

312-nanometer Ultraviolet B Light (Narrow-Band UVB) Induces Apoptosis of T Cells within Psoriatic Lesions

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Summary

Narrow-band (312 nm) ultraviolet B light (UVB) is a new form of therapy for psoriasis, but its mechanism of action is unknown. In a bilateral comparison clinical study, daily exposure of psoriatic plaques to broad-band UVB (290–320 nm) or 312-nm UVB depleted T cells from the epidermis and dermis of psoriatic lesions. However, 312-nm UVB was significantly more depleting in both tissue compartments. To characterize the mechanism of T cell depletion, assays for T cell apoptosis were performed on T cells derived from UVB-irradiated skin in vivo and on T cells irradiated in vitro with 312-nm UVB. Apoptosis was induced in T cells exposed to 50–100 mJ/cm² of 312-nm UVB in vitro, as measured by increased binding of fluorescein isothiocyanate (FITC)–Annexin V to CD3⁺ cells and by characteristic cell size/granularity changes measured by cytometry. In vivo exposure of psoriatic skin lesions to 312-nm UVB for 1–2 wk also induced apoptosis in T cells as assessed by the terminal deoxynucleotidyl transferase–mediated dUTP–biotin nick end labeling (TUNEL) reaction in tissue sections, by binding of FITC–Annexin V to CD3⁺ T cells contained in epidermal cell suspensions, and by detection of apoptosis-related size shifts of CD3⁺ cells. Induction of T cell apoptosis could be the main mechanism by which 312-nm UVB resolves psoriasis skin lesions.

Key words: psoriasis • immunosuppression • T lymphocyte • apoptosis • ultraviolet light

Psoriasis vulgaris is a chronic inflammatory skin disease characterized by infiltration of T cells and hyperproliferation of keratinocytes in focal skin areas. In clinical studies, disease-related pathology is reversed by lymphocyte-targeted drugs (1–6), whereas in xenotransplant systems, psoriasis is induced or sustained by intradermal injection of activated T cells (7, 8). Therefore, psoriasis is mediated by activated T cells. In the xenotransplant model, an epidermal hyperplasia response appears to arise from cytokines derived from T cells infiltrating into skin (7). These T cells are probably reacting to a cutaneous antigen, as clonal CD8⁺ T cell populations have been identified (9) and as systemic administration of the costimulation blocker, CTLA4Ig, reverses clinical and pathological disease features (6). Hence, skin infiltration by activated cutaneous lymphocyte–associated antigen–positive T cells appears to cause a complex inflammatory tissue phenotype that includes (a) the presence of several types of activated leukocytes in skin lesions—CD4⁺ and CD8⁺ T cells, neutrophils, and dendritic antigen presenting cells, (b) a diverse array of cytokines produced by activated leukocytes and keratinocytes, (c) proliferation of small blood vessels and epidermal keratinocytes, and (d) in-

creased expression of leukocyte–trafficking adhesion molecules, e.g., intercellular adhesion molecule 1 (ICAM-1), P-selectin, E-selectin, by hyperproliferative endothelial cells or keratinocytes (10).

Ultraviolet B light (UVB, 290–320 nm) is used as a therapeutic modality for psoriasis and other inflammatory skin disorders. Recently, a new UVB source which emits mostly 311/312-nm light (narrow band [NB]-UVB)¹ has been introduced for treatment of psoriasis and other inflammatory dermatoses (11–14). UVB irradiation in a variety of animal and human model systems inhibits cutaneous delayed-type hypersensitivity responses to haptens (15, 16), and its therapeutic mechanism in psoriasis has been attributed to these immunosuppressive properties. Interestingly, irradiation of psoriatic skin lesions with standard UVB light

¹Abbreviations used in this paper: AnV, Annexin V; APC, allophycocyanin; BB, broad band; FSC, forward scatter; NB, narrow band; PerCP, peridinin chlorophyll protein; PI, propidium iodide; SSC, side scatter; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP–biotin nick end labeling.

causes rapid depletion of intraepidermal T cells (17) and induction of Fas ligand on keratinocytes (18). As apoptosis is induced by *in vitro* UVB irradiation of T cells, or by incubation of T cells with keratinocytes expressing UVB-induced Fas ligand, it has been proposed that UVB may have immunosuppressive effects in psoriasis through induction of apoptosis in disease-mediating T cells (17, 18). Although cytotoxic effects of UVB on lymphocytes *in vivo* are unproven, irradiation of atopic dermatitis skin lesions with high-dose UVA (the delivered dose is ~1,000-fold higher than for therapeutic UVB) has produced detectable apoptosis in dermal lymphocytes (19). We now report that 311/312-nm UVB is directly cytotoxic for T cells *in vivo* in psoriatic skin lesions. Furthermore, this new UVB source depletes T cells to a greater extent than broad-band (BB)-UVB from both epidermis and dermis of affected skin.

Materials and Methods

Reagents. The following antibodies were used by FACSCalibur[®] analysis (Becton Dickinson): peridinin chlorophyll protein (PerCP)- or allophycocyanin (APC)-anti-CD3, PE-anti-CD4, FITC-anti-CD8 (Becton Dickinson), and isotype mouse IgG mAbs.

NB-UVB Treatment. 23 adult patients (16 men and 7 women) received irradiation with NB-UVB (312 nm, Philips TL01) on one vertical half of the body and BB-UVB (290–320 nm, FST72T12) on the other vertical half. Five additional patients received NB-UVB on the whole body. The minimum erythema dose for each patient was established before irradiation. On each subsequent day, NB- and BB-UVB irradiations were increased by 15% unless marked erythema developed. Treatment was terminated upon attaining clinical resolution of psoriatic lesions, typically after 4–5 wk of daily treatment (20).

Histopathological Analysis. Skin punch biopsies were taken from psoriatic lesions of both NB- and BB-UVB-treated sites before and after UVB treatment. Biopsies were frozen in OCT solution (Miles Diagnostic Division) for histological analysis. Unconjugated anti-CD3 mAb was applied to cryosections for 60 min at room temperature. After washing with PBS, bound antibody was visualized by the avidin-biotin complex detection system (Vectastain ABC; Vector Laboratories, Inc.) with 3-amino-9-ethylcarbazole as the chromagen. For double staining, alkaline phosphatase substrate (Vector Laboratories, Inc.) was also used. Number of CD3⁺ cells was counted on the image analyzer using NIH image public domain software. Detection of apoptosis in tissue sections was performed by the TUNEL (TdT-mediated dUTP-biotin nick end labeling) reaction as described (17). TdT (Amersham Pharmacia Biotech) and biotin-16-dUTP (Boehringer Mannheim) are used for this technique.

Psoriatic Epidermal Cell Suspensions. The shave biopsies were taken from eleven patients immediately before the start of treatment and after 1 and 2 wk of treatment with NB-UVB. The tissue was washed twice with sterile PBS, incubated in PBS with 1 mg/ml gentamicin (Life Technologies) for 1 h at 4°C, washed twice in PBS, and floated in 0.5% dispase (Sigma Chemical Co.) overnight at 4°C. The epidermis was removed and teased into a cell suspension after brief trypsinization.

NB-UVB Irradiation *In Vitro*. Narrow-band fluorescent UVB lamps (TL01) were used as a light source. PBMCs were prepared from heparinized venous blood of healthy volunteers by Ficoll

sedimentation and then irradiated in uncovered tissue culture plates (10⁶ cells/well) in PBS. After irradiation, they were suspended in RPMI 1640 with 5% heat-inactivated normal human serum (C-six Diagnostics, Inc.) with antibiotics.

Flow Cytometric Detection of T Cell Apoptosis. T cell apoptosis was detected using FITC-Annexin V (AnV) staining in conjunction with a membrane integrity probe (propidium iodide [PI]). *In vitro*-irradiated PBMCs, or epidermal cell suspensions obtained from NB-UVB-treated psoriatic lesions, were stained with APC-anti-CD3 mAb and FITC-AnV (Kamiya Biomedical Co.). After washing, the cells were stained with PI, and fluorescence intensity was measured by a four-color FACSCalibur[®].

Flow Cytometric Phenotyping of T Cells in Psoriatic Lesions. Psoriatic epidermal cell suspensions were stained with selected mAbs. After washing with PBS/0.1% sodium azide/2% FBS, cells were fixed with PBS/3.75% formaldehyde and analyzed by flow cytometry within 1 wk. Flow cytometric analysis was done using CellQuest software (Becton Dickinson).

Results

NB-UVB Depletes T Cells From Psoriatic Lesions More than BB-UVB. Psoriatic plaques in 23 patients were treated daily with NB-UVB or BB-UVB in a bilateral comparison study (20). The ability of NB-UVB or BB-UVB to deplete T cells from psoriatic lesions was studied with CD3 antibodies and quantitative image analysis using immunohistochemistry (Fig. 1 A). Both forms of UVB reduced intraepidermal T cells from lesional skin, but quantitative reductions were greater with NB-UVB (96 vs. 85% reduction, $P = 0.01$; Fig. 1 B). Reductions in dermal CD3⁺ cells were also greater with NB-UVB (54 vs. 29% reduction, $P < 0.01$; Fig. 1, A and B). Flow cytometric analysis confirmed that rapid decreases in the number of CD3⁺, CD4⁺, and CD8⁺ T cell subsets in psoriatic epidermis occur during the first 2 wk of treatment (Fig. 1 C).

T Cell Apoptosis Is Induced by NB-UVB Irradiation. To establish the mechanism of T cell depletion from psoriatic lesions by irradiation, we examined NB-UVB-treated lymphocytes for DNA fragmentation and membrane changes that typify apoptosis. TUNEL reactions showed more apoptotic cells in NB-UVB-treated psoriatic lesions compared with contralateral lesions treated with BB-UVB (Fig. 2). To confirm that apoptotic cells detected in psoriatic tissue after irradiation were T cells, we performed double staining (CD3 and TUNEL). Fig. 2 (bottom) shows a psoriatic lesion taken after 2 wk of daily NB-UVB treatment. TUNEL⁺ cells stain red, and CD3⁺ cells stain blue. Double-stained cells (indicated with arrows) were observed in the treated skin, whereas no double-positives were noted before treatment.

As detection of apoptotic cells in psoriatic epidermis is problematic using the current TUNEL method (21), we used flow cytometry to examine isolated intraepidermal CD3⁺ lymphocytes for (a) increased binding of AnV, a protein which binds to phosphatidyl serine that is expressed on T cell plasma membranes in early stages of apoptosis; and (b) forward scatter (FSC) and side scatter (SSC) alterations that typify cytoplasmic shrinkage and organelle compaction in T cells undergoing apoptosis. As a control for

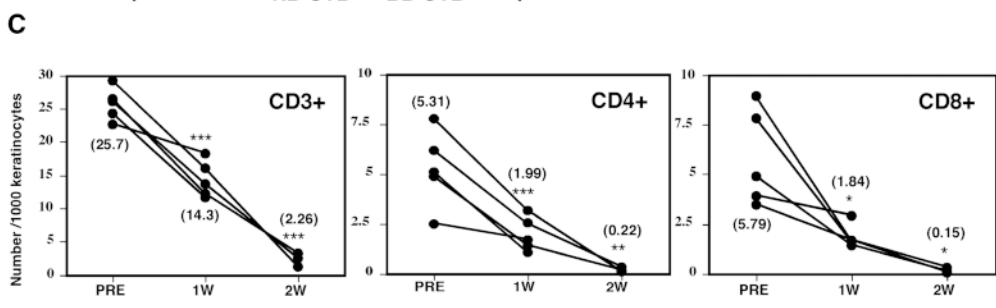
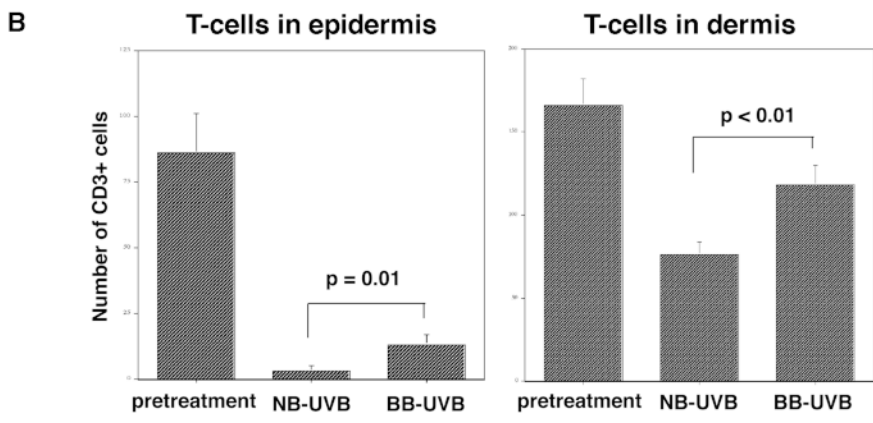
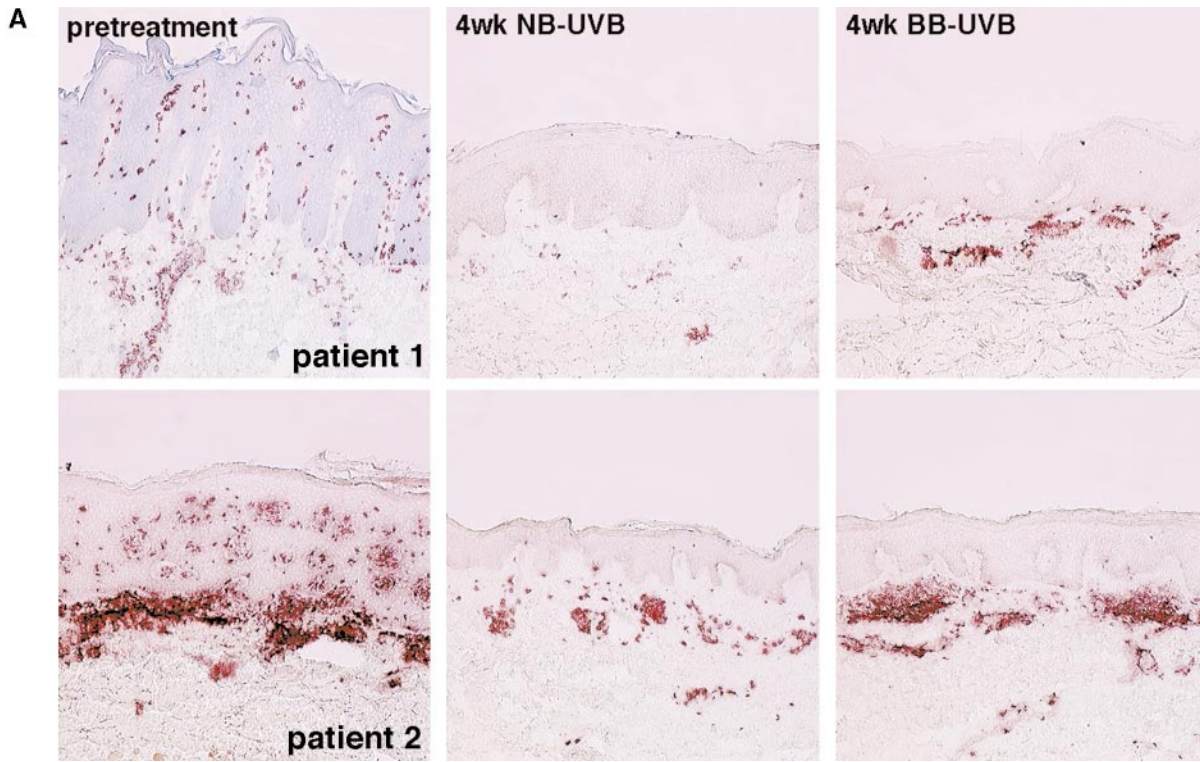


Figure 1. (A) Immunohistochemical detection of T cells in psoriatic lesional skin from two patients before and after 4 wk of UVB treatment. Left, untreated psoriatic lesions; middle, 4-wk NB-UVB-treated lesions; right, 4-wk BB-UVB-treated lesions (original magnification: $\times 100$). (B) Quantitative analysis of T cell infiltration in psoriatic skin. Panels show mean numbers of CD3⁺ cells in the epidermis and dermis of psoriatic lesional skin before and after treatment. $n = 23$. SE is shown for each mean value. Cell numbers are expressed per image analysis field. (C) Phenotypic characterization of T cells infiltrating psoriatic epidermis before and after NB-UVB treatment. Flow cytometric analysis was performed on epidermal suspensions from five psoriatic patients for phenotyping of intraepidermal T cells. Panels display the number of T cells (and also CD4 or CD8 subset) in psoriatic epidermis during treatment. The numbers are normalized to per 1,000 keratinocytes. Mean percentage is shown in parentheses. Paired t test was done. P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

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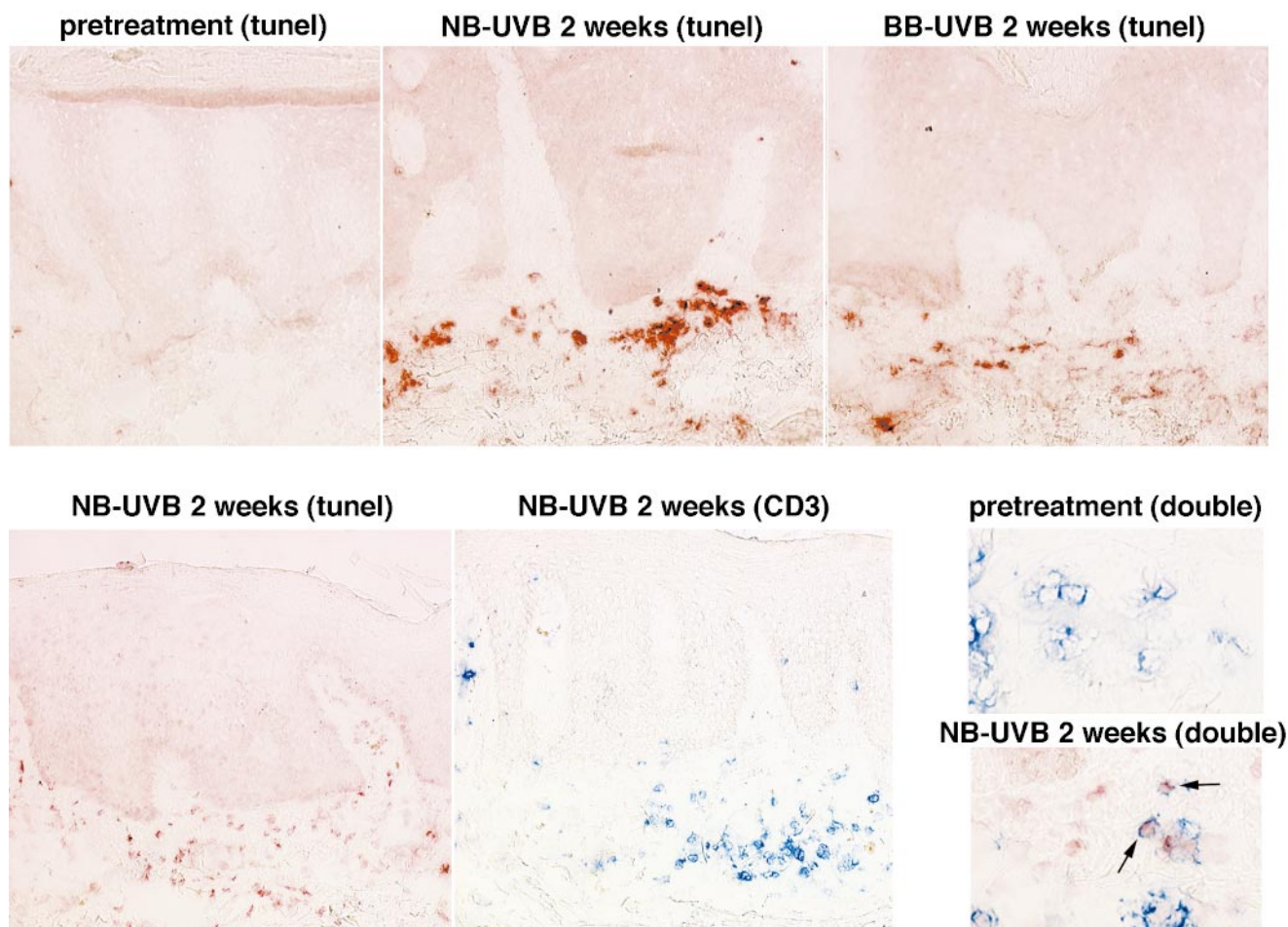


Figure 2. TUNEL reactions to sections of psoriatic skin during UVB treatment. Top: left, before treatment; middle, after 2 wk of NB-UVB treatment; right, after 2 wk of BB-UVB treatment (original magnification: $\times 100$). Bottom: double (CD3 and TUNEL) staining on sections of psoriatic skin from another patient after 2 wk of NB-UVB treatment. TUNEL⁺ cells stain red (left, original magnification: $\times 100$); CD3⁺ cells stain blue (middle, original magnification: $\times 100$); double-positive cells are indicated with arrows (original magnification: $\times 200$).

these experiments, PBMCs were irradiated with increasing amounts of NB-UVB (Fig. 3 A). Dual measurement of AnV binding and membrane integrity with PI makes it possible to distinguish early (AnV⁺/PI⁻) and late stages of apoptosis (AnV⁺/PI⁺). Hence, a flow cytometer with two lasers and four-color detection was used to simultaneously assess CD3-APC, AnV-FITC, and PI staining (PE/PerCP channels) on individual cells. As shown in Fig. 3 A, dose- and time-dependent increases in binding of AnV to T cells occurred after exposure of cells to NB-UVB ranging from 25 to 100 mJ/cm². Cells progressed from an early stage of apoptosis (AnV⁺/PI⁻) to a late stage of apoptosis (AnV⁺/PI⁺) during 20 h after irradiation. Analysis of FSC and SSC in CD3⁺ cells showed dose-dependent increases in a size-shifted population (blue). Cells in this size-shifted population are displayed as blue dots in Fig. 3 A, which indicates that size-shifted cells are mostly AnV⁺/PI⁺, or late-stage apoptotic cells.

Fig. 3 B shows analysis of T cell death in lesional epidermis from a psoriatic patient after irradiation with NB-UVB in vivo. After 2 wk of daily treatment of NB-UVB, 45% of

intraepidermal T cells were AnV⁺ vs. 14% in unirradiated psoriatic epidermis (panels for patient 1). Cells in the early stage of apoptosis (AnV⁺/PI⁻) were increased by fourfold in this patient. The mean value of four patients is listed in Table I. As shown in panels for patient 2, we also tracked cytotoxic effects of NB-UVB on intraepidermal T cells of 11 psoriatic patients using flow cytometry with ethidium homodimer (a vital fluorescent dye) and by examining T cells for cell shrinkage (reduced FSC signal) and increased granularity (increased SSC signal), changes that occur in apoptosis (22). Using ethidium homodimer staining, increased T cell death was detected in 10 out of 11 patients, and the mean value was $19.6 \pm 3.25\%$ before treatment and $30.3 \pm 4.10\%$ after treatment (listed in Table I). Using FSC and SSC analysis, the percentage of apoptotic T cells in 11 patients was $35.7 \pm 12.2\%$ before treatment and $56.3 \pm 18.5\%$ after treatment (paired *t* test; $P < 0.001$, Table I). Hence, three independent assays (AnV binding, membrane integrity, and cell morphology assays) demonstrate cytotoxic effects of NB-UVB on a significant fraction of intraepidermal T cells.

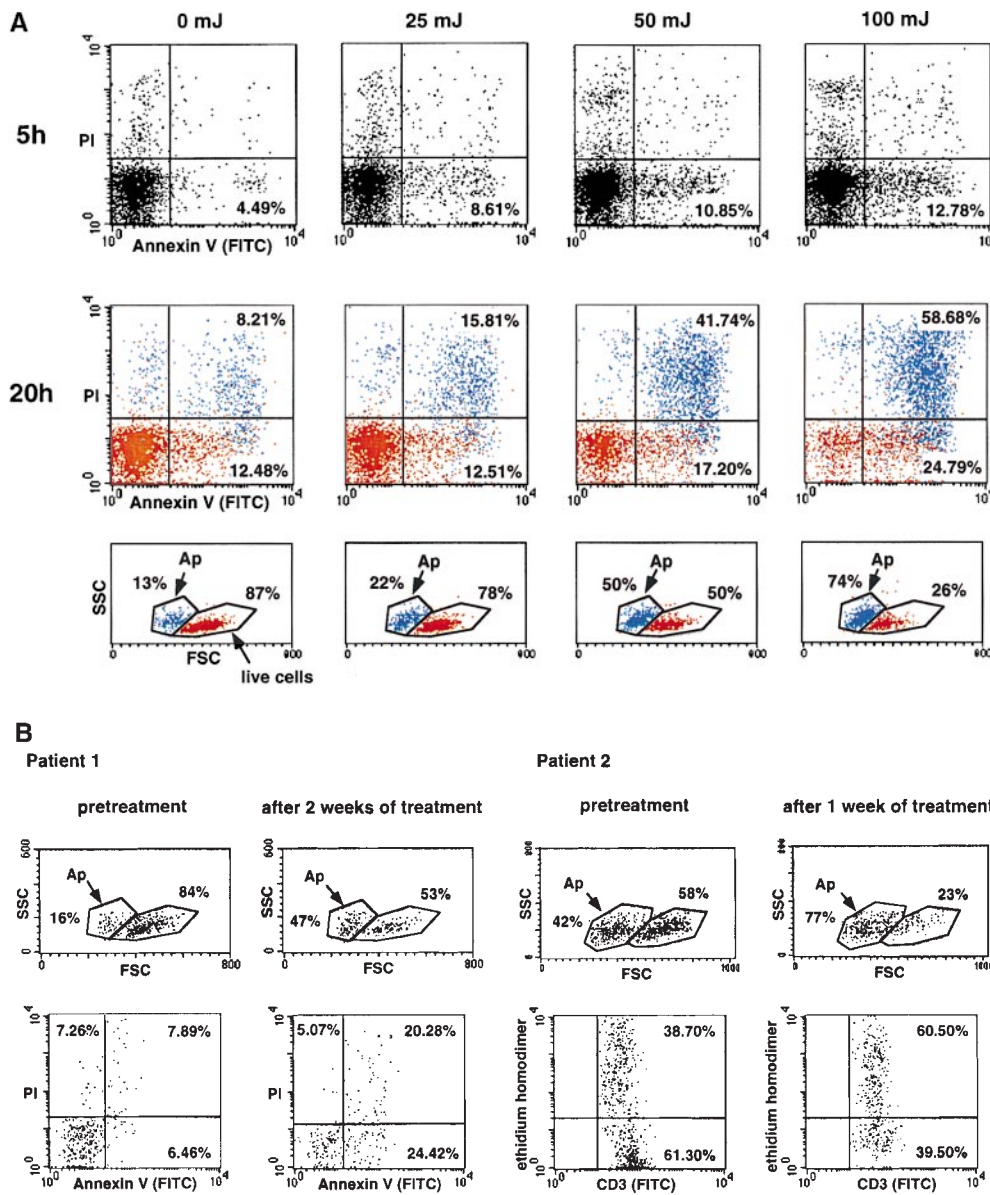


Figure 3. Flow cytometric assessment of T cell death induced by NB-UVB irradiation. All plots show only CD3⁺ T cells as identified by FITC-CD3 or APC-CD3 staining. (A) In vitro irradiation of T cells. Peripheral blood T cells were irradiated with increasing amounts of NB-UVB, and apoptosis was assessed at either 5 or 20 h after irradiation. The top panels show simultaneous FITC-AnV and PI staining on the ordinate and abscissa (AnV⁺/PI⁻). The quadrants of these plots indicate live cells (AnV⁻/PI⁻), early-stage apoptosis (AnV⁺/PI⁻), or late-stage apoptosis (AnV⁺/PI⁺). Results show dose- and time-dependent increases in AnV⁺/PI⁻ and AnV⁺/PI⁺ cell populations. The bottom panels are plots of FSC (cell size) and SSC (granularity) for CD3⁺ cells that are illustrated in AnV/PI plots of 20-h cells. Large CD3⁺ cells with minimal granularity are colored red and are predominantly live cells as shown in the panel above (the same cell population is colored red in the AnV/PI plot). Smaller CD3⁺ cells with increased granularity are colored blue. This population is apoptotic cells (Ap) as shown for blue-colored cells in the AnV/PI plots above. (B) In vivo irradiation of epidermal T cells. T cell apoptosis was assessed in epidermal cells obtained from psoriatic skin. Plots illustrate only CD3⁺ cells in the epidermis. The panels of patient 1 quantify apoptosis in intraepidermal T cells before treatment or after 2 wk of daily irradiation with NB-UVB using the assays shown in A. The plots for patient 2 show ethidium homodimer uptake as an alternative measure of cell death (ethidium homodimer⁺ cells are apoptotic and correspond to size-shifted [Ap] cells in FSC/SSC plots for this patient).

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Discussion

Paradoxically, the UVB spectrum (290–320 nm) causes inflammation in irradiated skin (the sunburn reaction), whereas exposure of inflamed skin to similar amounts of UV energy resolves a number of immunologically mediated dermatoses. An action spectrum study in patients with psoriasis established that wavelengths from 290 to 300 nm produced the sunburn reaction (intense erythema and keratinocyte necrosis) but had no therapeutic benefits, whereas 313-nm UVB showed mostly therapeutic, but minimally erythemogenic potential (23). Based on this action spectrum, a fluorescent UVB source was developed for psoriasis

therapy that delivers 311/312-nm (“narrow-band”) UVB (11). NB-UVB has been shown to be superior for clearing psoriasis and other inflammatory disorders compared with BB-UVB sources that emit over most of the UVB spectrum (11, 12, 24). Although BB-UVB produces a wide variety of cellular and immunosuppressive effects in skin (15, 25–29), it has been suggested that the therapeutic effectiveness in inflammatory skin disorders could be mediated by the cytotoxic effects of UVB on infiltrating lymphocytes (17). However, the only direct evidence that UV light can produce lymphocyte apoptosis in human skin comes from a

Table I. Measurement of Intraepidermal T Cell Apoptosis in Psoriatic Skin Treated with NB-UVB by Three Different Flow Cytometry-based Assays

Measurement in CD3 ⁺ cells*	No. of patients	Pretreatment	Posttreatment
		%	%
Apoptotic shift (FSC/SSC)	11	35.7 ± 12.2	56.3 ± 18.5 [‡]
FITC-AnV binding	4	10.8 ± 4.17	46.7 ± 14.8 [§]
Ethidium homodimer uptake	11	19.6 ± 3.25	30.3 ± 4.10 [§]

*All assays were performed with trypsinized epidermal cell suspensions. An electronic gate was set on T cells labeled with FITC-CD3 (for ethidium homodimer experiments) or APC-CD3 (for FITC-AnV experiments). The apoptotic shift indicates cell shrinkage and increased cytoplasmic complexity measured by FSC/SSC as illustrated in Fig. 3. The table lists mean values for each parameter ± SD. P values: [‡]P < 0.001, [§]P < 0.025.

study of high-dose UVA (320–400 nm) used to treat patients with atopic dermatitis (19).

Surprisingly, immune-modulating effects of 311–312-nm UVB have not been studied previously in psoriatic skin or skin lesions of other inflammatory diseases. The results of this study show that NB-UVB causes greater depletion of T cells in psoriatic tissue than BB-UVB, and establish direct cytotoxic actions of UVB on T cells infiltrating skin lesions. The *in vivo* results are paralleled by *in vitro* experiments where exposure of T cells to moderate doses of 312-nm UVB light induced rapid apoptosis. In fact, given that ~10% of UVB energy incident on skin penetrates the epidermis, there is reasonably good agreement between doses of 312-nm UVB required to kill T cells (50–100 mJ/cm²) and therapeutic amounts of 312-nm UVB delivered to psoriatic lesions (300–1,200 mJ/cm²). The greater effectiveness of 312-nm UVB in depletion of dermal T cells is probably related to (a) somewhat deeper penetration of this wave-

length in dermis compared with the composite of wavelengths present in BB-UVB sources (30, 31), and (b) the ability to deliver more Joules of UVB energy with NB-UVB sources due to a reduction in its burning potential.

These investigations do not define biochemical pathways mediating the cytotoxic or immunosuppressive effects of NB-UVB. Previous studies with BB-UVB have suggested that apoptosis of epidermal T cells might be induced by interaction of Fas on T cells and upregulated Fas ligand on irradiated keratinocytes (18). Although this mechanism could also pertain to 312-nm UVB, rapid apoptosis was produced in isolated leukocytes by direct irradiation *in vitro* with relatively small amounts of UVB (17, 32). Potential molecular targets of UVB in T cells include p53, which is upregulated or stabilized after UVB exposure in some cells and can potentially mediate apoptosis in the setting of UVB-induced DNA damage (33, 34), or calmodulin-dependent protein kinase II, which is activated after UVB exposure and leads to activation of AP24, 24-kD apoptotic protease (35, 36). In addition, low-dose UVB can inactivate signal transducer and activator of transcription 1 (STAT-1), a critical component of signal transduction, which then inhibits gene transcription (37, 38).

In most cases, direct irradiation of normal skin with erythemogenic amounts of UVB prevents delayed-type hypersensitivity reactions to topical sensitizers. This altered immune reactivity has been attributed to UVB-induced depletion of Langerhans cells from epidermis (39). In a prior study, therapeutic amounts of BB-UVB did not appreciably reduce the abundance of CD1a⁺ epidermal cells in lesional psoriatic skin (17). We detected no obvious reductions in CD1a⁺ cells after irradiation of psoriatic lesions with NB-UVB (data not shown). Hence, depletion of T cells from psoriatic lesions appears to be selective for a restricted set of leukocytes. Accordingly, our studies suggest that the major therapeutic mechanism of UVB light in inflammatory dermatoses is a cytotoxic effect on skin-infiltrating T cells, where the mechanism of death is most likely through apoptosis.

We thank Ms. Henrietta Carbonaro for help with immunohistochemical staining, Ms. Joan Hofmann for manuscript preparation, and Dr. Ian B. Walters and Ms. Patricia Gilleaudeau for clinical assistance.

This research was supported in part by a General Clinical Research Center Grant (M01-RR00102) from the National Center for Research Resources at the National Institutes of Health; by National Institutes of Health grant AI39214; and by grants or gifts from The Carl J. Herzog Foundation, the American Skin Association, The Carson Family Charitable Trust, Dr. James Murphy, and Jean Stein. L.H. Burack and T.R. Coven were supported as Rockefeller University Clinical Scholars from May 1996 through June 1997 and March 1997 through June 1998, respectively.

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Received for publication 15 September 1998 and in revised form 11 December 1998.

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