ANALYSIS OF THE MECHANISM OF UNRESPONSIVENESS PRODUCED BY HAPTENS PAINTED ON SKIN EXPOSED TO LOW DOSE ULTRAVIOLET RADIATION*

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It has been acknowledged for decades that ultraviolet B (UVB)[1] (290–320 nm) radiation may have adverse effects on normal cutaneous processes. Acutely, UVB exposure produces cutaneous erythema and edema (sunburn); chronically, it leads to premature cutaneous aging and to the induction of skin cancer. Recently, UVB radiation has been recognized to alter normal immunologic processes (1). Kripke et al. (2) showed that large doses of UVB delivered chronically to mice will not only induce cutaneous neoplasms but will also create a receptive immunologic milieu in which grafts of such UVB-induced neoplasms will grow. Analysis of the immunologic defects that follow high dose UVB exposure has revealed two features that may be relevant: the generation of suppressor T lymphocytes (3, 4) and the failure of Ia positive spleen cells to present antigens to T lymphocytes (5–7). Extrapolation of these findings from mice to man has been difficult because the large UVB doses employed, 180 kJ/m^2, are as much as 400 times the minimal phototoxic UVB dose (MPD) in untanned Caucasian skin. By contrast, Toews et al. (8) have shown that local in vivo administration of a relatively low UVB dose (0.2 kJ/m^2, corresponding to 1 human MPD daily) for four consecutive days also produced an altered immune response in C57BL/6 mice. When the contact-sensitizing agent DNFB was applied to the UVB-irradiated sites (but not to unirradiated sites) antigen-specific unresponsiveness rather than sensitization ensued. The mechanism(s) by which low doses of UVB radiation produce this unresponsiveness to contact sensitizers is unknown. In this study we show that the same dose of UVB radiation that produces a local alteration in antigen-presenting function of skin also permits suppressor T cells to emerge when hapten is placed on the irradiated site. We propose that these suppressor T cells are responsible at least in part for the hapten-specific unresponsive state.

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[1] *Abbreviations used in this paper:* DNBS, dinitrofluorobenzene sulfate; DNFB, 2,4-dinitro-1-fluorobenzene; ETAF, epidermal cell-derived thymocyte-activating factor; FUdR, 5-flourodeoxyuridine; IUdR, I^{125}-iododeoxyuridine; i.v., intravenous; UVA, ultraviolet A; UVB, ultraviolet B.

Materials and Methods

Animals. Female C3H mice were obtained from Jackson Laboratories (Bar Harbor, ME), Cumberland View Farms (Clinton, TN), or Charles River Breeding Laboratories (Wilmington, MA). They were used for experiments when between 2 and 4 mo of age.

Reagents. 2, 4 dinitro-1-fluorobenzene (DNFB) and 4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone) were purchased from Sigma Chemical Co. (St. Louis, MO). 5-fluorodeoxyuridine (FUdR), I^{125}-iododeoxyuridine (IUdR), and monoclonal anti-Thy-1.2 antibody were obtained from New England Nuclear (Boston, MA). Arsanilate-conjugated monoclonal anti-Lyt-1 and anti-Lyt-2 antibodies, and rabbit anti-arsanilate antibody were obtained from Becton-Dickinson (Sunnyvale, CA). Low toxicity rabbit complement was purchased from Cedarlane Laboratories (Hornby, Ontario). Monoclonal anti-arsanilate antibody was a gift of Dr. J. Donald Capra (Dept. Microbiology, U.T.H.S.C., Dallas, Texas).

Assessment of Contact Hypersensitivity. Induction and elicitation of contact hypersensitivity was performed according to the method of Phanuphak et al. (9). Briefly, mice were immunized to hapten by the epicutaneous application of 25 μl of 0.5% DNFB in an acetone/olive oil (4:1) solution to the razor-shaved abdominal wall skin on days 0 and 1. Reactions were elicited on day 5 by challenging one ear of each mouse with 20 μl of 0.2% DNFB. The increment in ear swelling was used as a measure of the development of contact hypersensitivity. Ear thickness was measured with an engineer's micrometer 1, 2, and 3 d (days 6, 7, and 8) after challenge and compared to the ear thickness just before the challenge.

The induction and elicitation of contact hypersensitivity to oxazolone was accomplished in the same manner as with DNFB with the exception that mice were sensitized with 50 μl of 5% oxazolone (in 4:1 acetone/olive oil) and ear challenged with 20 μl of 1% oxazolone solution.

Induction of Unresponsiveness to Hapten. Mice were made unresponsive to DNFB using the method of Toews et al. (8). Following sedation with chloral hydrate, animals were restrained in the supine position on a wooden platter. The razor-shaved abdominal skin was exposed to UV radiation from a bank of four FS-20 fluorescent lamps with a tube to target distance of 46 cm. These bulbs have an emission spectrum primarily in the UVB range (10). Irradiance ranged from 0.11 to 0.14 mW/cm^2 as measured by an IL700 UVB research radiometer with an SEE 240 UVB photodetector. Mice were exposed to UVB daily for 4 consecutive days (days −3 to 0). Immediately following the final exposure and 24 h later (days 0 and 1) 25 $1 of 0.5% DNFB was placed within the irradiated site.

In some experiments, mice were made unresponsive to DNFB by the intravenous inoculation of 750 mg/kg body weight of dinitrofluorobenzene sulfate (DNBS) (9).

Adoptive Transfer of Unresponsiveness. Mice were sacrificed 7 d after either the epicutaneous application of DNFB on UVB irradiated skin or the intravenous administration of DNBS. Spleens and lymph nodes (axillary, brachial, inguinal, cervical, and mesenteric) were removed and single cell suspensions prepared from (*a*) lymph nodes by gentle squeezing through a wire mesh screen and (*b*) from spleen by mincing with forceps. One hundred million viable cells (50% lymph node, 50% spleen) were then inoculated into the tail vein of naive syngeneic recipients that had received 240R x-irradiation from a cesium source (gamma cell 40, Atomic Energy of Canada, Ltd.) 2–4 h earlier (11). Recipient mice were then painted with an immunizing dose of DNFB on the abdomen on days 0 and 1, ear challenged on day 5, and measured for ear swelling on days 6, 7, and 8. The percent suppression was calculated according to the formula:

$$\% \text{ suppression} = \frac{\text{ear swelling (positive control)} - \text{ear swelling (experimental)}}{\text{ear swelling (positive control)} - \text{ear swelling (negative control)}} \times 100\%.$$

Co-transfer of Immune Cells and Unresponsive Cells. Immune lymph node cells were obtained from donors immunized by the epicutaneous application of 0.5% DNFB (25 μl to central body wall, 5 μl to each forepaw) on days −4 and −3. On day 0, the mice were

sacrificed and cell suspensions were prepared from their peripheral lymph nodes (cervical, axillary, brachial, and femoral). Thirty million immune peripheral lymph node cells were then inoculated intravenously along with 100×10^6 spleen and lymph node cells from UVB irradiated and hapten skin-painted (unresponsive) mice into naive syngeneic recipients who had received 240R x-irradiation. Recipient mice were ear challenged 1 h after adoptive transfer. The increment in ear swelling was measured over the succeeding 3 d.

Antibody Treatment of Cell Suspensions. In certain experiments, spleen and lymph node cell suspensions were depleted of T lymphocytes by pretreatment with antibody and complement before injection into recipient mice. Spleen and lymph node cells at a concentration of 5×10^7 cells/ml (in RPMI 1640 [Gibco Laboratories, Grand Island, NY] containing 2% fetal calf serum) were incubated with monoclonal anti-Thy-1.2 antibody (1 μl/ml) at 4°C for 30 min. The cell suspensions were then washed and incubated at a concentration of 1×10^7 cells/ml with a 1:10 dilution of rabbit complement at 37°C for 45 min. Treatment of the cell suspensions in this manner completely abrogated their proliferation response to Con A, but had no effect on their response to LPS (data not shown).

Depletion of Lyt-1+ and/or Lyt-2+ lymphocytes was accomplished by incubating identical members of cells (10^7/ml) with either arsanilate-conjugated anti-Lyt-1 or anti-Lyt-2 monoclonal antibody (8.3 μl/ml) for 1 h at 4°C. A third aliquot of cells was incubated with normal mouse serum under similar conditions. After washing the suspensions once, cells were resuspended in RPMI-FCS at 10^7/ml and incubated with rabbit anti-arsanilate antibody (11 μg/ml) for 1 h at 4°C. The cells were again washed, and then incubated at 2×10^7/ml for 1 h at 37°C with a 1:10 dilution of rabbit complement.

Incorporation of IUDR Into Regional Lymph Nodes. Panels of mice were skin painted with an immunizing dose of DNFB on days 0 and 1. On day 4, each mouse received 5×10^{-8} moles of FUdR intraperitoneally followed 10 minutes later by 2 μCi of ^{125}I-labeled IUdR. Draining inguinal, axillary, and brachial lymph nodes were taken 2 h later. After three 24-h washings in 70% alcohol, IUdR incorporation in the nodes was measured using a gamma spectrometer (12).

Results

Effect of UVB Radiation on Induction of Contact Hypersensitivity in C3H Mice. We have previously reported that local, acute, low dose UVB irradiation of abdominal wall skin of C57BL/6 mice alters the immune capabilities of these animals such that specific unresponsiveness, rather than sensitization, is produced (8). A similar set of experiments was conducted in C3H mice. Panels of mice were exposed to 0.7 kJ/m^2 daily for four consecutive days, skin painted with DNFB, and then challenged 4 d later. As the data in Table I reveal, UVB-treated animals had only minimal ear swelling responses. 14 d after initial skin painting with hapten, the dorsal body wall skin of these animals was painted with an immunizing dose of DNFB, which was repeated 24 h later. Appropriate positive and negative control panels were included. When the ears of these mice were challenged with DNFB 4 d later (19 d after first application of DNFB), the panel originally treated with UVB failed to respond. This result indicates that C3H mice resemble C57BL/6 mice in their susceptibility to the effects of UVB on the induction of contact hypersensitivity. Epicutaneous application of hapten to UVB-treated skin produces a state of profound unresponsiveness.

Adoptive Transfer of Unresponsiveness by Spleen and Lymph Node Cells. Both suppressor cell and nonsuppressor cell mechanisms have been identified as possible means by which unresponsiveness to contact-sensitizing agents can be produced and maintained (13). In order to determine whether suppressor cells develop following in vivo low dose UVB radiation and DNFB skin painting,

TABLE I

Unresponsiveness to DNFB Follows Low Dose UVB and Hapten Skin Painting in C3H Mice

Group	UV radiation	DNFB immunization	Increment in ear swelling ($\times 10^{-4}$ inches ± SEM)	Suppression
				%
I	−	+	22.5 ± 2.2	
II	+	+	12.2 ± 1.1	79
III	−	−	9.4 ± 1.2	

The shaved abdominal skin of C3H mice was exposed to 700 J/m^2 ultraviolet radiation daily for 4 consecutive days. Immediately after the last exposure and 24 h later 25 μl of 0.5% DNFB was applied epicutaneously to the irradiated site. 5 d after the initial application of hapten mice were ear challenged with 20 μl of 0.2% DNFB. The mean change in ear thickness was measured over the ensuing 72 h. Increment in ear swelling represents peak response recorded.

TABLE II

Unresponsiveness That Follows Low Dose UVB and Hapten Skin Painting Can Be Adoptively Transferred

Group	Tolerization procedure of donor mice	Cells transferred	DNFB sensitization	Increment in ear swelling ($\times 10^{-4}$ inches ± SEM)	Suppression
					%
I		None	+	36.1 ± 3.6	
II	UVB + DNFB	+	+	16.6 ± 4.6	70
III	IV DNBS	+	+	18.4 ± 0.7	64
IV		None	−	8.4 ± 2.7	

Recipient naive syngeneic mice received 100 × 10^6 spleen and lymph node cells from donor mice made unresponsive by low dose UV and hapten skin painting (group II) or by prior i.v. injection of DNBS (group III). Recipient mice were then immunized by two daily applications of 25 μl of 0.5% DNFB and ear challenged 4 d later with 20 μl of 0.2% DNFB. Comparison was made to positive controls who received no cells but were sensitized and ear challenged (group I) and negative controls who were ear challenged only (group IV). Responses were measured as described in Table I.

adoptive transfer experiments were undertaken. 6 d after the second skin painting of irradiated abdominal skin, donor mice were sacrificed and cell suspensions prepared from their spleens and lymph nodes. One hundred million of these spleen and lymph node cells were injected intravenously (i.v.) into normal syngeneic recipients, which were then skin painted with DNFB and challenged 4 d later. The increment in ear swelling for this experimental group was compared with that of (*a*) mice that received no cells but were skin painted and ear challenged (positive controls), (*b*) mice that were ear challenged only (negative controls), and (*c*) mice that received cells from donors made unresponsive to DNFB by the prior (i.v.) injections of DNBS. Table II shows the results of a representative experiment. Mice that received spleen and lymph node cells from donors made unresponsive to DNFB by in vivo low dose UVB and DNFB skin painting were 70% suppressed compared with positive controls. This amount of suppression was similar to that observed in recipients of cells from donors

made unresponsive by the i.v. injections of DNBS. Furthermore, panels of mice that received cells either from untreated donors or from donors exposed to UVB but not skin painted, responded in a manner identical to positive controls (data not shown). From these experiments, we conclude that the unresponsiveness to DNFB produced by low dose in vivo UV can be adoptively transferred, indicating that suppressor cells are generated by this regimen.

Action Spectrum for the Generation of Suppressor Cells. The FS-20 fluorescent sunlamps used in these studies have an emission spectrum primarily in the UVB range; however, these bulbs also emit a significant amount of UVA radiation (10). To determine whether radiation in the UVB range was required for the production of suppressor cells, a window glass filter was interposed between the fluorescent tubes and the mice during UV exposure. Donor mice were exposed to UV from the light source with the filter in place and were compared with donors exposed to unfiltered UV. This glass filter removed >97% of radiation in the UVB range and 33% of radiation within the UVA range. To compensate for diminished UVA exposure, times for mice irradiated through the filter were increased 33% to deliver UVA dose equivalent to that administered without the filter. With the exception of this increased irradiation time, the adoptive transfer protocol was identical to that described above. As shown in Table III, panels of mice that received cells from donors painted with DNFB on irradiated skin in which the UVB radiation had been removed by the window glass filter developed normal contact hypersensitivity responses. By contrast, mice that received cells from donors who were irradiated with unfiltered UV were again suppressed. Thus, wavelengths of UV radiation required for the generation of suppressor cells lie within the UVB range.

Phenotypic Analysis of Suppressor Cells. Because macrophages (14), B lymphocytes (15, 16), and T lymphocytes (11, 17) have all been implicated as suppressor cells in contact hypersensitivity, we next examined the cell type responsible for the suppression observed when DNFB is applied to low dose UVB-irradiated skin sites. Donor mice were made unresponsive to DNFB by the typical in vivo low dose UVB and hapten skin-painting protocol. They were sacrificed 7 d later and spleen and lymph node cell suspensions were prepared. Aliquots of this cell

TABLE III

Ultraviolet Radiation in the UVB Range is Required for the Generation of Suppressor Cells

Group	Treatment of donors of cell suspensions	DNFB immunization	Increment in ear swelling ($\times 10^{-4}$ inches ± SEM)	Suppression
				%
I		+	26.4 ± 7.2	
II	Filtered UV + DNFB	+	28.1 ± 3.3	None
III	Unfiltered UV + DNFB	+	11.7 ± 2.8	85
IV		−	9.2 ± 1.7	

Recipient mice received cells from donors exposed to UV radiation filtered by window glass (group II) or to UV radiation without the filter (group III). Groups I and IV received no cells. Responses were measured as described for Table I.

suspension were then treated either with anti-Thy-1.2 monoclonal antibody and complement or with the irrelevant monoclonal anti-arsanilate antibody and complement as a control. Groups of mice were injected i.v. with either of these treated cell suspensions immediately before epicutaneous application of DNFB. 4 d later, these mice as well as unsensitized controls were challenged with DNFB. As demonstrated in Table IV, treatment of cell suspensions with anti-Thy-1.2 plus complement abrogated the adoptive transfer of suppression, whereas treatment with anti-arsanilate plus complement had no effect. Thus, the cells that mediate adoptive transfer of the suppression produced by low-dose UVB and DNFB skin painting are T lymphocytes.

Subpopulations of suppressor T lymphocytes may express the Lyt-1+ phenotype, the Lyt-2+ phenotype, or both (18). In an attempt to determine the Lyt phenotype of the T lymphocytes that mediate suppression following low dose UVB irradiation and DNFB skin painting, depletion experiments with anti-Lyt-1 and anti-Lyt-2 monoclonal antibodies were performed. Before spleen and lymph node cell suspensions obtained from low dose, UV-treated, DNFB-painted donor mice were inoculated into naive recipients, the cells were pretreated with either an arsanilate-conjugated anti-Lyt-1 or anti-Lyt-2 monoclonal antibody, followed by rabbit anti-arsanilate antibody and then with rabbit complement. As demonstrated in Table V, sequential treatment with normal mouse serum, anti-arsanilate antibody and complement had no effect on the unresponsiveness observed. Alternatively, recipients of cell suspensions treated with anti-Lyt-1 antibody plus complement responded in a manner identical to positive controls, i.e., this pretreatment totally eliminated suppressor cell activity. Anti-Lyt-2 plus complement pretreatment resulted in partial reversal of the unresponsiveness (67% of the suppression seen in the normal serum-treated control suspension). In a parallel experiment in which similar conditions for cell lysis were employed, pretreatment of cells obtained from 5-d mixed lymphocyte reactions of C3H spleen cells vs C57BL/6 x-irradiated spleen cells with anti-Lyt-1 plus complement had no effect on their capacity to lyse ^{51}Cr-labeled EL4 target cells in a standard 4-h cytotoxicity assay. By contrast, specific cytotoxicity of similar aliquots of

TABLE IV

Suppressor Cells Generated by Low Dose UVB and Hapten Skin Painting Are T Lymphocytes

Group	Treatment of cell suspension	DNFB immunization	Increment in ear swelling ($\times 10^{-4}$ inches ± SEM)	Suppression
				%
I		+	34.5 ± 5.2	
II	Anti-Thy, complement	+	39.3 ± 5.1	−22
III	Anti-Ars, complement	+	17.0 ± 3.6	79
IV		−	12.4 ± 1.9	

Cell suspensions from unresponsive donor mice were treated in vitro with anti-Thy-1.2 and complement (group II) or with anti-arsanilate and complement (group III). The cells remaining following treatment were then injected i.v. into recipient mice. Groups I and IV received no cells. Responses were measured as described in Table I.

TABLE V
Ly-Phenotype of Suppressor Cells Generated by Low Dose UVB Radiation and DNFB Skin Painting

Group	Treatment of donor cell suspension	DNFB immunization	Increment in ear swelling ($\times 10^{-4}$ inches ± SEM)	Suppression
				%
I		+	24.2 ± 3.9	
II	Anti-Lyt-1	+	21.9 ± 4.1	10
III	Anti-Lyt-2	+	12.8 ± 1.0	48
IV	NMS	+	6.9 ± 1.4	72
V		−	0.3 ± 1.6	

Cell suspensions from unresponsive donors were treated with either arsanilate-conjugated Lyt-1+ antibody, arsanilate conjugated Lyt-2+ antibody or normal mouse serum (NMS). Each cell suspension was then treated with rabbit anti-arsanilate antibody and complement. The cells remaining after antibody and complement treatment were injected into naive recipients which were then immunized and ear challenged with DNFB. Groups I and V received no cells. Responses were measured as described in Table 1.

cultured cells was only partially reduced (from 30% seen in controls to 16%) by pretreatment with anti-Lyt-2 plus complement. This result strongly implies that the lysis of Lyt-2+ cells under the experimental conditions employed was incomplete. We therefore conclude that T cells bearing the Lyt-1 marker are crucial for suppression to occur. We more tentatively conclude that Lyt-2+ cells also participate in this suppression. Whether the suppression is mediated through a single population of Ly1+23+ cells or through distinct T cell subsets, one of which the Ly1+23− and the other of which is Ly1+23+ and Ly1−23+, cannot be determined from the studies performed to date.

Antigen Specificity of Suppressor Cells. Suppressor cells in intact hypersensitivity reactions may act specifically against cells reactive to the antigen that induces them, or they may act nonspecifically. We chose next to examine the specificity of the suppressor cells generated following low dose UVB and hapten skin painting. Donor mice were made unresponsive to DNFB, sacrificed, and suspensions of spleen and lymph node cells prepared according to the typical protocol. These cells were injected into two panels of naive recipient mice. One panel was then skin painted and ear challenged with DNFB; the other was skin painted and challenged with oxazolone, a non-cross-reacting contact-sensitizing agent. Recipient mice that received cells from DNFB unresponsive donors and were then sensitized and challenged with DNFB showed suppressed ear-swelling responses. By contrast, mice that received cells from DNFB unresponsive donors and were then skin painted and ear challenged with oxazolone developed normal contact hypersensitivity responses (Table VI). This result indicates that the suppressor cells generated by low dose UVB and hapten skin painting are specific for the antigen that elicited them.

Analysis of Locus of Action of Suppressor Cells. Suppressor cells in contact hypersensitivity that act within the afferent limb of the response to inhibit the generation of effector cells (19), and others that operate within the efferent limb

TABLE VI

Suppressor Cells Generated by Low Dose UVB and Hapten Skin Painting are Antigen Specific

Group	Recipients of DNFB unresponsive cells	Hapten used for immunization	Hapten used for ear challenge	% of positive control	Suppression
					%
I	−	DNFB	DNFB	100	
II	+	DNFB	DNFB	35	77
III	−	—	DNFB	16	
IV	−	Ox	Ox	100	
V	+	Ox	Ox	92	12
VI	−	—	Ox	31	

Cell suspensions from donor mice made unresponsive to DNFB were injected into two groups of recipient mice. Group II was immunized and ear challenged with DNFB. Group V was immunized and ear challenged with oxazolone (Ox). Responses were measured as the percent of the appropriate positive control.

to impair the function of effector cells (20), have been described. In order to determine whether the suppressor cells generated by low dose UVB and DNFB skin painting could effect efferent suppression, a co-transfer of immune lymph node cells with unresponsive spleen and lymph node cells into naive recipients was performed. In this experiment, one panel of donor mice was immunized to DNFB by applying the hapten to abdominal skin and forepaws on days −4 and −3. Another panel of donor mice was made unresponsive to DNFB by the low dose UVB and hapten skin-painting protocol beginning on day −10. On day 0, both panels of donor mice were sacrificed. Cell suspensions were prepared from the peripheral lymph nodes of the immune donors; spleen and lymph node cell suspensions were prepared from the unresponsive donors. In preliminary experiments, it was determined (*a*) that 30×10^6 peripheral lymph node cells from immune donors consistently could transfer contact hypersensitivity reactions to naive recipients and (*b*) that efferent suppression could be demonstrated by co-transferring 30×10^6 immune cells with 100×10^6 suppressor cells generated by the epicutaneous application of supraoptimal doses of DNFB (data not shown). In this experiment, 30×10^6 immune cells were inoculated with 100×10^6 UVB-induced suppressor cells into naive recipients that were ear challenged 1 h after cell transfer. This response was compared with panels of mice that received immune cells only, and to negative controls. As can be seen in Table VII, no suppression was observed in the panel that received immune cells and suppressor cells. Therefore, the suppressor cells generated by low dose UVB and DNFB skin painting do not appear to act on the efferent limb of the contact hypersensitivity response.

These results suggest that the suppressor cells might act on the afferent limb of the contact hypersensitivity response. We examined next the ability of suppressor cells to inhibit the proliferation of cells in draining lymph nodes of recipient mice painted with DNFB. Though proliferating cells in lymph nodes draining skin painted with hapten may not be the actual effector cells, the incorporation of IUdR by draining lymph nodes has been shown to correlate

TABLE VII

Suppressor Cells Fail to Inhibit Ear Swelling After Co-transfer of Immune Lymph Node Cells

Group	Cells transferred	Increment in ear swelling ($\times 10^{-4}$ in ± SEM)
I	Immune	27.9 ± 3.3
II	Unresponsive + Immune	26.9 ± 3.8
III	None	13.7 ± 5.9

Cell suspensions from unresponsive donors were transferred with (group II) or without (group I) immune lymph node cells. Recipient mice were ear challenged one hour after cell transfer. Responses were measured as described in Table I.

TABLE VIII

Suppressor Cells Inhibit In Vivo DNA Synthesis in Draining Lymph Nodes

Group	Type of cell suspension received	DNFB immunization	IUdR incorporation	Suppression
			%	%
I	Naive cells	+	0.2	
II	DNFB unresponsive	+	0.1	59
III	None	−	0.03	

Cell suspensions from unresponsive donors (group II) or from naive donors (group I) were injected into naive recipient mice who were then immunized to DNFB. 3 d after immunization each mouse was given 2 μCi IUdR i.p. The mice were sacrificed 2 h later and the 1% incorporation of IUdR into draining lymph nodes was measured.

with the induction of contact hypersensitivity (19). In this experiment, naive recipients of spleen and lymph node cells from donors made unresponsive to DNFB by low dose UVB and hapten skin painting were skin painted with an immunizing dose of DNFB. 3 d later each mouse received an intraperitoneal injection of FUdR followed by an intraperitoneal injection of I^{125}-labeled IUdR. Mice were then sacrificed and the percent IUdR incorporated into draining lymph nodes was measured. As demonstrated in Table VIII, incorporation of IUdR into draining lymph nodes of mice that received spleen and lymph node cells from mice made unresponsive to DNFB was significantly suppressed compared with animals that received cells from naive syngeneic donors. We conclude that the suppressor cells generated by low dose UVB and DNFB skin painting act on the afferent limb of the immune response, and thereby inhibit the induction of effector cells for contact hypersensitivity.

Discussion

Local irradiation of murine skin with doses of UVB radiation that are comparable to typical human exposure to sunlight profoundly alters the capacity of that skin to serve as a medium for the induction of contact hypersensitivity to

simple haptens. Epicutaneous application of haptens such as DNFB and oxazolone to body wall skin normally produces a state of intense contact hypersensitivity. By contrast, similar doses of DNFB applied to UVB-irradiated skin produces profound, specific unresponsiveness. As we demonstrated previously in Syrian hamsters, hapten-specific unresponsiveness induced in this manner in mice is infectious (21). Adoptive transfer of the unresponsive state is achieved by Thy-1+ suppressor cells that act on the afferent limb of the immune response, but fail to suppress immune effector cells. Our results indicate that suppression requires a cell population that is Lyt-1+, and further suggest the participation of cells bearing the Lyt-2+ marker. More definitive characterization of the responsible suppressor T cells will require analysis of such factors as their idiotypic orientation and the genetic restrictions on transferring suppression (including analysis of the role of I-J). While antibodies capable of identifying a DNP-specific idiotypic network are unavailable to us at present, experiments employing monoclonal anti-I-J antibodies are currently in progress.

During the past several years a large number of laboratories using a variety of model systems have studied regulatory phenomena in specific immune suppression (18, 22, 23). A number of related but apparently nonidentical suppressor regulatory pathways that involve interactions among cells with different phenotypes have been described. Recently, Germain and Benacerraf (24) raised the question whether the described pathways could represent fragments of larger single sequence; they then developed a model system that attempted to integrate the data from several of the more extensively defined suppressor systems into a "consensus" suppressor pathway. In this model three distinct suppressor T cells and their secretory factors sequentially conspire to regulate both humoral and cell-mediated immune reactions.

An incompletely developed suppressor network, in which only first order suppressor T cells (T_{s1} or $T_{s\,aff}$), which can only inhibit the afferent limb of an immune response, are detectable, appears to be a frequent result when soluble hapten or hapten-derivatized lymphoid cells are introduced intravenously on a single occasion. Thus a single i.v. injection of DNBS results within 7 d in the appearance of afferent, but not efferent T suppressor cells (reference 23, see also Tables II, VII, and VIII). In the "consensus" system, in order for efferent suppressor cells (T_{s2} and T_{s3}) to be activated, antigen re-exposure is usually necessary. In the model described in this report, hapten applied to UVB-irradiated skin results in the appearance of suppressor cells that act only on the afferent limb of the contact hypersensitivity response. This model superficially mimics that induced by intravenous introduction of DNBS. However, the suppressor cells in the two systems apparently have distinctive Lyt phenotypes: suppression in our UVB radiation model was eliminated by removing Lyt-1+ cells from the suspension; in the i.v. DNBS system, afferent suppressors are not removed by anti-Lyt-1, but are eliminated by anti-Lyt-2 (J. Moorhead, personal communication).

The suppressor cells generated in the present UVB-hapten model are also apparently different from those generated in another model that also involves introduction of reactive hapten (DNFB) to the animal via epicutaneous application. The suppressor T cells generated in mice after epicutaneous application of

supraoptimal amounts of DNFB can suppress both the afferent and efferent limbs of the response in adoptive recipients (23), whereas the present model generates only afferent limb suppressor activity. Thus, the suppressor network activated by hapten application to UVB-irradiated skin is both incomplete and also apparently distinct from other related models. This realization implies that the type of antigen processing and presentation via the skin that leads to unresponsiveness may also be relatively unique.

The line of investigation that this series of experiments advances concerns the role of epidermal Langerhans cells in the induction of contact hypersensitivity. At least two types of epidermal cells—Langerhans cells and keratinocytes—may participate in cutaneous immune processes. Langerhans cells are bone marrow-derived cells (25, 26) that bear C3b and Fc surface membrane receptors (27) and express large amounts of Ia determinants (28, 29, 30). They appear to act as epidermal antigen-presenting cells and are capable of stimulating allogeneic lymphocytes in mixed epidermal cell-lymphocyte reactions (31). Keratinocytes may participate by elaborating the soluble mediator, epidermal cell-derived thymocyte activating factor (ETAF), a substance with immunobiologic properties that are similar to interleukin-1 (32, 33). Since the doses of UVB used in these experiments cause massive morphologic alterations in exposed Langerhans cells, it is tempting to conclude that the unresponsiveness to haptens results from the failure of these Langerhans cells to present epicutaneous haptens effectively to T cells. However, this circumstantial argument can not exclude the possibility that keratinocytes play an important role in the UVB radiation effects observed in the present studies. It has been reported recently that ETAF will partially reconstitute the UVB-induced loss of stimulatory capacity of epidermal cells in the mixed epidermal cell lymphocyte reaction (34). The potential tolerance (suppression)-promoting effect of diminished ETAF production at the site of UVB radiation and hapten application has not been examined. The fact that a tolerogenic signal emerges from the skin that has been UVB radiated and painted with hapten raises the possibility that the source of the signal may be keratinocytes and/or products derived therefrom. We have recently demonstrated that hapten-derivatized keratinocytes largely depleted of Langerhans cells produce profound unresponsiveness when inoculated intravenously into Syrian hamsters. This effect is overcome when significant numbers of viable, haptenated Langerhans cells are included in the inoculum (35).

Chronic and/or high-dose UVB radiation of the type described and studied by Fisher et al. (4) and Spellman et al. (3) and their respective associates results in the induction of systemic unresponsiveness, mediated by suppressor T cells. However, these suppressor cells differ substantially from the cell(s) we have identified after acute, low dose UVB exposure. Induction of the latter cells is completely dependent upon epicutaneous application of hapten to the radiated sites (8). There is no generic, systemic impairment in immune responsiveness since mice irradiated only on abdominal skin can be sensitized to hapten readily if it is painted on dorsal body wall skin. Moreover, mice receiving local, low dose, UVB radiation are fully capable of developing typical delayed hypersensitivity when immunized subcutaneously with hapten-derivatized syngeneic lymphoid cells (36). These findings indicate that locally irradiated mice have no systemic

impairment in antigen-presenting cells—a characteristic feature of mice that have received chronic and/or large exposures to UVB radiation. Our evidence suggests that local, acute, low dose UVB radiation causes a perturbation of cutaneous immune capabilities that is distinct from that induced by chronic, high dose UVB radiation.

The adverse effects of actinic radiation on normal cutaneous processes can be interpreted in terms of the experimental results we have described in this report. Exposure of skin to UVB causes a transient impairment in the capacity of that skin to present epicutaneous antigens in an immunogenic form. Antigens introduced at that critical time are perceived as tolerogens, and an antigen-specific systemic network of suppression is established. If for purposes of discussion, the offending antigen happens to be an opportunistic virus or a neoantigen on a malignant epidermal cell, synchronous exposure to antigen and UVB radiation may evoke unresponsiveness and rob the host of the precise armamentarium needed to ward off the attack.

Summary

Acute, low dose ultraviolet B radiation of murine body wall skin followed by local application of DNFB produces a state of antigen-specific unresponsiveness. This state is maintained at least in part by an Lyt-1+ T cell that effects unresponsiveness by impairing the induction phase of contact hypersensitivity. The absence of suppressor cells capable of acting at the effector stage of immunity suggests that tolerogenic signals derived from the skin establish suppressor networks that are incomplete and perhaps different from networks that are induced by systemic administration of tolerogens. It is proposed that ultraviolet radiation produces its effects by impairing the antigen-presenting potential of resident Langerhans cells in whose absence hapten-derivatized keratinocytes (or their products) are able to deliver a tolerogenic signal.

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References

1. Elmets, C. A., and P. R. Bergstressser. 1982. Ultraviolet radiation effects on immune processes. *Photochem. Photobiol.* 36:715.
2. Kripke, M. L. 1974. Antigenicity of murine skin tumors induced by ultraviolet light. *J. Natl. Cancer Inst.* 53:1333.
3. Spellman, C. W., and R. A. Daynes. 1977. Modification of immunological potential by ultraviolet radiation. II. Generation of suppressor cells in short-term UV-irradiated mice. *Transplantation.* 24:120.
4. Fisher, M. S., and M. L. Kripke. 1977. Systemic alteration induced in mice by ultraviolet irradiation and its relationship to ultraviolet carcinogenesis. *Proc. Natl. Acad. Sci. USA.* 74:1688.
5. Greene, M. I., M. S. Sy, M. Kripke, and B. Benacerraf. 1979. Impairment of antigen-presenting cell function by ultraviolet radiation. *Proc. Natl. Acad. Sci. USA.* 76:6591.
6. Levitan, N. L., M. I. Greene, B. Benacerraf, and R. N. Germain. 1980. Immunologic effects of whole-body ultraviolet irradiation. Selective defect in splenic adherent cell function *in vitro*. *Proc. Natl. Acad. Sci. USA.* 77:2881.
7. Letvin, N. L., I. J. Fox, M. I. Greene, B. Benacerraf, and R. N. Germain. 1980.

Immunologic effects of whole body ultraviolet (UV) irradiation. II. Defects in splenic adherent cell antigen presentation for stimulation of T cell proliferation. *J. Immunol.* 125:1402.

8. Toews, G. B., P. R. Bergstresser, and J. W. Streilein. 1980. Epidermal Langerhans cell density determines whether contact hypersensitivity of unresponsiveness follows skin painting with DNFB. *J. Immunol.* 124:445.
9. Phanupak, P., J. W. Moorhead, and H. N. Claman. 1974. Tolerance and contact sensitivity to DNFB in mice. I. In vivo detection by ear swelling and correlation with in vitro cell stimulation. *J. Immunol.* 112:115.
10. Task Force on Photobiology. 1974. Report on ultraviolet light sources. *Arch Dermatol.* 109:833.
11. Phanuphak, P., J. W. Moorhead, and H. N. Claman. 1974. Tolerance and contact sensitivity to DNFB in mice. III. Transfer of tolerance with "suppressor T cells." *J. Immunol.* 113:1230.
12. Wood, P., G. L. Asherson, B. Mayhew, W. R. Thomas, and M. Zembala. 1977. Control of the immune reaction: T cells in immunized mice which depress the *in vitro* DNA synthesis response in the lymph nodes to skin painting with the contact sensitizing agent picryl chloride. *Cell. Immunol.* 30:25.
13. Miller, S. D., M. S. Sy, and H. N. Claman. 1977. The induction of hapten-specific T cell tolerance using hapten-modified lymphoid membranes. II. Relative roles of suppressor T cells and clone inhibition in the tolerant state. *Eur. J. Immunol.* 7:165.
14. Ptak, W., M. Zembala, G. L. Asherson, and J. Marcinkiewicz. 1981. Inhibition of contact sensitivity by macrophages. *Int. Arch. Allerg.* 65:121.
15. Katz, S. I., D. Parker, and J. L. Turk. 1974. B-cell suppression of delayed hypersensitivity reactions. *Nature (Lond.)* 251:550.
16. Asherson, G. L., M. Zembala, M. A. C. C. Perera, B. Mayhew, and W. R. Thomas. 1977. Production of immunity and unresponsiveness in the mouse by feeding contact sensitizing agents and the role of suppressor cells in Peyer's patches, mesenteric lymph nodes and other lymphoid tissues. *Cell. Immunol.* 33:145.
17. Zembala, M., and G. L. Asherson. 1973. Depression of the T cell phenomenon of contact sensitivity by T cells from unresponsive mice. *Nature (Lond.).* 244:227.
18. Benacerraf, B., M. I. Greene, M. S. Sy, and M. E. Dorf. 1982. Suppressor T cell circuits. *Ann. N. Y. Acad. Sci.* 392:300.
19. Moorhead, J. W. 1976. Tolerance and contact sensitivity to DNFB in mice. VI. Inhibition of afferent sensitivity by suppressor T cell in adoptive tolerance. *J. Immunol.* 117:802.
20. Asherson, G. L., and M. Zembala. 1974. Suppression of contact sensitivity by cells in the mouse. I. Demonstration that suppressor cells act on the effector stage of contact sensitivity; and their induction following *in vitro* exposure to antigen. *Proc. R. Soc. Lond. B. Biol.* 187:329.
21. Streilein, J. W., and P. R. Bergstresser. 1981. Langerhans cell function dictates induction of contact hypersensitivity or unresponsiveness to DNFB in Syrian hamsters. *J. Invest. Dermatol.* 77:272.
22. Asherson, G. L., M. Zembala, W. R. Thomas, and M. A. C. C. Perera. 1980. Suppressor cells and the handling of antigen. *Immunol. Rev.* 50:3.
23. Claman, H. N., S. D. Miller, M. S. Sy, and J. W. Moorhead. 1980. Suppressive mechanisms involving sensitization and tolerance in contact allergy. *Immunol. Rev.* 50:105.
24. Germain, R. N., and B. Benacerraf. 1981. A single major pathway of T-lymphocyte interactions in antigen-specific immune suppression. *Scand. J. Immunol.* 13:1.
25. Katz, S. I., K. Tamaki, and D. H. Sachs. 1979. Epidermal Langerhans cells are

derived from cells originating in bone marrow. *Nature* (*Lond.*). 282:324.
26. Frelinger, J. G., L. Hood, S. Hill, and J. A. Frelinger. 1979. Mouse epidermal Ia molecules have a bone marrow origin. *Nature* (*Lond.*). 282:321.
27. Stingl, G., E. C. Wolff-Schreiner, W. J. Pichler, F. Gschnait, W. Knapp, and K. Wolff. 1977. Epidermal Langerhans cells bear Fc and C3 receptors. *Nature* (*Lond.*). 268:245.
28. Stingl, G., S. I. Katz, E. M. Shevach, E. Wolff-Schreiner, and I. Green. 1978. Detection of Ia antigens on Langerhans cells in guinea pig skin. *J. Immunol.* 120:570.
29. Rowden, G., T. M. Phillips, and T. L. Delovitch. 1978. Expression of Ia antigens by murine keratinizing epithelial Langerhans cells. *Immunogenetics.* 7:465.
30. Stingl, G., S. I. Katz, L. D. Abelson, and D. L. Mann. 1978. Immunofluorescent detection of human B cell alloantigens on S-Ig positive lymphocytes and epidermal Langerhans cells. *J. Immunol.* 120:661.
31. Stingl, G., S. I. Katz, L. Clement, I. Green, and E. M. Shevach. 1978. Immunologic functions of Ia-bearing epidermal Langerhans cells. *J. Immunol.* 121:2005.
32. Sauder, D. N., C. S. Carter, S. I. Katz, and J. J. Oppenheim. 1982. Epidermal cell production of thymocyte activating factor (ETAF). *J. Invest. Dermatol.* 79:34.
33. Luger, T. A., B. N. Stauder, S. I. Katz, and J. J. Oppenheim. 1981. Epidermal cell (keratinocyte)-derived thymocyte-activating factor (ETAF). *J. Immunol.* 127:1493.
34. Sauder, D. N., F. P. Noonan, E. C. DeFabo, and S. I. Katz. 1982. UV alteration in alloantigen presenting ability of epidermal cells: restoration by the soluble epidermal cell product, epidermal cell derived thymocyte activating factor (ETAF). *J. Invest. Dermatol.* 78:337. (Abstr.).
35. Sullivan, S., P. R. Bergstresser, and J. W. Streillein. 1983. Evidence that suspensions of TNP-derivitized Langerhans cells promote while keratinocytes impair the induction of contact hypersensitivity in Syrian hamsters. *J. Invest. Dermatol.* 80:(in press).
36. Bergstresser, P. R., C. A. Elmets, and J. W. Streilein. 1983. Local effects of ultraviolet radiation on immune function in mice. *In* The Effects of Ultraviolet Light on the Immune System. J. A. Parrish, editor. Plenum Publishing Corp. New York. pp 73–86.