Ultraviolet Radiation Decreases the Granulomatous Response to Lepromin in Humans

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Ultraviolet radiation (UVR) modulates cellular immunity in humans and experimental animals and can interfere with immune responses against infectious agents in animal models. We used the lepromin reaction, a cell-mediated immune response to antigens of *Mycobacterium leprae*, to determine whether UVR affects the cellular immune response to an infectious agent in humans. We selected 29 healthy, lepromin-positive contacts of leprosy patients and determined their minimal erythema dose (MED) of UVR. Immediately afterward, each subject was injected with 0.1 ml of lepromin in two areas of the buttocks: one at the site that had received twice the MED of UVR and the other on the contralateral, unirradiated site. The irradiated site was given twice the MED every 4 d for a total of five treatments. One week after the last irradiation, both lepromin reactions were measured and biopsied. The size of the lepromin-induced granulomas was significantly reduced in the irradiated site, as was the number of lymphocytes. Immunohistochemical analysis showed a depletion in the number of infiltrating cells and a lower percentage of T cells, particularly the $CD4^+$ subpopulation, in granulomas formed in UV-irradiated skin. This study demonstrates that local UV irradiation reduces the granulomatous reaction to lepromin in sensitized individuals. These findings are of clinical relevance because of the fundamental role played by the delayed-type hypersensitivity response in defense against intracellular pathogens and because of potential increases in the amount of UVR in sunlight reaching the earth's surface. *Key words: photoimmunology/delayed-type hypersensitivity/Mycobacterium leprae/leprosy/tuberculoid granuloma. J Invest Dermatol 105:8–13, 1995*

In addition to its barrier and thermoregulatory functions, the skin contains populations of immune cells and acts as a peripheral immunologic organ [1]. A cutaneous immune surveillance system is probably vital for resistance against invasion by infectious agents. The influence of sunlight on human skin was noted almost 50 years ago in the pioneering studies of solar urticaria [2] and photoallergy [3]. Clinical observation has documented that sunlight can trigger certain skin diseases, such as lupus erythematosus, porphyria cutanea tarda, and polymorphic light eruption [4]. More recently, ultraviolet radiation (UVR), particularly wavelengths in the UVB (280–320 nm) range, has been shown in experimental animals to interfere with the development of cellular immune responses. Both contact and delayed-type hypersensitivity (DTH) responses to antigens applied locally, within UV-irradiated skin and at distant sites, are impaired by UVB irradiation [5–7]. In humans, UVR suppresses the induction of contact hypersensitivity (CHS) reactions [8–10] and alters the proportions of circulating blood leukocytes [9,11,12]. Inhibition of the CHS response can be achieved with suberythemal doses of UVR [10]. However, it is not known whether immunosuppression by UVR can increase the incidence or severity of infections in humans.

Manuscript received October 19, 1994; final revision received March 9, 1995; accepted for publication March 27, 1995.

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Abbreviation: CHS, contact hypersensitivity.

The DTH response is a fundamental immune defense mechanism against certain infections, and one of its manifestations in humans is the granuloma reaction [13,14]. The granulomatous response limits or suppresses infections and is considered to be an expression of individual immunologic reactivity [13,15]. A good example of an intracellular granuloma-producing infectious agent in humans is the leprosy bacillus [16]. The pathogenesis of leprosy is highly dependent on the immunologic status of the host, and susceptibility to the disease is genetically determined [17]. The majority of the population is immunologically resistant to leprosy, and immunocompetence is expressed by a positive DTH reaction (lepromin skin test or Mitsuda reaction) in individuals previously exposed to mycobacteria [16]. The lepromin reaction is used routinely to evaluate contacts of leprosy patients for the intensity of their cell-mediated immune reactivity against *M. leprae* [18].

We were interested in studying the effects of UVR on the human immune response. A particularly important objective is to determine whether UV irradiation can decrease immunity to infectious agents, thereby increasing the incidence or severity of human diseases. Because ethical considerations preclude studies to assess the effects of experimental UV irradiation on the pathogenesis of infectious diseases in humans, we examined the effect of UV irradiation on the local DTH response and granuloma formation elicited by lepromin in the skin of immune subjects.

MATERIALS AND METHODS

Subjects Healthy adult Caucasians (16 women, 13 men), 18–62 years old, residing in the state of Rio Grande do Sul, Brazil, participated in this

0022-202X/95/$09.50 • SSDI0022-202X(95)00208-3

study. None were currently taking any medication, and all were bacillus Calmette-Guérin (BCG)-vaccinated, lepromin-positive reactors and household contacts of lepromatous leprosy patients. They were informed about the objectives and requirements of the investigation, and all gave informed consent to participate.

Irradiation Device UVR was administered with a Psora-Comb Dermalight 80 (Dr. K. Hönle GmbH, Munich, Germany) equipped with two fluorescent bulbs that emit a continuous spectrum with a peak emission at 290 nm and an irradiance of 250 W/m^2 UVB (280–320 nm) and 130 W/m^2 UVA (320–400 nm).

Lepromin We used a suspension of heat-killed *M. leprae* bacilli at a concentration of 1.6×10^7/ml, obtained from human tissues (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil).

Experimental Design Before the experiment, the minimal erythema dose (MED) was determined as follows. Five non–sun-exposed areas of 1.0 cm^2 of the left buttock were irradiated at a distance of 2.5 cm with doses between 150 and 450 mJ/cm^2 of UVB radiation. The MED was considered to be the smallest dose giving a distinct erythema with sharp margins 24 h after irradiation. Immediately after the MED was determined, 0.1 ml of lepromin was injected intradermally into the area that had been given twice the MED on the left buttock and in an unirradiated site of the right buttock. The injected area of the left buttock and an adjacent normal skin site (control) were subsequently irradiated with twice the MED every 4 d for a total of five irradiations.

The diameters of the papules formed at both sites of lepromin injection were measured 24–28 d later, a time considered adequate for granuloma formation. Six-millimeter punch biopsy specimens of both injection sites were taken 7 d after the last irradiation. In addition, for some individuals, we obtained specimens of unirradiated normal skin and, from others, irradiated normal skin to evaluate the effects of UV alone.

Histopathology The biopsy samples were sectioned in two parts: one was formalin fixed and paraffin embedded, and the other was embedded in OCT compound (Miles Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at −70°C for immunohistochemical studies. The samples were coded before being processed and analyzed.

Antibodies Mouse anti-CD1a (cortical thymocytes, fetal B cells, Langerhans cells), −CD3 (pan T cells), −CD4 (helper/inducer T cells), −CD8 (suppressor/cytotoxic T cells), −CD22 (pan B cells), −CD68 (macrophages, monocytes), and Dako Universal rabbit peroxidase anti-peroxidase kit system 40 were all purchased from Dako Corp. (Carpinteria, CA). Rabbit anti-BCG (B124) was obtained from Dako A/S, Denmark; gold-labeled goat anti-mouse IgG Auroprobe was purchased from Amersham Corp. (Arlington Heights, IL).

Analysis of Paraffin Sections Sections were routinely processed and stained with hematoxylin and eosin and Ziehl-Neelsen. At least five sections per sample were analyzed and read blindly by two of the authors (TFC and LB). The following parameters were evaluated: area of the granuloma, number of lymphocytes surrounding the granuloma, number of lymphocytes inside the granuloma, presence of epithelioid giant cells, presence of necrosis, and presence of acid-fast bacilli.

The area of the granuloma was measured using an image analysis system consisting of a BH-2 Research Microscope (Olympus Optical Co. Ltd., Tokyo, Japan), a TEC-470 CCD video camera (Optronics Engineering, Goleta, CA), and a GEM 486DX/33mHz 16 RAM computer with a 220-megabyte hard drive and a Digital color printer (Sony Corp., Tokyo, Japan). The image analysis software was Bioscan Optimas (Bioscan Inc., Edmond, WA); calculations were made using Microsoft Excel (Microsoft Corp., Redmond, WA); the image editor was Picture Publisher (Micrografx Inc., Richardson, TX).

Each section was examined under a 2× objective, and the area of the infiltrate was circumscribed electronically. The resultant area was expressed in mm^2. Each sample was measured at least five times, and the average and SD of these measurements were calculated. The numbers of lymphocytes around the granuloma and inside the granuloma were scored as follows: 0 = absence of lymphocytes; + = minimal number of lymphocytes (representing less than 25% of the cells in each 10× field); ++ = moderate number (25% to 50% lymphocytes); and +++ = maximum number (more than 50% of the cells were lymphocytes).

The presence of acid-fast bacilli was detected by Ziehl-Neelsen staining and immunohistochemistry, and the number of bacilli was counted according to the index of Ridley and Hilson [19]. Immunohistochemical detection of mycobacterial antigens was performed using a peroxidase anti-peroxidase technique according to the manufacturer's instructions (Dako Universal rabbit peroxidase anti-peroxidase kit system 40). Positive controls were biopsy samples from lepromatous leprosy patients. Negative controls consisted of serial sections incubated with normal swine serum instead of anti-BCG antibody.

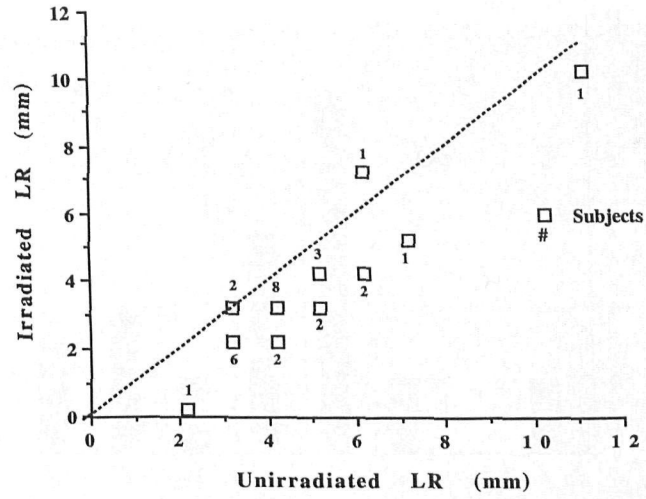

Figure 1. Lepromin reactions (LR) are smaller in UV-irradiated skin than in unirradiated skin of the same subject. *Squares* represent lesion diameters obtained from the same healthy volunteer. Adjacent *numbers* indicate the number of subjects with similar measurements. *Dotted line* corresponds to the theoretic distribution of a null hypothesis ($p = 0.0157$, analysis of variance).

Immunohistochemistry of Frozen Sections Cryostat sections of 8 μm were air-dried and fixed sequentially in cold acetone, acetone:chloroform mixture (1:1), and acetone for 5 min each. The sections were rinsed with phosphate-buffered saline, and adjacent sections were stained with the panel of mouse anti-human monoclonal antibodies listed previously.

Immunogold labeling was carried out as described previously [20]. To maximize contrast in the image analysis, no counterstain was used. Negative controls consisted of substituting normal horse serum (Vector Laboratories, Inc., Burlingame, CA) for the primary antibody. Optimal dilutions of antibodies were used, and positive controls were performed on sections of lymph nodes and unirradiated lepromin reactions.

It was not feasible to count all positively stained cells in the granulomas; therefore, we developed a reproducible and precise method for estimating the number of cells present in the granulomas. Because the antibodies used for immunostaining all detected membrane markers, which delimited the boundaries of the positively stained cells, we were able to use image analysis to determine the percent positive reactivity per field [21]. Preliminary experiments demonstrated that in this system, the color intensity closely correlates with the number of cells present in the microscopic field. For these studies, we preselected significantly cellular areas of the granulomas under 100× magnification to be sure that a difference in granuloma size would not bias the evaluation of the cellular distribution in the granulomas. A minimum of eight fields, representing at least 30% of the area of the granuloma, were measured for each section, and the mean ± SEM of the measurements was obtained. Because of differences in staining intensity, a black and white threshold was set for each antibody.

Statistical Analyses Two-way analysis of variance and Kruskall-Wallis nonparametric tests were used to assess the significance of differences in clinical and histologic results. The χ^2 test was also used where appropriate. A paired Student t test was used for immunohistochemical measurements. In all cases, $p \leq 0.05$ was considered statistically significant.

RESULTS

Lepromin Reactions Are Smaller in UV-Irradiated Skin Most of the 29 volunteers who participated in the study had skin type 3 or 4 (19 of 29), and the most frequent twice-MED doses were 190 mJ/cm^2 (seven subjects) and 270 mJ/cm^2 (five subjects). **Figure 1** summarizes the results of the size measurements of the lepromin reactions in unirradiated and UV-irradiated sites. In this figure, the diameter of the lepromin reaction in the UV-irradiated skin is plotted against that in unirradiated skin of the same individual. Reactions more than 4 mm in diameter occurred

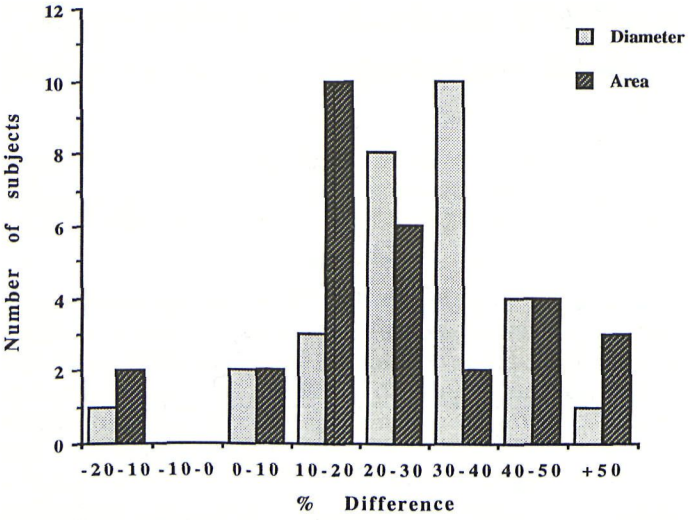

Figure 2. Maximum diameter and area of lepromin reactions are at least 20% larger in unirradiated than in UV-irradiated skin. Area measurements were obtained by image analysis of paraffin sections bisecting the granuloma. Percent difference was calculated using the following formula: % difference = 100 − (UV × 100)/(non-UV). One subject showed a larger diameter and two showed larger areas in the UV-irradiated site.

infrequently in the UV-irradiated skin (eight of 29) but were common (20 of 29) in unirradiated sites ($p < 0.01$, χ^2 test). The lepromin reaction in individual subjects was larger in the unirradiated site than in the UV-treated skin in 27 of 29 cases. In these subjects, the lepromin reaction was at least 20% larger in the unirradiated skin than in the UV-irradiated site **(Fig 2)**. Thus, UV irradiation decreased the size of the lepromin reaction, as determined by gross measurement of the skin test reaction. However, UV irradiation had no effect on the incidence of ulceration. There was no detectable relation between size of the lepromin reaction and skin type or between size of the reaction and MED, suggesting

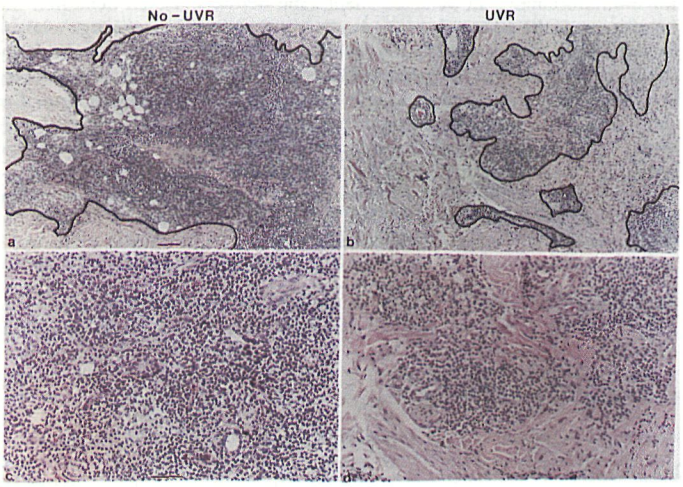

Figure 3. Lepromin reactions in unirradiated skin are larger and contain more lymphocytes than those in UV-irradiated skin. Hematoxylin and eosin–stained paraffin sections of granulomas from unirradiated (*a,c*) and UV-irradiated (*b,d*) skin. *a,b*) Low magnifications showing the area occupied by the granuloma. Areas were measured within the *solid lines*. Area = 10.1 mm^2 (*a*) and 5.4 mm^2 (*b*). *Bar*, 100 μm. *c,d*) High magnifications showing the predominance of lymphocytes in granulomas from unirradiated skin. *Bar*, 50 μm.

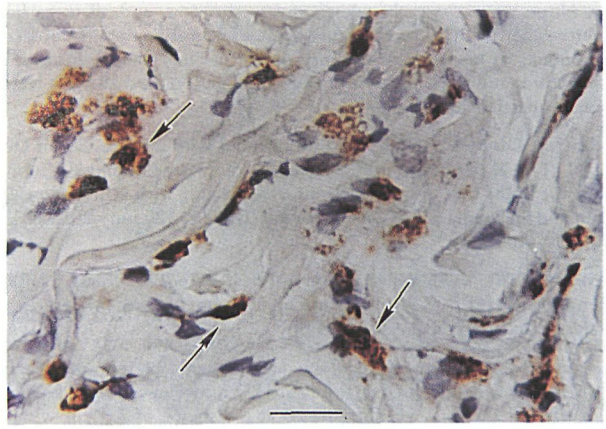

Figure 4. *M. leprae* antigens are present inside macrophages. Anti-BCG antibody was used for immunoperoxidase staining of paraffin sections; positive reactivity appears as brown granules. *Bar*, 20 μm.

that the decreased lepromin reaction was unrelated to skin pigmentation or sunburn sensitivity of the skin.

In addition to the gross measurements, we calculated the area occupied by the granuloma microscopically using image analysis of paraffin sections **(Fig 3)**. The mean area (± SEM) of the granulomas induced in the unirradiated skin was 5.39 ± 0.44 mm^2, whereas that in the UV-irradiated sites was 3.99 ± 0.38 mm^2. In 17 of 27 individuals, this difference in size of the two reactions was at least 20% larger in the unirradiated site **(Fig 2)**.

UV Irradiation Alters the Cellular Composition of Lepromin Reactions All specimens showed a well-defined dermal tuberculoid granuloma, characterized by epithelioid cells and lymphocytes concentrated around skin appendages **(Fig 3)**. Some of the reactions were ulcerated, but ulceration did not correlate with granuloma size. There was also no correlation between size and the presence of giant cells or acid-fast bacilli. As expected, most bacilli were degraded; intact or fragmented organisms were found in only a few samples. However, 93% of the reaction sites reacted positively with anti-BCG antibody, confirming the presence of mycobacterial antigens **(Fig 4)**. Reaction sites in UV-irradiated or unirradiated skin did not differ with respect to the presence or quantity of mycobacterial antigen.

The most striking difference between the granulomas produced in UV-irradiated and unirradiated skin was the intensity of the lymphocytic infiltrate **(Table I)**. Both inside and around the granulomas, more lymphocytes were present in the unirradiated sites. The difference was most apparent for lymphocytes surrounding the granulomas: in UV-irradiated skin, the majority of the samples received a score of 1+ (86%), whereas the majority (65%) of those from unirradiated sites received a score of 2+ or more ($p = 0.001$, analysis of variance). Comparing the reaction sites within

Table I. Fewer Lymphocytes Are Present in Granulomas Induced in UV-Irradiated Skin

Location of Lymphocytes[a]	Number of Subjects With Score of: 0	1+	2+	3+	p[b]
Surrounding granuloma					
No UVR	0	10	16	3	0.004
UVR	2	23	4	0	
Inside granuloma					
No UVR	0	10	17	2	0.043
UVR	2	16	9	2	

[a] Determined by microscopic evaluation of at least five sections from each sample.
[b] Kruskal-Wallis test.

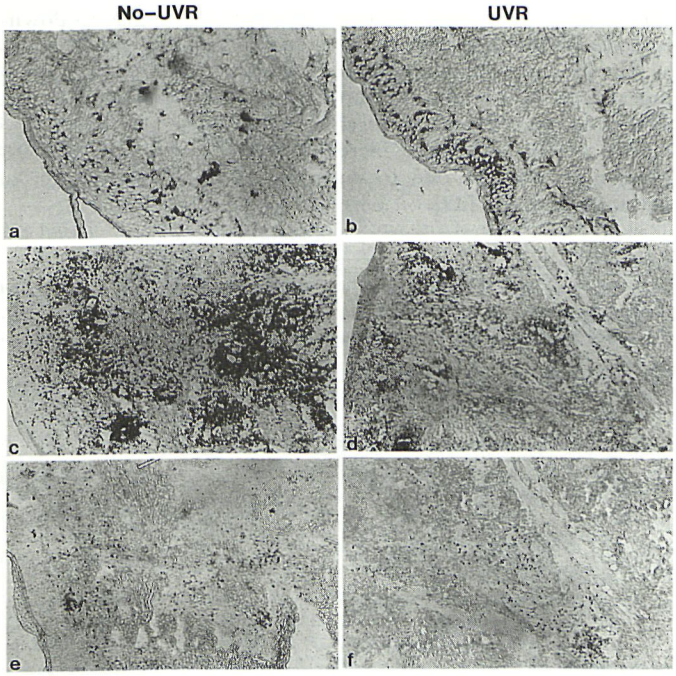

Figure 5. More CD1a$^+$ cells and fewer CD3$^+$ cells are present in granulomas in UV-irradiated skin. Immunogold staining of frozen sections of granulomas from lepromin reactions in unirradiated (*a,c,e*) and UV-irradiated (*b,d,f*) skin, showing immunoreactivity of infiltrating cells to CD1a (*a,b*), CD3 (*c,d*), and CD22 (*e,f*) antibodies. *Bar*, 70 μm.

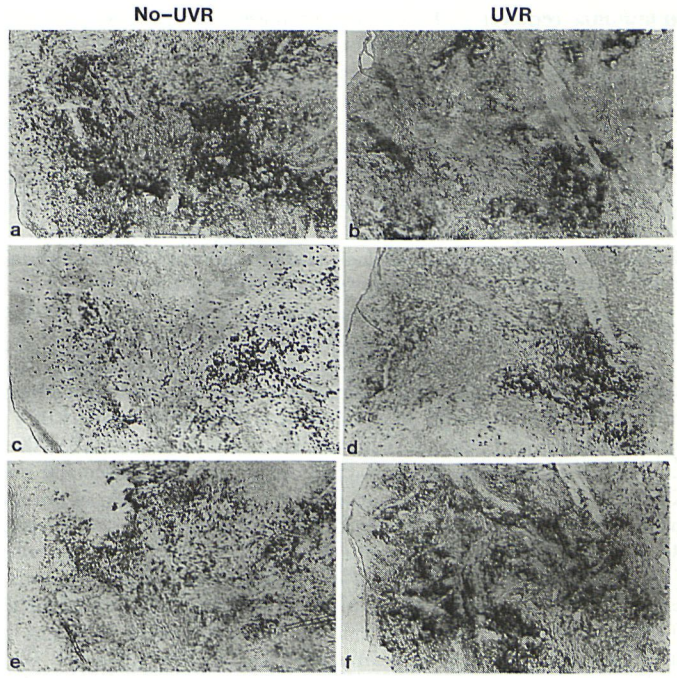

Figure 6. More CD68$^+$ cells and fewer CD4$^+$ cells are present in granulomas in UV-irradiated skin. Immunogold staining of frozen sections of granulomas from lepromin reactions in unirradiated (*a,c,e*) and UV-irradiated (*b,d,f*) skin, showing immunoreactivity of infiltrating cells to CD4 (*a,b*), CD8 (*c,d*), and CD68 (*e,f*) antibodies. *Bar*, 70 μm.

the same individual, 65% of the cases showed fewer lymphocytes in the UV-irradiated site than in the unirradiated site. There also seemed to be fewer basophilic cells in the UV-irradiated sites, but no attempt was made to quantitate this difference.

T Cells Are Decreased in UV-Irradiated Granulomas We next characterized the hematopoietic mononuclear cells in the granulomas using monoclonal antibodies against surface markers present on various types of hematopoietic cells. All samples showed the morphology and cellular distribution characteristic of the lepromin reaction **(Figs 5,6)**. Using image analysis, it was possible to determine the extent of antibody labeling by calculating the percentage of the field exhibiting a positive reaction. There was a significantly decreased number of T lymphocytes in the granulomas produced in the UV-irradiated site (14.8 ± 0.8 in UV-irradiated skin versus 23.4 ± 1.4 in unirradiated skin; **Table II**). Most T lymphocytes in the granulomas were CD4$^+$. The numbers of CD1a$^+$ Langerhans cells and CD68$^+$ macrophages were increased in the UV-irradiated sites. A small but significant increase in the number of these cells was also observed in UV-irradiated skin in the absence of lepromin injection, compared with that in normal skin.

DISCUSSION

Decreases in the concentration of stratospheric ozone are increasing the proportion of UVB radiation in sunlight reaching the earth's surface. This increase in UVB flux is projected to have a major impact on human health by increasing the incidence of skin cancer and ocular damage [22]. Of growing concern is the possibility that increased UVB might also decrease cell-mediated immunity, especially to infectious diseases. Doses of UVB sufficient to suppress the induction of CHS in humans can be easily achieved during normal outdoor activities in the tropics [10]. Furthermore, in contrast to the situation with nonmelanoma skin cancer, melanin does not protect against local immune suppression induced by UVR.

Vermeer *et al* [23] found that the proportion of individuals susceptible to suppression of the CHS reaction by a low dose of UVR was independent of skin pigmentation. This finding indicates that the population at risk for immunosuppressive effects of UVR is much larger than that at risk of developing nonmelanoma skin cancer.

For ethical reasons, it is difficult to study the effects of experimental UV irradiation on the pathogenesis of infectious diseases in humans; nevertheless, the induction and elicitation of CHS and the elicitation of DTH to recall antigens can be used as tests of cell-mediated immune responses. In the present study, we investigated the effects of UVR on the cell-mediated immune response to a recall antigen in human volunteers who were known to be lepromin positive. We chose to evaluate the formation of granulomas in response to lepromin. Granuloma formation is important in the management of infections produced by intracellular microorganisms, and this process may be altered by many components of the microenvironment, such as cytokines, reactive oxygen and nitrogen intermediates from macrophages and other cells, antigen-antibody complexes, complement, or toxic products released by dead bacilli [14]. The full development of a hypersensitivity

Table II. UV Irradiation Alters the Cellular Composition of the Lepromin Reaction[a]

Antibody	Unirradiated Granulomas[b]	UVR Granulomas[b]	Normal Skin[c]	UV-Irradiated Skin[c]
CD1a	1.5 ± 0.1	2.9 ± 0.2[d]	0.7 ± 0.3	1.0 ± 0.2[d]
CD3	23.4 ± 1.4	14.8 ± 0.8[d]	<0.1	<0.1
CD4	23.0 ± 1.3	17.4 ± 0.8[d]	<0.1	<0.1
CD8	5.4 ± 0.6	6.7 ± 0.6	<0.1	<0.1
CD22	0.9 ± 0.1	0.5 ± 0.1	<0.1	<0.1
CD68	21.2 ± 1.0	28.7 ± 1.8[d]	0.5 ± 0.1	0.9 ± 1.1[d]

[a] Percentage of field with positive reactivity to various antibodies in the lepromin reaction and in normal skin determined by image analysis.
[b] Mean of 23 samples ± SEM.
[c] Mean of eight samples ± SEM.
[d] $p < 0.001$ *versus* unirradiated controls, paired Student t test.

granuloma requires a T-lymphocyte–mediated response and the continuous recruitment of monocytes and their differentiation into epithelioid cells [24]. There is an autoamplification process maintained by tumor necrosis factor-α. This process is indispensable for the containment and elimination of bacteria, as well as for the persistence of a well-defined granuloma. The lepromin reaction is characterized histologically by a highly organized tuberculoid granuloma, composed of a central core of mature macrophages and an infiltrate of predominantly $CD4^+$ T lymphocytes [16,25–27]. Other diseases such as tuberculosis and sarcoidosis elicit the formation of granulomas with similar characteristics [28].

In our study, we found that exposure to UVR decreased the lepromin reaction in sun-protected skin areas, which rules out any contribution from chronic solar damage as a prerequisite for the effect of UV on elicitation of DTH. Moreover, the results described here show that although the granulomas that developed in UV-irradiated skin had characteristics qualitatively similar to those that developed in unirradiated skin, the number of cells, especially the $CD4^+$ T lymphocytes, was markedly decreased in the granulomas produced in UV-irradiated skin. Because this T-helper subset plays a critical role in the induction phase of the granulomatous response [28], our results suggest that UV irradiation can have a detrimental effect on the number and possibly the function of this critical T-lymphocyte population. In contrast, macrophages were found in increased numbers in granulomas produced in UV-irradiated skin and in UV-irradiated skin in the absence of lepromin injection. This increment in the number of macrophages therefore seems to be a response to UV irradiation. It would be interesting to determine whether these macrophages represent the antigen-presenting cells in the epidermis reported to activate a T-cell subset with inhibitory function [8,29]. It is possible that the increased number of macrophages present in skin exposed to UV irradiation simply constitutes part of an inflammatory response to UV. Clearly, additional phenotypic and functional studies are required to determine the role of these macrophages in the immune response of UV-irradiated skin and to assess their microbicidal activity.

An unexpected result was our finding of an increase in the number of $CD1a^+$ Langerhans cells in UV-irradiated skin regardless of exposure to lepromin. Previous studies have reported a decreased number of these cells in skin exposed to acute UV irradiation [10], chronic sun exposure [30], and therapeutic regimens [31]. It is important to note that Langerhans cells start to reappear at 5 d and reach normal numbers by 2 weeks after UV irradiation [31]. Because the skin specimens in our study were obtained 7 d after the last UV irradiation, it is possible that the increased number of Langerhans cells observed may reflect the period of recovery of these cells after exposure to UV. It is interesting that relatively high numbers of Langerhans cells in lepromin reactions [16] and other infectious granulomas [32] have been reported, and it is possible that both UV irradiation and the response to lepromin may account for the increased numbers of Langerhans cells that we observed in UV-irradiated, lepromin-injected sites. The role of Langerhans cells in the formation and function of the granuloma has not yet been elucidated; consequently, it is not possible at present to determine whether this regimen of UV irradiation alters the function of these cells in human skin.

Previous studies conducted in experimental animals have demonstrated that UV irradiation can decrease the induction of CHS to haptens applied locally [5] and the induction and elicitation of DTH to alloantigens [33–35], leishmania [36], herpes simplex virus [37], BCG [38], *M. lepraemurium* [39], and *Candida albicans* [40]. Moreover, the pathogenesis of systemic infections with leishmania [36], *M. bovis*, [37], *M. lepraemurium* [39], and *C. albicans* [41] is significantly altered by UVR. Our results provide further evidence for the *in vivo* immunosuppressive effects of UV irradiation and extend it to the granulomatous response to a recall antigen in humans. Consistent with our results, it was recently reported that UV irradiation decreased the formation of hypersensitivity granulomas in mice [42].

In summary, our results demonstrate that UV irradiation downregulates the immune response to microbial antigens and decreases the formation of granulomas in human skin. These findings are of clinical relevance because of the fundamental role played by the DTH response in defense against intracellular pathogens and because of the anticipated increases in the proportion of UVB radiation in sunlight due to decreases in the concentration of stratospheric ozone [22].

We thank Drs. Absalom Filgueira, Amminikutty Jeevan, and Kevin D. Cooper for helpful discussions on the design and implementation of this study; Dr. Ronald Palacios for his help in reviewing the manuscript; Ricardo Sanchez and Donna Reynolds for technical assistance; and Kenneth Dunner, Jr., for the photographic work.

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