A Monoclonal Antibody to *cis*-Urocanic Acid Prevents the Ultraviolet-Induced Changes in Langerhans Cells and Delayed Hypersensitivity Responses in Mice, Although Not Preventing Dendritic Cell Accumulation in Lymph Nodes Draining the Site of Irradiation and Contact Hypersensitivity Responses

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Ultraviolet B (UVB) irradiation of C3H mice causes suppression of delayed hypersensitivity and contact hypersensitivity (CH) to antigens encountered following exposure, and is accompanied by a reduction in Langerhans cell (LC) numbers in the epidermis, loss of epidermal antigen-presenting cell function, and accumulation of dendritic cells in lymph nodes draining the site of irradiation. Various photoreceptors and mediators of these changes have been proposed, one of which is *cis*-urocanic acid (*cis*-UCA) formed from the naturally occurring *trans*-UCA in the epidermis on UV irradiation. A monoclonal antibody that reacts with *cis*-UCA has become available recently and has been used in this study to clarify the role of UCA. Pretreatment of C3H mice with the monoclonal antibody abrogated the UVB-induced and *cis*-UCA–induced reduction in epidermal LC numbers. It also prevented the UV-induced suppression of epidermal antigen-presenting cell ability as measured by the mixed skin lymphocyte response. However, it had no effect on the accumulation of dendritic cells in lymph nodes draining the site of UV exposure. With regard to hypersensitivity responses, it did not prevent UV-induced suppression of CH to oxazolone at a range of concentrations but it restored to normal the UV-suppressed delayed hypersensitivity to herpes simplex virus, if administered before exposure. Thus *cis*-UCA is involved in some UV-induced changes in murine skin but not in others, where alternative mediators, such as tumor necrosis factor-α, may be more important. ***Key word: immunosuppression. J Invest Dermatol 105:264–268, 1995***

It has been recognized recently that an increasing amount of ultraviolet (UV) radiation is reaching the earth's surface due to thinning of the ozone layer [1]. This may lead to an enhanced risk of developing skin cancers and, as exposure to UV is known to suppress selected immune responses, the effect on immunity may be significant (reviewed in [2]). It has been shown that UVB causes a reduction in the density of Langerhans cells (LC) in human and murine epidermis [3]. In addition, the antigen-presenting capacity of LC is inhibited following *in vitro* UVB exposure of isolated murine epidermal cells [4] or *in vivo* irradiation of mice [5,6]. A suberythemal dose of UV-B also causes an increased accumulation of dendritic cells (DC) in the lymph nodes draining the irradiated area [7] reaching a peak about 48 h after exposure. UV irradiation is recognized to suppress the induction of the contact hypersensitivity (CH) response to a variety of contact sensitizers in susceptible mice [8,9] as well as the delayed hypersensitivity (DH) response to antigens such as *Candida albicans* [10] and herpes simplex virus (HSV) [11]. Both local and systemic suppression of CH has been reported depending on UV dose. Low-dose UVB irradiation (400 J/m^2 on four consecutive days) suppresses only the local CH response within the UV-irradiated site, whereas a dose of 30 kJ/m^2 of UVB results in systemic suppression of CH at non-irradiated sites [12]. Only low-dose irradiation was used in this study.

It is likely that at least one epidermal photoreceptor is required to mediate these UV-induced changes in immune function, and urocanic acid (UCA) has been suggested to play such a role [9,13]. UCA is formed from histidine in a one-step deamination by the enzyme histidase and accumulates in the upper layers of the epidermis. Upon UV irradiation, the naturally occurring *trans*-isomer of UCA absorbs UV energy and photo-isomerizes to *cis*-UCA [14]. In several murine systems *cis*-UCA has been shown to modulate immune responses and to mimic the effects of UV [15–17]. Thus, for example, an epicutaneous (topical) application

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Abbreviations: CH, contact hypersensitivity; *cis*-MoAb, monoclonal antibody to *cis*-UCA; DC, dendritic cells; DH, delayed hypersensitivity; HSV, herpes simplex virus; MSLR, mixed skin lymphocyte reaction; UCA, urocanic acid.

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of 20 μg of *cis*-UCA caused a reduction in the number of ATPase-positive LC [16] as well as suppression of the DH response to HSV [18]. In addition, the action spectrum of UV-induced immunosuppression of the CH response was found to resemble the *in vivo* UV-absorption spectrum of *trans*-UCA in the spectral range 250–320 nm [9]. However, there is also good evidence to support a role for other photoreceptors in the skin, such as DNA [19] and mediators such as tumor necrosis factor (TNF)-α [20], interleukin-10 [21], and prostaglandins [12]. Some of these factors may interact or may be acting sequentially. For example, *cis*-UCA has been shown to stimulate prostaglandin production [22] and *cis*-UCA suppression of CH has been reported to be mediated by TNF-α [15].

To clarify the involvement of UCA, a monoclonal antibody to *cis*-UCA (*cis*-MoAb) was developed [23]. It was produced by immunizing mice with *cis*-UCA–keyhole limpet hemocyanin conjugate and was shown to have high specificity for *cis*-UCA. In a competitive enzyme-linked immunosorbent assay using *cis*-UCA–bovine serum albumin as antigen, it reacted with *cis*-UCA itself and both *cis* and *trans*-UCA–keyhole limpet hemocyanin conjugates, but not with *trans*-UCA itself, histidine, histamine, or a variety of other *cis* and *trans*-UCA analogues. The monoclonal antibody was injected *in vivo* prior to UV irradiation of C3H mice to ascertain whether it abrogated the effect of UV on epidermal LC numbers, epidermal antigen presentation, DC accumulation in draining lymph nodes, CH to oxazolone, and DH to HSV.

MATERIALS AND METHODS

Mice Female C3H HeN mice, 6–9 weeks of age, were used in all experiments. They were bred and maintained in the departmental animal house where they were kept in a 12-h light-dark cycle (fluorescent light tubes with covers) in standard mouse boxes, and had unlimited access to food and water. Cervical dislocation was performed to kill the mice. Female B/b C3H × CD1 mice, aged 8–10 weeks, were used to provide responder cells for the mixed skin lymphocyte reaction (MSLR), outlined below.

***Cis*-UCA and the Monoclonal Antibody to *cis*-UCA** *Trans*-UCA was purchased from Sigma Ltd., dissolved at 10 mg/ml in methanol, and UVB irradiated for 3 h (8640 J/m^2). The *cis*-isomer was purified by thin-layer chromatography as previously described [18]. High-performance liquid chromatography analysis was used to confirm the complete separation of the *cis* and *trans* isomers. The production of a monoclonal antibody specific to *cis*-UCA (*cis*-MoAb) has been described elsewhere [23]. Ascitic fluid was produced in female Balb/c mice (8 weeks old, pretreated intraperitoneally with 0.5 ml of pristane). It had a titre of 1:32,000 to *cis*-UCA by ELISA and contained approximately 0.15 mg/ml IgG_1. An isotype-matched irrelevant monoclonal antibody hybridoma to border disease virus was kindly supplied by Dr. Peter Nettleton and was used to produce a negative control ascitic fluid containing an equivalent amount of IgG_1. After some preliminary optimizing experiments the following protocol was used: a volume of 300 μl of 1/500 dilution of the *cis*-MoAb ascitic fluid (equivalent to 0.1 μg IgG) or the irrelevant ascitic fluid was injected intraperitoneally (IP) into each mouse, 2 to 4 h prior to UV treatment. In addition, a range of concentrations between 0.1 and 1 μg IgG was used in the CH experiments.

UV Irradiation The mice were irradiated on their dorsal surfaces immediately following shaving with electric clippers. They were placed in a small perspex box designed to minimize the mobility of the mice without using anesthesia. The ears of the mice were not protected from UV radiation; this is known not to affect the extent of ear swelling on challenge. Broadband UVB from a bank of two TL20W/12 lamps (Philips) was used with an output range of 270–350 nm (peak, 308 nm). The irradiance was 80 μW/cm^2 measured inside the perspex box. A 20-min exposure corresponded to a UVB dose of 960 J/m^2 (0.6 minimal erythemal dose for this mouse strain) and a 30-min exposure to 1440 J/m^2 (0.9 minimal erythemal dose).

ATPase Staining of Epidermal Sheets The *cis*-MoAb or the irrelevant antibody was injected into groups of four C3H mice 2 h prior to UVB irradiation or painting with *cis*-UCA. Twenty-four hours later, the ears of the mice were removed and split, and epidermal sheets from the dorsal surface only were stained for ATPase as previously described, using ADP as substrate [24]. The number of ATPase$^+$ cells were counted in at least 50 fields per group (1 field = 0.1 mm^2).

MSLR Two C3H mice per group were injected IP with phosphate-buffered saline (PBS), the *cis*-MoAb or irrelevant monoclonal antibody and 2 h later received 1440 J/m^2 UVB. The mice were killed immediately after the UV treatment and their ears removed for isolation of epidermal cells (EC) using a 1% trypsin solution. Other groups received 20 μg *trans*-UCA or 20 μg per ear *cis*-UCA epicutaneously, dissolved in 20 μl ethanol. Spleen cells (4 × 10^5 cells/well from B/b C3H × CD1 mice) and EC (1 × 10^5 cells/well from C3H ears), were incubated together at 37°C in 96-well plates as previously described [6]. ^{3}H-thymidine incorporation was measured after 4 d in culture. The net proliferative response was calculated as the mean of 10 replicates after subtracting the negative control proliferation consisting of spleen cells alone cultured with 100 μl of complete RPMI medium. The relative proliferation as a percentage of the normal non-irradiated MSLR response was calculated to represent separate experiments in one figure.

Accumulation of Dendritic Cells (DC) in Draining Lymph Nodes Four mice per group were injected with the *cis*-MoAb or PBS and 2 h later were UVB irradiated with a dose of 1440 J/m^2. Forty-eight hours after irradiation, the mice in each group were killed and their auricular, axillary, and inguinal lymph nodes, draining the UV-irradiated skin, were removed and pooled. A single cell suspension was made and DC were purified on a 14.5% metrizamide cushion as previously described [7]. The number of DC per lymph node was deduced after a mean of four cell counts was calculated for each group.

CH Assay The CH response to oxazolone was measured by a standard technique. Briefly, nine mice per group received the *cis*-MoAb, irrelevant antibody, or PBS 4 h prior to UVB irradiation with 960 J/m^2. Twenty-four hours later, the injections and the UV irradiation were repeated. One day later all mice received a sensitizing dose of 50 μl 1% oxazolone on their shaved backs, except the negative control group, which received vehicle alone. Six days after the sensitization step, ear thicknesses were measured and all the mice were challenged with 25 μl per ear of 0.25% oxazolone on the dorsal surface. One day after the challenge, the ear thicknesses were measured again. The mean increase in ear thickness for each individual mouse was first calculated and then the mean increase for each group of mice. All the groups were coded.

DH Assay The DH response to HSV was measured as described elsewhere [18]. Briefly, 7–9 mice per group received the *cis*-MoAb, negative ascitic fluid, or PBS 4 h prior to UVB irradiation with 960 J/m^2. Three days later, all mice except the negative control group were infected with 5 × 10^6 plaque forming units of HSV type 1 subcutaneously in the back of the neck. Ten days after the sensitization step, ear thicknesses were measured and all the mice were challenged with 7.5 × 10^5 erstwhile plaque-forming units per ear of inactivated HSV in a 10 μl volume. One day after the challenge, the ear thicknesses were measured again. The mean increase in ear thickness for each individual mouse was first calculated and then the mean increase for each group of mice. All the groups were coded. This experiment was repeated twice and the combined data of the three experiments are presented here. The background control increase in ear thickness in a group of mice not infected with HSV but challenged with HSV was subtracted.

RESULTS

***cis*-MoAb Prevents the UVB and *cis*-UCA–Induced Reduction in Langerhans Cell Numbers** This experiment was performed twice and the pooled results are presented. A single suberythemal dose of 960 J/m^2 of UVB resulted in a 35% reduction in the number of ATPase$^+$ cells in the epidermis of C3H mice 24 h later **(Fig 1)**. This confirmed previously reported results [6]. An IP injection of 0.1 μg of the *cis*-MoAb, prior to UVB treatment, resulted in the total abrogation of this reduction. Similarly, the application of 20 μg of *cis*-UCA on the ears of C3H mice resulted in a reduction in the number of ATPase$^+$ cells that could be inhibited by a prior injection of the *cis*-MoAb **(Fig 1)**. Using an irrelevant monoclonal antibody had no effect in either system. Thus, the administration of the *cis*-MoAb was able to block a UV-induced event *in vivo*.

***cis*-MoAb Prevents the UVB–Induced Reduction in Langerhans Cell Function** A single dose of 1440 J/m^2 of UVB immediately prior to killing the mice and removing EC caused a 32% reduction in the MSLR. The *cis*-MoAb prevented this reduction in antigen-presenting function whereas the irrelevant monoclonal antibody had no effect **(Fig 2)**. In a separate experiment where no *cis*-MoAb was injected and no UV given, an application

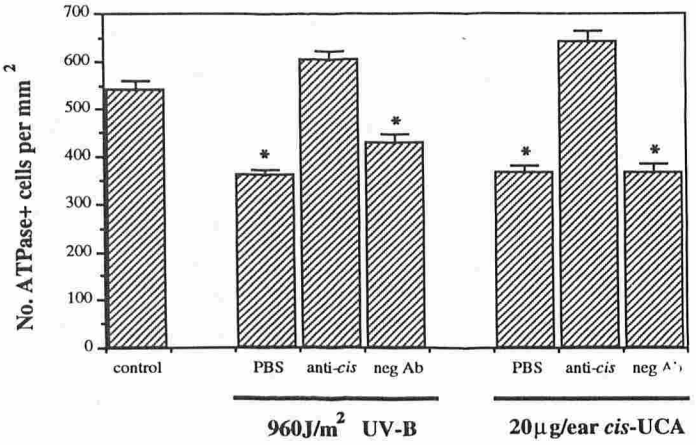

Figure 1. The *cis*-MoAb prevents the UV-B– and *cis*-UCA–induced reduction in Langerhans cell numbers. Four C3H mice per group were injected intraperitoneally with PBS, 0.1 μg of the *cis*-MoAb (anti-*cis*) or irrelevant monoclonal antibody (neg Ab) and 2 h later received 960 J/m^2 UVB or 20 μg per ear *cis*-UCA epicutaneously. Control mice were untreated. The number of ATPase$^+$ cells per mm^2 was counted 24 h later. The *bars* depict the SEM. *$p < 0.01$ compared with the control group.

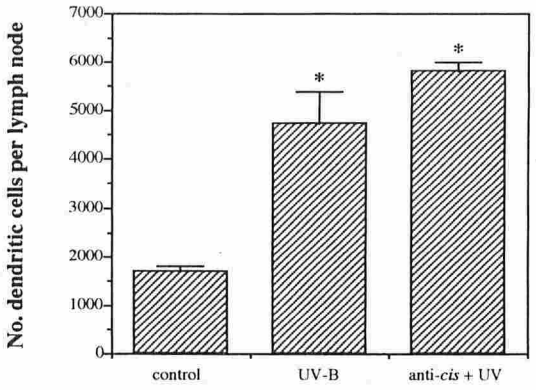

Figure 3. The *cis*-MoAb has no effect on the UVB-induced accumulation of dendritic cells in draining lymph nodes. The number of dendritic cells per lymph node 48 h after a single dose of 1440 J/m^2 UVB (UV-B) and after *cis*-MoAb pretreatment plus UVB (anti-*cis* + UV) is shown. The *bars* indicate SEM. *Significant difference from the control unirradiated group ($p < 0.01$). The difference between the "UV-B" and the "anti-*cis* + UV" groups was not significant ($p = 0.162$).

of *cis*-UCA alone to the ears 24 h prior to removing EC also resulted in a significant suppression of the MSLR. *Trans*-UCA, however, had no such effect **(Fig 2)**. The control proliferation in these experiments was relatively low, indicating that freshly isolated LC are poor stimulators as already reported [25]. The same pattern was obtained when the first part of this experiment (the blocking of the UV-induced reduction in the MSLR) was repeated.

***cis*-MoAb Has No Effect on UV-Induced Accumulation of DC in Lymph Nodes Draining Irradiated Sites** The average number of DC in one lymph node of a normal 7-week–old C3H mouse is around 1600. Forty-eight hours after a single UVB dose, this number increased to approximately 4700 per lymph node. This increase was not significantly altered when *cis*-MoAb was injected prior to UV treatment **(Fig 3)**. A similar result was obtained when this experiment was repeated.

***cis*-MoAb Has No Effect on the UVB-Induced Suppression of the CH Response** The CH response to oxazolone following two doses of UVB was reduced in comparison with non-irradiated controls. Following an intraperitoneal injection of a range of concentrations of the *cis*-MoAb or the irrelevant monoclonal antibody this UV-induced reduction was maintained **(Fig 4)**. The response of all the UV-irradiated groups was significantly lower than the positive control group, whether the animals had been pretreated with monoclonal antibody or not. The *cis*-MoAb therefore was unable to abrogate the UV-induced reduction in the CH response.

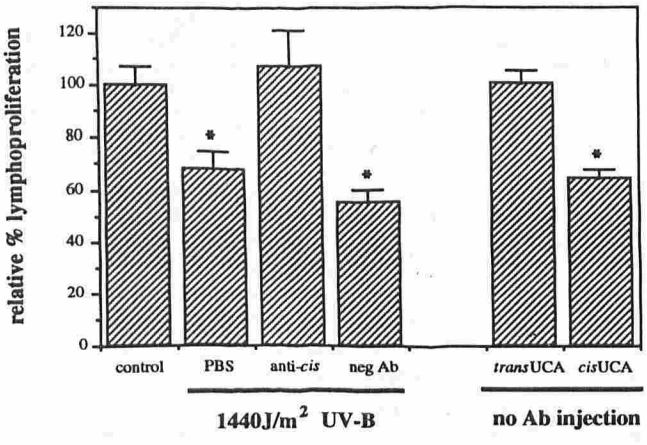

Figure 2. The *cis*-MoAb prevents the UVB-induced reduction in Langerhans cell function in the MSLR. Mice were injected intraperitoneally with PBS, 0.1 μg of the *cis*-MoAb (anti-*cis*) or irrelevant MoAb (neg Ab) and 2 h later received 1440 J/m^2 UVB. Other groups received 20 μg *trans*-UCA or 20 μg per ear *cis*-UCA epicutaneously. The relative lymphoproliferation as percentage of control MSLR is shown. The *bars* indicate the SEM. *Statistical significance ($p < 0.01$) of the data when compared with the control group. The 100% control proliferation (EC from untreated mice mixed with responder cells) was 1785 cpm in one experiment and 5506 cpm in another. Background proliferation of spleen cells alone was 597 and 552 cpm, respectively. Background proliferation of EC alone was 258 and 271 cpm, respectively.

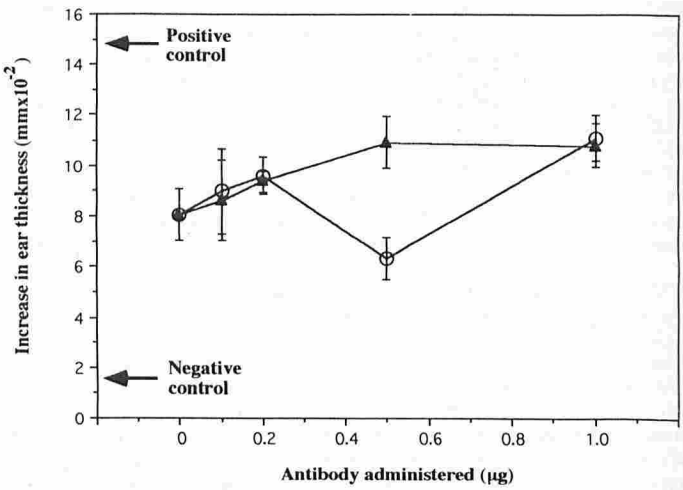

Figure 4. The *cis*-MoAb has no effect on the UVB-induced suppression of the CH response. The mean increase in ear thickness of nine mice per group and the SEM are shown. Mice were administered intraperitoneally 0.1 to 1 μg IgG 4 h prior to UVB irradiation with 960 J/m^2 on two consecutive days. Twenty-four hours later mice were sensitized with 1% oxazolone and 6 d later challenged with 0.25% oxazolone. The positive control group was sensitized and challenged whereas the negative control group received only the challenge. *Open circles* represent the *cis*-MoAb and the *closed triangles* represent the irrelevant monoclonal antibody.

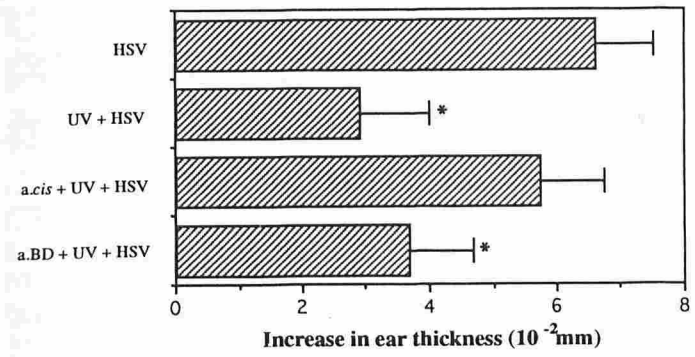

Figure 5. The *cis*-MoAb prevents the UVB-induced reduction in the DH response to HSV. Mean increase in ear thickness as a measure of the DH response and the SEM are shown. All mice were challenged with inactivated HSV. HSV, positive control group infected with 5×10^6 PFU HSV. This group received an intraperitoneal injection of PBS prior to infection with HSV. UV + HSV, UVB irradiated (960 J/m^2) once 3 d prior to infection with HSV. a.*cis* + UV + HSV, 0.1 μg of the *cis*-MoAb was administered 4 h prior to UVB irradiation. a.BD + UV + HSV, the irrelevant MoAb was administered prior to UVB irradiation. *$p < 0.05$ compared with the HSV group.

***cis*-MoAb Prevents the UVB-Induced Reduction in the DH Response to HSV** UVB irradiation has been previously shown to reduce the DH response to HSV by 54–92% [11]. A similar result was obtained in this series of experiments. Following an injection of PBS or the irrelevant monoclonal antibody, UV irradiation resulted in a reduction in the DH response **(Fig 5)**. However, when the *cis*-MoAb was used prior to irradiation, the DH response was restored to its normal level **(Fig 5)**. This represented an abrogation of the UV-induced reduction of the DH response. The data presented in this figure are the means of three repeat experiments.

DISCUSSION

The *cis*-isomer of UCA has been implicated as a photoreceptor for the UV-induced alterations to the immune system following irradiation (reviewed in [13,26]). In attempting to determine which UV-induced immunosuppressive events are mediated by *cis*-UCA, the MoAb to *cis*-UCA was administered prior to UVB irradiation and modulation in various immune parameters was measured. Epicutaneous application of *cis*-UCA alone mimics some of the effects of UVB. Thus, topical application of 20 μg *cis*-UCA reduces the number of ATPase-positive cells (LC) in murine ears [16], as does UVB exposure. The *cis*-MoAb prevented the UVB-induced and *cis*-UCA–induced reduction in LC density, indicating that *cis*-UCA is acting as an important mediator for the loss of ATPase-positive cells from the epidermis. As already demonstrated for UVB irradiation [6], a topical application of 20 μg *cis*-UCA was also capable of reducing LC function, as measured by the MSLR. The *cis*-MoAb prevented the UVB-induced reduction in MSLR response. This may occur through an inhibition of ICAM-1 expression on LC [4] or another co-stimulatory molecule such as B7/BB1 [27], thus reducing antigen-presenting capacity. Topical or subcutaneous application of *cis*-UCA has no detectable effect on DC accumulation in lymph nodes, whereas UVB induces DC accumulation in draining lymph nodes [7]. Therefore it is perhaps not surprising that the *cis*-MoAb did not affect the UV-mediated migration. It is interesting to note that a topical application of *cis*-UCA reduces the density of LC in the epidermis but does not cause an increase in DC numbers in draining lymph nodes. Perhaps *cis*-UCA alone is not sufficient signal to induce migration to lymph nodes. Alternatively, the DC accumulating in lymph nodes following UV irradiation may have come from sites other than the epidermis. It may be that multiple stimuli or mediators are required for some suppression events.

Previously we have been unable to demonstrate that topical or subcutaneous application of 20–200 μg *cis*-UCA per mouse significantly suppresses the CH response to oxazolone [Moodycliffe AM: Mechanism of immunosuppression induced by ultraviolet-B light irradiation. PhD thesis, University of Edinburgh, 1993]. Others, however, have reported a suppression of CH to dinitrofluorobenzene following intradermal inoculation of 200 μg *cis*-UCA [15]. The route of administration of *cis*-UCA, the strain of mouse, the contact sensitizer as well as the sensitizing dose used [28] may play a role in determining the outcome of such CH experiments. In our CH experiments, the *cis*-MoAb was unable to prevent the UV suppression of the CH response to oxazolone at a wide range of concentrations, indicating that *cis*-UCA is unlikely to be directly involved in the UV suppression of this hypersensitivity response. On the other hand, topical application of as small a quantity as 1–10 μg *cis*-UCA is effective in suppressing the DH response to HSV in a similar manner to UVB irradiation [18], and 0.1 μg of the *cis*-MoAb per mouse abrogated the UVB-induced suppression of the DH response. There is evidence that the mechanisms of CH and DH are different [29]. For example, LC are involved in inducing a CH response [30] whereas other antigen-presenting cells, such as dermal dendrocytes or tissue macrophages, may be important in the initiation of a DH response to a complex antigen administered subcutaneously. In addition, interleukin-10 has been implicated in the UV suppression of DH, but not CH [21]. Several attempts in our laboratory at using similar protocols for initiating and UV suppressing the CH and DH responses have proved unsuccessful. It is not surprising, therefore, that the means by which UV suppresses these two responses is also mediated differently.

Cis-UCA is not the only mediator of UV-induced immunosuppression that has been proposed and, amongst the others, TNF-α has been the most studied. It is released by murine keratinocytes following UV irradiation *in vivo* and *in vitro*. It has been previously proposed that *cis*-UCA mediates its suppressive function *via* TNF-α [15,20]. Our findings disagree with this conclusion. It has also been reported that the genetic susceptibility to UV radiation in inbred strains of mice is restricted to polymorphisms in the *Lps* and *Tnfa* loci [8], which influence the amount of TNF-α produced in response to UV. However, more recent studies on the control of genetic susceptibility to UV immunosuppression have not confirmed the role of these loci [31]. In addition, intradermal injection of *cis*-UCA has been shown to be capable of suppressing the CH responses in both UV-susceptible and UV-resistant strains [15], indicating that *cis*-UCA and TNF-α probably act independently.

Although polyclonal antibody to TNF-α totally abrogates the reduction in LC numbers in the epidermis following UV irradiation [20], it only partially restores the reduction following *cis*-UCA administration [15]. This, in conjunction with our results, may indicate that *cis*-UCA and TNF-α mediate the reduction in LC density by two overlapping mechanisms. TNF-α does not inhibit LC accessory cell function and TNF-α antibodies do not reverse the inhibitory effects of UVB irradiation on antigen presentation [4]. It is likely therefore that *cis*-UCA, but not TNF-α, is a mediator of the loss of this LC function following irradiation. Antibody to TNF-α substantially abrogates the UVB-induced accumulation of DC in lymph nodes [32], indicating that TNF-α, and not *cis*-UCA, mediates the migration of DC to lymph nodes. However, antibody to TNF-α does negate the UVB-induced suppression of CH [32]. Thus, in this response, TNF-α, and not *cis*-UCA, seems to play an important modulating role. Moreover, DNA damage [19] and prostaglandins [12] have both been implicated in the UV suppression of the CH response reflecting a complex interaction of mediators, some of which may function by overlapping mechanisms.

Previously, using the same murine model of HSV infection as reported here, we showed that neutralizing TNF-α antibodies partially abrogated the UVB-induced suppression of the DH response but had no effect on the suppression induced by *cis*-UCA [17]. It seems therefore that both *cis*-UCA and TNF-α may be implicated in the suppression of the DH response to HSV following UV exposure, possibly by two different mechanisms. In addition,

DNA damage mediates the suppression of the DH response by UV radiation *via* yet another mechanism [19]. These data point to a complex interaction of several mediators following UV irradiation with considerable redundancy in the systems that have evolved for suppressing immune responses in the skin. Perhaps certain wavelengths and/or doses of UV promote particular pathways and protect against excessive responses to antigens encountered frequently through the skin. In addition, it may have been necessary to develop several means to prevent immune responses to neoantigens induced in the skin as a result of the mutagenic activity of UV exposure.

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