INVESTIGATIVE REPORT

Occurrence of Neutrophils and Activated Th1 cells in UVB-induced Erythema

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We investigated the sequential changes in infiltrating inflammatory cells and several cytokine levels over a period of 48 h in human back skin exposed to 3 minimal erythematous doses of UVB. The measurement of blood flow, using a laser Doppler method, indicated that UVB-induced erythema reached a peak 12–24 h after irradiation. Immunohistochemically, an increase in the number of $CD4^+$ T cells was observed in perivascular areas 6 h after the UVB treatment and continued for up to 48 h. $CD8^+$ T cells were scarce until 24 h, but their numbers gradually increased thereafter. $HLA-DR^+$ cells were detected perivascularly and interstitially in parallel with the pattern of $CD4^+$ T-cell infiltration. In contrast, neutrophils were found 3 h after UVB exposure and reached a peak at 24 h. Using a RT-PCR method, we demonstrated that mRNAs for the Th1 cytokines (interferon-γ and interleukin-2), together with a pro-inflammatory cytokine (interleukin-8), became detectable at 6 h, whereas mRNA for the Th2 cytokine (interleukin-4) was not found at all during the first 48 h. In contrast, we found an increase in mRNA levels for C3 and tumor necrosis factor-α even at 3 h, suggesting a relationship between complement activation and accumulating neutrophils. Our results suggest that neutrophils and $CD4^+$ T cells in UVB-induced inflammation play different roles: neutrophils are more closely related to UVB-induced erythema, while T cells appear to be involved in subsequent dermal and epidermal inflammation accompanied by epidermal hyperproliferation. *Key words: cytokine; human; keratinocyte; UVB.*

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Exposure of human skin to UV light in the 280–320 nm range (UVB) results in both an acute inflammatory response, characterized by erythema and edema, and chronic inflammation associated with photoaging and carcinogenesis (1). The UVB-induced erythema can be characterized by sequential and overlapping events (2). The earliest event is a vasodilatory response, which is mediated by prostaglandins-E2 (3) and nitric oxide (4, 5). Subsequently, an inflammatory infiltration appears which consists of neutrophils and lymphocytes (6–7). Several investigators have reported the role of T lymphocytes as a source of UVB-induced inflammation (8–10). Among them, Di Nuzzo et al. (9) demonstrated that UVB irradiation induces a preferential recruitment of $CD4^+$ T cells in the dermis at 1 and 2 days after low doses of UV treatment (8).

They further indicated that intra-epidermal memory $CD4^+$ T cells can be found at 14 days after low-dose UV radiation (10).

UVB exposure upregulates a wide variety of inflammatory cytokines (11), which are part of the cascade leading to the UVB-induced inflammation, including the induction of erythema in the final phase. The mechanism of this induction involves a leukocyte infiltration mediated by the UVB upregulation of cytokines such as interleukin-1 (IL-1) (12) and tumor necrosis factor-α (TNF-α) (13–16) and also by the upregulation of two other cytokines, melanoma growth-stimulating factor and IL-8 (17), both of which are chemoattractants for T cells and neutrophils.

Other investigators have reported that complement activation occurs in UVB-exposed skin, resulting in deposition of complement components in the skin (18). We recently found that C3, a key complement component secreted by interferon-γ (IFN-γ)-stimulated cultured human keratinocytes (19), was enhanced by UVB exposure (20), suggesting a relationship between complement activation and UVB erythema.

The present study was designed to investigate the sequential changes of cell infiltration and their relationship with expression of mRNA for Th1 and Th2 lymphokines, pro-inflammatory cytokines and C3 in human skin exposed to 3 minimal erythematous doses (MED) of UVB.

MATERIAL AND METHODS

Chemicals and reagents

Murine monoclonal antibodies against human CD4, CD8, CD3 and HLA-DR were purchased from Becton Dickinson Labware (Franklin Lakes, NJ). Anti-human neutrophil-specific elastase antiserum was obtained from DAKO Japan (Kyoto, Japan).

Conditions of UVB irradiation and skin exposure to UVB

Five fluorescent sunlamp tubes (FL20 E-30/DMR; Toshiba Medical Supply, Tokyo, Japan) were used as the UVB source. These tubes emit light with wavelengths of 280–340 nm, with a peak of 305 nm. The spectral radiation output is as follows: total UV, 80.09%; UVA, 25.09%; UVB, 54.26%; UVC 0.74%. Irradiances of UVA and UVB were quantified using an UV radiometer (UVR-305/365D II; Topcon, Tokyo, Japan).

The skin of the mid-back of 2 normal Japanese male subjects (aged 26 and 42 years, respectively) was exposed to 3 MED of UVB after obtaining informed consent. Blood flow was assessed using a laser Doppler device and 4 mm punch biopsies were taken before and at various periods of time after the UVB exposure. Each specimen was cut into 2 pieces. One piece was snap-frozen for immunohistochemistry, and the other was prepared for RT-PCR.

Immunohistochemistry

Frozen sections were stained using an avidin–biotin peroxidase technique with anti-human monoclonal antibodies against CD4, CD8, CD3 and HLA-DR (1:40 to 1:50 dilutions at the final concentration) and antiserum against neutrophil-specific elastase (1:50). The sections (6 μm thick) were first blocked with rabbit serum (diluted 1:10) for 30 min and then exposed to primary antibodies at 4°C overnight. After washing, they were incubated with biotin-conjugated anti-murine antibody at room temperature for 30 min and then with peroxidase-labeled streptoavidin at room temperature for 30 min using a Histofine SAB-PO kit (Nichirei Co., Tokyo, Japan). Finally, they were developed with diaminobenzidine solution and 1% hydrogen peroxide, and counterstained with Mayer's hematoxylin.

Immunostaining intensity was evaluated arbitrarily as follows: 0, negative; 1, very weak; 2, weak; 3, moderate; 4, strong; 5, very strong. The changes in staining intensity in both subjects were plotted in the same diagram (Fig. 1).

RT-PCR

Total RNA was isolated from each biopsied skin sample by using ISOGEN (Wako, Osaka, Japan) according to the manufacturer's instructions. The concentration of total RNA was determined by reading its absorption at 260 nm. Single-stranded cDNA was prepared from 5 μg of total RNA in a 33 μl reaction mixture using 300 ng of random hexamer (Takara, Osaka, Japan) and Ready-To-Go You-prime First-strand Beads (Pharmacia, Milwaukee, WI), which contained 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 7.5 mM dithiothreitol, 10 mM $MgCl_2$, 0.08 mg/ml bovine serum albumin, 2.4 mM dNTPs and Moloney murine leukemia virus reverse transcriptase. The resultant cDNA solution was diluted to 250 μl. Subsequent PCR was performed with 2.5 μl of cDNA in a 25 μl reaction mixture containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.625 U Taq polymerase and 0.5 mM concentrations of each primer set. Specific PCR primer sets for cDNA of each cytokine and β-actin were obtained from Clontech (Palo Alto, CA) (Table I). We also used the primers for human C3 as reported previously (20). These primer sets are designed to span exon–intron boundaries. The reaction mixtures were placed in a thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT). After initial denaturing at 94°C for 2 min, the samples were cycled as follows: 94°C for 45 s, 60°C for 45 s and 72°C for 45 s. Thereafter, the samples were kept at 72°C for 2 min and then cooled to 4°C. For each experiment, PCR cycles, as well as primers and RNA concentrations, were titrated to establish standard conditions and to choose the optimal ones for linear PCR amplification. The numbers of PCR cycles are indicated in Fig. 2. PCR products were visualized on 1.2% agarose gels containing 0.5 mg/ml ethidium bromide.

RESULTS

Blood flow in the UVB-exposed erythematous skin

Blood flow was measured using a laser Doppler device to assess the intensity of UVB-induced inflammatory changes. The results showed that UVB-induced erythema reached a peak between 12 and 24 h in both subjects after UVB irradiation (data not shown).

Immunohistochemical study of inflammatory cells infiltrating the UVB-exposed skin

The results are summarized in Fig. 1. Immunohistochemically, we observed an infiltration of both CD4$^+$ and CD8$^+$ T cells. In untreated skin, some CD4$^+$ T cells were found in the perivascular area of the dermis and a few in the interstitial area. The infiltration of CD4$^+$ T cells started to occur 6 h after UVB exposure and continued to increase for up to 48 h. In contrast, CD8$^+$ T cells were observed in smaller numbers in the upper dermis compared with CD4$^+$ cells (Fig. 1). A gradual increase in numbers was seen for up to 48 h. Although there were few HLA-DR$^+$ cells in the unexposed skin, they increased in number interstitially as well as perivascularly for up to 48 h in the UVB-exposed skin. CD3$^+$ T cells were detectable in the perivascular area. As few other HLA-DR$^+$ cells, such as Langerhans' or dendritic cells (data not shown), were observed, we assume that most of the HLA-DR$^+$ cells were CD4$^+$ and CD8$^+$ T cells.

As for neutrophils, they were not found any earlier than T cells, i.e. they were detected as early as 3 h after UVB exposure and reached a peak at 24 h. At 48 h they infiltrated the epidermis.

UVB exposure of the skin results in the expression of mRNAs for Th1 lymphokines, pro-inflammatory cytokines and C3

We evaluated the time course of the mRNA expression of T-cell lymphokines, pro-inflammatory cytokines and C3 using a RT-PCR method. Because the infiltration of HLA-DR$^+$

Table I. *Primer sequences*

| Primers used	Primer sequence	Primer location
IL-2 5′ primer	5′CATTGCACTAAGTCTTGCACTTGTCA3′	504
IL-2 3′ primer	5′CGTTGATATTGCTGATTAAGTCCCTG3′	808
IFN-γ 5′ primer	5′GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC3′	164
IFN-γ 3′ primer	5′CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG3′	590
IL-4 5′ primer	5′CGGCAACTTTGACCACGGACACAAGTGCGATA3′	117
IL-4 3′ primer	5′ACGTACTCTGGTTGGCTTCCTTCACAGGACAG3′	460
TNF-α 5′ primer	5′GAGTGACAAGCCTGTAGCCCATGTTGTAGCA3′	404
TNF-α 3′ primer	5′GCAATGATCCCAAAGTAGACCTGCCCAGACT3′	847
IL-8 5′ primer	5′ATGACTTCCAAGCTGGCCGTGCT3′	1
IL-8 3′ primer	5′TCTCAGCCCTCTTCAAAAACTTCTC3′	289
C3 5′ primer	5′TCGGATGACAAGGTCACCCT3′	4,627
C3 3′ primer	5′GACAACCATGCTCTCGGTGA3′	5,034
β-Actin 5′ primer	5′CTACAATGAGCTGCGTGTTGG3′	311
β-Actin 3′ primer	5′AAGGAAGGCTGGAAGAGTGC3′	838
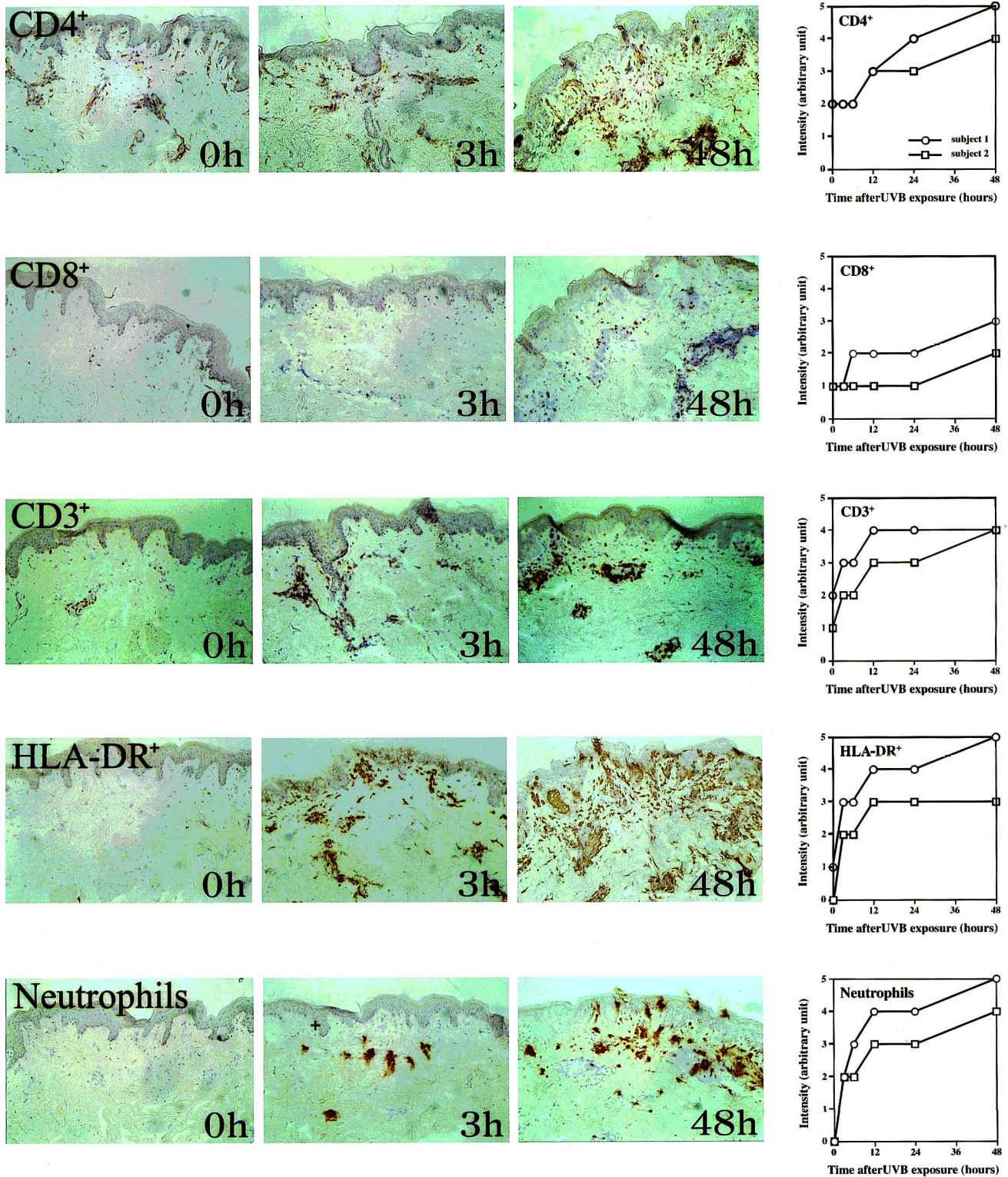
Fig. 1. Time-course study of infiltrated CD4^{+}, CD8^{+}, CD3^{+} and HLA-DR^{+} cells and neutrophils after exposure of human back skin to 3 MED UVB. The color images show the results in 1 subject at 2 selected time points after irradiation. The line diagrams show the combined results for both subjects.

CD4^{+} T cells was observed several hours after the UVB treatment, we first focused on the expression of mRNAs for both Th1 and Th2 lymphokines. As shown in Fig. 2, mRNAs for the Th1 cytokines, IL-2 and IFN-γ, were detected >6 h after the exposure of the skin to 3 MED of UVB. These two signals continued to be found until 48 h, although with a lower intensity. In contrast, mRNA for a Th2 cytokine, IL-4, was not detectable at any time (Fig. 2), even with 50 cycles of

PCR (data not shown). Next, mRNAs for the pro-inflammatory cytokines, TNF-α and IL-8, were evaluated. Some variations were observed in the expression of TNF-α, i.e. mRNA for TNF-α was detectable at 3 h in 1 subject and at 6 h in the other (Fig. 2). A very faint signal for IL-8 was observed 6–24 h after the UVB treatment. This demonstrated that an infiltration of T cells, rather than of neutrophils, correlated with IL-8 mRNA expression.

As reported previously (20), mRNA for C3 is constitutively expressed, although the signal was faint in both subjects. The signal for C3 showed an increase at 3–12 h after UVB exposure in both subjects (Fig. 2). This time point corresponds to that when the neutrophil infiltration showed an increase (Fig. 1). Thus, the changes in C3 expression seem to precede and correspond to the UVB-induced inflammation, when assessed by changes of erythema and blood flow.

DISCUSSION

It is well known that T cells and neutrophils infiltrate the dermis of human skin exposed to UVB. In this study, we demonstrated that CD4^+ T cells predominated over CD8^+ T cells for up to 48 h after 3 MED of UVB treatment and that most of them were HLA-DR-positive. Neutrophils were found at 3 h after UVB exposure and increased in number for up to 24 h. Interestingly, our results indicated that UVB irradiation caused the expression of mRNAs of Th1 cytokines (IL-2 and IFN-γ) and a pro-inflammatory cytokine (IL-8) at 6 h, whereas the signals for C3 and TNF-α were detectable even at 3 h. These results suggest that neutrophils and CD4^+ T cells play different roles in the generation of the inflammatory changes in UVB-exposed skin.

It has been reported that UVB itself can induce the production of many cytokines by epidermal keratinocytes (12–16). This suggests that UVB-induced inflammatory changes are in part mediated by these cytokines. For example, both TNF-α and IL-8 are known to facilitate the accumulation of neutrophils and T-lymphocytes (13). TNF-α stimulates the expression of adhesion molecules such as endothelial leukocyte adhesion molecule-1 and interstitial cell adhesion molecule-1 on vascular endothelial cells (13, 21). The expression of these molecules on endothelial cells is critical to trans-endothelial migration (22). IL-8 is reported to be chemotactic for both neutrophils and T cells (23). Thus, these pro-inflammatory cytokines are likely to promote UVB-induced inflammatory responses. The results of our study showed that neutrophil infiltration preceded the development of the early part of the cell-mediated event. The accumulation of neutrophils paralleled the expression of mRNA for TNF-α and C3. This suggests the importance of vascular endothelial cell expression of cell adhesion molecules mediated by TNF-α and complement activation, where C3 is a key molecule.

It is not however apparent how the production of cytokines and the cell function are orchestrated. One possible explanation is that the regulation of their production occurs through the interaction of infiltrating cells, such as neutrophils and T-lymphocytes, with epidermal keratinocytes. Many authors have focused on the role of neutrophils in the acute inflammation of UVB-exposed skin (6, 7, 13, 24–27), while there have been only a few reports concerning T-lymphocytes in animal models (7) and in human skin (7, 9). Needless to say, the UVB-induced inflammation changes phase-by-phase, although these phases are overlapping and cannot be clearly separated.

Lymphokines, including IFN-γ, have been reported to have an influence on cytokine production by keratinocytes (28). In addition, several recent reports have strongly suggested an association between T cells and UVB-induced inflammatory changes (8, 9). Although this suggests a relationship between keratinocyte proliferation and UVB-induced T-cell activation,

Fig. 2. mRNA signals for Th1 lymphokines (IFN-γ and IL-2), pro-inflammatory cytokines (TNF-α and IL-8) and C3 were detected in the UVB-induced erythematous sites of 2 subjects. mRNAs were assessed using a RT-PCR method.
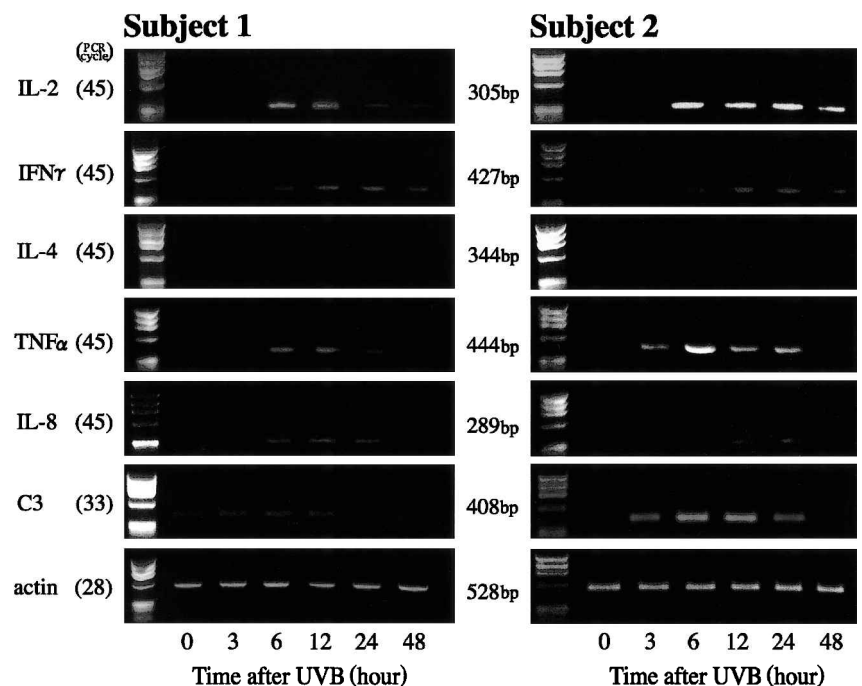
the experiments did not directly address the question of whether T cells are involved in UVB-induced inflammation. In this study, we demonstrated that signals for the Th1 cytokines IFN-γ and IL-2 were detectable from 6 to 48 h. It is known that it takes 12–24 h for IFN-γ to stimulate cultured keratinocytes to produce detectable levels of pro-inflammatory cytokines (data not shown) and C3 (19). Overall, it is unlikely that IFN-γ produced by activated T cells can exert potent effects on UVB-induced erythema. IFN-γ is known to induce keratinocyte proliferation, as suggested in the case of psoriasis (29). In addition, in a murine model, altered stratum corneum barrier function started to appear 72 h after UVB irradiation. These observations might suggest a role for Th1 cells in the repair process of UVB-damaged skin and in the subsequent immunological unresponsiveness.

In conclusion, this study suggests different roles played by neutrophils and CD4^{+} T cells in the cell-mediated event of UVB-induced inflammation. Neutrophils are more involved in the earlier part of this event, in which the pattern of changes in their infiltration corresponds to the extent of UVB-induced erythematous changes. In contrast, T cells appear to be involved in the later part of the cell-mediated event in which, via epidermal proliferation, they play an important role in the elimination of UVB-damaged keratinocytes.

ACKNOWLEDGEMENTS

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