

Aloe Barbadensis Extracts Reduce the Production of Interleukin-10 After Exposure to Ultraviolet Radiation

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Cutaneous exposure to ultraviolet radiation suppresses the induction of T cell mediated responses such as contact and delayed type hypersensitivity (DTH) by altering the function of immune cells in the skin and causing the release of immunoregulatory cytokines. Extracts of crude *Aloe barbadensis* gel prevent this photosuppression. Because the regulation of contact hypersensitivity and DTH responses differ, we investigated whether protection was afforded by a single or multiple agents in *Aloe* and the mechanism by which this material prevents suppression of DTH immunity. The ability of *Aloe* gel to prevent suppression of contact hypersensitivity responses to hapten decayed rapidly after manufacture. In contrast, agents that protected against systemic suppression of DTH responses to *Candida albicans* were stable over time. Oligosaccharides prepared from purified *Aloe* polysaccha-

ride prevented suppression of DTH responses *in vivo* and reduced the amount of IL-10 observed in ultraviolet irradiated murine epidermis. To assess the effect of *Aloe* extracts on keratinocytes, Pam 212 cells were exposed *in vitro* to ultraviolet radiation and treated for 1 h with *Aloe* oligosaccharides. Culture supernatants were collected 24 h later and injected into mice. Supernatants from ultraviolet irradiated keratinocytes suppressed the induction of DTH responses, whereas *Aloe* oligosaccharide treatment reduced IL-10 and blocked the suppressive activity of the supernatants. These results indicate that *Aloe* contains multiple immunoprotective factors and that *Aloe* oligosaccharides may prevent ultraviolet induced suppression of DTH by reducing keratinocyte derived immunosuppressive cytokines. **Key words:** cytokines/delayed type hypersensitivity/skin/tolerance/suppression/UV radiation. *J Invest Dermatol* 110:811–817, 1998

Excessive exposure to ultraviolet (UV) radiation is harmful, causing sunburn, premature aging of the skin, and mutations leading to skin cancer (Urbach, 1978; Harber and Bickers, 1989). Studies in laboratory animals have shown that UV radiation in the “B” range (280–320 nm) of the electromagnetic spectrum also contributes to the growth of highly antigenic skin cancers by suppressing T cell mediated immune responses (Kripke, 1974; Fisher and Kripke, 1977). Model systems established to study the link between UV induced immune suppression and tumor development showed that cutaneous exposure to low (suberythemal) doses of UV radiation inhibits the induction of contact hypersensitivity (CHS) response to hapten applied at the site of irradiation (Toews *et al*, 1980). Higher doses of UVB radiation induce systemic suppression of CHS responses to hapten applied to unirradiated sites and delayed type hypersensitivity (DTH) responses to infectious agents, including herpes simplex virus, leishmania, *Candida albicans*, and mycobacteria (Giannini, 1986; Otani and Mori, 1987; Denkins *et al*, 1989; Jeevan and Kripke, 1989). Susceptibility to UV induced

immune suppression of CHS responses may be a risk factor for the development of skin cancer in humans (Yoshikawa *et al*, 1990).

Whereas the precise manner by which UV suppresses T cell immunity is still unclear, CHS and DTH responses appear to be suppressed by different mechanisms (Kripke and Morison, 1986; Kim *et al*, 1990; Rivas and Ullrich, 1994). Suppression of CHS responses involves alterations in Langerhans cell functions and the release of soluble factors such as tumor necrosis factor- α , interleukin (IL)-1, prostaglandin-E₂, and *cis*-urocanic acid (Chung *et al*, 1986; Robertson *et al*, 1987; Harriott-Smith and Haliday, 1988; Vermeer and Streilein, 1990; Simon *et al*, 1991). Recent studies have demonstrated that UV induced keratinocyte derived IL-10 systemically suppresses DTH responses to alloantigen by downregulating antigen presenting cell functions and triggering the formation of antigen specific suppressor T cells (Noonan and De Fabo, 1992; Ullrich, 1994). A central role for IL-10 in mediating suppression of DTH responses has been shown using neutralizing anti-IL10 antibody (Rivas and Ullrich, 1994). Injection of the antibody into UV irradiated mice blocks the suppression of DTH responses. Furthermore, absorption of supernatants from UV irradiated keratinocyte cultures with anti-IL-10 antibody removes their *in vivo* suppressive activity. The role of IL-10 in the suppression of CHS immunity is less clear because injection of the cytokine blocks the induction of CHS responses in mice, but the treatment of UV irradiated mice with anti-IL-10 antibody fails to restore their CHS responses (Rivas and Ullrich, 1994; Niizeki and Streilein, 1997).

Therapeutic intervention to prevent immune suppression by blocking one or more of these pathways may be beneficial in reducing the risk of skin cancer. An understanding of the immunoregulatory mechanisms

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Abbreviation: CHS, contact hypersensitivity.

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can be aided by the use of agents that block the pathway at discrete steps, e.g., minimizing the effects of DNA damage by accelerating its repair, by the use of antioxidants to prevent cellular injury, and by using antibodies or other agents to block the action of immunosuppressive cytokines.

Extracts of *Aloe barbadensis* are widely used as therapeutic agents for the treatment of minor cutaneous injuries. We have recently found that topical application of crude *Aloe barbadensis* gel prevents UV induced suppression of both local and systemic CHS and DTH immune responses (Strickland *et al*, 1994). In this report, we examine some mechanisms by which crude and partially purified *Aloe* extracts prevent UV induced immunosuppression.

MATERIALS AND METHODS

Mice Specific-pathogen-free female C3H/HeN Cr (MTV⁻) mice were purchased from the Animal Production Area of the Frederick Cancer Research Facility (Frederick, MD) and were maintained in a pathogen-free barrier facility in accordance with the National Institutes of Health and the American Association for Assessment and Accreditation of Laboratory Animal Care International guidelines. The mice were housed in filter protected cages and provided with National Institutes of Health open formula mouse chow and sterile water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee. Each experiment was performed with aged matched mice that were 10–12 wk old.

***Aloe barbadensis* materials** The *Aloe barbadensis* gel preparations used were "Aloe Research Foundation Standard Gel Samples" prepared for the Aloe Research Foundation by AloeCorp (Harlingen, TX), as previously described (Strickland *et al*, 1994). These "Process A" materials were from lots ARF'91 A, ARF'94B, ARF'94G, and ARF'94K (Pelley *et al*, 1993). In general, the composition of matter of these lyophilized materials was 9.6% polysaccharides, 11% glucose, 27% divalent metal cations and multivalent organic acids, and less than 5% materials extractable with organic solvents. The remainder consisted of univalent metal cations, chloride, and univalent organic acids. At the time of lyophilization, the total bacterial content was always under 30,000 per ml of gel. Although this extract was prepared on an industrial scale using commercial equipment, it does not correspond to any commercial product.

An oligosaccharide-rich material was prepared from crude "Process A" gel by activation with cellulase followed by separation of small molecules from enzyme and polysaccharide by ultrafiltration. Frozen ARF 94K crude *Aloe* gel (2 liters, solids content 14 g) was thawed and 2.3 mg of crude commercial cellulase (Cellulase 4000, Valley Research, South Bend, IN) was added. The enzyme-*Aloe* mixture was ultrafilter fractionated by multiple cycles through a 5000 dalton cut-off polysulfone hollow fiber apparatus (2790 cm², A/G Technology, Needham, MA). Each cycle consisted of passing the material, at ambient temperature, through the ultrafilter at a pressure sufficient to produce diffusate at a rate of 2 liters per h. Retentate volume was kept constant by the addition of deionized water. As each 2 liter portion of diffusate was produced, it was removed and concentrated by lyophilization. Four cycles of enzymatic treatment-ultrafiltration were performed. The first diffusate yielded 60% of the total dialyzable mass, the second yielded 24%, and the third and fourth diffusates combined accounted for 12% of the total dialyzable mass.

More highly purified oligosaccharides were prepared by cleavage of purified *Aloe* polysaccharides with partially purified cellulase followed by separation of oligosaccharides from enzyme and polysaccharide by alcohol precipitation. Polysaccharide was purified from lyophilized "Process A" *Aloe*, lot ARF94K by modification of the method of Gowda using exhaustive dialysis followed by precipitation at 80% vol/vol with absolute ethanol at 4°C (Gowda *et al*, 1979). This yielded (14.2% of mass) a polysaccharide of expected sugar composition (7% glucose, 85% mannose, 4% galactose), almost all of which was in excess of 2,000,000 Da molecular weight. Cellulase was purified from crude *T. reesei* concentrated culture supernatants (lot ZPED, gift of Valley Research, South Bend, IN) by ethanol precipitation (50–80% fraction) and gel filtration upon Biogel P-200 (Bio-Rad, Richmond, CA). The protein concentration of the partially purified cellulase was determined by the Coomassie Blue dye binding assay (Bio-Rad, Richmond, CA). Oligosaccharide was produced by incubating 400 mg of purified polysaccharide with 12 µg of partially purified cellulase in 5 mM citrate buffer (pH 6) at ambient temperature for 2 h. This treatment reduced the viscosity of the solution by 50% but resulted in only a minor shift in the molecular weight distribution of the *Aloe* polysaccharides. Oligosaccharides were separated from precursor polysaccharide and enzyme by addition of absolute ethanol to 80% vol/vol and chilling to 4°C. The oligosaccharide containing supernatant was then separated from the precipitate (which contained the enzyme and polysaccharide) by centrifugation. Oligosaccharide, measured as hexose, constituted only 1.5% of the mass of the supernatant after stripping and lyophilization (the vast bulk of the supernatant consisting of the sodium

citrate buffer). Oligosaccharides used for *in vitro* culture were diluted in serum-free minimal essential medium (MEM; Gibco, Grand Island, NY) and filter sterilized through a 0.22 µm membrane.

UV radiation UV radiation was administered *in vivo* using a bank of six unfiltered FS40 sunlamps (National Biological, Twinsburg, OH). Approximately 65% of the energy emitted from these lamps is within the UVB range (280–320 nm) and the peak emission is at 313 nm. The average irradiance of the source was ≈4.5 W per m² at 20 cm distance, as measured by an IL700 radiometer with a SEE280 filter and a W quartz diffuser (International Light, Newburyport, MA). A single FS-40 bulb was used to irradiate cultured keratinocytes. The output of the lamp was 4.7 J per m² per s, at a tube-to-target distance of 23 cm.

***In vitro* UV irradiation of keratinocytes** The spontaneously transformed murine keratinocyte cell line, Pam 212, was obtained from Dr. Stuart Yuspa (National Cancer Institute, Bethesda, MD). The cells were maintained in our laboratory in MEM supplemented with 10% fetal bovine serum from Gibco at 37°C in a 5% CO₂ balanced air humidified atmosphere as previously described (Ullrich, 1994). To determine the effects of UV and *Aloe* extracts on cytokine production, keratinocytes were removed from subconfluent cultures by treatment with 0.25% trypsin (Gibco) and plated into sterile 100 mm plastic tissue culture-grade petri dishes (Corning Glass Works, Corning, NY) at a cell density of 2.5 × 10⁶ cells per dish, and incubated in MEM/10% fetal bovine serum overnight at 37°C. The monolayers were washed three times with phosphate buffered saline (PBS), overlaid with 5 ml of PBS, and exposed to 300 J per m² of UVB radiation. The cells were washed with PBS, overlaid with a solution of filter sterilized *Aloe* oligosaccharides in serum-free medium at 37°C. Endotoxin contamination of the *Aloe* was measured using the *Limulus* amoebocyte lysate assay (Cape Cod Associates, Woods Hole, MA) and was found to be below the limit of detection (0.125 ng per ml). After 1 h the supernatant was removed, the cells were washed three times with PBS, and the incubation was continued for 24 h in serum-free medium. Control cultures were identically treated but not exposed to UV radiation. The supernatants were collected and the protein concentration was determined by the Coomassie Blue dye-binding assay using a bovine serum albumin standard. For cytokine quantitation, the culture supernatants were concentrated using the centrprep concentrating system (Amicon, Beverly, MA) and stored at -20°C.

Treatment of mice Groups of five mice were anesthetized with Nembutal (sodium pentobarbital, 0.01 ml per g body weight) ip and their shaved ventral skin was exposed to a single dose of 2 kJ UVB radiation per m². Within 5 min of UV irradiation, the UV exposed skin was treated with *Aloe* extract in PBS or a control polysaccharide, methylcellulose (Sigma, St. Louis, MO) in PBS. Control animals were treated in an identical manner but were not exposed to UV radiation. Three days later the mice were sensitized on their shaved abdominal skin by applying 400 µl of 0.5% fluorescein isothiocyanate (Molecular Probes, Eugene, OR) in acetone:dibutylphthalate (1:1 vol:vol). Five days after sensitization, the mice were challenged by applying 5 µl of 0.5% fluorescein isothiocyanate on both the dorsal and the ventral surfaces of each ear. In some experiments, animals were sensitized with 0.3% 2,4-dinitrofluorobenzene (DNFB; Aldrich, Milwaukee, WI) in acetone. In those experiments, the mice were challenged with 0.2% DNFB applied to the ears as described above. Control mice were challenged with the hapten but were not sensitized. Ear thickness was measured using an engineers' micrometer (Production Tools, Houston, TX) immediately before challenge and 24 h later.

Systemic suppression of the DTH response was induced using a single exposure to UVB radiation as follows. The dorsal fur of the mice was shaved with electric clippers, the animals were put into cages with plexiglas dividers, one mouse per chamber, and the cage covered with a wire lid. The incident light received by the animals under these conditions was reduced to 2.6 W per m², by the shielding from the wire cage top. The animals were given a 5 kJ per m² dose of UVB radiation in a single exposure. Within 5 min of UV irradiation, the UV exposed skin was treated with *Aloe* extract in PBS or a control polysaccharide, methylcellulose (Sigma) in PBS. Three days later, the mice were injected subcutaneously in each flank with 1 × 10⁷ formalin fixed *C. albicans* cells. Ten days after sensitization, the mice were challenged with 50 µl of commercially prepared soluble *Candida* antigen, supplied as a 1:100 dilution (ALK Laboratories, Wallingford, CT) in each hind footpad. Footpad thickness (dorsal to plantar aspect) was measured immediately before challenge and 24 h later. Control mice were not sensitized with yeast cells but were challenged in both hind footpads with the *Candida* antigen. Specific footpad swelling was determined by subtracting the average values obtained from mice challenged but not sensitized.

The percentage restoration of immunity in UV irradiated animals treated with oligosaccharides was calculated using the following formula:

$$\frac{(\mu\text{m swelling unirradiated Aloe treated}) - (\mu\text{m swelling UV irradiated Aloe treated})}{\mu\text{m swelling unirradiated Aloe treated}} \times 100\%$$

The response of UV irradiated, untreated mice was set as 0% restoration, whereas values for unirradiated, *Aloe* treated groups were considered as 100% response.

In vivo activity of Pam 212 supernatants Groups of five C3H/HeN mice were injected intravenously with Pam 212 keratinocyte supernatants. Each animal received 0.5 ml of supernatant containing 15–20 μg of protein. Three days later, the mice were immunized by subcutaneous injection of 10^7 formalin fixed *C. albicans* cells and their DTH response was elicited 10 d after sensitization using 50 μl of *Candida* antigen in each hind footpad.

Quantitation of cytokines by enzyme linked immunosorbent assay (ELISA) Murine IL-10 and IL-4 were measured using commercial kits or kit reagents purchased from PharMingen (San Diego, CA). Murine IL-13 was quantitated using a kit purchased from R&D Systems (Minneapolis, MN). All analyses were performed according to the manufacturers' instructions. The optical density was measured using a MR 5000 microplate reader (Dynatech, Chantilly, VA).

Immunohistochemistry and image analysis Dorsal skin of C3H/HeN mice was shaved with electric clippers and exposed to 15 kJ UVB radiation per m^2 , followed immediately by treatment with a solution of *Aloe* extract in PBS. Control mice were similarly treated but were not exposed to UV radiation. Four days later, the mice were killed and the remaining fur in the treatment area was removed with a razor blade. The skin was excised, subcutaneous fat and connective tissue was removed, and the samples were frozen in liquid nitrogen. Cryostat sections were fixed with 2% paraformaldehyde (Electron Microscopy Sciences, Ft Washington, PA) in PBS and stained as previously described (Nishigori *et al.*, 1996). Pam 212 keratinocytes, cultured on 18 \times 18 mm glass coverslips (Fisher Scientific, Pittsburgh, PA), were exposed to 300 J UVB per m^2 , and treated as described above. Twenty-four hours later the cells were fixed with 2% paraformaldehyde and stained for IL-10 protein. The cells were counterstained with one drop of Gill's hematoxylin for 30 s, rinsed with distilled water until the wash was clear, and mounted in universal mount (Research Genetics, Huntsville, AL).

Microscopic images were recorded by digitizing the images using a 3CCD color camera (Sony, Tokyo, Japan), 24 bit True color frame grabber, and imaging system (Meyer Instruments, Houston, TX). The images were stored on 65 MB optical disks (Pinnacle Micro, Irving, CA) and analyzed using Optimas image-analysis software (Optimas, Bothell, WA). Images were printed using a digital printer (Sony).

Statistics Statistical analyses were performed using ANOVA or two tailed Student's *t* test. A *p* value of < 0.05 was considered significant. Analyses were performed using Statview SE + Graphics software (Abacus Concepts, Berkeley, CA) on a Macintosh LC II microcomputer.

RESULTS

Differential rates of decay of CHS and DTH protective activities in crude *Aloe barbadensis* gel

We have previously shown that cutaneous application of a crude extract of *Aloe barbadensis* gel protects both CHS and DTH responses in mice from suppression by UV radiation (Strickland *et al.*, 1994). This study investigates the mechanism of immune protection by first determining whether protection of CHS and DTH responses are mediated by the same or different agents in *Aloe* gel. Protection against UV induced local immune suppression of CHS was measured by exposing the shaved ventral skin of C3H/HeN mice to 2 kJ UVB radiation per m^2 and applying a solution of 5 mg *Aloe* gel extract per ml (lot ARF91 A, "Process A") in PBS to the irradiated skin immediately after exposure. The animals were sensitized 3 d later by applying hapten through their ventral skin. Unirradiated control groups were treated with the *Aloe* gel and sensitized.

The data obtained from three lots of *Aloe* gel (ARF91 A, ARF94B, and ARF94G) were combined and expressed as the mean \pm SEM percentage unirradiated matching control. The responses to antigen varied between experiments; however, typical positive control values for DTH responses to *C. albicans* ranged from 200 to 300 μm footpad swelling above background (Strickland *et al.*, 1994; not shown). Responses to hapten ranged from 70 to 150 μm ear swelling for fluorescein isothiocyanate and 150–180 μm for DNFB. In groups of

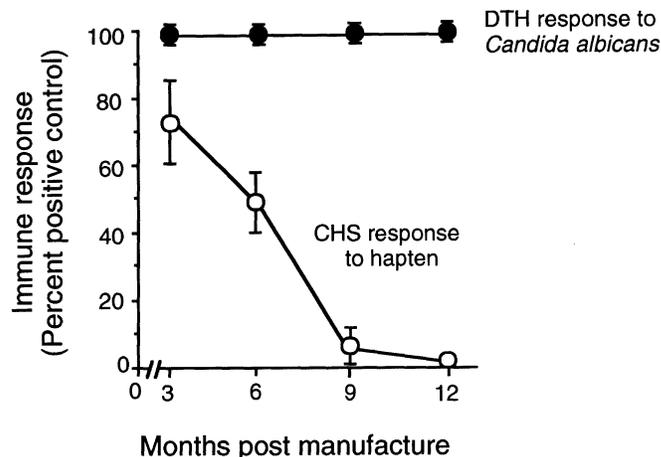


Figure 1. CHS and DTH protective activities in *Aloe* gel extract exhibit different rates of decay.

The ability of *Aloe* gel extracts to prevent suppression of CHS and DTH responses was measured using three lots (ARF91 A, ARF94B, and ARF94G) of "Process A" gel at various times after manufacture. CHS response: ventral skin of groups of five mice was exposed to 2 kJ UVB per m^2 and their irradiated skin was treated with 5 mg gel extract per ml in PBS. Three days later, UV irradiated and unirradiated control mice were sensitized with hapten and challenged 5 d later, as described in the *Materials and Methods*. DTH response: the shaved dorsal skin of mice was exposed to 5 kJ UVB radiation per m^2 and the exposed skin was treated with *Aloe* or PBS as above. UV irradiated and unirradiated animals were sensitized 3 d later with 2×10^7 formalin fixed *C. albicans* cells subcutaneous; their DTH response was elicited 10 d later. The *Aloe* extracts were stored as a lyophilized powder at -20°C until use. The data are normalized and are expressed as a percentage CHS or DTH response in unirradiated homologous controls (100%) and UV irradiated mice treated with PBS (0%) according to the formula described in the *Materials and Methods*. The data from the three lots of gel were combined and expressed as the mean \pm SEM.

UV irradiated mice treated with PBS alone, a 50–80% reduction in CHS and DTH responses was observed compared with their unexposed, matching controls. This level of response was considered as 0% restoration whereas the response of unirradiated, *Aloe* treated matching controls was set as 100%.

The results presented in **Fig 1** show that treatment of UV irradiated skin with the *Aloe* extract partially prevented suppression of the CHS response to hapten. The protection afforded by the three different lots of gel varied. For example, 3 mo after manufacture, application of ARF91G gel to UV irradiated animals provided only 43% restoration of their CHS response, whereas the ARF91A lot of gel extract completely restored the CHS response. The activity of ARF91B was intermediate between these two values. The levels of protection provided by the gel were maximal at a dose of 5 mg per ml (wt:vol) and could not be improved by increasing the dose of *Aloe* used (not shown). The activity of all three lots of *Aloe* gel extract decayed with time, despite their storage as lyophilized powder. After 9 mo, none of the extracts prevented UV induced suppression of CHS responses to hapten (**Fig 1**). Other lots of *Aloe* gel "Process A" extract gave similar results except that the levels of CHS protection ranged from 30 to 100% and longevity of CHS protective activity ranged from 3 to 9 mo post-manufacture. Commercially prepared (non-"Process A") *Aloe* gels from the same source were uniformly inactive even when tested within 1 mo of manufacture.

Systemic suppression of DTH responses was measured in mice whose shaved dorsal skin was exposed to 5 kJ UVB per m^2 , treated with the same lot of gel extract used for CHS protection studies, and sensitized by a subcutaneous injection of *C. albicans* 3 d later. In contrast to the partial protection of the CHS responses to hapten, *Aloe* gel completely prevented systemic suppression of DTH to *C. albicans*. The immunoprotective activity of all three lots of lyophilized extract remained unchanged after 12 mo of storage (**Fig 1**). At each time point at which the *Aloe* gel was tested, the experiment was repeated at least once in order to confirm the results. Restoration of immunity

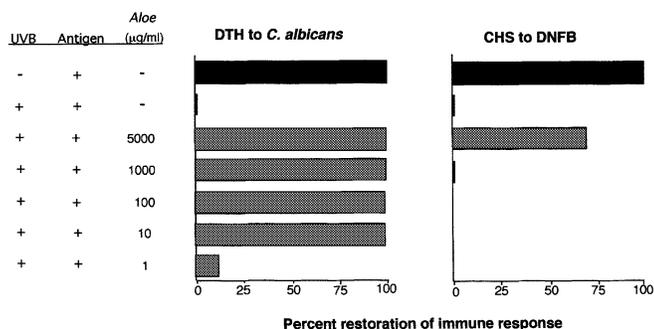


Figure 2. Protection of CHS and DTH responses from suppression by UV radiation requires different doses of *Aloe barbadensis* gel. Groups of five mice were exposed to 2 kJ (CHS) or 5 kJ (DTH) UVB radiation per m² followed immediately by topical application of 1 µg to 5 mg of *Aloe* gel in PBS. Control groups were shaved and treated identically but were unirradiated. Three days later the mice were sensitized with DNFB or *C. albicans* and challenged as described in the *Materials and Methods*. The data were combined from three experiments and expressed as percentage restoration of immune response using the formula described in the *Materials and Methods*. The mean ± SEM control immune responses to challenge with DNFB (CHS) and *C. albicans* (DTH) are as follows. CHS: unsensitized control, 55 ± 17 µm; positive control, 188 ± 10; unirradiated *Aloe* treated, 181 ± 22; UV irradiated untreated, 117 ± 18. DTH: unsensitized control, 161 ± 16 µm; positive control, 324 ± 18; unirradiated *Aloe* treated, 309 ± 20; UV irradiated untreated, 205 ± 17.

was not due to nonspecific immunostimulation by the *Aloe* gel, because neither CHS nor DTH responses were affected in unirradiated control animals at any dose tested (Strickland *et al.*, 1994; not shown). Also, the structurally unrelated polysaccharide, methyl cellulose failed to protect CHS and DTH responses against UV induced suppression (not shown).

The different decay rates observed for CHS and DTH protective activity in crude *Aloe* gel suggested that these activities are mediated by different factors. Additional evidence for the presence of distinct factors was obtained by performing a dose response. From 0 µg to 5000 µg *Aloe* gel in PBS was applied to unirradiated controls and to the UV irradiated skin of mice immediately after exposure. Three days later the animals were sensitized with hapten through UV irradiated skin (local CHS model) or injected with formalin fixed *C. albicans* cells (systemic DTH model). The data presented in **Fig 2** show that protection of CHS responses against suppression by UV radiation was mediated by only the highest dose (5000 µg) of *Aloe* gel. In contrast, as little as 10 µg *Aloe* gel completely protected DTH responses. These experiments were performed using ARF Standard *Aloe* gel 4 mo after manufacture. The levels of protection were consistent with those observed in **Fig 1** for gel of that age. Similar results were observed using lots ARF94G and ARF94B of crude *Aloe* gel, and an oligosaccharide enriched fraction prepared from cellulase treated ARF 94G *Aloe* polysaccharide by hollow fiber ultrafiltration. Taken together, the results indicate that protection of CHS and DTH immune responses from suppression by UV radiation is mediated by at least two separate factors in crude *Aloe* gel. The remaining studies, described herein, focused only on the DTH response and on mechanisms by which agent(s) from *Aloe* gel prevent systemic photosuppression.

***Aloe* oligosaccharides prevent suppression of DTH and reduce IL-10 production by UV irradiated keratinocytes**

Studies *in vitro*, using crude gel extracts, are difficult to perform and interpret because of the complexity of the material (Waller *et al.*, 1978; Gowda *et al.*, 1979). The DTH protective activity has recently been found to be concentrated in a fraction that is rich in oligosaccharides (molecular weight ≤ 5000 d) and polysaccharide-poor (RPP, manuscript in preparation). An oligosaccharide fraction of gel was prepared by cleavage of purified *Aloe* polysaccharide (lot ARF94K) with partially purified cellulase. Ethanol precipitation of the mixture separated the soluble oligosaccharides from precipitated "cellulase" and polysaccharides. Low microgram quantities of ethanol soluble *Aloe* oligosaccharides, applied to the UV exposed skin of mice, were effective

Table I. Treatment of UV irradiated skin with *Aloe barbadensis* purified oligosaccharides prevents systemic suppression of the DTH response to *C. albicans*

Treatment	Footpad swelling ^a (µm)	% Specific suppression ^b	p ^c
Negative control ^d	121 ± 26		
Positive control ^e	344 ± 26		
UV only ^f	210 ± 7	60	0.02
<i>Aloe</i> oligosaccharides 10 µg per ml ^g	353 ± 8		
UV + <i>Aloe</i> oligosaccharides 10 µg per ml ^h	320 ± 11	11	NS ⁱ

^aData represent the mean ± SEM of groups of five mice.

^bCompared with matching unirradiated control group.

^cDetermined by ANOVA.

^dUnsensitized mice challenged with *C. albicans* antigen.

^eC3H/HeN mice were sensitized with a subcutaneous injection of 2 × 10⁷ formalin fixed *C. albicans* cells. Ten days later the mice were challenged in the hind footpads with soluble *C. albicans* antigen.

^fMice exposed to 5 kJ UVB per m² and sensitized with 2 × 10⁷ formalin fixed *C. albicans* 3 d later.

^gOne milliliter of oligosaccharides prepared from purified *Aloe barbadensis* polysaccharide, as described in the *Materials and Methods*, was applied in PBS to the dorsal skin of each mouse. Animals were sensitized with 2 × 10⁷ formalin fixed *C. albicans* 3 d later.

^hMice were exposed to 5 kJ UVB per m² and each animal was treated with 10 µg oligosaccharides in PBS immediately after irradiation. Three days later the animals were sensitized with 2 × 10⁷ formalin fixed *C. albicans*.

ⁱNS, not significant (p > 0.05).

in preventing UV induced systemic suppression of the DTH response to *C. albicans* (**Table I**). Oligosaccharide treatment of unirradiated mice had no effect on their DTH response. Thus, the increase in DTH reactivity in UV irradiated mice appears to be a restoration of the immune response in these animals rather than a nonspecific stimulation of immune activity.

The target of action of the protective factor(s) was investigated by examining the effect of *Aloe* oligosaccharides on IL-10 production in UV irradiated skin. The dorsal skin of unirradiated and UV irradiated mice was treated with the oligosaccharides and examined 4 d later for IL-10 protein by immunohistochemical staining by digital image analysis. Sections of UV irradiated dorsal skin exhibited the expected epidermal hyperplasia. Low levels of IL-10 staining were detected in the epidermis of untreated or *Aloe* treated, unirradiated mice (**Fig 3**). High levels (9.12 ± 0.77 mean ± SD arbitrary gray scale log intensity units, n = 40 fields) of IL-10 protein were detected in the cytoplasm of epidermal cells. Oligosaccharide treatment of UV irradiated skin greatly diminished epidermal IL-10 production (7.13 ± 0.78 units) but cellular hyperplasia was unaffected.

To determine whether the same effect on keratinocyte IL-10 protein could be achieved *in vitro*, a transformed murine keratinocyte line, Pam 212, was exposed to 300 J UVB radiation per m² and treated for 1 h with the *Aloe* oligosaccharides. Twenty-four hours after treatment, supernatants from the cultures were collected for analysis and the cells were stained for detection of intracellular IL-10 protein. Unirradiated control and oligosaccharide treated Pam 212 keratinocytes showed background levels (1.80 ± 0.51 and 1.84 ± 0.42 units, respectively) of diffuse cytoplasmic staining. Keratinocytes exposed to UV radiation *in vitro* demonstrated darker perinuclear staining with diffuse cytoplasmic staining or ring formation that is consistent with cytokine production (4.67 ± 1.03 units). After treatment of the UV irradiated cultures with *Aloe* oligosaccharides, cytoplasmic IL-10 was reduced (2.21 ± 0.47 units) to near background values. The reduction of IL-10 protein in UV irradiated skin and Pam 212 keratinocytes was reproducible using other lots of partially purified *Aloe* oligosaccharide, but was not observed using structurally unrelated oligosaccharides such as methyl cellulose (not shown).

Treatment of cultures with *Aloe* oligosaccharides resulted in a slight reduction in the amount of protein observed in culture supernatants from either unirradiated or UV exposed cultures (**Table II**). Cell viability after *Aloe* treatment was 85–95% by dye exclusion (not shown).

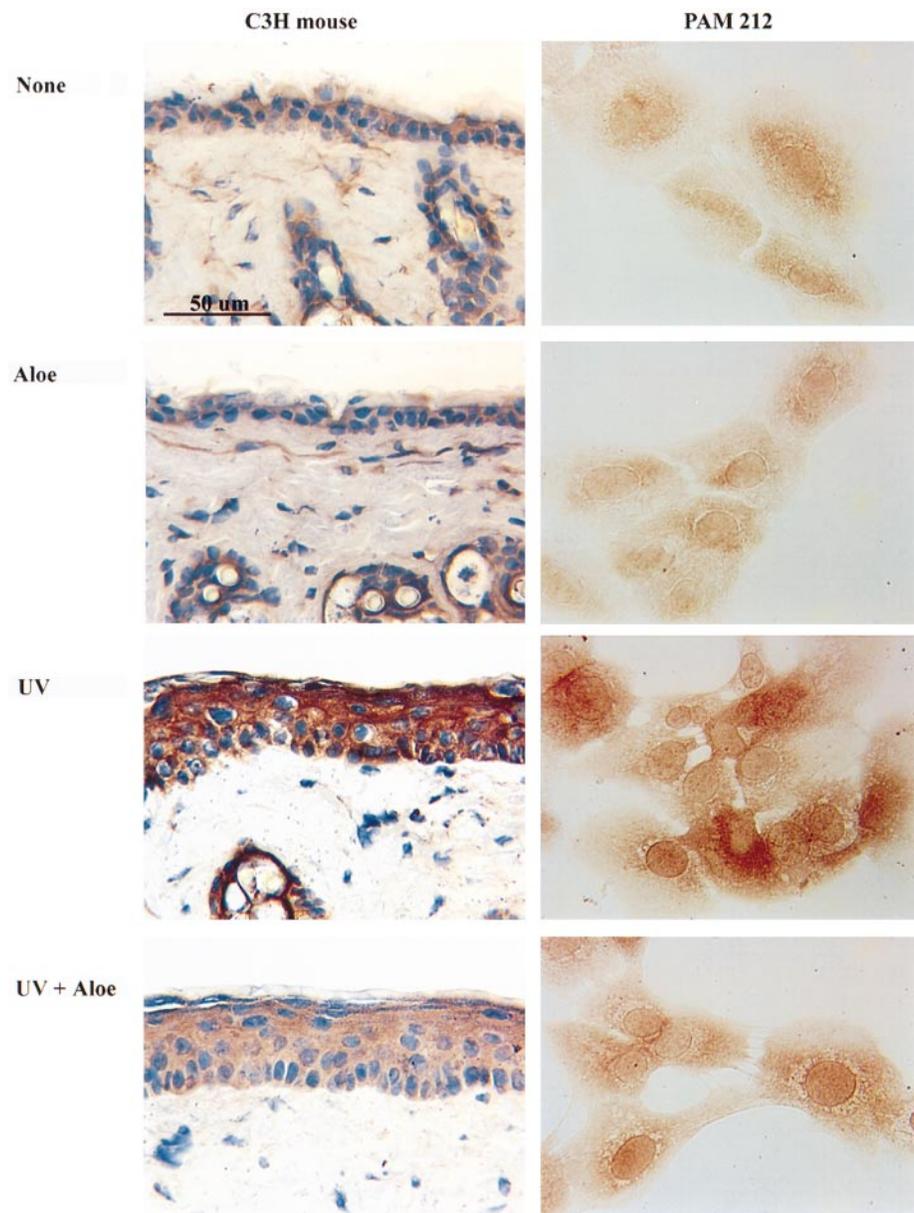


Figure 3. *Aloe* oligosaccharides decrease IL-10 production by UV irradiated murine keratinocytes. Shaved dorsal skin of C3H/HeN mice was exposed to 15 kJ UVB radiation per m² and treated with PBS or 500 µg oligosaccharides per ml purified from lot ARF94K *Aloe* polysaccharide. Cryosections of dorsal skin were prepared 4 d later. The transformed keratinocyte cell line, Pam 212, was exposed *in vitro* to 300 J UVB radiation per m² and treated for 1 h with 10 µg *Aloe* oligosaccharides per ml in PBS. The cells were washed, fresh serum-free medium was added, and the incubation continued at 37°C. Twenty-four hours later, the cells were washed, fixed, and stained for IL-10 using rat monoclonal anti-mouse IL-10 IgG, biotinylated anti-rat IgG, peroxidase labeled streptavidin, and diaminobenzidine substrate. The samples were counterstained with Gill's hematoxylin dye and examined under light microscopy using an ×20 objective.

Table II. *Aloe barbadensis* oligosaccharides reduce the production of IL-10 by UV irradiated Pam 212 keratinocytes

Experiment	Treatment	Protein ^b (µg)	Interleukin-10 ^a		p
			pg per 20 µg protein	Per cent reduction	
I	Untreated ^c	502	<5		
	<i>Aloe</i> oligosaccharides 10 µg per ml ^d	338	<5		
	UV ^e	996	253		
	UV + <i>Aloe</i> oligosaccharides 10 µg per ml	942	92	64 ^f	< 0.01 ^g
II	Untreated	673	<4		
	<i>Aloe</i> oligosaccharides 10 µg per ml	575	<4		
	UV	1020	24		
	UV + <i>Aloe</i> oligosaccharides 10 µg per ml	970	7	71	< 0.01

^aMeasured by ELISA using a murine rIL-10 standard.

^bDetermined by the Bio-Rad protein assay using concentrated supernatants. Data represent the total protein content in supernatants pooled from six cultures.

^cTwo × 10⁶ Pam 212 keratinocytes per 100 mm petri dish were cultured in serum-free MEM for 24 h at 37°C. Supernatants were collected, concentrated, and analyzed.

^dPam 212 cells were incubated in 10 µg *Aloe* oligosaccharides per ml in PBS for 1 h at 37°C. The medium was discarded, the cells were washed, and the fresh serum-free medium was added to the cultures. Supernatants were collected 24 h later and concentrated.

^ePam 212 cells in PBS were exposed to 300 J UVB radiation per m². Following exposure the cells were washed and incubated for 24 h in serum-free medium, after which the supernatants were collected and concentrated.

^fCompared with supernatant from cultures treated with UV alone.

^gDetermined by ANOVA.

Table III. *Aloe barbadensis* oligosaccharides decrease the *in vivo* immunosuppressive activity of supernatants from UV irradiated keratinocytes

Treatment of mice		Experiment 1			Experiment 2		
Sensitization	Supernatant	Footpad swelling (μm)	% Specific suppression	p	Footpad swelling (μm)	% Specific suppression	p
None	None	168 \pm 8 ^a	54 \pm 15				
<i>C. albicans</i> ^b	None	371 \pm 9	289 \pm 11				
<i>C. albicans</i>	Untreated ^c	373 \pm 8	353 \pm 31				
<i>C. albicans</i>	<i>Aloe</i> treated	365 \pm 10	319 \pm 35				
<i>C. albicans</i>	UV	253 \pm 18	58 ^d	< 0.01 ^e	154 \pm 17	66	< 0.001
<i>C. albicans</i>	UV + <i>Aloe</i>	418 \pm 21	0	NS ^f	317 \pm 60	1	NS

^aMean \pm SEM of groups of five C3H/HeN mice that were challenged but not sensitized.

^bSensitized with a subcutaneous injection of 2×10^7 formalin fixed *C. albicans* cells and challenged 10 d later.

^cAnimals were given a single intravenous injection of 20 μg supernatant protein from groups described in **Table II** and sensitized 3 d later.

^dCompared with matching unirradiated control group.

^eDetermined by ANOVA.

^fNS, not significant ($p > 0.05$).

Amounts of IL-10 protein, as measured by ELISA, were below the levels of detection in supernatants from unirradiated and untreated, or *Aloe* oligosaccharide treated cultures. Following UV irradiation, the protein content of the Pam 212 keratinocyte culture supernatants rose by ≈ 1.5 -fold. The UV exposed cells released nanogram quantities of IL-10 into the culture supernatant; however, *Aloe* treatment following UV irradiation decreased IL-10 levels by 64–71% compared with cultures treated with UV radiation alone. The total amount of IL-10 cytokine detectable in the culture supernatants of UV irradiated keratinocytes varied from experiment to experiment by as much as 10-fold; however, these experiments have been repeated an additional four times and in each case *Aloe* treatment significantly reduced IL-10 production by the UV irradiated keratinocytes (not shown).

***Aloe* oligosaccharides prevent the suppression of DTH by supernatants from UV irradiated Pam 212 cells** The effect of immunosuppressive activity of UV irradiated keratinocyte supernatants was assessed by injecting mice with 20 μg protein from the cultures described in **Table II**. The animals were sensitized with formalin fixed *C. albicans* cells 3 d after injection with culture supernatants. Supernatant from unirradiated, untreated, or *Aloe* oligosaccharide treated cultures had no significant effect on the DTH response to *C. albicans* (**Table III**). In contrast, supernatants from UV irradiated keratinocytes significantly suppressed the induction of DTH ($p < 0.01$) in both experiments. Treatment of UV irradiated keratinocytes with *Aloe* oligosaccharides completely blocked the immunosuppressive activity in these supernatants. Taken together these results indicate that *Aloe* oligosaccharides may prevent UV induced suppression of DTH responses *in vivo* by blocking the production and/or action of an immunosuppressive cytokine(s), such as IL-10.

DISCUSSION

We have previously shown that cutaneous application of a crude extract of *Aloe barbadensis* gel protects both CHS and DTH responses in mice from suppression by UV radiation (Strickland *et al.*, 1994); however, whether the protection in both models of T cell immunity was mediated by the same molecule is unknown, as is the chemical stability of the immunoprotective agent(s). In this study, these questions were addressed by assessing the immunoprotective activity of individual lots of *Aloe* gel extract over time. The different decay rates observed for CHS and DTH protective activity indicate that *Aloe* gel contains multiple factors that act on the two different models of UV induced immune suppression. The lability of the factor that prevents suppression of CHS responses varies in different lots of the specially prepared gel extract and was influenced by the manufacturing process used to produce the extracts. The finding that biologically active components in *Aloe* can be labile helps to explain some of the difficulties that investigators have reported in reproducing results using unfractionated materials from this plant.

Prevention of UV induced suppression of DTH responses to *C.*

albicans is afforded by an oligosaccharide fraction of *Aloe barbadensis* gel (**Table I** and RPP, manuscript in preparation). The material has a molecular weight of ≤ 5000 d and is relatively stable, although the activity can be degraded and is absent in most commercial preparations, even those purporting to be "pure *Aloe*." *Aloe* contains many compounds in addition to those utilized herein (Waller *et al.*, 1978). Whether protection of CHS is mediated by a fragment of the same agent that prevents suppression of DTH responses or is chemically distinct, is currently under investigation. The point of these studies is not the purification and characterization of the active components in *Aloe*. Rather, this degree of isolation was necessary to remove other components with possible confounding modes of action.

UV radiation has been shown to suppress CHS and DTH responses by different mechanisms. Cytokines such as tumor necrosis factor- α and IL-1 are produced by several cells following cutaneous exposure to UV irradiation and appear to be involved in the suppression of CHS responses, whereas IL-10 suppresses DTH immunity (Robertson *et al.*, 1987; Vermeer and Streilein, 1990; Rivas and Ullrich, 1994; Ullrich, 1994). The factor *cis*-urocanic acid suppresses both CHS and DTH responses and may act indirectly via the cytokines tumor necrosis factor- α and IL-10 (Moodycliffe *et al.*, 1996). In our study, IL-10 was detected immunocytochemically in the epidermis of mice following exposure to UV radiation (**Fig 3**). *Aloe* oligosaccharide treatment decreased IL-10 levels in UV irradiated epidermis and prevented immune suppression induced by whole-body irradiation.

Murine keratinocytes exposed to UV radiation *in vitro* produce soluble factors that mimic some of the immunosuppressive effects of whole-body UV irradiation (Rivas and Ullrich, 1994). Injection of culture supernatants containing IL-10 into normal mice induces suppression of DTH but not CHS responses (Kim *et al.*, 1990; Rivas and Ullrich, 1994). In those studies, immunoprecipitation of IL-10 removed all the suppressive activity from the supernatant. In our study, the murine keratinocyte cell line Pam 212 produced IL-10 following *in vitro* exposure to UV radiation. Treatment of UV irradiated cells with *Aloe* derived oligosaccharides reduced IL-10 production by these cells. Thus, protection against UV induced suppression of DTH responses roughly correlated with decreased IL-10 production both *in vivo* and *in vitro*.

The amounts of IL-10 protein present in the culture supernatants varied between experiments, although supernatants from cultures treated with UV alone contained sufficient amounts of the cytokine to induce suppression of DTH responses when injected into mice. In contrast, supernatants from UV irradiated, *Aloe* treated cultures consistently failed to induce suppression of DTH responses, even though they contained levels of IL-10 that, if present in supernatants from cultures treated with UV radiation alone, would be sufficient to induce suppression. These findings, together with the results on *in vivo* IL-10 discussed above, suggest that *Aloe* oligosaccharides, in addition to reducing the production of IL-10, may neutralize its bioactivity

without affecting its binding to antibodies in ELISA. Alternatively, there may be another immunosuppressive factor, either independent or induced by IL-10, that is also decreased by *Aloe* treatment. These possibilities are not mutually exclusive. Evidence for other immunosuppressive cytokines comes from reports showing that injection of antibodies to IL-4 can also block the induction of suppression of DTH responses (Rivas and Ullrich, 1994). Furthermore, there are functional similarities between the cytokines IL-13 and IL-4 and the receptors for these proteins share a common subunit (Malabarba *et al*, 1996). The ability of keratinocytes to produce IL-13 is unknown. We have been unable to detect IL-13 in the supernatants of UV irradiated Pam 212 cells by ELISA or to consistently measure IL-4 in the same keratinocyte supernatants that contain significant amounts of IL-10. Alternatively, *Aloe* might protect DTH responses indirectly by inducing keratinocytes to produce immunostimulatory cytokines, such as IL-12 (Aragane *et al*, 1994). Recently, IL-12 was shown to be involved in overcoming UV induced immune suppression, and in preventing the induction of suppressor T cells (Schmitt *et al*, 1995); however, we have not observed measurable amounts of IL-12 from Pam 212 cells treated with *Aloe* oligosaccharides, UV radiation, or a combination of the two agents. Thus, in light of the many studies demonstrating the central role of IL-10 as an immunoregulator of DTH reactions and the correlation between restoration of immunity to *C. albicans* and reduction in the levels of this cytokine, *Aloe's* action on IL-10 appears to be a major mechanism of its protective activity.

In summary, our data demonstrate that crude extracts of *Aloe barbadensis* gel contain at least two distinct agents that differentially protect CHS and DTH responses from photosuppression. An oligosaccharide fraction generated from *Aloe barbadensis* polysaccharide, prevented UV induced immunosuppression of DTH responses, reduced IL-10 production in UV irradiated skin and a keratinocyte cell line, and blocked the immunosuppressive activity of the supernatants. Whether CHS responses are protected from suppression by a similar mechanism and the ability of agents in *Aloe* to prevent photosuppression in humans can only be determined by further investigations.

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REFERENCES

- Aragane Y, Riemann H, Bhardwaj RS, *et al*: IL-12 is expressed and released by human keratinocytes and epidermoid carcinoma cell lines. *J Immunol* 153:5366, 1994
- Chung HT, Burnhan DK, Robertson B, Roberts LK, Daynes RA: Involvement of prostaglandins in the immune alterations caused by the exposure of mice to ultraviolet radiation. *J Immunol* 137:2478, 1986
- Denkins YD, Fidler IJ, Kripke ML: Exposure of mice to UV-B radiation suppresses delayed hypersensitivity to *Candida albicans*. *Photochem Photobiol* 49:615, 1989
- Fisher MS, Kripke ML: Systemic alteration induced in mice by ultraviolet light irradiation and its relationship to ultraviolet carcinogenesis. *Proc Natl Acad Sci USA* 74:1688, 1977
- Giannini MSH: Suppression of pathogenesis in cutaneous leishmaniasis by UV-irradiation. *Infect Immunol* 51:838, 1986
- Gowda DC, Neelissiddaiah B, Anjaneyalu YV: Structural studies of polysaccharides from *Aloe vera*. *Carb Res* 72:201, 1979
- Harber LC, Bickers DR: *Photosensitivity Diseases*, 2nd edn. BC Decker, Toronto, 1989, p. 112
- Harriott-Smith TG, Haliday WJ: Suppression of contact hypersensitivity by short term ultraviolet irradiation. II. The role of urocanic acid. *Clin Exp Med* 72:174, 1988
- Jeevan A, Kripke ML: Effect of a single exposure to UVB radiation on *Mycobacterium bovis* bacillus Calmette-Guerin infection in mice. *J Immunol* 143:2837, 1989
- Kim T-Y, Kripke ML, Ullrich SE: Immunosuppression by factors released from UV irradiated epidermal cells: selective effects on the generation of contact and delayed hypersensitivity after exposure to UVA or UVB radiation. *J Invest Dermatol* 94:26, 1990
- Kripke ML: Antigenicity of murine skin tumors induced by UV light. *J Natl Cancer Inst* 53:1333, 1974
- Kripke ML, Morison WL: Studies on the mechanism of systemic suppression of contact hypersensitivity by UVB radiation. II. Differences in the suppression of delayed and contact hypersensitivity. *J Invest Dermatol* 86:543, 1986
- Malabarba MG, Rui H, Deutsch HH, Chung J, Kalthoff FS, Farrar WL, Kirken RA: Interleukin-13 is a potent activator of JAK3 and STAT6 in cells expressing interleukin-2 receptor-gamma and interleukin-4 receptor alpha. *Biochem J* 319:865, 1996
- Moodycliffe AM, Bucana CD, Kripke ML, Norval M, Ullrich SE: Differential effects of a monoclonal antibody to *cis*-urocanic acid on the suppression of delayed and contact hypersensitivity following ultraviolet irradiation. *J Immunol* 157:2891, 1996
- Niizeki H, Streilein JW: Hapten-specific tolerance induced by acute, low-dose ultraviolet B radiation of skin is mediated via interleukin-10. *J Invest Dermatol* 109:25, 1997
- Nishigori C, Yarosh DB, Ullrich SE, Vink AA, Bucana CD, Roza L, Kripke ML: Evidence that DNA damage triggers interleukin 10 cytokine production in UV irradiated murine keratinocytes. *Proc Natl Acad Sci USA* 93:10354, 1996
- Noonan FP, De Fabo EC: Immunosuppression by ultraviolet B radiation: initiation by urocanic acid. *Immunol Today* 13:250, 1992
- Otani T, Mori R: The effects of ultraviolet irradiation of the skin on herpes simplex virus infection: Alteration in immune function mediated by epidermal cells and in the course of infection. *Arch Virol* 96:1, 1987
- Pelley RR, Wang Y-T, Waller TA: Current status of quality control of *Aloe barbadensis* extracts. *SOPWJ* 119:255, 1993
- Rivas JM, Ullrich SE: The role of IL-4, IL-10, and TNF- α in the immune suppression induced by ultraviolet radiation. *J Leuk Biol* 56:769, 1994
- Robertson B, Gahring L, Newton R, Daynes RA: In vivo administration of IL-1 to normal mice depresses their capacity to elicit contact hypersensitivity responses: prostaglandins are involved in this modification of immune function. *J Invest Dermatol* 88:380, 1987
- Schmitt DA, Owen-Schaub L, Ullrich SE: The effects of Interleukin-12 on immune suppression and suppressor cell induction by ultraviolet radiation. *J Immunol* 154:5114, 1995
- Simon JC, Tigelaar RE, Bergstresser PR, Edelbaum D, Cruz PD: Ultraviolet B radiation converts Langerhans cells from immunogenic to tolerogenic antigen-presenting cells. *J Immunol* 146:485, 1991
- Strickland FM, Pelley RP, Kripke ML: Prevention of ultraviolet radiation-induced suppression of contact and delayed hypersensitivity by *Aloe barbadensis* gel extract. *J Invest Dermatol* 102:197, 1994
- Toews GB, Bergstresser PR, Streilein JW: Epidermal Langerhans cell density determines whether contact hypersensitivity or unresponsiveness follows skin painting with DNFB. *J Immunol* 124:445, 1980
- Ullrich SE: Mechanisms involved in the systemic suppression of antigen-presenting cell function by UV irradiation: keratinocyte-derived IL-10 modulate antigen-presenting cell function of splenic adherent cells. *J Immunol* 152:3410, 1994
- Urbach F: Evidence and epidemiology of ultraviolet-induced cancers in man. *Nat'l Cancer Inst Monogr* 50:5, 1978
- Vermeer M, Streilein JW: Ultraviolet B light-induced alterations in epidermal Langerhans cells are mediated in part by tumor necrosis factor- α . *Photodermatol Photoimmunol Photomed* 7:258, 1990
- Waller GR, Mangiáfico S, Ritchey CR: A chemical investigation of *Aloe barbadensis* miller. *Proc Okla Acad Sci* 58:69, 1978
- Yoshikawa T, Rae V, Bruins-Slot W, Van den Berg J-W, Taylor JR, Streilein JW: Susceptibility to effects of UVB radiation on induction of contact hypersensitivity as a risk factor for skin cancer in humans. *J Invest Dermatol* 95:530, 1990