1 expression may be necessary for the development of LC into effective antigen-presenting cells.[35]

Unlike UV-B irradiation, there was little, if any, effect on DC migration induced by the UCA isomers even at larger than physiological doses (100–200 μg/mouse). C3H mice contain approximately 20 μg/cm^2 UCA in the epidermis, most as the *trans*-isomer, and about 50% converts into the *cis*-isomer after UV-B irradiation of 144 mJ/cm^2.[36] The result indicates that *cis*-UCA may mediate its effect on the immune system in a rather different way than via TNF-α production and DC migration. Indeed *cis*-UCA is a poor suppressor of CH compared with UV-B irradiation, while it is highly effective at suppressing DH responses. In a murine model of HSV infection *cis*-UCA suppresses DH even at doses of as little as 1 μg/mouse.[28] By using structural analogues of UCA and histamine receptor agonists and antagonists, we have shown that *cis*-UCA is likely to act via histamine-like receptors in the epidermis.[28,37] In addition there is some evidence that immune responses generated in DH and CH may be different. For example irradiation of keratinocytes with UV of different wavebands induced the release of mediators which were selective in their ability to induce suppression of CH and DH.[38]

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Characterisation of a monoclonal antibody to cis-urocanic acid: detection of cis-urocanic acid in the serum of irradiated mice by immunoassay

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Abstract

Cis-urocanic acid (cis-UCA), which is formed from the naturally occurring trans-isomer on ultraviolet (UV) irradiation, has been suggested as a photoreceptor for and mediator of the suppressive effects of UV irradiation on systemic immune responses. Trans-UCA is located predominantly in the stratum corneum, and the extent of isomerization to cis-UCA may be analysed by high performance liquid chromatography of skin extracts. Such an analysis is not suitable for other tissues. In this study a murine monoclonal antibody to cis-UCA was prepared and tested by ELISA using UCA isomers conjugated to protein as antigens. The interaction of the antibody with structural analogues of UCA was assessed by competitive inhibition ELISA which indicated that the antibody had a high specificity for cis-UCA. Screening of sera at various times after UVB irradiation of mice by competitive inhibition ELISA using the monoclonal antibody showed that cis-UCA was present, probably in an unbound form, for at least 2 days after the exposure. Thus, cis-UCA produced in the epidermis following UVB irradiation reaches the serum a few hours later. The implications of this finding for the generation of suppressed immune responses are discussed.

Key Words.

Urocanic acid isomers

Urocanic acid analogues

Monoclonal antibody

ELISA and competitive inhibition ELISA.

Introduction

Irradiation with ultraviolet-B light (UVB) results in suppression of some T cell mediated immune responses to antigens encountered within a short period after the exposure. This has been shown in a variety of species including man [reviewed in 1] and mice [reviewed in 2]. Several hypotheses have been suggested to explain the immunomodulation, one of which involves initiation by a specific photoreceptor, urocanic acid (UCA), in the skin which then mediates immunosuppression[3]. UCA is produced in the stratum corneum during keratinization by the action of the enzyme histidine ammonia-lyase[4]. It occurs naturally as the trans-isomer and represents a major UV absorbing component of skin. On UV irradiation in vitro or in vivo, trans-UCA converts to cis-UCA in a dose-dependent manner until a photostationary state is reached[5-7]. Evidence from a variety of experimental systems, most in mice, indicates that cis-UCA mimics a number of the effects of UV irradiation on immune responses. These include modification of antigen presenting cell function in vivo[8], suppression of contact hypersensitivity[9,10] and delayed hypersensitivity[11] responses, delay in rejection of transplant allografts[12] and enhancement of UV-induced tumour yield and malignancy[13].

It is not known exactly how cis-UCA alters immune function or, indeed, if it is confined solely to the epidermis after irradiation. High performance liquid chromatography (HPLC) has been used successfully to quantify UCA isomers in skin extracts[14-16]. However, it has not proved possible to analyse other tissues easily by HPLC because of the complexity of substances present and the fact that UCA represents a major UV absorbing constituent only in the skin. One approach has been to label UCA with ^{14}C and to follow its distribution after topical application in mice[17]. The novel approach, used in the present study, was to prepare a monoclonal antibody with specificity for cis-UCA and to use this to detect cis-UCA in serum following UVB irradiation of mice.

Materials and Methods

UCA isomers and analogues

Trans-UCA was purchased from Sigma (Poole, Dorset, UK) and cis-UCA prepared from it as outlined in Norval et al [18]. The following analogues of UCA were synthesized as described previously[18]: cis and trans-2-methyl urocanic acid, cis and trans-3-furanacrylic acid, cis and trans-2-thiopheneacrylic acid, cis and trans-3-thiophenoacrylic acid and dihydrourocanic acid. Histidine and histamine were purchased from Sigma. The trans-isomer of pyridine-2-acrylic acid was prepared by Knoevenagel condensation of pyridine-2-carboxaldehyde with malonic acid in the presence of pyridine. Trans-pyridine-3-acrylic acid was prepared similarly from pyridine-3-carboxaldehyde. Cis-pyridine-2-acrylic acid was prepared from the corresponding trans-isomer by irradiation followed by thin layer chromatography using a solvent system of 90% ethyl acetate, 8% methanol and 2% formic acid. The solvent was removed under vacuum and the cis-isomer purified on a dry-flash column eluted with ethyl acetate followed by sublimation after removal of the solvent. The cis-isomer of pyridine-3-acrylic acid was prepared similarly from the corresponding trans-isomer except it was recrystallised from ethanol in the final step. The identity and purity of the compounds were established as outlined in Norval et al[18]. The structures of these molecules are shown in Fig. 1.

Conjugates

Cis and trans-UCA were coupled to keyhole limpet haemocyanin (KLH; Sigma) and bovine serum albumin (BSA; Sigma) using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide as a condensing agent[19] followed by purification by FPLC using a Sephadex G25 column eluted with sodium borate buffer (200mM, pH 9). The hapten-carrier conjugate was obtained by freeze drying of the protein-containing fractions. Histamine was coupled to BSA similarly.

Trans-[2,5-^{3}H] UCA was prepared from L-[2,5-^{3}H] histidine using the enzyme histidine ammonia-lyase (Sigma), then irradiated and the labelled isomers separated by HPLC[14] and counted. Each was reacted with KLH and BSA in the presence of the relevant cold UCA isomer, and the final conjugate counted to give an estimate of the number of hapten molecules per molecule of protein. The epitope density was found to be 62 trans-UCA molecules and 29 cis-UCA molecules per KLH molecule, and 4.5 trans-UCA molecules and 3.7 cis-UCA molecules per BSA molecule.

Preparation of monoclonal antibody

Female Balb/c mice, aged 6 weeks and bred in the Department of Medical Microbiology Animal House, were immunized by subcutaneous injection of 200 μg cis-UCA-KLH conjugate in 0.1 ml Freund's incomplete adjuvant. A first booster injection consisting of 200 μg cis-UCA-KLH conjugate in 0.2 ml sterile 0.01M phosphate buffered saline pH 7.2 (PBS) given intraperitoneally was administered after 36 days, followed by a second after 61 days and a third after 166 days. Mice were bled 7 days after the second booster and sera screened for anti-cis-UCA antibody by ELISA (see below). Mice showing antibody titres to cis-UCA were killed 3 days after the third booster and spleen cells fused with NS-0 mouse myeloma cells (a gift from Dr M. McCann, Department of Surgery, Edinburgh University) using a standard fusion technique. Culture supernatants were screened 14 days after fusion for antibodies to cis-UCA. The single positive culture was cloned and re-cloned by limiting dilution. The immunoglobulin subclass of the monoclonal antibody was determined by an isotyping kit (Amersham).

ELISA

ELISA plates (Gibco) were coated overnight at 4°C with cis-UCA-BSA conjugate diluted in 0.1M carbonate-bicarbonate buffer pH 9.6 (5 μg in 100μl per well). After extensive washing

with PBS-Tween pH 7.2, 100μl hybridoma culture supernatant or serum diluted as appropriate in PBS-Tween containing 1% BSA was added and incubation continued for 3h at room temperature, followed by washing, incubation with anti-mouse IgG alkaline phosphatase conjugate (Sigma) and development with p-nitrophenyl phosphate. Absorbance was measured at 405nm. Tissue culture medium or pre-bleed serum, as appropriate, were used as negative controls. The test was considered positive if the absorbance was more than the mean of the negative control + 3 SD of the mean.

In other assays trans-UCA-BSA conjugate, histamine-BSA conjugate, KLH or BSA were used as antigen, all at 5 μg in 100μl per well.

Competitive inhibition ELISA

Wells of an ELISA plate were coated with cis-UCA-BSA conjugate at 0.08 μg per well and incubated overnight at 4°C. After extensive washing with PBS-Tween, 50μl of the appropriate inhibitor was added to triplicate wells or more, followed by 50μl of a 1/400 dilution of the hybridoma culture supernatant. The inhibitors were dissolved in DMSO at 37°C for 5 min at concentrations of 20, 5 or 1 mg/ml and then diluted appropriately in PBS-Tween containing 1% BSA. The plates were incubated for 3h at room temperature, washed and developed as above.

The negative control contained 100μl PBS-Tween with 1% BSA. The positive control contained 50μl of the diluted hybridoma culture supernatant and 50μl of a dilution of DMSO in PBS-Tween containing 1% BSA, prepared as for the inhibitors above. The inhibition of binding of the monoclonal antibody was calculated as:

$$\% \text{ inhibition} = 100\text{x } 1-\left[\frac{(\text{mean absorbance test} - \text{mean absorbance -ve control})}{(\text{mean absorbance +ve control} - \text{mean absorbance -ve control})}\right]$$

The SD from the mean absorbances of test and positive samples was less than 10%.

Screening of murine sera for cis-UCA

C3Hf Bu/Kam mice were bred and maintained in the Department of Medical Microbiology Animal House. The dorsal sides of female mice, aged 7-8 weeks, were shaved and they were irradiated once with 216 $mJcm^{-2}$ UVB or 144 mJm^{-2} UVB twice with a 24 h interval between exposures. The conditions of irradiation and the lamp used have already been described[20]. Five hours before irradiation the mice were eye bled and the sera pooled to give the pre-bleed sample. At various times following irradiation, the mice were bled from the vena cava, serum samples from 2 mice being pooled for each time point.

ELISA plates were coated with cis-UCA-BSA conjugate at 0.08 μg/well as already described. After washing, pre-bleed serum or sera from irradiated mice were diluted 1:3 in PBS-Tween with 1% BSA and 50 μl added to each of 6 wells or more followed by 50 μl hybridoma culture supernatant diluted appropriately. The plates were incubated for 3 h at room temperature, washed and developed as above. One control contained 50 μl pre-bleed serum diluted 1:3 in PBS-Tween with 1% BSA and 50 μl PBS-Tween with 1% BSA. This value was subtracted from the test value. Another control consisted of hybridoma culture supernatant and pre-bleed serum diluted 1:3 and containing various concentrations of cis or trans-UCA.

In a further experiment a group of 10 mice were eye bled and the serum pooled. After 5 hours the shaved mice were UVB irradiated with 216 $mJcm^{-2}$ and 25 h later they were bled from the vena cava and the serum pooled. Control mice were treated similarly but without irradiation. Half of each serum sample was dialysed against PBS at 4°C for 14 h using Visking tubing with molecular weight cut off in the range 12,000 to 14,000. The volume of the dialysed samples was measured and they were diluted appropriately in PBS-Tween with 1% BSA to be equivalent to 1:3 of the original volume. The undialysed samples were also diluted 1:3 in the same diluent. A competitive inhibition ELISA was carried out as above.

Results

Preparation of monoclonal antibody against cis-UCA

Mice were immunized with cis-UCA-KLH and spleen cells from mice showing antibody titres to cis-UCA-BSA by ELISA were fused with NS-0 cells. One hybridoma was found which produced an antibody recognising cis-UCA-BSA conjugate on ELISA. After cloning by limiting dilution twice, dilutions of the hybridoma culture supernatant were tested by ELISA, using 5 μg cis-UCA-BSA per well as antigen. The highest dilution which gave a mean absorbance more than the mean of an equivalent dilution of culture medium + 3 SD of the mean was 256,000. Using an isotyping kit, the monoclonal antibody was found to be IgG_1 Kappa.

This culture supernatant was tested for activity against other conjugates and proteins as antigens as shown in Table 1. Trans-UCA-BSA and histamine-BSA were not recognized, nor was BSA or KLH.

Specificity of the monoclonal antibody

A checkerboard titration was performed with doubling dilutions of both cis-UCA-BSA starting at 1.25 μg per well, and the monoclonal antibody starting at 1:100 (Fig. 2). Antigen at 0.08 μg per well and antibody at a dilution of 1:400 gave an absorbance of 0.30. These conditions were chosen for the following competitive inhibition ELISAs to demonstrate the specificity of the monoclonal antibody. In these tests UCA analogues or conjugates were added to the well together with the monoclonal antibody and the inhibition of binding of the monoclonal antibody to cis-UCA-BSA calculated. The results are shown in Table 2 where it can be seen that, while cis-UCA inhibited the binding of the monoclonal antibody to the antigen down to a level of 0.1 μg per well, trans-UCA had little effect in comparison; inhibition was only apparent if trans-UCA was present at 5 μg per well. The only UCA

analogue to show any inhibition in binding was cis-2-methyl-UCA, again at the high concentration of 5 μg per well. Trans-2-methyl-UCA had no effect. Cis and trans-UCA-KLH conjugate both had inhibitory activity.

Screening of murine sera for cis-UCA

Initially various amounts of cis and trans-UCA were added to murine serum to obtain a standard curve for the quantification of UCA isomers using the competitive inhibition ELISA. The result of one such experiment is shown in Fig.3. It may be seen that amounts of cis-UCA between 0.75 μg and 0.1 μg may be detected quantitatively by this method, while trans-UCA is not detected at all.

The assay was then applied to the measurement of cis-UCA in serum at various times after UVB irradiation of mice (Table 3). In the first experiment, after a single UVB exposure, the inhibition in binding of the monoclonal antibody indicated a maximum amount of cis-UCA in the serum 25 h after irradiation, equivalent to approximately 0.1 μg cis-UCA in each well of the assay i.e. about 6 μg cis-UCA per ml serum. In the second experiment after two exposures to UVB, cis-UCA was detected in the serum 1 h after the second irradiation and reached a maximum at 5 h of approximately 30 μg per ml serum.

It was not known whether the cis-UCA in the serum was present in a free form or conjugated to another molecule. To test this, the serum from irradiated mice was dialysed and used in a competitive inhibition ELISA (Table 4). It can be seen that there was inhibition of binding of the monoclonal antibody to the antigen in the presence of serum from irradiated animals but this inhibition was removed by dialysis of the serum. Therefore cis-UCA in the serum of irradiated animals was in an unbound form or conjugated to a molecule with molecular weight below 12,000.

Discussion

To define the role of cis-UCA in UV-induced immunosuppression, it is necessary to identify its site of action and cellular target. In the present study, a monoclonal antibody with specificity for cis-UCA was developed and applied to the detection of UCA in serum of irradiated mice.

UCA isomers were conjugated to KLH and BSA by the carbodiimide method which was chosen for its convenience and simplicity. One problem which can occur is the precipitation of protein which has become denatured by cross-linking but very little precipitation was, in fact, observed. As UCA is amphoteric with both a basic imidazole ring and an acidic carboxylic acid group, there is the possibility of coupling through either group to the carrier protein to form two different structures (Fig. 4). It is not known which of these forms was synthesized or which the monoclonal antibody recognized. The epitope density of the conjugates was fairly light, as measured by radioactive labelling, but the cis-UCA-KLH used for immunization elicited a good antibody response and the conjugates used as antigens in ELISAs proved sensitive and specific. A minimum number of haptens per protein of 15-30 is recommended for the induction of antibodies[19], yet as few as 5 have been reported to give good IgG responses[21].

From Table 1, it may be seen that the monoclonal antibody recognized only cis-UCA-BSA conjugate as antigen and this was corroborated in the competitive inhibition ELISAs (Table 2) where cis-UCA competed, even at low concentrations, with the antigen bound to the well for binding of the antibody. Trans-UCA only competed when it was present at high concentration. The only UCA analogue out of the fifteen tested which showed any inhibitory activity was cis-2-methyl UCA, although it was not so active as cis-UCA itself. When the UCA-KLH conjugates were used as competitors, both cis-UCA-KLH and trans-UCA-KLH demonstrated inhibitory activity. From the inhibition data in Table 2, the UCA-KLH conjugates were recognised approximately 1000-fold better in molar terms than cis-UCA

itself. As KLH itself does not interact with the monoclonal antibody (Table 1), this result implies that the antibody is specific for the combined structure of UCA and the region on the carrier to which it is bound. In this state it cannot distinguish trans or cis-UCA-KLH conjugates. However, the isomers alone or conjugated to another protein were distinguished and we conclude that the monoclonal antibody is specific for cis-UCA unless trans-UCA is present in large quantities or trans-UCA is conjugated to KLH.

Analysis of UCA isomers in skin extracts by HPLC is straightforward and quantitative; in our hands the sensitivity of detection in homogenised ears is about 0.14 μM, representing 1 ng/mg wet weight tissue[14]. The identificationof such isomers in serum by this method proved impossible due to the complexity of UV absorbing substances present. Use of the monoclonal antibody gave us the opportunity to assay cis-UCA in serum of irradiated mice by competitive inhibition ELISA. The sensitivity of detection was about 40 μM. However, despite the low sensitivity in comparison with HPLC, cis-UCA was detected in serum soon after irradiation, its concentration diminishing over the next two days. By dialysis of the serum, cis-UCA was demonstrated to be in a dialysable form and therefore unbound or conjugated to a small molecule, and unlikely to be conjugated to serum proteins or membranes. It has been reported that radiolabelled cis-UCA can be detected in urine of mice 24 h after topical application [D. Hug and J.K. Hunter, quoted in[22]]. In addition Reeve et al.[17] have found low but significant levels of ^{14}C in serum of mice after painting them with ^{14}C-UCA synthesized from [2-^{14}C]-malonic acid. The counts were highest in animals that were UV-B irradiated after painting, and were present 2 h after exposure and not 6 days later. Electrophoresis of serum proteins suggested that ^{14}C was associated with specific proteins.

Thus there is evidence that a proportion of cis-UCA formed in the epidermis on UV irradiation reaches the serum either through capillaries or lymph, and may also be excreted in the urine. The cellular target for cis-UCA may be in the skin but, as a result of the systemic spread of cis-UCA after irradiation, it may act in lymph nodes or spleen, there to influence interactions of antigen presenting cells and T cells. We are in the process of

analysing murine tissues from these sites for cis-UCA and also have the opportunity to use the monoclonal antibody in experiments to block the effects of UV irradiation on the immune system.

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Table 1

Binding of monoclonal antibody (1:1600 dilution) to various antigens (5 μg per well) measured by ELISA.

	Absorbance ± SD
cis-UCA-BSA	0.408 ± 0.015
trans-UCA-BSA	0.044 ± 0.005
histamine-BSA	0.049 ± 0.011
BSA	0.037 ± 0.005
KLH	0.060 ± 0.005

Table 2

Competitive inhibition ELISA to test effects of UCA analogues and conjugates on binding of monoclonal antibody (1:400 dilution) to cis-UCA-BSA conjugate (0.08 μg per well).

	% inhibition of binding			
μg analogue or conjugate per well	5	1	0.1	0.03
cis-UCA	100	80	16	0
trans-UCA	27	0	0	0
histamine	ND	0	0	0
histamine-BSA	ND	0	0	ND
cis-UCA-KLH	ND	96	60	21
trans-UCA-KLH	ND	83	6	2
cis-2-methyl-UCA	59	6	0	0
trans-2-methyl-UCA	0	0	0	0

Cis and trans pyridine-2-acrylic acid, cis and trans pyridine-3-acrylic acid, cis and trans-2-furanacrylic acid, cis and trans-2-thiophenoacrylic acid, cis and trans-3-thiopheneacrylic acid, dihydrourocanic acid and histidine (all at 1 μg and 0.1 μg per well) did not inhibit the binding of the monoclonal antibody.

ND = not done

Table 3.

Competitive inhibition ELISA to assay cis-UCA in serum at various times after irradiation of mice. The monoclonal antibody was used at a dilution of 1:400, and the cis-UCA-BSA conjugate at 0.08 μg per well.

Experiment 1				Experiment 2			
Time after irradiation (h) (one exposure of 216 $mJcm^{-2}$)	Absorbance	Significance*	% inhibition of binding	Time after second irradiation (h) (two exposures of 144 $mJcm^{-2}$ with 24 h between)	Absorbance	Significance*	% inhibition of binding
Prebleed serum	0.157			Prebleed serum	0.355		
14	0.124	NS	21	1	0.294	p<0.01	17
25	0.116	p<0.001	26	5	0.225	p<0.01	35
39	0.139	NS	11	11	0.256	p<0.001	28
48	0.142	p<0.01	10	24	0.291	p<0.01	18
114	0.161	NS	0	29	0.283	p<0.01	20
				47	0.341	NS	4
				114	0.338	NS	5
Prebleed + 0.5μg cis-UCA	0.050	p<0.001	68	Prebleed + 0.5μg cis-UCA	0.224	p<0.001	37
Prebleed + 0.5μg trans-UCA	0.163	NS	0	Prebleed + 0.5μg trans-UCA	0.340	NS	4
Prebleed + 0.1μg cis-UCA	0.112	p<0.001	29	Prebleed + 0.1μg cis-UCA	0.291	p<0.001	18
Prebleed + 0.1μg trans-UCA	0.171	NS	0	Prebleed + 0.1μg trans-UCA	0.335	NS	6

* Significance of difference from prebleed serum (Student's t test)

Table 4

Competitive inhibition ELISA (conditions as in Table 3) to assay cis-UCA in dialysed and undialysed serum 25 h after irradiation of mice with 216 $mJcm^{-2}$

Serum sample	Absorbance	% inhibition of binding
Prebleed : undialysed	0.149[1]	
Prebleed : dialysed	0.109[2]	
Post-irradiation : undialysed	0.085[3]	43
Post-irradiation : dialysed	0.108[4]	0

[3] significantly different from [1] ($p < 0.001$; Student's t test)

[4] not significantly different from[2] ($p > 0.01$; Student's t test)

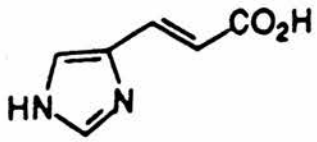
Legends for Figures

Fig. 1. Structure of urocanic acid isomers and analogues.

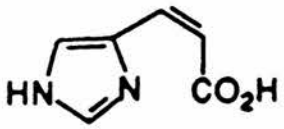
Fig. 2. Binding of monoclonal antibody to cis-UCA-BSA conjugate at concentrations of 1.26 (□), 0.63 (○), 0.32 (■), 0.16 (△), 0.08 (▲) and 0.04 (×) μg per well by ELISA.

Fig. 3. Standard curve showing the effect of varying concentrations of cis-UCA in serum in the competitive inhibition ELISA. The monoclonal antibody was used at a dilution of 1:400 and the cis-UCA-BSA con jugate at 0.08 μg per well. Trans-UCA, added at 1 μg and 0.5 μg per well, showed absorbances of 0.176 and 0.188 respectively.

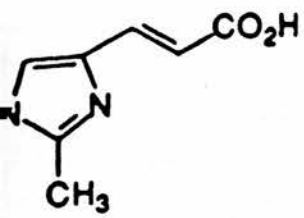
Fig. 4. Possible modes of coupling of trans-UCA to a protein carrier.



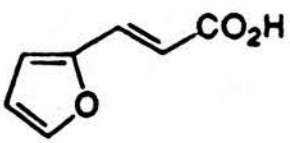
trans - urocanic acid



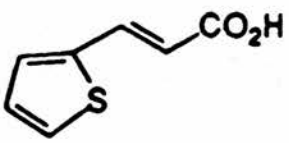
cis - urocanic acid



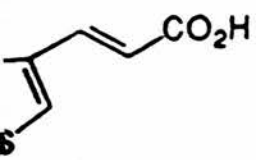
-methylurocanic acid



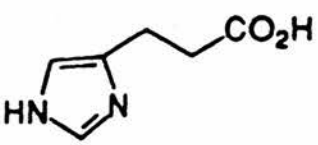
trans - 2 - furanacrylic acid



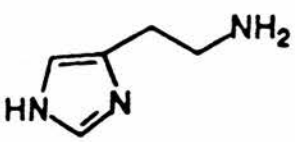
trans - 2 - thiopheneacrylic acid



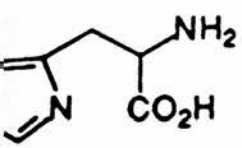
- thiopheneacrylic acid



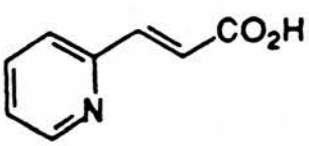
dihydrourocanic acid



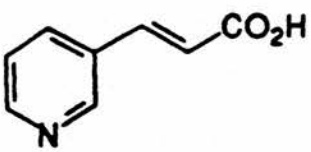
histamine



tidine



trans - 2 - pyridineacrylic acid



trans - 3 - pyridineacrylic acid

Fig 1.

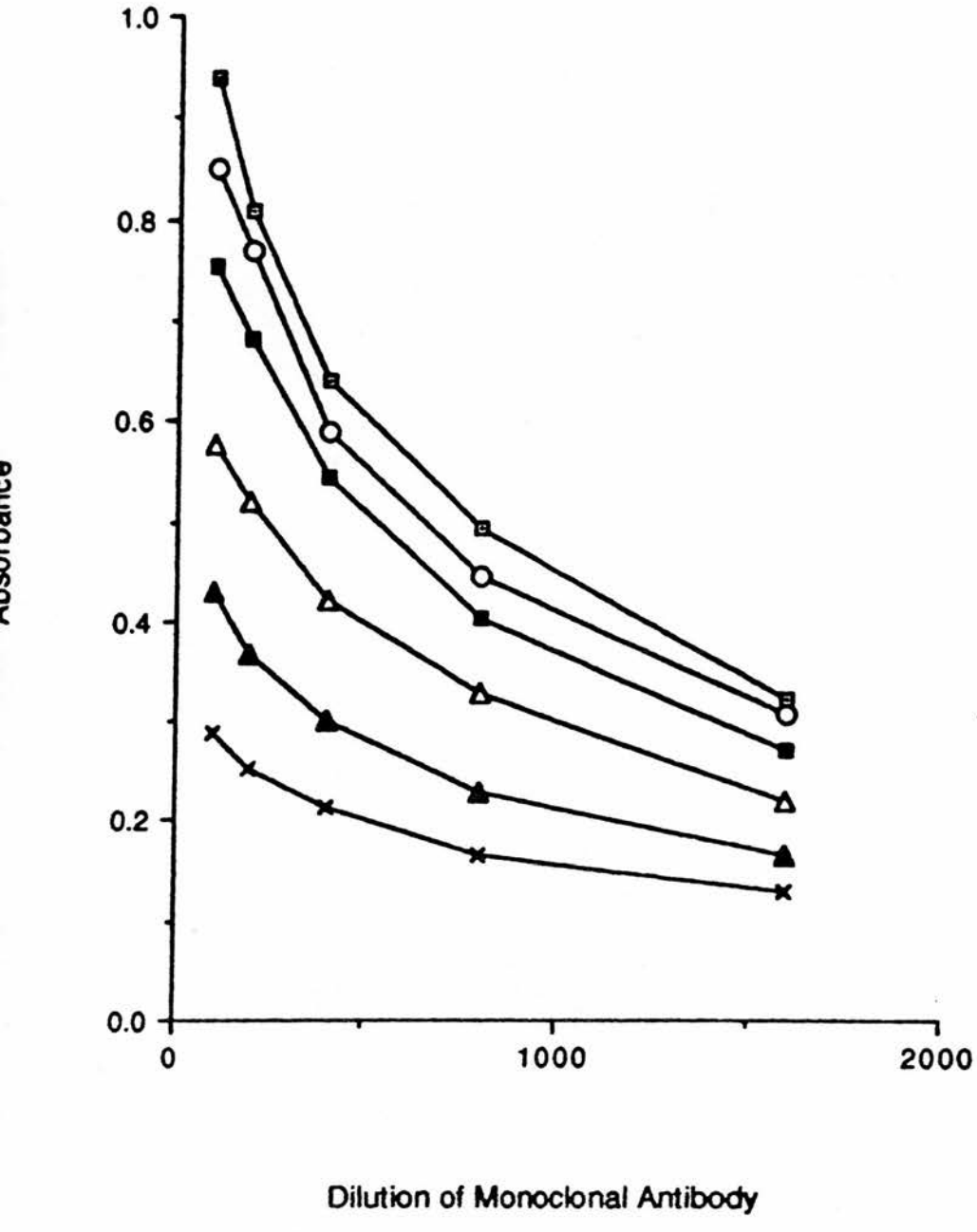

1.0
0.8
0.6
0.4
0.2
0.0
0
1000
2000
Absorbance
Dilution of Monoclonal Antibody

Fig 2

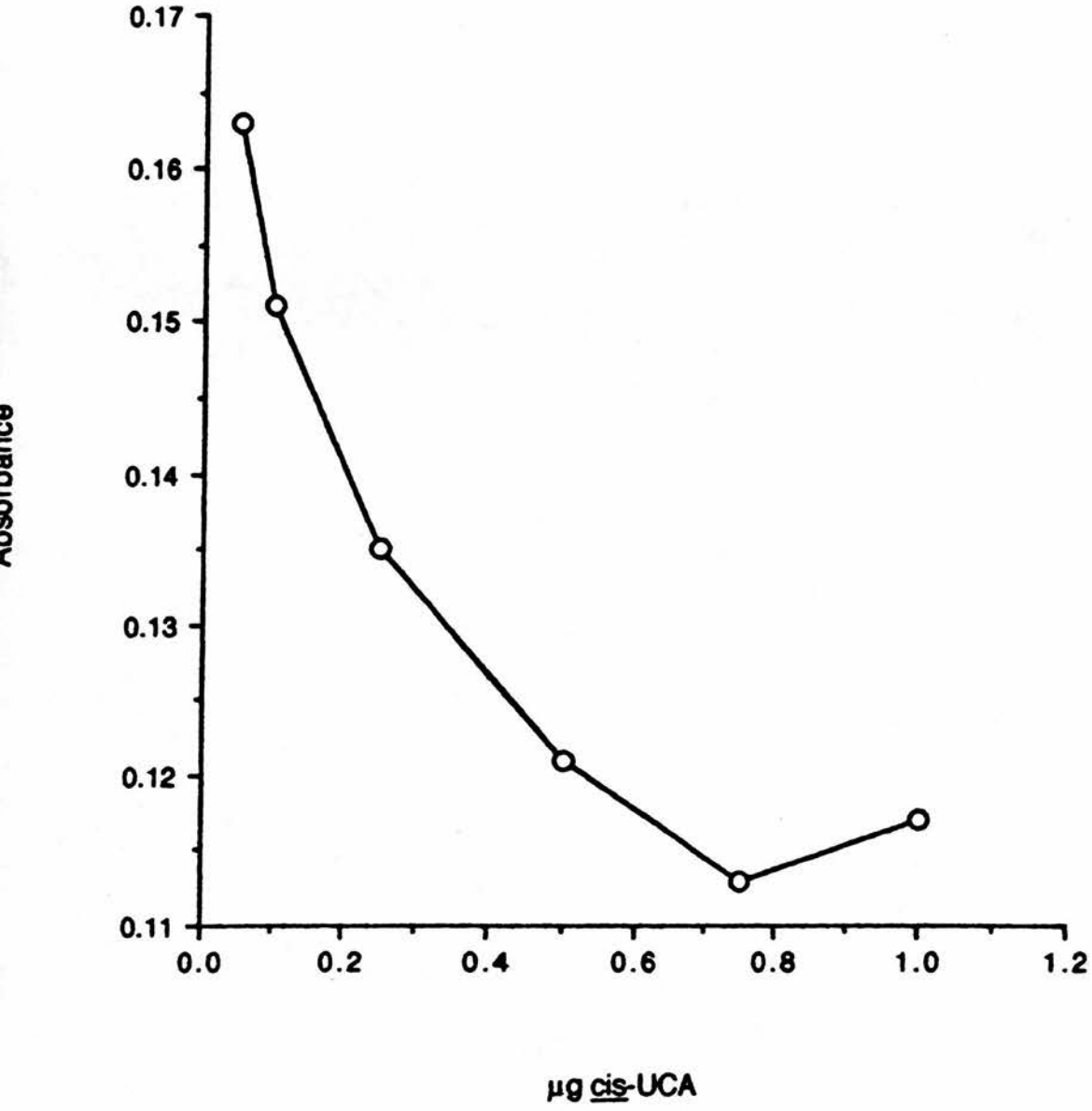


Fig 3

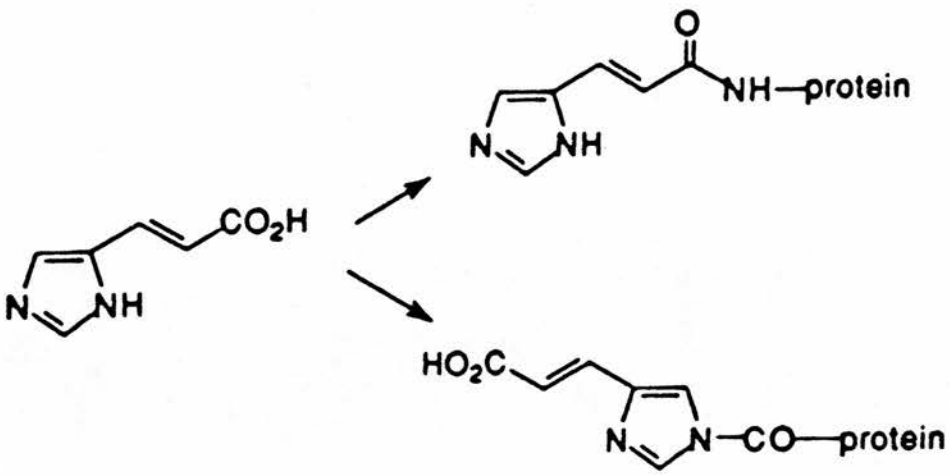


Fig 4.