Modulation of T helper cell activity by ultraviolet B irradiation

Analysis of cytokines and genetic susceptibility factors

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Modulatie van T helper cel activiteit door blootstelling aan ultraviolet B

Analyse van cytokinen en genetische aspecten

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Analysis of cytokines and genetic susceptibility factors

CONTENTS

Chapter 1

UVB irradiation and immune reactivity

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1. INTRODUCTION

Over the last decades, human exposure to ultraviolet B (UVB) irradiation has increased due to the reduction of the protective atmospheric ozone layer and a change in human behavior (e.g. more sun holidays). This increasing UVB exposure has resulted in concern for the consequences for human health.

Photo-immunology was initiated by the observation that chronic UVB irradiation induced skin tumors in mice (Kripke, 1974; 1981). These tumors were highly antigenic, since they were rejected when transplanted onto syngeneic recipients. Moreover, when the recipients received immunosuppressive drugs or low doses of UVB, the tumor rejection failed to occur. This was the first observation that UVB irradiation induced immunosuppression (Kripke, 1983). Subsequently, many epidemiological studies did indicate that sun exposure is the major environmental agent implicated in induction of non-melanoma skin cancer (Kricker, 1995; Urbach, 1991; 1997).

The immunosuppressive effects were further demonstrated by showing that UVB suppressed contact hypersensitivity (CHS), delayed type hypersensitivity (DTH) and alloantigen responses (Toews, 1980; Rivas, 1992; Rattis, 1995). As a consequence of the observed impairment of cellular immunity a reduced resistance to infections (such as *Herpes simplex virus*, *Candida albicans* and *Mycobacterium bovis*) was observed in both mice and humans (Garssen, 1998; Duthie, 1999). Other effects of UVB include erythema, sunburn and photoaging of the skin, as well as photokeratitis, pytergium and cataract of the eye (Fisher, 1997; Longstreth, 1998; de Gruijl, 1997).

The adverse effects of UVB have stimulated research on the mechanisms underlying the immunosuppressive effects of UVB in order to define its contribution to the prevalence of infections and skin carcinogenesis. In addition, much research was conducted in order to improve the therapeutical use of UVB light for treatment of diseases (e.g. psoriasis and cutaneous T cell lymphoma), and the protection efficacy of sunscreens. These studies have clearly demonstrated that the interaction between the antigen presenting cells (APC) and CD4$^+$ T helper cells play an important role in UVB-induced immunosuppression, as will be discussed below.

2. T CELL ACTIVATION

2.1. Th1-Th2 subsets

Naive CD4$^+$ T helper (Th) lymphocytes recognize antigen by their specific T cell receptor (TCR) in the context of major histocompatibility complex (MHC) class II molecules on the APC which initiates their activation, proliferation and the release of cytokines. Further differentiation results in effector cells that are capable of modulating specific immune

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responses. Naive Th cells produce only small amounts of interleukin-2 (IL-2). Upon stimulation, these cells can develop into at least two subpopulations, each with a distinct cytokine profile (Mosmann, 1989; Nakamura, 1997; Romangani, 1997). Th type I (Th1) cells secrete IL-2, interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), but not IL-4, IL-5 and IL-13, whereas Th type 2 (Th2) cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, but not IL-2 and IFN-γ. Both subpopulations secrete IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). Th type 0 (Th0) cells have also been identified secreting both Th1 and Th2 cytokines. It is still uncertain whether these Th0 cells are the common precursors of Th1 and Th2 cells or comprise a third effector population. It should be stressed that Th1 and Th2 subsets are extremes of a spectrum of T cells and do not comprise discrete subsets. Moreover, within a polarized Th1 or Th2 phenotype, individual Th cells possess differential rather than coordinated gene expression (Kelso, 1995).

Th1 cells efficiently induce cell-mediated responses including phagocytosis and cytotoxicity mainly through the action of IFN-γ and are active during bacterial and viral infections. Th2 cells, on the other hand, provide help for antibody production (mediated by IL-4) and enhance eosinophil proliferation and function (mediated by IL-5). Furthermore, Th2 cells control the eradication of extracellular helminthic pathogens (Abbas, 1996; O'Garra, 1998). The ratio of these Th cell subpopulations determines whether or not the immune system is able to respond appropriately to specific stimuli. Disturbance of the balance may result in various clinical manifestations. Excessive Th1 stimulation can result in enhanced rejection of grafts, inflammatory responses and some forms of auto-immunity (e.g. diabetes type I), whereas excessive Th2 activation can induce enhanced antibody synthesis, including IgE involved in allergic responses.

Table 1: Cell surface markers reported to be expressed specifically on Th1 or Th2 cells.

markers	Th1	Th2	reference
IFN-γR β chain	+	-	Groux, 1997
IL-12R β2 chain	+	-	Szabo, 1997
IL-18R	+	-	Xu, 1998
T1/ST2	-	+	Lohning, 1998
CCR5	+	-	Sallusto, 1998; Bonecchi, 1998
CXCR3	+	-	Sallusto, 1998; Bonecchi, 1998
CCR3	-	+	Sallusto, 1997
CCR4	-	+	D'Ambrosio, 1998
CCR8	-	+	Zingoni, 1998
CD30	-	+	Del Prete, 1995

The classification of Th cells into Th1 or Th2 cells is based on the detection of intracellular cytokines (IFN-γ and IL-4, respectively). Recently, a number of studies described the preferential association of particular cell surface molecules with Th1 or Th2 cells (see Table 1). These markers include cytokine receptors, as will be discussed below, and chemokine receptors. The differential expression of chemokine receptors affects the migration pattern of Th cells, and may selectively guide Th1 or Th2 cells to enter the appropriate sites of inflam-

mation (O'Garra, 1998; Annunziato, 1999; Zlotnik, 2000). Chemokines are also involved in the development of Th1 and Th2 cells. For instance, monocyte-chemoattractant protein-1 (MCP-1) gene targeted mice show an almost complete inhibition of Th2 responses (Gu, 2000). However, because of the tightly regulated expression of chemokine and cytokine receptors and the ability to respond to external factors (like cytokines), it is not clear whether these molecules are useful markers for discriminating Th1 and Th2 cells in a given immune response.

2.2. Polarization of the T helper cell phenotype

An important question is which events determine the induction of either a Th1 or a Th2 response. There is general agreement that this process is influenced by tightly regulated, multi-factorial events, such as the type of antigen, the antigen dose, the route of antigen entry as well as the genetic background of the host (Hsieh, 1995; Abbas, 1996). These factors influence the production of cytokines by the APC, which are now known to play a key role in determining the Th cell subset polarization into either Th1 or Th2 type responses (O'Garra, 1998).

IL-12 is a dominant factor promoting Th1 development. Professional APC, like dendritic cells (DC), macrophages and Langerhans cells (LC), are able to produce IL-12 immediately after recognition of specific antigens, particularly microbial antigens (Trinchieri, 1995). The bioactive form of IL-12 (p70) consists of the p40 and p35 subunits. p40 monomers and homodimers were found to inhibit the activity of IL-12 by blocking its receptor (Mattner, 1993). IL-12 is a potent inducer of IFN-γ production by natural killer (NK) cells and T cells (both CD4^{+} and CD8^{+}). On binding to its receptor, consisting of the IL-12R β1 and IL-12R β2 chains, IL-12 activates a.o. the signal transducer and activator of transcription-4 (STAT4). Binding of this transcription factor to the IFN-γ promoter induces the expression of IFN-γ. The crucial role for IL-12 in the development of Th1 responses was confirmed in gene targeted mice with disruptions in the IL-12p40, IL-12R β1 and STAT4 genes (Kaplan, 1996; Magram, 1996; Wu, 1997). Inhibitors of IL-12 induction have been reported, including retinoic acid, acetylsalicylic acid, 1,25-dihydroxyvitamin D3 and glucocorticoids (D'Ambrosio, 1998; Mazzeo, 1998; Vieira, 1998; Na, 1999). At least *in vitro*, the consequence of the reduction of IL-12 production is a reduced Th1 development.

IL-18 has been shown to act synergistically with IL-12 to induce the development of Th1 cells (Ahn, 1997; Robinson, 1997; Okamura, 1998). IL-18 gene targeted mice show strongly reduced IFN-γ production *in vitro* (Takeda, 1998). When T cells from IL-18^{-/-} mice are cultured in the presence of excess amounts of IL-12 *in vitro*, almost identical levels of IFN-γ are produced as compared to wildtype mice, showing that IL-12 is a central factor in Th1 cell development (Takeda, 1998). Besides its effect on Th1 development, IL-18 has a growth stimulatory effect on Th1 cells. Furthermore, IL-12 is able to upregulate the expression of the IL-18 receptor, thereby rendering these cells more permissive for the effects of

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IL-18. Polarized Th2 cells lack expression of the IL-18 receptor (Ahn, 1997).

IFN-γ has been shown to increase the IL-12 production by DC and macrophages, and it has been suggested that IFN-γ may enhance the expression of functional IL-12 receptors on naive T cells (Wenner, 1996). In addition, IFN-γ directly suppresses the proliferation of Th2 cells, but not of Th1 cells, thereby favoring the outgrowth of Th1 cells.

The role of IFN-γ in driving Th1 polarization is not entirely clear. *In vitro* studies have shown that addition of IFN-γ alone during priming of uncommitted, naive T cells is not sufficient for Th1 development. Besides, IFN-γ receptor knock-out mice are still able to elicit Th1 responses (Schijns, 1998). In addition, it has been reported that Th1 cells, in contrast to Th2 cells, are unable to respond to IFN-γ in the absence of the IFN-γ receptor β chain (Groux, 1997). This explains why IFN-γ inhibits the proliferation of Th2 cells, but not of Th1 cells.
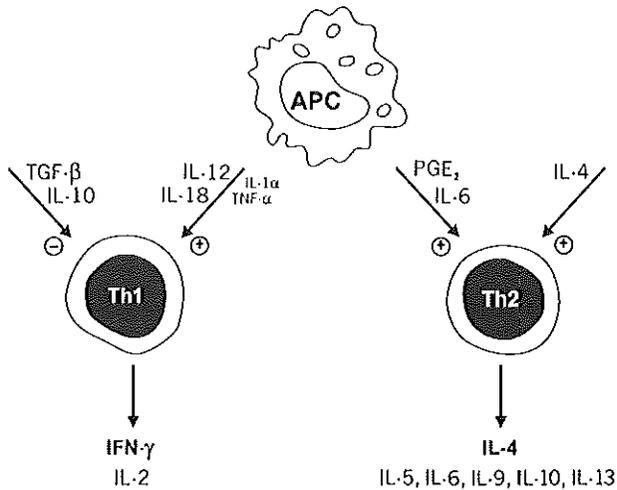

Figure 1: The regulation of the Th cell subsets, Th1 and Th2, by cytokines.

Polarization of Th2 cells is critically dependent on the presence of IL-4 (20). Ligand binding of the IL-4R activates STAT6. Both IL-4 and STAT6 gene targeted mice show defective Th2 responses (Kuhn, 1991; Kaplan, 1996). The source of the initial endogenous IL-4, which initiates the polarization of Th2 cells, is still a matter of debate. Candidate cells include naive T cells, NK1.1^{+} T cells and non-lymphoid cells like basophils and mast cells. Moreover, APC-derived IL-6 has been shown to induce the synthesis of small amounts of IL-4 in CD4^{+} T cells, which might trigger autocrine IL-4 production by these cells (Rincon, 1997). IL-4 functions as a growth factor for Th2 cells, thereby clonally expanding Th2 cells and consequently augmenting the IL-4 production. On the other hand, IL-4 inhibits the production of IFN-γ and IL-12. Besides IL-4, the APC-derived prostaglandin E_2 (PGE_2) and IL-10 strongly reduce the production of IL-12 by APC and consequently inhibit the Th1 development (Gately, 1998). Interestingly, IL-12 cannot inhibit priming for IL-4 production,

demonstrating a dominant effect of IL-4 on the phenotype of the induced immune response.

The existence of positive and negative feedback mechanisms suggests that once a Th1 or Th2-type immune response is established, this response is irreversibly committed to a particular cytokine profile. In several systems it has been shown that early in their development Th1 cells can be converted to IL-4 producers with IL-4, but that Th2 cells display a stable phenotype when cultured in the presence of IL-12 (Perez, 1995). By repeated *in vitro* stimulation, both Th1 and Th2 cells become irreversibly committed (Murphy, 1996). The inability of IL-12 to reverse an established Th2 phenotype into Th1 cells was suggested to be due to the differential expression of the IL-12R subunits. Polarized Th1 cells express both the IL-12R $\beta1$ and $\beta2$ chain, whereas committed Th2 cells lack expression of the IL-12R $\beta2$ chain, which is essential for IL-12 signaling (Rogge, 1997; Szabo, 1997). Recent studies, however, demonstrate that ectopic expression of the IL-12R $\beta2$ subunit in developing or committed Th2 cells did not reverse their phenotype into IFN-γ producing Th1 cells when cultured in the presence of IL-12 (Heath, 2000). This indicates that activation of IL-12 signaling does not switch off Th2 cell development. Interestingly, ectopic expression of STAT6 in developing Th1 cells was sufficient for the induction of Th2-specific cytokines as well as suppression of the production of IFN-γ (Kurata, 1999). However, introduction of the STAT6 construct into committed Th1 cells demonstrated a less efficient capacity to develop a Th2 phenotype.

It should be emphasized that these mechanisms were clarified *in vitro*. Under physiological conditions, it is likely that cytokines only transiently shift the balance along the Th1-Th2 axis, without permanently fixing the Th phenotype (Oriss, 1999). The extreme situation of a fixed Th1 or Th2 phenotype is only seen in severe pathological conditions.

Additional differences between Th1 and Th2 cells exist at the level of the transcriptional regulation of cytokine genes. Th2 cells specifically express the transcription factors c-Maf and GATA-3. c-Maf increases the expression of IL-4 (Ho, 1996), while GATA-3 can induce the expression of a broad spectrum of Th2 specific cytokines (Zhang, 1997) and inhibit the production of IFN-γ (Ferber, 1999). Recently, a Th1 specific transcription factor, T-bet, was isolated which induces the expression of IFN-γ, but also represses Th2 specific cytokines (Szabo, 2000). In addition, the Th specific Ets family transcription factor, ERM, was found to be induced by IL-12 and involved in the enhanced expression of IFN-γ (Ouyang, 1999). Furthermore, the class II MHC transactivator (CIITA) is selectively expressed in Th1 cells. CIITA represses IL-4, but has no direct effect on IFN-γ transactivation. In the presence of IL-12, CIITA$^{-/-}$ CD4^{+} cells produced not only IFN-γ but also IL-4 (Gourley, 1999).

2.3. Antigen presentation to T helper cells

Besides the crucial role of cytokines in directing the development of Th1 and Th2 responses, other factors have been implicated as well. A number of studies have shown that the dose of antigen as well as the strength of the interaction between the TCR and the MHC-

antigen complex, influences the Th cell development. Low antigen doses were found to favor Th1 responses, whereas high doses give rise to Th2 responses (Hosken, 1995). Furthermore, a high level of triggering of the TCR favored Th1 activation, whereas low levels resulted in Th2 activation (Constant, 1995; Iezzi, 1999). The mechanisms underlying the role of antigen dose and TCR triggering in the Th cell subset development are as yet unclear.

Other factors suggested to differentially influence Th cell development include co-stimulatory molecules such as B7-1, B7-2 and CD40 (Kuchroo, 1995). These molecules stabilize the physical interaction between the TCR and the MHC/antigen complex and deliver additional signals resulting in complete T cell activation. Both B7-1 (CD80) and B7-2 (CD86) are expressed by the APC and interact with the same ligand, CD28, on the T cell (Lenschow, 1996). Antibodies to B7-1 and B7-2 inhibited Th1 and Th2 responses, respectively. The mechanisms for these modulating effects on Th cell polarization are not clear. First of all, the level of B7-1 and B7-2 expression depends on the activation state of the APC; resting APC have high B7-2 expression, whereas B7-1 is increased after triggering (Lenschow, 1996). It is most likely that the difference in the affinity of B7-1 and B7-2 for CD28 results in a variable strength of co-stimulation. This suggests that the timing of expression and the strength of the co-stimulatory signal are more important than the presence or absence of a particular molecule.

Recent progress in the study on phenotypic and functional heterogeneity of macrophages and DC suggests the involvement of different DC subsets in the skewing of Th cells, both in mouse and human (Reid, 2000). In the mouse, lymphoid DC (CD8α^+DEC-205$^+$) facilitate a Th1 response, whereas myeloid DC (CD8α^-DEC205$^-$) facilitate a Th2 response (Maldonado, 1999). In support of this, it was shown that CD8α^+ DC produce IL-12, whereas CD8α^- DC do not. In humans, DC subsets with similar activity were identified, termed DC1 and DC2 (Rissoan, 1999).

2.4. The induction of tolerance by T helper cells

As outlined above, Th subset polarization is achieved by two basic principles. Firstly, each subset produces its own autocrine growth factors and, secondly, cytokines are produced that cross-regulate the outgrowth of the counter-acting subset. The activity of Th cells is influenced by yet another level of regulation, which main effect is to dampen and control ongoing Th cell responses by active suppression mediated by regulatory Th cells. These Th cells have been suggested to be involved in the pathogenesis of a number of (experimental) autoimmune diseases, like experimental autoimmune encephalomyelitis (EAE), diabetes in NOD mice, experimental inflammatory bowel disease and skin allograft rejection (O'Garra, 1997). Regulatory Th cells comprise classical Th2 cells, capable of inhibiting Th1 responses, but also alternative T cell populations (Mason, 1998). One of the primary mechanisms of action is via secretion of immunosuppressive cytokines like IL-10, IL-4 and transforming growth factor-β (TGF-β), which are potent inhibitors of Th1 driven responses.

Regulatory CD4$^+$ T cell clones, called Tr1 (human and murine), were obtained from *in vitro* culture of CD4$^+$ T cells in the presence of IL-10. These clones produced low levels of IL-2 and no IL-4, but high levels of IL-10 and TGF-β (Groux, 1997). When co-cultured with naive CD4$^+$ T cells, these antigen-specific clones suppressed the proliferative response to the same antigen. The Tr1 clones themselves exhibited a low proliferative capacity which was overcome by neutralization of IL-10 and TGF-β. Furthermore, *in vivo* injection of Tr1 cells was found to prevent the induction of inflammatory bowel disease (Groux, 1997; 1999).

TGF-β secreting regulatory T cells, termed Th3 cells, have been shown to be induced under some circumstances during oral tolerance. These Th3 cells secrete high levels of TGF-β but lower levels of IL-4 and IL-10, and were shown to protect mice from EAE (Chen, 1994). TGF-β-deficient mice suffer from inflammation that affects multiple organ systems, mainly the heart, liver and lungs (Shull, 1992; Gorelik, 2000), whereas IL-10 knock-out mice develop a spontaneous chronic enterocolitis (Kuhn, 1993). This demonstrates the importance of these cytokines in regulating and dampening the immune response.

Another Th cell subset which was found to act as suppressor cells *in vitro* and *in vivo* are the CD4$^+$CD25$^+$ cells comprising about 10% of CD4$^+$ cells in normal mice. These cells do not proliferate and display similar inhibitory effects as the Tr1 cells. Their suppressive activity is not mediated by cytokines, but requires cell-cell contact, which in an as yet undefined way results in inhibition (Thornton, 1998; Takahashi, 1998; Chai, 1999; Thornton, 2000; Seddon, 2000).

Besides unresponsiveness due to the active suppression by regulatory T cells, also passive mechanisms regulate immune responses, like clonal deletion and T cell anergy. The process of T cell anergy is best defined as a state of cellular unresponsiveness in which the cell is alive, but unable to perform functional responses upon stimulation. Anergic cells can be generated by antigen-specific T cell activation in the absence of co-stimulatory molecules. This partial activation signal renders these cells unable to proliferate and to secrete IL-2. The main contributors to the co-stimulatory signals are CD28 on the T cell and B7-1 and B7-2 on the APC. The interaction between these molecules enhances the transcription rate and stabilization of IL-2 mRNA and increases the sensitivity of TCR triggering. Another ligand for B7 molecules, CTLA-4, has been shown to negatively regulate T cell responses and to evoke a complete block in the cell cycle progression and IL-2 production. The importance of CTLA-4 triggering is evident in CTLA-4 gene targeted mice, which display a fatal lymphoproliferative disease (Waterhouse, 1995). CD28 expression on the T cell is constitutive, whereas CTLA-4 is induced early after T cell activation. The differences in affinities and binding kinetics with B7-1 and B7-2 determine whether or not a cell becomes activated or remains unresponsive.

Such passive and active mechanisms resulting in unresponsiveness are not mutually exclusive, but may act synergistically.

3. PHOTO-IMMUNOLOGY

UV radiation is defined as electromagnetic radiation with a wavelength ranging from 200 to 400 nm. As depicted in Figure 2, UV radiation is subdivided into UVC, UVB and UVA. The energy of a photon of UV radiation is inversely related to its wavelength ($E=hc/\lambda$). The energy-rich UVC photons (200-290 nm) react readily with stratospheric ozone and are therefore completely absorbed by the ozone layer, whereas UVA rays (320-400 nm) are not. Most of the UVB rays are filtered out in the stratosphere. However, since its photons are relatively energy-rich, solar UVB may reach the earth and is held responsible for the negative health effects of UV.
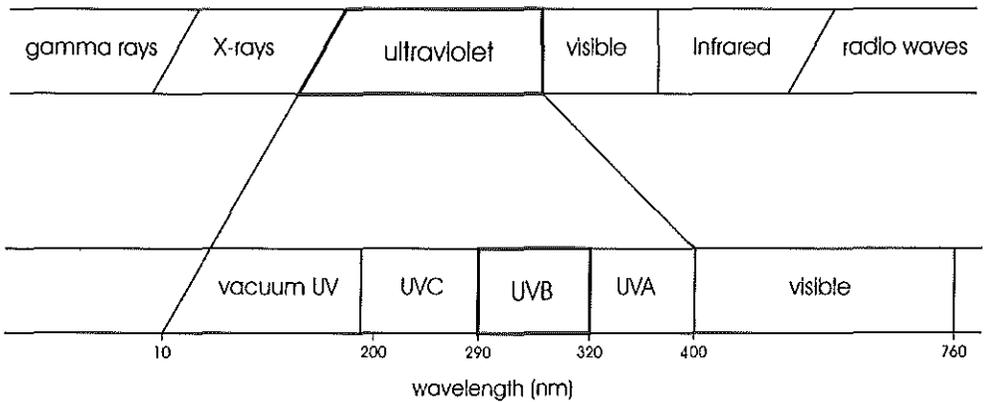

Figure 2: Schematic presentation of the classification of electromagnetic radiation and their corresponding wavelengths (in nanometers).

3.1. Photoreceptors in the skin

The energy load of the photons of UVB enables them to penetrate only the epidermis and the upper layers of the dermis. Within these layers the energy is absorbed by photoreceptors and transduced into biological effects. Wavelengths below 320 nm are not strongly absorbed by proteins and nucleic acids. Consequently, moderate doses of UVA have no adverse effects on the normal skin. Several candidate molecules have been proposed which absorb UVB light in the skin and subsequently modulate cellular responses. Of the candidate molecules urocanic acid (UCA) and DNA have received most attention.

UCA is a naturally occurring component of the superficial cornifying epidermis (Noonan, 1992). Upon absorption of UVB light, UCA isomerizes from its naturally occurring *trans* configuration to a *cis* isoform in the stratum corneum (Figure 3). Similar to UVB, intravenous (i.v.) injection of *cis*-UCA also suppressed the DTH and CHS responses (Ross, 1986; Kurimoto, 1992). Furthermore, pretreatment of mice with anti-*cis*-UCA antibodies followed

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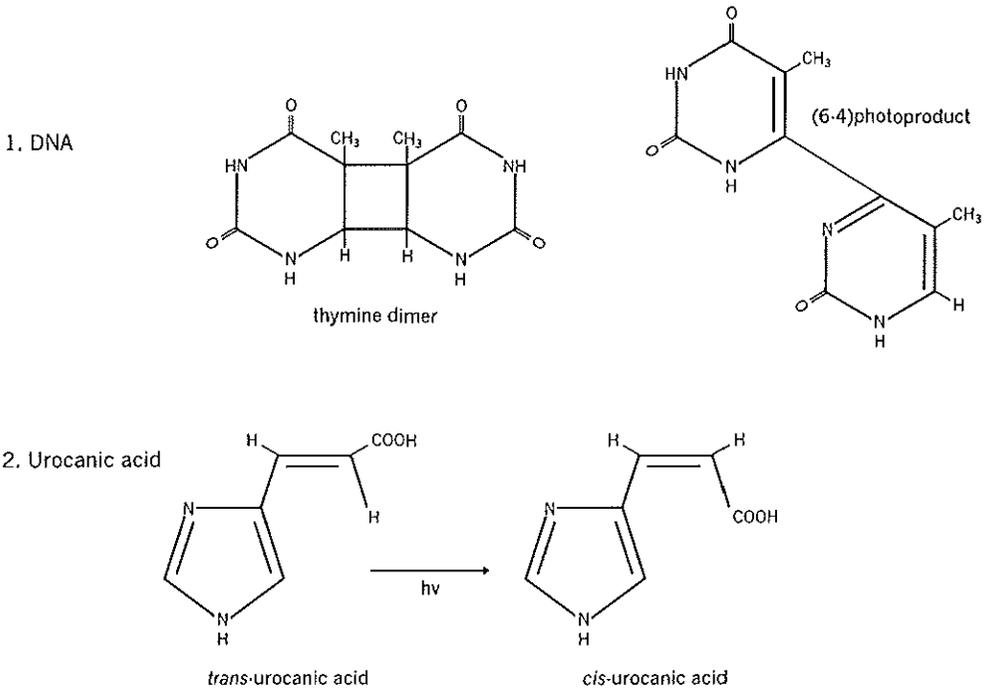
Figure 3: Photoproducts formed after UVB exposure of DNA molecules and trans-UCA.

by UVB irradiation did not result in suppression of DTH. However, the CHS response was suppressed, showing that *cis*-UCA did not play a role in the UVB-induced suppression of the CHS response (Moodycliffe, 1996). Interestingly, *in vitro* studies showed that *cis*-UCA enhances the production of IL-10 by splenic T cells (Holan, 1998). An effect on other cytokines (like IL-4 and IFN-γ) was not reported. The modulation of systemic responses by *cis*-UCA can be explained by its presence in serum and urine (Kanmeyer, 1997).

A second photoreceptor for UVB is DNA. UVB irradiation of cellular DNA has been found to generate a large variety of photoproducts (e.g. cyclobutyl pyrimidine dimers and 6-hydroxy-dihydropyrimidine) as well as DNA double-strand breaks. Formation of pyrimidine dimers occurs most frequently (Bykov, 1996; Figure 3). These mutagenic effects result in the transcriptional upregulation of numerous genes, including genes encoding proto-onco-genes (c-fos, c-jun) and cytokines. Indeed, enhanced production of TNF-α, IL-6 and IL-10 has been shown to be the result of the induction of DNA damage in murine skin *in vivo* and keratinocytes *in vitro* (Nishigori, 1996; O'Conner, 1996; Petit Frere, 1998).

The biological consequences of UVB-induced DNA damage are evident in patients suffering from the rare hereditary diseases xeroderma pigmentosum (XP) and Cockayne syndrome (CSA or CSB). XP patients are divided into eight different genetically defined subgroups (XP-A to XP-G and a variant) characterized by a specific defect in their nucleotide excision repair (NER) mechanism of DNA-repair (Chu, 1996; Vermeulen, 1997). XP-A is the

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most common form of these diseases. These patients are extremely sensitive to UV light and have a more than 1000-fold increased chance of developing skin cancer, and exhibit neurological abnormalities (Kraemer, 1997; Bootsma, 1998; Table 2). There is little information on the immune status of these patients, and due to the small study groups the data are conflicting. In recent years, gene targeted mice have been generated with similar genetic defects as the XP and CSB patients (de Vries, 1995; Cheo, 1997; van der Horst, 1997; de Boer, 1998). This makes it possible to study the link between UVB irradiation, DNA damage, DNA-repair and immune reactivity in more detail.

Table 2: Clinical features of human NER deficient syndromes.

	Xeroderma pigmentosum	Cockayne syndrome
Skin cancer	++	-
UV sensitivity	++	+
Neuronal degeneration	+	?
Neurodemyelination	-	+
Mental retardation	-	+
Dwarfism	-	+

3.2. UVB-induced immunosuppression and tolerance

The immunosuppressive effects of UVB have been extensively studied by measuring CHS and DTH responses. Sensitization to an antigen for inducing CHS is performed by applying the antigen epicutaneously, whereas DTH sensitization involves injection of the antigen either subcutaneously or intradermally. CHS is a T cell mediated response in the epidermis against a hapten coupled to cell membrane proteins. During sensitization of the skin, LC carrying the hapten migrate to the skin-draining lymph node (LN), where they activate hapten-specific T cells. Challenge with the hapten results in cutaneous inflammation by a.o. hapten primed T cells. These T cells mediate the CHS response by releasing IFN-γ and TNF-α. Both CD4$^+$ and CD8$^+$ T cells are implicated in the CHS response (Dilulio, 1996; Grabbe, 1996), whereas the DTH response is mainly dependent on CD4$^+$ cells (Cher, 1987).

Exposure to low doses of UVB, followed by application of the hapten on the irradiated skin, results in a markedly reduced CHS response upon challenge with the same hapten at an unexposed site one week later. This suggests that UVB induces local immunosuppression. Furthermore, when mice are exposed to UVB and sensitized with hapten on non-irradiated skin, subsequent challenge also reveals a reduced CHS response, which demonstrates that immune responses at non-exposed sites are also affected by UVB exposure. In addition, the transfer of purified CD4$^+$ T cells from UVB irradiated sensitized mice could pass on this reduction of the CHS response, showing that the reduced CHS response is mediated by CD4$^+$ T cells (Daynes, 1977; Ullrich, 1990; Shreedhar, 1998).

Different mice strains are not equally susceptible to UVB-induced immunosuppression. In order to obtain 50% suppression of the CHS response, BALB/c mice require a dose of UVB which is nearly six times higher than C57BL/6 mice (Noonan, 1990). The susceptibility is assumed to be a genetically determined trait, which is governed by polymorphic alleles at the *tnf-α* and *lps* loci. Resistance to the effects of UVB is a recessive trait and can be conferred by homozygosity at either the *tnf-α* or the *lps* locus (Streinlein, 1993). It has been proposed that polymorphisms in the non-coding sequences of the *tnf-α* locus result in distinct cytokine mRNA stabilization in UVB susceptible and resistant mice. Consequently, activation of keratinocytes of susceptible mice by UVB results in the generation of excessive amounts of intracutaneous TNF-α (Streinlein, 1994).

Although it is generally accepted that the UVB susceptibility differs between mouse strains, the classification depends on a number of experimental parameters, like the UVB dose, the concentration of the hapten and whether local or systemic immunosuppression is assessed. Comparison of BALB/c and C57BL/6 mice showed that the UVB dose response curves for local and systemic immunosuppression are identical (Noonan, 1990). The C3H/HeJ (carrying a defective *lps* gene) mouse was found to be resistant compared to its congenic strain C3H/HeN with respect to local UVB-induced suppression (Yoshikawa, 1990). In contrast, the systemic UVB immunosuppression was identical in both strains (Noonan, 1990). Moreover, it was found that when the concentration of hapten was reduced, the C3H/HeJ mouse was also sensitive to the effects of local UVB suppression (Yamawaki, 1997). These findings demonstrate that the classification of mouse strains with regard to UVB susceptibility may depend on the experimental set-up.

3.3. Local effects of UVB irradiation

Following exposure of the skin to UVB a local inflammatory response takes place. Erythema of the skin occurs within 24 hours and is caused by increased vasodilatation of dermal capillary blood vessels. The erythema response is used as a biological read-out of the local UVB-induced effects which differ with skin type. One minimal erythemal dose (MED) is the minimal amount of energy required to induce a uniform, demarcated erythema reaction at 24 hours. The UVB-induced inflammatory response is mediated by the release of a variety of factors which affect the activity of epidermal cells (keratinocytes, LC and melanocytes). Excessive UVB irradiation results in blister formation accompanied by high numbers of sunburn cells (i.e. apoptotic keratinocytes). Chronic irradiation eventually results in premature skin aging due to the alterations in the structure and composition of elastin and collagen in the dermal extracellular matrix (Fisher, 1997).

3.3.1. Effect of UVB on keratinocytes

Within hours after UVB exposure, keratinocytes are triggered to produce and/or release a plethora of cytokines. These factors mediate the local and systemic inflammatory reactions and modulate various immune responses. As shown in Table 3, UVB induces the transcription of a variety of keratinocyte-derived cytokines. Following UVB radiation,

Table 3: Modulation of the expression of various cytokines by keratinocytes after UVB irradiation.

cytokine	origin	effect	reference
IL-1α/ß	human	upregulation	Kupper, 1987
IL-1α	mouse	upregulation	Ansel, 1988
IL-1ra	human	upregulation	Hirao, 1996
EC-contra IL-1	mouse	upregulation	Schwartz, 1987
IL-3	human	upregulation	Danner, 1987
	mouse	upregulation	Gallo, 1991
IL-6	human	upregulation	Kirnbauer, 1991
IL-7	mouse	downregulation	Takashima, 1995
IL-8	human	upregulation	Kondo, 1993
IL-10	human	upregulation	Enk, 1995
		not detected	Teunissen, 1997
	mouse	upregulation	Rivas, 1992
IL-12	human	upregulation	Enk, 1996
IL-15	human	upregulation	Mohamadzadeh, 1995
IL-18	human	upregulation	Nakagawa, 1999
GM-CSF	human	upregulation	
	mouse		Gallo, 1991
TNF-α	human	upregulation	Kock, 1990
	mouse	upregulation	Simon, 1991
α-MSH	human	upregulation	Schauer, 1994
TGF-β1	human	upregulation	Lee, 1997
PGE$_2$	human	upregulation	Grewe, 1990
NGF	mouse	upregulation	Tron, 1990
IFN-α/β	human	upregulation	Fujisawa, 1997
ST2	human	upregulation	Kumar, 1997
CGRP	mouse	upregulation	Niizeki, 1997
bFGF	human	upregulation	Halaban, 1988

autocrine stimulation of keratinocytes by IL-1α results in an enhanced production of IL-1α, IL-6, TNF-α and GM-CSF. Some of the cytokines released upon UVB irradiation (IL-1, IL-6, TNF-α and IL-10) have been detected in serum and are thought to play a role in the UVB-induced suppression of systemic immunity (see below) (Ansel, 1983; Kock, 1990; Urbanski, 1990; Enk, 1993). Interestingly, upregulation of the IL-1 receptor antagonist and a 40-kD protein which inhibits the activity of IL-1 have been reported as well (Schwarz, 1987; Hirao, 1996). These anti-inflammatory factors are considered relevant mediators of immuno-suppression in the skin. UVB also affects the expression of IL-1 receptors type I and II (IL-1RI and IL-1RII) on human keratinocytes (Grewe, 1996). It was shown that within 1 hour after UVB exposure IL-1RII was upregulated, and returning to background levels within 24 hours, whereas the IL-1RI expression initially decreased and later increased. Since IL-1RII probably functions as a scavenger for IL-1, the resulting effect of UVB is an initial unre-sponsiveness to IL-1, followed by enhanced sensitivity of keratinocytes for IL-1. A novel member of the IL-1 family, ST1, was also upregulated in UVB irradiated keratinocytes (Kumar, 1997).

Murine keratinocytes upregulate the expression and produce IL-10 after UVB irradia-tion and are considered the major source of IL-10 in murine skin (Enk, 1995). In humans, however, keratinocytes do not appear to produce IL-10 and the main sources of IL-10 in UVB irradiated skin are melanocytes and infiltrating macrophages (Teunissen, 1997; Kang, 1998).

IL-7 is the only cytokine that is down-regulated after UVB irradiation. In normal skin, IL-7 is a growth factor for dendritic epidermal T cells (DETC) and has an anti-apoptotic activity. As a consequence of the down-regulation of IL-7 by UVB irradiation, the density of DETC in the skin is reduced due to enhanced apoptosis (Takashima, 1995). Interestingly, it was found that UVB suppressed the IFN-γ-induced IL-7 expression by reducing the tyrosine phosphorylation of STAT1 (but not STAT3), thereby reducing IRF-1 binding and conse-quently IL-7 expression (Aragane, 1997a; 1997b).

3.3.2. Effect of UVB on Langerhans cells

After UVB irradiation, the morphology of LC changes from a dendritic appearance into cells with a roundish shape. Furthermore, the LC loses characteristic markers (e.g. mem-brane ATPase) and are reduced in numbers in the epidermis either through apoptosis or enhanced migration to the draining LN of the skin (Toews, 1980; Spangrude, 1983; Moodycliffe, 1992). The migration of LC to the skin-draining LN is thought to be primarily mediated by TNF-α and IL-1β, both known to be produced by keratinocytes in the UVB irra-diated skin (Kimber, 2000). During their journey of the LC to the LN, the cells mature and develop into efficient APC. The reduced numbers of LC are the major reason for the unre-sponsiveness of the UVB exposed skin. The activity of LC is modulated by the autocrine effects of UVB-induced cytokines (including IL-1β, IL-6, IL-12), but also by the cytokines produced by keratinocytes and other epidermal cells, like melanocytes.

One of the UVB-induced cytokines that received much attention is TNF-α. It was shown that intradermal injection of low doses of TNF-α alters the function of LC, and impairs the CHS response (Yoshikawa, 1990). These effects are similar to those observed after UVB irradiation. Furthermore, in the local model for immunosuppression, UVB-induced CHS impairment could be reversed by anti-TNF-α mAb (Vermeer, 1990). This suggests that UVB exposure impairs CHS induction by a mechanism that requires TNF-α. In addition, using TNF-R-p55 and TNF-R-p75 knock-out mice, it was shown that the UVB-induced impairment of CHS acted via the TNF-R-p75 (Kurimoto, 1999).
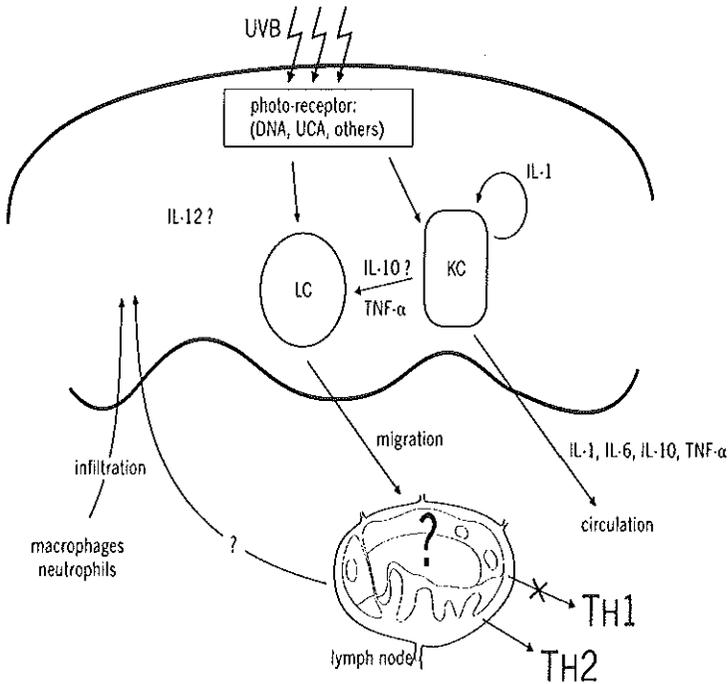

Figure 4: Simplified schematic presentation of local effects induced by UVB exposure. UVB is transduced into biological effects via absorption by DNA, urocanic acid (UCA) or others. This results in the release of various cytokines by keratinocytes (only IL-1, IL-10 and TNF-α are shown). These keratinocyte-derived cytokines may have local (autocrine or paracrine) or systemic effects (e.g. by means of circulating cytokines). LC migrate to the LN where they present antigens to Th2 cells. Subsequently, the skin becomes populated with infiltrating macrophages, neutrophils and possibly activated T lymphocytes. Whether IL-12 plays a role in this process is still unclear.

Recently, a number of studies have identified the dermal mast cell as the major source of UVB-induced TNF-α in the skin. It was shown that after UVB irradiation, calcitonin gene related peptide (CGRP) was released from cutaneous nerve endings which triggers mast cells to release TNF-α (Niizeki, 1997). Interestingly, an antagonist of CGRP abrogated the UVB-induced systemic immunosuppression (Garssen, 1998). In addition, in mast cell deficient mice no UVB-induced suppression of the CHS response was observed (Alard, 1999).

Mixed leukocyte reactions performed with UVB irradiated epidermal cells or spleno-cytes as stimulator cells are suppressed, which suggests an impairment of the antigen-pre-senting function of LC (Rattis, 1995). Since UVB as well as IL-10 have no effect on MHC II expression (Enk, 1993), much attention has focussed on the potential modulation of costim-ulatory signals. In this respect, two candidate molecules on the APC are ICAM-1 and B7. ICAM-1 expression on LC has been reported to be reduced following UVB, thereby reduc-ing the costimulation to the T cell via this pathway. With respect to B7, it was demonstrated that freshly isolated LC do not express B7-1 and B7-2 (Rattis, 1996; Weiss, 1995). Culture of LC upregulates the expression of B7 molecules, whereas UVB irradiation prevents the upreg-ulation of B7-1 and B7-2 (Weiss, 1995). Inhibition of mixed epidermal cell leukocyte responses in this system could be overcome by anti-CD28 mAb, indicating a role for B7 mol-ecules in the UVB-induced suppression of antigen-presenting functions. Interestingly, IL-10 reduces the expression of B7 on the APC (Ding, 1993), suggesting the involvement of IL-10 in the modulation of B7 expression on LC by UVB.

As a result of *in vitro* UVB treatment of murine LC, the capacity to activate Th1 cells was reduced. T cells isolated from the draining LN of such mice showed a decreased pro-duction of IFN-γ and an increased IL-4 production (Araneo, 1989). The inhibition of IFN-γ production was confirmed by Simon et al (Simon, 1991). They demonstrated that UVB irra-diated purified LC had lost the ability to present keyhole limpet hemocyanin (KLH) to Th1 cells, whereas the capacity to activate Th2 cells was not altered. The unresponsive Th1 cells were unable to produce IL-2 and to proliferate upon stimulation with non-irradiated APC, but they responded normally when exogenous IL-2 was added. This suggests that activation of Th cells by UVB irradiated LC results in clonal anergy of Th1 cells, rather than deletion.

3.3.3. UV induced recruitment of inflammatory cells

As a consequence of the inflammatory reaction in the UVB irradiated skin, LC are depleted from the skin, while granulocytes and macrophages repopulate the dermis and epi-dermis. Following UVB irradiation, ICAM-1 on keratinocytes and E-selectin on endothelium are upregulated with different kinetics (Norris, 1991). Upregulation of E-selectin on endothe-lium is generally considered an important early event in inflammation. In UVB injured skin this is followed by infiltration of neutrophils and macrophages (Kang, 1994). It is likely that also chemokines are involved in the inflammatory process in the UVB irradiated skin. However, apart from the UVB-induced upregulation of IL-8, no data are reported on the expression of chemokines. Recently, CXCR-2, a receptor for a.o. IL-8, was shown to be down-regulated on keratinocytes by UVB (Kondo, 2000). The functional significance of this finding is not clear yet.

The inflammatory process in the UVB exposed human skin is accompanied by the influx of monocytic/macrophagic cells (CD1a⁻, HLA-DR⁺, CD11b⁺CD36⁺) appearing in the dermis 6 hours after UVB exposure. These cells then express relatively high levels of IL-10,

and low levels of IL-12 (Kang, 1994; 1998), differentiate into macrophages, and migrate to the epidermis while still maintaining their IL-10^{high}/IL-12^{low} phenotype. The numbers of these cells in the epidermis peak at about 72 hours. The time course of the presence of this population corresponds to that of the local immunosuppression (Kang, 1998). One could speculate that this IL-10 producing population not only contributes to the immunosuppressive environment after UVB, but also to the resolution of the inflammatory response in the skin. In UVB exposed murine skin, a similar population of MHC II$^+$/CD11b$^+$ macrophages was observed (Hammerberg, 1994; 1996). The infiltrating macrophages are considered to prefer-entially expand suppressor-inducer CD4$^+$ T cells, which in turn activate suppressor CD8$^+$ T cells (Baadsgaard, 1990), thereby further contributing to the unresponsiveness of the UVB exposed skin. Following a single dose of UVB on the skin of human volunteers, the epider-mis showed increased numbers of non-activated memory CD4$^+$ and CD8$^+$ T cells 2 weeks after UVB exposure, demonstrating the long-lasting effects of UVB in the skin (di Nuzzo, 1998).

3.4. Systemic effects of UVB

UVB-induced systemic immunosuppression is thought to be mediated by soluble fac-tors which are produced in the UVB irradiated skin and released into the circulation. A num-ber of observations support this concept. First, i.v. injection of supernatant from UVB irradi-ated keratinocytes as well as plasma from irradiated mice suppressed the CHS and DTH response in mice (Schwarz, 1986; Kim, 1990). In addition, splenic adherent cells incubated *in vitro* with supernatant of UVB irradiated keratinocytes preferentially activate Th2 cells.

The systemic effects of UVB irradiation have been tested in a number of animal mod-els showing that UVB aggrevates the course of disease after infections, such as *Leishmania, Trichinella spiralis, Herpes simplex virus, Candida albicans, Mycobacterium bovis* and *Listeria* (Giannini, 1986; Hayashi, 1986; Denkins, 1989; Jeevan, 1990; Goettsch, 1994; 1996). Apart from the systemic UVB effects on T cell mediated immunity, important in the response against these infectious agents, also phagocytosis and NK cell activity are known to be affected by UVB exposure in both humans and animals (Jeevan, 1995; Yaron, 1995; Goettsch, 1996; Leino, 1999). Humoral immunity has been shown to be affected by UVB after infection of mice with *Borrelia burgdorfei* and *Herpes simplex virus*. In both cases a reduction of the Th1 associated specific IgG2a production was observed (Brown, 1995; El-Ghorr, 1998).

The mechanisms responsible for the UVB-induced systemic immunosuppression are not well understood, although much progress was made in recent years. First of all it was shown that IL-10 plays a central role in the systemic effects of UVB. For example, the sys-temic suppression of DTH by UVB can be rèversed by injecting anti-IL-10 antibodies (Rivas, 1994). Also, the UVB-induced reduction of the activation of Th1 cells by APC is restored by anti-IL-10 treatment of irradiated mice. Moreover, in IL-10 gene-targeted mice no suppres-

sion of DTH was observed following UVB irradiation, whereas a normal suppression of the CHS response was demonstrated (Beissert, 1996). This confirms the central role of IL-10 in the UVB-induced inhibition of the DTH, and also shows that the suppression of the DTH and CHS responses are mediated via different pathways.

Table 4: Survey of the literature summarizing the immune mediators demonstrated to be involved in the UVB-induced immunosuppression. *In vivo* treatment with neutralizing mAb or the use of particular gene targeted mice abrogated the suppressive effect of UVB on the CHS or DTH response. * only skin effects were observed.

treatment	reference
anti-IL-10	Rivas, 1994
anti-IL-4	Rivas, 1994
anti-TNF-α	Rivas, 1994
anti-CD86	Ullrich, 1998
anti-CD11b	Hammersberg, 1996
anti-*cis*-UCA	Moodycliffe, 1996
indomethacin	Chung, 1986
anti-C3b	Hammersberg, 1998
CGRP antagonist	Garssen, 1998
IL-6$^{-/-}$ mice*	Nishimura, 1999
CTLA-4 Ig Tg mice	Beissert, 1999
IL-10$^{-/-}$ mice	Beissert, 1996
IL-4$^{-/-}$ mice	Hart, 2000
TNF-R-p75$^{-/-}$ mice	Kurimoto, 1999
gld mice (FasL defect)	Hill, 1999

Recently, it was reported that UVB induces local immunosuppression of CHS responses in IL-4$^{-/-}$ mice, whereas systemic suppression of CHS and DTH was not observed (Hart, 2000). Shreedhar et al. showed that the systemic effects of UVB could be initiated by the release of PGE_2 by irradiated KC. This PGE_2 induced an increase of serum IL-4 and IL-10 levels (Shreedhar, 1998). The authors suggested that this cascade initiated in the skin is responsible for the systemic immune suppression. Treatment of mice with the PGE_2 inhibitor indomethacin as well as anti-IL-4 or anti-IL-10 mAb restored the suppressed DTH response.

In the systemic model, antigen-specific suppressor T cells were demonstrated in the LN and spleen, which could adoptively transfer the specific tolerance to syngeneic naive recipients (Elmets, 1983). This transfer of tolerance is mediated by the induction of antigen-specific CD3$^+$CD4$^+$CD8$^-$ suppressor cells (Ullrich, 1990). Yagi et al. reported on a suppressor T cell line isolated from UV irradiated mice, that secretes IL-4 and IL-10, but not IFN-γ. This clone therefore resembles a Th2 clone (Yagi, 1996). Shreedhar et al. cloned FITC-specific CD4$^+$ T cells from UVB irradiated mice (Shreedhar, 1998). These clones produced IL-10, but not IL-4 or IFN-γ. Injection of low numbers of these cloned T cells into untreated mice suppressed the induction of CHS against FITC.

As described before, IL-12 has also been recognized as a critical mediator in the cross-

regulation of Th1 and Th2 responses and to promote the development of Th1 cells. Injection of IL-12 in UVB irradiated mice was found to restore the suppressed systemic immune functions (Schmitt, 1995). This may be due to a blockade in the production of IL-10 or an increase of Th1 cells, thereby downregulating Th2 responses.

4. AIM AND OUTLINE OF THE THESIS

As described in the preceding paragraphs, the effects of UVB irradiation on the local immune responses involving keratinocytes and Langerhans cells have been extensively studied. Although it is known that UVB irradiation also results in systemic immunosuppression, the mechanisms are less well understood. Therefore, the aim of this thesis was to investigate the mechanisms that underlie the systemic effects of UVB exposure and the consequences for Th cell driven immune responses.

The first part of this thesis focuses on the UVB-induced modulation of cytokine production by various leukocyte subpopulations, especially CD4$^+$ T cells, and the consequences for Th1 and Th2 driven immune responses. In chapter 2 we investigate whether UVB exposure affects the development of CD4$^+$ T cells. In addition, the consequences for Th1 and Th2 associated immunoglubulin isotype production were studied *in vivo*. Chapter 3 describes the effect of UVB exposure on the cytokine production by splenic APC, and the consequences for CD4$^+$ and B cells responses. In chapter 4 the effects of UVB on systemic Th responses were investigated. In this chapter, we tested the hypothesis that UVB inhibits Th1 responses and stimulates Th2 responses by examining the effects in a mouse model for occupational asthma (Th1 dependent) and a model for allergic asthma (Th2 dependent).

Since different mouse strains are not equally susceptible to the effects of UVB exposure, the second part of this thesis compares the effects of UVB irradiation on cytokine production in various mouse strains with defined mutations in specific genes. In chapter 5 we determined the effects of a mutation of the *lps* gene on the susceptibility to UVB with respect to immunosuppression. These effects were examined in the systemic protocol for inhibition of the CHS response. In chapter 6, the consequences of mutations in genes encoding nucleotide excision repair proteins were investigated with regard to the UVB-induced immunomodulation. This was done by comparing the cytokine responses in three DNA-repair deficient mouse strains.

In chapter 7 our findings are discussed in the context of the present literature.

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Chapter 2

UVB irradiation suppresses the production of

immunoglobulin isotypes associated with Th1 and Th2 responses:

the involvement of CD4$^+$ T cells and IL-10

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SUMMARY

Ultraviolet B (UVB) irradiation suppresses the rejection of skin cancers and the resistance to infectious agents. It is generally accepted that UVB-induced systemic immune suppression is mediated by inhibition of Th1 responses. However, the effect on Th2 responses is not clear. Since Th2 cells provide help in B cell responses, we analyzed the effects of UVB irradiation on antibody formation after immunization with TNP-KLH. A suberythemal UVB dose inhibited the production of TNP-specific IgM, IgG2a, IgG1 and IgE in BALB/c mice. The inhibitory effect on IgG1 and IgE production suggests that UVB irradiation may also affect the Th2 type response. However, when studying the effect of *in vivo* UVB exposure, we found an increase in IL-4 production accompanied by a reduction in the production of IFN-γ by splenic CD4$^+$ T cells. This preferential development of Th2-like cells was confirmed by intracellular cytokine staining. Although the IL-10 levels secreted *in vitro* by CD4$^+$ T cells were not significantly increased after *in vivo* UVB irradiation, we did find an increase in the frequency of CD4$^+$ T cells producing IL-4 and IL-10 simultaneously, and of cells producing IL-10 only.

The finding that UVB irradiation inhibits not only the IgG2a, but also the IgG1 and IgE antibody levels after immunization, while it simultaneously promotes the development of Th2 cells, suggests the involvement of additional regulatory factors. Since IL-10 has been described to inhibit both Th1 and Th2 type responses, we examined its role in the UVB-induced suppression of antibody formation. Blocking the activity of IL-10 was able to reverse the UVB-induced suppression of TNP-specific IgG2a production and the contact hypersensitivity response, but was unable to restore the suppression of the Th2 associated isotypes IgG1 and IgE. Taken together, these data demonstrate that IL-10 is involved in the UVB-induced inhibition of Th1-like responses, such as the contact hypersensitivity and the IgG2a production. However, our findings that (1) UVB exposure results in enhanced development of Th2 cells and (2) neutralization of IL-10 can not overcome the UVB-induced suppression of IgG1 and IgE production suggest the involvement of additional factors that specifically inhibit Th2-like antibody responses.

INTRODUCTION

The immunosuppressive effect of ultraviolet B (UVB) irradiation has long been recognized [1]. Relatively low doses of UVB irradiation increase the incidence of skin tumors, but also lead to a higher susceptibility to infectious diseases, in both animal models and in humans [2, 3]. Sensitization to haptens through UVB exposed skin results in modulation of the antigen presenting cell (APC) function and the development of a systemically active, antigen-specific tolerance [4, 5].

The mechanisms involved in local immunosuppression in the UVB irradiated skin are

well documented [6-8]. Suberythemal doses of UVB induce the production of various cytokines by keratinocytes and inhibit the capacity of Langerhans cells to stimulate allogeneic T cells [9]. The effects of UVB on Langerhans cells are thought to be largely responsible for the reduction of delayed type hypersensitivity responses and the induction of antigen-specific tolerance as observed after UVB exposure.

The suppression of systemic immune responses by UVB is less well understood. A number of studies suggest that the functional activity of helper T (Th) cells may be involved in the UVB-induced systemic immune suppression [7, 10]. Based on their cytokine production profile, Th cells can be divided into Th1 and Th2 cells. The Th1 population produces interleukin (IL)-2, lymphotoxin (LT) and interferon-gamma (IFN-γ), whereas the Th2 cells produce IL-4, IL-5, IL-6 and IL-10 [11]. Araneo was the first to describe that T cells obtained from the lymph nodes of UVB irradiated mice showed a reduced production of IFN-γ and IL-2, and increased levels of IL-4 when stimulated in an antigen-specific fashion [12]. *In vitro* UVB irradiated APC showed reduced activation of Th1 clones [6]. Similar data were obtained by Ullrich using splenic adherent cells from irradiated mice as APC [13]. Furthermore, these authors demonstrated augmented IL-4 production by Th2 clones. Other studies using primary cells from the lymph nodes or the spleen of UVB irradiated mice showed a reduction of IFN-γ production with undetectable IL-4 [14], or a reduction of both the IFN-γ and IL-4 production by Th1 and Th2 cells after antigen-specific stimulation of lymph node cells [15]. Taken together, from these studies using Th cell clones and primary cell suspensions it is convincingly demonstrated that UVB irradiation results in reduced IFN-γ production. However, with regard to the effect of UVB irradiation on Th2 cell activity and IL-4 production the data are conflicting.

The cytokines produced by CD4$^+$ T cells regulate the process of immunoglobulin (Ig) isotype switching by murine B cells, in that IL-4 facilitates IgG1 and IgE production, whereas IFN-γ enhances the production of IgG2a [16]. Much research has focussed on the effect of UVB on cellular immunity, whereas little attention has been given to its modulatory effect on humoral immunity. Araneo et al. described the modulation by UVB of antibody responses after immunization with ovalbumin (OVA) [12] and found reduced production of all OVA-specific isotypes. Recently, the effect of UVB on *Herpes simplex virus* (HSV)-specific antibodies was studied [17]. Total HSV-specific IgG titers were not affected by UVB, while a decrease in IgG2a levels to HSV was observed. Treatment of rats with UVB prior to infection with *Trichinella spiralis* had no effect on specific IgM, IgG or IgE levels. However, when UVB was applied more than 16 days after infection a reduction of specific IgE was observed, whereas IgM and IgG levels were not affected [18]. In another study, UVB-induced suppression of primary specific IgG2a and IgG2b responses was observed after immunization with inactivated *Borrelia burgdorferi* [19]. However, no significant levels of specific IgE, IgG1, IgG3, IgA and IgM were detected. These studies demonstrate the inhibitory effect of UVB on Ig isotypes induced during a Th1 response, but do not provide conclusive information on the effect on Th2 associated isotypes.

Much research has focussed on the role of IL-10 in UVB-induced immunomodulation.

Murine keratinocytes produce IL-10 after UVB irradiation, as has been detected in serum of UVB irradiated mice [20]. In addition, injection of anti-IL-10 mAb in UVB irradiated mice restored the affected antigen presentation of spleen cells resulting in normal activation of Th1 clones [13]. Furthermore, anti-IL-10 mAb treatment reversed the UVB-induced suppression of delayed type hypersensitivity (DTH) responses [20, 21]. In addition, Yagi et al. reported cloning of a CD3$^+$CD4$^+$CD8$^-$ suppressor cell line isolated from the spleen of UV irradiated mice [22]. This cell line secreted IL-4 and IL-10, but not IL-2, IFN-γ or TGF-β. The authors suggested that the activity of this Th2-like clone could prevent *in vivo* Th1 activation, result-ing in suppression of delayed type hypersensitivity (DTH) responses by virtue of the produc-tion of the immunosuppressive cytokine IL-10. With respect to antibody responses, it was found that the administration of anti-IL-10 mAb *in vivo* blocked the UVB-induced suppres-sion of *Borrelia*-specific IgG2a and IgG2b antibody responses [19]. In summary, anti-IL-10 mAb treatment appears to reverse the inhibition induced by UVB on Th1 effector responses.

To resolve the conflicting data on UVB modulation of Th2 driven immune responses, we examined the effect of UVB exposure on the various Ig isotypes in order to ascertain whether its effects are reflective of Th1 or Th2 responses. Furthermore, we studied the effect of UVB on the capacity of CD4$^+$ T cells from UVB irradiated mice to differentiate into Th1 and Th2 cells. To determine the role of IL-10 in mediating the UVB-induced modulation of a generalized immune response, we examined the effect of neutralization of IL-10 in both a CHS and an antibody response. After immunization with a well-defined T cell dependent antigen (TNP-KLH), we found a reduced production of both Th1 (IgG2a) and Th2 (IgG1 and IgE) associated Ig isotypes, despite observations that CD4$^+$ T cells showed a highly Th2 polarized phenotype. Only the UVB-induced suppression of the IgG2a and the CHS response were restored by blocking the activity of IL-10, whereas levels of IgE were unaffected.

MATERIAL AND METHODS

Animals

Female BALB/c mice were bred at the animal facility at our department and kept under specific-pathogen-free conditions. Mice were at an age of 8-10 weeks at the start of the experiments. The experiments were approved by the Animal Experiments Committee of the Erasmus University Rotterdam.

mAb and other reagents

All cultures were performed in RPMI 1640 medium supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM pyruvate, 50 μM 2-ME and 10% heat inactivated FCS. All recombinant mouse cytokines used were obtained from R&D

Systems (Minneapolis, MN, USA). MAb used in the various assays were directed against CD3 (145.2C11), CD28 (37.51), MHC class II (M5/114), CD11b (M1/70), B220 (RA3.6B2), CD8 (YTS-169) and CD40 (FGK4.5). For the detection and neutralization of cytokines, mAb against IL-4 (11B11; BVD6.24G2), IFN-γ (XMG1.2; R46A2) and IL-10 (SXC-1; JES-2A5.1) were used. The anti-IL-12p40 clone C17.8 was a kind gift from Dr. G. Trinchieri, and the anti-IL-10R mAb (1B1.2) was a kind gift from Dr. A. O'Garra [30]. For FACS staining, anti-IL-4 PE, anti-IFN-γ FITC, anti-IL-10 FITC and the isotype control mAb GL113 PE and GL117 FITC were used (all obtained from PharMingen, San Diego, CA, USA). Picrylchloride (PCl) was used as a contact sensitizer (Chemotronix, Swannanoa, NC, USA) and was recrystallized three times from methanol/H_2O before use and protected from light during storage at 4°C.

UVB irradiation

One day prior to UVB irradiation, the dorsal hair of mice was shaved using electric clippers. Mice were exposed on 4 consecutive days to 1500 J/m^2/day UVB using two Philips TL-12 tubes (40W), which represents a suberythemal dose for BALB/c mice [31]. The tubes had a broad emission spectrum (280 - 350 nm) with a maximum emission at 306 nm. 60% of the energy emitted was within the UVB range (280 - 320 nm). The tube to target distance was 25 cm. Irradiation measurements were calibrated using a Waldmann UV meter (Waldmann, Schwenningen, Germany). Twenty-four hours after the last exposure mice were sacrificed. Control mice were not exposed to UVB.

Immunization of mice

KLH (Pierce, Rockford, IL, USA) was trinitrophenylated to a level of approximately 25 TNP residues per 100 kDa of KLH by using trinitrobenzenesulfonic acid (Sigma, St. Louis, MO, USA). Mice were i.p. injected 3 days after the last UVB treatment [32] with 10 μg TNP-KLH adsorbed on 2 mg alum, and bled at day 14 after immunization. As a T-cell independent antigen, DNP-Ficoll was used and prepared as described previously [33]. Mice were immunized i.p. with 50 μg DNP-Ficoll and bled at day 9 after immunization.

Preparation of spleen cells and purified CD4$^+$ cells

Spleens of control and UVB irradiated mice were removed under aseptic conditions and single cell suspensions were prepared. Purified CD4$^+$ T cells from the spleen were obtained by magnetic activated cell sorting with a cocktail of biotinylated mAb against CD11b, B220, CD8, MHC II and CD40, followed by incubation with streptavidin-conjugated microbeads (Milteny Biotech, Bergisch Gladbach, Germany). CD4$^+$ cells used for exper-

iments were always 90-95% pure as determined by flow cytometry.

Stimulation of CD4$^+$ T cells

CD4$^+$ cells were seeded at a concentration of 1 x 10^6 cells/ml and stimulated with plate-bound anti-CD3 (145-2C11; 25 µg/ml) in combination with soluble anti-CD28 mAb (37.51; 5 µg/ml). At 48h, the supernatants were harvested and the concentration of cytokines was determined by ELISA. Cultures were performed in triplicate or quadruplicate.

Th polarization assay

For primary stimulation, purified CD4$^+$ T cells were cultured at 5x10^5 cells/ml in flat-bottom plates (Nalge Nunc Int., Naperville, IL, USA), and stimulated with plate-bound anti-CD3 mAb (145-2C11; 25 µg/ml) in the presence of soluble anti-CD28 mAb (37.51; 5 µg/ml) and IL-2 (50 U/ml). For differentiation of Th1 cells, anti-IL-4 mAb (11B11; 10 µg/ml) and IL-12 (5 ng/ml) were added to the cultures. Differentiation of Th2 cells was with IL-4 (35 ng/ml), anti-IL-12 (C17.8; 10 µg/ml) and anti-IFN-γ mAb (XMG 1.2; 10 µg/ml) [34]. Neutral primed cultures contained only anti-CD3, anti-CD28 and IL-2. All doses were optimized in preliminary experiments. After 4 days of culture, the cells were washed 3 times and transferred to new anti-CD3-coated 96 well plates and restimulated in the presence of IL-2 (50 U/ml) and anti-CD28 (5 µg/ml). Forty-eight hours later, supernatants were collected and assayed for IL-4, IFN-γ and IL-10 production by ELISA as a read-out for Th1 versus Th2 polarization.

Intracellular cytokine staining

For intracellular cytokine staining, CD4$^+$ T cells were cultured as described above. On day 4 after primary stimulation the cells were transferred to a new plate and cultured for another 6 days in medium supplemented with IL-2 without anti-CD3/anti-CD28 mAb. On day 10, the cells were harvested and resuspended at a concentration of 10^6 cells/ml in RPMI medium supplemented with IL-2 (50 U/ml), PMA (1 µg/ml; Sigma) and ionomycin (2 µg/ml; Sigma) and incubated at 37°C. After 2h, Brefeldin A (10 µg/ml; Epicentre Tech, Madison, WI, USA) was added and the cells were incubated for another 2h. The cells were then fixed with 2% formaldehyde, permeabilized with 0.5% saponin and stained as described before [35]. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using CellQuest software (Becton Dickinson).

Cytokine ELISA

Flat bottom microplates (96-wells, Falcon 3912, Microtest II Flexible Assay Plate; Becton Dickinson, Oxnard, CA, USA) were coated with capture antibody diluted in PBS (1 µg/ml SXC-1; 5 µg/ml 11B11 or XMG1.2) at 4°C for 18h. After coating, plates were washed (PBS, 0.1% BSA, 0.05% Tween-20) and blocked with PBS supplemented with 1% BSA at room temperature for 1h. After washing, samples and standards were added and incubation was continued for at least 4 h at room temperature. Thereafter, plates were washed and biotinylated detection antibodies were added (0.1 µg/ml 2A5.1 or BVD6.24G2; 1 µg/ml R46A2) and incubated overnight at 4°C. After washing, streptavidin-peroxidase (1/1500 diluted, Jackson Immunoresearch, West Grove, PA, USA) was added. After 1h, the plates were washed and the reaction was visualized using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, 1 mg/ml, Sigma). Optical density was measured at 414 nm, using a Titertek Multiscan (Flow Labs, Redwood City, CA, USA). The detection limits of the various ELISA were for IL-4: 80 pg/ml; IFN-γ: 0.4 ng/ml; and IL-10: 80 pg/ml.

Antigen specific isotype ELISA

TNP-specific IgG1, IgE, IgG2a, IgA and IgM levels were measured by isotype specific ELISA as described previously [36]. As coating antibodies, goat-anti-mouse-IgM, -IgG1, -IgG2a (1 µg/ml; Southern Biotechnology, Birmingham, AL, USA), and the rat-anti-mouse-IgE mAb (2 µg/ml; clone EM95) were used. Serially diluted serum samples were incubated for at least 4 h. For determination of TNP-specific Ig levels, the plates were incubated with biotinylated TNP-OVA followed by peroxidase-coupled streptavidin (Jackson Immunoresearch), and the reaction was visualized using ABTS as described above. Total Ig levels were determined by ELISA as described before [37].

Contact hypersensitivity response

The mice were skin-sensitized 4 days after the last UVB irradiation by topical application of 150 ml of 5% picrylchloride (PCl) in ethanol/acetone (3:1) to the non-UVB-irradiated shaved abdomen, chest and feet. Control mice were sham sensitized by topical application of ethanol/acetone (3:1). Four days after the sensitization, both ears of the mice were challenged by topical application of one drop (27-gauge needle) of 0.8% PCl in olive oil. 24 hours after challenge, duplicate measurements of the ear thickness were made using an engineer's micrometer (Mitutoyo digimatic 293561, Veenendaal, the Netherlands).

Statistical analysis

Data were analyzed by Student's T test and differences were considered significant at $p < 0.05$.

RESULTS

Effect of UVB irradiation on *in vivo* Ig production and particularly on isotypes reflective of a Th1 or Th2 phenotype

To examine the effect of UVB on humoral immunity and to determine whether UVB irradiation results in a generalized Th1-Th2 switch, we immunized BALB/c mice with the T cell dependent antigen TNP-KLH. Mice were pre-exposed to UVB, immunized with TNP-KLH and at day 14 blood was collected. As shown in Figure 1A, UVB irradiated mice demonstrated significantly reduced TNP-specific IgG2a, IgG1, IgE and IgM responses. The total levels of the individual isotypes were not altered due to UVB treatment (data not shown).
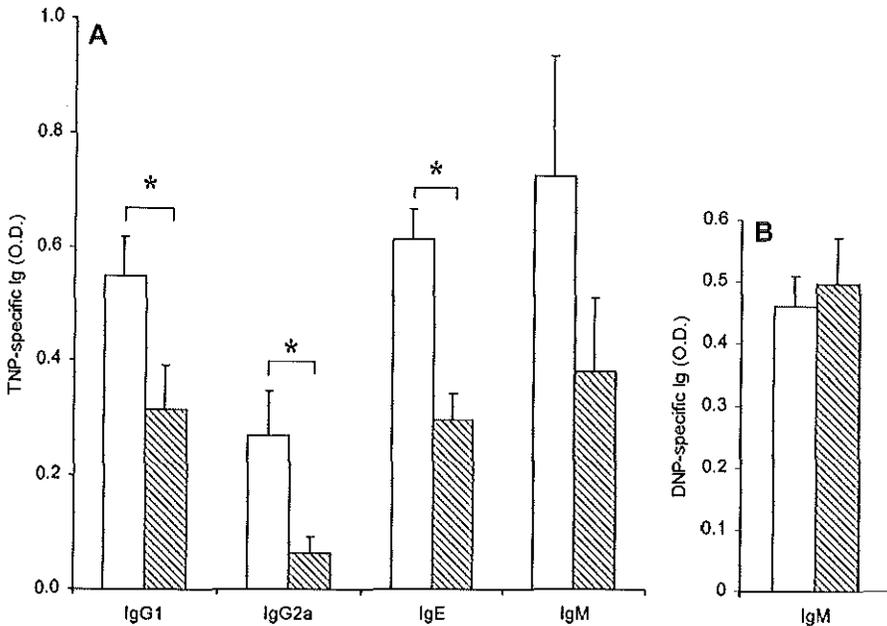
Figure 1: Effect of UVB irradiation on the antibody response to the T-cell dependent antigen TNP-KLH (panel A) and the T-cell independent antigen DNP-Ficoll (panel B). BALB/c mice were irradiated for 4 consecutive days (1500 J/m^2/day) and immunized 3 days later. Mice were immunized with 10 μg TNP-KLH adsorbed on alum and bled at day 14, or with 50 μg DNP-Ficoll and bled at day 9. Open bars represent control mice and hatched bars UVB irradiated mice. Results are expressed as OD414 values corrected for the blanc value. * indicates p<0.05.

To determine whether the UVB-induced reduction of antigen-specific antibody responses were caused by an effect at the B- and/or the T-cell level, we also evaluated the response against a T-cell independent antigen. To that end, we pretreated BALB/c mice with UVB followed by immunization with DNP-Ficoll. As depicted in Figure 1B, the levels of DNP-specific IgM antibodies in serum were not altered in UVB irradiated mice as compared to control (non irradiated) mice. DNP-specific IgG was not detected using this immunization protocol (data not shown). Thus, UVB irradiation causes a reduction of all isotypes produced in response to a T-cell dependent antigen. In contrast, UVB irradiation has no effect on the humoral response to a T-cell independent antigen. Together, these findings indicate that the effect of UVB irradiation at the humoral immune response is primarily via an effect at the T cell level, albeit in part via effects on the APC, resulting in a reduction of both Th1 and Th2 associated isotypes.

Effect of UVB irradiation on cytokine production by CD4$^+$ cells

CD4$^+$ cells mediate B cell help, in particular the regulation of isotype switching and Ig production, via the cytokines that they produce. The finding that UVB irradiation caused a reduction of all isotypes, rather than selected Ig isotypes, suggesed that *in vivo* a generalized reduction of Th activity was induced by UVB rather than a selective shift in the Th response. Therefore, we determined whether the intrinsic capacity of purified CD4$^+$ cells to produce IFN-γ, IL-4 and IL-10 was affected by *in vivo* UVB irradiation. For this purpose, purified splenic CD4$^+$ T cells obtained from control and UVB irradiated BALB/c mice were
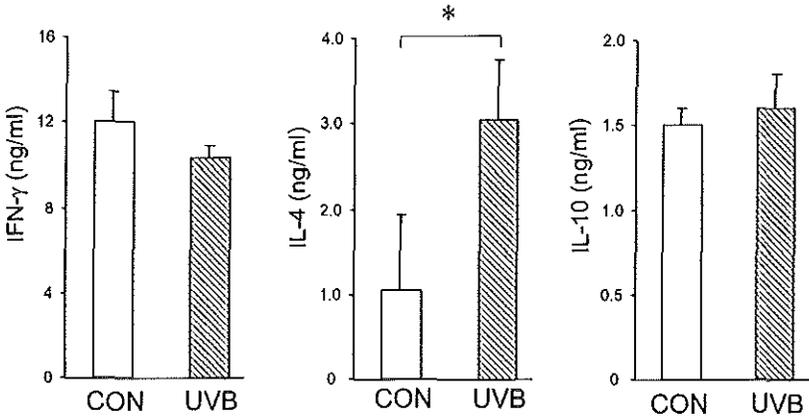
Figure 2: Effect of UVB irradiation on the cytokine production of stimulated CD4$^+$ T cells obtained from BALB/c mice. Mice were irradiated for 4 consecutive days (1500 J/m^2/day). On day 5, spleens were dissected and CD4$^+$ T cells were purified. CD4$^+$ T cells were stimulated with plate-bound anti-CD3 (25 μg/ml) and soluble anti-CD28 mAb (5 μg/ml). Supernatants were collected after 48h and analyzed for IL-4, IFN-γ and IL-10 levels by ELISA. Open bars represent control mice and hatched bars UVB irradiated mice. * indicates p<0.05.

polyclonally stimulated with anti-CD3 mAb in combination with anti-CD28 mAb. Supernatants were collected at 48h, and analyzed by ELISA for the cytokine levels. As shown in Figure 2, purified CD4$^+$ cells from UVB irradiated mice showed a significant increase in IL-4 production and a minor, but consistent reduction of the levels of IFN-γ as compared to control mice. The levels of IL-10 produced by CD4$^+$ cells were not altered after UVB treatment in these short-term cultures. No significant differences were observed in anti-CD3/anti-CD28 mAb-induced proliferation of CD4$^+$ cells from irradiated and control mice (data not shown). Taken together, these data suggest that UVB irradiation does not cause a generalized suppression of Th activity, but rather a stimulation of the IL-4 production coinciding with a slight reduction of the IFN-γ production.

In order to accentuate the differential cytokine production by CD4$^+$ cells obtained from control and UVB irradiated mice, we cultured purified CD4$^+$ T cells in an APC independent system for 4 days. Splenic CD4$^+$ cells were stimulated with anti-CD3 and anti-CD28 mAb. After 4 days of culture, the cells were restimulated for 48h, the supernatants were harvested and analyzed for IFN-γ, IL-4 and IL-10 levels by ELISA. Control cultures were included containing IL-4 and anti-IFN-γ/anti-IL-12 (Th2-skewing) or IL-12 and anti-IL-4 (Th1-skewing). All cultures were supplemented with IL-2 to ensure cell viability and proliferation. The result of a representative experiment using CD4$^+$ T cells obtained from UVB irradiated and control BALB/c mice is shown in Figure 3. Under neutral priming conditions, CD4$^+$ cells from control mice produced both IL-4 and IFN-γ upon restimulation. After *in vivo* UVB treat-
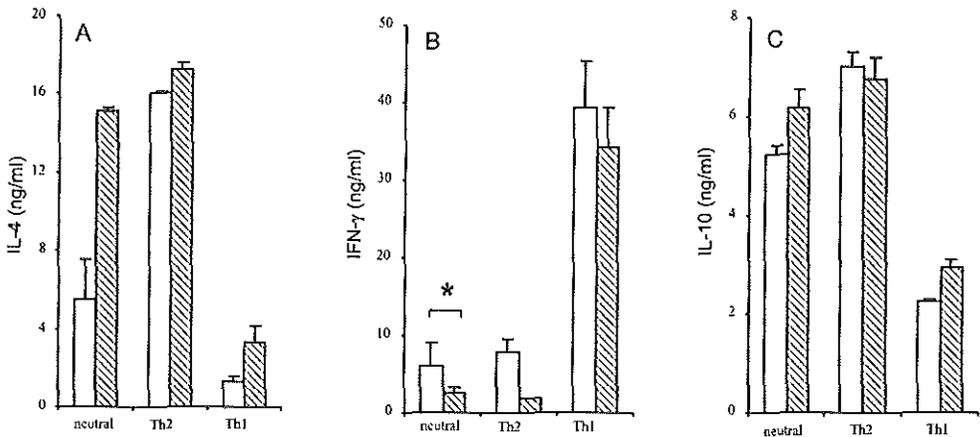

Figure 3: Effect of UVB irradiation on the development of CD4$^+$ cells into Th1 or Th2 effector cells. BALB/c mice were irradiated for 4 consecutive days (1500 J/m^2/day). On day 5, spleens were dissected and CD4$^+$ T cells purified. CD4$^+$ T cells were stimulated with anti-CD3/anti-CD28 and IL-2. During the primary stimulation, the conditions of the cultures were as follows: no additions, or IL-4 plus anti-IFN-γ (Th1-skewing), or IL-12 plus anti-IL-4 mAb (Th2-skewing). After 4 days of culture, the cells were harvested, washed and restimulated with only anti-CD3/anti-CD28 mAb and IL-2. Supernatants were collected after 48h and analyzed for IL-4 (panel A), IFN-γ (panel B) and IL-10 (panel C) levels by ELISA. Open bars represent control mice and hatched bars UVB irradiated mice. * indicates $p<0.05$.

ment, cultured CD4$^+$ cells produced more IL-4 (panel A), whereas the production of IFN-γ was significantly reduced (panel B). Interestingly, the levels of IL-4 produced by CD4$^+$ T cells from UVB irradiated mice cultured under neutral conditions were as high as the levels of IL-4 production seen when CD4$^+$ T cells from UVB irradiated or control mice were cultured *in vitro* under Th2 polarizing conditions. Priming for the development of IFN-γ producing cells by IL-12 and anti-IL-4 mAb (Th1 conditions) could overcome much of the effect of UVB exposure, although a small reduction of IFN-γ production was still reproducibly seen after restimulation when comparing the UVB treated group with the control group. Taken together, these findings suggest that UVB irradiation induces the development of Th2 cells in BALB/c mice to its maximal potential as reflected by the levels of IL-4. However, under neutral conditions, the production of IL-10 was slightly, but consistently, increased in the UVB irradiated group as compared to the control group (panel C). Under Th2 skewing conditions, no differences were observed between both experimental groups.

Altered cytokine profile of CD4$^+$ T cells at the single cell level in UVB irradiated mice

Having demonstrated that UVB irradiation affects the development of CD4$^+$ T cells, as reflected by their cytokine production at the population level, we examined the phenotype of these cells at the cellular level by intracellular cytokine staining. Figure 4 shows the results of two-color staining of purified CD4$^+$ cells from UVB irradiated and control BALB/c mice stimulated with anti-CD3/anti-CD28 mAb and cultured for 10 days under neutral, or Th1- or Th2-polarizing culture conditions to ensure significant detectibility. Intracellular staining for IL-4 and IFN-γ in CD4$^+$ cells from control mice cultured under neutral conditions resulted in a population in which 6.5% of the cells synthesized IL-4, but few cells produced IFN-γ ($<1\%$).

Treatment of mice with UVB irradiation resulted in an increase of IL-4 producing CD4$^+$ cells from 6.5% to 16.8% when cultured under neutral conditions, whereas no change in the percentage of IFN-γ producing cells was observed after UVB irradiation (panel A). Under neutral conditions, the frequency of IL-10 producing cells increased from 20.5% in the control group to 35.1% in the UVB irradiated group (panel B).

The percentage of the IL-4 producing CD4$^+$ cells from UVB irradiated mice cultured under neutral conditions was almost as high as the percentage of IL-4 producing cells seen when CD4$^+$ cells from control irradiated mice were cultured *in vitro* under Th2-promoting conditions. Culture of CD4$^+$ cells from UVB irradiated mice under Th2-promoting conditions increased the percentage of IL-4 producing cells even further. Identical effects were observed for the effects of UVB irradiation on IL-10 producing cells in these polarizing cultures. Although few IFN-γ producing cells were observed under neutral conditions, the percentage was increased under Th1-promoting culture conditions, and this showed no consistent differences when comparing the control and the UVB irradiated group.

The two color staining for IL-4 and IL-10 showed an increase after UVB irradiation
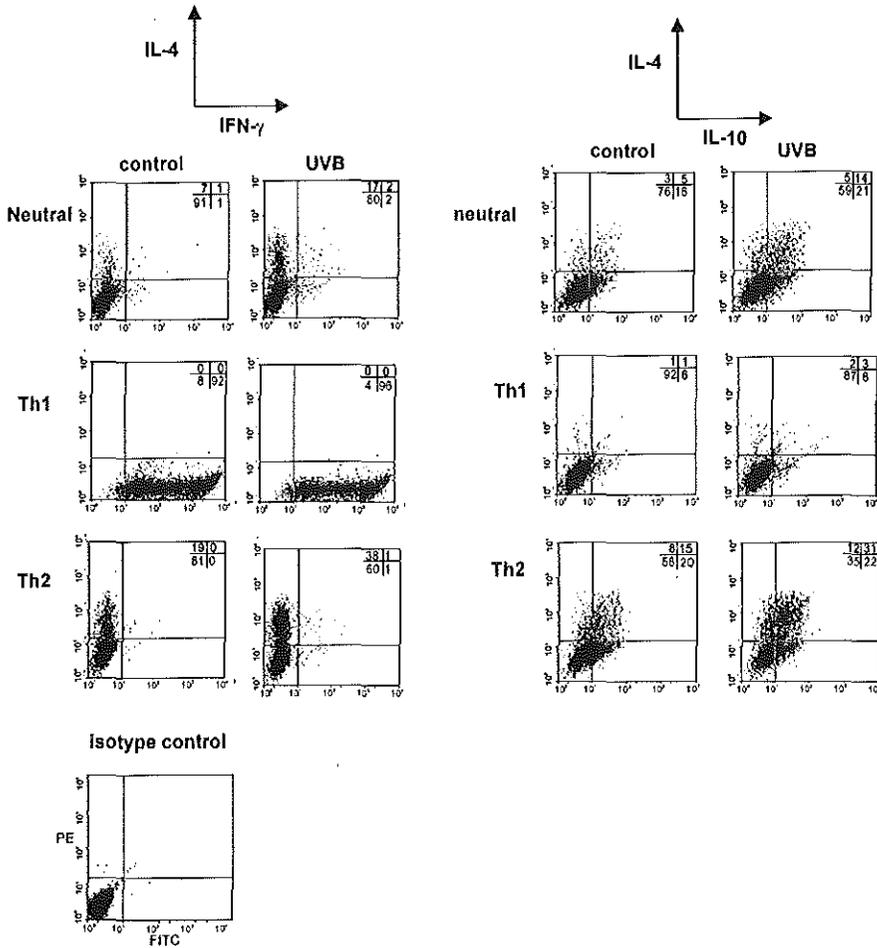
Figure 4: Effect of UVB irradiation on the cytokine profile of CD4^{+} cells at the single cell level. CD4^{+} cells obtained from UVB irradiated and control BALB/c mice were stimulated as described in the legend of Figure 3. However, on day 4 of culture, the cells were transferred to a new plate and cultured for another 6 days in medium supplemented with IL-2 in the absence of anti-CD3/anti-CD28 mAb. Cells were diluted every 2 days to ensure cell viability. On day 10, cells were restimulated with PMA, ionomycin and IL-2 for 4h, with brefeldin A added for the last 2h. Intracellular synthesis of IFN-γ, IL-4 and IL-10 was determined by flow cytometry. The left panel shows the anti-IL-4 PE/anti-IFN-γ FITC staining. The right panel shows the anti-IL-4 PE/anti-IL-10 FITC staining.

of the percentage of IL-4/IL-10 double positive cells as well as of the IL-10 single positive cells when cultured under neutral conditions. As expected, under Th1 priming conditions the number of positive cells for IL-4 and IL-10 was low. Under Th2-priming conditions, the percentage of IL-10 positive cells increased from 34.8% in the control group to 52.6% in the UVB irradiated group.

In conclusion, these experiments at the cellular level also demonstrate that UVB irradiation induces a major stimulation of Th2 development. Although no differences in IL-10 production were detected, we did show an increase of the percentage of IL-10 producing cells after UVB irradiation, which could be due to the prolonged culture time or increased sensitivity by FACS.

Effect of neutralization of IL-10 on the modulation of *in vivo* immune responses by UVB irradiation

The increased number of IL-10 producing splenic CD4$^+$ cells after UVB irradiation prompted us to investigate the involvement of IL-10 in UVB-induced changes on *in vivo* responses. First, we tested this in a contact hypersensitivity (CHS) response. The results of a representative experiment are depicted in Figure 5. Each bar represents the net antigen-specific ear swelling response (i.e. ear swelling in the sensitized mice minus ear swelling in the non-sensitized mice). Using our experimental procedure we found a significant reduction of the ear swelling in UVB irradiated as compared to non-irradiated mice. In UVB irradiated mice the CHS response was suppressed to 13% of the response in non-irradiated mice. Treatment of non-UVB-irradiated mice with anti-IL-10 mAb suppressed the ear swelling response as compared to the sham-injected or isotype matched mAb-injected mice, demon-
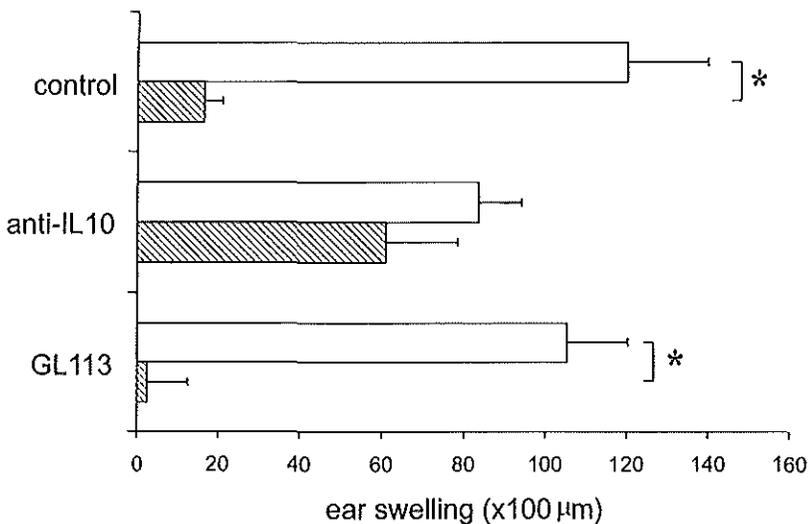
Figure 5: Effect of anti-IL-10 mAb treatment on the UVB-induced systemic suppression of the CHS response. BALB/c mice were injected i.p. with 1 mg anti-IL-10 mAb (2A5.1), isotype control (GL113) or PBS on day -16 and day -10. Mice were exposed to UVB from day -8 to -4. Sensitization to picrylchloride of the chest, abdomen and footpads was at day 0. Four days after the sensitization, the ears of the mice were challenged and 24h later the ear thickness was measured. Open bars represent control mice and hatched bars UVB irradiated mice. * indicates p<0.05.
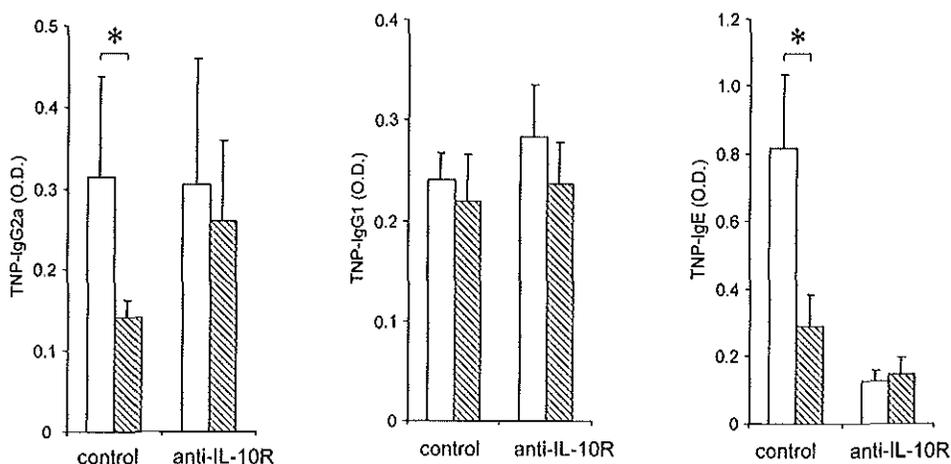
Figure 6: Effect of neutralization of IL-10R on UVB-induced modulation of antibody responses. On day -9, BALB/c mice were i.p. injected with 1 mg anti-IL-10R mAb or isotype control. From day -8 to -4 mice were UVB irradiated. On day 0, 10 µg TNP-KLH was injected i.p. adsorbed on alum, and 14 days later blood was collected, and serum was analyzed for TNP-specific Ig production by ELISA. Open bars represent control mice and hatched bars UVB irradiated mice. * indicates p<0.05.

strating a regulatory role for IL-10 in the CHS response. Injection of the UVB irradiated mice with anti-IL-10 mAb reversed the induction of UVB-induced suppression of the CHS response to almost that of control mice.

Furthermore, we were interested to determine whether IL-10 also played a role in the observed suppression of Th1 and Th2 associated Ig isotype responses as observed after UVB irradiation. For prolonged *in vivo* neutralization of the IL-10 responses, we chose to treat the mice with anti-IL-10R mAb which may have greater neutralizing capacity (A. O'Garra, personal communication). We treated BALB/c mice with anti-IL-10R mAb, followed by exposure to UVB and subsequently immunized the mice with TNP-KLH. As shown in Figure 6, the reduction of TNP-specific IgG2a levels in UVB irradiated mice could be overcome by blocking of the IL-10R. TNP-specific IgG1 and IgE levels were reduced by UVB exposure. No effect of anti-IL-10R treatment was observed on TNP-specific IgG1 levels, whereas anti-IL-10R treatment suppressed the specific IgE levels in both experimental groups.

Taken together, these data demonstrate that neutralization of IL-10 can overcome the UVB-induced inhibition of Th1 like responses (CHS and IgG2a formation), whereas it is not involved in the UVB-induced suppression of the production of the Th2 associated Ig isotypes IgG1 and IgE.

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DISCUSSION

It is generally accepted that UVB irradiation inhibits Th1 responses which is most convincingly shown by the suppression of DTH and CHS responses accompanied by a reduced production of IFN-γ [15, 21, 23]. A number of studies using bacterial or viral antigens have previously reported the UVB-induced reduction of IgG2a levels. However, these antigens were unable to evoke significant production of IgG1 and IgE as would be compatible with a Th1-Th2 shift induced by UVB irradiation [17, 19]. To determine whether UVB irradiation affects both Th1 and Th2 responses, it is important to measure IgE, as this isotype is considered to be the hallmark for Th2 associated antibody responses [24]. In our study we therefore immunized mice with a well-studied antigen, TNP-KLH, which is known to induce a Th0 like immune response accompanied by isotype switching into IgG2a as well as IgG1 and IgE [25]. We demonstrated that UVB irradiation reduces the production of both the Th1 (IgG2a) and the Th2 (IgG1 and IgE) associated antigen-specific isotypes. Since in UVB irradiated mice, immunization with the T-cell independent antigen DNP-Ficoll did not reveal reduced IgM antibody production, it is likely that the UVB effect on T cell dependent humoral immunity is an indirect effect mediated by modulation of T cell activity, rather than a direct effect on B cell activity.

The finding that UVB irradiation reduced the production of IgG2a as well as IgG1 and IgE antibodies suggested a generalized reduction of Th activity rather than a preferential activation of Th1 or Th2 cells. In order to examine this, we determined the production of Th1 and Th2 specific cytokines by freshly isolated CD4^{+} T cells and by CD4^{+} T cells cultured for 4 days to accentuate any small differences. The cytokine profiles, reflective of the effects of UVB irradiation *in vivo* versus controls, were also compared to that observed when CD4^{+} T cells from control or UVB irradiated mice were cultured under Th1 or Th2 polarizing conditions. We found that the IL-4 production was significantly increased, whereas the IFN-γ production was consistently reduced after UVB exposure *in vivo*. We showed previously that UVB irradiation inhibits the *in vivo* production of IL-12p70 (submitted). Others showed that injection of IL-12 was able to overcome UVB-induced systemic immune suppression [26]. Our findings expand these data by showing augmented differentiation into Th2 cells comparable to the levels when T cells are driven with IL-4, and the inhibition of Th1 cells after UVB irradiation, which we show is also overcome *in vitro* by culture with IL-12.

With respect to the production IL-10 no significant changes were observed, although there was a trend to increased IL-10 production by CD4^{+} T cells from UVB irradiated mice. Interestingly, the frequency of IL-10 producing cells was dramatically increased. The discrepancy between the IL-10 levels and the number of IL-10 producing cells can be explained by the increased sensitivity of flow cytometry or the longer culture period required for the intracellular cytokine staining. By performing two color staining for IL-4 and IL-10 we found that UVB irradiation increased the population that simultaneously produced IL-4 and IL-10 (the classical Th2 cell) as well as a population that produced IL-10 only. So far, two groups have reported on the cloning of putative UVB-induced suppressor T lymphocytes. Yagi et al.

cloned an antigen-specific T cell line from the spleen of UVB irradiated mice by *in vitro* stimulation in the presence of IL-4 [22]. This clone produced both IL-4 and IL-10, but not IL-2, IFN-γ or TGF-β. More recently, Shreedhar et al. reported on the cloning of a suppressor CD4+ T cell line from the lymph nodes of irradiated mice, which produced IL-10 but not IL-4 or IFN-γ and suppressed the CHS response *in vivo* [5]. The increased frequency of single IL-10 producing cells in our primary cultures corresponds to the phenotype of this suppressor T cell and to the phenotype of the regulatory Tr1 cells described by Groux et al [27]. By assessing the polarization of CD4+ cells from SJL and C3H/HeJ mice exposed to UVB we found that the results obtained in this study are applicable to other mouse strains as well (data not shown). These findings suggest that genetic differences determining the Th differentiation capacity of mice do not affect the capacity of UVB to induce Th2 polarization of CD4+ T cells selectively.

The UVB-induced shift of the Th cells into a highly Th2 polarized population could explain the suppression of IgG2a production, but not of the reduced IgG1 and IgE responses. Apparently, another factor is involved in the inhibition of Th2 driven antibody responses. Since IL-10 can in some cases inhibit both Th1 and Th2 responses [28], we examined the involvement of IL-10 in the UVB-induced modulation of immune responses. In the Th1 driven CHS response we found that the reduced ear swelling by UVB exposure could be reversed by neutralization of IL-10, suggesting that UVB-induced IL-10 was indeed able to inhibit Th1 driven responses. These data appear to be in conflict with studies performed by Rivas et al., who showed that neutralization of IL-10 did not reverse the suppression of the CHS response but only of the DTH response [20]. On the other hand, the data are in agreement with studies performed by Enk et al., who showed that administration of IL-10 can suppress the induction of CHS [29]. The differences between our findings and the study of Rivas et al. may be due to the different mouse strains used (BALB/c versus C3H/HeN mice) and the different UVB doses. Furthermore, we used two i.p. injections of 1 mg anti-IL-10 mAb administered 8 days and 2 days before exposure to UVB to ensure that all biologically active IL-10 was neutralized. Thus, additional technical differences possibly explain the conflicting results.

Finally, we examined whether UVB-induced IL-10 was also involved in the observed reduced production of the Th1 and Th2 associated Ig isotypes. Blockade of the IL-10R could overcome the UVB-induced suppression of TNP-specific IgG2a production. IL-10 did not overcome the UVB-induced suppression of the IgG1 and the IgE responses, but actually reduced the IgE response. This could result indirectly by enhancement of the Th1 response which is known to inhibit IgE. This indicates that UVB-induced IL-10 production suppresses only the Th1 driven antibody response, whereas it is not involved in the inhibition of the production of Th2 associated isotypes. At this stage, we can only speculate on the mechanisms resulting in the reduction of IgG1 and IgE production after UVB irradiation. One possible candidate cytokine involved is TGF-β. Yet, because of the complex regulation of the production and activity of TGF-β, neutralization studies *in vivo* are necessary to further evaluate the possible involvement of TGF-β in UVB-induced immunomodulation.

In conclusion, this study demonstrates that UVB irradiation inhibits the production of

both Th1 and Th2 associated Ig isotypes. Since the CD4$^+$ T cell population displays a highly Th2 polarized phenotype, it is unlikely that the classical Th2 cells are involved. We found that neutralization of IL-10 is very efficient in reversing the UVB-induced inhibition of the Th1 driven response, whereas it could not overcome the effect on the suppressed Th2 response.

ACKNOWLEDGEMENTS

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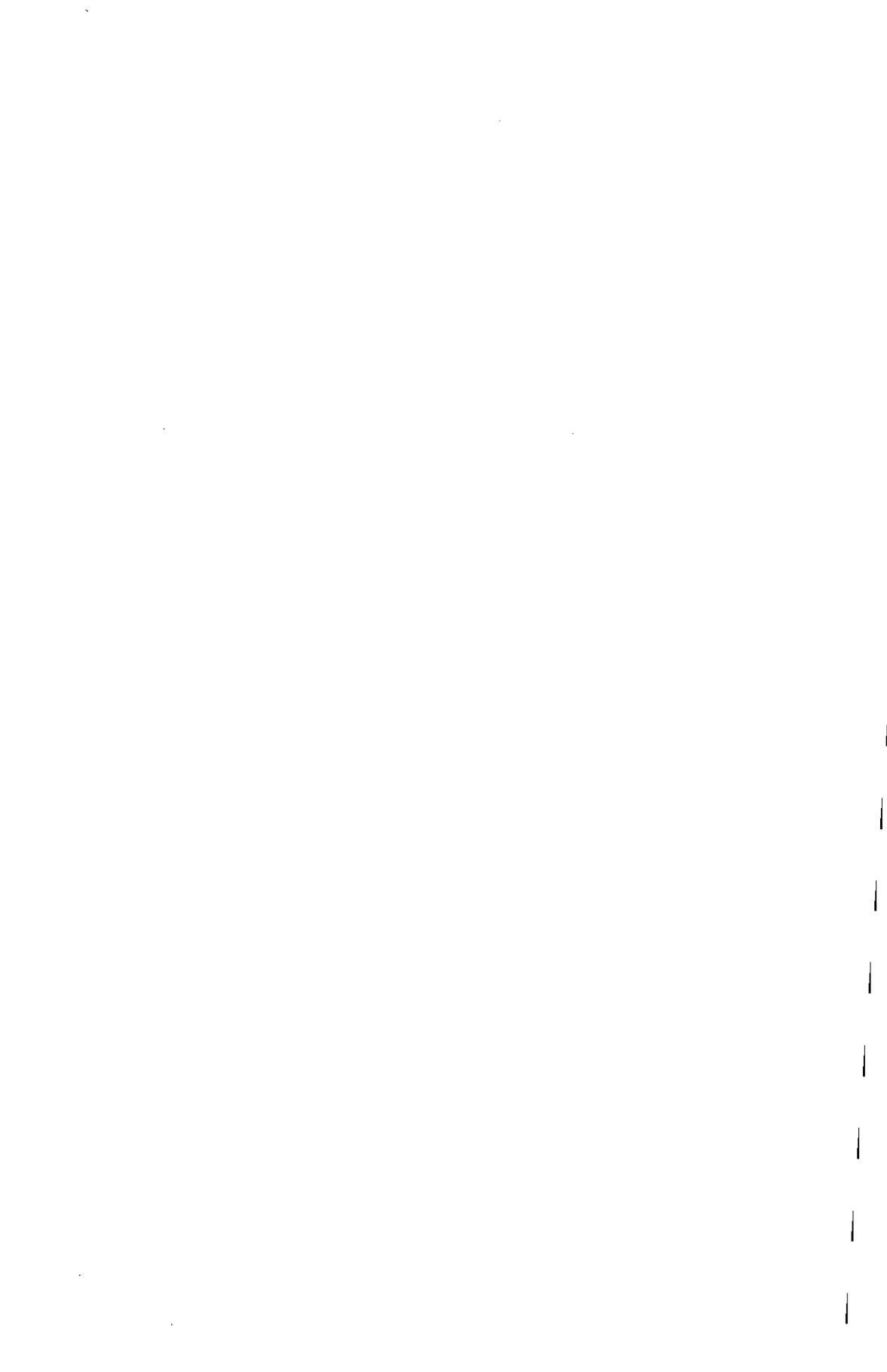
Chapter 3

UVB irradiation modulates systemic immune responses by affecting cytokine production of antigen-presenting cells

SUMMARY

The immunosuppressive effects of UVB irradiation have been well documented. The production of cytokines by keratinocytes is considered to play a major role in the induction of local as well as systemic immunosuppression. It is thought that partly due to the interaction of locally produced cytokines with antigen-presenting cells (APC), systemic effects, like antigen-specific tolerance, can be induced. In this study we examined the effect of UVB irradiation on cytokine profiles of peripheral APC as well as the functional consequences. Our results indicate that UVB irradiation impairs Th1 mediated immune responses *in vivo* by suppression of the systemic IL-12p70 production. Splenic APC from UVB exposed euthymic BALB/c mice, but also from athymic BALB/c nu/nu mice showed an enhanced production of PGE_2, IL-1, IL-6 and TNF-α after *in vitro* stimulation. Also spleen cells from UVB irradiated IL-4$^{-/-}$ mice showed increased IL-6 levels. These APC were less efficient in inducing IFN-γ production by CD4$^+$ T cells and suppressed IgM production by B cells. We conclude that the altered cytokine profile of peripheral APC can be responsible for the systemic effects of UVB irradiation on the Th1/Th2 balance as well as on B cell responses.

INTRODUCTION

Low doses of ultraviolet B (UVB) irradiation are known to suppress local cellular immune responses in humans and rodents, like the rejection of UVB-induced skin tumors, contact hypersensitivity and delayed type hypersensitivity (DTH) to allergens as well as micro-organisms (1-4). In addition, sensitization to haptens through UVB exposed skin results in the development of a systemically active antigen-specific tolerance due to the activity of antigen-specific suppressor T cells (5, 6).

The mechanisms responsible for local immunosuppression in the UVB irradiated skin are well documented (7, 8). Keratinocyte-derived cytokines are generally considered to be the initiators of the local effects of UVB resulting in the production of a plethora of cytokines within the epidermis (7) and crucial for the observed local immunological unresponsiveness. Keratinocyte-derived interleukin (IL)-10 has been demonstrated to affect B7 expression on Langerhans cells directly, thereby modifying the presentation of antigen to T helper (Th) cells in the draining lymph nodes (8). Based on their cytokine secretion patterns, the CD4$^+$ Th cells can be divided into at least two effector populations: Th1 and Th2 cells. The Th1 population produces IL-2, lymphotoxin (LT) and interferon-gamma (IFN-γ), whereas the Th2 cells produce IL-4, IL-5, IL-6 and IL-10 (9). It is generally assumed that especially Th1 mediated responses are sensitive to UVB exposure (10-12), but the mechanisms by which UVB affects Th1 responses and the consequences for Th2 mediated responses are not yet clear.

The effects of UVB irradiation on Th cells are thought to be partly caused by altered antigen presentation. When spleen cells from UVB irradiated mice were used as antigen pre-

senting cells (APC), the cytokine production by Th1 clones was suppressed whereas the production by Th2 clones was increased. This effect could be reversed by injection of anti-IL-10 mAb in UVB irradiated mice (13). Furthermore, anti-IL-10 mAb treatment inhibited UVB-induced suppression of DTH responses (13,14). Also, treatment of UVB irradiated mice with anti-CD86 mAb, but not with anti-CD80, was shown to overcome the UVB-induced impairment of antigen presentation to Th1 cells (15). However, the expression of CD86 or CD80 was not modulated on splenic APC after UVB irradiation. In addition, both the injection of IL-12 as well as the neutralization of prostaglandin E_2 (PGE_2) were shown to restore the UVB-induced suppression of DTH responses (16, 17). These *in vivo* studies therefore clearly showed that the APC activity of UVB irradiated animals is modulated. However, at present it is unknown by which mechanisms the splenic antigen presenting capacity is altered by UVB irradiation resulting in the suppressed activation of Th1 cells.

Apart from the UVB-induced effects of the APC on Th cells, it was shown that specific macrophage functions, like phagocytosis and killing of intracellular organisms were impaired (18, 19). The suppressed activity of macrophages could be restored by *in vivo* treatment with anti-IL-10 and anti-TGF-β mAb (20). Interestingly, it was reported recently that after whole body irradiation human neutrophils showed an impaired phagocytosis and a reduced adherence *in vitro* (21).

Although the involvement of cytokines in the modulation of APC activity by UVB irradiation is recognized, the source and targets of these factors are not well understood. In this study we address the UVB-induced modulation of cytokine production by APC, both *in vivo* and *in vitro*, as well as the possible consequences of the altered APC activity on the micro-environment of the spleen.

MATERIAL AND METHODS

Mice

Female BALB/c mice, IL-4 gene targeted mice (22) and their wildtype (C57BL/6) were bred at the animal facility of the Erasmus University Rotterdam and kept under specific-pathogen-free conditions. BALB/c athymic nude (nu/nu) mice were purchased from Harlan (Horst, the Netherlands). Mice were 8-10 weeks at the start of the experiments. The experiments were approved by the Animal Experiments Committee of the Erasmus University Rotterdam.

mAb and reagents

All cultures were performed in RPMI 1640 supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 µg/ml streptomycin, 1 mM pyruvate, 50 M 2-mercapto-ethanol and 10%

heat inactivated FCS. mAb used in the various assays were directed against CD3 (145.2C11), CD28 (37.51), heat stable antigen (HSA; J11D.B1), CD16/32 (2.4G2), MHC class II (M5/114), GR-1 (RB6.8C5), CD11b (M1/70), B220 (RA3.6B2), CD8 (YTS-169) and CD40 (FGK4.5). For the detection and neutralization of cytokines, mAb against IL-4 (11B11; BVD6.24G2), IFN-γ (XMG1.2; R46A2), IL-6 (32C11; 20F3), IL-12p40 (C15.6; C17.15, kindly provided by G. Trinchieri) and IL-10 (SXC-1; 2A5.1) were used. Indomethacin (Sigma, St. Louis, MO, USA) was used at a concentration of 10^{-6} M. LPS was obtained from Difco Laboratories (Detroit, MI, USA; LPS E. coli O26:B6).

UVB irradiation

UVB irradiation was essentially performed as described previously (23). Briefly, one day prior to UVB irradiation, the dorsal hair of mice was shaved using electric clippers. Mice were exposed on 4 consecutive days to 1500 J/m^2/day UVB using two Philips TL-12 tubes (40W), which represents a suberythemal dose for the mouse strains tested. The tubes had a broad emission spectrum (280 - 350 nm) with a maximum emission at a wavelength of 306 nm. 60% of the energy emitted was within the UVB range. The tube to target distance was 25 cm. Irradiation measurements were calibrated using a Waldmann UV meter (Waldmann, Schwenningen, Germany). Twenty-four hours after the last exposure mice were sacrificed and spleens were dissected. Control mice were shaven but not exposed.

LPS-induced cytokine production *in vivo*

Mice were injected intraperitoneally with 5 µg LPS in a volume of 200 ml. After 4 hours mice were sacrificed and blood was collected. The dose and time point of collection were found to be optimal for the detection of IL-12 levels.

Preparation of spleen cells and purified CD4$^+$ cells

Spleens of control and UVB irradiated mice were removed under aseptic conditions and single cell suspensions were prepared. Erythrocytes were removed by incubating with Gey's medium for 5 minutes on melting ice. Total spleen cells were either used as a source to purify CD4$^+$ cells or stimulated with LPS to determine cytokine levels or immunoglobulin production by splenic cells. Purified CD4$^+$ T cells from the spleen were obtained by complement depletion with mAb to HSA, CD16/32, MHC class II and GR-1. Cells were further purified using magnetic activated cell sorting with a cocktail of biotinylated mAb against CD11b, B220, CD8 and CD40, followed by incubation with streptavidin-conjugated microbeads (Milteny Biotech, Bergisch Gladbach, Germany). CD4$^+$ cells used for experi-

ments were always 90-95% pure as determined by flow cytometry.

In vitro stimulation of splenocytes

Splenic cell suspensions were stimulated with LPS (10 µg/ml) in the presence of different reagents and anti-cytokine mAb as indicated in the text. Cells were cultured in 96-well flat bottom tissue culture plates at a concentration of 2.5 $\times 10^5$ cells/ml. After incubation for 48 hours, supernatant was harvested and assayed for cytokine content by ELISA. Cultures were performed in triplicate or quadruplicate.

Flow-cytometric analysis

2 $\times 10^5$ cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide. For the staining of surface antigens, spleen cells were incubated with FITC-or PE-conjugated mAb against CD80, CD86, CD40, F4/80 and MHC-II (all obtained from PharMingen, San Diego, CA, USA). After washing twice with PBS-BSA-azide, the cells were resuspended and analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA). 10 µl propidium iodide (0.2 µg/ml) was added to evaluate the viability of the cells. 10^4 events were collected and the expression of the markers analyzed using CellQuest software (Becton Dickinson).

Cytokine ELISA

Flat bottom microplates (96-wells, Falcon 3912, Microtest II Flexible Assay Plate; Becton Dickinson, Oxnard, CA, USA) were coated with capture antibody diluted in PBS (1 µg/ml 20F3 or SXC-1; 5 µg/ml 11B11, XMG1.2 or C15.6) at 4°C for 18h. After coating, plates were washed (PBS, 0.1% BSA, 0.05% Tween-20) and blocked with PBS supplemented with 1% BSA at room temperature for 1h. After washing, samples and standards were added and incubation was continued for at least 4 hours at room temperature. Thereafter, plates were washed and biotinylated detection antibodies were added (1 µl/ml 32C11; 0.1 µg/ml 2A5.1 or BVD6.24G2; 1 µl/ml R46A2; 2 µg/ml C17.15) and incubated overnight at 4°C. After washing, streptavidin-peroxidase (1/1500 diluted, Jackson Immunoresearch, West Grove, PA, USA) was added. After 1h, plates were washed and the reaction was visualized using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS, 1 mg/ml, Sigma).

Optical density was measured at 414 nm, using a Titertek Multiscan (Flow Labs, Redwood City, CA, USA). The amounts of IL-12p70 and TNF-α were measured with commercially available ELISA kits (Genzyme Corp, Cambridge, MA, USA) according to the protocols provided by the manufacturer. PGE_2 levels were determined as previously described (24). The detection limits of the ELISA were: IL-4 (80 pg/ml), IFN-γ (0.3 ng/ml), IL-10

(80 pg/ml), IL-12p70 (12.5 pg/ml), IL-12p40 (75 pg/ml), IL-6 (3.9 U/ml) and TNF-α (15.6 pg/ml).

IL-1 bioassay

IL-1 activity was measured by bioassay using a sub-line of the murine T cell line D10.G4.1, designated D10(N4)M (D10) (kindly provided by Dr. S.J. Hopkins, Manchester, UK) (25). Proliferation of the D10 cells was measured via [^{3}H]-thymidine incorporation. Recombinant IL-1β (UBI, Lake Placid, NY, USA) served as a positive control. IL-1 activity was corrected for background activity of the culture medium, and expressed in counts per minute (cpm).

Isotype specific ELISA

Total supernatant IgM was measured by isotype specific ELISA as described previously (26). Goat-anti-mouse-IgM (Southern Biotechnology, Birmingham, AL, USA) was used at 1.0 µg/ml and biotinylated goat-anti-mouse-IgM antibody at 0.5 µg/ml as second step. The detection limit for the IgM ELISA was 0.2 ng/ml.

In vitro APC exchange cultures

Cell suspensions were prepared from the spleens of UVB irradiated and control BALB/c nude mice. 2 x 10^5 cells from both experimental groups were added to 96-wells flat-bottom plates and incubated for 2 hours at 37°C allowing the APC to adhere to the plastic surface of the culture plates. The adherent cells were washed gently to remove remaining non-adherent cells. To assess the effect on Th cells, purified CD4$^+$ cells were added to the cultures in the presence of 100 ng/ml soluble anti-CD3 mAb. After 2 days, supernatant was collected and IFN-γ levels were determined by ELISA. To assess the effect on immunoglobulin production, cultures were prepared containing adherent cells from UVB or control mice, and incubated with non-adherent B cells from UVB or control mice in all possible combinations. These cultures were stimulated with 25 µg/ml LPS. After 7 days, supernatant was collected and immunoglobulin production was determined by ELISA.

Statistical analysis

Data were analyzed by Student's T test and differences were considered significant at $p < 0.05$.

RESULTS

In vivo LPS-induced IL-12 levels in serum

UVB irradiation is known to inhibit Th1 mediated immune responses. Since IL-12 is the dominant factor in directing the development of Th1 cells, we determined if the *in vivo* production of this cytokine was affected by UVB irradiation. To examine the ability to produce IL-12, we exposed BALB/c mice to UVB and injected LPS intraperitoneally 24 hours after the last UVB treatment. Serum was collected from blood at 4 hours after injection, since at that time point IL-12p70 levels were found to be maximal in serum. Control samples were obtained simultaneously from mice that were shaved but not exposed to UVB. Figure 1 shows that LPS-induced IL-12p70 production in serum was significantly inhibited by treatment with UVB, whereas no effect on IL-12p40 was observed. At this time-point, no serum IL-10 could be detected (data not shown). Also, using this UVB irradiation protocol no serum IL-10 could be detected in the absence of LPS injection.
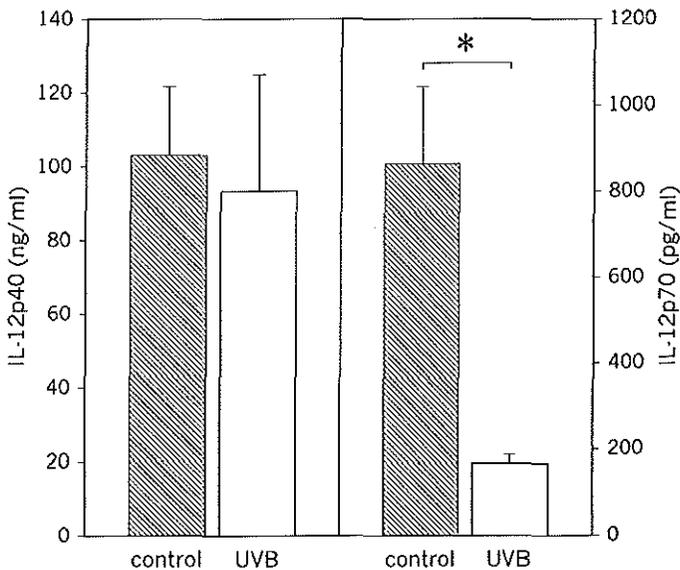
Figure 1: UVB irradiation inhibits LPS-induced IL-12p70 production *in vivo*. BALB/c mice were irradiated for 4 consecutive days with a daily dose of 1500 J/m^2. On day 5, LPS (5 µg) was injected i.p. and blood was collected 4 hours later. IL-12p40 and IL-12p70 production were determined in serum by ELISA. * p<0.05.

In vitro LPS-induced cytokine production by splenocytes

Since we observed a reduction of IL-12p70 production upon LPS challenge of UVB treated mice *in vivo*, we analyzed whether this occurs *in vitro* as well. For this purpose, spleen

cell suspensions from UVB treated BALB/c mice were stimulated with an optimal concentration of LPS (10 µg/ml) and IFN-γ (1000 U/ml). These conditions have been shown to be optimal for IL-12p70 production (27, 28). At 24 hours after stimulation with LPS, supernatants were harvested and cytokine levels determined. As shown in Figure 2, we could not detect consistent effects of UVB on the IL-12p70 production after stimulation with LPS and IFN-γ *in vitro*. Also no changes were observed in the production of IL-10. However, we did find a significant increase of LPS-induced IL-1, IL-6, TNF-α and PGE$_2$ production by stimulated spleen cells from UVB exposed mice as compared to control mice. These systemic effects were not strain specific since similar data were obtained using SJL and C57BL/6 mice (data not shown).
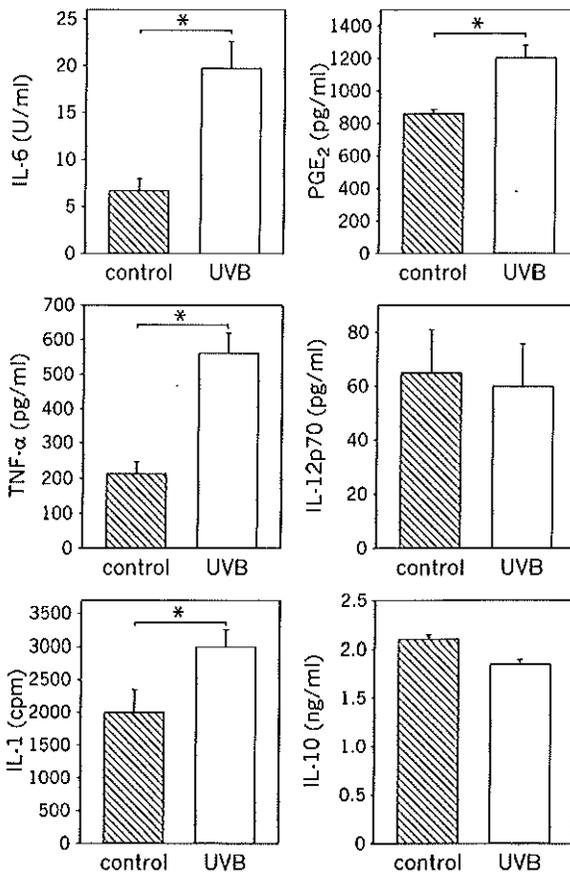
Figure 2: UVB irradiation affects LPS-induced cytokine production by total splenocytes *in vitro*. BALB/c mice were irradiated for 4 consecutive days with a daily dose of 1500 J/m^2. On day 5, spleens were removed and the single cell suspensions were stimulated with LPS (10 µg/ml), or LPS (10 µg/ml) in combination with IFN-γ (1000 U/ml) for the detection of IL-12p70. After the cells were cultured for 24h, supernatants were harvested and tested by ELISA (IL-6, IL-12p70, TNF-α, IL-10), by bio-assay (IL-1) or RIA (PGE$_2$). * p<0.05.

It has been demonstrated by a number of studies that UVB affects Th cells by inhibition of IFN-γ production and possibly an increase of IL-4 production. One could speculate that the observed effect on cytokine production by the APC is an indirect effect mediated by increased numbers of Th2 cells either *in vivo* or *in vitro*. To examine this, IL-4 gene-targeted mice were irradiated and the spleen cells stimulated with LPS. As shown in Figure 3a, upon UVB exposure and *in vitro* stimulation of whole spleen cells, IL-4$^{-/-}$ mice showed an increased production of IL-6 as compared to non-irradiated IL-4$^{-/-}$ mice, suggesting that the elevated LPS-induced IL-6 production is not primarily due to the presence of IL-4. The UVB-induced increase of IL-6 production was even more enhanced in IL-4$^{-/-}$ mice as compared to the wildtype C57BL/6 mice. To further address the possible involvement of T cells we examined the effects in BALB/c nu/nu mice, which are devoid of functional T cells. As shown in Figure 3b, BALB/c nu/nu mice still show an increased LPS-induced IL-6 production after UVB irradiation. Also the production of TNF-α and IL-1 were increased (data not shown).
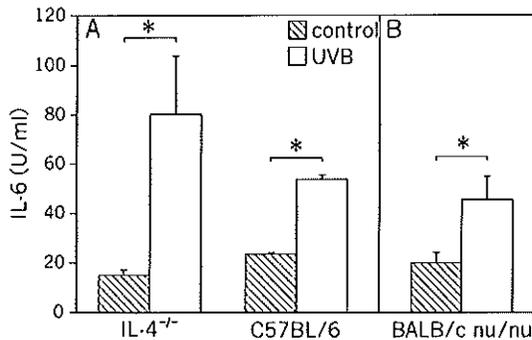
Figure 3: Augmented IL-6 production by UVB irradiation in IL-4$^{-/-}$ mice (A) and BALB/c nu/nu mice (B). Mice were irradiated for 4 consecutive days with a daily dose of 1500 J/m^2. Stimulations of spleen cells were performed as described in the legend of figure 2. * p<0.05.

Role of other cytokines in the increased LPS-induced cytokine production by spleen cells from UVB exposed mice

PGE$_2$, IL-4 and IL-10 have been implicated in the UVB-induced systemic immunosuppression (29). We therefore examined if neutralization of these cytokines could prevent the observed UVB-induced increase of the IL-6 production by the APC in our system. To determine the involvement of other cytokines in the observed upregulation of IL-6 production, we added neutralizing anti-cytokine mAb to the LPS-stimulated spleen cell cultures. As shown in Figure 4, addition of anti-IL-10 mAb strongly enhanced the production of IL-6. Anti-IL-4 mAb had no effect on the IL-6 production by spleen cells from control mice, but did increase the IL-6 production when added to cells from *in vivo* UVB irradiated mice. Incubation with the prostaglandin-inhibitor indomethacin significantly inhibited the IL-6 production in both experimental groups.
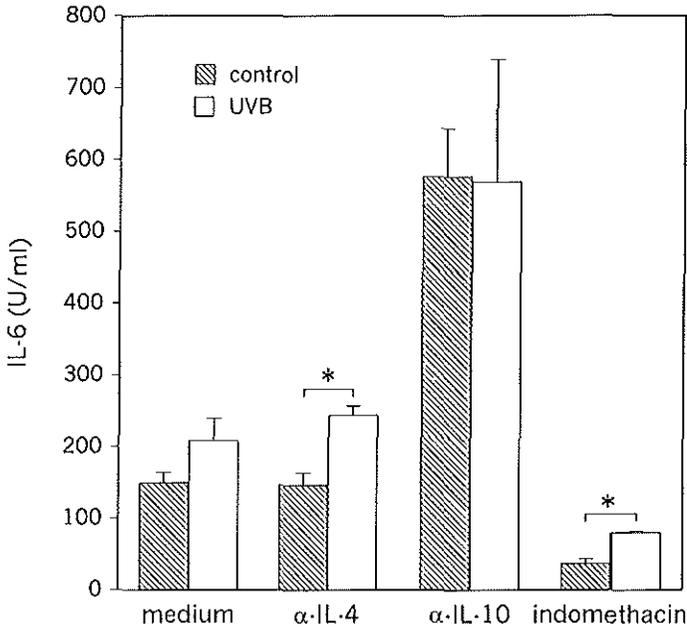
Figure 4: The effect on LPS-induced IL-6 production by BALB/c splenocytes treated *in vivo* by UVB for 4 consecutive days with a daily dose of 1500 J/m^2. Neutralizing antibodies against IL-10 (10 µg/ml 2A5.1), IL-4 (10 µg/ml 11B11) or indomethacin (10^{-6} M) were added to the cultures. After 24h incubation, IL-6 production was measured by ELISA as described in the Material and Methods section. * p<0.05.

Effect of UVB exposure on activation of APC

Upon stimulation, macrophages modulate the membrane expression of a number of activation markers. We therefore tested whether UVB treatment of BALB/c mice altered this expression. Freshly isolated spleen cells from control mice and UVB irradiated mice were compared for their expression of CD80, CD86, MHC class II, and CD40 using flow cytometry. No differences in expression levels of these activation markers were observed between both experimental groups (data not shown). In addition, the number of F4/80$^+$ cells was not affected by UVB irradiation. When testing their expression after stimulation with LPS *in vitro* for 1, 2 or 3 days, again, we did not find modulation of the expression of these markers after *in vivo* UVB treatment (data not shown).

Modulation of the Th cell activity by APC from UVB irradiated mice

Having demonstrated that the splenic APC compartment is altered by UVB irradiation with regard to their cytokine profile, we tested whether this affected the activation of Th cells.

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To examine this we incubated adherent APC with purified $CD4^+$ cells in the presence of a suboptimal concentration of soluble anti-CD3 mAb (100 ng/ml). After 48 hours, supernatant was collected and IFN-γ levels were determined. Figure 5 shows that the IFN-γ production after co-culture with APC from UVB exposed mice was significantly reduced as compared to culture with APC from control mice. The IFN-γ levels of cultures containing UVB-APC as well as cultures containing only $CD4^+$ cells and soluble anti-CD3 were below the detection limit of the ELISA.
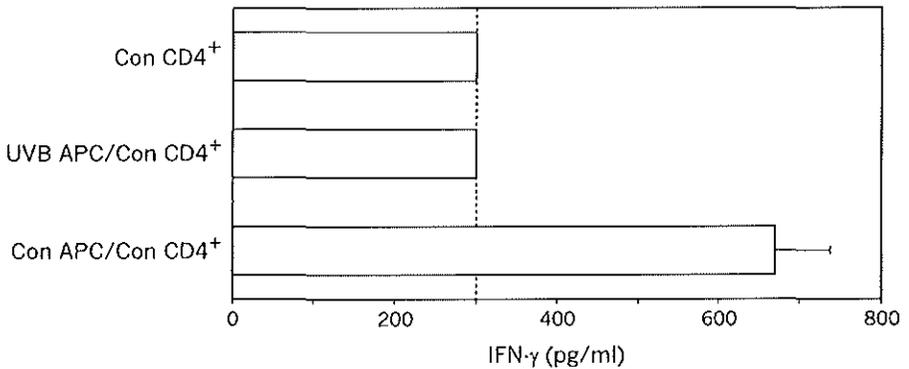
Figure 5: The effect of splenic adherent cells from UVB irradiated and control mice (Con) on the IFN-γ production by control $CD4^+$ T cells. Mice were irradiated with UVB for 4 consecutive days with a daily dose of 1500 J/m^2. The cultures were stimulated with a suboptimal concentration of soluble anti-CD3 mAb (145-2C11, 100 ng/ml). Supernatant was harvested after 2 days and IFN-γ levels were determined by ELISA.

Modulation of B cell activity by APC from UVB irradiated mice

The effect of APC from UVB irradiated mice on B cell activity was tested by LPS-stimulated co-culture of splenic adherent cells and B cells. After 7 days, the cultures were harvested and an IgM ELISA was performed on the supernatant. Figure 6 shows the results of a representative experiment. Comparison of the IgM levels in supernatants of total spleen cell cultures from UVB irradiated and control mice showed a reduction due to UVB treatment. Addition of splenic B cells to wells containing the adherent APC showed similar IgM levels as detected for the total spleen suspensions. However, co-culture of control B cells with APC from UVB irradiated mice led to a significant reduction of the IgM production as compared to co-culture with control APC. Interestingly, also culture of B cells from UVB irradiated mice with control APC showed a reduced IgM production as compared to co-culture of control APC with control B cells.
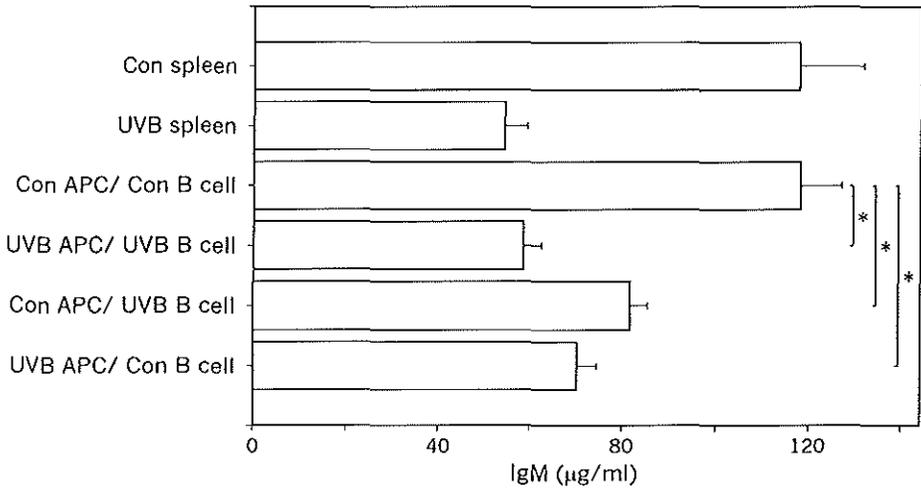
Figure 6: The effect of splenic adherent cells obtained from UVB irradiated and control mice on the *in vitro* IgM production by splenic B cells. Mice were irradiated with UVB for 4 consecutive days with a daily dose of 1500 J/m². Splenic adherent cells (Con or UVB) were cultured with non-adherent BALB/c nu/nu cells (Con or UVB) for 7 days in the presence of LPS. After 7 days, supernatant was harvested and IgM levels determined by ELISA. * p<0.05.

DISCUSSION

Low doses of UVB irradiation modulate the immune system in both humans and animals. Importantly, Th cells are known to be affected by UVB irradiation. The majority of studies dealing with the modulation of Th cell responses by UVB have shown that Th1-mediated responses in particular are sensitive to the effects of UVB (11, 12). *In vivo* disease models have shown that UVB irradiated animals are more susceptible to viral and bacterial infections than control animals (4, 18, 20).

IL-12 is a key regulatory cytokine in directing the development of Th1 cells producing high levels of IFN-γ (30). In the present study, we showed that UVB irradiation reduced the LPS-induced IL-12p70 production. This reduction of the bioactive form of IL-12 is a likely explanation for the reduction of systemic Th1 responses, such as the responses to bacterial and viral infection after UVB irradiation. Since it was shown that injection of IL-12 *in vivo* overcame UVB-induced immunosuppression (31), our data fit in the current dogma and show for the first time the effect on IL-12 production in UVB-induced systemic immunomodulation. *In vitro* UVB irradiation of human monocytes also showed a reduction of IL-12p70 production (32). However, this system using *in vitro* irradiation of cells has limited value for the evaluation of *in vivo* systemic immune responses. Murine IL-12p40 was found to inhibit the bioactivity of IL-12 (33). Since we did not observe any changes in the production of LPS-induced IL-12p40 after UVB exposure, it is likely that the inhibition of Th1 responses after

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UVB exposure is primarily due to a reduced IL-12p70 heterodimer formation.

Several studies reported on the induction of IL-10 after UVB irradiation, both locally in the skin as well as systemically (29, 34, 35). In the skin, cross-regulation of IL-12 and IL-10 is important in establishing a Th2 dominated environment after UVB irradiation (35). Furthermore, neutralization of IL-10 *in vivo* was found to overcome systemic UVB-induced immunosuppression (14).

IL-10 has been demonstrated to inhibit the production of a.o. IL-12 (36). One could speculate that the reported increased levels of serum IL-10 may negatively affect the production of IL-12, which might explain our findings. However, using our protocol of UVB irradiation (suberythemal dose) we were unable to detect modulation of the IL-10 levels in serum of UVB irradiated animals, which is not in agreement with other studies (34, 37). In these studies, however, a supra-erythemal, single dose of UVB was given.

In vitro stimulation of splenocytes from UVB irradiated mice with LPS showed no effect on the production of IL-10 and IL-12 as compared to the control group. Interestingly, we found that following UVB treatment the LPS-induced production of IL-1, IL-6, TNF-α and PGE$_2$ was consistently increased. This was rather surprising since previous reports described a reduction of the number of Ia$^+$ F4/80$^+$ cells in the spleen and a reduced APC function (20, 38). We therefore expected a reduction in the production of these immunoregulatory and pro-inflammatory cytokines.

Enhanced production of PGE$_2$, IL-1, IL-6 and TNF-α may have important implications for the priming conditions of CD4$^+$ T cells *in vivo* resulting in enhanced Th2 polarization, since all of these cytokines have been described to be involved in the process of Th cell differentiation. Since we observed this increased LPS-induced cytokine production in BALB/c nu/nu mice as well as in IL-4$^{-/-}$ mice, we exclude the possiblity that the modulated cytokine profile is due to an effect on T cells rather than a direct UVB effect on the APC. We anticipate that UVB irradiation will affect (sub)populations of APC differentially, depending on the phase of the immune response, type and dose of antigen etc.

Both IL-1 and IL-6 are known as growth factors for Th2, but not for Th1 cells (39). IL-6 has been reported, by means of its transcription factors, to activate the transcription of the IL-4 gene, thereby promoting the development of Th2 cells (40). PGE$_2$ is a potent inhibitor of IL-12 production, but also facilitates Th2 development by directly inhibiting IFN-γ production (41, 42). In addition, PGE$_2$ stimulates the differentiation of dendritic cells towards effective APC with Th2-skewing capabilities (43).

The enhanced systemic production of PGE$_2$, IL-1, IL-6 and TNF-α after UVB exposure, as shown in our *in vitro* studies, may therefore have important implications for the Th cell differentiation and the maintenance of a micro-environment promoting Th2 activity as observed after UVB exposure.

Interestingly, PGE$_2$ has been detected in serum of UVB irradiated mice and inhibition of PGE$_2$ production abrogates the induction of immunosuppression (17) by a mechanism involving the induction of IL-4 and subsequent IL-10 production (29). We found that inhibition of the production of PGE$_2$ reduced the production of IL-6 as well as TNF-α and IL-1 by

splenocytes (data not shown). Since we observed increased production of PGE_2 by spleno-cytes of UVB irradiated mice, our findings suggest that PGE_2 plays a role in the enhanced APC derived cytokine production following UVB irradiation, thereby contributing to the enhanced Th2 polarization. Besides the reported upregulation of the IL-4 production, we showed that PGE_2 may well be responsible for the enhanced production of pro-inflammato-ry, immunoregulatory cytokines in the spleens of UVB irradiated mice. These activities sug-gest multiple mechanisms whereby PGE_2 favors the development of Th2 cells.

We and others showed that antigen presentation by splenic APC to Th cells is modu-lated by UVB exposure as demonstrated by the reduced IFN-γ production (13). Since the expression of the activation markers tested on APC was not altered, it is likely that the mod-ulated cytokine profile of the APC is the most important mechanism that affects Th cell activ-ity. Although obtained from *in vitro* culture systems, these data are likely representative for the APC-Th cell interaction in the micro-environment of the spleen, as *in vitro* and *in vivo* data are consistent.

Furthermore, we showed that the effect of UVB on the splenic APC compartment resulted in a reduction of the IgM production by B cells *in vitro*. The production of the other isotypes was reduced as well (data not shown). We could not detect a selective reduction of Th1-associated isotypes (IgG2a) as compared to Th2-associated isotypes (IgG1 and IgE). Interestingly, our studies show that not only APC, but also B cells are intrinsically modulat-ed by UVB exposure. This effect on B cells has not been described before. Since B cells are rarely found in the skin, they are likely affected by a yet undefined indirect mechanism, involving soluble factors.

There are two possible mechanisms that may account for the UVB-induced alteration of the cytokine profile of splenic APC. Firstly, it is possible that the cytokine production of the individual APC is altered, influenced by immunoregulatory mediators like PGE_2. Secondly, it is possible that the APC population as such in the spleen has changed. It is known that UVB irradiation causes a redistribution of APC due to migration to peripheral lymphoid tissues and a subsequent replenishment by newly immigrating cells in the skin (38). Due to the extensive functional and phenotypic heterogeneity of the APC it is tempting to speculate that after UVB irradiation the spleen is repopulated with functionally distinct subpopulations of APC (44). We are currently testing these possibilities.

In conclusion, we showed that UVB irradiation affects the APC-derived cytokines IL-1, IL-6, TNF-α, IL-12p70 and PGE_2. The coordinated action of IL-1, IL-6, TNF-α and PGE_2 together with the reduced IL-12p70 production may well be responsible for the UVB-induced systemic selective suppression of Th1 driven immune responses as well as the effects on B cell activity.

ACKNOWLEDGEMENTS

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Chapter 4

UVB exposure alters respiratory allergic responses in mice

SUMMARY

We have tested the hypothesis that exposure to ultraviolet light inhibits Th1 responses and stimulates Th2 responses. We therefore examined whether in a mouse model of allergic asthma (i.e. extrinsic; using ovalbumin as the allergen) increased symptoms would be observed after UVB irradiation, while in a model of Th1 dependent occupational asthma (with picrylchloride as the allergen) decreased symptoms would be observed.

In UVB pre-exposed picrylchloride sensitized and challenged mice, we observed reduced IFN-γ production, decreased inflammatory responses in the airways, and reduced airway reactivity to non-specific stimuli. The results in the ovalbumin model were less clear. In this model, increased IL-10 production together with unchanged IL-4 and IFN-γ production were observed as a result of UVB exposure. In addition, decreased ovalbumin-specific IgG1 and IgE titers were noted, as well as decreased non-specific airway hyperreactivity. Eosinophilic inflammatory responses were not influenced.

The results indicate that UVB exposure has systemic effects that influence ongoing immune responses in the respiratory tract. The effects are not restricted to immune responses that are predominantly Th1 dependent (i.e. pulmonary DTH and IFN-γ production in response to picrylchloride) but also hold for immune responses that are predominantly Th2 dependent (decreased specific IgE titers).

INTRODUCTION

Over the last two decades it has become clear that UVB exposure (280-320 nm) can affect specific and non-specific immune responses (1, 2). Since UVB is not able to penetrate beyond the first few cell layers of the epidermis, UVB-induced immunosuppression must be mediated by cells or their products or photoactive factors present in the skin. Both UVB-induced DNA damage and UVB-induced photoisomerization of urocanic acid (present in the stratum corneum) have been implicated to play a role in the induction of immunosuppression (3-8). In both mechanisms altered expression of several inflammatory mediators (including prostaglandins, NO, and cytokines) have been shown to be involved (9-14). Neither DNA damaged cells (11), nor *cis*-urocanic acid (15, 16) nor inflammatory mediators necessarily remain in the skin after UVB-irradiation. For this reason effects of UVB are not restricted to the skin, but also include systemic effects (1, 17-19).

Cytokines are crucial in the regulation of immune responses. Based on their cytokine profiles T helper cells can be subdivided into at least two major subsets of T helper effector cell populations. The T helper-1 (Th1) subpopulation produces especially IL-2 and IFN-γ. In contrast, T helper-2 cells (Th2) produce IL-4, IL-5, IL-6, and IL-10 (20). It is not always possible to reconcile all observations with respect to cytokine responses associated with immune reactions into the Th1/Th2 paradigm (21). However, the proposed dichotomy in responses has

provided a framework to understand immune responses as such, and the modulation of such immune responses, among others by UVB (22, 23). The majority of studies dealing with immunomodulation by UVB that have been performed so far indicated that especially Th1 mediated immune responses are sensitive to UVB exposure (24, 25). The effects of UVB exposure on Th2 mediated immune responses have gained less attention. In 1989 Araneo et al. (24) demonstrated that UV exposure reduced the production of IL-2 and IFN-γ. The production of the Th2 cytokine IL-4 was augmented. The conclusion of these authors was that Th1 mediated immune responses are suppressed by UVB exposure, leading to augmented Th2 mediated responses.

If UVB exposure not only leads to suppression of Th1 responses, resulting in for example a reduced resistance to several types of infections, but also to increased Th2 responses, then an additional risk associated with UVB exposure is evident. Specifically, a systemic immunomodulatory effect of UVB might exacerbate respiratory allergy, that is associated with systemic Th2 immune responses (23).

We have addressed this hypothesis in two models of respiratory allergy. One model is a mouse model of respiratory allergy to protein allergens, in which mice are sensitized with ovalbumin (OVA) in such a fashion (intraperitoneally, followed by inhalatory exposure) that Th2 type of responses, including OVA-specific IgE responses, eosinophilic inflammatory responses, and increased airway reactivity to non-specific stimuli are induced (26). Respiratory allergy to protein allergens, such as house dust mite, is the most common form of respiratory allergy, and is often associated with asthma, i.e. extrinsic allergic asthma. In occupational asthma, i.e. asthma induced by low-molecular weight chemicals, a role of IgE and thus of Th2 responses is less evident, and Th1 responses may be crucial (27). The second model to test the hypothesis that UVB exposure would lead to altered expression of respiratory allergy was a model for occupational asthma. In this model, mice are skin-sensitized to the contact sensitizer picrylchloride (PCl), and subsequently challenged intranasally with the allergen to induce Th1 type of immune responses, i.e. mononuclear type infiltrations in the airways, and in addition increased airway reactivity to non-specific stimuli (28, 29).

MATERIAL AND METHODS

Mice

Specific pathogen free BALB/c mice (male, 6-8 weeks of age) were obtained from the breeding colony at the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. The mice were housed in macrolon cages with unlimited access to standard mouse chow and tap water. Each experimental group consisted of at least 6 mice.

Reagents

PCl was used as contact sensitizer (Chemotrix, Swannanoa, NC, USA) and was recrystallized three times from methanol/H_2O before use, and protected from light during storage at 4°C. Picrylsulphonic acid (PSA) and TNP-KLH were purchased from Jackson Immunoresearch, West Grove, PA, USA). PCl, PSA, and TNP-KLH are all recognized by the same B cell receptor (TNP-specific). OVA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Carbachol (carbamylcholinechloride) and methacholine were obtained from Onderlinge Pharmaceutische Groothandel (OPG; Utrecht, The Netherlands).

UV irradiation

The UV source was a Kromayer UV lamp (Hanovia Ltd., Slough, UK) equipped with a Schott WG 305 filter. This lamp had an irradiance of 140 (J/m^2)/second in the UV range (wavelengths 305-400 nm), as measured by a Kipp E11 thermopile. After dorsal hair was removed with electrical clippers, two circular areas (6.3 cm^2 in total) on the backs (ventral skin) of the mice were irradiated, and the ears were protected from exposure. Mice were irradiated for 16 seconds on the shaved backs on four consecutive days. The last UV exposure was 4 days prior to the sensitization procedure. The dose of UV received by the mice was 2.2 kJ/m^2 at each exposure, a dose that was suberythemal for this species.

Sensitization

PCl: The mice were skin-sensitized 4 days after the last day of (sham) irradiation by topical application of 150 µl of 5% of the hapten PCl in ethanol/acetone (3:1) to the *non*-UV-irradiated shaved abdomen chest and four feet. Seven days after epicutaneous sensitization the mice were challenged intranasally by application of 50 µl of 0.6% PSA (the water soluble form of the hapten) in phosphate buffered saline (PBS, pH 7.2) on the nostrils of the animals under light ether anesthesia.

OVA: The sensitization protocol started 4 days after the last (sham) irradiation. The mice were sensitized on alternate days by intraperitoneal injection of 10 µg OVA in 0.5 ml pyrogen-free saline for 13 days. Four weeks after the last intraperitoneal injection the mice were exposed to 8 OVA (2 mg/ml) aerosol treatments on consecutive days (1 treatment per day). The aerosol was generated using an ultrasonic nebulizer (Medix 8001, particle size 305 µm) connected to a plexiglas exposure chamber of 5 liters. The animals were exposed for 5 min each time. Non-sensitized control mice only received the last OVA aerosol exposure as a challenge.

Histological examination

Twenty-four hr after PCl or OVA challenge, the lungs were removed after lethal anesthesia with 50 µl of a cocktail consisting of 7 ml of 50 mg/ml ketalar (Parke Davis, Spain), 3 ml 2% rompun (Bayer, Leverkusen, Germany), and 1 ml of 1 mg/ml atropine (OPG) injected intramuscularly. Before removal the lungs were perfused with a solution of 0.5% BSA (bovine serum albumin) and 5 mM glucose in PBS. Hereafter the lungs were removed and filled intratracheally with formalin fixing solution and embedded in paraffin. Tissue sections were stained according to routine procedures (haematoxilin-eosin).

Evaluation of the number of mononuclear cells was performed. The parameters were scored according to the scoring method described by Enander et al. (30), as indicated in Table 1. In addition to this method, the number of patchy cellular infiltrates (hot spots) per section was counted.

Table 1: Histological score of lung DTH-like responses to picrylchloride according to the method of Enander et al. (30).

		score 1	score 2	score 3
accumulation of mononuclear cells	diffusely around bronchioli	< 10 cell layers thick	> 10 cell layers thick	> 10 cell layers thick
	around blood vessels	3 cell layers thick	4-10 cell layers thick	> 10 cell layers thick
	interstitial	scattered distribution of single cells	dense accumulation covering < 25% of specimen surface	dense accumulation covering > 25% of specimen surface

Isometric measurement of bronchial reactivity

Tracheal reactivity was measured *ex vivo in vitro* as described previously (29). Mice were killed 24 hr after PCl or OVA challenge. The tracheas (9 trachea rings just beneath the larynx) were immediately slipped onto two supports in an organ bath, of which one was connected to the isometric transducer. Isometric tension was measured with a force displacement transducer (isometric transducer, Harvard Bioscience, Boston, USA) and a two-channel recorder (Servogor type SE-120) and is expressed as changes in grams force. The optimal basic force for the mouse trachea is 1 g. After equilibration cumulative dose-response curves were prepared for carbachol or methacholine.

Measurement of Ig isotypes

Total serum IgG1, IgE and IgG2a were measured by isotype-specific ELISA as

described previously (31). TNP-specific IgG3 and IgM were determined by ELISA. Plates were incubated overnight with rat-anti-mouse IgG3 and IgM (both obtained from Southern Biotechnology, Birmingham, AL, USA), washed with PBS and incubated with 1% BSA in PBS. Diluted serum samples were added and incubated for at least 4 hours at room temperature. After washing, the plates were incubated overnight with biotinylated TNP-KLH. Finally, the plates were incubated with peroxidase-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA, USA) and developed using 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) (Sigma) as substrate. The optical density was read at 414 nm.

OVA-specific IgG1, IgE and IgG2a were measured on rat anti-mouse IgG1, IgE or IgG2a coated plates (Southern Biotechnology, Birmingham, AL, USA). Non-specific protein binding sites were blocked with 1% BSA in PBS. Again diluted serum samples were added and incubated for at least 4 h. After washing, the plates were incubated overnight with 1 µg/ml OVA VII-coupled digoxigenin. Next, 150 mU/ml peroxidase-coupled sheep anti-digoxigenin Fab fragments (Boehringer, Mannheim, Germany) were added and the reaction was visualised as described above using ABTS as substrate.

Cell preparation

Single spleen cell suspensions were prepared by pressing the organs through a sterile cell strainer. The cells were washed once (300 g, 10 min, 4°C) and resuspended with complete RPMI medium (10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.05 µg/ml 2-mercaptoethanol). The lymphocytes were cultured in 6- or 24-wells culture plates for 24 with concanavalin A (ConA; 5 µg/ml) in complete RPMI medium at 37°C and 5% CO_2. After culture, the cell suspensions were pelleted (300 g, 10 minutes, 4°C). The supernatants were pipetted off and immediately frozen and stored at -70°C until analysis.

Cytokine analysis

Cytokine concentrations were determined by ELISA as described earlier (32). Anti-mouse IFN-γ (R4-6A2, rat IgG1; Pharmingen, San Diego, CA, USA), recombinant mouse IFN-γ (Biosource, Camarillo, CA, USA), anti-mouse IL-4 (11B11, rat IgG1; Pharmingen), recombinant mouse IL-4 (Peprotech, Rocky Hill, NJ, USA) and IL-10 commercial ELISA kits from Biosource were used. The manufacturer's instructions were followed point by point.

Statistics

For results of analysis of cytokine levels or antibodies, significance was calculated using the two-tailed Student's t test. Trachea reactivity curves were compared using ANOVA.

For comparing specific curve parameters the concentration-response curves of the tracheal rings were analyzed by means of a computerized curve-fitting technique based on the 4-parameter logistic equation (32).

Histology data were compared using the non-parametric Wilcoxon rank test. $P<0.05$ was taken as a significant difference between groups. Each group consisted of at least 6 mice.

RESULTS

Effects of UVB exposure on histopathology in the lungs due to sensitization and challenge

PCl model

In animals challenged with PCl only, no inflammatory responses were seen. Intranasal challenge to PCl induced mononuclear leukocyte infiltrates around bronchioli and vessels in sensitized mice. As expected according to earlier publications (28), UVB pre-exposure diminished these responses in PCl sensitized and challenged mice (Figure 1).
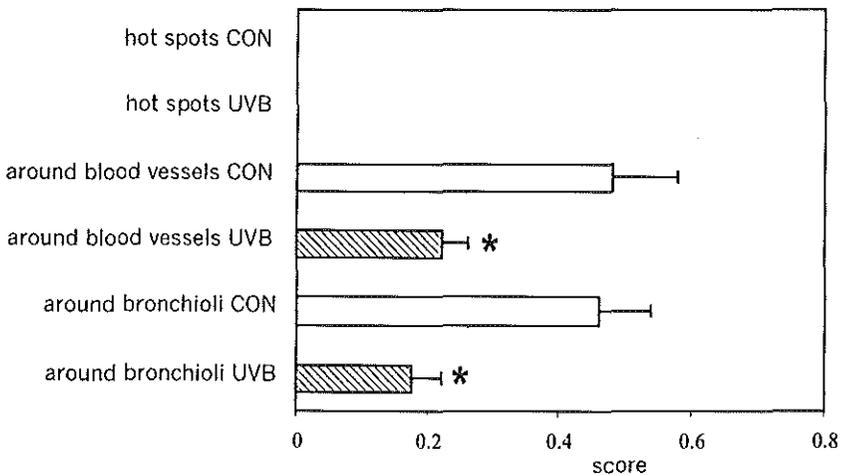
Figure 1: Effect of UVB exposure on histopathology of the lung due to sensitization and challenge with PCl. Perivascular and peribronchial mononuclear infiltrates were scored according to Table 1. *p<0.05 (UVB sensitized and challenged vs. non-irradiated sensitized and challenged control).

OVA model

In contrast to the PCl model, sensitization and challenge to OVA induced only a marginal mononuclear infiltrate around bronchioli and vessels, but produced profound patchy
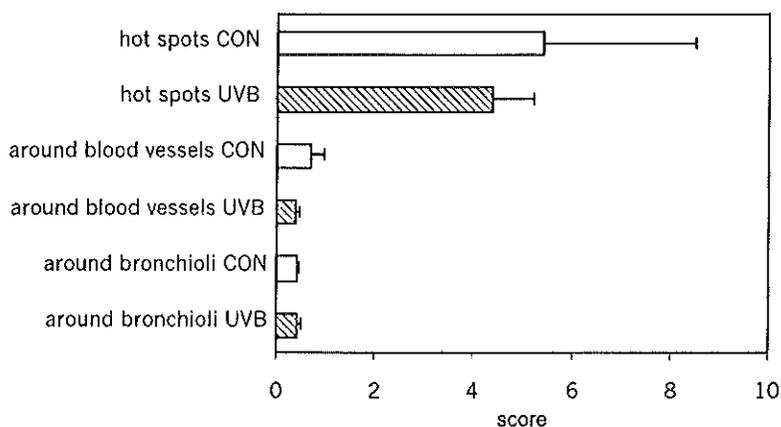
Figure 2: Effect of UVB exposure on histopathology of the lung due to sensitization and challenge with OVA. Inflammatory eosinophilic infiltrates: number of hot spots per section. Infiltrates were scored according to Table 1.

cellular infiltrates (including eosinophils), also called hot spots, which is in accord with earlier publications (26). UVB pre-exposure had no effect on these responses (Figure 2).

Effects of UVB on tracheal reactivity

PCI model

In vitro reactivity of the isolated trachea to carbachol was increased in PCI sensitized and challenged mice, as compared to challenged non-sensitized control mice (Figure 3, left), reproducing earlier observations (29). In UVB pre-exposed PCI sensitized and challenged mice this increment was not found; in contrast, a (non-significant) decrease was observed (Figure 3, right).

OVA model

In vitro reactivity of the isolated trachea to carbachol was increased in OVA sensitized and challenged mice, as compared to challenged non-sensitized control mice (Figure 4, left), reproducing earlier observations (26). As in the case of PCI, also in this OVA model the effect of sensitization could not be observed in UVB pre-exposed mice (Figure 4, right).
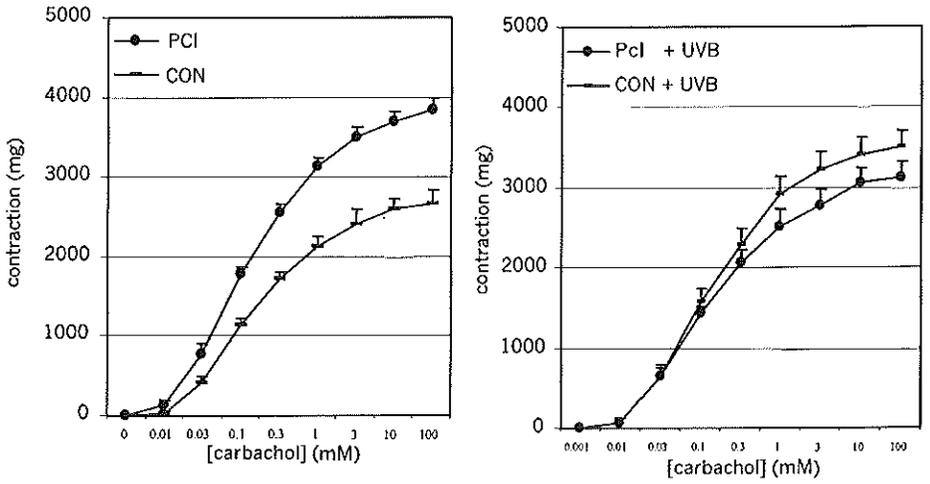
Figure 3: Effect of UVB on tracheal reactivity to carbachol after sensitization and challenge with PCl. Left: sensitized and challenged vs challenged control: Right: UVB irradiated sensitized and challenged vs UVB irradiated and challenged control.
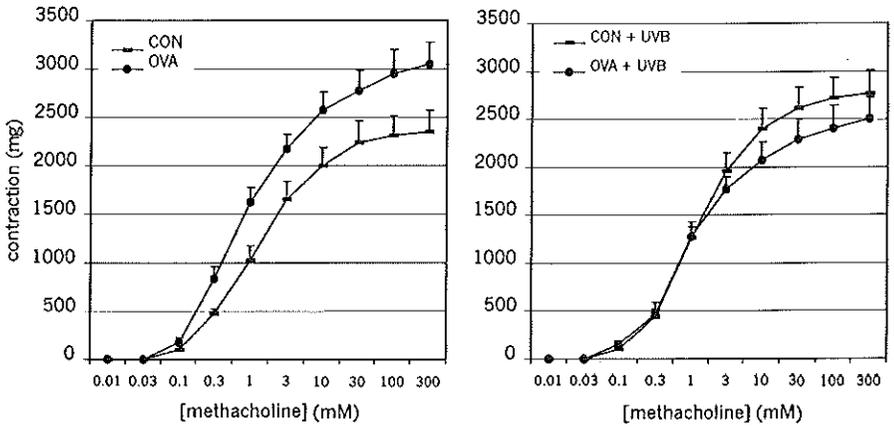

Figure 4: Effect of UVB on tracheal reactivity to methacholine after sensitization and challenge with OVA. Left: sensitized and challenged vs challenged control: Right: UVB irradiated sensitized and challenged vs UVB irradiated and challenged control.

Effects of UVB exposure on antibody levels

Picryl chloride model

No PCl-specific IgG3 and IgM antibodies could be detected in serum of mice in any of the experimental groups.

OVA model

In the OVA protocol we assessed total as well as OVA-specific IgG1, IgE and IgG2a. As can be seen in figure 5, UVB irradiation did not affect the total IgG1 and IgE levels in OVA sensitized and challenged mice. However, OVA-specific IgG1 and IgE were significantly ($p<0.05$) reduced in the UVB irradiated mice as compared to the sensitized and challenged control group. No OVA-specific IgG2a was detected using this immunization protocol.
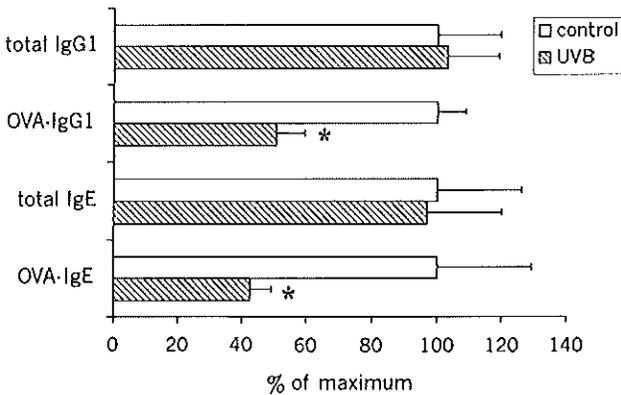
Figure 5: Effects of UVB exposure on antibody levels after sensitization and challenge with OVA. The antibody levels of control mice have been set at 100%. *$p<0.05$ (UVB sensitized and challenged vs. non-irradiated sensitized and challenged control).

Effects of UVB exposure on cytokine levels

PCl model

IFN-γ and IL-4, but not IL-10, were detectable in the supernatants of spleen cells cultured with ConA. This was observed after culture of spleen cells from all groups of mice. In the PCl-sensitized and challenged animals, significantly increased levels of IFN-γ were noted as compared to non-sensitized challenged controls (Figure 6). Pre-exposure to UVB diminished these IFN-γ levels, whereas no further increase in IL-4 was observed (Figure 7).

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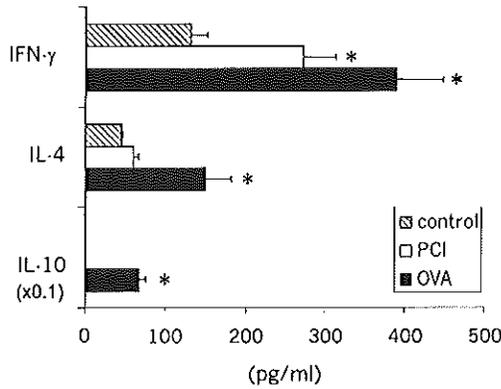
Figure 6: Effects of sensitization and challenge with PCl or OVA on cytokine levels in supernatants of spleen cell cultures. The cells were triggered to produce cytokines *in vitro* using ConA, and supernatant was harvested after 24h. *p<0.05.
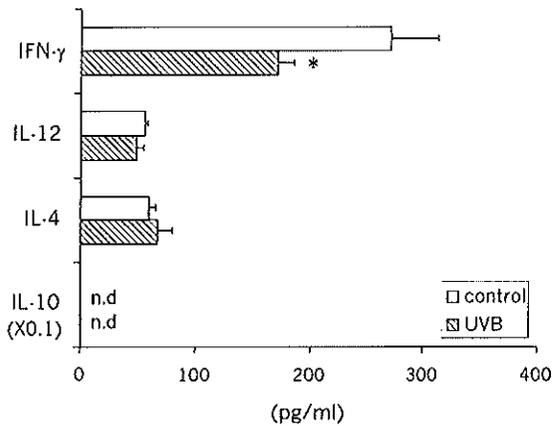

Figure 7: Effects of UVB exposure on cytokine levels in the supernatant of spleen cell cultures after *in vivo* sensitization and challenge with PCl. The cells were triggered to produce cytokines *in vitro* using ConA and supernatant was collected after 24h. *p<0.05 (UVB sensitized and challenged vs. non-irradiated sensitized and challenged control). nd: not detectable.

OVA model

IFN-γ levels were significantly increased in the supernatant of ConA-stimulated spleen cell cultures from OVA sensitized and challenged mice (Figure 6). UVB pre-exposure did not affect these levels (Figure 8). Sensitization and challenge with OVA also led to an increased level of IL-4 in the spleen cells. This increment was relatively more pronounced than the increased IFN-γ level (Figure 6). UVB pre-exposure did not affect this increased level. IL-10 production could not be observed in spleen cells from non-sensitized challenged control mice.

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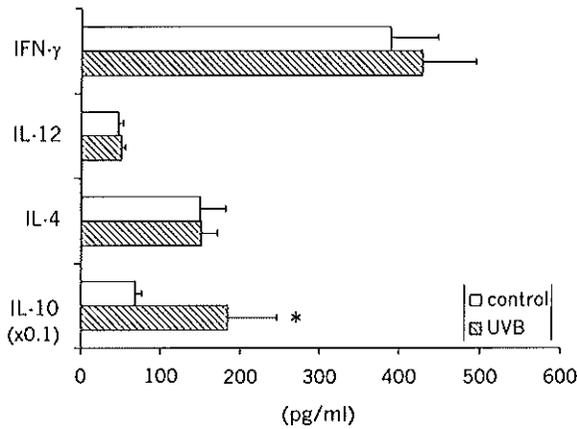
Figure 8: Effects of UVB exposure on cytokine levels in the supernatants of spleen cells cultured after *in vivo* sensitization and challenge with OVA. The cells were triggered to produce cytokines *in vitro* using ConA, and supernatant was collected after 24h. *p<0.05 (UVB sensitized and challenged vs. non-irradiated sensitized and challenged control).

Sensitization with OVA led to detectable IL-10 production, which was further enhanced by UVB pre-exposure (Figure 8). This latter UVB effect was statistically significant.

DISCUSSION

We have found an effect of UVB pre-exposure in two models of respiratory hypersensitivity. The working hypothesis was that UVB would decrease Th1 responses, and stimulate Th2 responses. This would lead to reduced Th1-mediated allergic responses in the respiratory tract as is the case with PCl, as a model for occupational asthma. It would lead to an increased Th2-mediated allergic respiratory response in OVA sensitized animals, as a model for extrinsic asthma.

UVB pre-exposure decreased the PCl induced inflammatory responses in sensitized and challenged mice, as well as the reactivity to carbachol in such mice. Since both these inflammatory responses and airway reactivity were dependent on non-IgE-dependent immunity (29), these data are consistent with the hypothesis that UVB decreases Th1 type responses, and that thus UVB decreases respiratory hypersensitivity that depends on Th1 immunity.

In this PCl model, increased IFN-γ levels were induced by sensitization. This level was decreased if sensitized mice were pre-exposed to UVB, which is in line with the hypothesis. Suppression of the IFN-γ production was also found by Simon et al. (25) in dinitrofluorobenzene sensitized and UVB exposed mice. Since in the PCl protocol TNP-specific IgG3 as well as TNP-specific IgM were below detection levels in the sensitized group as well as the UVB-irradiated group (data not shown), no conclusions can be drawn concerning these

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isotypes.

The effects of UVB in the OVA model were surprising, and more difficult to interpret. The histopathology that was observed in OVA sensitized and challenged mice was not affected by UVB pre-exposure. The tracheal reactivity to carbachol in sensitized and challenged mice, however, was diminished by UVB pre-exposure. In addition, it was surprising that OVA-specific IgG1 and IgE responses, characteristic of Th2 type immunity, were significantly reduced in the UVB irradiated as compared to the non-irradiated group. Using a parasite infection model, we have observed previously that the levels of the Th2 associated isotype IgE were significantly reduced (33). In a study by Araneo et al. (24) OVA-specific antibodies were measured in UVB exposed and non-exposed animals. These authors found suppressed IgG1 titers as well. In a recent study (34) we have demonstrated that both Th1 and Th2 mediated immune responses in spleen and lymph nodes can be affected by UVB as evidenced by changes in cytokine patterns. These data suggest a general immunosuppression rather than a selective suppression of the Th1 or Th2 compartment determined on basis of isotype switching.

Inflammatory responses due to OVA sensitization are IL-4 dependent (26). For the OVA model it has been suggested that IFN-γ may be involved in the induction of tracheal hyperreactivity (26). OVA sensitization induced increased IFN-γ as well as IL-4 levels. Relatively, IL-4 levels were stimulated in a more pronounced fashion. It should be noted that interleukin responses were analyzed in the supernatants of lymphoid cells stimulated with mitogens. Yet, similar results were noted in supernatants of lymphocytes that were stimulated antigen-specifically (34). In addition, sensitization with OVA induced detectable amounts of IL-10, that could not be observed in spleen cells of non-sensitized animals. These data are compatible with the Th2 nature of the model, even though Th2 responses do not occur exclusively. UVB pre-exposure did not affect the IFN-γ and IL-4 levels. However, in the OVA model the production of IL-10 was significantly increased by UVB, which is compatible with the hypothesis that UVB increases Th2 responses. The fact that UVB did not decrease the IFN-γ expression in the OVA model, in contrast to the PCl model, was surprising, especially since IL-10 is known to inhibit Th1 mediated immune responses (35-37). On the other hand, in some cases IL-10 can also inhibit Th2 responses (38).

UVB seems to reduce some Th2 associated responses (such as specific IgG1 and IgE), but not all (i.e. eosinophilic infiltrates). IgE and IgG1 production are associated with Th2 immune function (39), however, no strict association in the OVA model seems to be apparent with the cytokine profiles. IL-4, IL-10, and IFN-γ production are all increased due to OVA sensitization and challenge, whereas diminished IgG1 and IgE responses are seen in association with unchanged IL-4 and increased IL-10 production after UVB exposure. These data illustrate that results cannot always be reconciled with the Th1/Th2 paradigm, so that the validity of the paradigm may be questioned (21). The conclusion must be that the regulation in this OVA model is complex, and that UVB does not uni-directionally stimulate Th2 associated interleukins and suppresseses Th1 interleukins, but rather suppress Th2 responses, i.e. IgE suppresses production.

The precise mechanisms of the interaction of UVB with respiratory immune responses to different types of antigen, pertaining to cytokine patterns, the production of specific antibodies, the induction of pathology, and lung function alterations is not clear. Yet, the findings in this paper indicate that such systemic effects of UVB may occur, and thus pose a concern for the human population.

ACKNOWLEDGEMENTS

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Chapter 5

Differential UVB-induced immunosuppression in mice differing at the *lps* locus: disparate effects on Th1 cell activity

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SUMMARY

Irradiation of mice with low doses of ultraviolet B suppresses contact hypersensitivity (CHS) to a different degree in different mouse strains. UVB susceptibility is assumed to be a genetically determined trait, which is governed by the *tnfa* and *lps* loci. In this study we compared the LPS-responder C3HeB/FeJ with the congenic, LPS-nonresponder C3H/HeJ strain for their UVB sensitivity in the systemic suppression of CHS. We found that the LPS-responder mice were more sensitive to UVB than the LPS-nonresponder mice.

In LPS-responder mice, the reduction of CHS responses coincided with lymph node hyperplasia and a reduction of IFN-γ production by lymph node cells upon stimulation *in vitro*. In contrast, in LPS-nonresponder mice we demonstrated suppression of the CHS response and lymph node hyperplasia at relatively high UVB doses, but these effects were not accompanied by an effect on IFN-γ production capacity.

We conclude that in C3H/HeJ mice, UVB-induced suppression of CHS and inhibition of Th1 activity are unlinked events. The absence of inhibition of Th1 activity in C3H/HeJ mice suggests that a reduced migration or activation potential of APC from LPS-nonresponder mice may cause the differences in UVB susceptibility.

INTRODUCTION

Exposure to ultraviolet B (UVB) irradiation can lead to deleterious effects on human health, a.o. by affecting the immune system (Longstreth, 1998; Duthie, 1999). Even suberythemal UVB doses can significantly impair both local and systemic immune responses in both animals and humans. It has been shown that UVB-induced immunomodulation plays a role in photocarcinogenesis. In addition, an increased susceptibility to viral, bacterial and fungal infections has been reported after UVB irradiation (Garssen, 1998).

Exposure to low doses of UVB, followed by application of a hapten on the *irradiated* skin, results in a markedly suppressed contact hypersensitivity (CHS) response upon challenge with the same hapten a week later (Streilein, 1988; Yoshikawa, 1990). This demonstrates that UVB induces local immunosuppression. Furthermore, UVB-irradiated mice sensitized with hapten on *non-irradiated* skin, also showed a suppressed CHS response upon challenge, which demonstrates that systemic immune responses are also affected by UVB exposure (Noonan, 1994).

Comparison of UVB-induced suppression of the CHS response of different inbred mouse strains revealed differential susceptibility to the immunosuppressive effects of UVB. In the 'local' model, some strains like C57BL/6, C3H/HeN and SJL showed significant suppression of the CHS response at relatively low doses of UVB. These strains were assigned as UVB sensitive. Other strains, like BALB/c, C3H/HeJ and DBA/2J mounted a normal CHS response at the same UVB dose, and were termed UVB resistant (Yoshikawa, 1990; Noonan,

1994). A similar differential susceptibility to UVB was also observed in humans (Streilein, 1994a, 1994b; Skov, 1998).

Subsequent studies on the factors determining the UVB phenotype have demonstrated the genetic basis for UVB sensitivity by showing that polymorphisms at the *tnfa* and *lps* loci may dictate the biological response to UVB (Vincek, 1993; Streilein, 1994). The *tnfa* locus consists of a number of genes within the H-2 complex on chromosome 17. Recently, the number of candidate genes conferring UVB susceptibility in humans was narrowed down to seven, including the TNF-α gene itself (Handel, 1999). Furthermore, a number of studies have convincingly shown that TNF-α is a key regulator of at least the local unresponsiveness induced by UVB (Yoshikawa, 1990; Vermeer, 1990; Shimizu, 1994; Hart, 1998).

The role of the *lps* locus in UVB susceptibility was indicated by studies with C3H/HeJ mice which was found to be resistant to the induction of local suppression by UVB, whereas the congenic strain C3H/HeN was susceptible (Yoshikawa, 1990). These mice differ only at the *lps* locus, as the C3H/HeJ carries a missense mutation in the *lps* locus, resulting in hyporesponsiveness to LPS. The *lps* gene was found to correspond to the gene encoding the Toll-like receptor-4 (Tlr-4; Poltorak, 1998; Qureshi, 1999; Hoshino, 1999). This was supported by the phenotype of the Tlr-4 gene targeted mice, which resembled the phenotype of C3H/HeJ mice (Hoshino, 1999). The mechanisms whereby the *lps* locus is involved in UVB susceptibility are as yet unclear. It was demonstrated that although C3H/HeJ mice did not produce TNF-α after stimulation with LPS, UV irradiation of epidermal cells and macrophages from C3H/HeJ elicited high TNF-α production (Bazzoni, 1994).

Studies on the parameters that dictate UVB susceptibility are important to our understanding of the mechanisms causing the deleterious effects of UVB irradiation. In this study, we therefore compared LPS-responder and nonresponder mouse strains in the systemic CHS model for UVB-induced immunosuppression using UVB dose response curves. Furthermore, we determined if the suppressed CHS response correlated with lymph node size and composition and with the modulatory effect of UVB on Th1-associated IFN-γ production.

MATERIAL AND METHODS

Mice

Specific pathogen free C3HeB/FeJ and C3H/HeJ mice (female, 10 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The C3HeB/FeJ and the C3H/HeJ are congenic and have a common origin. The mice were housed in macrolon cages with unlimited access to standard mouse chow and tap water. Each experimental group consisted of at least five mice. The experiments were approved by the Animal Experiments Committee of the National Institute of Health and the Environment.

UVB irradiation

One day prior to UVB irradiation, dorsal hair was removed by electric clippers. Two circular areas (6.3 cm^2 total) on the backs of the mice were irradiated and the ears were protected from exposure. The UV source was a Kronmayer UV lamp (Hanovia Ltd., Slough, UK) that was equipped with a Schott WG 305 filter. The lamp had an irradiance of 140 $J/m^2/s$ in the UV range. Mice were irradiated for 4, 8 or 16 sec on the shaved backs on 5 consecutive days. The UVB dose received by the mice was suberythemal for the mouse strains used. Control mice were treated similarly but were not exposed to UV.

Contact sensitization

Picrylchloride (PCl) was used as a contact sensitizer (Chemotronix, Swannanoa, NC, USA) and was recrystallized three times from methanol/H_2O before use and protected from light during storage at 4°C. The mice were skin sensitized 4 days after the last day of irradiation by topical application of 150 µl of 5% PCl in ethanol/acetone (3:1) on the abdomen ('systemic' protocol). Control mice were sham sensitized by topical application of 150 µl of ethanol/acetone (3:1). Four days after the sensitization, both ears of the mice were challenged by topical application of one drop (27-gauge needle) of 0.8% PCl in olive oil. Prior to challenge and 24h after challenge, duplicate measurements of ear thickness were made using an engineer's micrometer (Mitutoya digimatic 293561, Veenendaal, the Netherlands).

Stimulation of spleen and lymph node cell suspensions

Skin-draining lymph nodes (inguinal and axillary) and spleens of control and UVB-irradiated mice were removed under aseptic conditions and single cell suspensions were prepared. All cultures were performed in RPMI 1640 medium supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 µg/ml streptomycin, 1 mM pyruvate, 50 µM 2-ME and 10% heat inactivated FCS.

The cells were seeded at a concentration of 1 x 10^6 cells/ml in flat bottom plates (96-wells) and stimulated with plate-bound anti-CD3 mAb (145-2C11; 25 µg/ml). At 48h, the supernatants were harvested and the concentration of IFN-γ was determined by ELISA. Spleen cell cultures were stimulated with 10 µg/ml LPS (Difco Laboratories, Detroit, MI, USA; LPS E. coli O26:B6) and were harvested at 24 h. The concentration of TNF-α, IL-6, IL-1 and IL-10 were measured in the supernatant by ELISA. Cultures were performed in triplicate or quadruplicate.

Cytokine ELISA

For the detection of cytokines by ELISA, mAb against IFN-γ (XMG1.2; R46A2), IL-6 (MP5-32C11; MP5-20F3, IL-10 (SXC-1; JES5-2A5.1) were used. Flat bottom microplates (96-wells, Falcon 3912, Microtest II Flexible Assay Plate; Becton Dickinson, Oxnard, CA, USA) were coated with capture antibody diluted in PBS (1 μg/ml 20F3 or SXC-1 or XMG1.2) at 4°C for 18h. After coating, the plates were washed (PBS, 0.1% BSA, 0.05% Tween-20) and blocked with PBS supplemented with 1% BSA at room temperature for 1h. After washing, samples and standards were added and incubation was continued for at least 4h at room temperature. Thereafter, the plates were washed and biotinylated detection antibodies were added (1 μg/ml 32C11; 0.1 μg/ml 2A5.1; 0.1 μg/ml BVD6.24G2 or 1 μg/ml R46A2, respectively) and incubated overnight at 4°C. After washing, streptavidin-peroxidase (1/1500 diluted, Jackson Immunoresearch, West Grove, PA, USA) was added. After 1h, the plates were washed and the reaction was visualized using 2,2'-azino-bis(3-ethylbenz-thiazo-line-6-sulfonic acid) (ABTS; 1 mg/ml, Sigma). Optical density was measured at 414 nm, using a Titertek Multiscan (Flow Labs, Redwood City, CA, USA). The amounts of TNF-α were measured with a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA). The detection limits of the various ELISA were for IFN-γ: 400 pg/ml, TNF-α: 15 pg/ml, IL-10: 80 pg/ml and IL-6: 4 U/ml.

IL-1 bioassay

IL-1 activity was measured by bioassay using a sub-line of the murine T cell line D10.G4.1, designated D10(N4)M (D10) (kindly provided by Dr. S.J. Hopkins, Manchester, UK; Hopkins, 1989). Proliferation of the D10 cells was measured via [^{3}H]-thymidine incorporation. Recombinant IL-1β (UBI, Lake Placid, NY, USA) served as a positive control. IL-1 activity was corrected for background activity of the culture medium, and expressed in counts per minute (cpm).

Statistics

Levels of significance were calculated using the two-tailed Student's t-test. $p < 0.05$ was taken as a significant difference between groups. Each group consisted of at least 5 mice.

RESULTS

C3H/HeJ mice are LPS-nonresponder mice

In order to verify their LPS-respondership, spleen cells from both C3H/HeJ and C3HeB/FeJ strains were stimulated *in vitro* by LPS and the resulting cytokine production was determined in the supernatant. The results showed (Table I) that the C3HeB/FeJ mice produced TNF-α, IL-6, IL-1 and IL-10 after LPS stimulation, whereas spleen cells from C3H/HeJ mice responded only minimally to LPS with the production of cytokines.

Table 1: Spleen cell suspensions from C3HeB/FeJ and C3H/HeJ mice were stimulated with LPS and cultured for 24h. The supernatant was tested by ELISA (TNF-α, IL-6, IL-10) or bio-assay (IL-1). The results are expressed as mean production levels ($\pm$ S.D.) of 4 replicate wells.

		C3HeB/FeJ		C3H/HeJ	
TNF-α	(pg/ml)	405.2	(56.0)	82.3	(23.1)
IL-6	(U/ml)	3.6	(1.0)	0.0	(0.0)
IL-1	(U/ml)	0.43	(0.18)	0.13	(0.03)
IL-10	(pg/ml)	348.0	(80.1)	60.3	(12.5)

Systemic effects of UVB on CHS responses

To assess the UVB sensitivity of C3HeB/FeJ and C3H/HeJ mice to systemic immuno-suppression we measured CHS responses. C3HeB/FeJ and C3H/HeJ mice were irradiated on the dorsal skin with varying doses of UVB for 5 consecutive days. The systemic suppression of the CHS response was determined by applying PCl on the non-irradiated abdominal skin 4 days after UVB irradiation. Another 4 days after sensitization the ears were challenged and the ear swelling was measured 24h later. The results of a representative experiment are presented in Figure 1. The CHS response in non-irradiated C3H/HeJ mice (0 sec UVB) was more vigorous than the response in C3HeB/FeJ mice. A UVB-dose dependent reduction of the CHS response was observed for both strains. This UVB-induced reduction was more prominent in C3HeB/FeJ than in C3H/HeJ mice. After 8 sec of irradiation, the CHS response was reduced by 84% in C3HeB/FeJ mice, whereas it was only mildly suppressed in C3H/HeJ mice (28% suppression). After 16 sec the CHS responses in both strains were almost completely suppressed to baseline levels.

These results indicate that C3HeB/FeJ mice are more sensitive than C3H/HeJ to the effects of UVB on the CHS response in the systemic protocol.
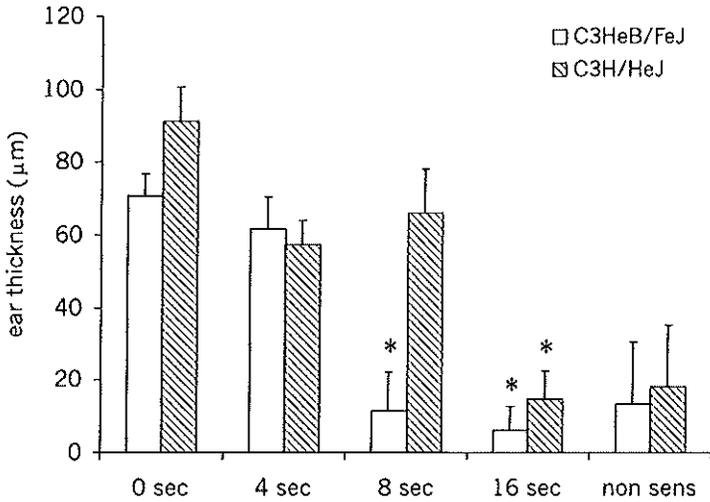
Figure 1: CHS response to picrylchloride in C3HeB/FeJ (open columns) and C3H/HeJ (hatched columns) mice 24h after topical ear challenge. *p<0.05 compared with non-irradiated mice (0 sec). Mice were exposed on 5 consecutive days for 0, 4, 8 or 16 sec to UVB (representing 0, 560, 1120, 2240 J/m^2/day) on the shaved dorsal skin, and sensitized on the abdominal skin (systemic protocol).

Effect of UVB on number of lymph node cells

Next, we determined if the differential susceptibility to UVB in these strains was also reflected in the number of lymph node cells after UVB irradiation. Figure 2 shows the results of a representative experiment. In the systemic protocol, C3HeB/FeJ mice respond with an increase in the number of lymph node cells after 4 sec, whereas C3H/HeJ mice show the first significant increase after 16 sec. These data are inversely related to the magnitude of the CHS response. Sensitization and challenge of mice resulted in a strong increase of cell number, suggesting that hapten-loaded APC are present in the LN, and evoke antigen-specific T cell activation.

In all experimental groups no significant changes were observed in the distribution of total lymphocytes (CD3, B220) and their subsets (CD4, CD8) after UVB exposure as determined by flow cytometry (data not shown).

Effect of UVB on IFN-γ production by lymph node cells

A number of studies have shown that the UVB-induced inhibition of CHS responses was accompanied by a reduction of IFN-γ production (Simon, 1994; Garssen, 1999). We therefore examined the production of IFN-γ after stimulation with plate-bound anti-CD3 mAb
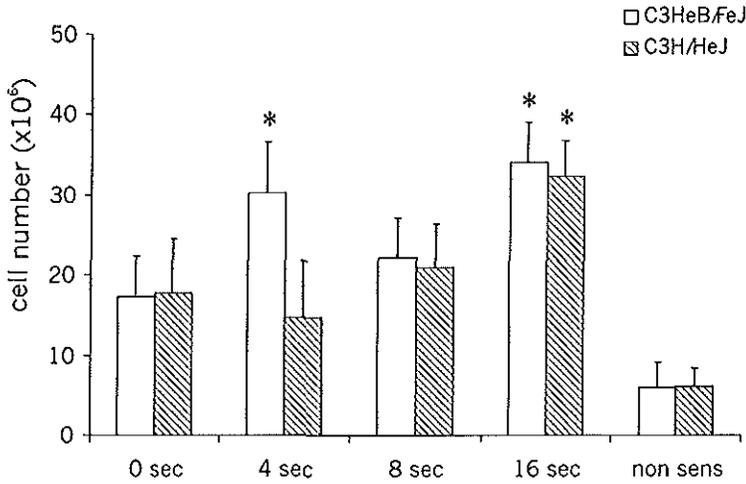
Figure 2: Total numbers of draining lymph node cells were determined of C3HeB/FeJ (open columns) and C3H/HeJ (hatched columns) mice treated by the systemic protocol for UVB-induced immunosuppression (see legend to Figure 2). * p<0.05.

for both C3H/HeJ and the C3HeB/FeJ mice in the systemic model for UVB-induced immuno-suppression. As depicted in Figure 3, a reduction of the IFN-γ production by lymph node cells was observed in C3HeB/FeJ mice starting at a daily exposure of 8 sec. C3H/HeJ mice did not
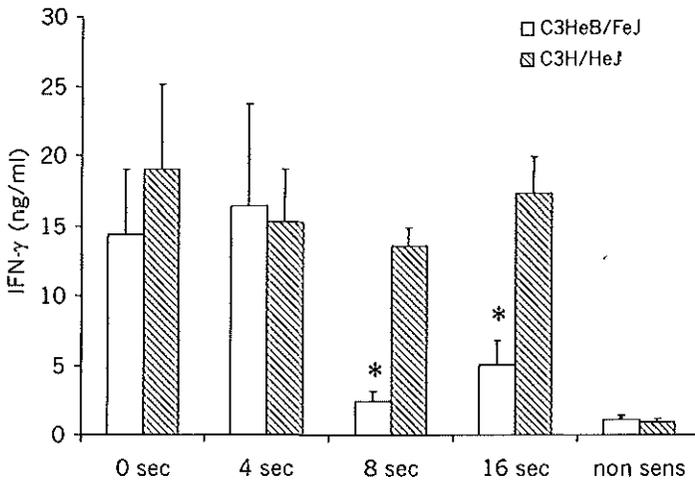

Figure 3: IFN-γ production of anti-CD3 mAb stimulated lymph node cells from C3HeB/FeJ (open columns) and C3H/HeJ (hatched columns) mice. The supernatant was harvested after 48h and IFN-γ levels determined by ELISA. The mice were treated by the systemic protocol for UVB-induced immunosuppression (see legend to Figure 2). * p<0.05.

show any reduction in IFN-γ production at these UVB doses.

Also when assessing the IFN-γ production in the spleen, a similar pattern of UVB-induced modulation of the IFN-γ levels was observed (Figure 4). Stimulated splenocytes of C3HeB/FeJ mice showed a reduction of IFN-γ production after 8 sec of UVB, whereas, again, no significant UVB-induced changes were observed after stimulation of C3H/HeJ spleno-cytes.
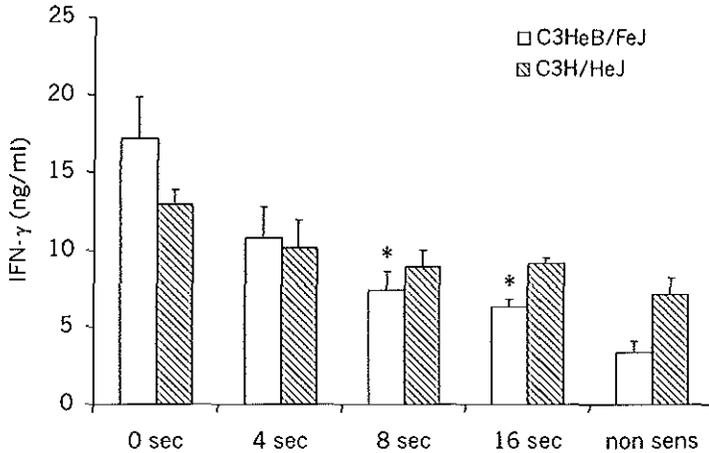

Figure 4: IFN-γ production of anti-CD3 mAb stimulated spleen cells from C3HeB/FeJ (open columns) and C3H/HeJ (hatched columns) mice. For details see legend to Figure 3. * p<0.05.

DISCUSSION

The results described in this report show a number of phenomena that have not received much attention in photo-immunology. First of all, we determined UVB-dose response curves for LPS-responder and nonresponder mice in the systemic CHS protocol. Secondly, we compared the UVB-induced effects on the CHS response with the effects on IFN-γ production and the degree of lymph node hyperplasia.

Using varying UVB doses and the same amount of hapten, we found that the C3H/HeJ strain was more resistant in the systemic protocol as compared to the congenic, LPS-respond-ing C3HeB/FeJ strain. Since we observed that LPS-responder mice were more susceptible to UVB than LPS-nonresponder mice, we examined the cellular responses in the lymph nodes. One of the effects of UVB irradiation on the skin is an increase of the number of cells in the skin-draining lymph nodes. A UVB-dose dependent increase of the numbers of lymph node cells was detected in both strains, yet with different sensitivity, which reflected the suppres-sion of the CHS responses. This demonstrates that triggering of the immune system occurs in both strains after UVB irradiation.

It is generally accepted that UVB exposure inhibits the activity of Th1 cells, which is reflected by reduced production of IFN-γ by lymph node cells (Garssen, 1999). Indeed, when we examined C3HeB/FeJ mice we found a UVB-dose dependent reduction of IFN-γ production. This effect on IFN-γ production showed the same dose-response pattern as the suppression of CHS and the hyperplasia of the lymph nodes. This simultaneous effect of UVB on these 3 parameters was also observed in BALB/c and C57BL/6 mice (data not shown). However, no significant effect of UVB exposure on IFN-γ production was observed in C3H/HeJ mice. Even after 16 sec of UVB, when the CHS respsonse was inhibited dramatically, no significant effect was seen on the production of IFN-γ. Apparently, CHS responses can be uncoupled from the capacity for Th1 differentiation in C3H/HeJ mice after UVB irradiation. We also determined the effect of UVB irradiation on IFN-γ production in the spleen and found similar results.

In contrast to our findings, Noonan et al. found that the C3H/HeJ and the C3H/HeN strains did not differ significantly as determined by UVB dose response curves (Noonan, 1994). These authors applied a single UVB dose, whereas we exposed mice for 5 consecutive days. Furthermore, the dose and choice of hapten used for sensitization is another important factor (Kurimoto, 1993; Miyauchi, 1995). It was found by Yamawaki et al. that by lowering the sensitizing dose of hapten, UVB irradiation was able to suppress the local CHS response in C3H/HeJ mice, showing that these mice are not entirely resistant to the effects of UVB (Yamawaki, 1997). Furthermore, the UVB sources used in Noonan's and our study were different (FS40 vs. Kronmeyer lamps). Together, these experimental differences may explain the differences between our study and that of Noonan et al.

It therefore appears that the mutation of Tlr-4 at the *lps* locus also has consequences for the susceptibility to the systemic UVB-induced suppression. At present, no evidence has been provided on the underlying mechanism of how the *lps* locus (i.e. the mutation in the Tlr-4 gene) is involved in UVB sensitivity. A study by Yoshikawa et al. suggested a possible defect in the production of TNF-α as the main contributor to the different susceptibility (Yoshikawa, 1990). However, the TNF-α production by murine epidermal cells after a single dose of UVB was identical for LPS-responder and LPS-nonresponder mice (Kochevar, 1994). In another study using C3H/HeJ macrophages, it was demonstrated that no TNF-α was produced after LPS stimulation, but significant amounts of TNF-α were produced after exposure *in vitro* to UV light. Thus, the LPS mutation has no effect on the cellular sensitivity to UV light (Bazzoni, 1994). After intradermal injections of TNF-α, the density of Ia^{+} cells in the epidermis was less reduced (Vermeer, 1990) and the CHS less inhibited (Yoshikawa, 1990) in LPS-nonresponder as compared to LPS-responder mice. This suggests that although C3H/HeJ mice are able to produce TNF-α, their response to this cytokine is diminished. Recently, it was reported that heat shock protein 60 (hsp60) is a putative endogenous ligand of Tlr-4, and that macrophages of C3H/HeJ mice are nonresponsive to hsp60 (Ohashi, 2000). Since hsp is expressed in the skin and liberated during cell damage, e.g. damage induced by UVB (Maytin, 1995; Zhou, 1998; Birk, 1999), it is likely that lack of hsp60 signaling results in deviant cellular responses after UVB exposure of the skin.

A number of studies have demonstrated that the mutation of the Tlr-4 gene in C3H/HeJ mice has severe consequences for the immune status of these mice (Vogel, 1992). Especially, the APC compartment of these mice is affected due to the inability to respond to LPS. The systemic UVB-induced suppression of the CHS response is a complex model, since the response involves migration of non-primed APC from the UVB-irradiated skin and hapten-primed APC from the non-UVB-irradiated skin. In the lymph nodes of C3HeB/FeJ mice, the activation of T cells by these two APC populations results in immune activation, indicated by increase in lymph node size, concommitant with suppression of IFN-γ production. Bystander activation of T cells by the non-hapten primed UVB-irradiated APC apparently alters the IFN-γ productionpotential by T cells in the lymph nodes. This could either be achieved by cytokines (like IL-10, IL-12, TNF-α) produced by the UVB-irradiated APC or by modified expression of surface molecules (like CD80, CD86, CD40). In the lymph nodes of C3H/HeJ mice, immune activation takes place as demonstrated by the increase in lymph node size, whereas no detectable effects of UVB on the IFN-γ production are observed. Apparently, APC from the UVB-irradiated skin of C3H/HeJ can not affect the Th1 cell responses in the lymph node. This can be explained by either reduced migration of these cells towards the lymph nodes, or reduced ability of these cells to modulate the hapten-specific response.

Taken together, the reduced UVB sensitivity in C3H/HeJ mice appears to be due to the inability of APC from the UVB-irradiated skin to modulate the Th1 cell activity in the lymph nodes. Furthermore, our findings that reduction of CHS responses in C3H/HeJ mice and inhibition of IFN-γ production are not necessarily linked, suggest that in LPS-nonresponder mice alternative mechanisms, like a direct effect of APC or keratinocyte derived immunosuppressive cytokines, may be involved in the UVB-induced reduction of the CHS response.

The results presented in this study demonstrate that the Tlr-4 mutation influences the systemic UVB-induced immunosuppression. The lack of signaling via Tlr-4 results in reduced sensitivity to UVB. Exposure to relatively high UVB doses can suppress the CHS response and evoke lymph node hyperplasia, but is unable to inhibit the activity of Th1 cells in Tlr-4 defective mice. These findings suggest that the differential susceptibility to UVB in LPS-responder and LPS-nonresponder mice is due to modulated migration or activating-potential of APC by UVB, rather than specific defects in the production of pro-inflammatory cytokines in the skin.

ACKNOWLEDGEMENTS

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Chapter 6

Differential UVB-induced modulation of cytokine production in XPA, XPC and CSB DNA-repair deficient mice

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SUMMARY

Ultraviolet B irradiation has dramatic consequences for cellular immunity and can suppress the rejection of skin tumors and the resistance to infectious diseases. DNA damage plays a crucial role in these immunomodulatory effects of UVB, since repair of DNA damage has been shown to prevent immunosuppression. UVB-induced DNA damage is repaired by the nucleotide excision repair (NER) mechanism, which comprises two subpathways: transcription-coupled and global genome repair. In this study we examined the immunological consequences of specific defects of NER genes in 3 mouse models: XPA, XPC and CSB mutant mice. XPA carries a specific defect in both NER subpathways, while XPC and CSB mice only lack global genome and transcription-coupled NER, respectively.

Our data demonstrate for the first time that cellular immunity in XPA, XPC and CSB mice is normal as compared to wildtype mice. This indicates that the reported altered cellular responses in XP patients are not constitutive effects but are due to external factors, like UVB.

Of the three DNA-repair deficient mouse strains tested, only XPA mice are sensitive to UVB-induced inhibition of Th1-mediated contact hypersensitivity responses and IFN-γ production. LPS-stimulated TNF-α and IL-10 production, as well as the cellularity in the lymph nodes, were significantly augmented in both XPA and CSB mice after UVB exposure. XPC mice did not exhibit enhanced UVB susceptibility with regard to immune responses.

These data indicate that both global genome repair and transcriptional coupled repair are needed to prevent immunomodulation by UVB, while transcription coupled repair is the major DNA-repair sub-pathway of NER that prevents the acute UVB-induced effects.

INTRODUCTION

Exposure of the skin to ultraviolet B (UVB) light is well known to have deleterious effects on human health. Epidemiological studies indicate that exposure to UVB increases the incidence of non-melanoma skin cancer (Kricker, 1995; Urbach, 1997). In addition, UVB irradiation impairs specific and non-specific immune responses locally (in the skin) as well as systemically. UVB irradiation can induce antigen-specific unresponsiveness by mechanisms involving impaired antigen presentation to T helper (Th) cells, resulting in a shift from Th1- to Th2- mediated responses (Boonstra, 1997; Schwarz, 1999). This may explain the observed UVB-induced suppression of Th1-driven cellular immune responses, like contact hypersensitivity (CHS) and delayed type hypersensitivity (DTH) responses.

Numerous studies have demonstrated that the induction of DNA damage plays a crucial role in the UVB-induced immunosuppression. The repair of UV-induced DNA damage by T4 endonucleases prevents local immunosuppression and inhibits the induction of cytokine production (Kripke, 1996). Furthermore, immunosuppression could be induced by

the introduction of DNA strand breaks in epidermal cells by restriction endonucleases (O'Conner, 1996; Nishigori, 1998). DNA damage alone is sufficient to trigger epidermal cells to produce tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10, thereby modulating the activity of Langerhans cells and subsequently the local immune response (O'Conner, 1996; Nishigori, 1996; Petit-Frere, 1998).

The majority of DNA lesions induced by UVB are cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6-4) photoproducts. These lesions are repaired by the nucleotide excision repair (NER) mechanism, a complex 'cut and paste' reaction involving more than 25 proteins.

Two subpathways of NER are known: transcription-coupled repair (TCR) and global genome repair (GGR). TCR is designed to clean the transcribed strand of active genes from lesions that block transcription. GGR eliminates lesions anywhere in the genome, including the non-transcribed strand. UV-induced CPD are hardly removed by the GGR subpathway, in contrast to (6-4) photoproducts which are removed even faster by GGR than by TCR (Cleaver, 1997; Bootsma, 1998; Citterio, 2000).

The importance of the NER system in repair of DNA damage is illustrated by the rare, hereditary human NER-deficient syndromes Xeroderma pigmentosum (XP) and Cockayne syndrome (CS). At least 8 different gene defects have been described in XP (XPA to XPG and a variant), of which XPA is the most common form of the disease. XP patients are extremely sensitive to UV light and have a more than 1000-fold higher risk of developing skin cancer. In addition, they exhibit pigmentation abnormalities in sun-exposed areas of the skin (Kraemer, 1987; Bootsma, 1998). CS is characterized by photosensitivity, neuronal dysmyelination and skeletal abnormalities. CS patients are not cancer-prone (Nance, 1992).

A number of studies indicate that XP patients have an impaired cellular immunity with reduced DTH and CHS responses (Dupey, 1974; Salomon, 1975; Wysenbeek, 1986). However, there are some discrepancies on the immunological alterations in XP patients. Normal numbers of peripheral T cells (Norris, 1990; Gaspari, 1993), as well as reduced numbers have been reported (Mariani, 1992). In contrast to XP patients, no specific immune defects have been reported for CS patients (Norris, 1990; 1991; Nance, 1992). It is difficult from patient studies to conclude whether the possible immune defects in XP patients are either UV-induced effects or related to active disease (some patients had skin tumors) or constitutive effects due to the defect in the NER genetic machinery. Moreover, in CS patients interpretation may be even more complicated due to the multi-systemic character of the disease.

To discriminate between the different possible causes of immune suppression in NER-deficient individuals, we examined the effect of UVB irradiation on various immune parameters in the transgenic mouse models for XPA, XPC and CSB (de Vries, 1995; Cheo, 1997, van der Horst, 1997). Comparison of these 3 mouse models permits a systemic analysis of the contribution of the TCR and the GGR to the UVB-induced immunomodulation. XPA mice have a complete NER defect, meaning that both TCR and GGR are deficient. XPC mice carry a specific deficiency in the GGR, whereas CSB mice have impaired TCR with a normal, func-

tional GGR. We focussed on the cytokine production by T cells and antigen presenting cells (APC). Recently, we and others showed that XPA and CSB mice were very sensitive for the local UVB effect like erythema, while only XPA mice were susceptible to UVB-induced immunosuppression (Miyauchi, 1996; Garssen, 2000). XPC mice did not show any signs of augmented UVB sensitivity as compared to normal littermates.

Our findings show that the immune status of non-irradiated XPA, XPC and CSB mice is not detectably affected by the mutation. However, the UVB-induced cytokine production is severely affected in XPA mice, moderately affected in CSB mice and similar to wildtype littermates in XPC mice. These findings obtained from mouse models suggest that the immune defects in XPA, XPC and CSB patients are UVB-induced and are not intrinsic effects due to the NER mutation. Furthermore, the data indicate that transcription coupled repair is the major repair pathway that prevents the UVB-induced acute effects.

MATERIAL AND METHODS

Mice

XPA, XPC and CSB mice refer to animals homozygous for the targeted allele in the respective genes (de Vries, 1995; Cheo, 1997, van der Horst, 1997). C57BL/6 or hybrid 129-C57BL/6 littermates of homozygous knockout mice were used as control animals (Garssen, 2000). The genotype of each mouse was determined by PCR as described in the original references. All mice were bred at the animal facility of the National Institute of Public Health and the Environment (Bilthoven, the Netherlands). The mice were kept under specific-pathogen-free conditions and were 8-10 weeks old at the start of the experiments. The room was illuminated with yellow fluorescent tubes (Philips TL40W/16), which did not emit any measurable UV radiation. No daylight entered the animal facilities. The experiments were approved by the ethical committee of the National Institute of Public Health and the Environment.

mAb and reagents

All cell cultures were performed in RPMI 1640 medium supplemented with 25 mM HEPES, 100 IU/ml penicillin, 50 µg/ml streptomycin, 1 mM pyruvate, 50 µM 2-mercapto-ethanol and 10% heat-inactivated fetal calf serum. For polyclonal T cell stimulation, con-canavalin A (ConA; Janssen Chimica, Beerse, Belgium) or anti-CD3 mAb (145-2C11) were used. For the detection of cytokines by ELISA, mAb against IFN-γ (XMG1.2; R46A2), IL-10 (SXC-1; JES-2A5.1) and IL-4 (11B11; BVD2.4G2) were used. All recombinant mouse cytokines were obtained from R&D Systems (Minneapolis, MN, USA). LPS was obtained from Difco Laboratories (Detroit, MI, USA; LPS E. coli O26:B6). Picrylchloride (PCl) was

used as a contact sensitizer (Chemotronix, Swannanoa, NC, USA) and was recrystallized three times from methanol/H_2O before use and protected from light during storage at 4°C.

UVB irradiation

The animals were shaven (on the back) one day prior to UV exposure using an electric clipper under light ether anesthesia. The UV source was a Kronmayer UV lamp (Honovia Ltd, Slough, UK) equipped with a Schott WG 305 filter. The lamp had an irradiance of 140 $(J/m^2)/s$ in the UV range (280-400 nm), as measured by a Kipp E11 thermopile. Two circular areas (6.3 cm^2 in total) were irradiated for 1, 2, 4 or 6 seconds each day for 5 consecutive days. Twenty-four hours after the last exposure mice were sacrificed and spleens and lymph nodes were dissected. Control mice were shaven but not exposed to UV.

CHS responses

The mice were skin-sensitized 4 days after the last UVB irradiation by topical application of 150 μl of 5% PCl in ethanol/acetone (3:1) to the non-UVB-irradiated shaved abdomen, chest and feet. Control mice were sham-sensitized by topical application of ethanol/acetone (3:1). Four days after the sensitization, both ears of the mice were challenged by topical application of one drop (27-gauge needle) of 0.8% PCl in olive oil. 24 hours after challenge, duplicate measurements of the ear thickness were made using an engineer's micrometer (Mitutoyo digimatic 293561, Veenendaal, the Netherlands).

In vitro stimulation of lymph node and spleen cells

Draining lymph nodes (inguinal and axillary) and spleens of control and UVB-irradiated mice were removed under aseptic conditions and single cell suspensions were prepared. Erythrocytes were removed by incubating with Gey's medium for 2 minutes on melting ice. The isolated lymphocytes were seeded at a concentration of 1 x 10^6 cells/ml and stimulated with ConA (5 μg/ml) or plate-bound anti-CD3 mAb (10 μg/ml) in 96-wells flat bottom plates. At 48h, the supernatants were harvested and the concentration of cytokines was determined by ELISA. To assess anti-CD3 mAb and LPS induced proliferation, [^{3}H]-thymidine was added to separate cultures after 48h and 72h, respectively. Twenty hours later, the cells were harvested and [^{3}H]-thymidine incorporation was measured. LPS-induced cytokine production was determined in supernatant of lymphocyte cultures stimulated for 24h (10 μg/ml LPS). The cells were cultured in 24-well flat bottom tissue culture plates at a concentration of 2.5 x10^5 cells/ml. All cultures were performed in triplicate or quadruplicate.

Flow-cytometric analysis

2×10^5 cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide. For the staining of surface antigens, spleen cells were incubated with FITC- or PE-conjugated mAb against CD3 (145-2C11), CD4 (GK1.5), CD8 (Lyt-2), B220 (RA3.6B2), F4/80, NK1.1 and MHC-II (M5/114; all obtained from PharMingen, San Diego, CA, USA). After washing twice with PBS-BSA-azide, the cells were resuspended and analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA). 10 µl propidium iodide (0.2 µg/ml) was added to evaluate the viability of the cells. 10^4 events were collected and the expression of the markers analyzed using CellQuest software (Becton Dickinson).

Cytokine ELISA

IFN-γ and IL-10 were determined by ELISA as described previously (MacNeil, 1990; Chatelain, 1992). Briefly, 96-wells microplates were coated with capture antibody (1 µg/ml SXC-1; 5 µg/ml XMG1.2) at 4°C for 18h. Incubation of the samples and standards lasted 4 hours. After addition of the biotinylated detection antibodies (0.1 µg/ml 2A5.1; 1 µg/ml R46A2), the plates were incubated overnight at 4°C. The reaction was visualized using streptavidin-peroxidase (1/1500 diluted; Jackson Immunoresearch, West Grove, PA, USA) and ABTS (1 mg/ml, Sigma). Optical density was measured at 414 nm, using a Titertek Multiscan (Flow Labs, Redwood City, CA, USA). The TNF-α concentrations were measured with a commercially available ELISA kit (DuoSet, R&D Systems) according to the protocols provided by the manufacturer. The detection limits of the ELISA were: IFN-γ (20 pg/ml), IL-10 (5 pg/ml) and TNF-α (15 pg/ml).

Statistical analysis

Levels of significance were calculated using the two-tailed Student's t-test. $P< 0.05$ was taken as a significant difference between groups. Each group consisted of at least 5 mice.

RESULTS

Normal T, B and APC activity in NER-deficient mice

Since only limited data are available on the immune status of non-irradiated DNA-repair-deficient mice, a broad scala of immune parameters was determined for XPA, XPC and CSB mice in cell suspensions obtained from their spleens and lymph nodes. We tested *in vitro* ConA- and anti-CD3 mAb-induced T cell proliferation and cytokine production (IFN-γ,

IL-4, IL-10), B cell proliferation, *in vitro* immunoglobulin production, serum immunoglobulin levels (IgM, IgG1, IgG2a, IgE) and LPS-induced cytokine production (TNF-α, IL-6, IL-10). We did not find major changes in any of these parameters in XPA, XPC and CSB mice as compared to their wildtype littermates (data not shown). In addition, subset ratios were determined by flow cytometry (CD3, CD4, CD8, B220, NK, F4/80) and again no significant differences were found when comparing the mutant mice and their respective wildtype littermates. These findings suggest that the *in vivo* APC and T cell compartments of XPA, XPC and CSB mice are not detectably affected by the different NER gene defects.

Contact hypersensitivity responses in DNA-repair deficient mice

Besides these *in vitro* assays, we also examined the *in vivo* CHS responses to picrylchloride. As shown in Figure 1, ear swelling in non-irradiated DNA-repair deficient mice (0 J/m^2) was similar to the response in control littermates. We recently showed that besides the CHS reaction to picrylchloride also the DTH response to *Listeria monocytogenes* was similar in XPA, XPC and CSB mice as compared to their wildtype littermates (Garssen, 2000). Together, these data support the finding that both APC- and T cell-driven responses are normal in these mice.

With respect to the sensitivity to UVB, Figure 1 shows that XPA mice were extremely sensitive and demonstrate suppression of the CHS responses at doses as low as 140 J/m^2.
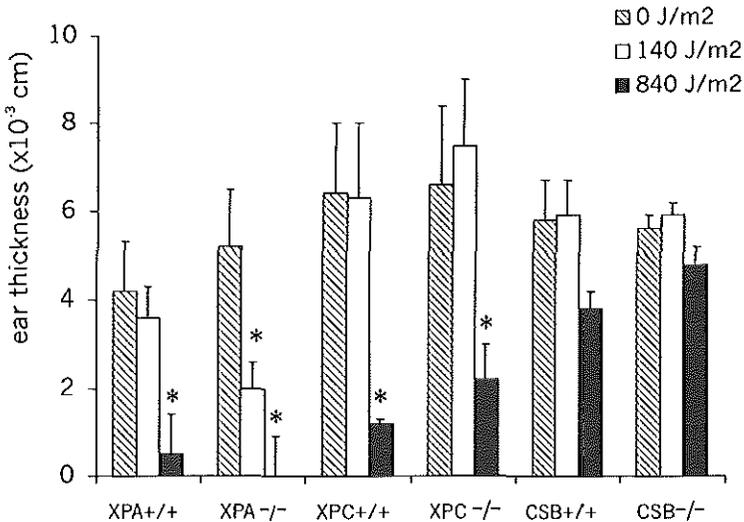
Figure 1: UVB-induced systemic suppression of CHS to picrylchloride in NER-deficient mice and their repair-competent littermates. Each bar represents the mean value of the corrected ear swelling (i.e. swelling in sensitized minus swelling in non-sensitized animals of the same strain) of at least 5 mice ± SEM. Units are 10^{-3} cm. P-values reflect the comparison with the non-irradiated group (0 sec UVB). * p< 0.05.

At this dose both XPC and CSB mice displayed normal CHS responses. A significant reduction was seen at 840 J/m^2 UVB in both XPC and, to a lesser extent, CSB mice, as well as their respective littermates. It can be concluded that XPC and CSB mice did not show an enhanced sensitivity to UVB exposure with respect to the suppression of CHS responses, whereas XPA mice were extremely sensitive.

Effect of UVB on the IFN-γ production in DNA-repair deficient mice

A number of studies have indicated that UVB irradiation inhibits the activation of Th1 cells in the lymph nodes as demonstrated by the reduced production of IFN-γ.

We determined the UVB-induced modulation of ConA-induced IFN-γ production in the lymph nodes of XPA, XPC and CSB mutant mice to examine their differential UVB susceptibility. Figure 2 shows the results of a representative experiment. The IFN-γ production by XPA lymphocytes displayed a significant reduction after a dose of 140 J/m^2 UVB, whereas the levels in the wildtype littermates were not significantly changed. The IFN-γ production of both XPC and CSB lymphocytes were not inhibited after 140 J/m^2 UVB, but showed a reduction after a UVB dose of 840 J/m^2, which is similar to the response seen in littermates. Taken together, the UVB-induced changes of the IFN-γ production in these mice reflected the pattern seen in the CHS responses.
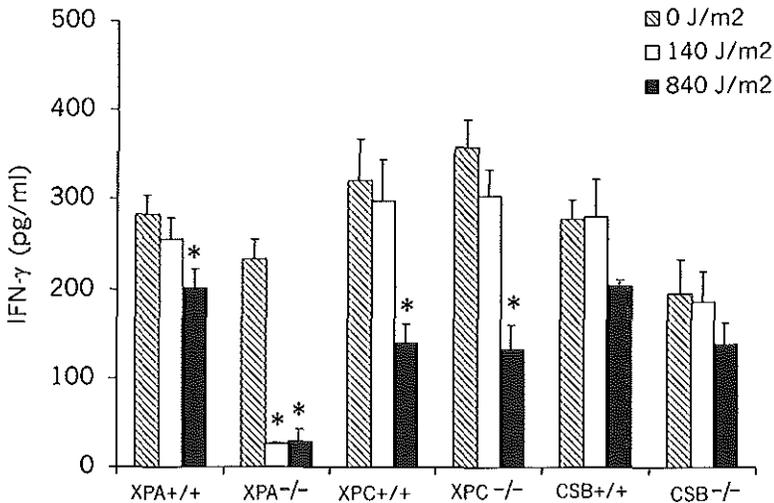

Figure 2: UVB-induced effects of IFN-γ production by lymph node cells stimulated with ConA in the picrylchloride-sensitized mice (see Figure 1). Each bar represents the mean value of at least 5 mice ± SEM. P-values reflect the comparison with the non-irradiated group (0 sec UVB). * p< 0.05.

Effect of UVB on lymph node hyperplasia in DNA-repair deficient mice

Next, we determined the effect of UVB on the degree of hyperplasia in the skin-draining lymph nodes of XPA, XPC and CSB mice. As depicted in Figure 3, already after 140 J/m^2 UVB a threefold increase in the number of lymph node cells was observed in XPA mice, whereas no increase was observed in wildtype littermates at any UVB dose used. In XPC mice a slight increase in the number of lymph node cells was seen after UVB irradiation. However, this was also observed in wildtype littermates. Finally, CSB mice showed an increase in the number of lymph node cells after 140 J/m^2 UVB. At higher doses no further increase was detected. No change was seen in the control littermates. The pattern of cellular increase in XPA and CSB mice was identical: a sharp increase after 140 J/m^2 UVB to a plateau value. The augmented response was more vigorous in XPA as in CSB mice.

Flow cytometric analysis of the lymph node cells of all groups for CD4, CD8, CD3, B220 and MHC II did not reveal any significant changes in the ratio of a specific leukocyte subset, indicating that the increase in cell numbers in the lymph node is not due to selective expansion of one or a few specific subsets.
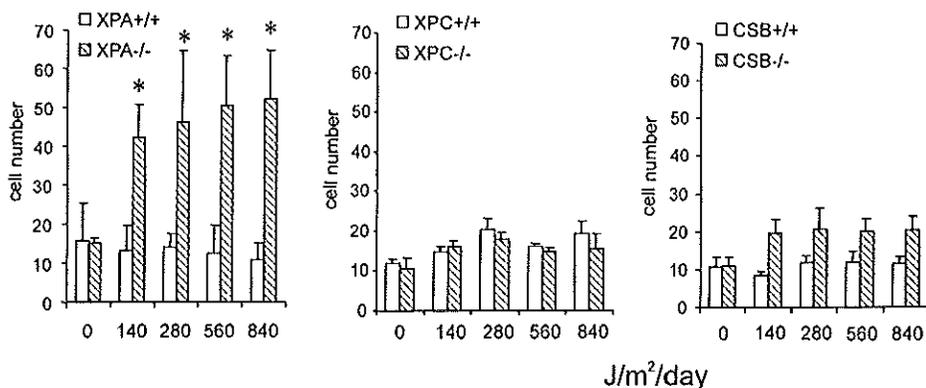

Figure 3: The effect of UVB irradiation on the cellularity of the draining lymph nodes in non-treated NER-deficient mice and their littermates after UVB irradiation. Cell numbers are expressed as mean values x 10^6 ± SEM. P-values reflect the comparison with the non-irradiated group (0 sec UVB). * p< 0.05.

Effect of UVB on TNF-α and IL-10 production in the LN of DNA-repair deficient mice

Since APC (macrophages and dendritic cells) play a pivotal role in governing lymphocyte responses, we determined the effect of UVB on cytokine production by APC in the lymph nodes. In order to detect possible differences between DNA-repair deficient mice and their respective littermates, lymph node cells were stimulated *in vitro* with LPS and the levels of TNF-α and IL-10 were determined by ELISA. As depicted in Figure 4, LPS-stimulat-
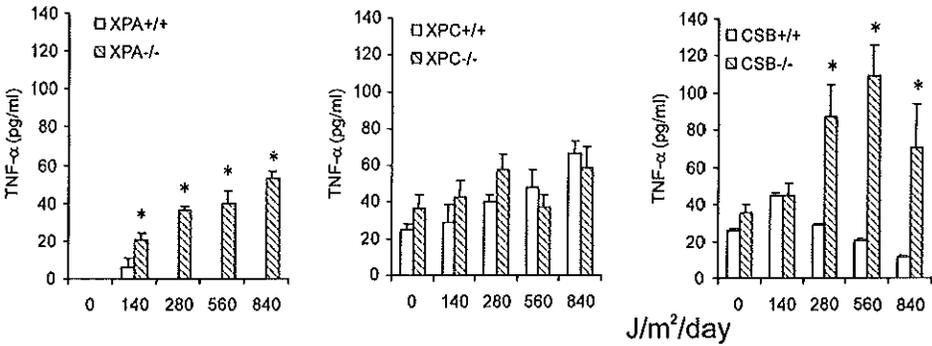
Figure 4: TNF-α production by draining lymph node cells from UVB-irradiated and non-irradiated NER-deficient mice and their littermates. The cells were stimulated with LPS *in vitro*, and the supernatant was harvested after 24h. The levels of TNF-α were determined by ELISA. Each bar represents the mean value of at least 5 mice ± SEM. P-values reflect the comparison with the non-irradiated group (0 sec UVB). * p< 0.05.

ed lymph node cells from XPA and CSB mice showed a significant increase in the production of TNF-α as compared to their littermate controls. In XPA mutants, this increase was already demonstrated after 140 J/m^2 UVB, whereas in CSB mice the response started at 280 J/m^2 UVB. Also XPC mice showed an increase, but again this was also observed in littermates.

The same pattern as described for TNF-α was found for IL-10 (Figure 5): a UVB-dose-dependent increase of IL-10 production in the XPA and CSB mice, whereas the control littermates demonstrated only a mild increase in IL-10 production. In XPC mice, IL-10 was not detectable in any of the cultures. Similar effects on TNF-α and IL-10 production were demonstrated after stimulation of splenocytes (data not shown), indicating that it is not only
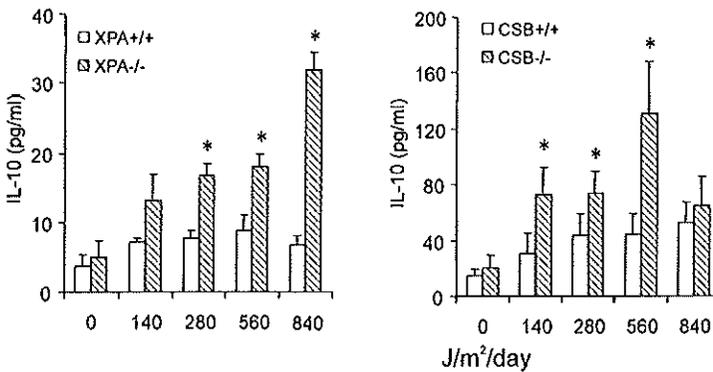

Figure 5: *In vitro* IL-10 production by LPS stimulated lymph node cells from UVB irradiated and non-irradiated NER deficient mice and their littermates. For details see the legend to Figure 4. Stimulation with LPS of lymph node cells from XPC$^{+/+}$ and XPC$^{-/-}$ mice resulted in IL-10 values below the detection limit of the ELISA, and are therefore not graphically depicted. * p< 0.05.

a local event in skin-draining lymph nodes, but has systemic consequences as well.

DISCUSSION

In the present study we examined the immune status of DNA-repair deficient XPA, XPC and CSB mice as well as the UVB-induced immune effects in these mice. We demonstrated that all three transgenic mouse strains have normal APC, T and B cell responses which are identical to their wildtype littermates. After UVB exposure, we found that XPA mice are extremely sensitive to UVB-induced immunomodulation, as demonstrated by the effect on lymph node cellularity and cytokine production by stimulated T cells and APC. Moreover, CSB mice react intermediate and XPC mice are even insensitive to these UVB-induced effects as compared to their respective wildtype littermates.

A number of studies showed immunological abnormalities in XP patients. These included weaker DTH and CHS responses, low *in vitro* response to PHA, reduced IFN-γ production, decreased CD4/CD8 ratios and reduced natural killer cell activity (Morison, 1985; Wysenbeek, 1986; Mariani, 1992; Gaspari, 1993; Norris, 1990). No such immune defects have been described for CS. One of the problems encountered when studying these immune responses in NER-deficient patients is that the differences in their immune status may be either constitutive or due to external factors, such as UVB irradiation. Furthermore, the multisystemic character of the diseases, the small study groups and the intervariability (caused by gender, ethnicity etc.) further complicate the interpretation of data obtained from patient studies. Examination of the NER deficient mouse models for XPA, XPC and CSB provides an excellent tool to determine if the defined genetic defects of NER affect the immune system. Our findings that cytokine production and activity of APC, T and B cells were similar in non-irradiated NER deficient transgenics and their wildtype littermates, suggests that the presence of mutated NER genes by itself has no consequences for the capacity of these cells to generate cellular immunity.

Exposure of DNA-repair deficient mice to UVB revealed that XPA mice are extremely susceptible to systemic suppression of CHS responses and IFN-γ production at low UVB doses, whereas CSB and XPC mice are not more sensitive than wildtype littermates, consistent with data obtained previously (Garssen, 2000). These effects of UVB are well described and reflect an inhibition of the initiation and effector phase of Th1-mediated immune responses, important in a.o. the response to pathogens.

Exposure to UVB has been shown to increase the number of peripheral lymphocytes that migrate into the skin-draining lymph nodes (Spangrude, 1983; Samlowski, 1988). The enhanced migration correlated with an increase in the number and density of high endothelial venules within the lymph nodes (Samlowski, 1988). Both XPA and CSB mice showed an enhanced sensitivity to UVB with respect to the lymph node cellularity.

Furthermore, the LPS-induced TNF-α and IL-10 production by lymph node cells was also enhanced in XPA and CSB mice exposed to low UVB doses. In XPC mice and litter-

mates no UVB-induced changes were detected in these parameters. Since we also observed a comparable modulation of the cytokine production in the spleen, our data extend these findings by showing that the effects are not limited to the local level, but have consequences for the systemic immunosuppression as well. The important role of TNF-α and IL-10 in modulating immune responses has been described extensively (Rivas, 1994; Boonstra, 1997). In the UVB-irradiated skin augmented production of these cytokines has been reported, which was induced by DNA damage directly. Since Langerhans cells migrate to the draining lymph nodes after UVB exposure, and since CPD$^+$ dendritic-like cells are detected in the lymph nodes (Vink, 1996), it is likely that they are the producers of TNF-α and IL-10 in the lymph node. The persistence of DNA damage in these cells may result in long-lasting cytokine expression. The inhibitory activity of TNF-α and IL-10 may add to the immunosuppression as observed after UVB irradiation both locally and systemically. These findings are in agreement with our previous observations that UVB-induced acute skin-reactions (edema and erythema) are absent in XPC mice, while more vigorous in XPA and CSB mice (Garssen, 2000), which is most likely caused by enhanced keratinocyte-derived cytokine production.

The UVB-induced immune effects in XPA mice were more vigorous than in CSB mice, and absent in XPC mice. XPA mice lack both TCR and GGR, whereas XPC and CSB mice are deficient in GGR and TCR, respectively. Functionally, GGR has been associated mainly (although not exclusively) with preventing mutagenesis and carcinogenesis, while TCR activity prevents apoptosis after genotoxic injury (de Boer, 2000). The work presented here clearly demonstrates an additional involvement of these pathways in the UVB-induced immunomodulation. Both TCR and GGR are involved in the UVB-induced immunomodulation (as deduced from the synergistic effects seen in XPA mice) but with the strongest input from TCR (as apparent from the partial effects seen in CSB and the absence of significant abnormality in XPC mice).

Introduction of DNA lesions in the absence of TCR (as evident in XPA and CSB mice) results in a transcriptional block of specific genes. It is likely that the arrest of RNA synthesis of critical genes in epidermal cells dysregulates the homeostasis in the skin and evokes the observed immune effects. Furthermore, apoptosis is another likely mechanism explaining the severe immune effects in XPA and CSB mice. Arrest of RNA synthesis initiates p53-dependent apoptotic pathways (Ljungman, 1996, Yamaizumi, 1994). Therefore one could speculate that the immune system of TCR-deficient mice (i.e. XPA and CSB) mice is triggered by stress-signals, evoked by massive death of keratinocytes in the absence of apoptosis-preventing TCR-activity. Indeed, enhanced numbers of sunburn cells and extensive inflammatory infiltration of lymphocytes have been reported in the UVB irradiated skin of XPA mutant mice (Miyauchi, 1996). Furthermore, the ability of XPC mice to regulate apoptosis of damaged keratinocytes, by means of a functional TCR subpathway, prevents the triggering of immune responses. Indeed, no studies have reported an enhanced UVB sensitivity of XPC mice with respect to immune modulation. It is of interest to note that susceptibility to epidermal hyperplasia and skin cancer is enhanced in XPA and CSB, but also in XPC mice (de Vries, 1995; Sands, 1995; van der Horst, 1997). Apparently, these effects are the consequence

of DNA lesions in either the transcribed or the non-transcribed strand.

Taken together, our data demonstrate that 1) a mutation of the XPA, XPC and CSB genes in non-irradiated gene targeted mice has no significant constitutive effects on immune responses (APC, T or B cell responses). 2) The immunological alterations seen in patients can be ascribed to external factors, like UVB, since we observed susceptibility to UVB in XPA and CSB mice as determined by changes in T cell and APC derived cytokine production. 3) XPC mice showed no increased susceptibility to UVB as compared to their wildtype littermates. 4) Both GGR and TCR are needed to prevent immunomodulation by UVB, while TCR is the major DNA-repair sub-pathway of NER that prevents the acute UVB-induced effects.

ACKNOWLEDGEMENTS

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Chapter 7

General discussion

GENERAL DISCUSSION

The skin forms the principal barrier against environmental assaults, like mechanical stress, chemicals, pathogens and, of course, UVB irradiation. The skin is a dynamic tissue in which also the immune system is well organized (Bos, 1993). Exposure of the skin to UVB irradiation causes DNA strand breaks and mutations, which give rise to neo-antigens expressed on keratinocytes. Damage of the UVB irradiated skin due to autoimmune responses directed against these neo-antigens and excessive inflammation, are prevented by a temporary immunosuppression in the skin. The balance between UVB-induced inflammatory responses and immunosuppression in the skin is delicate. Extremes at both ends may result in adverse reactions. These balances are likely to be disturbed when high doses of UVB are received or when the body is attacked by pathogens. Cytokines are the main determinants of this balance.

In this thesis we examined how UVB affects the cytokine balances systemically. We focussed on the effects of UVB on APC and Th cell subset differentiation and function, and the consequences for systemic Th-driven responses.

Effect of UVB on Th cell subset development

It is generally accepted that UVB irradiation affects the cytokine production by CD4^{+} T cells. We showed that UVB exposure results in the inhibition of IFN-γ production in both the spleen and the LN (chapter 2). Furthermore, we demonstrated in a number of models (CHS, model for occupational asthma, Th1-associated Ig isotype production) that Th1 driven responses are actively suppressed after exposure to UVB. Other studies documented suppression of DTH and a reduced resistance to pathogens as models for UVB-induced reduction of Th1 responses. Thus, our findings support the current thinking.

Based on the described effects on Th1 cells, feedback mechanisms controlling the Th1/Th2 balance might suggest an additional effect on Th2 cells. However, in the literature, the effect of UVB on Th2 cells is less clear (Simon, 1994; Saijo, 1996; Ullrich, 1996). Our results show a strong effect on Th2 cells. We found significantly enhanced IL-4 production and an increase in the percentage of IL-4 producing cells by examining CD4^{+} cells from the spleen of UVB-irradiated BALB/c mice (chapter 2). Other studies, however, could not detect effects of UVB exposure on the cytokine production by Th2 cells (Simon, 1994; Saijo, 1996). In these studies, however, total LN or spleen cell suspensions of C3H and C57BL/6 mice were stimulated and the IL-4 production was assessed. Since these strains have default Th1 dominated responses, high IFN-γ but no IL-4 could be detected in these cultures. We therefore suggest that the inability to detect an effect of UVB irradiation on IL-4 production in these studies was due to the selected mouse strains.

The effect of UVB on Th1 and Th2 driven immune responses

A great body of literature describes effects of UVB on Th1 driven responses, like DTH, CHS and the resistance to pathogens, whereas the effect of UVB on non-skin associated Th1-mediated autoimmune diseases have not gained much interest. A number of such autoimmune diseases, like EAE and IDDM, are due to strong Th1 dominated responses. It is likely that UVB might abrogate these Th1 responses and thereby improve the clinical outcome. Indeed, prevention of the development EAE by UVB irradiation was reported in mice (Hauser, 1984). Also we found, using our experimental set-up with suberythemal doses of UVB, a mild reduction of the clinical severity of the EAE symptoms (PLP model in SJL mice; data not shown). No such studies have yet been conducted on diabetes or other autoimmune diseases in mice.

Because Th1 and Th2 subsets cross-regulate each other, it is conceivable that UVB might exacerbate Th2 driven allergic diseases. We tested this hypothesis and found, to our surprise, that UVB does inhibit Th2 mediated allergic immune responses (chapter 4). More specifically, we found that UVB inhibits the characteristic features of allergy: allergen-specific IgE production and tracheal hyperreactivity. Therefore, the hypothesis that UVB inhibits Th1 and augments Th2 mediated immune responses needs to be adjusted; our data suggests that UVB exposure results in a general immunosuppression. In support of this, we found inhibition of the production of both the Th1 and the Th2 associated immunoglobulin isotypes. We showed that IL-10 was involved in the suppression of the Th1 associated isotypes, but not in the suppression of Th2 isotypes (chapter 2). Inhibition of Th2 driven responses by IL-10 has been reported previously (Grunig, 1997), but apparently this is not applicable to Th2 driven IgE and IgG1 production. We did not examine whether neutralization of IL-10 had an effect on allergy-related symptoms in UVB irradiated mice, like tracheal hyperreactivity.

The UVB-induced suppression of Th2 responses might well be related to TGF-β. Neutralization of TGF-β in cultures of CD4$^+$ cells obtained from UVB irradiated mice increased the IFN-γ production, whereas no effect was observed in cultures with control CD4$^+$ cells (data not shown). These findings point out that TGF-β is produced by CD4$^+$ cells from UVB exposed mice and affects the Th1-Th2 balance. Indeed, a number of studies have shown that a TGF-β secreting CD4$^+$ subset exists, which inhibits Th1 driven (Powrie, 1996), or both Th1- and Th2 mediated autoimmune diseases (Bridoux, 1997). Furthermore, a number of studies have demonstrated that TGF-β plays an important role in the regulation of a.o. the IgE production (Stavnezer, 1995; Letterio, 1998). Testing of the hypothesis that the suppression of IgG1 and IgE after UVB exposure is mediated by TGF-β produced by CD4$^+$ cells, is currently hampered by the absence of neutralizing anti-TGF-β antibodies.

TGF-β secreting CD4$^+$ cells have been described as alternative Th cells involved in the regulation of immune responses (Chen, 1994). This subset, called Th3, is different from Th1 and Th2 cells. One might speculate that UVB induces these Th3 cells, which might play a role in the suppression of Th2 responses. In addition, IL-10 secreting regulatory CD4$^+$ cells have been described, called Tr1 cells, which have been implicated in the induction of toler-

ance in various disease models (Groux, 1997; Mason, 1998). We found an important role for IL-10 in UVB-induced immunosuppression, and that UVB irradiation results in an increase of both the classical Th2 cells as well as IL-10$^+$IL-4$^-$ CD4$^+$ cells. The latter resemble the Tr1 subset, suggesting that Tr1 cells are also involved in the UVB-induced immunosuppression.

Similar findings were obtained by Shreedhar et al. who cloned suppressor CD4$^+$ cells from UVB irradiated mice (Shreedhar, 1998). In their experimental setting only a minority of the clones obtained were suppressor Th cells, whereas the vast majority were the classical Th2 cells. Combined, Shreedhar's data obtained from cloned CD4$^+$ cells and our data from primary CD4$^+$ cells suggest the induction of alternative CD4$^+$ subsets after UVB irradiation. The fact that only a minority of the total CD4$^+$ population are putative Tr1 cells underscores their functional activity in that even low numbers of these cells are able to exert highly suppressive effects, as demonstrated by the UVB-induced suppression of the CHS and the reduction of the resistance to infectious diseases.

Assuming that such alternative Th cells indeed occur, a valid question is when these cells become activated. Since UVB induces high numbers of Th2 cells and inhibits the development of Th1 cells, the shift towards Th2 mediated responses is likely sufficient to inhibit the responses examined thus far, like resistance to pathogens, the DTH and CHS responses. Therefore, what is the additional value of developing alternative Th cells after UVB irradiation? Is it to inhibit specific responses or to dampen the UVB-induced development of Th2 cells? No data are available on these issues yet. Therefore, these questions have to be addressed in future experiments.

The effect of UVB on the APC in directing Th driven responses

Also with regard to the effect of UVB on APC the majority of the studies performed so far have focussed on skin-related effects. It is known for many years that the antigen presenting function of splenic APC is modulated by UVB, but no studies were conducted to unravel the mechanisms. Chapter 3 of this thesis describes the effect of UVB on cytokine production by APC. At first glance these results are quite puzzling. From the literature it was known that UVB causes a reduction in the number of APC as well as a reduction of the antigen presentation and phagocytosis. We therefore expected an overall reduction of the cytokine production due to reduced numbers of APC in the spleen of UVB treated mice. Instead, the production of the LPS induced cytokines (IL-1, IL-6, TNF-α and PGE$_2$) in the spleen was augmented after UVB exposure. This is spite of, or maybe due to, the extensive migration of macrophages towards the skin and the draining lymph nodes. This, and the enhanced supply of macrophages from the bone marrow, is likely to affect the composition of the macrophage/DC subsets in the spleen and to result in the altered cytokine pattern as observed after UVB exposure.

We demonstrated that the modulated cytokine profile has consequences for the interaction of the APC with CD4$^+$ cells and B cells, as demonstrated by the inhibition of the

splenic IFN-γ production and IgM production, respectively.

We also found an enhanced production of PGE_2 in LPS stimulated spleen cell cultures obtained from UVB irradiated mice. PGE_2 is a potent regulator of Th polarization. It is known to inhibit the production of IL-12, resulting in suppressed Th1 responses. PGE_2 production by keratinocytes has been reported to be upregulated after UVB irradiation. It was suggested that keratinocyte derived PGE_2 is the initiator of a cascade reaction involving IL-4 and IL-10 (Shreedhar, 1998). The same cascade might also be active in the spleen. Consequently, the enhanced production of PGE_2 by splenic APC after UVB irradiation could induce a change into an environment favoring Th2 responses. The reduced serum IL-12 levels after UVB further add to these Th2 promoting conditions.

Neutralization of IL-10 resulted in reversal of the UVB-induced suppression of the systemic CHS response. The involvement of IL-10 might indicate that it also interferes with the Th1-Th2 balance, e.g. by inhibiting the IL-12 production. Although we found an increase in the number of IL-10 producing $CD4^+$ cells, the LPS induced IL-10 production by splenic APC was not affected by UVB. In the local UVB response it has been convincingly shown that murine keratinocytes produce IL-10 and that infiltrating macrophages produce high amounts of IL-10 at a later stage. IL-10 appears to be able, at an early stage, to modulate the antigen presentation of LC, and at a later stage to dampen the local UVB-induced inflammation. Systemically, the source of IL-10 is not known. Some studies conclude that keratinocytes are the main contributors by releasing high amounts of IL-10 in the circulation. This is still controversial, as two recent studies on this issue are conflicting. Shreedhar et al. concluded that keratinocytes are not the major source of IL-10, since neutralization of IL-4 after UVB irradiation prevented the induction of IL-10 in serum, suggesting that $CD4^+$ cells are the producers (Shreedhar, 1998). Hart et al. found similar UVB-induced IL-10 levels in wildtype and IL-4 knock-out mice, suggesting that non-T cells are the major contributors to serum IL-10 (Hart, 2000). Both studies used FS-40 sunlamps, and the dose of UVB was
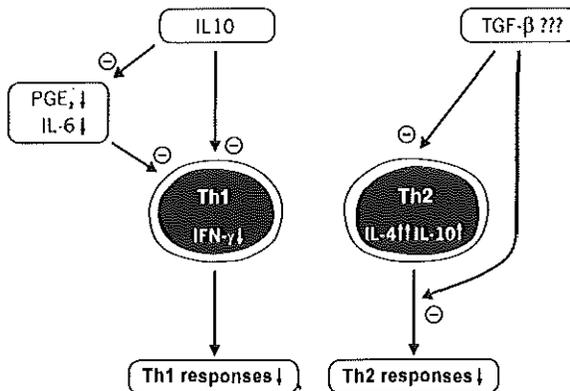

Figure 1: Proposed interaction of cytokines involved in the UVB-induced inhibition of both Th1 and Th2 driven immune responses.

15 kJ/m^2 and 30 kJ/m^2, respectively. In our study, using suberythemal UVB doses, we were unable to detect any IL-10 in serum of UVB irradiated mice (4x 1.5 kJ/m^2, TL-12/40 tubes). Since neutralization of IL-10, also in our models, abrogates some of the UVB-induced effects, it is important to identify the cell type, which produces the IL-10 that is responsible for the systemic effects of UVB. Furthermore, it is important to determine whether physiological UVB doses cause IL-10 to appear in the serum, or whether IL-10 is only locally produced at low levels, thereby modulating cell-cell interactions in the local microenvironment.

Effect of UVB irradiation on humoral immunity

A classical dogma in photoimmunology is that UVB affects cellular immunity, but not humoral immunity. However, a number of studies, including ours, do not support this. In chapter 2, we showed that UVB does have dramatic effects on the Ig production after immunization with TNP-KLH *in vivo*. Suppression by UVB of both the Th1 and Th2 associated Ig isotypes was found. This suppression appeared to be T cell mediated since responses to T cell-independent antigens were not affected by UVB. Besides the involvement of T cells, antigen uptake and presentation after immunization could also be affected by UVB. APC may indeed be involved in the observed UVB-induced effects on antibody formation as activation of B cells by T cell-independent antigens usually does not rely on APC. Nevertheless, the finding that the effect of UVB on T cells (and possibly on the APC) results in inhibition of B cell responses, is an important finding. The data are in line with reports that Th1 associated isotypes are suppressed after immunization with *Borrelia* and HSV, and consequently show that UVB suppresses the protective humoral immunity to pathogens (Brown, 1995; El-Ghorr, 1998). Secondly, it might have consequences for the combat against parasites. To date, the effect of UVB irradiation on IgE and consequently on the resistance to parasitic infections has not been examined extensively. In animal models, cutaneous leishmaniasis was found to improve by UVB, whereas the systemic disease was exacerbated (Giannini, 1986). On the other hand, no effect of UVB was described on *Schistosoma mansoni* (Jeevan, 1992), while recently an increased susceptibility of mice to malarial infection was reported after UVB exposure (Yamamoto, 1999).

Another important issue concerning the effect of UVB on B cell responses is the efficacy of vaccines. Seasonal and regional influences due to sunlight exposure may be important factors contributing to the effectiveness of vaccinations to hepatitis, measles etc. Therefore further research on this issue is needed.

Role of LPS respondership in the susceptibility to UVB

The ability of UVB to impair CHS responses is genetically determined. The loci involved include the *tnfa* and *lps* loci (Vincek, 1993; Streilein, 1994). With respect to the *tnfa*

locus is has been convincingly shown that increasing doses of UVB induce increasing levels of TNF-α, which in turn mediates the increased UVB susceptibility (Vermeer, 1990; Shimizu, 1994). With regard to the *lps* locus, the mechanisms of differential susceptibility are unclear.

It was reported that LPS responder mice (C3H/HeN) are UVB sensitive, whereas LPS non-responder mice (C3H/HeJ) are UVB resistant in the local CHS response (Yoshikawa, 1990). Neutralization of TNF-α *in vivo* in C3H/HeN mice results in a UVB resistant phenotype, similar to the C3H/HeJ (Yoshikawa, 1990). However, this does not provide any clues on the involvement of the *lps* gene. The fact that UVB irradiation of C3H/HeJ epidermal cells and macrophages mounted a normal cytokine response suggests that the initiating responses induced by UVB are not affected in LPS non-responder mice. Some years ago, it was believed that relatively low doses of UVB resulted in local immunosuppression, whereas relatively high doses caused systemic immunosuppression. This appeared not to be true, since systemic immunosuppression is observed in UVB susceptible mice also at low UVB doses.

In our experimental setting we found that the C3H/HeJ LPS non-responder mice were sensitive in the local protocol (data not shown), and resistant to UVB in the systemic protocol for the UVB-induced suppression of the CHS. This clearly shows that UVB susceptibility depends on many factors and is not easily defined. Comparison of our data with other reports suggests that the sensitivity may depend on the source of UVB, the choice and dose of sensitizing hapten and the UVB irradiation protocol employed. In order to further clarify this issue, it would be of interest to extend these studies, a.o. by employing another LPS-non-responder strain (C57BL/10ScCr) as well.

Our data also show that LPS responder and non-responder mice with a C3H background have significant differences in their T cell and APC compartments, and we showed in chapters 2 and 3 that especially T cell and APC responses are affected by UVB exposure. It is therefore likely that differences in the default settings of the immune system result in a different UVB-induced immune modulation, which explains the deviating UVB susceptibility in C3H/HeJ and C3HeB/FeJ mice. Furthermore, the fact that the C3H/HeJ is sensitive in the local model and resistant in the systemic model, demonstrates that the mechanisms responsible for the UVB-induced effects are different in both mouse strains. Thus, whether a mouse strain is classified as UVB susceptible or resistant depends on the protocol used, which was also suggested on basis of other studies (Yoshikawa, 1990; Noonan, 1994). Furthermore, we showed that also the type of immune response measured (CHS, cytokine production, lymph node cellularity) may be an important criterium in the classification of UVB susceptibility.

DNA-repair deficiency and UVB-induced immunusuppression

NER deficient mice are particularly suitable to study the differential sensitivity to UVB. The defined mutations in the three different mouse strains examined in this thesis, XPA, XPC and CSB, enabled us to determine the qualitative and quantitative contribution of a specific DNA-repair pathway to UVB-induced immunomodulation. From our studies two

important conclusions were drawn (chapter 6). First, DNA-repair deficient mice have normal T and APC mediated immune responses. Defective responses can only be observed after UVB irradiation. Secondly, mice lacking the TCR (XPA and CSB) show enhanced sensitivity to UVB-mediated immunomodulation, while a mutation of the XPC gene has no effect.

The first conclusion is important since for XP patients several immune defects have been described, like weaker DTH responses, low response to PHA, reduced IFN-γ production and decreased CD4/CD8 ratios (Morison, 1995; Wysenbeek, 1986; Mariani, 1992; Gaspari, 1993). Extrapolation of the data obtained from the mouse models therefore suggest that these immune defects are due to increased susceptibility to environmental factors, like UVB exposure, rather than an inevitable consequence of the genetic defect itself.

The disturbed immune surveillance due to UVB irradiation as well as the high numbers of persistent UVB-induced mutations in the skin likely contribute to the enhanced skin cancer susceptibility as seen in these patients. It was reported recently that in XPA mice the NK cell cytotoxicity is increased, whereas UVB exposure resulted in a strong reduction of the NK cell response as compared to the wildtype. This inhibitory effect of UVB on NK cell activity has been reported by others in both animals and men (Yaron, 1995; Goettsch, 1996). In contrast, we found that in XPA mice back-crossed for at least 8 generations on the C57BL/6 background, a reduction of the NK cell cytotoxicity to YAC-1 cells was detected even in non-irradiated XPA mice (An, 1999). These findings are suggestive for a reduced anti-tumor activity in XPA mice. Similar effects have been described in patients (Gaspari, 1993). At present, we are pursuing these findings by examining the underlying mechanisms resulting in the NK cell defect.

The second conclusion drawn from the studies on NER mice relates to the role of the TCR and the GGR in the UVB-induced immunosuppression. The majority of DNA lesions induced by UVB irradiation are cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6-4) photoproducts. The NER mechanism is one of the repair pathways that is very efficient in correcting these lesions. We showed that mice with defective TCR are more sensitive to UVB-induced immunosuppression than wildtype mice. Furthermore, when both the TCR and the GGR pathways were defective, mice became extremely sensitive to UVB. In the skin of C3H mice, cells with CPD mutations were still detected 4 days after UVB exposure. In the draining LN, the number of MHC class II^{+} cells with CPD mutations increased as early 1 hour after UVB exposure. The number of CPD^{+} cells in the draining LN was maximal at 24h, and declined thereafter. The cells were found to originate from the skin and were phenotyped as dendritic cells (Sontag, 1995; Vink, 1996). The decline of CPD^{+} cells in both the skin and the draining LN could be due to a number of processes, including DNA-repair , cell death and emigration. Since the introduction of DNA strand breaks and mutations by UVB was reported to induce cytokine production, it is likely that in NER deficient mice long-lasting cytokine production is found in the epidermis and LN due to persistent mutations caused by slow DNA-repair . Extrapolation of our results in the NER deficient mice suggests that possible differences between mouse strains in the efficacy of DNA-repair may account for their differential susceptibility to UVB-induced immunosuppression. Efficient DNA-

repair will eliminate the modulated production of cytokines and other immunoregulatory components (e.g. signaling proteins) induced by DNA damaged genes.

Since DNA damage is considered to be the initiator of the cascade of cytokines produced by UVB-damaged epidermal cells, repair of DNA damage is crucial in abrogating the UVB-induced response. Therefore, the process of repair of DNA damage by the NER pathway plays an important role in prevention of the long-lasting disturbance of the local and systemic immune response and in normalizing the homeostasis in the skin.

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SUMMARY

Exposure to UVB irradiation can have serious consequences for human health. Of particular concern are the adverse effects of UVB resulting in skin carcinogenesis and immunosuppression, which contributes to an increased prevalence of skin tumors and infections.

In this thesis we investigated the mechanisms that underlie the systemic effects of UVB exposure and the consequences for Th driven immune responses. For this purpose, we used several different mouse models and we irradiated mice with suberythemal UVB doses. We examined *in vivo* immune responses, and isolated the spleen and lymph nodes of the experimental mice to assess *in vitro* immune parameters, thereby focussing on cytokine production and cytokine-mediated responses.

In *chapter 2* we described the effects of UVB exposure on the peripheral development of Th1 and Th2 cells, and showed that UVB irradiation results in an enhanced development of Th2 cytokines as demonstrated at the single cell level. However, this shift towards a Th2 cytokine profile was not reflected in antibody production, since we demonstrated that UVB exposure inhibits both Th1- (IgG2a) and Th2- (IgE and IgG1) associated immunoglobulin isotype production. The UVB-induced reduction on Th1 associated isotypes was found to be mainly mediated by IL-10. The inability of IL-10 to affect the UVB-induced reduction of Th2 associated isotypes indicates the involvement of additional, yet unidentified, factor(s).

In *chapter 3* we described that also the APC population in the spleen was functionally altered due to UVB exposure of the skin. We found enhanced cytokine production (PGE_2, IL-1, IL-6, and TNF-α) after *in vitro* stimulation of spleen cells. These effects, combined with a reduced LPS-induced *in vivo* production of IL-12 (as measured in serum), may explain the inhibition of Th1 responses as observed after UVB exposure. Furthermore, we demonstrated that the APC population obtained from UVB irradiated mice affected both B cell and T cell functions, as demonstrated by suppressed IgM and IFN-γ production, respectively.

Chapter 4 describes the results obtained from *in vivo* experiments in mouse models for respiratory allergy. We studied the effect of UVB in two different mouse models that evoked either a Th1-driven or a Th2-driven immune response. We found that both allergic responses were inhibited by UVB exposure, i.e. airway hyperreactivity responses as well as antibody production. These data provide additional evidence that UVB irradiation induces a general immunosuppression rather than a selective suppression of only Th1-driven responses.

The effects of UVB on APC, CD4^{+} cells and B cells appeared to be a general effect of UVB irradiation. However, the degree of UVB-induced immunosuppression differs in different mouse strains. This variation in UVB susceptibility is a genetically determined trait. The *lps* locus, encoding the Tlr-4 protein, is one of the loci involved. In *chapter 5* we demonstrated that in mutant mice lacking Tlr-4 signaling, systemic immunosuppression occurred only at relatively high UVB doses. Furthermore, we showed in these mutant mice that UVB-induced suppression of the CHS response was not linked to inhibition of Th1 activity. Apparently, other mechanisms account for the immunosuppression in these mice.

Another approach to study UVB susceptibility was focussed on the repair of UVB-induced DNA damage. We examined three different mutated mouse strains with specific NER gene defects, XPA, XPC and CSB mice (*chapter 6*). With respect to the immune status of these mice, we demonstrated T, B and APC responses similar to their wildtype littermates. However, the UVB-induced immunomodulation in these mice was differentially affected. Only mice with defective GGR and TCR (XPA mice) displayed an augmented sensitivity to systemic UVB-induced suppression of the CHS response. The acute UVB-induced effects were enhanced in XPA and CSB mice, demonstrating the crucial importance of TCR to prevent immunosuppression.

In conclusion, we demonstrated that UVB causes a systemic immunosuppression. This immunosuppression was of generalized nature as shown by the inhibition of Th1 as well as Th2 driven immune responses. This was not limited to cellular immunity, but also involved humoral immunity. The Th2 priming conditions provided by the APC after UVB, as well as the UVB-induced production of immunoregulatory cytokines (a.o. IL-10), facilitate the development of Th2 cells and possibly alternative regulatory subsets. Furthermore, the differences in UVB susceptibility between mouse strains were shown to be related to the immune status of the mice and the integrity of the NER mechanism of DNA-repair .

The findings described in this thesis have increased our knowledge on the immunomodulatory effects of UVB exposure. Furthermore, relevant immune parameters were selected which will be the basis for ongoing research on the consequences of UVB-induced DNA damage on systemic and local immunoregulation.

SAMENVATTING VOOR NIET-BIOLOGEN EN NIET-MEDICI

Regelmatig wordt in de media gewaarschuwd voor de gezondheidsrisico's van blootstelling aan zonlicht. Met name huidkanker en versnelde veroudering van de huid (rimpels) worden als belangrijke risico's genoemd. De veroorzaker van dit alles zijn de ultraviolette stralen in het zonlicht, en dan met name de ultraviolet-B straling (UVB) in het zonlicht.

Het laatste decennium is veel onderzoek verricht naar de effecten van UVB straling op het afweersysteem. Zo is aangetoond dat de kans op infecties toeneemt door overmatige blootstelling aan UVB licht. Dit geldt niet alleen voor huid-gerelateerde infecties, maar ook voor infecties van dieper liggende organen.

In dit proefschrift wordt een aantal studies beschreven naar de mechanismen die verantwoordelijk zijn voor de negatieve effecten van UVB licht op het afweersysteem.

Het afweersysteem is een zeer ingewikkeld systeem dat bestaat uit tientallen verschillende soorten cellen, met elk hun eigen specifieke functies. Zo'n complex systeem is noodzakelijk omdat het lichaam ziekmakende bacteriën en virussen moet kunnen herkennen en verwijderen, en tegelijkertijd moet voorkomen dat gezonde eigen lichaamscellen aangevallen worden.

Centraal in de eerste fase van een specifieke afweerreactie staan de antigeenpresenterende cel en de T-helper cel. Antigeenpresenterende cellen zijn in staat om bacteriën 'op te eten' en moleculen ('deeltjes') hiervan op hun oppervlak te binden. T-helper cellen kunnen deze moleculen herkennen en worden vervolgens daardoor geactiveerd. Tegelijkertijd instrueert de antigeenpresenterende cel de T-helper cel en bepaalt deze of die T-helper cel moet helpen bij het doden van bacteriën en virussen (Th1), of moet helpen bij het maken van antistoffen en het doden van parasieten (Th2). Op basis van de eiwitten (cytokinen) die ze produceren, worden Th cellen ingedeeld in Th1 en Th2 cellen.

In dit proefschrift is onderzocht wat de oorzaak is van het feit dat bestraling met UVB licht resulteert in een verhoogde kans op infecties.

Hiertoe is gebruikt gemaakt van verschillende muizenmodellen, waarbij de muizen bestraald werden met een lage dosis UVB. Naast het bepalen van *in vivo* afweerreacties, werden de milt en de lymfklieren van muizen onderzocht. De effecten van UVB op de milt en de lymfklieren werden onderzocht door de cellen van deze organen in celkweek te bestuderen. Daarbij bleek dat de communicatie tussen de antigeenpresenterende cellen en de T helper cellen, die verloopt via cytokinen, verstoord is. De antigeenpresenterende cellen afkomstig van UVB bestraalde muizen scheiden andere cytokinen uit dan die van controle muizen. Dit beïnvloedt de T-helper cellen zodanig dat de balans tussen Th1 en Th2 cellen verstoord raakt; er ontwikkelen zich dan te veel Th2 cellen. Als er in deze situatie een infectie op zou treden, zijn er op dat moment te weinig Th1 cellen aanwezig en kan de infectie niet adequaat bestreden worden.

Th2 cellen zijn met name actief tijdens infecties met parasieten, en zij spelen een belangrijke rol bij allergieën. Op grond van bovenstaande resulten zou verwacht worden dat

bestraling met UVB (als gevolg van een toename van het aantal Th2 cellen) de symptomen van allergisch astma verergert. Dit is onderzocht in een muizenmodel, en bleek niet het geval te zijn. Twee belangrijke symptomen van astma, reactiviteit van de luchtwegen en anti-stofvorming tegen allergenen (IgE antistoffen), bleken beide geremd te worden door de bestraling met UVB licht. In dit model remt UVB straling dus ook Th2 reacties.

In hoofdstuk 2 is beschreven dat een specifiek cytokine, interleukine-10 geheten, de activiteit van Th1 cellen remt. Wij hebben aanwijzingen dat een ander cytokine, TGF-β, met name de activiteit van Th2 cellen kan remmen. Dus de remming, die we aangetoond hebben van Th1 en Th2 activiteit, kan mogelijk verklaard worden door de productie van deze cytoki-nen.

Het tweede deel van het proefschrift richt zich op de genetische factoren die een rol spelen bij de individuele verschillen in gevoeligheid voor UV straling. Hierbij is onder andere gebruik gemaakt van muizenmodellen voor de huidziekte xeroderma pigmentosum, een erfe-lijke ziekte die wordt gekarakteriseerd door extreme gevoeligheid voor zonlicht, en door de sterk verhoogde kans op het ontwikkelen van huidkanker. Deze patiënten hebben een speci-fiek defect in het DNA-herstelmechanisme en kunnen daardoor de DNA schade, die ontstaat door UVB bestraling, niet herstellen. Door diverse muizenstammen te vergelijken waarin ver-schillende delen van het DNA-herstel mechanisme uitgeschakeld waren, is hun aandeel in de remming van het afweersysteem vastgesteld.

Kort samengevat is in dit proefschrift in verschillende modellen aangetoond dat UVB bestraling zowel de Th1 activiteit (o.a. de afweer tegen infecties) als de Th2 activiteit (o.a. allergisch astma) remt. Tevens is aangetoond dat naast de T cel responsen ook de anti-stofvorming geremd wordt door UVB. In de verschillende hoofdstukken van het proefschrift is uitvoerig ingegaan op de mechanismen waardoor UVB het afweersysteem remt. Daarbij bleek dat de verschillen in UVB gevoeligheid gerelateerd kunnen worden aan de mate van functioneren van het afweersysteem en aan de effectiviteit van het DNA herstelmechanisme.

De bevindingen zoals beschreven in dit proefschrift hebben onze kennis over de effecten van UVB op het afweersysteem sterk vergroot. Tevens vormt dit proefschrift de basis voor vervolgonderzoek naar de gevolgen van UVB geïnduceerde DNA schade op het func-tioneren van het afweersysteem.

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ABBREVIATIONS

APC	antigen presenting cell(s)
BSA	bovine serum albumin
CCR	CC chemokine receptor
CD	cluster of differentiation
CGRP	calcitonin gene related peptide
CHS	contact hypersensitivity
CIITA	class II MHC transactivator
ConA	concanavalin A
CPD	cyclobutane pyrimidine dimer(s)
CSB	Cockayne Syndrome B
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell(s)
DETC	dendritic epidermal T cell(s)
DTH	delayed-type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FITC	fluorescein isothiocyanate
GGR	global genome repair
GM-CSF	granulocyte-macrophage colony stimulating factor
HSA	heat stable antigen, CD24
hsp	heat shock protein
HSV	*Herpes simplex virus*
i.p.	intraperitoneal
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.v.	intravenous
KLH	keyhole limpet hemocyanin
LC	Langerhans cell(s)
LN	lymph node(s)
LPS	lipopolysaccharide
LT	lymphotoxin
mAb	monoclonal antibody
MCP-1	monocyte-chemoattractant protein-1
MED	minimal erythemal dose
MHC	major histocompatibility complex
mRNA	messenger RNA
NER	nucleotide excision repair
NK	natural killer
OVA	ovalbumin

PBS	phosphate-buffered saline
PCl	picrylchloride
PE	phycoerythrin
PGE_2	prostaglandin E_2
PMA	phorbol-12-myristate-13-acetate
SD	standard deviation
SEM	standard error of the mean
STAT	signal transducer of activated T cells
TCR	T cell receptor
TCR	transcription coupled repair
TGF	transforming growth factor
Th	T helper
Tlr	Toll-like receptor
TNF	tumor necrosis factor
TNP	trinitrophenol
UCA	urocanic acid
UVB	ultraviolet B
XP	xeroderma pigmentosum

DANKWOORD

CURRICULUM VITAE

The author of this thesis was born on July 16, 1969. After finishing high school in 1987, he studied Biology at the University of Groningen. During this period, practical training in medical biology was conducted at the University Hospital Groningen on tumor-immunology (Prof. L. de Leij) and the Free University in Amsterdam on the molecular biology of human papillomaviruses (Prof. J. Walboomers). His degree in Biology was obtained in August 1993. In stead of military service, he conducted research on the Vh gene locus at the University of Groningen (Dr. N.A. Bos) and on immunotoxicology of flatfish at the National Institute of Public Health and the Environment (Prof. J.G. Vos). In 1996, the author started his PhD study at the Department of Immunology of the Erasmus University in Rotterdam supervised by Prof. H.F.J. Savelkoul and Prof. R. Benner. The studies described in this thesis were performed during this period. From September 2000 onwards he will work in the lab of Dr. A. O'Garra at the DNAX Research Institute, Palo Alto, USA.

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