# THE IMMUNOREGULATORY EFFECTS OF ULTRAVIOLET EXPOSURE: EARLY EVENTS

by

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## ABSTRACT

Ultraviolet (UV)-irradiation of mice is known to cause a modification in their immune potential such that exposed mice lose their ability to reject transplanted UV-induced tumors. Further, this tumor susceptibility is mediated by a population of suppressor T lymphocytes (Ts) found in the spleen and lymph nodes of UV-exposed animals which will adoptively transfer tumor susceptibility to normal animals. As the induction of these Ts cells is undoubtedly a complex process, my studies were initiated to elucidate the early events involved in this process. It was found that sunscreen agents such as para-aminobenzoic acid (PABA), while able to prevent the pathological skin damage associated with the UV exposure, were without affect on the induction of tumor susceptibility. To date, however, I have been unable to detect $T_s$ cells in PABA-treated UV-irradiated mice.

Animals exposed to six daily UV exposures (about 3000 $J/m^2/day$) have been reported to exhibit decreased antigen-presenting and accessory cell function (assayed solely from the spleen). My investigations have confirmed this finding. Further, the results demonstrated that this is: 1) probably due to UV-induced inflammation and; 2) the result of a migration of antigen-presenting cells (APC) from the spleen to the peripheral lymph nodes. These results are consistent with previous studies which found that UV-irradiation does not cause a generalized suppression in immune responses.

While regional differences in APC activity may be attributable to cellular migration, a direct inactivation of epidermal Langerhans cells (the epidermal APC) also occurs (as assessed in vitro) following UV exposure. My results demonstrated that the APC function of epidermis was immediately lost in a dose-dependent manner following UV-irradiation. Further, treatment of skin with PABA prior to the UV exposure did not significantly alter the rate of Langerhans cell inactivation. Abrogation of contact sensitivity responses after a single UV exposure, however, required two to three days between exposure and sensitization. Collectively, these data indicate that early in the course of the treatments, UV-irradiation causes both a direct inactivation of epidermal APC and a migration of splenic APC to peripheral lymph nodes. Hence, these events may play a role in the cellular interactions involved in the induction of the Ts cells which mediate the UV-induced tumor susceptible state.

CONTENTS

# INTRODUCTION

The exposure of mice to ultraviolet (UV) radiation results in a multitude of effects which range from subtle immunoregulatory changes to overt carcinogenesis (1-5). The intent of this dissertation is to describe some of the early events which are involved in these changes and to relate these events to our current understanding of cellular immunology.

The generation of an immune response is a complex and dynamic process which is characterized by a high degree of specificity for the immunizing antigen. This specificity is due to activation of antigen-specific clones of lymphocytes by a complex system of cellular interactions. The principal cell types involved in this process are antigen-presenting cells (APC), helper T lymphocytes, effector cells and suppressor cells (6).

The cell type first involved in the generation of an effective immune response is the macrophage-like, antigen-presenting cell (APC). These cells are responsible for processing and presentation of antigen to the lymphocytes. Effective antigen recognition and activation of antigen-specific lymphocytes also requires the presence of products of immune response genes (Ia antigens), an interaction molecule, and the secretion of a soluble factor termed interleukin one (IL-1). These conditions then lead to stimulation of antigen-specific clones of lymphocytes.

Helper T lymphocytes ($T_H$) recognize both Ia and antigen on the surface of the APC and, in the presence of IL-1, are activated. These cells then proliferate resulting in the expansion of the antigen-reactive clones. Following activation, $T_H$ cells (perhaps a subpopulation) also secrete a second soluble factor termed interleukin two (IL-2), or T cell growth factor, which is required for the proliferation of both helper and effector T lymphocytes (7). Different subpopulations of $T_H$ cells apparently are involved in different aspects of the ongoing immune response such as stimulation of the different effector cells, i.e., the stimulation of precursors for cytotoxic T cells ($T_K$) versus antibody producing cells, B cells. Another factor, different from IL-2, termed B cell growth factor, has recently been described as necessary for B cell proliferation (8). Thus, with appropriate antigenic stimulation and the presence of the soluble factors, the precursors of the effector cells, T prekillers and small B lymphocytes also undergo proliferation and differentiation into mature effector cells. It should be noted that, for clarity, I have described these interactions individually. In reality, however, many of these reactions are occurring simultaneously during the generation of an immune response.

The regulation of these specific but potentially destructive responses is the domain (although not exclusively) of the suppressor T lymphocytes ($T_s$). The induction of these $T_s$ cells probably occurs as a normal consequence of the generation of an immune response. In addition though, certain conditions can lead to the

preferential induction of $T_s$ cells and thus prevent the appearance of effective immunity. One interesting example of this phenomenon is the response generated by certain mice to hen egg lysozome (HEL). In this system, it has been demonstrated that one part of the molecule causes the generation of $T_s$ cells which are able to prevent an immune response toward other immunogenic parts of HEL (9). This phenomenon, termed associative recognition, has been observed in other systems including the UV-induced tumor system described below (10,11).

The role of $T_s$ cells in the regulation of immune responses has led to a number of studies on the mechanisms involved in the induction of these cells. One possibility, suggested by both in vitro and in vivo studies, is that in the absence of effective antigen-presentation by APC, $T_s$ cell precursors are preferentially stimulated. Thus, the antigenic stimulation of lymphocytes in vitro in the absence of Ia positive adherent cells (characteristics of APC), has been shown to result in the induction of $T_s$ cells in a number of systems (12-15). These results have led, in turn, to the suggestion that some of the immunoregulatory modifications caused by UV radiation (discussed below) may be a direct result of UV-inactivation of antigen-presenting cells (4,5,16). In this regard, several studies have demonstrated that UV-irradiation of immunocytes in vitro causes both a functional inactivation and, a later dramatic reduction in cell viability (17-20). Further, one recent study on antigen-presenting function suggests that UV-irradiation destroys the cells' (APC) ability to provide

both IL-1 and the necessary processing and presentation (21). This ability of UV radiation to inactivate APC functionally is, in fact, one of the central themes of this dissertation.

The carcinogenic potential of UV radiation has been recognized for decades(2,3). Only in the past decade, however, have we come to recognize that UV exposure also causes an inability to mount a normal anti-tumor immune response(4,5). The discovery of this tumor susceptibility was a direct result of the unusual growth characteristics of the majority of UV-induced tumors. Approximately three-quarters of the tumors which arise as a result of repeated UV treatment ( > 20 weeks of 3000 $J/m^2$/day, 5 days/week) are rejected when transplanted into normal syngeneic mice. These UV regressor tumors are capable of progressive growth, however, in immunosuppressed mice or mice which have received at least two weeks of UV treatment(22-25).

The tumor susceptibility, once induced, is a long lasting state mediated by a population of $T_s$ cells specific for tumor antigen (26-29). It is not the result of a pan-immune suppression. Thus, it has been repeatedly demonstrated that tumor susceptible mice are able to mount normal immune responses to numerous antigens (other than tumors) both in vitro and in vivo (30,31). One noted exception to this is the response of these mice to contact sensitizing agents topically applied to irradiated skin. Under these circumstances a decreased contact sensitivity response has been observed (32,33). This phenomenon is important and will be discussed further below.

The role of $T_s$ cells in the UV-induced tumor system was demonstrated by adoptive transfer experiments. Specifically, the tumor susceptible state can be transferred from UV-irradiated mice to normal mice with a population of T cells which are nylon wool non-adherent, Ia positive and functionally very sensitive to gamma radiation (34). The functional life-span of these adoptively transferred $T_s$ cells is only 3-4 weeks (29). UV-exposed mice, however, remain tumor susceptible long after cessation of the UV treatments (23, 29). Because of this finding and other experiments it has been postulated that UV exposure causes a permanent somatic change which 1) results in the appearance of new antigenic determinant(s) in the exposed skin (the antigenic stimulus for Ts cell induction) and 2) maintains the population of Ts cells long after the cessation of UV-irradiation.

Functional assays of tumor growth in vivo or tumor killing in vitro have demonstrated that certain responses recognize only the tumor used for challenge while other responses show cross-reactivity between tumors (4). To distinguish between the specific versus the cross-reactive response, we refer to these as anti-TSTA (tumor-specific transplantation antigen) and anti-TAA (tumor-associated antigen) responses respectively. However, it must be remembered that these are functional definitions. The actual differences between the different antigenic determinants are not certain. With this caveat in mind, the specificity of the $T_s$ cells induced by UV exposure appears to be anti-TAA while cytotoxic T cells ($T_k$) demonstrate both anti-TSTA and anti-TAA speci-

ficity. Thus, in vivo, the presence of the $T_s$ cells prevents the appearance of both specificities of $T_k$ cells. This scheme is illustrated in Figure 1. As shown in the figure, recent evidence suggests that the $T_s$ cells do not affect the afferent of inductive phases of immunity. Rather the available evidence suggests that it is the final differentiation or functional expression of the $T_k$ cell that is inhibited by the $T_s$ cells present in UV-exposed mice (35).

As mentioned above, the other immune response which is altered by UV treatment is the induction of contact sensitivity responses. This phenomenon occurs after only one week of UV treatment and is localized to the irradiated skin even after five weeks of UV treatment (33). Thus, mice which have received five weeks of UV treatment generate normal contact sensitivity if the sensitizing agent is applied to non-exposed ventral skin. Further, application of the contact sensitizing agent to irradiated skin results in a state of antigen-specific tolerance which is transferable from tolerant to naive animals with spleen cells (32, 33).

The loss of reactivity toward contact sensitizers, following UV exposure, is thought to reflect an inactivation of antigen-presenting cells in the irradiated epidermis. This was suggested by studies showing that UV radiation also caused an apparent loss in both the expression of Ia antigens and ATPase activity of Langerhans cells (the epidermal APC) in the exposed epidermis (reviewed in 36, 37). The Langerhans cells (L.C.) are thought to be the antigen-presenting cells in epidermis as they are a migra-

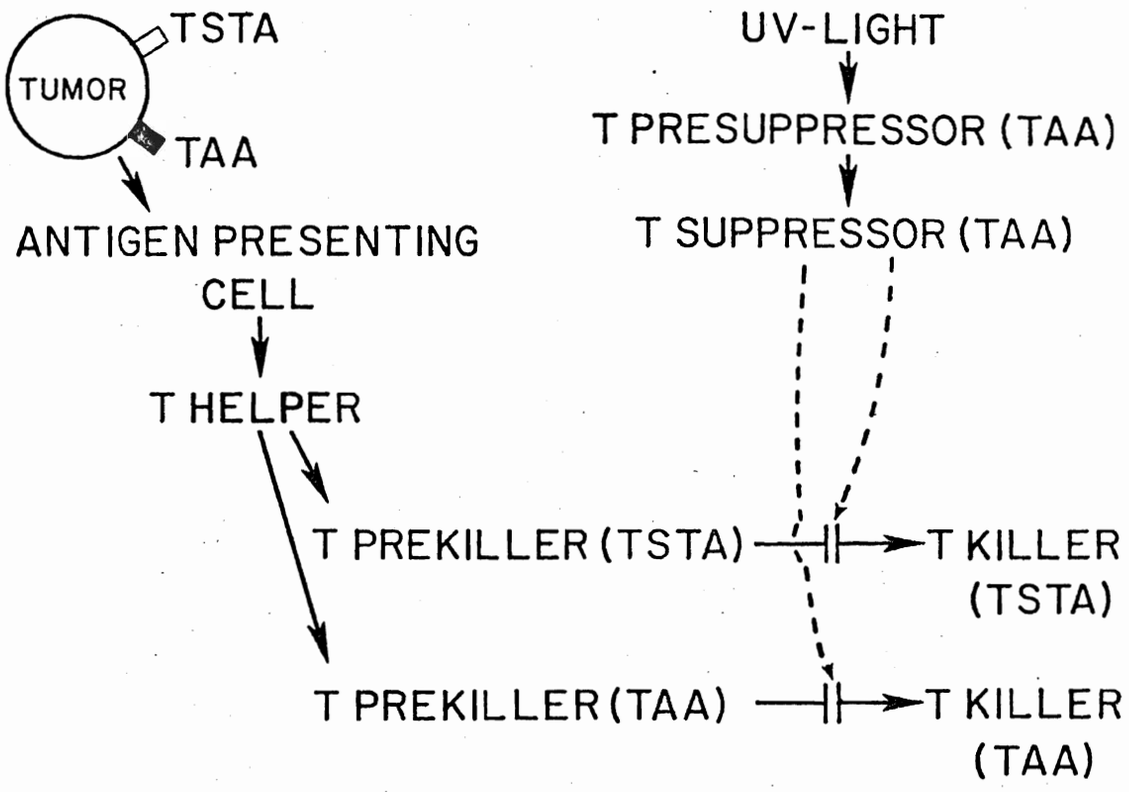


Figure 1 illustrates the cells involved in the induction of an effective anti-tumor immune response and the level at which UV-induced T suppressor cells are felt to block this induction

tory, bone-marrowed derived, macrophage-like cell. In addition, L.C. are the predominant (perhaps the only) Ia positive epidermal cell under normal conditions. It should be noted, however, that keratinocytes secrete an IL-1 like molecule (epidermal cell-derived, thymocyte activating factor; ETAF) and, although APC activity is contained in suspensions of epidermal cells enriched for L.C., L.C. have not been obtained in a homogenous suspension (38). Hence, it is possible that epidermal APC activity is a result of synergism between Ia positive LC, which present the antigen to lymphocytes, and keratinocytes, which produce the second signal, ETAF. Verification of the ability of L.C. to both present antigen and to provide the second signal awaits better purification procedures. In any case, the similarity between the tumor susceptibility and the reduction in contact sensitivity responses caused by UV-irradiation (both transferable states of antigen-specific tolerance) suggests a model for the former phenomenon based on inactivation of epidermal Langerhans cells by UV radiation.

The working hypothesis to explain induction of the $T_s$ cells by UV radiation is divided into 3 basic parts. The first step in this process is proposed to be the inactivation by UV of APC function in the exposed epidermis. This is followed by the appearance of new or re-expressed antigen(s) in the exposed skin which cross-reacts with determinants found later on tumors. The appearance of the skin-associated antigen(s) in the presence of functionally inactivated APC, then leads to the preferential

induction of antigen-specific $T_s$ cells as occurs with contact sensitizing agents when applied to the irradiated epidermis.

This hypothesis presents two testable results. The first is the effect of UV-irradiation of skin on epidermal APC activity. The second is the evaluation of exposed skin for the appearance of antigens which cross-react with tumor antigens. In my studies, I have confined the investigations to evaluating the effects of UV radiation on epidermal APC activity.

Recently, it has been reported that six daily UV treatments cause a loss of APC activity in the spleens of exposed mice (16). Inasmuch as this phenomenon also occurs prior to the induction of the tumor susceptible state, it warrants further consideration. Thus, I have evaluated whether this 1) reflects a localized or systemic loss in APC function; 2) is a permanent or reversible modification; and 3) could be due to UV-induced inflammation which causes a systemic migration of cells with APC function.

The final aspect considered in these investigations is the role of the skin damage caused by UV treatment. By employing various sunscreen agents such as para-aminobenzoic acid, (PABA), it has been shown that both histological skin damage and the appearance of skin tumors following UV-irradiation are inhibited (39-42). However, it has also been shown that photoprotected (with PABA), UV-irradiated animals are unable to support the induction of normal contact sensitivity responses when the sensitizing agent is applied to irradiated skin (33). Thus, if the

decrease in contact sensitivity responses is due to inactivation of epidermal APC activity by UV radiation and, this loss also plays a role in the induction of $T_s$ cells as proposed, PABA should have little or no effect on 1) epidermal APC function following UV exposure, nor on 2) the induction of the tumor susceptible state. Both of these predictions have been tested and the results are presented in the following chapters.

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# CHANGES IN ANTIGEN-PRESENTING CELL FUNCTION IN THE SPLEEN AND LYMPH NODES OF ULTRAVIOLET-IRRADIATED MICE[1]

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**It has been previously reported that mice exposed to ultraviolet (UV) radiation exhibit a decrease in splenic antigen-presenting cell (APC) function. The results presented here confirm this observation and further demonstrate that animals exposed daily to UV for extended periods of time (5 weeks instead of 6 days) no longer exhibit this depressed capability. In spite of the depression in splenic APC activity found in 6-day UV-irradiated mice, lymph node APC function from these same animals was elevated compared with that found in the lymph nodes from normal animals. Lymph node APC activity in animals that were splenectomized prior to the UV irradiation, however, was not enhanced over controls. Treatment of animals with a chemical irritant (turpentine) also caused a depression in splenic APC function without modifying lymph node activity. Collectively, our findings suggest that the observed decrease in splenic APC activity, found after the first week of UV exposures, may be attributable to the migration of splenic APC to peripheral lymphoid tissue which drain the site of epidermal inflammation.**

Exposure of animals to UV radiation can cause numerous biological effects. Even suberythemal doses of UV are capable of inhibiting the ability of exposed mice to mount normal contact sensitivity responses to reactive chemicals applied to the irradiated sites (*1, 2*). This diminished contact sensitivity reactivity appears to be attributable to a functional inactivation of the epidermal APC, the Langerhans cell (*1, 2*). In addition, the alteration is reversible since irradiated skin regains full potential 2 weeks after cessation of the UV treatments (*1, 2*).

Exposure to erythemogenic doses of UV results in inflammation, leukocytic infiltration, hyperkeratosis, hyperplasia, and hyperpigmentation of the exposed skin. In mice, repeated exposure (greater than 20 weeks) eventually results in tumor formation (*3*). Long before the occurrence of neoplasias however, UV-irradiated mice lose the ability to mount effective antitumor responses (*4, 5*). Further studies demonstrated that the unresponsive state was specific for tumor-associated antigens present on UV-induced tumors since UV-irradiated mice were capable of responding normally to numerous systemically administered antigens (*6, 7*). This unresponsive state is now known to be mediated, at least in part, by a population of T suppressor cells (Ts) which are found in the spleens and lymph nodes of mice after only 5 weeks of UV treatment (*8*).

The mechanism underlying the induction of Ts cells by UV irradiation is not fully understood. Because of the potential implications on host-tumor relationships, however, this represents an active research area. It has been recently determined that following six daily UV exposures, irradiated mice possess decreased numbers of Ia-positive, adherent cells in their spleens (*9*). Concomitantly, these mice also demonstrate decreased levels of splenic APC activity (on a per cell basis) when compared with unirradiated littermates (*9–12*). The loss of this activity has been suggested to be attributable to the direct effect of UV irradiation on the APC precursors as they pass through the peripheral circulation before homing to the spleen and has been implicated in the afferent phase of Ts cell induction (*9*). An alternate possibility, however, is that the loss of $Ia^+$ APC from the spleen is a normal consequence of the inflammatory response caused by the UV exposures. Hence, the observed depression in splenic APC activity may be caused by a directed migration of APC to the sites of inflammation and not attributable to their functional inactivation within the spleen.

In this investigation we have tested this latter possibility. To do so, APC activity in animals exposed to either 6 days or 31 days of UV irradiation, or to a nonspecific inflammatory agent, was evaluated. The longer exposure periods to UV radiation was chosen as this represents a time when the compensatory changes (hyperpigmentation, hyperplasia, and hyperkeratosis) are well established and observed inflammation is minimal. This is also a dose which has been found to produce the maximal inhibition of antitumor responses and the consistent generation of Ts cells (*4, 5*). An antigen-primed T cell proliferation assay was used to assess APC activity in both the spleen and lymph nodes of UV-exposed animals. This procedure provides a means to evaluate, in a semiquantitative manner, the ability of a cell population to effectively present antigen to antigen-primed T cells. Possible influences of contaminating non-APC present in the splenic adherent cell preparations were also evaluated, as their consideration was imperative for accurate interpretation of the experimental protocols.

## MATERIALS AND METHODS

*Animals.* Four- to 6-week-old female C3Hf/HeN (MTV−) mice were obtained from the animal production facility of the National Cancer Institute, Bethesda, Maryland. All mice were housed at a maximum density of five animals per 7- x 11-inch cage and maintained on Wayne sterilizable lab blox and acidified water ad libitum. The mice were age matched (6 to 8 weeks old) at the onset of each experiment. A minimum of three animals per group was utilized in each experiment.

*UV irradiation.* The UV light source and preparation of mice for UV treatment have been previously reported (*4*). Briefly, the UV source consists of a bank of six FS40 Westinghouse fluorescent sunlamps emitting their energy principally (>60%) at wavelengths between 280 and 320 nm. The energy output of

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(6, 7). However, since APC function was not evaluated in these studies, we next compared the splenic APC activity of 6-day and 5-week UV-irradiated animals in parallel. In addition, most investigators who have used the T cell proliferation assay have inactivated the APC population (γ irradiation or mitomycin C treatment) just prior to coculturing with the primed T cells. The object of these studies was to compare differences between APC populations. We are aware that SAC preparations (used here as the sources of APC) can be heavily contaminated with lymphocytes and other irrelevant cell types (*13*), and felt that the presence of contaminating non-APC needed to be considered since their presence might lead to artifactual results. To test these possibilities, SAC preparations from the different treatment groups were γ-irradiated either just before (referred to simply as SACs) or 18 to 20 hr before (termed radiation-insensitive SACs) coculturing with antigen-primed T cells. We found that SACs prepared from both 6-day and 5-week UV-irradiated animals exhibited decreased APC activity (Table 2).

TABLE 1. Ability of SACs from 6-day UV-irradiated animals to present antigen to antigen-primed T cells

| Animal | Treatment[a] | Radiation-insensitive SACs/well[b] | |
|---|---|---|---|
| | | 2 × 10⁴ | Average Δcpm ± SD |
| 1 | None | 61,460[c] | 49,371 ± 11,350 |
| 2 | | 38,944 | |
| 3 | | 47,710 | |
| 1 | 30-min UV | 23,421 | 26,993 ± 5,881 |
| 2 | | 23,777 | |
| 3 | | 33,780 | |
| 1 | 60-min UV | 23,272 | 24,448 ± 7,883 |
| 2 | | 17,220 | |
| 3 | | 32,853 | |

[a] Animals were UV-irradiated concomitantly for 6 consecutive days for either 30 min or 60 min/day.

[b] SACs were prepared as described in Materials and Methods except that cells from individual animals were not pooled.

[c] Values represent Δcpm as described in Materials and Methods. SEM for triplicate cultures were less than 10%. The cpm in wells containing T cells alone or T cells plus KLH (50 μg/ml) were 1314 and 3017, respectively.

When radiation-insensitive SACs are used as the source of APC, however, only the cells from 6-day UV-irradiated animals exhibited decreased splenic APC function. Radiation-insensitive SACs prepared from 5-week UV-exposed mice possessed normal levels of APC activity. These results demonstrate that contaminating non-APC present in a SAC population can influence the level of APC activity of that population. These findings, however, do not explain the consistent reduction in splenic APC activity in animals exposed to 6 days of UV irradiation.

Several investigations have shown that prostaglandins can markedly influence certain T cell functions in vitro (*15, 17*). These observations suggested to us that prostaglandin-secreting cells in the SAC preparations from 6-day UV-irradiated animals could possibly account for the decrease in splenic APC activity observed in these animals. To test this possibility, it was first necessary to evaluate whether APC preparations containing prostaglandin-secreting cells would affect our T cell proliferation assay. We used PECs (*L. monocytogenes* induced) as an APC source known to be contaminated with prostaglandin-secreting cells. The drug indomethacin was used to inhibit prostaglandin synthesis as described by Nussenzweig et al. (*15*). In the presence of indomethacin, PECs demonstrated twice as much APC activity as cells cultured in its absence (27,166 Δcpm versus 11,849 Δcpm, respectively at $10^5$ PECs/well). The APC activity of SAC preparations from normal animals was unaffected by the addition of indomethacin to the cultures (32,353 Δcpm in the presence of indomethacin versus 33,583 Δcpm in the absence of indomethacin). Since these results demonstrated that indomethacin-sensitive cells could influence observed APC activity of a heterogeneous cell population, we next evaluated the splenic APC activity of 6-day UV-irradiated animals in the presence and absence of indomethacin. In the absence of indomethacin, radiation-insensitive SACs from 6-day UV-irradiated animals had depressed APC activity when compared with normals (9125 Δcpm versus 14,395 Δcpm). The presence of indomethacin did not reverse this reduced activity (9886 Δcpm versus 16,896 Δcpm). We conclude from these results that prostaglandin-secreting cells are not responsible for the decrease in splenic APC activity observed in UV-exposed animals.

*Relationship between epidermal inflammation and splenic APC function.* Mice exposed to the UV regimen used in these

TABLE 2. Ability of SACs from normal and UV-irradiated animals to present antigen to antigen-primed T cells

| Experiment | SAC donor | Viable cells added/well[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | SACs[b] | | | Radiation-insensitive SACs[b] | | |
| | | 2 × 10⁴ | 10⁴ | 5 × 10³ | 2 × 10⁴ | 10⁴ | 5 × 10³ |
| 1 | Normal | 23,439[c,d] | 16,437 | 8,059 | 65,559 | 35,563 | 13,690 |
| | 6-day UV | 12,440 | 3,999 | 1,316 | 27,578 | 15,244 | 6,461 |
| | 5-week UV | 6,961 | 4,567 | 1,131 | 45,308 | 49,831 | 24,994 |
| 2 | Normal | 32,661[c,d] | 15,075 | 2,311 | 35,491 | 26,004 | 6,490 |
| | 6-day UV | 13,099 | 3,949 | 1,015 | 15,209 | 4,788 | 884 |
| | 5-week UV | 23,849 | 8,699 | 1,264 | 35,680 | 26,188 | 6,563 |

[a] Purification, antigen pulsing, and γ irradiation of SAC preparations are described in Materials and Methods.

[b] SACs: SACs received 1000 rad of γ irradiation immediately prior to being set-up in culture with the primed T cells. Radiation-insensitive SACs: SACs received the γ irradiation 20 hr before addition to the T cells.

[c] See footnotes to Table 1.

[d] Values (cpm) for the T cell proliferation in the presence of nonantigen-pulsed SACs (2 × 10⁴ added per well) were: experiment 1, SACs: normal = 3115, 6-day UV = 2158, 5-week UV = 2526; radiation-insensitive SACs: normal = 6164, 6-day UV = 3830, 5-week UV = 3877; experiment 2, SACs: normal = 4743, 6-day UV = 3820, 5-week UV = 4677; radiation-insensitive SACs: normal = 5022, 6-day UV = 3655, 5-week UV = 4539.

[e] In experiment 1, SACs were also pulsed with ovalbumin (100 μg/ml overnight) to evaluate the antigenic specificity of the response. At 2 × 10⁴ ovalbumin-pulsed SACs added per well, the values (cpm) obtained were: SACs: normal = 2833, 6-day UV = 2456, 5-week UV = 2913; radiation-insensitive SACs: normal = 6290, 6-day UV = 2962, 5-week UV = 3284.

these lamps, measured at the dorsal surface level, was 3 J/m$^2$/sec. UV treatments were 30 min in duration unless indicated otherwise, and the animals were used within 2 days of the last exposure.

Short-term (6-day) UV-irradiated animals were exposed for 6 consecutive days, one treatment per day. Long-term (5-week) UV-irradiated animals were treated once daily, 5 days per week for 4 weeks. For the 5th week of treatment, the animals were treated concomitantly with the short-term UV-irradiated animals.

*Adherent cell preparation.* Spleens were excised and placed individually in complete media as previously described (*6*). Cell recoveries per spleen were determined for both total splenocytes and splenic adherent cells. Erythrocytes were lysed by a 1-min exposure to 0.83% ammonium chloride which resulted in minimal lymphoid cell loss (always less than 15% with greater than 95% viability).

Splenic adherent cells (SACs) were prepared by adherence to glass Petri dishes (one spleen equivalent in 5 ml of complete medium per 100- x 15-mm dish). The cells were allowed to adhere for 2 hr at 37 C, followed by removal of the nonadherent cells by gently washing four times with phosphate-buffered saline. The glass-adherent cells were released by a 15-min incubation in 5 ml of Versene (1:5000; Grand Island Biological Co., Grand Island, New York) at 37 C and recovered by vigorous washing with two volumes of complete medium.

SACs from a minimum of three animals were pooled (unless indicated), suspended in 10 ml of complete medium, and divided into two tubes. SAC preparations were then subjected to one of two protocols. Protocol A: Antigen (keyhole limpet hemocyanin (KLH)) was added to one tube to a final concentration of 100 μg/ml. The cells were gently rocked overnight at 37 C, washed four times with large excesses of medium, and then given 1000 rad of γ irradiation just prior to coculturing with the T cells. Protocol B: The SACs were prepared as described above with the exception that they received the γ irradiation just before addition of antigen and 18 to 20 hr before coculturing with the T cells. This protocol was used to inactivate radiosensitive cells, since SACs are known to be heavily contaminated with lymphocytes (*13*). SACs prepared by this latter protocol are referred to as radiation-insensitive SACs.

Lymph node-adherent cells (LNACs) were prepared from a pool of the axillary, brachial, and inguinal nodes of at least five animals by the same procedure described above under protocol B.

*Peritoneal exudate cells (PECs).* PECs were induced by injecting 3 × 10$^4$ viable *Listeria monocytogenes* i.p. Three days later the animals were killed and PECs were aspectically harvested. PECs were γ-irradiated (1000 rad) and washed just prior to the incubation with antigen.

*Primed T cells.* KLH-primed T cells were prepared essentially as described by Cowing et al. (*14*). Mice were given injections of 20 μg of KLH emulsified in 50 μl of complete freunds adjuvant (Difco) in the hind footpads. Fourteen to 30 days later, the inguinal and popliteal lymph nodes were removed, dissociated in complete medium, and the cells passed over a nylon-wool column. The nylon-nonadherent cells were then treated with anti-Ia$^k$ (ATH anti-ATL) plus rabbit complement for 45 to 60 min at 37 C. This resulted in a T cell-enriched population (overall yield ~30%) which was antigen specific (see footnote *e*, Table 2) and required antigen presentation to induce a significant proliferative response (see footnote *c*, Table 1).

*Antigen-specific T cell proliferation.* Antigen-induced stimulation was assessed by measuring T cell proliferation. Two × 10$^5$ antigen-primed T cells were added to various numbers of either antigen-pulsed or nonantigen-pulsed SACs (in triplicate) in flat bottomed microtiter plates (M. A. Bioproducts, Los Angeles, California) in a final volume of 0.2 ml. The plates were incubated at 37 C in a humidified atmosphere of 6% $CO_2$ in air. After 3 days, 1 μc of $^3$H-thymidine (2 c/mmol; New England Nuclear, Boston, Massachusetts) was added to the wells in a total volume of 0.01 ml. The cells were harvested with a MASH II (M. A. Bioproducts) 8 to 10 hr later. Radioactivity incorporated into DNA was assessed as previously described (*6*). The values presented represent the difference in cpm in wells containing antigen-pulsed SACs minus the cpm in wells containing an equal number of nonpulsed SACs. The cpm in wells containing nonpulsed SACs were similar regardless of the SAC source (see footnote *d*, Table 2). All SEM were less than 10% for the triplicate cultures.

*Indomethacin.* Indomethacin (Sigma, St. Louis, Missouri) was added to some cultures to inhibit prostaglandin synthesis, and was prepared and used according to a previously described protocol (*15*).

*Turpentine oil treatment.* Rectified turpentine oil was used to induce a local inflammatory response. Animals were shaved and treated with a commercial depilatory agent (Nair, Carter Products, New York), followed by washing the treated epidermal surfaces with a 1% boric acid solution. These manipulations produced no observable inflammation by themselves. Turpentine (0.1 ml) was then topically applied to the depilated sites for 6 consecutive days. Spleens of animals treated with this protocol were excised 24 hr after the last treatment and their APC activity was evaluated. Evaluation of skin biopsies showed little or no hyperplasia 1 week after treatment with only the depilatory agent. Marked epidermal hyperplasia and dramatic cellular infiltration into the dermis resulted from the daily application of turpentine.

## RESULTS

*APC function of splenic adherent cells isolated from UV-irradiated mice.* Antigen-primed T cell proliferation was used to evaluate effective antigen presentation. With this assay we first evaluated the splenic APC activity from animals given 30 min or 60 min of UV irradiation per day for 6 consecutive days. SACs were used as the source of APC and each animal was individually evaluated. The SACs were γ-irradiated (1000 rad) 18 to 20 hours before coculturing with the primed T cells in order to inactivate radiosensitive cells and thus are termed radiation-insensitive SACs.

The results (Table 1) demonstrate that, compared with nonirradiated littermates, both groups of UV-irradiated animals exhibited decreased splenic APC activity when evaluated on a per cell basis. The average decrease in observed proliferation was equivalent at both dosages (30 min versus 60 min) and represented approximately a 50% reduction in incorporated radioactivity. Similar results were obtained when unfractionated spleen cells (instead of SACs) were used as the source of APC (data not shown). Since the lower dosage of UV is less traumatic to the exposed animals (*16*), a daily 30-min exposure was chosen for all subsequent experimentation.

Previous studies have shown that 5 weeks of UV treatment produces no general decrease in immunological competence other than an inability to generate effective antitumor responses

TABLE 3. Decreased antigen-presenting potential of SACs isolated from animals undergoing an acute inflammatory response in the dorsal epidermis

| SAC donor | Radiation-insensitive SACs/well[a] | | |
|---|---|---|---|
| | $2 \times 10^4$ | $10^4$ | $5 \times 10^3$ |
| Normal | 50,824[b] | 20,085 | 6,403 |
| Turpentine treated[b] | 24,164 | 10,989 | 3,448 |

[a] See footnotes to Table 1.

[b] An inflammatory response was induced by the topical application of turpentine for 6 days prior to analysis of APC function.

TABLE 4. Comparison of antigen-presenting potential of adherent cells from the lymph nodes of normal and UV-irradiated animals

| Experiment | Lymph node donor | Viable adherent cells/well[a] | | | |
|---|---|---|---|---|---|
| | | $2 \times 10^4$ | $1 \times 10^4$ | $5 \times 10^3$ | $2.5 \times 10^3$ |
| 1 | Normal | 27,315[b] | 10,200 | 1,962 | 603 |
| | 6-day UV | 51,260 | 28,557 | 5,060 | 2,156 |
| | 5-week UV | 17,219 | 7,272 | 1,072 | 348 |
| 2 | Normal | | ND[c] | 2,038 | 448 |
| | 6-day UV | | 34,529 | 15,703 | 4,090 |

[a] Purification, antigen pulsing, and γ irradiation of lymph node-adherent cells are described in Materials and Methods.

[b] See footnotes to Table 1.

[c] ND, not done.

experiments exhibited an acute inflammatory response in the dorsal epidermis during the first 2 weeks of treatments. With further exposures, the normal compensatory mechanisms (increased melanization and hyperplasia) provide a degree of protection against the damaging, inflammatory effects of UV radiation. The observation that the UV-induced inflammatory response occurred at approximately the same time as the depression in APC function suggested an interrelationship between these two effects. To test whether an acute inflammatory response in the skin could influence splenic APC activity, animals were treated with turpentine for 6 days and then evaluated for splenic APC function. This treatment resulted in a marked epidermal hyperplasia and cellular infiltration of the dermis in these mice. The results (Table 3) demonstrate that the induction of an inflammatory response in the dorsal skin by an agent other than UV light could also result in decreased splenic APC activity when evaluated by the T cell proliferation assay.

*APC potential of LNACs from normal, UV-irradiated, and turpentine-treated animals.* During necropsies of UV-irradiated animals, we observed that the peripheral lymph nodes in these animals (axillary, brachial, and inguinal) were hyperplastic, giving cell yields of approximately two times that found in normal mice. This was just the opposite of what was observed in the spleens of these animals, which were consistently smaller than normal. These observations led us to evaluate whether the decrease in APC activity was localized to the spleen. Glass-adherent cells were prepared from the axillary, brachial, and inguinal lymph nodes of normal and UV-irradiated animals and assayed for APC activity. The APC function of the LNACs from either normal or 5-week UV-irradiated animals was found to be similar while the LNACs from 6-day UV-irradiated animals demonstrated enhanced APC function (Table 4). To evaluate whether the increase in lymph node APC activity might be a consequence of the skin inflammation regardless of how it was induced, both splenic and lymph node APC activity was evaluated in turpentine-treated animals and compared to 6-day UV-irradiated and normal animals. The results (Table 5) demonstrate that while splenic APC activity was consistently depressed in both turpentine-treated and UV-irradiated animals, only the UV-irradiated animals showed elevated levels of lymph node APC activity.

*Splenectomy prevents the increase in APC activity of LNACs from short-term UV-irradiated animals.* One possible explanation for the increase in APC activity in the lymph nodes of 6-day UV-irradiated animals is that the spleen is acting as a reservoir of APC which can migrate to sites draining an ongoing inflammatory response. To test this possibility, a group of 12 animals was splenectomized and one-half was exposed to 6 days of UV irradiation. LNACs were prepared from these animals and compared with LNACs obtained from nonsplenectomized animals that had been treated in parallel. The results (Table 6) demonstrate that splenectomy prior to UV irradiation prevents the elevation in lymph node APC activity. These observations are consistent with the hypothesis that splenic APC migrate to the lymph nodes draining the site(s) of the epidermal inflammation induced by UV exposure.

## DISCUSSION

Numerous investigations into the immunological consequences of UV irradiation have demonstrated that the effects are complex and may vary depending on the dosage, time, and method of evaluation. Recent studies on splenic APC function have demonstrated that after 6 days of UV irradiation, this activity is markedly depressed (*9–12*). Although these studies

TABLE 5. Comparison of APC activity in the spleen and lymph nodes of normal, UV-irradiated, and turpentine-treated animals

| Treatment of donor[a] | APC source[b] | Viable adherent cells/well[c] ($10^4$) |
|---|---|---|
| None | SACs | 53,288 |
| 30-min UV | SACs | 23,418 |
| Turpentine | SACs | 18,463 |
| None | LNACs | 40,664 |
| 30-min UV | LNACs | 54,958 |
| Turpentine | LNACs | 37,363 |

[a] All animals were treated concomitantly for 6 consecutive days as described in Materials and Methods.

[b] Adherent cells were γ-irradiated 18 hr before coculture with the primed T cells.

[c] See footnotes to Tables 1 and 4.

TABLE 6. Splenectomy prevents the increase in APC activity observed in the draining lymph nodes of animals subjected to 6 days of UV irradiation

| Treatment of lymph node donor | Viable adherent cells/well[a] | | |
|---|---|---|---|
| | $2 \times 10^4$ | $1 \times 10^4$ | $5 \times 10^3$ |
| Normal | 7,518[a] | 2512 | 1968 |
| Splenectomy[b] | ND[c] | 2862 | 688 |
| 6-day UV | 12,570 | 7283 | 1140 |
| Splenectomy plus 6-day UV[b] | 6,948 | 3231 | 757 |

[a] See footnotes to Table 1.

[b] All animals were splenectomized at one time. Three days later, one-half was selected to be given 6 days of UV treatment.

[c] See footnotes to Table 4.

did not evaluate animals subjected to longer irradiation protocols, earlier investigations using mice that had received at least 5 weeks of similar UV treatments demonstrated normal immunological reactivity (*6, 7*). In these latter studies, APC activity was not specifically evaluated but presumably was required for many of the responses. Collectively, these observations provided a number of testable hypotheses.

The first hypothesis suggested by these studies is that the decrease in splenic APC function is reversible and, therefore, should not be observed in animals exposed to chronic UV irradiation. The results presented herein verified this suggestion. When only radiation-insensitive SACs were used as the source of APC, 5-week UV-irradiated animals consistently possessed normal APC activity. Conversely, 6-day UV-irradiated animals consistently demonstrated decreased APC activity. These results confirm and extend the findings of previous investigations (*6, 7, 9-12*).

The time of the decrease in splenic APC function approximately coincides with the time of greatest epidermal inflammation. This observation suggested a possible correlation between these two effects, a hypothesis which was tested by chemically inducing an epidermal inflammatory response. The results demonstrated that a topically applied chemical irritant could also induce a decrease in splenic APC activity. It should be noted, however, that the effects of physical and chemical agents on the epidermis are not equivalent and therefore the mechanism involved may be different for the different agents (UV versus turpentine). This possibility is further supported by the results of the evaluation of APC activity in the lymph nodes of these animals as discussed below.

A third hypothesis suggested by the results of the previous studies was that the decrease in APC activity was localized to the spleen and should not be observed in peripheral lymphoid tissue. In fact, when APC activity from the lymph nodes of 6-day UV-irradiated mice was evaluated, it was consistently greater than that found in normal lymph nodes. The lymph nodes from turpentine-treated animals, however, did not demonstrate this consistent elevation in APC activity even though inflammation of the treated site was evident. This result may reflect either qualitative or quantitative differences in the effect of these different agents on murine epidermis. Nonetheless, the decrease in splenic APC function in 6-day UV-irradiated animals appears to be attributable to migration of APC from the spleen to the peripheral lymph nodes since splenectomy prior to the UV treatments prevented the elevation in lymph node APC activity.

In summary, the results presented herein confirmed the existence of a decrease in splenic APC function following 6 days of UV irradiation. They also further substantiate the findings of previous studies which showed that no decrease in general immunological reactivity is observed in animals subjected to at least 5 weeks of UV irradiation (*6, 7*). In addition, the decrease in splenic APC function may be attributable to a response to the epidermal trauma since (1) APC activity is elevated in the lymph nodes of animals exposed to 6 days but not 5 weeks of UV irradiation; (2) a decrease in splenic APC function is also observed in animals treated with a chemical irritant; and (3) the increase in lymph node APC activity is not observed if the animals are splenectomized prior to the UV treatments.

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# The Effect of Various Sunscreen Agents on Skin Damage and the Induction of Tumor Susceptibility in Mice Subjected to Ultraviolet Irradiation

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**Sunscreen preparations containing various chemical UV absorbers, para-aminobenzoic acid (PABA), 2 PABA derivatives, benzophenone or a combination of these were topically applied to the backs of C3H/HeN mice prior to their being irradiated with ultraviolet light in the UVB range. In all cases this treatment was effective in preventing the pathological skin changes associated with UVB irradiation. Histological evaluation of skin biopsies from mice treated with the sunscreen preparations and UVB irradiation showed little or no difference from normals in amount of hyperplasia, melanization or parakeratosis present. These histologic changes were observed in animals receiving UVB irradiation in the absence of any sunscreen agent.**

**Pretreatment with the various sunscreen agents did not, however, prevent the induction of tumor susceptibility as measured by the sustained growth of a UV-induced tumor which is immunologically rejected in normal syngeneic mice. These data show a clear distinction between the effects of UVB irradiation leading to histological changes in the epidermis and those leading to the state of tumor susceptibility in mice. The distinction was further corroborated by the finding that epidermal hyperplasia induced by repeated applications of croton oil had no significant enhancing or inducing effects on the induction of tumor susceptibility. In addition, the induction of tumor susceptibility is due to wavelengths of light less than 320 nm since this effect was abrogated when the UVB radiation was filtered through glass.**

**Possible mechanistic differences between the tumor susceptibility generated in UVB and photoprotected UVB irradiated animals were observed, however, when we attempted to adoptively transfer the state of tumor susceptibility to normal animals. While it was readily transferable with splenic lymphoid cells from UVB irradiated animals, all attempts to transfer the tumor susceptibility from photoprotected animals have, to date, been unsuccessful.**

It is well known that ultraviolet light (200–400 nm) is capable of producing numerous effects on biologic systems. Environmentally, most of the wavelengths capable of causing direct genetic damage (wavelengths less than 280 nm) are screened out by the atmosphere. However, wavelengths greater than 280 nm are still capable of exerting many potent biological effects. It is the region between 280 and 320 nm (UVB) that has been found to be primarily responsible for Vitamin D production, melanization, sunburn (erythema and hyperplasia), premature aging of the skin and skin tumors [1,2]. In addition, more recent studies on mice have demonstrated that UVB irradiation is also capable of inducing a subtle alteration in the potential immunological reactivity of the host [3–7]. This change is evidenced by the progressive growth of UV-induced tumor implants in syngeneic mice which have received a subcarcinogenic dose of UV, while a similar implant is immunologically rejected in normal (unirradiated) litter mates. The dose of UV light necessary to induce this tumor susceptible state is at least 10-fold less than the amount of light energy needed to induce an overt tumor. Additional studies have also established that this state is not due to panimmunosuppression. Rather, the underlying mechanism appears to involve the induction and maintenance of regulatory, suppressor T-cells, ($T_s$) which demonstrate a functional specificity for common tumor associated antigen(s) (TAA) found on all UV-induced tumors [7–8]. These $T_s$ cells are able to inhibit the animal's ability to mount an effective immunological response against the tumor implant resulting in this state of tumor susceptibility [8,9].

Sunscreens, the majority of which employ para-aminobenzoic acid (PABA) (or one of its derivatives) and/or benzophenone as their active ingredient, have been reported to protect against a number of the effects of UV radiation. The ability of topically applied PABA containing sunscreens to reduce the erythema and parallel skin damage caused by prolonged or chronic UV exposure has been amply demonstrated [10–13]. In addition, sunscreens have been shown to protect against both the co-carcinogenic as well as the carcinogenic effects of UV light [12,13]. To date, however, no studies have evaluated the effect of these sunscreen agents on the more subtle and less well understood immunoregulatory modifications now known to be produced by UVB irradiation [3–9].

In this study we report the results of experiments designed to evaluate the potential protective effect of commercial sunscreen preparations on the induction of a tumor susceptible state by UVB. Our results confirm that each of the test agents employed provide excellent protection against the pathologic skin changes associated with chronic UVB exposure. In no case, however, did we observe that a sunscreen preparation was capable of preventing the induction of a tumor susceptible state in mice exposed to UVB irradiation.

## MATERIALS AND METHODS

### *Animals*

Four- to 6-week old female C3Hf/HeN mice were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). All mice were housed at a maximum density of 5 animals per standard 7 × 11 inch cage and maintained on Wayne Sterilizable Lab Blox and acidified water ad libidum. The mice were age matched (6 to 8 weeks old) at the onset of each experiment. All experimental groups contained 5 to 8 animals.

### *Ultraviolet Irradiation of Mice*

The UV light source, energy output and preparation of mice for UV-treatment have been previously reported [4]. Briefly, the UV light

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Abbreviations:
PABA: para-aminobenzoic acid
SALT: skin associated lymphoid tissue
SPF: sun protection factors
TAA: tumor-associated antigen(s)

source consists of a bank of 6 FS40 Westinghouse fluorescent sunlamps emitting principally (>60%) wavelengths between 280 and 320 nm with a total energy output of $1.79 \times 10^3$ ergs/cm$^2$/sec (0.179 mw/cm$^2$) measured at the dorsal surface level. Measurements were made using an International Light Inc. (Newburyport, Mass. 01950) UV-visible photometer, model #IL200 with detector model #2T101D. Precise measurements of the energy emitted and the calibration of this instrument were performed as previously described [14]. All UV irradiations consisted of 30 min of exposure, 5 times per week. All groups of animals employed in a particular experiment were irradiated concomitantly.

Glass filtered ultraviolet irradiation of animals was done by supporting a square pane of glass (3/16 in. thickness) on blocks of styrofoam just below the fluorescent sunlamps. The cages of mice were placed under the glass so that all light reaching the mice was filtered through the glass. The absorption spectrum of the glass was evaluated by placing a 1 × 4 cm section in a Beckman spectrophotometer model 35 and measuring transmittance at various wavelengths (280–400 nm). Greater than 95% of the light energy below 320 nm was effectively absorbed by employing the glass as a filter.

*Treatments*

All sunscreen preparations were obtained from commercial sources. The benzophenone-3 (oxybenzone), octyl dimethyl PABA, glyceryl PABA, and the sunscreen base were supplied by Allergan Laboratories (Irvine, California). PreSun (Westwood Pharmaceutical, N.Y.) was used as the source of PABA. Approximate sun protection factors (SPF) for these products were obtained from the supplier or from Sayre et al [15]. The SPFs for the various preparations are: PABA, 12; PABA esters, 13; oxybenzone, 5; PABA esters plus oxybenzone, 15. All the preparations containing PABA or its esters have a SPF of greater than 10, which implies that they absorb greater than 90% of the erythemogenic radiation. The oxybenzone preparation absorbs approximately 80% of the erythemogenic radiation. The various sunscreen preparations were liberally applied (approximately 0.3–0.5 ml) and rubbed on the shaved dorsal surface, ears and tail, one-half hour prior to the UV-irradiation of the test animals.

Croton oil (Sigma Chemical Co., St. Louis, Mo.) was used to induce a state of epidermal hyperplasia. It was first diluted to a 2% solution in acetone, and 50 microliters were applied to the shaved dorsal surface of animals, 3 times per week for 6 weeks.

*UV-Induced Tumors and Tumor Challenge*

All tumor challenges were performed with RD-87, a C3Hf/HeN, UVB-induced tumor which grows readily in syngeneic UVB irradiated animals but not in normal syngeneic mice [4]. This spindle cell tumor (fibrosarcoma) was maintained *in vivo* by serial subcutaneous passage of 1mm$^3$ tumor fragments into UVB irradiated animals as described previously [4]. All tumor challenges were done in this same manner. Tumor growth rates were determined by measuring 2 perpendicular diameters with calipers twice a week and plotting mean tumor area (the product of the 2 diameters) versus time postimplantation.

*Adoptive Transfer of Tumor Susceptibility*

Adoptive transfer of the tumor susceptibility with splenic lymphoid cells was performed as previously described [7]. Briefly, spleens were removed, dissociated and washed in media containing 5–10% calf serum. The lymphoid cells were resuspended at $4 \times 10^6$ per ml in serum free medium and 0.3 ml injected intravenously via the recipient's lateral tail vein. All adoptive recipients were challenged with a tumor implant within 6 hours following lymphoid cell transfer.

*Histology*

Animals were randomly chosen from each of the groups and small sections of skin were surgically excised from the ear, and shaved dorsal surface. The samples were surgically excised from the ear, and shaved dorsal surface. The samples were immediately fixed in 10% neutralized formalin. Parafin sections were stained with either hematoxalin and eosin, or Schmorl's melanin stain and evaluated microscopically for relative amounts of parakeratosis, melanin, hyperplasia and nuclear changes. The nuclear changes observed were peripheral condensation of nuclear material, atypical nuclei, frothy and prominent nucleoli.

## RESULTS

The results of the histological evaluation for the amount of hyperplasia, parakeratosis, melanization and nuclear changes seen after three and four weeks of treatment are presented in Table I. All of the sunscreen agents tested effectively prevented the pathological skin damage associated with UVB exposure. The animals which were photoprotected by prior application of a sunscreen showed no evidence of any parakeratosis or increased melanization, and only slight nuclear changes or increases in the number of epidermal cell layers. The unprotected animals, however, demonstrated marked changes in all of these areas.

An evaluation of tumor susceptibility was made by challenging all test animals with the UV-induced tumor RD87. The data presented in Fig 1 clearly demonstrate that photoprotection of the animals with PABA had no effect on the induction of the tumor susceptible state after 3 weeks of treatment. All test animals were found to be tumor susceptible. Furthermore, the tumor growth rate in the photoprotected mice was equivalent to that seen in the unprotected UVB irradiated animals.

An almost complete lack of protection against the acquisition of tumor susceptibility after 4 weeks of UVB irradiation was also demonstrated in animals treated with the mixture of PABA esters, benzophenone or all 3 in combination (Fig 2). A slight decrease in the tumor growth rate as well as a slight reduction in the percentage of tumor susceptible animals was seen in the animals treated with either the PABA esters alone or in combination with benzophenone. Animals pretreated with PABA, benzophenone or the sunscreen base alone all grew the tumor implant at a slightly increased rate. However, these differences were not significant when evaluated by the Student's *t*-test.

These data suggested that the pathological skin damage associated with UVB exposure does not play a role (or even serve as an indicator) for the induction of tumor susceptibility by UV light. To test for any potentiating or inductive effects due to epidermal hyperplasia, croton oil (2% v/v in acetone) was topically applied to the shaved dorsal surface of mice 3

TABLE I. *Evaluation of histologic changes in skin exposed to ultraviolet light with and without the prior application of various sunscreen agents*

| | No. of cell layers | | Para-keratosis[b] | Melanin[b,c] | Nuclear changes[b,d] |
|---|---|---|---|---|---|
| | Epider-mis | Granular layer | | | |
| Exp. 1 | | | | | |
| *Ear*[a] | | | | | |
| PABA alone | 3.0 | 1.0 | 0 | 1 | 0 |
| UVB alone | 6.0 | 3.0 | 4 | 4 | 4 |
| UVB & PABA | 3.5 | 1.5 | 0 | 1 | 1 |
| *Back*[a] | | | | | |
| PABA alone | 3.0 | 1.0 | 0 | 0 | 0 |
| UVB alone | 6.0 | 3.0 | 3 | 3 | 3 |
| UVB & PABA | 4.0 | 1.0 | 0 | 0 | 1 |
| Exp. 2 | | | | | |
| *Back*[a] | | | | | |
| UVB + Benzo-phenone + PABA Esters | 3.5 | 1.5 | 0 | 0 | 0 |
| UVB + PABA Esters | 3.5 | 1.5 | 0 | 0 | 1 |
| UVB + Benzo-phene | 3.0 | 1.0 | 0 | 0 | 0 |
| | 23 | | | | |
| UVB + PABA | 3.5 | 1.5 | 0 | 0 | 1 |
| UVB + Sun-screen | 6.0 | 3 | 2 | 3 | 3 |
| Base | | | | | |
| Benzophenone + PABA Esters alone | 3.0 | 1 | 0 | 0 | 0 |

[a] Results represent typical histologic findings on the skin of one animal taken at the time of tumor implantation.
[b] Quantitated via an arbitrary 0–4 scale: 0 = the amount seen in back skin of animals treated with sunscreen alone; 4 = the amount seen in ear skin of animals treated with UVB alone.
[c] Quantitated with Schmorl's melanin stain.
[d] Nuclear changes include: Peripheral condensation of nuclear material, atypical nuclei, frothy nucleoli, prominent nucleoli.

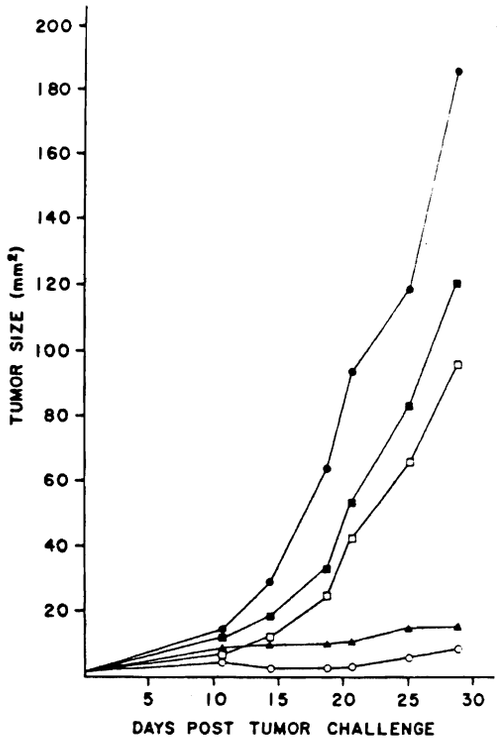


FIG 3. Comparison of tumor growth rates showing a lack of enhancement or induction of the tumor susceptible state due to hyperplasia produced by repeated applications of 2% croton oil. (●) 8-week UVB irradiated animals; (■) 2 weeks of UVB irradiation followed by 6 weeks of 2% croton oil applied 3 times/week; (□) 2 weeks of UVB irradiation, then rested for 6 weeks before tumor challenge; (▲) 2% croton oil applied 3 times/week for 6 weeks; (○) untreated control. Tumor size the product of 2 perpendicular diameters. Each *point* represents the mean of 6–8 animals.

TABLE II. *Abrogation of UVB-induced tumor susceptibility by employing a glass filter to eliminate wavelengths below 320 nm*

| Group | Treatment[a] | Glass Filtered[b] | # of TBA[c] per # animals challenged |
|---|---|---|---|
| 1 | 30′ UVB | - | 4/5 |
| 2 | 30′ UVB | + | 1/5 |
| 3 | 60′ UVB | + | 0/5 |
| 4 | - | - | 0/5 |

[a] All animals irradiated concomitantly, 5 × per week for 5 weeks.
[b] Glass (3/16 in. thickness) was placed over the cages so that all radiation reaching these animals had to first penetrate the glass.
[c] Number of animals with progressively growing tumors per number of animals receiving tumor implants. Data collected 25 days after tumor challenge.

susceptibility in mice, an effect which also appears to be mediated by an $Ia^+$ cell type [22]. Once again, it has been observed epidemiologically, that patients treated with PUVA show a greater risk of developing a malignancy than an environmentally matched population [23]. Thus, there is an increasing amount of suggestive evidence that UV-induced immunological modifications lead to an increased risk of malignancy. This has led us to investigate the underlying events resulting in, and the effect of photoprotective agents on the induction of the tumor susceptible state in mice.

In addition to its immuno-modulatory influences, UVB also produces numerous skin changes which can be assessed histologically. These include increased melanization, epidermal hyperplasia, parakeratosis and nuclear changes. Previous studies have shown that sunscreen agents are capable of providing good protection against these pathological changes [10–13]. The results presented here confirm and extend these observations, as good protection against these histological changes were noted when any of the sunscreens tested were applied one-half hour before UVB-irradiation. Surprisingly, animals which were photoprotected histologically, were still tumor susceptible as indicated by the progressive growth of the UV-regressor tumor implanted in these animals.

Prior studies have established that the minimum amount of UV treatment necessary to consistently induce the tumor susceptible state is approximately three weeks (30 min per day) and that the average tumor growth rate is proportional to the UVB dose up to approximately eight weeks of irradiation [4]. Our data demonstrate that the observed state of susceptibility in photoprotected animals is equivalent to that observed in the unprotected, irradiated animals, as the tumor growth rates are indistinguishable. This observation, that tumor growth rates are equivalent even though the minimal dose of UVB necessary to establish tumor susceptibility is employed, suggests that sunscreen agents do not significantly affect the mechanism underlying the induction of tumor susceptibility. Thus, the histologic changes in the skin associated with UVB exposure do not appear to play a significant role in the induction of the UVB-induced tumor susceptible state. In further support of this concept is the observation that a chronic state of hyperplasia induced by repeated croton oil treatment does not induce or enhance the tumor susceptible state.

From an initial consideration of the absorption spectrum of PABA, it was suggested that the induction of tumor susceptibility could be due to the wavelengths not effectively absorbed

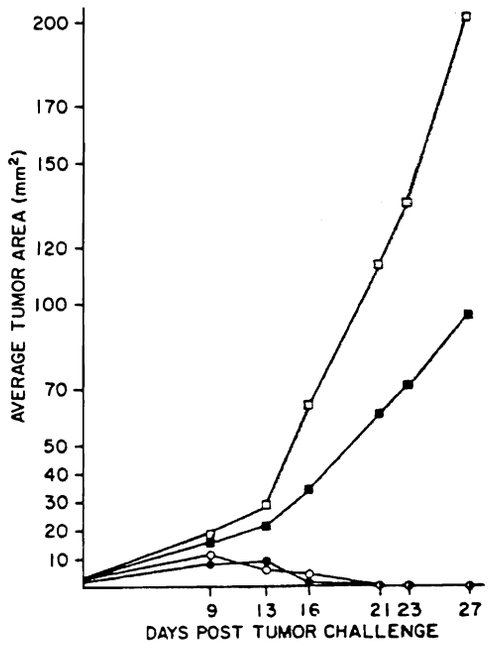


FIG 4. Growth rate of RD87 in animals given splenic lymphoid cells from either UVB or PABA + UVB irradiated animals. (□) 6 week UVB irradiated mice, (■) normals given $10^8$ lymphoid cells from 6 week UVB irradiated mice, (○) normals given $10^8$ lymphoid cells from 6 week PABA + UVB irradiated mice, (●) normal mice. Tumor area is the product of 2 perpendicular diameters. Each *point* represents the mean of 5 animals.

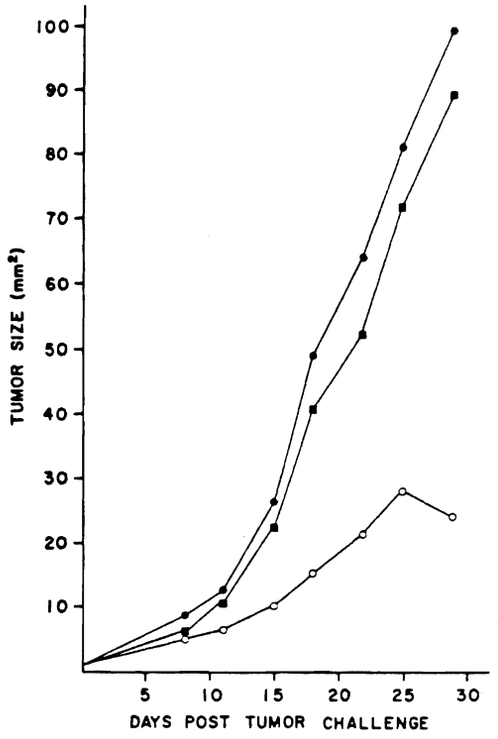


FIG 1. Comparison of growth rates of tumor RD87 in 3 week UVB irradiated animals, one-half of which were protected by prior application of Pre Sun containing 5% PABA. (●) 3-week UVB irradiated mice; (■) Pre Sun applied topically one-half hr before UVB irradiation; (○) Pre Sun applied topically without UVB irradiation. Tumor size is the product of 2 perpendicular diameters. Each *point* represents the mean of the 6–8 animals in each group.

times per week for 6 weeks. This treatment schedule was performed on 2 groups of mice: (1) normal and (2) age-matched animals given 2 weeks of UVB exposure just prior to beginning the croton oil regimen. For comparison, the 3 other groups in this experiment were: (1) animals given 8 weeks of UVB irradiation to achieve maximal tumor susceptibility, (2) animals given 2 weeks of UVB and then rested for 6 weeks; and (3) normal untreated animals. All groups were implanted with RD87 at the end of the eight-week treatment and subsequent tumor growth rates evaluated (Fig 3). Normal animals with or without the croton oil treatment did not permit tumor growth. Those animals given croton oil after two weeks of UVB did not exhibit a significantly different tumor growth rate from those animals given 2 weeks of UVB and then rested for 6 weeks before tumor challenge. The tumor susceptibility however, was maximal in those animals exposed to 8 weeks of UV as evidenced by the enhanced tumor growth rates in these animals.

The possibility that the induction of tumor susceptibility could be due to wavelengths greater than 320 nm was examined by irradiating the animals with light filtered through glass. The glass was determined to absorb >95% of the light energy below 320 nm. The results of this experiment (Table II) show that animals given 5 weeks of filtered light exposure, even with twice the normal daily dose (60 min) were not tumor susceptible. The control animals (unfiltered exposure) were found to be highly tumor susceptible. This experiment has been repeated with similar findings.

The induction of tumor susceptibility in UVB animals has also been shown to involve the concomitant induction of suppressor T lymphocytes which are transferable with splenic lymphoid cells [7]. Since PABA plus UVB treated animals were consistently found to be tumor susceptible to the same extent as UVB exposed controls, we examined whether the tumor susceptible state could also be adoptively transferred with spleen cells (Fig 4). Both normal animals given $10^8$ splenic lymphoid cells from UVB treated animals and UVB irradiated animals were determined to be tumor susceptible. Those animals given the same number of splenic lymphoid cells from PABA plus UVB treated animals, however, rejected the tumor implant at the same rate as the normal control group. This experiment has also been repeated with equivalent results.

## DISCUSSION

UV carcinogenesis is a complex phenomenon, part of which appears to involve a modification in the immunologic potential of the host. Early events which have been elucidated include decreased Langerhans cell function in the epidermis [16], a decrease in antigen presenting cell function in the spleen [17], and the induction and maintenance of a population of $Ia^+$ suppressor T cells ($T_s$) [8,9,18]. These $T_s$ cells appear to possess a functional specificity for common, tumor associated antigen(s) found on all UV-induced tumors [8,9]. In addition, UV irradiation, (and by implication the accompanying $T_s$ cells,) has also been shown to be capable of reducing the latency period for tumor induction by a chemical carcinogen administered at an unirradiated site [19]. This observation is distinct from the known cocarcinogenic potential of UV light where the chemical carcinogen is applied at a site receiving UV exposure [12,20], and may explain the observation in man that as many as one-third of all basal cell carcinomas arise on areas receiving very little UV radiation [21]. Furthermore, PUVA (8-methoxypsoralen potentiated UVA) has also been shown to induce tumor

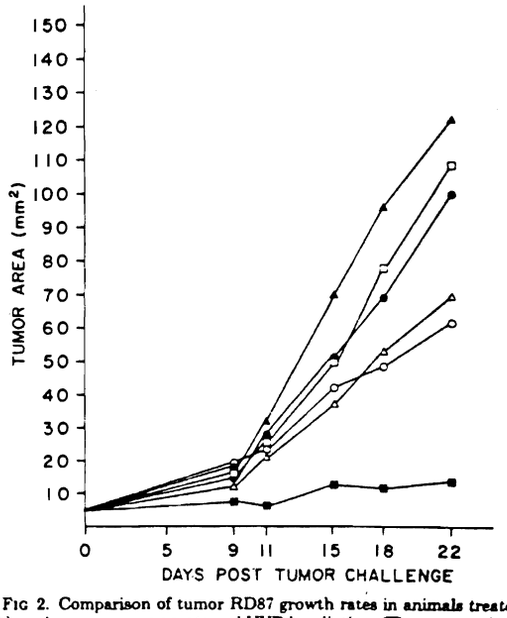


FIG 2. Comparison of tumor RD87 growth rates in animals treated with various sunscreen agents and UVB irradiation. (□) sunscreen base plus UVB; (▲) PABA plus UVB; (●) benzophenone-3 plus UVB; (○) octyl, dimethyl and glyceryl PABA (PABA esters) plus UVB; (△) PABA esters, and benzophenone-3 plus UVB; (■) PABA esters and benzophenone-3 alone. Tumor area is product of 2 perpendicular diameters. Each *point* represents the mean of the 5 animals in each group.

by PABA. The experiments utilizing benzophenone (which absorbs wavelengths up to approximately 340 nm), glass filters (which eliminate >95% of the light energy below 320 nm) as well as past studies (which have shown that even up to 10 weeks of treatment with UVA light does not induce a tumor susceptible state [22]) demonstrate that this phenomenon is not due to wavelengths of light greater than 320 nm.

Alternate explanations to account for our observations are currently being investigated. One is that the sunscreen agents may act as photosensitizers initiating a photochemical reaction in the skin which leads to the induction of tumor susceptibility. Evidence for this occurring in a prokaryotic system has been presented by Hodges, Moss and Davies [24] who found increased genetic damage when *E. coli* were irradiated in the presence of PABA. Another possibility is that the amount of light energy not absorbed by the sunscreen agents is sufficient to induce the tumor susceptible state.

While tumor growth rates and therefore the degree of tumor susceptibility appears equivalent in both UVB and PABA-UVB treated animals, some differences between the 2 states do exist as shown by the adoptive transfer experiments. Tumor susceptibility induced by UVB is easily transferred to normal syngeneic animals with as few as $3.5 \times 10^7$ nylon wool non-adherent T cells [8]. However, our attempts to adoptively transfer the tumor susceptible state using lymphoid cells from photoprotected UVB exposed donors have been uniformly unsuccessful.

Since with photoprotective agents we have essentially eliminated the UVB-induced skin damage and, concomitantly, the adoptively transferable tumor susceptibility, these 2 effects may be related. One possible explanation of this phenomenon is that the inflammation and actinic damage cause a systemic migration of the $T_s$ cells. Thus, while PABA plus UVB (and possibly PUVA) may cause an equivalent number of $T_s$ cells and therefore an equivalent tumor growth rate, the cells might be restricted to skin associated lymphoid tissue (SALT), which would explain our unsuccessful attempts to transfer the susceptibility with splenic lymphoid cells. Evidence for this type of restricted lymphoid circuit comes from a number of sources as previously discussed by Streilein [25].

Another important immunological component found in the epidermis which is affected by both UVB and PABA + UVB is the Langerhans cell. This ATPase positive, dentritic cell in the epidermis shares many characteristics with the antigen presenting macrophage including: expression of Ia determinants as well as Fc and C3b receptors, ATPase and nonspecific esterase positivity, bone marrow origin, and the ability to present antigen to macrophage depleted immune T cells [26–29]. The decreased function of Langerhans cells after UV irradiation has been shown by Toews, Bergstresser, and Streilein [16]. They found decreased numbers of ATPase positive cells in the murine epidermis after UVB irradiation corresponded to a decrease in the ability to contact sensitize the animal at the same site [16]. It was concluded from these data that the antigen presenting function of the Langerhans cell is depressed by UV irradiation. We have extended these observations and found that the same effect is also observed in animals treated with both PABA plus UVB and PUVA (Lynch et al, J Immunol in press). The inactivation of the Langerhans cell's antigen presenting capability may play an important role in the early events which lead to UV-induced tumor susceptibility.

In summary, the *in vivo* effects of UVB irradiation are extremely varied, including histologic skin damage, the induction of tumor susceptibility and even overt carcinogenesis. While pretreatment with various sunscreen agents eliminates most of the observed skin damage and significantly retards tumor development, they do not appear to affect the induction of the tumor susceptible state. Evidence indicates however, that the nature of the tumor susceptible state observed in photoprotected animals is not equivalent to that induced in UVB exposed animals since systemic involvement (splenic suppressor cells) appears to be lacking. Further investigations are in progress to more completely define the tumor susceptible state in photoprotected animals. We are currently involved in determining whether the state is transient and wanes with time (unlike the tumor susceptibility induced in UVB irradiated animals) and also whether it is mediated via a suppressor cell mechanism.

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# LOSS OF ANTIGEN PRESENTING FUNCTION BY EPIDERMAL CELLS FOLLOWING UV EXPOSURE *IN VIVO*[1]

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## Introduction

Langerhans cells (LC), discovered by Paul Langerhans in 1868 (1), have been the subject of an increasing number of investigations. Much of the recent work on this epidermal cell population has stemmed from the discovery that these cells share many of the characteristics of accessory cells involved in immune responses; C3b and IgG - Fc receptors, expression of immune response associated antigens (Ia) and the ability to stimulate both allogeneic T cells and syngeneic, antigen-specific T cells (reviewed in reference 2). Further investigations have demonstrated that the induction of contact sensitivity (CS) reactions through areas of skin naturally deficient in LC results in decreased reactivity to the contactant (3). In addition, UV-irradiation of normal skin prior to application of the contact sensitizing agent also results in decreased reactivity (3, 4). This latter phenomenon is thought to be due to a functional inactivation of the LC in the irradiated skin as UV-irradiation has also been shown to eliminate certain LC surface markers (3-5) although by electron microscopy, the cells can still be detected in exposed epidermis (5). This interpretation has been further supported by recent studies which have shown that UV-irradiation of a single cell suspension of epidermal cells eliminates the ability of these cells to 1) induce contact hypersensitivity to haptens coupled to the epidermal cell surface (6) and 2) present antigen to syngeneic, antigen-primed T cells (7).

These studies demonstrating an immunologic role for epidermal LC have led to much discussion as to possible involvement of LC in photocarcinogenesis, especially since it is now well established that photocarcinogenesis in mice is preceded by a suppression of certain immune responses. Specifically, mice exposed to subcarcinogenic doses of UV light develop an inability to reject certain UV-induced tumors which are readily rejected by unirradiated littermates. This phenomenon is referred to as UV-induced tumor susceptibility and has been shown to be mediated, at least in part, by a population of T suppressor cells ($T_s$; reviewed in 8). It should be noted that this tumor susceptibility is not due to a pan-immunosuppression however, as tumor susceptible animals have been shown to be capable of mounting normal immune responses to numerous other antigens (9, 10).

The UV-induced $T_s$ cells which are able to prevent the generation of an effective antitumor immune response have further been shown to be capable of adoptively transfering the tumor susceptible state to normal unirradiated animals. A similar induction of $T_s$ cells also may be responsible for the reduction in CS responses following exposure to UV light. Thus, the transfer of spleen cells from UV exposed, contact sensitized animals to normal, syngeneic mice resulted in a reduced CS reaction upon challenge of the recipients (4). This similarity between a) UV-induced tumor susceptibility and b) the decrease in CS responses in animals sensitized in areas exposed to UV light has led to the

suggestion that inactivation of LC function may be responsible for both of these modified responses (3,4,8).

The ability of UV exposure to cause skin damage has led to studies on the relationship of this change to alterations in immune responsiveness. One approach has been to investigate the effect of various sunscreen agents which are known to prevent the histological damage, on the systems mentioned above. These studies have demonstrated that the topical application of para-aminobenzoic acid (PABA) is able to prevent much of the measureable skin damage (i.e., hyperplasia, parakeratosis and decreased ATPase positive cell densities) but not the decrease in CS responses or the acquisition of the tumor susceptible state (4, 11). In fact, no protection against the immunoregulatory effects of UV light by PABA could be detected by functional assays. If the hypothesis that UV-irradiation causes a functional inactivation of LC in the epidermis is correct, then it follows that PABA should not prevent this inactivation.

The purpose of this investigation was to further test the hypothesis presented above and its prediction. To do so, we have evaluated the epidermal APC activity of murine ear skin following UV-irradiation. In addition, some ears were pretreated with PABA to evaluate whether this sunscreen agent afforded any protection against a UV-induced decrease in activity. The APC activity was evaluated by assessing antigen-specific proliferation of primed T cell <u>in vitro</u>. Furthermore, we compared the dose-response of the

APC inactivation to the inactivation of CS reactions by UV light. Finally, we evaluated the ability of epidermal cells to provide accessory cell function in a mitogen-induced stimulation of T cells *in vitro* and the effect of UV-irradiation on this accessory cell function of epidermal cells.

## Materials and Methods

### Animals

Normal 4-6 week old C3Hf/HeN (MTV-) mice were obtained from the animal production facility of the National Cancer Institute (Bethesda, MD). All mice were housed at a maximum density of five animals per 7 x 11 inch cage and maintained on Wayne Sterilizable Lab Blox and acidified water ad libidum.

### UV-Irradiation

The UV light source consists of a bank of three FS40 Westinghouse fluorescent sunlamps emitting principally (>60% of their energy) at wavelengths between 280 and 320 nm (UVB). The flux of 2.9 $J/m^2/sec$ was measured at a tube to target distance of ~21cm. The measurement of flux and spectrum of emissions have been described in detail previously (12). Animals were anesthetized by intraperitoneal injection of a solution of 4% chloral hydrate, 0.15% atropine sulfate (0.2 ml) prior to the UV treatments to decrease variations in the exposures due to animal movement during the treatment period. The anesthetized animals

were then placed on a bed of sawdust in the bottom of a 7 x 11 inch cage for the exposure period.

To evaluate the effects of para-aminobenzoic acid (PABA), animals' ears were each treated with approximately 50 µl of PreSun (Westwood Pharmaceuticals, Buffalo, N.Y.) with a sun protection factor of 8 (5% PABA). This was done just prior to the UV treatment while the anesthetic was taking effect.

In vitro UV-irradiation was performed by exposing either single cell suspensions of epidermal cells in 60 x 15 mm plastic petri dishes (Falcon #1007, Oxnard, Calif.) or by exposing the excised ear, split and laid dermal side down on PBS soaked cotton guaze. The single cells were washed once with modified Dutton's Balanced Salt Solution (DBSS without phenol red) to eliminate the fetal calf serum and resuspended in 5 mls of DBSS for the irradiation period (final cell concentration: $2 \times 10^5$/ml). The exposed ears were washed once in PBS following the exposure and then dissociated as described below.

## Cell Suspensions

Ear epidermis was used as the source of epidermal cells and only that portion of the ear opposing the light bulbs was used. To obtain epidermis, the ears were washed with 95% ethanol, excised, split, and placed dermal side down on a solution of EDTA (18mM) as described by Juhlin and Shelley (13). After incubation at 37°C for 75-90 minutes, the epidermis was carefully peeled off

the dermis and minced in a solution of 0.25% collagenase (Sigma, St. Louis, MO.), 0.25% Dispase (Boehringer-Mannheim, Indianapolis, Ind.) in $Ca^{++}$ $Mg^{++}$ free Hanks Balanced Salt Solution (Flow Laboratories, Inglewood, Calif.). This was incubated for one-half hour at 37°C, briefly aspirated in a pipet and then incubated for another 30-40 minutes at 37°C. Following the addition of 10 mls of complete medium (RPMI + 10% fetal calf serum + 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine 1 mM sodium pyruvate and $5 \times 10^{-4}$ M 2-mercaptoethanol) the suspension was repeatedly aspirated in a pipet. The debris was then allowed to settle out for 5 min. The resulting single cell suspension was washed twice in complete medium and gamma-irradiated (1000 rad) before use.

Spleens were aseptically excised, dissociated in complete medium and the red cells eliminated by hypotonic lysis as previously described (9). Prior to addition to co-cultures as APC or accessory cell sources, the cells were gamma-irradiated (1000 rads) and washed in complete medium.

Primed T Cells

Keyhole Limpet Hemocyanin (KLH) - primed T cells were prepared essentially as described by Cowing, et. al. (14). Mice were injected in each hind footpad with 20 µg of KLH emulsified in 50 µl of CFA (Complete Freunds Adjuvant, Difco). Fourteen to 30 days later, the inguinal and popliteal lymph nodes were removed,

dissociated in complete medium and the cells passed over a nylon wool column as described previously (15). In addition, we treated the nylon non-adherent cells with anti-$Ia^k$ serum (ATH anti-ATL) plus rabbit complement for 45-60 minutes at 37°C. This resulted in a T cell enriched population which required the addition of an exogeneous source of antigen presenting cells to induce a significant proliferative response to either KLH or the purified protein derivative of Mycobacterium tuberculosis and was antigen specific (no proliferative response to ovalbumin) as shown in a previous paper (16).

Antigen-Specific and Mitogen-Induced Proliferation of T Cells

Antigen-induced stimulation was assessed by measuring T cell proliferation in flat bottomed microtitre plates (#3596, Costar, Cambridge, MA). Two x $10^5$ antigen-primed T cells were added to various numbers of epidermal cells (in sextuplicate) in a final volume of 0.2 ml per well. We found that between 2.5 x $10^4$ and $10^5$ epidermal cells produced the best stimulation; greater numbers often proved inhibitory and fewer cells produced little stimulation. KLH was added to half of the wells to a final concentration of 50 µg/ml . Preliminary experiments demonstrated that this procedure gave equivalent stimulation and was easier to set-up than pulsing the epidermal cells with antigen and then washing them before co-culture with the primed T cells. The plates were incubated at 37°C, in a humidified atmosphere of 6%

$CO_2$ in air. After 5 days, 1 μCi of $^3H$-thymidine (2 Ci/m mole, New England Nuclear, Boston,-
Mass.) was added to the wells in a total volume of 0.01 ml. After 8-10 hours of additional incubation, the cells were harvested. The amount of radioactivity incorporated into DNA was assessed as previously described (16). The values presented, Δ counts per minute (Δ cpm), represent the difference in mean cpm in antigen-pulsed wells minus the mean cpm in non-pulsed wells containing equal numbers of epidermal cells. Standard errors of the mean (SEM) were generally less than 10%.

Mitogen-induced stimulation was evaluated as for antigen-induced stimulation except for the following changes. Concanavalin A (Con A) was added to the wells (in place of KLH) at a final concentration of 4 μg/ml. The assay was generally harvested after 4 days and only $10^5$ T cells were added per well.

Contact Sensitizations

Two sensitization protocols were used depending on whether the animals were sensitized on the dorsal surface or on the ears. Sensitizations on the ear were done on day 0 by carefully applying (with a cotton swab) a small amount (5-10 μl) of a 0.5% solution of 1-fluoro, 2, 4-dinitrobenzene (DNFB, Sigma Chemical Co., St. Louis, MO.), in acetone:olive oil (4:1). The application of DNFB was limited to the exposed anterior section of the ear. In addition, the animals were anesthetized before sensitization, to prevent them from rubbing their ears and spreading the DNFB to

unexposed sites. The anesthetic is effective for approximately 30-45 minutes. Sensitizations on the back were done on day 0 and day 1 by applying 25 µl of the DNFB solution to the shaved dorsal surface. All animals were challenged on day 5 with 10 µl of the same solution (stored at 4°C) on one hind footpad. Footpad swelling was assessed the next day as the difference in total thickness of the challenged foot versus the unchallenged foot as described previously (4).

Determination of Ia Positive Cells Densities

To evaluate the number of $Ia^+$ cells in a section of epidermis, the epidermis was separated from the dermis as described above. The epidermis was then fixed in acetone for 20 min at room temperature and rehydrated in complete media for 1 hour. To stain $Ia^+$ cells, the fixed epidermis was cut into 2 x 2 mm sections and incubated in complete media or supernatant from the anti-I-$A^k$ hybridoma clone 11-5.2 obtained from the Salk Institute for Biological Studies (La Jolla, Calif.). After 1 hour at room temperature or an overnight incubation at 4°C the epidermal sections were washed in 3 changes of PBS and incubated for another hour with a 1:40 dilution (in PBS with 1% bovine serum albumin) of a fluorescein conjugated, goat anti-mouse $IgG_2$ (Meloy Laboratories Springfield, VA). After 3 washes in PBS the epidermal sections were placed on a glass slide, mounted with tris-buffered glycerol (pH-8.0) and fluorescent cells enumerated

using a Zeiss microscope (model 62709) equipped for epifluorescence. To obtain a cell density, one eyepiece was fitted with a reticle which, using the 40X objective, corresponded to an area of 0.0365 $mm^2$. Six to 8 fields were counted to obtain a mean and standard deviation. This procedure resulted in fluorescent dendritic cells only in epidermis from C3H or B10.A ($I\text{-}A^k$) mice. B10 ($I\text{-}A^b$) epidermis or epidermis which was incubated in media (not hybridoma supernatant) were always negative with regards to fluorescent dendritic cells.

Histochemistry

When epidermis was also stained for ATPase activity, the procedure used, [described elsewhere (4)], was essentially as described by MacKenzie and Squier (17). ATPase positive cell densities were enumerated as for $Ia^+$ cells but with visible light.

## Results

We first established the parameters of the antigen presenting cell (APC) assay. The stimulation was antigen-specific (i.e., 9,000 Δcpm using KLH-pulsed epidermal cells versus 1000 Δcpm using ovalbumin-pulsed epidermal cells after only 3 days in culture) and reached a peak after 5 days in culture (Table I). We also found that similar levels of stimulation were obtained if the KLH were simply added to the co-cultures at the time of APC addition, rather than pretreating and washing the APC before co-culture.

TABLE I

Kinetic evaluation of antigen-primed T cell proliferation using murine epidermal cells as a source of antigen presenting cells (APC).

| Day[a] | APC Source[b] | | T cells alone[d] | |
|---|---|---|---|---|
| | Spleen | Epidermis | Media | +KLH |
| 3 | 10,258[c] | 502 | 135 | 130 |
| 4 | 22,704 | 4738 | 990 | 179 |
| 5 | 28,721 | 6845 | 361 | 253 |

a) Number of days in culture before harvesting. Cultures (in triplicate) were pulsed with $^{3}$H-thymidine as described in Materials and Methods.

b) APC were gamma irradiated (1000 rads) just prior to coculture with the antigen-primed T cells as described in Materials and Methods. Cell concentrations per well were $7.5 \times 10^{4}$ spleen or epidermal cells and $2 \times 10^{5}$ T cells.

c) Values presented are Δcpm as described in Materials and Methods. SEM were generally less than 10%.

d) As a control, the T cell enriched population was cultured in the presence and absence of antigen (KLH) without the addition of an exogenous source of APC. Values presented here are cpm not Δcpm.

Good stimulation was observed with a final concentration of KLH between 25 and 100 μg/ml (data not shown). Therefore, we chose a final concentration of 50 μg/ml and used this throughout the rest of the study.

If epidermis were first dissociated into a single cell suspension and then exposed to the UV light (see Table II), exposures of approximately 90 $J/m^2$ (9.0 $mJ/cm^2$) reduced the amount of $^3H$-Tdr ($^3H$-thymidine) incorporated by almost 60% (Table II). Even as little as 15 $J/m^2$ (1.5 $mJ/cm^2$) was capable of reducing the amount of stimulation (by approximately 16%). Two representative experiments (out of 5) are shown in Table II. These results are comparable to those of Stingl, et al., who found that exposures of 100 $J/m^2$ (10 $mJ/cm^2$) resulted in reductions which varied from 67-90% (7).

The exposure of single cell suspensions certainly does not reflect the situation *in vivo*, as the usual residence of the Langerhans cell in the epidermis is intercalating between prickle cells in a supra basal position (2). Hence, in the murine epidermis which is 2-3 cell layers thick, any UV photons must pass through at least the stratum corneum and often one other cell layer before striking an LC. These overlying cell layers present a potential "trap" for the UV radiation which may, in turn, dramatically reduce the inactivation of LC function by UV-irradiation. To evaluate this possibility two irradiation protocols were employed. In the first procedure, termed *in vitro*

Table II

Antigen presenting cell activity of murine epidermal cell suspensions exposed to UV-irradiation.

| UV Exposure[a] ($J/m^2$) | Δcpm (% Reduction)[b] Exp. 1 | | Exp. 2 | | Average %[d] Reduction |
|---|---|---|---|---|---|
| 0 | 15,510 | - | 16,620 | - | - |
| 15 | 14,223 | (8)[c] | 12,499 | (25) | 16.5 |
| 45 | 9,832 | (37) | 10,630 | (36) | 36.5 |
| 90 | 6,335 | (59) | 6,996 | (58) | 58.5 |

a) One million gamma irradiated (1000 rads) epidermal cells were suspended in 5 mls of balanced salt solution in a plastic petri dish and exposed to various doses of UV light.

b) Five x $10^4$ viable epidermal cells (as determined by trypan blue dye exclusion) were co-cultured for 5 days with 2 x $10^5$ antigen-primed T cells in the presence and absence of antigen (KLH). The cultures were pulsed with $^3$H-thymidine for 8-10 hours on day 5. The values (Δcpm) presented are the mean difference in $^3$H-incorporation in the presence versus the absence of added antigen for triplicate cultures. SEM were generally less than 10%.

c) Per cent reduction is calculated by:

$$\left(1 - \frac{\Delta\text{cpm UV-exposed}}{\Delta\text{cpm unexposed}}\right) \times 100$$

d) Average per cent reduction at each dosage for the two experiments presented.

UV-irradiation, ears were aseptically excised from the animal, split (along the cartilage) and placed dermal side down on PBS soaked cotton gauze in plastic petri dishes before the UV-irradiation. In the second procedure, termed *in vivo* UV-irradiation, the animals were irradiated prior to removal and dissociation of the ear epidermis.

Evaluation of epidermal APC activity following *in vitro* UV-irradiation demonstrated that approximately twice as much energy was required to inactivate the APC function of cells in skin as compared to a single cell suspension (Table III). A 50% reduction in activity required 175 $J/m^2$ (Expt. 2, Table III) whereas less than 90 $J/m^2$ was required when single cell suspensions were exposed to the UV light (Table II). Exposure to ~ 850 $J/m^2$ resulted in reductions of 66-94% in different experiments (see also Table VIII). Exposures of more than 2500 $J/m^2$ consistently resulted in greater than a 90% reduction in APC activity.

Following *in vivo* UV-irradiation the dose-response was shifted even further so that an increased dosage was required to obtain a reduction in activity similar to that obtained by *in vitro* UV-irradiation. Thus, 500 $J/m^2$ reduced the APC activity by approximately 45% and exposures of 3000 $J/m^2$ were necessary to reduce the epidermal APC activity by 80% or better (Table IV).

UV-irradiation has been shown to produce concomitant decreases in both $ATPase^+$ cell densities and in contact sensitivity (CS) responses. Both of these responses have been

Table III

Antigen presenting cell activity of murine epidermal cells after in vitro UV-irradiation of ear skin.

| Experiment # | Epidermal[a] Cell Source | UV[a] Exposure ($J/m^2$) | Epidermal Cells/Well[b] $5 \times 10^4$ | Epidermal Cells/Well[b] $2.5 \times 10^4$ | Average % Reduction |
|---|---|---|---|---|---|
| 1 | Normal | 0 | 9127[b,c] | 4307 | |
| | UV | 3250 | 358 (96)[b] | 448 (90) | 93 |
| | UV | 4850 | 397 (96) | 0 (100) | 98 |
| 2 | Normal | 0 | 2695 | 1237 | |
| | UV | 175 | 1552 (42) | 437 (62) | 52 |
| | UV | 875 | 437 (84) | 68 (94) | 89 |
| | UV | 2600 | 0 (100) | 0 (100) | 100 |

(a) Ears were aseptically excised, split and floated dermal side down on PBS in plastic petri dishes. UV dosage was monitored for each experiment.

(b) Values represent Δcpm with percent reduction in parentheses. See footnotes to Table II.

(c) Background values (cpm) for T cells alone, with and without KLH in Exp. 1 were: media = 95, + KLH = 325; in Exp. 2 were: media = 197, + KLH = 179.

Table IV

Antigen presenting cell activity of epidermal cells isolated from ear epidermis after *in vivo* UV-irradiation.

| Experiment # | Epidermal[a] Cell Source | UV[a] Exposure ($J/m^2$) | Epidermal Cells/Well[b] $5 \times 10^4$ | | $2.5 \times 10^4$ | | Average % Reduction |
|---|---|---|---|---|---|---|---|
| 1 | Normal | 0 | 22,306[b,c] | | 9,602 | | |
| | UV | 500 | 8,999 | (60)[b] | 4,264 | (56) | 58 |
| | UV | 1500 | 3,322 | (85) | 1,656 | (83) | 84 |
| | UV | 3000 | 284 | (99) | 240 | (97) | 98 |
| 2 | Normal | 0 | 10,897 | | 6,021 | | |
| | UV | 500 | 6,985 | (36) | 4,146 | (31) | 34 |
| | UV | 1500 | 3,970 | (64) | 1,736 | (71) | 68 |
| | UV | 3000 | 2,257 | (79) | 976 | (84) | 82 |

(a) Epidermis was isolated and dissociation begun within two hours after the UV treatment. All animals were anesthetized just prior to the exposure. UV dosage was monitored for each experiment.

(b) See footnotes to Table II and III.

(c) Background values (cpm) for T cells alone, with and without KLH in Exp. 1 were: media = 83, + KLH = 280; in Exp. 2 were: media = 121, + KLH = 563.

postulated to be indicative of a functional inactivation of LC in the irradiated epidermis (3, 4). As we have established the dosage of UV necessary to eliminate APC function in the epidermis, we compared this dose-response to that needed to inhibit the induction of CS responses to DNFB. The results demonstrate that epicutaneous sensitization, via the ear, immediately following UV exposures of less than 5000 $J/m^2$, does not affect the induction of CS responses. Furthermore, an exposure of 7200 $J/m^2$ results in only a marginal diminution (12%) in CS (Table V). When the animals were rested for 7 days between the UV exposure and contact sensitization, however, decreased CS responses were observed in a dose-related fashion. Although a 48% reduction in the CS response was observed at an exposure of 7200 $J/m^2$, a comparison with Table IV shows this to be greater than 10 times the amount of energy necessary to get a similar reduction in APC activity following *in vivo* UV-irradiation.

Epicutaneous sensitization with DNFB via the dorsal epidermis resulted in a slightly different picture from that presented above. A UV exposure of 4800 $J/m^2$ immediately prior to sensitization resulted in a 40% reduction in CS responses (Table VI). An exposure of 2400 $J/m^2$ (which is capable of eliminating the majority of epidermal APC activity, see Table IV) resulted in only a 12% depression in CS response when sensitization was performed immediately after UV treatment. With increasing intervals between the exposure and the DNFB application, however,

Table V

Contact sensitivity responses of animals sensitized on the ear either immediately or seven days after receiving a single UV exposure.

| UV treatment[a] Day | Dose ($J/m^2$) | DNFB[b] Sensitization | Footpad swelling[c] (mm x $10^2$ ± SEM) | Per Cent[d] Reduction |
|---|---|---|---|---|
| - | 0 | - | 5.0 ± 2.2 | 92 |
| - | 0 | + | 65.0 ± 13.4 | - |
| 0 | 2400 | + | 74.0 ± 7.2 | 0 |
| 0 | 4800 | + | 72.0 ± 6.3 | 0 |
| 0 | 7200 | + | 57.0 ± 7.2 | 12 |
| -7 | 2400 | + | 53.8 ± 10.3 | 17 |
| -7 | 4800 | + | 42.0 ± 8.5 | 35 |
| -7 | 7200 | + | 33.8 ± 4.0 | 48 |

a) UV-irradiated animals were anesthetized for the period of the single UV treatment. The treatment was administered either immediately prior to, or seven days prior to the epicutaneous sensitization.

b) Epicutaneous sensitization was performed on day 0 on the outer, anterior section of the ear as described in Materials and Methods. All animals were sensitized at the same time.

c) Animals were challenged on one footpad on day 5 and footpad swelling (measured 24 hours later) evaluated as the difference in thickness between the challenged and the unchallenged foot. Values are a mean of five animals per group.

d) Per cent reduction in footpad swelling was calculated by the formula:

$$\left( 1 - \frac{\text{Experimental value}}{\text{Normal value}} \right) \times 100$$

Table VI

Contact sensitivity responses of animals sensitized on the dorsal skin after a single UV exposure either 0, 1, 2 or 3 days before sensitization.

| UV treatment[a] | | DNFB[b] | Footpad swelling[c] | Per Cent[c] |
|---|---|---|---|---|
| Day | Dose ($J/m^2$) | Sensitization | (mm x $10^2$ ± SEM) | Reduction |
| - | 0 | - | 5.0 ± 2.0 | 95 |
| - | 0 | + | 107.5 ± 3.2 | - |
| 1-week[d] | 5 x 4800 | + | 28.3 ± 6.3 | 74 |
| -3 | 300 | + | 103.8 ± 8.7 | 3 |
| -3 | 800 | + | 93.8 ± 7.2 | 13 |
| -3 | 2400 | + | 18.8 ± 5.6 | 83 |
| -3 | 4800 | + | 22.5 ± 4.4 | 79 |
| -2 | 300 | + | 103.3 ± 10.1 | 4 |
| -2 | 800 | + | 103.8 ± 11.2 | 3 |
| -2 | 2400 | + | 65.0 ± 13.5 | 40 |
| -2 | 4800 | + | 37.5 ± 10.5 | 65 |
| -1 | 300 | + | 103.8 ± 13.9 | 3 |
| -1 | 800 | + | 95.0 ± 7.9 | 12 |
| -1 | 2400 | + | 71.3 ± 9.5 | 34 |
| -1 | 4800 | + | 58.8 ± 16.0 | 45 |
| 0 | 300 | + | 106.3 ± 10.9 | 1 |
| 0 | 800 | + | 97.5 ± 11.1 | 9 |
| 0 | 2400 | + | 95.0 ± 3.1 | 12 |
| 0 | 4800 | + | 61.2 ± 11.2 | 43 |

a) Dorsal hair was shaved and animals were exposed to a single UV exposure either 0, 1, 2 or 3 days before sensitization with DNFB.

b) Epicutaneous sensitization was performed by applying 25 µl of the 0.5% DNFB solution to the shaved dorsal surface on days 0 and 1.

c) See footnotes to Table V.

d) These animals received 5 daily exposures of 4800 $J/m^2$/day (one exposure/day) beginning on day -5.

sensitization was increasingly less effective. A 34% reduction in CS responses was observed when the animals were sensitized 24 hours after the UV treatment and a 83% reduction was observed if sensitization was begun 72 hours after the UV treatment. This is the maximal reduction observed in this system, being equivalent to the reduction observed in animals treated with 4800 $J/m^2$/day for 5 days prior to sensitization (74% reduction) and equivalent to that previously reported for long-term UV-irradiated animals (5 weeks of daily 30' treatments) (4). Surprisingly, a dosage of 800 $J/m^2$, which is sufficient to eliminate much of the epidermal APC activity (see Table IV), resulted in almost no diminution of CS responsiveness (i.e., maximum reduction equalled 13%) even if sensitization were begun 3 days post UV-irradiation. These findings are similar to the results of Noonan et al. who, in a study of systemic effects, found it was necessary to wait more than 24 hours between UV exposure and sensitization to obtain decreased CS responsiveness (18,19). From our results it is apparent that the level of epidermal APC activity (as evaluated in vitro) does not necessarily correlate with the level of CS responsiveness which can be induced in UV-irradiated animals. Reductions in CS responsiveness required greater dosages of UV exposure and exhibited differences in kinetics.

The ability to detect Ia positive cells in murine epidermis has been reported to be lost following exposure to 600-800 $J/m^2$ of UVB radiation (5). That study, however, was performed with wild

type, hairless mice. As the presence of hair has been shown to influence dramatically the amount of UV exposure required to induce tumor susceptibility in C3H mice (8), we evaluated the density of I-$A^k$ positive cells in irradiated ear epidermis following a single exposure to various doses of UV light. A dosage of 1600 J/$m^2$ did not reduce the density of $Ia^+$ cells in the exposed ear epidermis (Table VII) although this dosage had been able to reduce APC activity by an average of 76% in previous experiments (Table IV). Higher dosages of UV, however, did reduce the density of epidermal $Ia^+$ cells by 30-40%. We also found that the densities of $ATPase^+$ cells paralleled those of $Ia^+$ cells in irradiated skin (data not shown), as previously reported by Aberer et al. (5). Topical application of a solution containing 5% para-aminobenzoic acid (PABA) immediately prior to UV-irradiation prevented any significant loss of $Ia^+$ cells at exposures of 1600 or 2800 J/$m^2$ and resulted in only a slight decrease in $Ia^+$ cell densities (< 20%) at the higher dosage of 5600 J/$m^2$. Topically applied PABA also prevented any reduction in $ATPase^+$ cell densities (data not shown). Thus, these data demonstrate that the loss of APC activity cannot be attributed simply to loss of $Ia^+$ cells from the exposed epidermis.

The results of previous studies as well as those presented above, demonstrate that PABA is very effective in preventing much of the skin damage associated with experimental exposure to UV light (4, 11, Table VII). Despite this, PABA is ineffective in

TABLE VII

The effect of UV-irradiation and topically applied para-aminobenzoic acid (PABA) plus UV-irradiation on the density of Ia positive cells in ear epidermis.

| Experiment # | Treatment[a] | UV Exposure ($J/m^2$) | $Ia^+$ Cells/$mm^2$[b] day 0[c] | day 1[c] | day 2[c] |
|---|---|---|---|---|---|
| 1 | None | 0 | 897 | 794 | N.D.[e] |
| | UV | 1600 | 877 (2)[d] | 847 (0) | N.D. |
| | PABA + UV | 1600 | 1042 (0) | 759 (4) | N.D. |
| 2 | None | 0 | 980 | 733 | 857 |
| | UV | 2800 | N.D. | 514 (30) | 554 (35) |
| | UV | 5600 | 691 (29) | 477 (35) | 528 (38) |
| | PABA + UV | 2800 | 910 (7) | 866 (0) | 822 (4) |
| | PABA + UV | 5600 | 814 (17) | 625 (15) | 925 (0) |

(a) Animals were anesthetized and on one half of the animals, Pre Sun (containing 5% PABA) was rubbed on the ears just prior to exposure.

(b) I-$A^k$ positive cells were enumerated as described in Materials and Methods. SEM were less than 10%.

(c) Samples of ear skin were obtained within 2 hours of the UV exposure for day 0, at 20-24 hours later for day 1, and at 44-48 hours later for day 2.

(d) The number in parentheses is the percentage reduction in Ia positive cells/$mm^2$ based on the normal value obtained on that day.

(e) N.D. = not done

preventing the UV-induced depressions in immunologic responses to contact sensitizing agents or to UV-induced tumors (4,11). Hence, we evaluated the effect of PABA on the functional inactivation of epidermal cells following UV exposure. Application of a solution of 5% PABA before in vitro UV-irradiation of ear skin offered a small degree of protection against the inactivation of epidermal APC activity (Table VIII). While an exposure of 850 $J/m^2$ of UV alone resulted in approximately a 75% reduction in epidermal APC activity (see Tables III and VIII), in the presence of PABA, this dosage resulted in only a 45% reduction. At the higher dosage of 2500 $J/m^2$, a slightly lower reduction (~ 25%) was also observed in the PABA plus UV treatment group. Nonetheless, the reduction in the presence of PABA represents a significant elimination of APC activity. In addition, the dosages used here are well within the dosages used in previous studies which demonstrated that PABA was unable to provide significant protection against the UV-induced alterations in immunological function (4,11). At dosages less than 2500 $J/m^2$ the effect of PABA on in vivo UV irradiation of ear skin gave similar results. [i.e., at 1500 $J/m^2$ the average % reduction ± SEM for 5 experiments with PABA plus UV = 47 ± 8% (data not shown) compared with ~75% for UV alone]. Clearly, epidermal APC are rapidly inactivated by UV light, even in the presence of PABA.

The cell type responsible for APC function has been shown to bear Ia antigens regardless of the cell source (7,14,20). The

Table VIII

The effect of para-aminobenzoic acid (PABA) on epidermal antigen presenting cell activity following in vitro UV-irradiation of ear skin.

| Experiment # | Epidermal[a] Cell Source | UV[a] Exposure ($J/m^2$) | Epidermal Cells/Well[b] $10^5$ | $5 \times 10^4$ | $2.5 \times 10^4$ | Average % Reduction |
|---|---|---|---|---|---|---|
| 1 | Normal | 0 | 31,344[b,c] | 26,597 | 12,133 | |
| | UV | 850 | 10,656 (66)[b] | 8,506 (68) | 3,720 (69) | 68 |
| | UV | 2550 | 1,667 (95) | 2,766 (90) | 1,633 (87) | 91 |
| | PABA Plus UV | 850 | 14,953 (52) | 16,918 (36) | 8,026 (34) | 41 |
| | PABA Plus UV | 2550 | 11,814 (62) | 10,405 (61) | 5,843 (52) | 58 |
| 2 | Normal | 0 | 4,322 | 2,070 | 1,139 | |
| | PABA Plus UV | 870 | 2,525 (41) | 1,107 (47) | 500 (44) | 44 |
| | PABA Plus UV | 2600 | 1,108 (74) | 454 (78) | 219 (81) | 78 |

(a) Ears were aseptically excised from animals, split and placed dermal side down on PBS soaked cotton gauze for the UV exposure. Treatment of the ears with 5% PABA was done before the ears were excised from the animals. UV dosage was monitored for each experiment.

(b) See footnotes to Tables II and III.

(c) Background values (cpm) for T cells alone, with and without KLH in Exp. 1 were: media = 240, + KLH = 1202; in Exp. 2 were: media = 45,+ KLH = 64.

expression of Ia antigens is also a strict phenotypic characteristic of the accessory cell required for a mitogen-induced stimulation of T cells (21). The Langerhans cell (LC) in the epidermis, while known to bear Ia and possess good APC function, has not yet been evaluated for accessory cell activity. A kinetic evaluation of T cell stimulation by concanavalin-A in the presence and absence of epidermal cells demonstrated that: 1) these cells provide necessary and ample accessory cell function, and 2) stimulation is detectable as early as 48 hours after initiation of the co-cultures. Maximum stimulation, however, was not observed until 96 to 120 hours in culture, depending on the number of epidermal cells added (Table IX).

The finding that LC in the epidermis are functional accessory cells provides another means of assessing their immunological function following exposure to UV-irradiation. The results of this assessment (Table X) demonstrate that both the accessory cell function and APC function of epidermal cells were lost at comparable dosages following in vitro UV-irradiation of whole skin. Exposure to 850 $J/m^2$ caused a 90% reduction in accessory cell activity (Table X) while this same dosage resulted in a 66-94% reduction in APC activity (Tables III and VIII). The small degree of protection provided by PABA against UV-induced inactivation of APC activity (~ 20%) is also observed in the evaluation of accessory cell function.

Table IX

Kinetic evaluation of T cell stimulation by concanavalin-A in presence of epidermal cells as the accessory cell source.

| Day[a] | Viable epidermal cells per well[b] | | T cells alone[d] | |
|---|---|---|---|---|
| | $10^5$ | $5 \times 10^4$ | Media | + ConA |
| 2 | 4,544[c] | 2,691 | 162 | 885 |
| 3 | 22,227 | 14,649 | 231 | 353 |
| 4 | 148,628 | 96,885 | 273 | 518 |
| 5 | 114,894 | 128,570 | 219 | 1312 |

a) Number of days in culture before harvesting as described in Materials and Methods.

b) Various numbers of viable epidermal cells (as determined by trypan blue exclusion) were gamma-irradiated (1000 rads) and co-cultured with $10^5$ T cells in the presence and absence of concanavalin-A (4 µg/ml).

c) Values presented are Δcpm as described in Materials and Methods. SEM for triplicate cultures were less than 10%.

d) To evaluate the residual level of accessory cells in the T cell enriched population, the enriched population was always cultured alone in the presence and absence of concanavalin-A. Values presented here are cpm, not Δcpm.

Table X

Accessory cell function of epidermal cells in a proliferative response to Con A following exposure of murine ear skin to UV light *in vitro*.

| Epidermal[a] Cell Source | UV Exposure ($J/m^2$) | Viable epidermal cells/well[b] $10^5$ | $5 \times 10^4$ | $2.5 \times 10^4$ | Average % Reduction |
|---|---|---|---|---|---|
| Normal | 0 | 148,628[b] | 96,885 | 46,195 | |
| UV | 850 | 12,134 (91.8)[c] | 7,339 (92.4) | 3,482 (92.4) | 92 |
| UV | 2550 | N.D.[d] | 448 (100) | 524 (99) | 100 |
| PABA + UV | 850 | N.D. | 29,223 (70) | N.D. | 70 |
| PABA + UV | 2550 | 24,053 (83.8) | 13,100 (86.5) | 6,031 (86.9) | 86 |

a) Epidermal cells were isolated from ear epidermis after UV exposure as described in Materials and Methods. PABA was applied while the ear was still attached to the animal. Ears were excised, split and laid dermal side down on PBS-soaked sterile cotton gauze for exposure to the UV radiation.

b) See footnotes to Table IX. Background values for T cells alone (no accessory cells added) were: media = 273 cpm, + ConA = 518 cpm.

c) Values in parentheses are per cent reduction of $^3H$-incorporation as calculated by:

$$\left(1 - \frac{\Delta\text{cpm UV exposed}}{\Delta\text{cpm normal}}\right) \times 100$$

d) N.D. = not done.

## Discussion

The effects of UV radiation on immune responses in mice include decreased contact sensitivity (CS) reactions and decreased responsiveness to UV-induced tumors (3,4,8). Both of these phenomena are known to involve the induction of $T_s$ cells (4,8). The speculations that one mechanism of induction of $T_s$ cells is by bypassing the antigen processing step of the macrophage, has lead to the hypothesis that UV-induced inactivation of epidermal Langerhans cells by UV light is a critical step in both of these modified immune responses (22-24).

The results presented in this report verify the suggestion that Langerhans cells in UV-exposed skin lose immunologic function (2-4,8). The inactivation of both accessory cell function (evaluated by mitogen-induced stimulation of T cells) and of antigen presenting cell function (evaluated by antigen-specific stimulation of primed T cells) occurs rapidly and in a dose dependent manner. In addition, the results demonstrate that UV exposure of intact skin requires at least twice as much energy to achieve a comparable reduction in APC activity as the amount needed when the epidermal cells are in a single cell suspension. A further increase in energy is needed if the tissue source (ear skin) is left on the animal during the UV exposure (as opposed to being excised and placed on cotton gauze in a petri dish during the exposure). This latter increase in the energy needed for inactivation of APC activity may be due to the differences in

geometry associated with the two procedures. When the ear is excised prior to the UV exposure, it is laid flat in the petri dish and is therefore more exposed to the UV light. Irradiation of the ear while still attached to the animal, however, presents an entirely different geometry, i.e., a curved surface, all of which is not directly facing the light source, as well as being more protected by the sides of box in which the mice are kept during the irradiation.

The effect of the sunscreen agent, para-aminobenzoic acid (PABA), on the inactivation of epidermal APC and accessory cell function was also investigated in this study. The rationale for this evaluation was that previous reports have shown that despite this agent's ability to prevent much of the histologic skin damage caused by UV exposure, PABA does not prevent either the decreased ability to contact sensitize UV-irradiated animals or the induction of tumor susceptibility (4,11). The results of this present study demonstrate that PABA is also incapable of preventing the inactivation of epidermal APC and accessory cell function by UV light. Although the data indicate that there is a slight protective effect, it is at best marginal. Certainly, at the dosages of UV-radiation used in the previous studies which demonstrated alterations in immunological function following treatment with PABA plus UV light, APC function had been eliminated from the epidermis. Thus, our results support the hypothesis that UV-inactivation of APC in exposed epidermis plays a critical role in the immunosuppression (3,8).

The inactivation of cells following *in vitro* UV exposure has been shown to require an interval to manifest the effects, i.e., loss of function and death (12,25,26). This fact and the knowledge that immune responses require the interaction of multiple cell types may explain the differences in kinetics and, to a certain degree, the differences in dosage required to inactivate APC function when assessed by *in vitro* versus *in vivo* methods (T cell stimulation versus CS reactions respectively). Thus, we propose that LC in exposed epidermis are able to initiate a CS reaction if the contactant is applied soon enough after the exposure. Following initiation, the exposed LC may die, but other accessory cells are then able to support the induction of the CS reaction and allow for the normal response. Support for this proposal has been presented by Turk and Stone who found that excision of the site of an epicutaneous sensitization earlier than 24 hours after application of the contactant resulted in decreased CS reactions (27). If excision were performed 24 hours or more after sensitization, however, normal CS responsiveness was obtained. Hence, some critical step occurs within 24 hours after sensitization which may involve the initial antigen processing or presentation by LC.

The proposal, mentioned above, suggests that interactions can occur between APC and/or accessory cells involved in the generation of an immune response. Other evidence for this occurring in the development of CS reactions comes from the

studies of Greene and coworkers (28-32). These investigators found that the subcutaneous injection of hapten-modified SAC (splenic adherent cells) which were deficient in both APC and accessory cell activity into UV-irradiated mice resulted in decreased CS responses to the hapten, although the injection of haptenated normal SAC into these animals resulted in normal CS reactions. The injection of the haptenated, deficient SAC into normal animals however, gave normal responses. Hence, these results suggest that APC in the normal animals were able to reprocess the hapten, or in some way support the induction of a CS response following the injection of the deficient SAC.

In summary, this study has demonstrated that both APC function and accessory cell function in murine epidermis is rapidly lost following UV exposure. In addition, PABA, which has been shown to protect against histologic UV-induced skin damage, does not protect against the loss of either of these immunological functions. While the loss of immunologic function following UV irradiation of murine epidermis occurs very rapidly, it does not correlate kinetically with decreased CS responses. These results however, support the hypothesis that UV-induced inactivation of epidermal LC function is involved in the immunologic alterations observed following UV-exposure of mice.

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## ADDITIONAL EXPERIMENTS

### Persistent and transferable tolerance in photoprotected, UV-irradiated mice

The evidence presented earlier indicates that sunscreens prevent much of the UV-induced skin damage without affecting the induction of tumor susceptibility. Nonetheless, transfer of spleen cells from photoprotected, UV-irradiated animals to naive recipients did not effect the transfer of tumor susceptibility. These data suggest that either $T_s$ cells are not present in the spleen in numbers great enough to reverse the tumor resistent state or that via a mechanism such as depletion or inhibition of the tumor-antigen specific lymphocytes the animals are tolerant. In fact, these two mechanisms may not be distinct if the action of $T_s$ cells leads to clonal depletion or inhibition. To further evaluate the tolerance induced in photoprotected, UV-irradiated mice, I evaluated whether the tumor susceptible state is: a) reversible if the UV-irradiation is terminated after the minimum treatment schedule and the animals are rested for 5 weeks before the tumor challenge; b) reversible by the adoptive transfer of normal lymphocytes to photoprotected, UV-exposed mice or; c) adoptively transferable to lethally-irradiated mice using spleen cells from PABA plus UV-treated mice. As shown in Table I, all UV-treated animals exhibited comparable tumor growth regardless of whether or not the animals were: 1) treated with PABA; or 2) were rested for five weeks prior to the tumor challenge. The data in Table II demonstrate that the tumor susceptibility induced in PABA plus

UV-treated mice is not reversible by the adoptive transfer of normal spleen cells to these animals. Furthermore, spleen cells from PABA treated UV-irradiated animals, when used to reconstitute the hematopoetic system of lethally irradiated mice, allowed the growth of a UV-regressor tumor while normal spleen cells markedly inhibited tumor growth. Hence, the only detectable immunological difference between PABA plus UV-treated and UV-treated animals is the demonstrable presence of splenic $T_s$ cells in the UV-treated animals.

TABLE I

Growth of UV regressor tumor RD87 in animals treated with UV or PABA (para-aminobenzoic acid) plus UV after 3 weeks of treatment and 5 weeks of rest before the tumor challenge.

| w Group # | Treatment[a] | Day 37 post RD87 Tumor Challenge | |
|---|---|---|---|
| | | Avg Tumor Area[b] mm2 (SD) | # TBA[c] / # Animals Challenged |
| 1 | 3 wks UV + RD87 | 215 (79) | 4/4 |
| 2 | 3 wks UV + 5 wks rest + RD87 | 262 (87) | 5/5 |
| 3 | 3 wks (PABA + UV) + RD87 | 207 (57) | 4/4 |
| 4 | 3 wks (PABA + UV) + 5 wks rest + RD87 | 227 (102) | 5/5 |
| 5 | Normal + RD87 | 0 | 0/6 |

a) All animals were implanted with the UV regressor tumor RD87 concomitantly. PABA treatment consisted of a topical application of Pre-Sun just before each UV exposure.
b) Average tumor area is the mean of 4-6 animals; tumor area was estimated as the sum of two perpendicular diameters of the tumor mass measured 37 days after implantation of the tumor fragment.
c) The number of tumor bearing animals per total number of animals challenged.

TABLE II

The tumor susceptible state induced in photoprotected UV irradiated animals is not abrogated by splenectomy and reconstitution with normal spleen cells, but is adoptively transferable to lethally irradiated normal mice.

| Recipient | Treatment[a] | Adoptive Transfer[b] | Day 37 post RD87 Tumor Challenge Avg Tumor Area[c] ($mm^2$ (SD)) | # TBA[c] #Animals Challenged |
|---|---|---|---|---|
| Normal | Splenectomy | $10^8$ Norm Spleen Cells | 76 (143) | 2/6 |
| UV | Splenectomy | $10^8$ Norm Spleen Cells | 493 (111) | 5/5 |
| PABA + UV | Splenectomy | $10^8$ Norm Spleen Cells | 401 (110) | 5/5 |
| PABA + UV | Splenectomy | - | 392 (224) | 4/4 |
| Normal | Lethally Irradiated | Normal Spleen Cells | 55 (43) | 4/5 |
| Normal | Lethally Irradiated | UV Spleen Cells | 212 (57) | 6/6 |
| Normal | Lethally Irradiated | PABA + UV Spleen Cells | 167 (60) | 5/5 |

a) Mice were treated with UV light or PABA (para-aminobenzoic acid) plus UV light (30 min/day, 5 days/week) for 6 weeks and then splenectomized as described previously (see prior chapter). Lethally-irradiated normal mice were given 800 rads $\gamma$-irradiation plus $10^7$ normal bone marrow cells. All mice received a tumor fragment (RD87) subcutaneously on the ventrum at the same time.

b) Lethally-irradiated animals received $1.2 \times 10^8$ spleen cells as indicated. Other groups received $10^8$ spleen cells. Cells were injected intravenously.

c) See footnotes to Table I.

## Cooperation between UV-exposed and normal accessory cells

The data presented earlier demonstrated a difference in the dosage of UV radiation necessary to eliminate the APC activity of epidermal cells when measured *in vitro* versus *in vivo*. Thus, a reduction in the contact sensitivity response required a greater exposure than that necessary to cause a reduction in the stimulation of primed T cells *in vitro*. One possible explanation is that non-exposed accessory cells, present *in vivo*, were able to support the reaction by providing one or more of the functions lost after UV exposure. This possibility is supported by recent *in vitro* results (Table III). I used cell free supernatants from cultures of *Mycoplasma arthriditis* (termed MAS) known to contain a mitogenic factor which can 1) be taken up by accessory cells and 2) cause a stimulation of T cells *in vitro*. The results demonstrate that a significant proliferative response is observed when functional accessory cells are present in the co-cultures. If the accessory cells were UV-irradiated, they are still able to take-up the MAS but not support the proliferative response unless functional accessory cells are also added or present within the responding cell population. Thus, the presence of functional accessory cells are able to reconstitute a response to a mitogen presented by UV-inactivated accessory cells *in vitro*. This result supports the suggestion discussed above. However, further work is

obviously necessary to determine if a similar situation exists for defined antigens such as the hapten DNFB.

TABLE III

The Proliferative Response to Mycoplasma arthiditis Supernatant Requires the Presence of Functional Accessory Cells

| Responding Cells ($10^5$/well) | Accessory[b] Cells ($10^5$/well) | MAS Added | H-TdR cpm (SEM) |
|---|---|---|---|
| "T" | - | - | 1,484 (208) |
| | - | + | 11,756 (811) |
| | γ-spln | - | 2,612 (124) |
| | γ-spln | + | 83,655 (10,127) |
| | γ-spln* | - | 83,438 (2116) |
| | γ-UV-spln | + | 8,661 (2197) |
| | γ-UV-spln* | - | 7,557 (1818) |
| | γ-UV-spln* + γ-spln | - | 30,606 (1681) |
| Lymph node | - | - | 1,907 (119) |
| | - | + | 78,287 (11,734) |
| | γ-spln | - | 1,807 (373) |
| | γ-spln* | - | 41,204 (2441) |
| | γ-UV-spln* | - | 25,148 (2997) |
| | γ-epid | - | 2,023 (397) |
| | γ-epid* | - | 89,291 (1459) |
| | γ-UV-epid* | - | 58,989 (653) |

a) Balb/c responding cells were either unfractionated lymph node cells which had serially passed over two nylon wool columns to eliminate adherent accessory cells. These latter cells are labelled "T" cells.

b) Accessory cells were prepared from either Balb/c spleen or epidermis as described earlier. All accessory cell preparations received 1000 rads of gamma radiation to prevent their incorporation of $^3$H-thymidine. In addition, some accessory cells were exposed to ~90 seconds of UVB-irradiation (FS40 bulbs); a dose known to eliminate accessory cell function although not immediately cytotoxic. The astericks (*) indicate that these accessory cells were preincubated with MAS (final dilution = 1:50) for 30 minutes at 37°C, washed twice in complete medium to eliminate unbound MAS and then added to wells containing the responding cells.

c) Mycoplasma arthriditis supernatant (MAS) was added to the cocultures as indicated to a final dilution of 1:500.

d) Triplicate cultures in half-area, flat-bottomed microtitre plates (100 microliters per culture) were pulsed for five hours with 1 μCi of $^3$H-thymidine ($^3$H-TdR) on day 3. Values presented are the mean counts per minute (cpm) from the triplicate cultures, with the standard error of the mean (SEM) in parentheses.

## DISCUSSION

The purpose of these investigations was to begin to delineate the initial events involved in the induction of tumor susceptibility and of $T_s$ cells by UV radiation (1,2). Because antigen-presenting cells are believed to play a pivotal role in the generation of an immune response, a major part of these studies has been the evaluation of the effect of UV on APC function; both systemic and localized effects were considered (3-5). In addition, the effect of the sunscreen agent, PABA, was evaluated since this agent has been reported to inhibit significantly both the skin damage and appearance of skin tumors which occur following repeated UV exposure (6-9).

One recently reported effect of UV radiation was that six daily UV exposures caused a dramatic reduction in the activity of splenic APC (10,11). My studies have extended this finding and indicate that the phenomenon is a direct result of the migration of cells from the spleen to peripheral lymph nodes. The impetus for this migration appears to be the inflammation induced by the UV-irradiation. The role played by these localized changes in APC activity (decreased in the spleen, increased in the peripheral lymph nodes) in the induction of $T_s$ cells is uncertain at the present time. However, the available data suggest that the antigenic stimulus arises in the exposed skin. Hence, logically, the first lymphoid organ to perceive the hypothetical skin-associated

antigen would be a draining lymph node. And, unless too much APC activity leads to $T_s$ cell stimulation as is postulated for too little activity, one would not expect this situation to lead to the induction of the tumor susceptible state. Rather, the localized effect of UV radiation on epidermal APC function occurs very quickly and probably plays a more direct role in the induction of $T_s$ cells.

The in vitro assessment of epidermal APC function demonstrated that UV exposure of skin caused a rapid and dose-dependent inactivation of the APC activity of epidermal cells. In fact, at the dosages currently used to induce tumor susceptibility (~ $2000 J/m^2$) a dramatic reduction in activity ( > 60%) occurs after the first treatment. Kinetically, this is consistent with the model proposed earlier; that is, inactivation of APC function occurs at least concomitantly with the appearance of the skin-associated antigen so that effective antigen-presentation cannot take place. With an essentially total loss of APC activity probably occurring after only two UV treatments, the appearance of the antigenic change could occur within 48 hours of the first exposure and still lead to the induction of $T_s$ cells.

The evaluations conducted with PABA also support the proposed model. Thus, PABA did not inhibit significantly the functional inactivation of epidermal APC nor the induction of the tumor susceptibility, nor the reduction in contact sensitivity responses, as would be predicted by the model (13). It should be noted, however, that photoprotected, UV-exposed animals appear

equivalent to animals treated with UV alone in all respects except for the demonstrable presence of Ts cells in their spleens. In light of the finding that UV-induced inflammation can influence cell migration, it seems feasible that by inhibiting the inflammatory effects, PABA allows the $T_s$ cells to remain close to the site of their antigenic stimulus (14). Hence, the peripheral lymph nodes draining the UV-exposed skin may be a rich source of $T_s$ cells. This suggestion awaits further study. However, the influence of UV radiation on cell migration is currently under investigation and may provide some insight into this possibility.

Another observation was that contact sensitivity responses were normal if the animals were sensitized immediately after exposure to moderate doses of UV radiation. This occurred despite the fact that the in vitro assessment of APC activity indicated that the majority of this activity had been eliminated. As suggested in an earlier chapter, the normal response could be due to additional accessory cells present in vivo which support the development of contact sensitivity. Further work is currently in progress to investigate whether this phenomenon involves simply IL-1 production or, reprocessing, representation and IL-1 production by functional APC. Nonetheless, the results still suggest that a single UV exposure is capable of severely compromising antigen-presentation in the exposed epidermis. Furthermore, these data support the concept that ineffective antigen-presentation in vivo leads to the preferential induction of antigen-specific $T_s$ cells, as observed in vitro (3,4).

The next test of the model is, obviously, to determine if the skin-associated antigen is present in the exposed skin. Because the inactivation of APC activity is so rapid at the dosages employed to induce tumor susceptibility, the appearance of the antigen may also occur fairly rapidly. However, since more than one week of treatment is necessary to induce the tumor susceptible state, the change probably reaches optimal levels for the induction of $T_s$ cells during the second week of treatment (15). In any case, the change should also be observed in PABA plus UV-treated animals as this treatment also results in the induction of the tumor susceptible state.

In summary, the results presented here support the concept that in the absence of effective antigen-presentation, immune responses are characterized by the preferential induction of suppressor T cells. In addition, UV-irradiation of mice can result in both a systemic migration of APC and a functional inactivation of these cells in the exposed epidermis. Use of the sunscreen agent, PABA, does not prevent any of the UV-induced alterations in immune responsiveness including the induction of a tumor susceptible state, a reduction in contact sensitivity responses or the functional inactivation of APC in the exposed epidermis. Collectively, these results are consistent with the proposed model for the induction of tumor susceptibility and $T_s$ cells by a ultraviolet radiation.

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