SUPPRESSION OF ANTIBODY RESPONSES
TO TOPICALLY APPLIED ANTIGENS
BY ULTRAVIOLET LIGHT IRRADIATION
Induction of Phototolerance

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Ultraviolet (UV)[1] light (280–340 nm) irradiation of mice results in immunologic alterations that are dependent on the dose and time course of the radiation. Acute UV irradiation regimens (less than ~14 daily exposures) are associated with tolerance to topically applied skin contact sensitizers (1–8). If the acute UV regimen is performed with low-dose radiation (~0.01–0.35 J/cm^2 per exposure), the tolerance is "site specific" in that it is manifest only if the antigen is introduced through UV-irradiated skin (1, 2, 5). However, if high-dose UV radiation is employed (greater than ~0.5 J/cm^2 per exposure), systemic tolerance results (4, 5, 7), and the specific contact sensitizing antigen can be applied to unirradiated skin sites and no delayed-type hypersensitivity (DTH) reaction is elicited upon subsequent challenge. If UV exposures are administered chronically (4–8 wk), the capacity to manifest DTH through UV-irradiated skin reappears (3).

Our interest in immunological aberrations associated with UV irradiation stems from our studies in UV-induced susceptibility to skin tumors. It is reasonable that the immunological dysfunctions associated with acute UV irradiation, measured in terms of DTH unresponsiveness (1–8), diminished antigen presentation capacity (9–15), altered cell distributions (15), and elevated acute phase reactants (16), might play a role in the subsequent development of the tumor-susceptible state. The data to be presented in this report address another facet of early immunologic changes associated with acute low-dose UV irradiation. We have investigated the role of UV exposure in the induction of humoral tolerance to soluble protein antigens. Our results suggest that UV irradiation can inhibit antibody responses initiated by skin-priming with antigen (17). We have termed this phenomenon "phototolerance" (PT). Our rationale for selecting soluble protein antigens was the possible clinical relevance of such protocols where tolerance to a variety of soluble antigens might be inducd. Examples

[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; PBS, phosphate-buffered saline; PT, phototolerance.

include, but are not limited to, monoclonal antibodies or enzymes administered for therapeutic reasons and transplantation antigens to inhibit organ rejection.

Materials and Methods

Mice.   C3Hf/HeN female mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA. BALB/c mice were obtained from our own breeding colony. Groups of four age-matched animals, 6–8 wk old, were used in all experiments. Bleeding was performed by puncturing the retro-orbital plexus while the animals were under light ether anesthesia. Serum samples from the mice in each group were pooled and stored frozen until analysis.

UV Irradiation of Mice.   Details of the UV irradiation of mice have been published previously (18). Briefly, the UV source consisted of a bank of six FS-40 fluorescent sunlamps (Westinghouse Electric Corp., Pittsburgh, PA) emitting principally (>60%) between 280 and 320 nm. The energy output, from a distance of 20 cm, was ~2 J/m^2·s. Mice were irradiated for 30 min/d. Dorsal skin sites were exposed by removing the fur with clippers.

Skin Sensitization.   Antigen was introduced by scarification of several square millimeters of UV-irradiated skin. Our protocol involved placing 20 μl phosphate-buffered saline (PBS) containing 20 μg antigen on a patch of irradiated skin. A 27 gauge needle was used to stroke the area. Scarification was complete when the solution had been evenly distributed and absorbed onto the skin.

Induction of PT.   Mice were UV-irradiated four times, for 30 min/d, prior to being skin-sensitized with antigen. Mice that received multiple sensitizations with antigen were maintained on the UV irradiation regimen until the last sensitization was performed. The term "PT induction" is defined as the process of UV-irradiating and skin-sensitizing the mice.

Antigen Challenge.   Mice were challenged by injecting 0.2 ml PBS containing 50 μg antigen intraperitoneally. The particular immunization schedules used are indicated in each data table.

Antigens.   Human or rabbit IgG was purchased from Sigma Chemical Co. (St. Louis, MO). These materials were further purified by DEAE cellulose (Whatman, Ltd., Kent, England) and Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) chromatography. Conalbumin (Sigma Chemical Co.) was used without further purification.

During these investigations, we examined a range of antigen concentrations for inducing PT and eliciting antibody responses. 50 μg of human IgG, rabbit IgG, or conalbumin was optimal for antigen challenge. PT was readily induced over a dose range of 2–100 μg of antigen. The results presented here are based on the use of 20 μg of antigen for sensitization.

Quantitation of Antibody Activity.   The specific antibody titer of pooled mouse sera was determined using an enzyme-linked immunosorbent assay (ELISA) technique. Antigen was adsorbed onto Immulon microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) by incubation of 10 μg/ml of antigen in 0.05 M NaHCO$_3$, pH 9.6, for 18 h. After extensive washing of the wells with PBS/1% fetal calf serum (FCS), a 100-μl aliquot of the antiserum or doubling dilutions of the antiserum was added to individual wells. The wells were then filled with 200 μl of PBS/1% FCS. Following a 2.5-h incubation at room temperature, the wells were emptied, washed, and refilled with 300 μl of a rabbit anti-mouse Ig alkaline phosphatase conjugate optimally diluted in PBS/1% FCS. Following a second 2.5-h incubation, the wells were washed and refilled with 300 μl p-nitrophenyl phosphate (Sigma Chemical Co.), 0.2 mg/ml, dissolved in 0.1 M Tris-Cl buffer, pH 10. The optical density at 410 nm was determined with an automatic microplate reader (MR 600; Dynatech Laboratories, Inc.) after an appropriate incubation period. The reported titers are the end dilutions of the antiserum, which yield essentially prebleed levels of reactivity. The titers shown in all data tables reflect the average response based on pooled bleeds of four animals per group.

Rabbit Anti-Mouse Ig Conjugate.   For the ELISA procedure, the antibody-enzyme

conjugate was prepared as follows. Mouse IgG, purified by DEAE cellulose and protein A (Pharmacia Fine Chemicals) chromatography, was used to immunize rabbits. The rabbit antiserum was purified by affinity chromatography on CM-Bio Gel (Bio-Rad Laboratories, Inc., Richmond, CA) coupled mouse IgG. The enzyme-labeled rabbit antibody was prepared by the procedure of Avermeas (19). Briefly, one part of affinity-purified antibody was mixed with three parts of alkaline phosphatase (Sigma Chemical Co.) in PBS and glutaraldehyde was added to a final concentration of 0.05%. After 2 h incubation, the mixture was exhaustively dialyzed, filter-sterilized, and stored at 4°C in 50% glycerol.

Results

PT to Protein Antigens. UV-induced unresponsiveness to protein antigens is shown in Table I. Three comparisons should be noted. First, mice, skin-primed and challenged (groups 3, 7, 11, and 15), respond to the antigens. Second, normal and UV-irradiated mice exhibit equivalent responses to systemic challenge (groups 1, 2, 5, 6, 9, 10, 13, and 14). This indicates that the dose of UV radiation employed does not by itself cause immune suppression. Third, sensitization through the UV-irradiated skin site followed by intraperitoneal (or intravenous; data not shown) challenge fails to elicit an antibody response (groups 4, 8, 12, and 16); that is, it results in PT. These results demonstrate that skin sensitization through irradiated skin sites effectively inhibits responses to subsequent systemic antigen challenge. Unresponsiveness, caused by some type of suppression, is apparent because mice subjected to PT respond less than normal or UV-irradiated control mice.

To demonstrate that the effects of UV irradiation were directly related to the exposed skin site, experiments were performed in which mice were skin-sensitized at different sits. Our results suggest that PT is detected only if the antigen is introduced through UV-irradiated skin (Table II). Further, as shown in Table

TABLE I
PT to Protein Antigens

Group	Mice	Antigen	UV	Skin sensitization	Challenge	Titer
1	4 C3H*	HuIgG‡			+	40
2			+		+	40
3				+	+	1,280
4			+	+	+	0
5	4 BALB/c				+	20
6			+		+	40
7				+	+	640
8			+	+	+	0
9	4 C3H	RabIgG			+	20
10			+		+	20
11				+	+	160
12			+	+	+	0
13		CALB			+	20
14			+		+	20
15				+	+	160
16			+	+	+	0

* Animals were UV-irradiated on days 0–4, skin-sensitized on day 4, challenged on day 8, and bled on day 14.
‡ HuIgG, human IgG; RabIgG, rabbit IgG; CALB, conalbumin.

TABLE II

Induction of PT by Sensitization through UV-irradiated or Distant Skin Sites

Group	Mice	UV	Sensitization site	Challenge	Titer
1	4 C3H*			+	40
2			Dorsal	+	1,280
3			Ventral	+	1,280
4		+ (Dorsal)	Dorsal	+	0
5		+ (Dorsal)	Ventral	+	1,280

* Animals were UV-irradiated on days 0–4, skin-sensitized on day 5, challenged on day 9, and bled on day 14. The antigen was HuIgG.

TABLE III

Effects of Sensitization by Various Routes on PT Induction

Group	Mice	UV	Sensitization Cutaneous	Intradermal	Subcutaneous	Challenge	Titer
1	4 C3H*					+	40
2			+			+	1,280
3				+		+	1,280
4					+	+	640
5		+	+			+	0
6		+		+		+	1,280
7		+			+	+	1,280

* Animals were UV-irradiated on days 0–4, sensitized by scarification, or intradermal or subcutaneous injection on day 5, challenged on day 9, and bled on day 13. The antigen was HuIgG.

TABLE IV

Induction of PT with Different Doses of UV Radiation

Group	Mice	UV (days) 1	3	4	5	7	25	40	Skin sensitization	Challenge	Titer
1	4 C3H*									+	80
2									+	+	1,280
3		+							+	+	1,280
4			+						+	+	0
5				+					+	+	0
6					+				+	+	0
7						+			+	+	20
8							+		+	+	40
9								+	+	+	160

* Animals were UV-irradiated for the number of days indicated, skin-sensitized on the day after the last UV exposure, challenged 5 d after sensitization, and bled 10 d after sensitization. Starting times for irradiation were staggered so that all groups finished simultaneously. The antigen was HuIgG.

III, the sensitization must be via the cutaneous route. If antigen is injected intradermally or subcutaneously, priming occurs.

The induction of PT in mice receiving different amounts of UV irradiation (Table IV) suggests that at least 3 d of exposure is necessary for unresponsiveness

to occur. Mice irradiated for ~6 wk appear to be recovering the capacity to be primed through the skin. This observation parallels the data from DTH experiments, which also suggest that chronically irradiated mice recover the ability to be skin-sensitized (3). The data shown in Table V address two questions. First, how much time can elapse after PT induction before systemic challenge with antigen again results in a response? Our results indicate that responsiveness is manifest if the challenge is delayed ~2 wk after PT induction (see Table V, groups 4–8). Second, how much time can elapse between the termination of UV exposures and skin sensitization for PT to be induced? It appears that PT occurs if the animal receives antigen within 2 wk after cessation of UV exposures (groups 9–12).

While PT can inhibit primary responses, it does not appear to reverse an ongoing response. The experiment illustrated in Table VI was performed by first injecting the mice intraperitoneally with antigen and then subjecting them to the PT induction regimen. As shown, PT has no effect on the efferent phase of the antibody response.

Evidence was obtained that PT is antigen specific. The data presented in Table VII are based on immunizations with human IgG and conalbumin because these proteins do not elicit cross-reactive responses in the controls after primary or secondary encounter with the antigens (groups 9, 10, and 1–4). The experimental groups show that PT is induced in both antigen systems (groups 5 and 7), but that PT to one antigen does not inhibit the response to systemic challenge with the other antigen (groups 6 and 8). Because conalbumin is a weak antigen in this immunization regimen, specificity experiments were also performed with purified rabbit IgG, and similar results were obtained (data not shown).

TABLE V

Effects of Altering Time of Sensitization or Challenge on PT Induction

Group	Mice	UV	Skin sensitization (Days after UV irradiation)	Challenge (Days after sensitization)	Titer
1	4 C3H*			+	40
2			+	24	1,280
3			+	4	1,280
4		+	1	24	1,280
5		+	1	18	1,280
6		+	1	14	320
7		+	1	10	0
8		+	1	4	0
9		+	4	4	0
10		+	5	4	0
11		+	10	4	20
12		+	16	4	640

* Animals were UV-irradiated on days 0–4, skin-sensitized on day 1, 4, 5, 10, or 16 after UV irradiation, challenged on day 4, 10, 14, 18, or 24 after sensitization, and bled 5 d after challenge. Control groups 1–3 received no UV radiation. Group 1 received only intraperitoneal antigen challenge. Groups 2 and 3 were skin-sensitized and challenged either 24 or 4 d later, respectively. The antigen was HuIgG.

TABLE VI

Induction of PT Does Not Inhibit an Ongoing Primary or Secondary Response

Group	Mice	Challenge 1	Challenge 2	UV	Skin sensitization	Titer
1	4 C3H*	+				40
2		+		+		40
3		+		+	+	40
4	4 C3H‡	+	+			640
5		+	+	+		1,280
6		+	+	+	+	640

* Animals were challenged intraperitoneally on day 0, UV-irradiated on days 1–4, skin-sensitized through the irradiated skin site on day 5, and bled on day 10.
‡ Animals were challenged intraperitoneally on days 0 and 9, UV-irradiated on days 5–9, skin-sensitized through the irradiated skin site on day 10, and bled on day 14. The antigen HuIgG was used in both experiments.

TABLE VII

Specificity of PT

Group	Mice	UV	Skin sensitization	Challenge	Titer HuIgG	Titer CALB
1	4 C3H*		CALB	CALB	0	160
2			CALB	HuIgG	20	0
3			HuIgG	HuIgG	1,280	0
4			HuIgG	CALB	320	10
5		+	CALB	CALB	0	0
6		+	CALB	HuIgG	40	0
7		+	HuIgG	HuIgG	0	0
8		+	HuIgG	CALB	0	5
9			—	CALB	0	10
10			—	HuIgG	40	0

* Animals were UV-irradiated on days 0–4, skin-sensitized on day 5, challenged on day 9, and bled on day 16. The antigens were CALB and HuIgG.

TABLE VIII

Response to Multiple Cutaneous Sensitizations in Phototolerized Mice

Group	Mice	UV	Skin sensitization 1	Skin sensitization 2	Skin sensitization 3	Titer 1	Titer 2	Titer 3	Titer 4
1	4 C3H*		+	+	+	0	20	2,560	2,560
2		+	+	+	+	5	0	0	0

* Animals were UV-irradiated on days 0–9, skin-sensitized on days 5, 9, and 13, and bled on days 9, 13, 17, and 25. The antigen was HuIgG.

TABLE IX

Response to Multiple Challenges Following Single PT Induction

Group	Mice	UV	Skin sensi-tization	Challenge			Titer		
				1	2	3	1	2	3
1	4 C3H*			+	+	+	20	160	1,280
2		+		+	+	+	20	160	1,280
3			+	+	+	+	160	640	2,560
4		+	+	+	+	+	0	320	640

* Animals were UV-irradiated on days 0–4, skin-sensitized on day 4, given multiple challenges on days 8, 11, and 15, and bled on days 11, 15, and 18. The antigen was HuIgG.

TABLE X

Response to HuIgG in Mice Phototolerized Before Each Challenge

Group	Mice	UV	Skin sensitization			Challenge			Titer		
			1	2	3	1	2	3	Day 14	21	27
1	4 C3H*					+	+	+	80	1,280	2,560
2			+	+	+	+	+	+	640	2,560	2,560
3		+	+	+	+	+	+	+	0	0	160
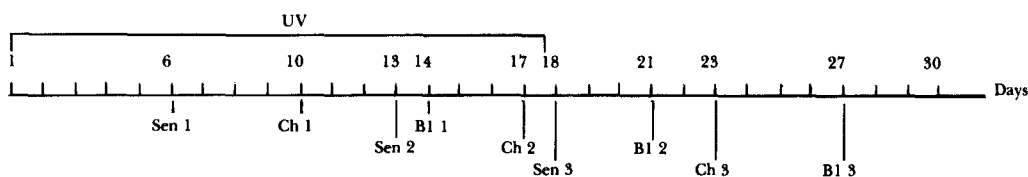
* Animals were UV-irradiated on days 1–18, skin-sensitized with HuIgG on days 6, 13, and 18, challenged intraperitoneally with HuIgG on days 10, 17, and 23, and bled on days 14, 21, and 27.

The data presented above (Tables I–IV, VI, and VII) were derived from single sensitization and challenge procedures within a 2-wk time frame. We also examined the effects of PT when antigen was repeatedly administered either intradermally or systemically. The results shown in Table VIII indicate that multiple skin sensitization in normal controls leads to vigorous antibody responses. However, if the sensitizations are performed on UV-irradiated skin, no reponse is elicited. Multiple challenges following a single PT regimen (Table IX) suggest that the unresponsiveness is short-lived. Specifically, no response is detected in the phototolerized mice after the first challenge, but successive challenges result in the emergence of a response (Table IX, group *4*). In contrast, if sensitization through a UV-irradiated skin site precedes each challenge, the antibody response is maintained at a low level (Table X).

Discussion

Several conclusions can be drawn from these studies. (*a*) PT can be demonstrated in C3H and BALB/c mice to several antigens, including both aggregated and deaggregated Ig. (*b*) PT induction is restricted to the irradiated skin site. (*c*) PT is elicited only if sensitization with antigen is via the cutaneous route. (*d*) Approximately 3–4 d of UV irradiation is sufficient for PT induction. (*e*) PT appears to be a short-lived phenomenon. Thus, suppression is observed only if

antigen challenge is given within 2 wk of sensitization and if skin sensitization is performed within 10 d of the UV irradiation. (*f*) If animals have been previously immunized, PT induction cannot inhibit an ongoing response. (*g*) PT appears to be antigen specific. (*h*) If antigen is given multiple times through the skin, UV-irradiated hosts do not appear to mount a response. If hosts are phototolerized once, subsequent systemic challenge with antigen multiple times will elicit a response. However, if PT induction is performed prior to each challenge, the response is maintained at a low level.

The mechanisms underlying PT are unknown. It appears that PT may involve a failure to prime for a response. This hypothesis is based on the observations that repeated cutaneous sensitizations through a UV-irradiated skin site do not elicit a response, but sensitization at a distant skin site during PT induction clearly elicits antibody. Further, multiple systemic challenges after PT eventually lead to specific antibody production. The emergence of this response is delayed, and when it appears, it resembles a primary response in that it is predominantly IgM (55–60%; data not presented). Thus, the animal does not respond to the sensitization, but can respond to some degree to the systemic challenge.

A defect in antigen presentation has a precedent in investigations of antigen-presenting cell function in UV-irradiated mcie (1, 9, 20–25). Other work (26, 27) has implicated antigen-specific suppressor cells in UV-induced DTH tolerance. Our attempts to demonstrate active suppression have included the use of adoptive transfer experiments and cyclophosphamide pretreatments (28) to reverse PT induction. Cyclophosphamide (200 mg/kg), administered 1–3 d before the initiation of UV irradiation, or 1–3 d before skin sensitization, was ineffective in restoring competence. Adoptive transfer experiments, using 5 × 10^7 cells from tolerant animals injected into normal syngeneic mice, also failed to demonstrate suppressor cells. Whole spleen, regional lymph node cells, and nylon-column-purified T cells from these tissues were used. Also, serum, does not appear to transfer suppression. Perhaps, however, UV irradition induces several defects and suppression may be observable only in a "prepared" host (4, 11, 27). Experiments currently in progress, using UV-irradiated recipients of transferred cells, suggest that a suppressor cell component may be present.

A potentially important practical application of this study is the induction of tolerance to foreign proteins. One of the major problems of therapy with mouse monoclonal antibodies is the eventual formation of an anti-mouse Ig response that not only limits the effectiveness of the therapeutic antibody, but also introduces the danger of an allergic response. Similar problems are encountered when other proteins (enzymes, hormones, etc.), derived from foreign species, are used in prolonged treatment regimens. Our data suggesting that small localized doses of UV plus "antigen" (given before each injection) can maintain tolerance are encouraging. We are currently investigating whether PT and high intravenous doses of antigen (simulating the condition used in monoclonal antibody therapy) may be effective in maintaining tolerance over long periods. Finally, it will be important to determine if soluble forms of transplantation antigens applied in a PT regimen induce sufficient suppression of the immune

response to be beneficial in organ transplantation. PT early in transplantation may prevent early rejection and allow other mechanisms of tolerance to become established.

Summary

C3Hf/HeN or BALB/c mice, exposed to acute ultraviolet (UV) irradiation and skin-sensitized through the irradiated skin site with soluble protein antigens, exhibit humoral tolerance to subsequent systemic challenge with antigen. We have termed this phenomenon "phototolerance" (PT). With the doses of UV radiation used, PT induction is retricted to the irradiated skin site and is observed only if sensitization is performed via the cutaneous route. PT is antigen specific and operates at the afferent level of the immune response. While single PT induction regimens result in transient humoral suppression, multiple inductions before each systemic challenge can maintain the response at low levels. The capacity to induce PT to a variety of soluble protein antigens may have potentially important clinical applications.

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