Mechanisms Underlying the Suppression of Established Immune Responses by Ultraviolet Radiation

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The ultraviolet radiation present in sunlight is immune suppressive. Recently we showed that solar-simulated ultraviolet radiation (ultraviolet A + B; 295–400 nm), applied after immunization, suppressed immunologic memory and the elicitation of delayed-type hypersensitivity to the common opportunistic pathogen, *Candida albicans*. Further, we found that wavelengths in the ultraviolet A region of the solar spectrum (320–400 nm), devoid of ultraviolet B, were equally effective in activating immune suppression as ultraviolet A + B radiation. Here we report on the mechanisms involved. Maximal immune suppression was found when mice were exposed to solar-simulated ultraviolet radiation 7–9 d post immunization. No immune suppression was found in ultraviolet-irradiated mice injected with monoclonal anti-interleukin-10 antibody, or mice exposed to solar-simulated ultraviolet radiation and injected with recombinant interleukin-12. Suppressor lymphocytes were found in the spleens of mice exposed to ultraviolet A + B radiation. In addition, antigen-specific suppressor T cells (CD3$^+$, CD4$^+$, DX5$^+$) were found in the spleens of mice exposed to ultraviolet A radiation. Applying liposomes containing bacteriophage T4N5 to the skin of mice exposed to solar-simulated ultraviolet A + B radiation, or mice exposed to ultraviolet A radiation, blocked immune suppression, demonstrating an essential role for ultraviolet-induced DNA damage in the suppression of established immune reactions. These findings indicate that overlapping immune suppressive mechanisms are activated by ultraviolet A and ultraviolet A + B radiation. Moreover, our findings demonstrate that ultraviolet radiation activates similar immunologic pathways to suppress the induction of, or the elicitation of, the immune response. *Key words: delayed type hypersensitivity/immune suppression/skin cancer/solar–simulated UV radiation/suppressor T cells. J Invest Dermatol 119:600–608, 2002*

The adverse effects of ultraviolet (UV) radiation on human health and well-being are well known (Ullrich, 2000). Besides being the primary cause of human nonmelanoma skin cancer (Urbach, 1997), the UV radiation present in sunlight is immune suppressive (Kripke, 1974; Kripke and Fisher, 1976). The immune suppressive effects of UV radiation contribute to skin cancer development by depressing cell-mediated immune reactions that normally serve to destroy the developing skin tumors. Epidemiologic studies with immune suppressed renal transplant patients (Penn, 1984), experiments with laboratory mice (Fisher and Kripke, 1982), and immunologic studies with skin cancer patients (Yoshikawa et al, 1990) support the hypothesis that the immune suppression induced by UV exposure is a major risk factor for skin cancer induction. In addition to suppressing tumor rejection, UV radiation interferes with a wide variety of immune reactions including contact hypersensitivity to chemical allergens applied to the skin (Noonan et al, 1981; Cooper et al, 1992) and delayed-type hypersensitivity (DTH) to bacterial (Jeevan and Kripke, 1989) and viral (Howie et al, 1986) antigens.

In the majority of studies documenting UV-induced suppression of the immune response to microbial and viral agents, the UV was administered to naive animals prior to immunization (i.e., suppressing the induction of immunity). Of equal concern, however, is the ability of UV exposure to suppress established immune responses. Perhaps the most important medical advance of the past century was the reduction, and in some cases the eradication, of microbial and viral infections through the widespread use of childhood vaccinations. Because UV radiation can suppress the elicitation of certain immune responses (Denkins et al, 1989; Magee et al, 1989; Damian et al, 1997; Moyal et al, 1997), sunlight exposure may compromise the ability of prior vaccination to control infectious disease.

Recently we reported that exposing mice to solar-simulated UV radiation suppressed immunologic memory and the elicitation of DTH *in vivo* (Nghiem et al, 2001). We found that UVA radiation, essentially devoid of UVB, was equally effective at suppressing the elicitation of DTH as was solar-simulated UV radiation. In

addition, we found that sunscreens that absorbed only UVB radiation were ineffective at protecting against immune suppression and immune protection was observed only with sunscreens that absorbed both UVB and UVA radiation. Moyal and Fourtanier (2001) came to a similar conclusion in a study using human volunteers and natural sunlight; UVA protection was required for maximal protection against UV-induced suppression of established immune reactions. Unfortunately little is known concerning the underlying immunologic mechanism(s) of UV-induced suppression of an established immune response. The focus of the experiments presented here is to understand the immunologic mechanisms involved.

MATERIALS AND METHODS

Antibodies and reagents Dr. Stanley Wolf, Genetics Institute (Cambridge, MA), provided us with the recombinant interleukin-12 (IL-12). The hybridoma secreting anti-IL-10 (JES-2A5.11) was kindly provided by Dr. Anne O'Garra, DNAX Research Institute, Palo Alto, CA. The hybridoma cells were grown in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% newborn bovine serum (HyClone Laboratories, Logan, UT). Supernatants were collected, the IgG fraction was enriched by 33% ammonium sulfate precipitation, and the IgG was further purified by passage over protein A/G columns (Pierce Immunochemicals, Rockford, IL). Protein concentration was determined by use of the bicinchoninic acid protein determination kit (Pierce Immunochemicals). Control rat IgG was purchased from Sigma (St. Louis, MO). Liposomes containing the bacteriophage DNA excision repair enzyme T4N5 were kindly provided by Dr. Dan Yarosh, AGI-Dermatics, Freeport, NY. They were prepared and used as described previously (Kripke et al, 1992). Keyhole limpet hemocyanin (KLH) was purchased from Pierce Immunochemicals.

Animals Specific-pathogen-free female C3H/HeNCr (MTV) mice were obtained from the National Cancer Institute Frederick Cancer Research Facility Animal Production Area (Frederick, MD). The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with current regulations and standards of the National Institutes of Health. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee. Within each experiment all the mice were age matched. The mice were 8–10 wk old at the start of each experiment.

Radiation source A 1000 W xenon UV solar simulator equipped with a Schott WG-320 atmospheric attenuation filter (1 mm thick), a visible/infrared bandpass blocking filter (Schott UG-11; 1 mm thick), and a dichroic mirror to further reduce visible and infrared energy (Oriel, Stratford, CT) was used to provide solar-simulated UV radiation (UVA + UVB). Replacing the WG-320 filter with a 3 mm thick WG-335 filter resulted in a UVA source deficient in UVB. The WG-320 and WG-335 filters were purchased from Oriel. The intensity and spectral output of the WG-320 and WG-335 equipped solar simulator were measured with an Optronics model OL 754 scanning spectrophotometer interfaced to an Acer model 330 notebook computer (Optronics Laboratories, Orlando, FL). The spectral output of both light sources has been published (Nghiem et al, 2001). During irradiation of the shaved dorsal skin, the mice were held individually in a specially constructed Plexiglas container with a quartz glass top, to prevent cage mates from climbing on top of each other and interfering with the UV dose applied. Spectrophotometer readings were taken through the quartz glass top. During the irradiation period (15–90 min in duration) the mice were conscious and had full range of movement.

Suppression of the elicitation of DTH by solar-simulated UV radiation Female C3H/HeN mice were immunized by subcutaneous injection of 10^7 formalin-fixed Candida albicans into each flank. Nine days later the immunized mice were shaved and exposed to solar-simulated UV radiation as described previously (Ananthaswamy et al, 1999). The next day each hind footpad was measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan) and then challenged by intrafootpad injection of 50 μl of Candida antigen (Alerchek, Portland, ME). Eighteen to 24 h later the thickness of each foot was re-measured and the mean footpad thickness for each mouse was calculated (left foot + right foot ÷ 2). Generally, there were five mice per group; the mean footpad thickness for the group ± the standard deviation of the mean was calculated. The background footpad swelling (negative control) was

determined in a group of mice that were not immunized but were challenged. The specific footpad swelling response was calculated by subtracting the background response observed in the negative controls from the mean footpad swelling found in mice that were immunized and challenged. Each experiment was repeated at least three times. Statistical differences between the controls and experimental groups were determined by use of the two-tailed Student's t test, with a probability of less than 0.05 considered significant (Prism Statistical Software, GraphPad, San Diego, CA). Percentage immune suppression was determined using the following formula: % immune suppression = 1 − (specific footpad swelling of the UV-irradiated mice ÷ specific footpad swelling of the positive control) × 100.

Isolation of T cell subsets Splenic $CD4^+$ T cells were purified by negative selection using antibody cocktails and magnetic microbeads (Stem Cell Technologies, Vancouver, Canada) as described previously (Moodycliffe et al, 2000). They were then stained with rat antimouse pan natural killer cell monoclonal antibody (DX5, IgM, PharMingen, San Diego, CA) followed by mouse antirat IgM (IgG). After staining, the T cells were mixed with antimouse IgG-coated magnetic beads, at a cell to bead ratio of 4:1 (Dynal, Great Neck, NY), and the mixture was enriched for $DX5^+$ cells by passing over a magnetic column. Relative purity of each population was determined by flow cytometry using monoclonal antibodies specific for T cell receptor αβ, CD3, CD4, and CD8.

Measurement of UV-induced cyclobutane pyrimidine dimers (CPD) Two methods were used to document the formation of CPD by UVA, immunohistochemistry and radioimmunoassay. The shaved dorsal skin of adult C3H/HeN was exposed to 80 kJ per m^2 of UV radiation supplied by the WG-320- or WG-335-filtered xenon solar simulator. Twenty-four hours after UV exposure the mice were killed and epidermal DNA was extracted according to the procedures described by Ananthaswamy et al (1999). The numbers of CPD present in the epidermal DNA of control nonirradiated animals, or found in the DNA of mice exposed to UVA + UVB or UVA only, were determined by use of a radioimmunoassay, as described previously (Mitchell, 1999). There were three mice per group. DNA was isolated from each animal and samples from each mouse were run in triplicate. The data are expressed as the mean number of CPD per 1 million bases of DNA ± the standard deviation of the mean. Statistical differences between the numbers of CPD found in the control DNA and the DNA isolated from the UV-irradiated mice were determined by use of Student's t test.

UVA-induced CPD were also measured by immunohistochemical analysis. Mice were exposed to 80 kJ per m^2 solar-simulated UVA + UVB radiation or 80 kJ per m^2 of UVA radiation. Skin sections from the UV-irradiated mice and sections from normal nonirradiated control mice were harvested 24 h after irradiation. They were embedded in Tissue-Tek OCT medium (Miles Laboratories, Elkhart, IN) and snap frozen in liquid nitrogen, and 5 μm thick sections were cut with a cryostat. A mouse monoclonal antibody (H3; IgG1λ) and goat antimouse IgG fluorescein-labeled secondary antibodies were used for CPD immunostaining of the skin sections. The antibody was developed against cyclobutane thymine dimers in single-stranded DNA (Roza et al, 1988) and has high affinity for 5′-T-containing dimers (Fekete et al, 1998). CPD were detected by fluorescent microscopy.

RESULTS

Time course for the suppression of established immune reactions by solar-simulated radiation From our previous experiments we know that exposure to solar-simulated UV radiation, given 9 d post immunization, suppresses the elicitation of DTH to the fungal antigen C. albicans. Exposing mice to 80 kJ per m^2 of solar-simulated UV radiation yielded 50% immune suppression (Nghiem et al, 2001). What was not clear was the timing between immunization and UV exposure required for optimal activation of immune suppression. Therefore, we set up an experiment in which groups of mice were immunized with C. albicans on day 0 and then exposed to 80 kJ per m^2 solar-simulated UV radiation on subsequent days. All mice were challenged with antigen on day 10 and DTH was measured 18–24 h after challenge. Data from this experiment are found in **Table I**. In this particular experiment, maximal immune suppression (74%, p < 0.01 versus the positive control) was observed when the mice were exposed to UV 9 d after immunization. Significant immune suppression (p < 0.05) was also noted when the mice were immunized 7 d post

Table I. Time course for suppression of the elicitation of DTH by solar-simulated UV radiation

Treatment[a]	Δ footpad[b] swelling	Specific swelling[c]	%[d] suppression	p[e]
Negative control	1.8 ± 1.5	–	–	
Positive control	18.5 ± 7.6	16.7	–	–
UV 3 d post immunization	11.4 ± 2.7	9.6	43	NS
UV 5 d post immunization	12.8 ± 3.9	11	34	NS
UV 7 d post immunization	9.1 ± 1.4	7.3	56	0.05
UV 9 d post immunization	6.1 ± 1.8	4.3	74	0.01

[a]Mice were immunized with *C. albicans* on day 0, exposed to 80 kJ per m^2 of UV radiation 3–9 d post immunization, and challenged with antigen on day 10. DTH was measured 18–24 h post challenge. Negative control refers to mice that were not immunized but were challenged; positive control refers to mice that were immunized and challenged.
[b]mm × 10^{-2} ± SD, N = 5.
[c]Change in footpad swelling of the positive control or experimental groups minus the background swelling found in the negative control.
[d]% immune suppression = 1 – (specific footpad swelling of the experimental groups ÷ specific footpad swelling of the positive control) × 100.
[e]p-values determined by two-tailed Student's *t* test *versus* the positive control; p > 0.05 considered not significant (NS).

immunization (56%). No significant immune suppression was noted when the mice were UV irradiated 3–5 d post immunization. This experiment was repeated three times, and in all cases significant immune suppression was observed when the UV was given 7–9 d post immunization. In all subsequent experiments, we irradiated the mice 9 d post immunization.

A role for cytokines in suppressing established immune reactions by solar-simulated UV radiation   First we tested the hypothesis that UV-induced IL-10 is involved in suppressing established immune reactions. Mice were immunized on day 0 and exposed to UV on day 9. Four hours following UV exposure, 100 μg of monoclonal anti-IL-10 or 100 μg of rat IgG was injected into the peritoneal cavity. The next day the mice were challenged with antigen and the effect anti-IL-10 had on the suppression of DTH was measured (**Fig 1**). Two different radiation sources were used in this experiment, the WG-320-filtered solar simulator (UVA + UVB) and the WG-335-filtered solar simulator (UVA). Exposing mice to 80 kJ per m^2 of solar-simulated radiation (UVA + UVB) suppressed the elicitation of DTH. Injecting the UV-irradiated mice with rat IgG had no effect on the degree of immune suppression. When the mice were injected with monoclonal anti-IL-10, however, the suppressive effect was lost (p > 0.05 *versus* the positive control).

Identical results were observed when the mice were exposed to UVA radiation and injected with monoclonal anti-IL-10. As shown previously, UVA suppresses the elicitation of DTH (Nghiem *et al*, 2001). Treating the UVA-irradiated mice with rat IgG failed to reverse the immune suppression, but when the UVA-irradiated mice were injected with neutralizing anti-IL-10, the DTH response generated was not significantly different from the positive control (p > 0.05). These data indicate that UV-induced IL-10 plays a role in suppressing the elicitation of DTH by UVA and by solar-simulated UV radiation.

Previously, we demonstrated that injecting UV-irradiated mice with recombinant IL-12 reversed the UV-induced suppression of the induction of immunity (Schmitt *et al*, 1995; 2000). Next we wished to determine if IL-12 would reverse the suppression of the elicitation of DTH by UV radiation. Mice were immunized with *C. albicans* and then exposed to solar-simulated UVA + UVB radiation. Four hours after UV exposure (80 kJ per m^2) recombinant IL-12 or vehicle (phosphate-buffered saline containing 1% fetal bovine serum) was injected into the peritoneal cavity. Significant immune suppression (p < 0.001) was observed in mice exposed to UV radiation, or exposed to UV and injected with the vehicle. When the UV-irradiated mice were injected with 1–2 μg of IL-12, doses of IL-12 that totally reversed the UV-induced suppression of the induction of immunity (Schmitt *et al*, 1995), no suppression of the elicitation of DTH was noted (**Figs 1, 2**) indicate that cytokines are involved in suppressing the elicitation of DTH by solar-simulated UV radiation.
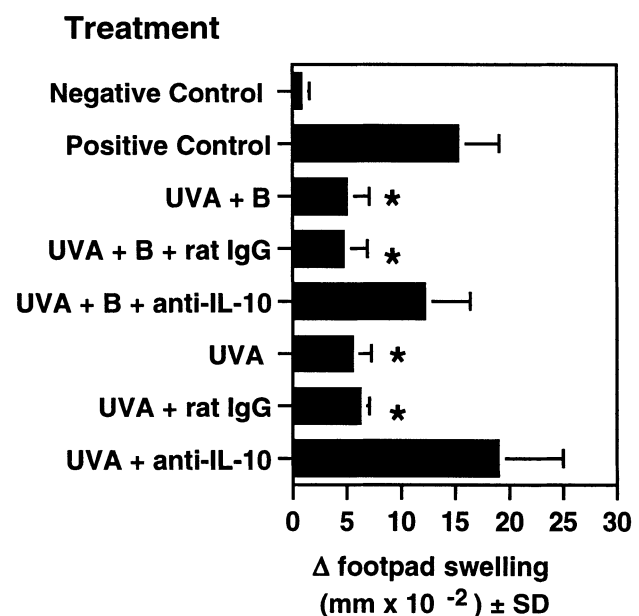

Figure 1. Neutralizing IL-10 activity *in vivo* blocks UV-induced suppression of the elicitation of DTH. Mice were immunized with *C. albicans* on day 0, and on day 9 they were exposed to 80 kJ per m^2 solar-simulated UV radiation (UVA + UVB) or to UVA only. Four hours following UV exposure, the mice were injected with monoclonal anti-IL-10 or rat IgG. DTH was measured on day 11. *Significant difference (p = 0.0001, two-tailed Student's *t* test) *versus* the positive control.

Activation of antigen-specific suppressor T cells by UV exposure   The activation of immune regulatory T cells is a prominent feature of the immune suppression induced by UV exposure. We wished to determine whether suppressor T cells can be found in the lymphoid organs of mice first immunized with *C. albicans* and then exposed to UV radiation. Donor mice were exposed to 80 kJ per m^2 of solar-simulated UV (UVA + UVB) radiation 9 d post immunization. In addition, one set of donor mice were injected with 100 μg of isotype-matched control IgG, and another set were injected with 100 μg of anti-IL-10 antibody. As shown above, significant immune suppression was noted in donor mice exposed to UVA + UVB radiation, or exposed to UVA + UVB radiation and injected with control IgG. The spleens of the donor mice were removed, single cell suspensions were prepared, and the cells (10^8 per mouse) were injected into the tail veins of recipient mice. The recipient mice were then
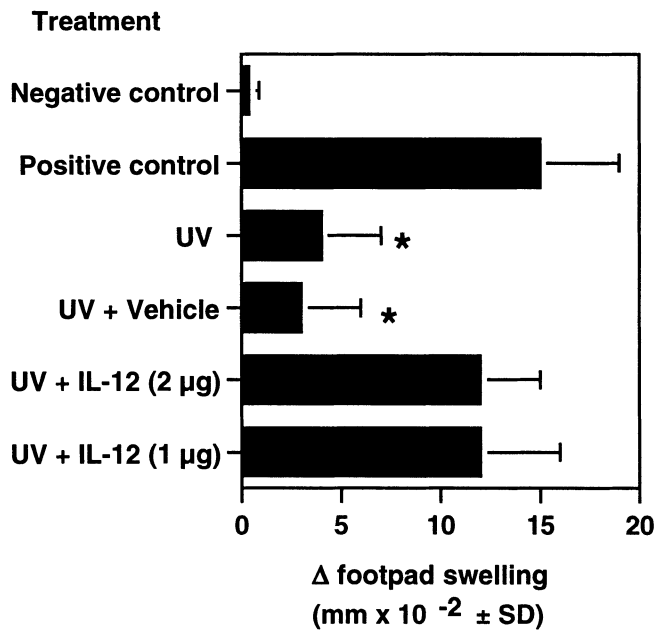
Treatment

Figure 2. Recombinant IL-12 restores immune function in UV-irradiated mice. Mice were immunized with *C.* albicans on day 0, and on day 9 they were exposed to 80 kJ per m^2 solar-simulated UV radiation. Four hours following UV exposure, the mice were injected with recombinant IL-12. DTH was measured on day 11. *Significant difference (p = 0.0001, two-tailed Student's *t* test) *versus* the positive control.

immunized with *C. albicans* and the DTH reaction was measured 11 d later. The data from this experiment are found in **Fig 3(A)**. The positive control for this experiment consisted of measuring the immune response in a group of mice that were simply immunized and challenged with antigen. An additional control consisted of injecting 10^8 spleen cells from mice immunized with antigen but not exposed to UV (NR-SC) into another group of recipient mice. The magnitude of the immune reaction observed in these two groups was identical, indicating that the simple transfer of spleen cells does not adversely affect DTH in the recipients. Significant immune suppression (p < 0.001) was observed in mice that received spleen cells from the solar-simulator-irradiated donors, indicating that UV exposure post immunization activates suppressor cells. Injecting UV-irradiated mice with control antibody did not affect the activation of suppressor cells. No immune suppression was noted when spleen cells were isolated from donor mice that were exposed to UV and then injected with anti-IL-10, however. These data indicate that IL-10 plays an essential role in the activation of suppressor cells.

Next we asked if UVA radiation could activate suppressor cells. Spleen cells were transferred from three different groups of donor mice. The first set of donors were immunized and challenged but not exposed to UV radiation. The second set of donors were exposed to solar-simulated UV radiation (UVA + UVB), 9 d post immunization. The third set of donor mice were exposed to 80 kJ per m^2 of UVA radiation 9 d post immunization. As shown above, irradiating the donor mice with UVA or UVA + UVB significantly suppressed DTH. The spleens of the donor mice were removed, single cell suspensions were prepared, and the cells (10^8 per mouse) were injected into the tail veins of recipient mice. The recipient mice were then immunized with *C. albicans* and the DTH reaction was measured 11 d later. The data from this experiment are found in **Fig 3(B)**. Significant (p < 0.005) immune suppression was observed when spleen cells from donor mice, exposed to solar-simulated UV radiation (UVA + B-SC), were injected into the recipient mice. In addition, significant immune suppression
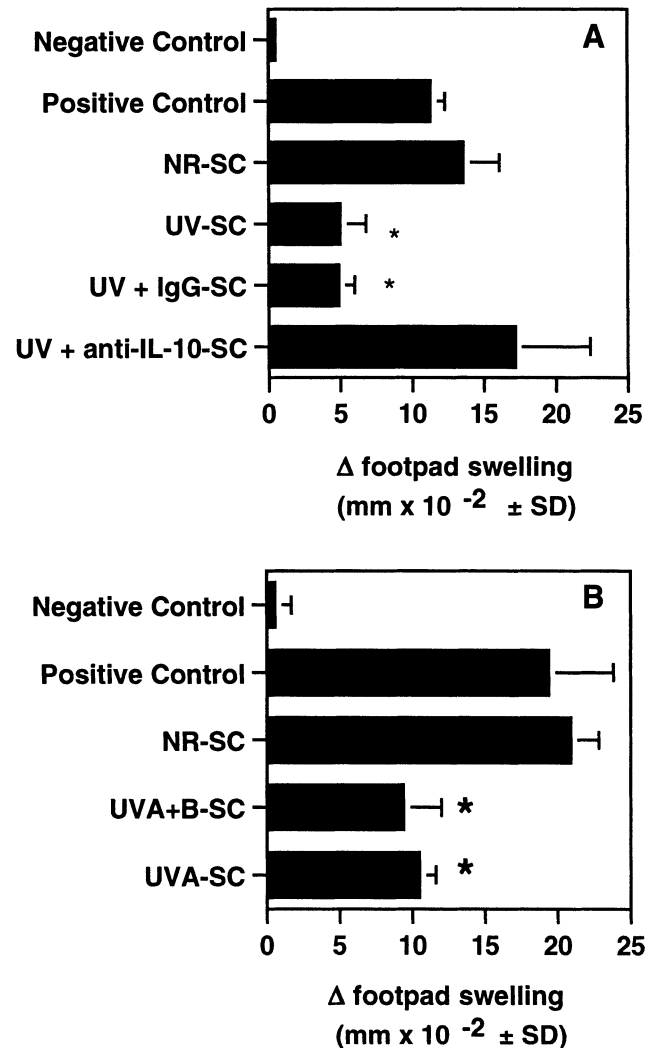

Figure 3. Suppressor cells are found in the lymphoid organs of mice exposed to UV radiation 9 d post immunization. *Panel A:*10^8 spleen cells from solar-simulated-UV-irradiated mice (UV-SC), spleen cells from solar-simulated-UV-irradiated mice injected with isotype-matched control antibody (UV + IgG-SC), spleen cells from solar-simulated-UV-irradiated mice injected with monoclonal anti-IL-10 (UV + anti-IL-10-SC), or spleen cells from nonirradiated control mice (NR-SC) were injected into recipient mice. The mice were immediately immunized and DTH was measured 11 d later. *Panel B:*the induction of suppressor cells by UVA radiation was measured. Recipient mice received spleen cells from donors exposed to 80 kJ per m^2 solar-simulated UV radiation (UVA + UVB) or 80 kJ per m^2 UVA radiation. *Significant difference (p = 0.005, two-tailed Student's *t* test) *versus* the positive control.

(p < 0.005) was observed when spleen cells from donor mice exposed to UVA were injected into recipient mice (UVA-SC). As before no immune suppression was observed when cells from non-UV-irradiated control mice (NR-SC) were injected into the recipient mice. These findings indicated that UVA radiation activates suppressor cells.

A second prominent feature of UV-induced immune suppression is the activation of antigen-specific suppressor cells. Years ago, we reported that exposing mice previously immunized with allogeneic histocompatibility antigen to UVB radiation suppressed the elicitation of DTH, in part through the activation of antigen-specific suppressor T cells (Magee *et al*, 1989). Are the suppressor cells found in the lymphoid organs of UVA-irradiated mice antigen-specific T cells? To address this question the following

Table II. Induction of antigen–specific suppressor cells by UVA radiation

Cells transferred[a]	Antigen used to immunize recipients	Δ footpad[b] swelling	Specific swelling[c]	%[d] suppression	p[e]
None	–	2.6 ± 0.9			
None	KLH	24.5 ± 2.3	21.9	0	–
KLH, no UVA	KLH	19.1 ± 1.1	16.5	25	0.07
KLH, UVA	KLH	8.9 ± 0.4	6.3	71	0.0002
C. albicans, UVA	KLH	18.6 ± 1.5	16	27	0.06
None	–	0.7 ± 0.2	–	–	
None	*C. albicans*	19 ± 1.3	18.3	0	–
C. albicans, no UVA	*C. albicans*	18.4 ± 1.9	17.7	3	0.8
C. albicans, UVA	*C. albicans*	5.7 ± 0.6	5	73	0.0001
KLH, UVA	*C. albicans*	23.6 ± 2.1	22.9	0	0.1

[a]Spleens were removed from mice that were immunized with *C. albicans* or KLH and exposed to 80 kJ per m^2 of UVA radiation, 9 d post immunization. 10^8 whole spleen cells were transferred into recipient mice that were then immunized with either *C. albicans* or KLH. The positive control for this experiment was a set of mice that received no cells, but were immunized and challenged. The negative control was a set of mice that received no cells, were not immunized, but were challenged.
[b]mm × 10^{-2} ± SD; $N = 5$.
[c]Change in footpad swelling of the positive control or experimental groups minus the background swelling found in the negative control.
[d]% immune suppression = 1 – (specific footpad swelling of the experimental groups ÷ specific footpad swelling of the positive control) × 100.
[e]p-values determined by two-tailed Student's *t* test *versus* the positive control.

experiment was performed. Groups of mice were immunized with one of two non-cross-reacting antigens, *C. albicans* or KLH (100 μg per mouse, subcutaneous injection). Nine days later the mice were exposed to 80 kJ per m^2 of UVA radiation. After ensuring that their DTH reaction was suppressed, the spleens of these mice were removed, single cell suspensions were prepared, and the cells (10^8 per mouse) were injected into the tail veins of recipient mice. The recipient mice were then immunized with either *C. albicans* or KLH and their DTH reaction was measured 11 d later. Data from this experiment are shown in **Table II**. As above, the positive control in this experiment consisted of measuring DTH in groups of mice that were simply immunized with the antigen. Also, as reported previously, transferring spleen cells from groups of mice that were immunized with antigen but not exposed to UVA did not induce immune suppression. When spleen cells, isolated from donor mice that were immunized with KLH and then exposed to UVA, were injected into recipient mice that were immunized with KLH, significant immune suppression was observed (41% suppression, p = 0.0002 *versus* the positive control). No immune suppression was observed when these same cells were injected into mice that were subsequently immunized with *C. albicans*, however.

The reciprocal experiment was also done (**Table II**). Spleen cells from mice first immunized with *C. albicans* and then exposed to UVA suppressed when the recipient mice were immunized with *C. albicans* (73% suppression, p = 0.0001 *versus* the positive control). No immune suppression was observed when these same cells were injected into mice that were subsequently immunized with KLH, however (p = 0.1 *versus* the positive control). These data indicate that UVA exposure activates antigen-specific suppressor cells.

The identity of the suppressor cells was addressed next (**Fig 4**). As described above, transfer of 10^8 whole spleen cells from UVA-irradiated mice (UV-SC) suppressed DTH in the recipient animals. We used a magnetic bead technique to isolate CD4$^+$ T cells from the spleens of mice immunized with antigen and then exposed to UVA radiation. When 3 × 10^7 CD4$^+$ T cells were injected into the recipient mice, significant immune suppression (p < 0.002) was noted, indicating that the UVA-induced suppressor cells are CD4$^+$ T cells.

Recently we reported that UVB-induced suppressor T cells belong to a unique subpopulation of T cells, generally known as natural killer T cells (Moodycliffe *et al*, 2000). Natural killer T cells represent a small subset of splenic T cells (1%–2% of total splenic T cells) and differ from conventional T cells by coexpressing markers
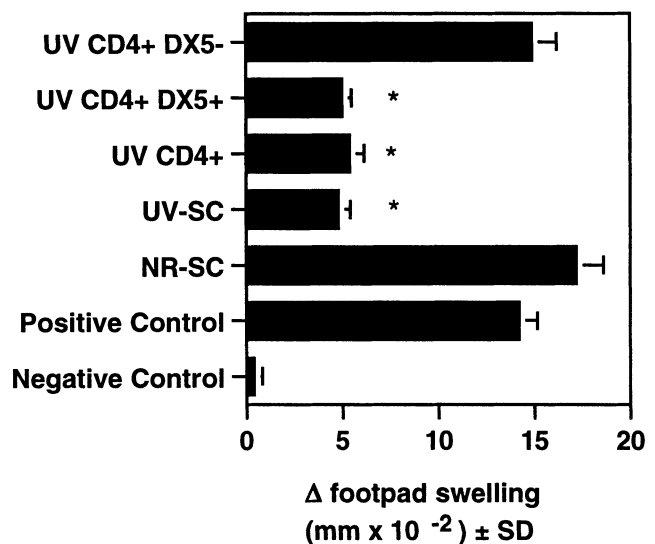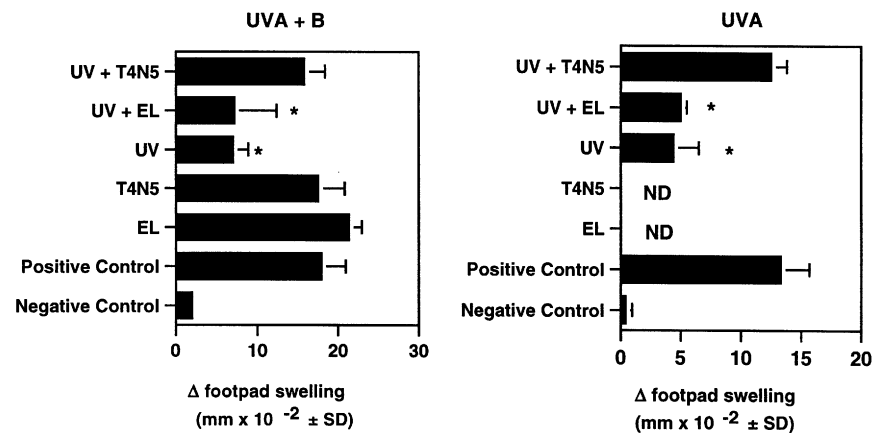
Cells Transferred

Figure 4. CD4$^+$, DX5$^+$ T cells are activated by exposing mice to UVA radiation 9 d post immunization. Mice were immunized with *C. albicans* on day 0, and on day 9 they were exposed to 80 kJ per m^2 UVA radiation. On day 11, spleen cells from these mice were removed and whole spleen cells (UV-SC) or the CD4$^+$, CD4$^+$ DX5$^+$, and CD4$^+$ DX5$^-$ subsets were isolated and injected into the tail veins of recipient mice. One group of mice received spleen cells from nonirradiated but immunized mice (NR-SC). The recipients were immediately immunized with *C. albicans* and DTH was measured 11 d later. *Significant difference (p = 0.0002, two-tailed Student's *t* test) *versus* the positive control.

found on both natural killer cells and T cells. One such marker is known as DX5. Therefore, we wished to determine if the T cells activated by UVA were similar in cell surface phenotype to those induced by UVB. To do this we separated the CD4$^+$ cells into two different subpopulations (DX5$^+$ and DX5$^-$) and asked which subpopulation transferred immune suppression. Injecting as few as 2 × 10^6 CD4$^+$ DX5$^+$ T cells into recipient mice transferred immune suppression (p < 0.0002 *versus* the positive control; **Fig 4**).

Figure 5. Liposomes containing DNA excision repair enzyme block UV-induced suppression of the elicitation of DTH. Mice were exposed to 80 kJ per m^2 solar-simulated UVA + UVB radiation (*left panel*) or 80 kJ per m^2 UVA radiation (*right panel*) 9 d post immunization. Immediately following UV exposure the mice were treated with empty liposomes (EL) or liposomes containing T4N5. DTH was measured on day 11. *Significant difference (p = 0.001, two-tailed Student's *t* test) *versus* the positive control. ND, not done.

Injecting a similar number of CD4$^+$ DX5$^-$ cells into the recipient mice had no suppressive effect. These data suggest that the suppressor cells induced by UVA radiation belong to a unique subpopulation of T cells known as natural killer T cells.

Repairing UV-induced DNA damage blocks the suppression of established immune reactions by UV radiation UV-induced DNA damage, particularly cyclobutane pyrimidine formation, has been shown to be the initiating step in the cascade of events that lead to immune suppression (Kripke *et al*, 1992). Therefore, we wanted to determine whether UV-induced DNA damage plays a role in suppressing established immune reactions (**Fig 5**). To do this, mice were first immunized with *C. albicans* and then exposed to UV radiation. Immediately after UV exposure, liposomes containing the excision repair enzyme T4N5 were applied to the skin of the UV-irradiated mice, according to procedures described previously (Kripke *et al*, 1992). Two different light sources were used in this experiment. In the left panel of **Fig 5**, the mice were exposed to solar-simulated UV radiation (UVA + UVB). In the right panel, the mice were exposed to UVA radiation. Regardless of the light source used, the results were similar. In both cases UV exposure suppressed the elicitation of DTH (p < 0.001 UVA + UVB; p < 0.0001 UVA). In both cases applying empty liposomes to the skin of the irradiated mice (UV + EL) had no effect; the immune suppression observed was indistinguishable (p > 0.05) from that found in UV-irradiated mice. When T4N5-containing liposomes were applied to the skin of UV-irradiated mice (UV + T4N5), however, no immune suppression was observed. Applying T4N5-containing liposomes (T4N5) or empty liposomes (EL) to the skin of normal mice had no effect on DTH.

These data imply that exposing mice to 80 kJ per m^2 of UVA radiation induces CPD formation. This was confirmed by immunohistochemistry and by measuring the numbers of CPD in epidermal DNA by radioimmunoassay. Skin samples were prepared from mice exposed to 80 kJ per m^2 of solar-simulated UVA + UVB radiation, or mice exposed to 80 kJ per m^2 of UVA only. The background staining was measured in normal mice that were not UV irradiated. As expected, few dimers were found in the nonirradiated skin (**Fig 6A**) and intense staining was found in the skin exposed to solar-simulated UV radiation (**Fig 6B**). Dimers were also found in the skin of mice exposed to UVA radiation (**Fig 6C**) indicating that the dose of UVA radiation used here to activate immune suppression can damage DNA. This was confirmed by radioimmunoassay where the numbers of CPD per 10^6 DNA bases was determined (Mitchell, 1999). As expected few dimers were found in the control DNA (9 ± 1 CPD per 10^6 bases). Exposing the mice to solar-simulated UV radiation (UVA + UVB) resulted in a large increase in CPD formation (199 ± 15 CPD per 10^6 bases). Although UVA irradiation did not result in as large an increase in CPD formation as solar-simulated radiation (17 ± 0.5 CPD per 10^6 bases), we did note a significant increase in CPD

formation compared to the nonirradiated control (p < 0.05, Student's *t* test). These data indicate that the dose of UVA used in this study is capable of activating CPD formation in the epidermis of UV-irradiated mice.

DISCUSSION

There are only a few examples in the literature demonstrating that UV radiation, given post immunization, can suppress established immune reactions. Moyal *et al* (1997) and Damian *et al* (1998) found that solar-simulated UV radiation can suppress DTH to recall antigens. In addition, UV radiation suppresses contact allergy in individuals presensitized to nickel (Damian *et al*, 1997). These experiments, done with human volunteers, not only confirmed the initial animal data showing that UV radiation suppresses established immune reactions (Denkins *et al*, 1989; Magee *et al*, 1989), but made an important contribution to photoimmunology by indicating that UVA radiation plays a role in activating immune suppression. Recently, we showed that the photobiologic characteristics of the suppression of established immune reactions in mice and humans are similar. Solar-simulated UV radiation suppressed immunologic memory and the elicitation of DTH. The effective wavelengths for immune suppression resided in the UVA region of the solar spectrum. Furthermore, applying a broad-spectrum sunscreen, but not a UVB-only absorbing sunscreen, afforded immune protection (Nghiem *et al*, 2001). Because of these similarities we decided to employ the animal model to study the immunologic mechanisms involved.

The first series of experiments were designed to test the hypothesis that UV-induced cytokines play an essential role in suppressing established immune reactions (**Figs 1, 2**). Although our focus was limited to IL-10 and IL-12, our data suggest that similar immunologic mechanisms are involved in suppressing the immune response in immunized and naive individuals. In our previous studies, where mice were exposed to UV radiation, injected with anti-IL-10, and then immunized with antigen, anti-IL-10 blocked the induction of immune suppression (Rivas and Ullrich, 1992). We see a similar situation here: anti-IL-10 blocks immune suppression in immunized mice.

The source of the IL-10 is not exactly clear. Most mouse experiments showing IL-10 production by epidermal cells have used FS-40 sunlamps (Rivas and Ullrich, 1992). In humans, IL-10 production was induced by two to three minimal erythema doses of solar-simulated radiation (Barr *et al*, 1999; Wolf *et al*, 2000). Whether IL-10 secretion is induced by UVB or UVA is controversial. Using cultured human keratinocytes, Grewe *et al* (1999) found IL-10 production following both UVB and UVA I exposure. On the other hand, Skov *et al* (1998) failed to find any IL-10 in human skin after UVA I exposure; IL-10 was only secreted in response to UVB. It may be wrong to assume that the UV-induced IL-10 is keratinocyte derived, however. Kang *et al* (1994) report that macrophages infiltrate human skin after UV
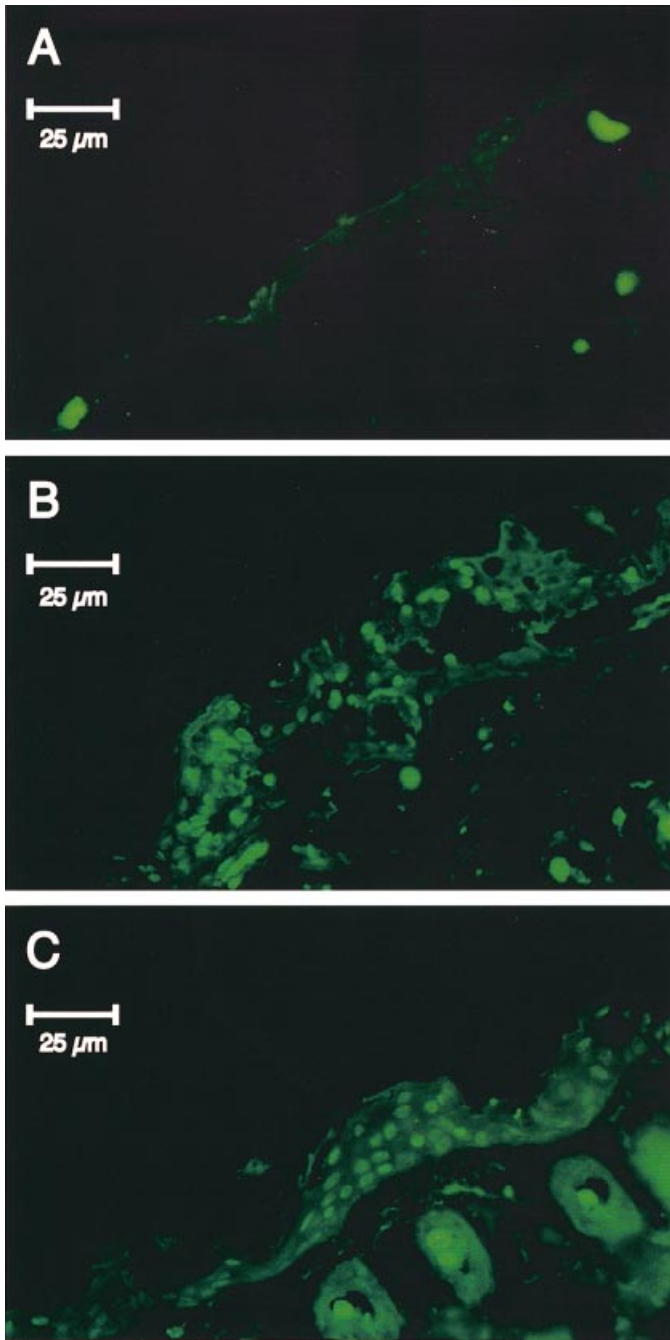
Figure 6. Detection of CPD in mouse skin after exposure to UVA radiation. Cryostat sections of skin from normal nonirradiated mice (*A*), mice exposed to 80 kJ per m² of solar-simulated UV radiation (*B*), or mice exposed to 80 kJ per m² of UVA radiation (*C*) were stained with a monoclonal antibody specific for CPD and counterstained with fluorescein isothiocyanate conjugated goat antimouse IgG. *Scale bar*: 25 μm.

exposure and secrete IL-10. In experiments using mice, we found serum IL-10 secretion was the result of a cytokine cascade involving prostaglandin E2 and IL-4 (Shreedhar *et al*, 1998). These data imply that UV-induced IL-10 can be derived from cells other than keratinocytes, so the observations by others that UVA does not induce keratinocytes to secrete IL-10 does not necessarily negate the findings presented here.

Our results with recombinant IL-12 also support a role for UV-induced cytokines in suppressing established immune reactions. Previously we reported that recombinant IL-12 blocked

UV-induced immune suppression (Schmitt *et al*, 1995), primarily by blocking cytokine secretion *in vivo* and *in vitro* (Schmitt *et al*, 2000). We suggest the same is occurring here. UV irradiation 9 d post immunization induces the production of cytokines, such as IL-10, that block effector cell function. We suggest that injecting mice with IL-12 immediately after UV irradiation interferes with IL-10 production *in vivo*. These data therefore support the hypothesis that cytokine production in response to UV irradiation is a fundamental feature involved in suppressing both the induction and the elicitation of the immune response.

The second set of experiments was designed to determine whether suppressor T cells were activated when mice were exposed to UV radiation post immunization. The activation of suppressor T cells by UV radiation is a well-recognized phenomenon. Previous studies from our laboratory demonstrated that exposing mice to UV radiation (FS-40 sunlamps) post immunization activates antigen-specific suppressor CD4$^+$ T cells (Magee *et al*, 1989). In the studies reported here, we confirmed our previous findings by demonstrating that solar-simulated UV radiation given post immunization will also activate suppressor cells. Furthermore, we found that injecting solar-simulator-irradiated mice with monoclonal anti-IL-10 will block suppressor cell activation. Here again, these findings are similar to the findings we published previously, examining the effects of UV radiation on the induction of an immune response (Rivas and Ullrich, 1994). The conclusion from these experiments supports the concept that UV irradiation activates similar immunologic mechanisms to suppress the induction or the elicitation of the immune response.

Rather than simply repeating many of our previous experiments by substituting solar-simulated UV radiation for FS-40 sunlamp exposure, we thought that asking whether UVA radiation could activate suppressor T cells would be more informative. Our findings clearly demonstrate that UVA irradiation activates suppressor cells. The suppressor cells activated following UVA radiation are specific for the antigen used to immunize the mice (**Table II**). In addition, the suppressor cells are CD4$^+$ T cells that coexpress a cell surface marker (DX5) found on the suppressor T cells induced by UVB radiation (Moodycliffe *et al*, 2000). These findings indicate that the suppressor T cells induced by UVA and UVB share a similar cell surface phenotype (CD4+, DX5+). They may also suggest that suppressor T cells belong to a unique subpopulation of regulatory T cells known as natural killer T cells. It must be noted that there is some debate with regard to the cell surface expression of DX5 on natural killer T cells. The DX5 antibody recognizes CD49b (very late antigen 2), a marker generally found on natural killer T cells (Arase *et al*, 2001), and DX5 positive staining has often been used to identify natural killer T cells in the past (Vicari and Zlotnik, 1996). Recently, however, using CD1 tetramers, some have shown that not all populations of natural killer T cells stain with DX5 (Hammond *et al*, 2001). Be that as it may, we previously demonstrated that the suppressor T cell activated after UVB exposure is a CD1-restricted, DX5+, CD4+ T cell that shows intermediate αβ T cell receptor staining and secretes large amounts of IL-4 rapidly after stimulation *in vitro*. These characteristics strongly suggest that the suppressor T cell is a natural killer T cell. Although additional studies are in progress to confirm that the UV-induced suppressor T cell is a natural killer T cell, it is important to note that both UVB (FS-40 sunlamps) and UVA (WG-335-filtered solar simulator) activate a suppressor T cell with similar cell surface characteristics, suggesting that UVB and UVA activate similar immunologic mechanisms to suppress DTH.

We also know from previous work that UV-induced DNA damage is the initiating event in the induction of immune suppression (Kripke *et al*, 1992). Furthermore, UV-induced DNA damage activates cytokine secretion (Nishigori *et al*, 1996; Kibitel *et al*, 1998). These conclusions were based primarily on the ability of liposomes containing the DNA excision repair enzyme T4N5 to reverse UV-induced immune suppression. Our third set of experiments used the same liposomes and asked whether we could reverse immune suppression when mice were exposed to

solar-simulated UVA + UVB radiation post immunization. Our findings indicate that UV-induced DNA damage initiates the suppression of the elicitation of immunity. Here again, these data support the hypothesis that similar mechanisms are activated to suppress the induction of, and the elicitation of, the immune response.

Somewhat surprising, however, was the observation that applying T4N5-containing liposomes reversed UVA-induced immune suppression. Because this enzyme excises UV-induced pyrimidine dimers, at first glance one may not expect it to have much of an effect on UVA-induced immune suppression. Others have shown, however, that UVA radiation can induce CPD formation (Ley and Fourtanier, 1997; Young *et al*, 1998). In addition, using a light source almost identical to the one used here (WG-335-filtered solar simulator), Woollons *et al* (1999) report that pyrimidine dimers could be induced, despite the fact that the light source used was devoid of UVB radiation. We show the same here. The dose of UVA used in this study is clearly capable of activating CPD formation in the epidermis of UV-irradiated mice. These data indicate that UVA-induced CPD formation can activate the suppression of established immune reactions.

In summary, we report on the immunologic mechanisms underlying the suppression of established immune reactions by solar-simulated UV radiation. The mechanisms involved are very similar to those described previously. Our data support a role for UV-induced DNA damage as the initiating event. Cytokines are involved in transmitting the immunologic signal from the skin to immune effector cells. Suppressor T cells are activated and these cells are identical, with regard to function and cell surface markers, to those that suppress the induction of immunity. Moreover, we find that similar immune regulatory mechanisms are activated regardless of whether we expose mice to solar-simulated UV (UVA + UVB) radiation or UVA only. This may reflect the ability of solar UVB and UVA to induce pyrimidine dimers in the DNA of target tissues. This observation supports earlier studies, by ourselves (Nghiem *et al*, 2001) and others (Moyal and Fourtanier, 2001), demonstrating that the critical wavelengths for suppressing established immune reactions and immunologic memory reside in the UVA portion of the solar spectrum. These data also demonstrate the need for continuing experimentation to clarify the role of UVA in photoimmunology and photodamage.

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