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Immunology

UV exposure reduces immunization rates and promotes tolerance to epicutaneous antigens in humans: Relationship to dose, CD1a$^-$DR$^+$ epidermal macrophage induction, and Langerhans cell depletion

(photoimmunology/ozone depletion/contact sensitivity/T lymphocytes/antigen presenting cells)

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ABSTRACT **Increasing UVB radiation at the earth's surface might have adverse effects on *in vivo* immunologic responses in humans. We prospectively randomized subjects to test whether epicutaneous immunization is altered by prior administration of biologically equalized doses of UV radiation. Multiple doses of antigens on upper inner arm skin (UV protected) were used to elicit contact sensitivity responses, which were quantitated by measuring increases in skin thickness. If a dose of UVB sufficient to induce redness (erythemagenic) was administered to the immunization site prior to sensitization with dinitrochlorobenzene (DNCB), we noted a marked reduction in the degree of sensitization ($P < 0.0006$) that was highly dose responsive ($r = 0.98$). Even suberythemagenic UV (less than a visible sunburn) resulted in a decreased frequency of strongly positive responses (32%) as compared to controls (73%) ($P = 0.019$). The rate of immunologic tolerance to DNCB (active suppression of a subsequent repeat immunization) in the groups that were initially sensitized on skin receiving erythemagenic doses of UV was 31% ($P = 0.0003$). In addition, a localized moderate sunburn appeared to modulate immunization with diphenylcyclopropenone through a distant, unirradiated site (41% weak responses) as compared to the control group (9%) ($P = 0.05$). Monitoring antigen presenting cell content in the epidermis revealed that erythemagenic regimens induced CD1a$^-$DR$^+$ macrophages and depleted Langerhans cells. In conclusion, relevant and even subclinical levels of UV exposure have significant down modulatory effects on the ability of humans to generate a T-cell-mediated response to antigens introduced through irradiated skin.**

With UVB comprising an increasing proportion of the sunlight reaching the earth's surface, the impact of such a change on human health becomes increasingly important to understand. In addition to causing photosensitivity diseases (i.e., lupus, porphyrias, medication reactions) and carcinogenic genetic mutations, mammalian UV exposure alters immunologic responses that normally handle microbial pathogens and UV-induced cancers (1, 2). For instance, although murine UV-induced cancers can be highly antigenic and are rejected upon transplantation into normal mice (3), mice exposed to subcarcinogenic doses of UV allow progressive tumor growth (4). Although most other immune functions remained intact, UV-exposed animals could no longer become immunized to normally potent contact allergens (5, 6). UV exposure resulted not only in a simple failure of immunization, but also in long-term, active suppression of subsequent immunizations to the contact allergen through normal skin (tolerance) (7–11). Both the increased UV-induced tumor susceptibility of UV-exposed mice and the unresponsiveness of UV-exposed mice to contact allergens were found to be due to antigen-specific suppressor T lymphocytes (12, 13).

UV regulation of murine contact sensitivity has held up well as a model of immunologic events occurring in photocarcinogenesis. Epidermal Langerhans cells, an antigen presenting population of dendritic cell lineage present in the epidermis (14), have a potent capacity to initiate contact sensitivity reactions (15, 16), as well as tumor rejection (17). However, purified Langerhans cells exposed to UV are no longer able to induce T-lymphocyte proliferation (18), possibly through alterations of adhesion molecule expression (19). The net result of UV exposure is that immunization (to contact, tumor, or microbial antigens) through the skin results in persistent antigen-specific unresponsiveness to the antigens (20–23). Depression in the ability of T cells to react to new peptide sequences generated as a result of a UV-induced genetic mutation could then result in tolerance rather than rejection of UV carcinoma cells bearing such abnormal gene products (24).

Previous studies on whether UV modulates the contact sensitization potential of human skin *in vivo* have suggested slightly reduced (25–31) or unaltered responses (32, 33). However, none of these studies is conclusive due to the use of subjective assessments, to testing of the response by using skin of patients with skin disease or skin that had also recently received UV exposure, to insufficient *n* for statistical analysis, or to lack of appropriate control groups.

We modified a highly quantitative and sensitive method for assessing dinitrochlorobenzene (DNCB) contact sensitivity in human skin (34). Prospectively randomized groups were sensitized through normal skin or skin irradiated with various doses of individually biologically equalized doses of UV. We found that UV exposure in humans resulted in highly significant, dose-responsive decreases in immunologic responsiveness. Of additional concern are our findings that levels of UV exposure below clinical detectability can impair immune responsiveness and that a localized sunburn can alter T-cell responses at distant, unirradiated sites.

MATERIALS AND METHODS

Demographics and UV Exposure of Study Populations. The Institutional Review Board approved the protocol, advertisements, and consent document. Upon recruitment, each subject was randomly assigned to a UV administration sched-

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Abbreviations: DNCB, dinitrochlorobenzene; MED, minimal erythemal dosage; DPCP, diphenylcyclopropenone.

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ule. Individuals were of skin type I, II, or III, without history of chronic disease and not currently on medication.

Individuals received either no UV radiation or one of three localized UV exposure regimens. A portable UVB phototherapy device (Dermacontrol, Frankfort, IL) containing six FS40 bulbs emitted 0.3 mJ/cm^2 at a 10-inch (1 inch = 2.54 cm) source-to-skin distance. An LMHO6C meter (National Biological, Cleveland) equipped with an IL SEE1240 detector fitted with a W wide-angle quartz diffuser and a SCS280 filter (International Light, Newburyport, MA) was used. The minimal erythemal dosage (MED) was determined for each subject, such that biologically equivalent amounts of UVB were administered. Except for the 3 × 5-inch exposure area on the left buttock, all areas of the body were draped. Two groups were exposed 4 days in a row, with the first group receiving 0.75 of the MED (0.75MED × 4) and the second group receiving 2 times the MED each day (2MED × 4) (Table 1). These subjects were sensitized to contact allergens immediately following the last UV exposure (day 4). A third group received 4 MED on the 1st day only (4MED × 1). These subjects were sensitized on the 3rd day after UV exposure (day 3). To control for diminished levels of contact sensitization that occurs in menstruating women except during midcycle (35), all female subjects were sensitized 14 days after the onset of menses.

Sensitization. DNCB was applied to the left buttock, which had received the UV exposure [or no UVB for the control group (Table 1)], and diphenylcyclopropenone (DPCP) was applied on the right (protected or non-UV exposed) buttock (Aldrich). Delivery of DNCB and DPCP was accomplished by soaking a petrolatum-backed 11-mm filter disk with 48 μl of 0.0625% DNCB in acetone (30 μg) or 32 μl of 0.0625% DPCP in acetone (20 μg). The filter disk was mounted inside a 12-mm aluminum Finn chamber (Hermal Pharmaceutical Laboratories, Oak Hill, NY) and the assembly was taped onto the skin and left in place for 48 hr.

Challenge. Three weeks after the initial sensitization, subjects received an antigenic challenge on unirradiated upper inner arm skin, because this skin is loose enough to be pinched between the calipers of a micrometer for objective quantitation of skin edema. Petrolatum-backed 7-mm filter disks were placed in 8-mm Finn chambers and soaked with 20 μl of antigen solutions of various strengths. Five patches were placed on the right arm, one containing an acetone control and four containing increasing amounts of DNCB (3.125, 6.25, 8.8, and 12.5 μg). Four patches were similarly placed on the left arm to deliver increasing amounts of DPCP (0.39, 0.78, 1.56, and 3.125 μg). These patches remained in place for 6 hr; at 48 hr, the sites were evaluated. The concentration sites were rotated in each successive subject. A fifth group of 14 people received the challenge patches only [no UV or sensitization (Table 1)] in order to quantify the irritating effects of these concentrations of DNCB and DPCP and to thus define the range of negative responses.

Scoring of Contact Sensitivity Elicitation. A visual subjective score (*i–v*) scales intensity as follows: (*i*) no reaction, (*ii*) mild, macular erythema, (*iii*) moderate erythema, occasionally with papulation, (*iv*) strong erythematous reaction (includes edematous vesicular changes), (*v*) extreme or spreading reaction (includes bullous or ulcerative reaction).

Table 1. Demographics and UV exposure of study population

Study group	Males/ females	Mean age, yr	Mean MED ± SEM, mJ/cm^2	Mean total UVB dose, mJ/cm^2
Challenge only	8/6	27.2 ± 1.8	NT	0
No UV	12/10	28.3 ± 2.5	NT	0
0.75MED × 4	14/8	27.8 ± 2.1	32.7 ± 1.7	98 ± 5.1
2MED × 4	14/6	23.9 ± 1.2	29.1 ± 1.9	222 ± 14.8
4MED × 1	14/8	29.1 ± 1.6	32.5 ± 1.7	127 ± 10.1

NT, not tested.

In addition to the subjective assessment, we used an objective assessment of skin edema. The skin fold thickness was determined by using a micrometer with spring-loaded calipers (Mitutoyo Manufacturing, Tokyo), recording the skin thickness in millimeters at each site before and 48 hr after the patch was applied. The increase in skin thickness over the 48-hr period was calculated for each site by subtracting the prechallenge thickness from the 48-hr postchallenge thickness. The "mm increase sum" is derived from the addition of the increases in skin thickness of each of the four concentrations for each allergen and allows the entire dose–response curve (see Fig. 1) to be approximated as a single value (see Fig. 2). Photographs were also obtained.

Langerhans Cell Quantitation in Sheets. Punch biopsies (4 mm) were taken on the day of sensitization—one from the exposed buttock and one from the protected buttock. A 1 M NaCl-split sheet of epidermis was fixed in acetone and stained with a combination of fluoresceinated OKT6 (anti CD1a; Orthomune, Raritan, NJ) and phycoerythrin-conjugated anti-HLA-DR (Becton Dickinson). Blinded quantitations were performed with the aid of an ocular grid.

Statistical Analysis. Differences in challenge response, as measured by increase (mm) in skin thickness and visual scores, among the different exposure groups for each antigen concentration, were determined by a one-way ANOVA followed by the Scheffe method for multiple comparisons. Differences in overall response among the different exposure groups, as measured by the sum of the increases in skin thickness across the four antigen concentration levels, were compared with a one-way ANOVA. Association between strength of response (strong, weak, none) and exposure requirements was performed with a Pearson's χ^2 test. A χ^2 test was also used to statistically compare the proportion of patients who became tolerant in the erythemagenic groups versus control. Comparisons of the numbers of Langerhans cells and macrophages between UV-exposed and unexposed sites on each patient were made with the paired *t* test.

Summary statistics are reported as means ± 1 SEM. All *P* values are two-tailed. The data were analyzed with the Michigan data analysis system (MIDAS, a statistical software package developed at the University of Michigan).

RESULTS

Quantifiable Assessment of Contact Sensitivity in Normal Human Volunteers. Among controls sensitized with DNCB on the unexposed left buttock, the elicitation response was linear whether assessed by using the visual scoring scale or an engineer's micrometer (Fig. 1, open bars), and the two methods correlated at $r = 0.88$. These values were substantially (10-fold) greater than the DNCB irritant response; the mean increase in skin thickness due to the irritant response of 14 unsensitized individuals to 12.5 μg of DNCB was 0.11 ± 0.03 mm, as compared to a mean increase of 1.19 ± 0.14 mm in sensitized individuals.

Sensitization of the right buttock with various doses of DPCP also resulted in responses in sensitized individuals that were substantially greater (1.44 ± 0.21 mm at 3.125 μg) than the irritant responses to the identical concentrations of DPCP applied to upper inner arms of unsensitized individuals (0.06 ± 0.04 mm at 3.125 μg).

Immunization Through Skin Exposed to Localized UV Results in Dose-Responsive Decreases in Induction of Contact Sensitivity. Individuals receiving four daily modest sunburns with 2MED UV on the left buttock immediately before sensitization with DNCB on the same site demonstrated a marked reduction in contact sensitivity responses. We observed statistically significant decreases at each of the challenge concentrations upon assessment with the visual scoring

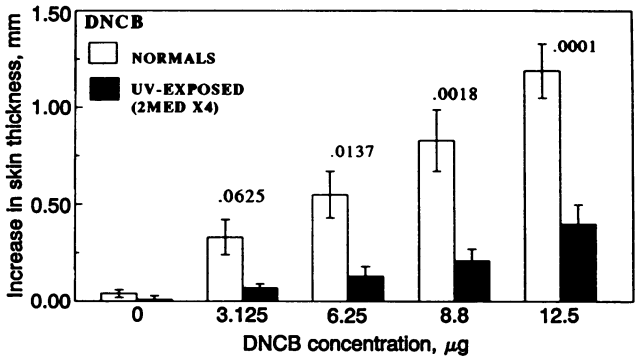

FIG. 1. Response to various concentrations of DNCB (in μg per filter disk) as assessed by the increase in skin thickness at the site. Open bars, group mean ± SEM of 22 normal subjects sensitized on normal skin; solid bars, group mean ± SEM of 20 subjects sensitized on buttock skin that had been exposed daily for 4 days to 2MED of UVB (2MED × 4).

system and by measuring the increase in skin thickness at the reaction site (Fig. 1). A single acute moderate sunburn with 4MED UV 3 days before application of the sensitizer also resulted in significant reductions in contact sensitivity responses, whether expressed as mean visual score ($P < 0.0003$ at the 12.5-μg test site) or as mean increase in skin thickness ($P < 0.0006$ at 12.5 μg) (data not shown).

Analysis of Overall Individual Responses Reveals Decreased Frequencies of Fully Successful Immunizations in All UV Exposure Groups. The overall response of each individual (sum of increases in skin thickness across the four challenge concentrations) was used to determine the frequency of clearly positive responses, weak responses, and negative responses. A negative response to DNCB was defined as that which is indistinguishable from irritant responses to DNCB in unsensitized subjects [a sum of mm increases in skin thickness that was <2 SD above the mean increase in skin thickness that occurs in response to DNCB in previously unsensitized individuals (challenge only)] (Fig. 2). To determine the expected range of positive responses in control subjects sensitized on normal skin (No UV; Fig. 2), the mean and SD of all responses above the negative cutoff (>0.81 mm) was calculated. The mean ± 1 SD of positive responses thereby falls between 1.75 and 5.35 mm, allowing responses > 1.75 mm to be defined as a fully successful immunization (Fig. 2, upper line). Only 9% of the DNCB-sensitized, no UV control group demonstrated weak responses; that is, sensitization on normal skin generally resulted in a strongly positive or a totally negative response.

The distribution of frequencies of DNCB responses of the UV exposure groups was significantly different from that of the control no UV group (Fig. 2). The percentage of strong positive responses dropped from 73% in the no UV control group to 32% in the suberythemagenic group ($P = 0.019$), to 5% in the 2MED × 4 group ($P < 0.0001$), and to 27% in the 4MED × 1 group ($P = 0.011$) (Pearson's uncorrected χ^2 test) (Table 2). The frequency of positive responses was reduced in direct proportion to the total number of mJ/cm^2 delivered to the skin in the immediately preceding period, with a high negative correlation coefficient of −0.98. These data indicate that virtually all individuals with skin types I–III are susceptible to the immunomodulatory effects of UV, that this is a dose-responsive effect, and that skin absorbing subclinically detectable levels of UV is also affected.

Increased Rate of Tolerance to DNCB in Subjects Receiving Primary DNCB Sensitization on Sunburned Skin. We attempted to resensitize all individuals identified as nonresponders after primary sensitization to DNCB. Those agreeing to undergo the procedure again were then resensitized

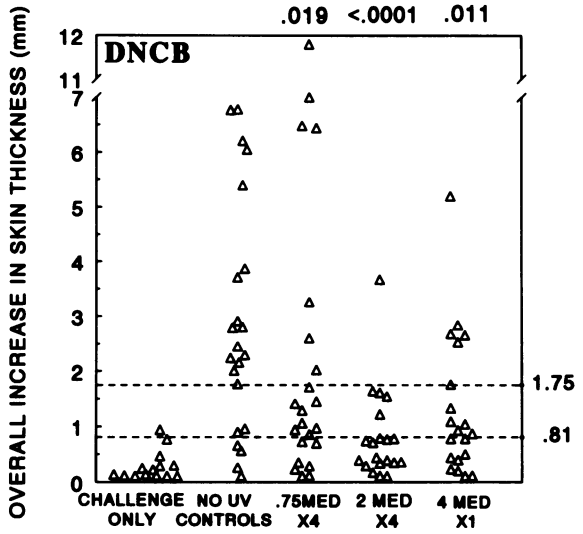

FIG. 2. Scattergram of overall individual responses to DNCB by sensitization group. Sum of the increases in skin thickness of <0.81 mm represents negative responses and values > 1.75 mm are defined as fully successful immunizations (defined in text).

(second sensitization) to DNCB on the normal skin of the right (never received UV) buttock and rechallenged on the upper inner arms. Individuals continuing to exhibit a negative response were defined as tolerant to DNCB. To estimate the frequency of tolerance in the original study population, the frequency of tolerance among tested nonresponders was multiplied by the frequency of nonresponders (Table 3). Whereas only 7.0% of subjects who received primary sensitization on normal skin were calculated to be tolerant to DNCB, 31% of individuals receiving primary sensitization through skin exposed to erythemagenic doses of UV were calculated to be tolerized (Table 3) ($P = 0.0003$ by χ^2 test). These data indicate that UV exposure in humans may indeed result in long-term (up to 4 months) active down regulation of the immune response to a normally highly immunogenic stimulus.

A 4MED UV Exposure Can Systemically Modulate Immunization Through Normal Skin. To determine whether UV exerts a distant effect on cutaneous immunizations, the subjects had been simultaneously sensitized with DPCP (does not demonstrate immunologic cross-reactivity with DNCB) on the contralateral, UV-protected right buttock.

Table 2. UV effects

Response	No UV	0.75MED × 4	2MED × 4	4MED × 1
Local UV effects: Increased occurrence of weak or negative DNCB responses in all UV irradiation groups				
Strong positive	73%	32%	5%	27%
Weak positive	9%	36%	20%	23%
Negative	18%	32%	75%	50%
		$P = 0.019$	$P < 0.0001$	$P = 0.011$
Systemic UV effects: Increased occurrence of weak DPCP responses in the 4MED × 1 UV irradiation group				
Strong positive	64%	54%	50%	41%
Weak positive	9%	14%	20%	41%
Negative	27%	32%	30%	18%
		$P = 0.81$	$P = 0.54$	$P = 0.05$

Response is the sum of increases in skin thickness at each challenge concentration. Strong positive responses are as defined in Figs. 1 and 2. Weak responses are >0.81 and <1.75 mm for DNCB and >0.72 and <1.97 mm for DPCP (Figs. 3 and 4). Negative responses are less than the mean + 2 SD of the subjects receiving antigen challenge without sensitization (irritant response).

Table 3. Increased rate of tolerance to DNCB in individuals initially immunized through erythemagenic UV-exposed skin

Primary sensitization group	Frequency of primary nonresponders*	Frequency of tolerance among primary nonresponders†	Overall frequency tolerance‡
No UV	20%	36%	7%§
UV	62%	50%	31%¶

*% negative responses after initial sensitization.
†Primary nonresponders resensitized to DNCB on normal skin. Tolerance is a negative response upon rechallenge.
‡Overall frequency of tolerance is the frequency of subjects who are both primary and secondary nonresponders.
§n = 3 tolerant among 11 primary nonresponders retested among 103 subjects initially sensitized on unexposed normal left buttock skin.
¶n = 5 tolerant among 10 primary nonresponders retested from 42 subjects initially sensitized to DNCB on left buttock skin that had been exposed to erythemagenic doses of UV ($P = 0.0003$ by χ^2 test).

The distribution of responses in the 0.75 and 2MED × 4 groups was not different from that of the no UV control group, which exhibited 64% strongly positive immunizations, 9% weakly positive, and 27% negative responses ($P = 0.81$ and 0.54, respectively) (Table 2) (Fig. 3). On the other hand, a single 4MED exposure did appear to modulate the distribution of contact sensitivity responses ($P = 0.05$). The frequency of 4MED × 1 weakly positive DPCP responses was 41%, whereas only 9% of the no UV control group had weakly positive responses (Table 2). Thus, a single localized sunburn in one site may systemically alter epicutaneous immunizations at a distant, UV-protected site. However, the modest intensity of the distant effect cannot account for the profound reductions that occur by contact sensitization directly through UV-exposed skin.

Reduced Langerhans Cell Density and Induction of CD1a$^-$DR$^+$ Cells in Sunburned Epidermis. Murine experiments have demonstrated differences in the ability to contact sensitize depending on whether the mice are exposed to low-dose UV (40–50 mJ/cm^2) or high-dose UV (≥100 mJ/cm^2) (7, 8). It is not possible to easily extrapolate these exposures to humans. However, one can compare the degree of Langerhans cell depletion achieved. Whereas suberythemagenic UV exposures for 4 days resulted in slight, but statistically significant, reductions in CD1a$^+$ cells (20% decrease) ($P = 0.003$), marked reductions in Langerhans cells only occurred in skin exposed to erythemagenic UV (71% decrease in each) ($P < 0.0001$) (Fig. 4). Thus, it appears that low-dose murine UV exposures that resulted in >70% depletion of Langerhans cells (7) were more equivalent to the human 2MED and 4MED exposure regimens.

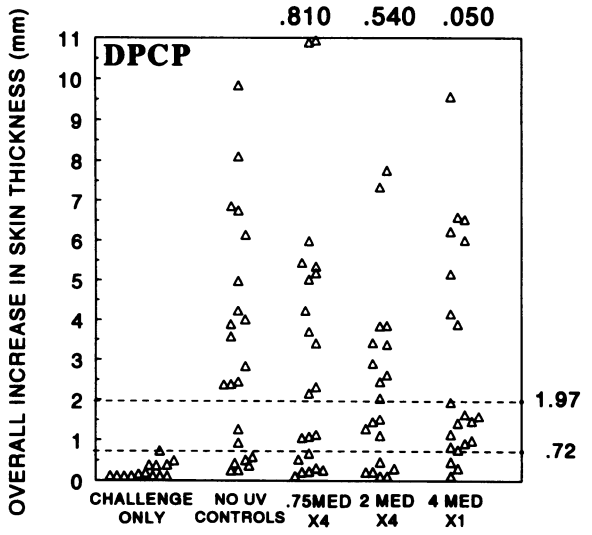

FIG. 3. Scattergram of overall individual responses to DPCP challenge by sensitization group as in Fig. 2.

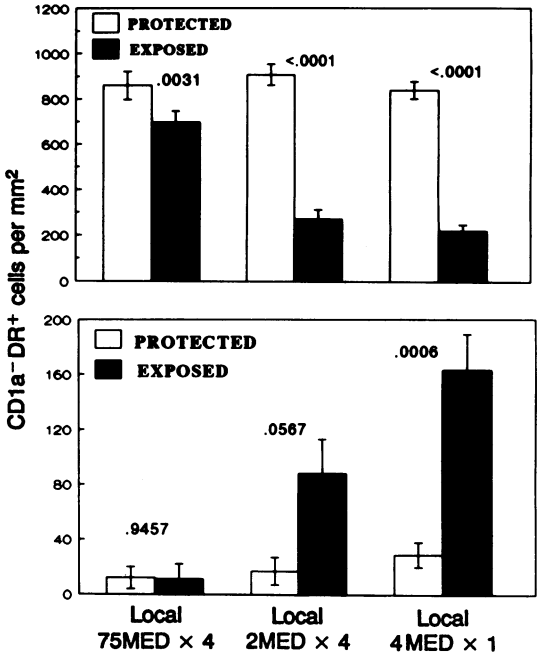

FIG. 4. (*Upper*) Decreased Langerhans cells in epidermis of UV-exposed skin. Langerhans cells were quantified in epidermal sheets as CD1a$^+$ cells per mm^2 in biopsies from the light-protected buttock (open bars) and from the UV-exposed buttock (solid bars). (*Lower*) Induction of CD1a$^-$DR$^+$ cells in epidermal sheets of volunteers subjected to erythemagenic doses of UVB. CD1a$^-$DR$^+$ cells were expressed as CD1a$^-$DR$^+$ cells per mm^2.

Langerhans cell depletion alone, however, may not totally account for UV induction of antigen-specific tolerance (36). A population of UV-induced macrophages appears in UV-exposed human (37) and murine (38) epidermis. In humans, the macrophages are responsible for preferential activation of suppressor cells (39) and in mice they are critical for tolerance induction (38). Induction of CD1a$^-$DR$^+$ epidermal cells was observed in both of the erythemagenic UV-exposure regimens ($P = 0.0006$ and 0.0567) but not with the suberythemagenic regimen ($P = 0.9457$) (Fig. 4).

DISCUSSION

Because of progressive thinning of stratospheric ozone, an ever-increasing proportion of solar energy reaching the earth's surface is composed of highly active UVB radiation. Increasing UVB flux is projected to have a major impact on skin cancer health expenditures in the near future (40). Our data demonstrating that UV exposure also has a major impact on the *in vivo* functioning of the human immune system suggest that additional expenditures may be engendered as a result of increasing immune dysfunction.

Even daily exposure to levels of UV below the erythemal threshold (0.75MED) appeared to down modulate immune responsiveness, which makes it difficult for people to gauge a "safe" level of exposure. Brief, inadvertent, midday summer or high-altitude exposures can easily deliver a 0.75MED dose. The daily 2MED erythemagenic dose of UV used here models the level of exposure received by individuals in whom a low-grade redness is evident as a result of outdoor exposure in association with school recess, work, conditioning, recreation, or cosmesis, or as a result of exposure in UVA tanning salons that are commonly equipped with UVB-contaminated bulbs. The 4MED erythemagenic dose more closely models

an acute weekend or vacation sunburn that is not severe enough to blister, but that may result in a slight peel, again a common and relevant occurrence. The ability of such a local sunburn to systemically modulate distal immune responses (Table 2) may be consonant with the work of Hersey *et al.* (33), which demonstrated suppressor cells for pokeweed mitogen-induced *in vitro* immune responsiveness in subjects exposed to "sun-baking" for 2 weeks in Australia. Of note is that the acquisition of malignant melanoma correlates with the occurrence of an acute sunburn in childhood (41); altered immune responsiveness to new peptide sequences produced by UV-mutated genes that might also function as tumor antigens (24, 42) could be expressed as tolerance rather than rejection (Table 3) (30).

The sensitizing dose of DNCB (30 μg in 48 μl) was chosen to have our data read out on the linear portion of the curve of sensitization rate vs. sensitizing dose (34). That this approach allowed detection of immunomodulation was confirmed by our finding of only 5% fully successful immunizations after four $\approx$30 mJ exposures (Tables 1 and 2). This result differs from a related study, which only demonstrated a trend toward a reduced immunization rate through skin previously exposed to four fixed doses of 144 mJ/cm^2 of UVB per day (6/10 successful immunizations) (30). A difference in sensitivity of the bioassay is likely; Yoshikawa *et al.* (30) sensitized and challenged their subjects to much higher doses of DNCB (2000 and 50 μg, respectively). Increased sensitivity of our assay may also be due to control of additional variables: MED testing to equalize optical penetration of UV between individuals, immunizing only midcycle females (35), and using a randomized study design.

What mechanisms occur in sunburned skin that might account for our findings? Acute erythemagenic UV injury to the skin is characterized by erythema and induration due to mediator release (43), endothelial ELAM-1 induction (44), leukocytic infiltration (45), keratinocyte necrosis and Langerhans cell depletion (7, 18). Infiltrating macrophages (37) preferentially activate CD4$^+$ (suppressor inducer) T lymphocytes to down regulate lymphocyte activation (39), and an analogous population plays a critical role in the induction of murine tolerance (38). In addition, UV-damaged Langerhans cells may deliver negative signals by inducing clonal anergy or preferential TH2 expansion (21). Whatever the mechanism, however, the common occurrence of UV-induced (46) failure to sensitize to DNCB in skin cancer patients (30) suggests that UV induces a relevant degree of immunologic injury in humans.

In conclusion, commonly experienced levels of UV exposure render the skin of 95% of individuals diminished in their ability to mount a form of delayed-type hypersensitivity (contact sensitivity) if the initial immunization occurs in the UV-exposed skin. Thirty percent appear particularly susceptible and become unable to generate a contact sensitivity response even after a repeated sensitization (tolerance). Increased understanding of this area may allow better planning of strategies to minimize adverse UVB effects on skin cancer, immunization programs, microbial immunity, and photosensitivity diseases.

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