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Immunology

Systemic alteration induced in mice by ultraviolet light irradiation and its relationship to ultraviolet carcinogenesis

(immune surveillance/tumor immunology/immunosuppression)

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ABSTRACT **Chronic irradiation of mice with ultraviolet (UV) light produces a systemic alteration of an immunologic nature. This alteration is detectable in mice long before primary skin cancers induced by UV light begin to appear. The alteration results in the failure of UV-irradiated mice to reject highly antigenic, transplanted UV-induced tumors that are rejected by unirradiated syngeneic recipients. The immunologic aspect of this systemic alteration was demonstrated by transferring lymphoid cells from UV-irradiated mice to lethally x-irradiated recipients. These recipients were unable to resist a later challenge with a syngeneic UV-induced tumor, whereas those given lymphoid cells from normal donors were resistant to tumor growth.**

Parabiosis of normal mice with UV-irradiated mice, followed by tumor challenge of both parabionts with a UV-induced tumor, resulted in the growth of the challenge tumors in both UV-irradiated and unirradiated mice. Splenic lymphocytes from tumor-implanted UV-treated mice were not cytotoxic *in vitro* against UV-induced tumors, whereas under identical conditions cells from tumor-implanted, unirradiated mice were highly cytotoxic. Our findings suggest that repeated UV irradiation can circumvent an immunologic mechanism that might otherwise destroy nascent UV-induced primary tumors that are strongly antigenic.

Most skin tumors induced in mice by chronic UV irradiation are highly antigenic and can be transplanted only in immunosuppressed recipients (1). Because a high proportion of these noncross-reacting tumors are immunologically rejected upon transplantation to normal syngeneic recipients, we wished to discover how these tumors are able to arise and grow progressively in their original hosts.

Our recent work suggested that chronic treatment with UV light altered the host in some way, and made it unable to eliminate these highly antigenic tumors (2). We found that long before primary tumors appeared, the UV-treated mice were unable to resist transplants of UV-induced tumors, even though such transplants were rejected by unirradiated animals. The progressive growth of these tumors in UV-treated mice was due to a systemic alteration in the animals, induced by a relatively short course of UV irradiation of the skin (3). Although this systemic alteration prevented the immunologic rejection of syngeneic UV-induced tumors, it did not affect the ability of UV-treated animals to reject skin and tumor allografts (2).

In the following experiments we are continuing our attempts to characterize this UV light-induced systemic alteration. Specifically, we question whether or not the systemic alteration has an immunologic nature.

Abbreviation: CMEM, Eagle's minimal essential medium supplemented with 10% fetal bovine serum, vitamin solution, sodium pyruvate, nonessential amino acids, L-glutamine, and penicillin-streptomycin. One rad equals 0.01 J/kg.

MATERIALS AND METHODS

Mice. Specific pathogen-free mice of the inbred strain C3H/HeN(MTV$^-$) were supplied by the Frederick Cancer Research Center Animal Production Facility. The mice were started on their regimen of UV irradiation at 6–8 weeks of age.

UV Irradiation. The light source was a bank of six Westinghouse FS40 Sunlamps, which delivered an average dose rate of 2.8 J/m^2 per s over the wavelength range of 280–340 nm. This range includes approximately 80% of the total energy output of the lamps. Five mice were housed per cage on a shelf 20 cm below the fluorescent lamps, and the cage order was systematically rotated before each treatment to compensate for the uneven lamp output along the shelf. The dorsal hair of the mice was removed with electric clippers once per week. The mice were irradiated for 1 hr, three times per week (Monday, Wednesday, and Friday) for a minimum of 3 months prior to their use. The mean time of tumor appearance for this regimen of UV treatment is approximately 32 weeks, with a range of 22 to 42 weeks. None of the animals had developed primary tumors from the UV irradiation at the time of these experiments. Age-matched, untreated mice served as controls for the UV-treated groups.

Tumor Transplantation. The tumors used for implantation were fibrosarcomas induced in C3H$^-$ mice by the UV treatment schedule described above. They were maintained by serial passage in immunosuppressed, syngeneic recipients. Immunosuppression was produced by adult thymectomy and sublethal whole-body x-irradiation (450 rads; 4.5 J/kg). The tumors were in the third to the fifth transplant generations at the time of these experiments. Mice were challenged with tumors that were excised, cut into 1 mm^3 fragments, and transplanted subcutaneously on the ventral side of the recipient with a trocar. The recipients were inspected once a week for at least 2 months for tumor growth, and the tumor sizes were measured.

Parabiosis. Formation of a common circulation between pairs of age-matched female mice was accomplished by the technique of Eichwald *et al.* (4). An incision was made through the skin and panniculus in each anesthetized partner from the knee to the elbow joints in the sides to be joined. A subpannicular space, extending approximately 1 cm around the perimeter of the incision, was formed by blunt dissection. The mice were tied together with heavy thread at the knee and elbow joints, and the skins of the partners were joined around the circumference of the incisions with a continuous row of wound clips. The mice were allowed to recover for 3 weeks before tumor challenge. At the conclusion of the experiment, the parabionts were tested for blood vessel anastomosis by injecting trypan blue dye intraperitoneally into one partner and

Table 1. Growth of transplanted UV-induced tumor (no. 1463) in parabiotic mice

Parabiotic partners	Untreated: untreated	UV-irradiated: UV-irradiated	Untreated: UV-irradiated
Tumor incidence*	1/9:0/9	10/10:10/10	15/16:15/16
Totals	1/18	20/20	30/32†

* Number of progressively growing tumors/number of mice challenged.
† The two mice that failed to develop tumors were not paired with each other.

checking the other for blue coloration 4 hr later. All pairs were shown to have a common circulation by this method.

Cell Transfer Studies. Tumor-immune donors were immunized by subcutaneous implantation of tumor fragments. These fragments regressed in about 2 weeks; 4 weeks after immunization the animals were reimplanted, and their lymphoid cells were collected 10 days later.

Lymphoid cell suspensions were prepared from the spleens and lymph nodes of groups of normal, UV-irradiated, and tumor-immune mice. The organs were collected in cold RPMI 1640 medium, pressed through a wire screen, and the suspension was filtered through nylon mesh. The cells were washed once and resuspended in RPMI 1640 medium. Their viability was >80% as judged by trypan blue exclusion. The cells were diluted to a concentration of 5×10^7 viable, nucleated cells in 0.5 ml, and were injected intravenously into recipient groups. Tumor challenges were given 24 hr after the cell transfers. Some recipient groups were given 850 rads (8.5 J/kg) whole-body x-irradiation with a Philips MG 301 x-ray therapy unit, 24 hr before cell transfer. The x-ray unit was operated at 300 kV constant potential and 10 mA. The beam characteristic was half-value layer, 8.70 mm aluminum.

Microcytotoxicity Test *In Vitro*. The establishment of tissue culture lines of the UV-induced tumors, their properties *in vitro*, and their antigenic characterization in this assay have been described (5). Cell cultures were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, vitamin solution, sodium pyruvate, nonessential amino acids, L-glutamine, and penicillin-streptomycin. The components of this complete medium (CMEM) were obtained from Flow Laboratories (Rockville, MD). Tumor cells in exponential growth were harvested by trypsin treatment for 1 min (0.25% trypsin) of the monolayers, then the cells were washed and resuspended in CMEM at a concentration of 1×10^6 viable cells per ml.

The test *in vitro* used to detect cell-mediated immunity against syngeneic UV-induced tumors was the microcytotoxicity assay described by Lucas and Walker (6), as modified by Fortner *et al.* (7) for use with syngeneic tumor target cells. Briefly, 2×10^3 tumor target cells in 2 μl CMEM were plated in microtiter plates (Falcon 3034 Microtest, Falcon Plastics, Oxnard, CA). The plates were incubated at 37° in a humidified atmosphere containing 5% CO_2. After the cells were attached to the plate, 5 μl of CMEM was added to the wells and incubation was continued for 24 hr.

Spleen cell suspensions were prepared as described above; erythrocytes were removed by lysis with 0.14 M NH_4Cl/0.17 M Tris-buffered, and the lymphoid cell suspension was incubated on a column containing glass wool for 15 min at room temperature to remove adherent cells. The eluate consisted of >95% viable lymphocytes. A suspension of 1×10^5 lymphocytes in 10 μl of CMEM was added to the tumor target cells, and incubation was continued for 40 hr. The plates were washed extensively, and 0.125 μCi ^{86}Rb (0.5 Ci/g, New England Nuclear, Boston, MA) was added to each well. After a 2-hr incubation, the extracellular ^{86}Rb was removed by washing the cells. The intracellular ^{86}Rb was released by saponin lysis and assayed for radioactivity in a liquid scintillation counter. The percentage of cytotoxicity was determined by comparing the amount of ^{86}Rb taken up by the residual tumor cells in wells containing normal lymphocytes versus wells containing lymphocytes from tumor-sensitized mice, according to the formula:

$$\% \text{ Cytotoxicity} = 100\left[1 - \frac{\text{cpm (tumor cells + sensitized lymphocytes)}}{\text{cpm (tumor cells + normal lymphocytes)}}\right],$$

where cpm is the average cpm of samples from five to eight replicate wells.

Table 2. Growth of UV-induced tumor (no. 1591) in mice injected with lymphoid cells

Treatment of lymphoid cell donors*	Treatment of recipients: None	850 rads†	UV	UV + 850 rads†
UV irradiation	0/10‡	17/17	—	16/17
None	—	5/18	10/10	19/19
Immunization with tumor no. 1591	—	0/12	14/25	7/27
Media control	1/25	—	26/26	—

* Viable, nucleated spleen and lymph node cells (5×10^7) were given to recipients intravenously 24 hr before challenge.
† Whole-body x-irradiation (850 rads) was given 24 hr before lymphoid cell transfer.
‡ Ratio of the number of mice with progressive tumor to the number challenged.

RESULTS

Parabiosis. In this experiment, a common circulation was established between pairs of UV-treated and untreated mice, and both members of the pairs were challenged 3 weeks after parabiosis with a tumor implant. Table 1 shows that the tumors regressed in most parabiotic, nonirradiated mice, while all tumors grew progressively in pairs of UV-treated animals. When unirradiated mice were joined parabiotically with UV-treated mice, the challenge tumors grew progressively in nearly all recipients. This suggested that the UV-treated mice might have exerted some type of suppressive influence on their unirradiated partners.

Cell Transfer Studies. To clarify this finding, cell transfer studies were performed (Table 2). The transfer of 5×10^7 lymphoid cells from UV-treated donors to untreated recipients had no effect on the ability of the latter to reject a tumor challenge. However, when the recipients were lethally x-irradiated prior to transfer of these lymphoid cells, they were unable to reject a tumor challenge, even though normal lymphoid cells restored the rejection response in 72% (13/18) of these mice. This demonstrates that the lymphoid cells from UV-treated mice lack the capacity to mediate tumor rejection *in vivo*. Lymphoid cells from untreated donors failed to effect tumor rejection in UV-treated and UV-treated lethally x-irradiated mice. Thus, normal lymphoid cells failed to function in UV-treated mice, even when these recipients' own lymphoid tissues were depleted by a lethal dose of x-rays. Adoptive transfer of

Table 3. Growth of UV-induced syngeneic (no. 1591) or allogenic (no. 112) tumors in mice after cell transfer

Recipients		UV-induced challenge tumor	
Strain	Treatment	C3H⁻ no. 1591	C57BL no. 112
C57BL	None	—	8/10*
C3H⁻	None	1/10	0/10
	850 rads + normal lymphoid cells†	2/8	0/10
	850 rads + UV-irradiated lymphoid cells‡	10/10	0/10
	UV, 850 rads + normal lymphoid cells	10/10	0/9
	UV, 850 rads + UV-irradiated lymphoid cells	8/8	0/10

* Ratio of the number of mice with progressive tumors to the number challenged.

† Viable, nucleated spleen and lymph node cells (5×10^7) were given to recipients intravenously 24 hr after x-irradiation and 24 hr before challenge.

‡ Spleen and lymph node cells (5×10^7) from 3-month UV-irradiated donors were given as above.

cells from immunized mice gave complete tumor immunity to non-UV-treated x-irradiated mice (12/12 resistant), partial immunity to UV-treated mice (44%), and nearly complete immunity to UV-treated x-irradiated mice (72%).

The preceding experiment demonstrated that lymphoid cells from UV-irradiated mice are unable to effect the rejection of a UV-induced syngeneic tumor. To test the specificity of this immunosuppression, we repeated the experiment, but challenged only half the mice with the syngeneic tumor. The other half received implants of allogeneic UV-induced tumor which grows progressively in its strain of origin (C57BL). The data in Table 3 show that although the mice given lymphoid cells from UV-irradiated donors were unable to reject the syngeneic tumor (no. 1591), they all rejected the allogeneic tumor transplants (no. 112). In addition, when UV-irradiated mice were treated with 850 rads and reconstituted with normal lymphoid cells, they also failed to reject the syngeneic tumor, but did reject the tumor allografts. Thus, the immunosuppression was specific for UV-induced tumor antigens, and did not include reactivity to major histocompatibility antigens.

The failure of lymphoid cells from UV-treated mice to mediate syngeneic tumor rejection in lethally x-irradiated recipients suggested two possibilities. Either the precursors for cell-mediated reactivity against UV-induced tumors were absent or inactivated, or a specific suppressor cell population was preventing the expression of reactivity. A test for the presence of suppressor cells is summarized in Fig. 1. Reconstitution of lethally x-irradiated mice with normal lymphoid cells restored their ability to reject tumor no. 1591 almost to the level exhibited by untreated mice (4/10 resistant versus 5/10 resistant). Reconstitution of lethally x-irradiated mice with cells from UV-treated donors again did not provide protection against tumor challenge (10/10 susceptible). A mixture of equal numbers of cells (5×10^7) from normal and UV-treated recipients gave the same tumor incidence (10/10) and pattern of tumor development as seen in the mice given 5×10^7 cells from UV-treated donors only. This suggested that the lymphoid cells from the UV-irradiated mice actively suppressed the reactivity of the normal lymphoid cells.

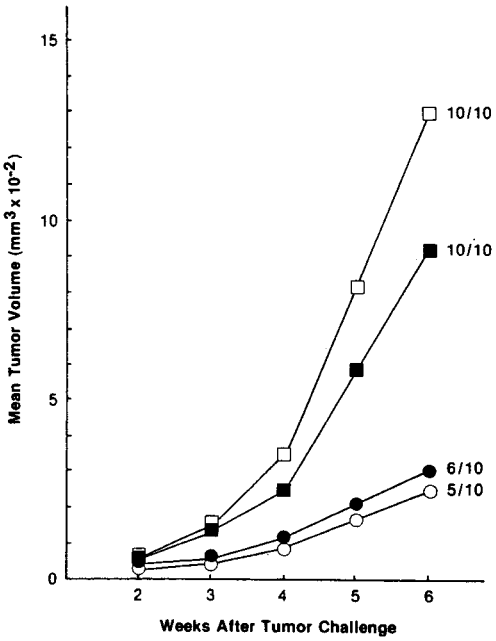

FIG. 1. Average growth rates of tumor no. 1591 in untreated mice (○), mice given 850 rads + 5×10^7 normal lymphoid cells (●), 850 rads + 5×10^7 lymphoid cells from UV-treated mice (□), or 850 rads + a mixture of 5×10^7 normal lymphoid cells and 5×10^7 cells from UV-treated mice (■). Lymphoid cells were injected 24 hr after x-irradiation, and tumor fragments were implanted 1 day later. Tumor volumes are the product of three tumor diameters, and the means include values of 0.

Reactivity *In Vitro*. Groups of untreated and UV-irradiated mice were given two implants of either tumor nos. 1591 or 1316. At various times thereafter, pooled spleen cells from two mice in each group were tested for cytotoxicity *in vitro*. Additional mice were kept for 2 months to determine the fate of the tumor grafts *in vivo*. All 10 implants (5 mice) of tumor no. 1591 and all 10 implants of tumor no. 1316 regressed in the unirradiated mice while the 10 implants of each of these tumors grew progressively in the UV-irradiated animals. Figs. 2 and 3 show the lymphocyte reactivity of these groups against cultured 1591 or 1316 target cells. Significant reactivity against the immunizing tumor was detected with lymphocytes from the unirradiated mice on days 8–14. The cytotoxicity was specific for the immunizing tumor, as there was no reactivity against the unrelated tumor line. Cells from tumor-implanted UV-irradiated donors showed no reactivity relative to lymphocytes from UV-treated mice not given tumor implants.

DISCUSSION

These experiments show that there is an immunologic defect in UV-treated mice that precedes the development of primary skin cancers. This defect seems to be specific for the neoantigens on UV-transformed cells, because reactivity to allogeneic tumors is unaltered. The defect was demonstrated *in vivo* by transferring lymphoid cells from UV-irradiated mice to lethally x-irradiated recipients. These recipients were unable to resist a later challenge with a syngeneic UV-induced tumor, whereas those given lymphoid cells from normal donors became resistant to tumor challenge. When tested *in vitro*, lymphocytes from tumor-implanted UV-treated donors failed to exhibit reactivity under conditions where cells from tumor-implanted unirradiated mice were highly cytotoxic. The generation of cytotoxic splenic lymphocytes after tumor implantation could not be

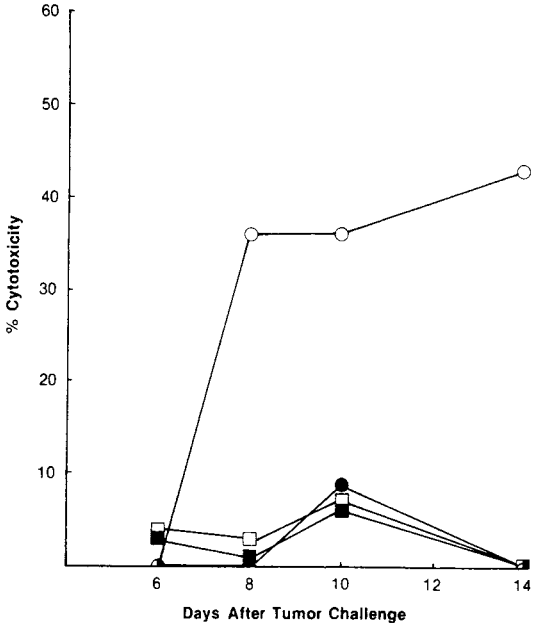

FIG. 2. Cytotoxicity *in vitro* against tumor no. 1316 of splenic lymphocytes from mice receiving implants of tumor no. 1316 on day 0 (○), tumor no. 1316 and UV treatment (●), tumor no. 1591 (□), or tumor no. 1591 and UV treatment (■). Reactivity at the four time points was measured in independent assays. On days 8, 10, and 14, the cytotoxicity of cells from mice implanted with tumor no. 1316 (○) is statistically significant ($P < 0.01$) by Student's t-test.

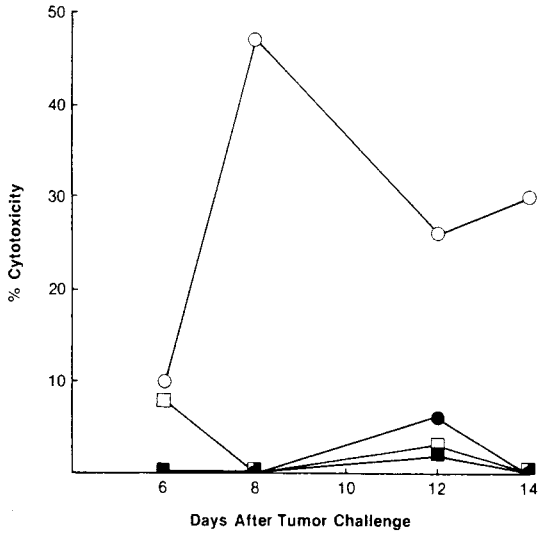

FIG. 3. Cytotoxicity *in vitro* against tumor no. 1591 of splenic lymphocytes from mice receiving implants of tumor no. 1591 on day 0 (○), tumor no. 1591 and UV treatment (●), tumor no. 1316 (□), or tumor no. 1316 and UV treatment (■). Reactivity at the four time points was measured in independent assays. On days 8, 12, and 14, the cytotoxicity of cells from mice implanted with tumor no. 1591 (○) is statistically significant ($P < 0.01$) by Student's t-test.

detected in UV-treated mice. Whether this defect resulted from the absence (real or functional) of reactive cells or the presence of suppressor cells could not be determined from the study *in vitro*.

The experiment in which lymphoid cells from normal and UV-treated mice were mixed prior to adoptive transfer suggests that specific suppressor cells are responsible for the lack of reactivity. In addition, parabiosis of normal and UV-treated mice resulted in both partners becoming susceptible to tumor challenge. This is reminiscent of the transfer of classical immunologic tolerance to skin allografts by parabiosis of normal and tolerant syngeneic mice (8), and suggests an active suppression, rather than an absence of reactive cells. One experiment was inconsistent with the suppressor cell interpretation. The injection of lymphoid cells from UV-treated mice directly into normal recipients did not render them susceptible to tumor challenge, as might have been expected were suppressor cells involved. However, the number of cells injected (5×10^7) might have been insufficient to suppress an intact, immunocompetent recipient.

Regardless of its underlying mechanism, the immunologic deficiency in the UV-treated mice seems to involve the early stages of the immune response against tumor antigens, rather than a direct inhibition of effector cells. Lymphoid cells from specifically-immunized donors transferred some degree of immunologic resistance to UV-treated recipients and a high level of immunologic reactivity to UV-treated mice given lethal x-irradiation earlier. This is consistent with our earlier finding that mice immunized to a UV-induced tumor and then irradiated with UV light retained their ability to reject the immunizing tumor, but were susceptible to challenge with unrelated UV-induced tumors (2). These results indicate that the effector cells responsible for tumor rejection can function in UV-irradiated hosts. Therefore, the immunologic defect must involve either the absence of antigen-reactive cells specific for UV-induced tumor antigens, or a block in the pathway of differentiation of these cells *in vivo* into effector cells.

The factors responsible for inducing the immunologic anergy have not been identified. The immunologic alteration is probably not due to the direct toxic effect of UV light on lymphoid cells demonstrated *in vitro* (9), because this *in vivo* anergy is selective and does not include reactivity to all antigens. In addition, the transfer of normal lymphoid cells to lethally x-irradiated UV-treated recipients failed to restore tumor rejection. This suggests that the factors responsible for inducing the immunologic defect are not destroyed by 850 rads, and, thus, may be inducing the same alteration in the newly transferred lymphoid cells. It seems most likely that the immunologic defect is secondary to a direct effect of UV radiation on the skin. For example, the production of soluble antigens as a result of UV-induced skin damage might lead to the induction of immunologic tolerance or suppressor cells, rather than to the production of effector cells (10).

The finding that a selective immunologic defect precedes the appearance of UV-induced primary tumors suggests that an immune surveillance system of the type postulated by Burnet (11) may actually exist for cells transformed by UV radiation. If so, one would expect these transformed cells to develop into visible tumors only after the surveillance system were circumvented. This might explain the observation that regardless of the UV dose, there is a long latent period before primary tumors begin to appear (12). Perhaps this represents a period during which there is immunologic elimination of nascent tumor cells. Thus, tumors would begin to grow progressively only after this immunologic mechanism were depressed, either selectively by UV irradiation, or nonspecifically by aging or immunosuppressive therapy. The reported increase in the incidence of UV-associated skin cancer and its early age of onset in immunosuppressed renal transplant patients (13) is consistent with this possibility.

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