Antigen-Presenting Cells in the Induction of Contact Hypersensitivity in Mice: Evidence that Langerhans Cells Are Sufficient but not Required

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One explanation for the fact that certain genetically defined strains of mice prove to be resistant to effects of low dose ultraviolet B radiation on the induction of contact hypersensitivity is that ultraviolet B resistant mice possess a second pathway for antigen presentation through the skin — a pathway that is independent of epidermal Langerhans cells and beyond the reach of the damaging effects of ultraviolet B light. As a corollary, ultraviolet-B susceptible mice would be expected to be deficient in this pathway. Several experimental strategies were employed to determine whether Langerhans cells are required for the induction of contact hypersensitivity by epicutaneously applied hapten. The results reveal that tape-stripped skin supports the induction of contact hypersensitivity, whereas surgical excision of hapten-painted skin within 1 h of application fails to permit the development of contact hypersensitivity. Because the former selectively eliminates epidermal Langerhans cells while the latter deletes both Langerhans cells and dermal antigen-presenting cells, we conclude that either Langerhans cells or dermal cells are sufficient to provide antigen presentation in the induction of contact hypersensitivity. When large amounts of hapten are painted epicutaneously, or when hapten is injected subcutaneously or painted on sub-dermal tissues, contact hypersensitivity also results, indicating that induction of contact hypersensitivity does not require that antigen processing and presentation be provided by cutaneous cells. Reasons are presented for concluding that under physiologic circumstances induction of contact hypersensitivity by epicutaneous hapten application relies primarily upon the antigen-presenting capabilities of epidermal (Langerhans cells) and dermal cells.
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Fifteen years after the observation by Silberberg [1] that lymphocytes were clustered around Langerhans cells *(LC) in contact hypersensitivity (CH) reactions in skin*, a staggering body of evidence has accumulated in support of the hypothesis that LC function as the critical antigen-presenting cells of skin, especially the epidermis. The evidence from in vivo studies has been based upon two types of observations. In the first, cutaneous surfaces naturally (hamster cheek pouch [2], mouse tail skin [3]) or artificially (ultraviolet B (UVB) radiation [3,4]) depleted of LC support poorly the induction of CH following epicutaneous application of hapten. In the second, LC-deficient allografts of skin (such as normal cornea [5], or UVB-treated [6,7] or tape stripped [8] body wall skin) have proven sometimes to be inefficient at inducing alloimmunity, especially that directed at class II Major Histocompatibility Complex (MHC) antigens. These in vivo-derived data are more than matched by a sizeable body of literature describing the role of LC as antigen-presenting cells in vitro [9]. Based on these experimental data, the conclusion that LC are the primary antigen-presenting cells of skin seems secure.

Recently, we have reported [10] that UVB irradiation of mouse skin, which severely depletes epidermal LC in all mouse strains tested [11], prevents induction of CH to epicutaneously applied hapten in only certain strains [C57BL/6, C3H/HeN (unpublished observation)]. In contrast, similar doses of UVB administered to the skin of BALB/c mice fail to impair CH induction, even though the extent of LC damage in the skin of these mice is comparable to that achieved in the so-called UVB-susceptible strains [11]. This provocative result was actually anticipated by the work of Sauder and Katz [12], who demonstrated that hapten painted on tail skin of C57BL/6 mice sensitizes poorly, whereas hapten painted on tails of BALB/c mice produces vigorous CH. Both sets of experimental results call into question whether LC are *always* required for the induction of CH. In an effort to explain the results of the UVB experiments, we have proposed that two antigen-presentation pathways for the induction of CH may exist in the skin [13]. One pathway, which is LC dependent, is present in all mice, but the other pathway, which is independent of LC, may be functional only in mice that are designated as UVB resistant. The nature and location of the non-LC responsible for the second pathway are unknown, but class II MHC positive dendritic cells/macrophages of the dermis are reasonable candidates (Ref 14 and unpublished observations).

Several years ago we used cellophane tape-stripping of mouse epidermis to demonstrate that a) LC could be completely (albeit transiently) removed from the epidermis by this technique, and b) tape-stripped skin failed to induce anti-class-II MHC alloimmunity when grafted to class II-disparate recipients [8]. We concluded that

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Abbreviations:
CH: contact hypersensitivity
DNFB: dinitrofluorobenzene
LC: Langerhans cells
MHC: major histocompatibility complex
SEM: standard error of mean
Thy 1+ DEC: Thy 1+ dendritic epidermal cell
UVB: ultraviolet B light

the impaired ability of stripped skin to sensitize recipients to class II alloantigens correlated with the severe depletion of class II-bearing LC from the epidermis. In light of the observation that UVB results in impaired CH induction in some strains of mice, it seemed reasonable to determine whether tape-stripped skin would support the induction of CH to haptens applied to the stripped surface. If the hypothesis concerning two antigen-presentation pathways (vide supra) is correct, one would predict that tape stripping would resemble UVB irradiation in the ability to rob the skin of UVB-susceptible animals of its capacity to support the induction of CH. The experiments and results presented here fail to realize the prediction in that sensitization to hapten through stripped skin took place even in UVB-susceptible mice. The results strongly suggest that LC are not required for induction of CH and that alternative antigen-presenting cells within the dermis and even deeper tissues possess similar antigen-presenting properties.

MATERIALS AND METHODS

Mice Adult (8–12 weeks), female mice of the following strains were obtained from our domestic breeding colony: BALB/c, C57BL/6, C3H/HeN.

Assessment of Contact Hypersensitivity Induction and elicitation of contact hypersensitivity was performed according to a modification of the method of Elmets et al [15]. Briefly, mice received epicutaneous application of 25 μl (125 μg) of 0.5% DNFB (2,4 dinitro 1-fluorobenzene; Sigma Chemical Co., St. Louis, MO) in an acetone/olive oil (4:1) solution on day 0. Each panel contained at least five mice. Reactions were elicited on day 6 by challenging one ear of each mouse with 20 μl of 0.2% DNFB. The increment in ear swelling was used as a measure of the development of contact hypersensitivity. Ear thickness was measured with an engineer's micrometer 1,2, and 3 d after challenge and compared to the ear thickness just before challenge. Mean and standard errors of the mean (SEM) were calculated for each panel.

Tape Stripping Mice were anesthetized with chloral hydrate, restrained in a supine position, and their abdominal skins dry shaven with a razor blade. The surface of the shaved abdomen was then stripped [8] by repeated application (15 times) of cellophane tape (Scotch-brand magic transparent tape, 810; 3M Co., St Paul, MN). This number of tape applications was sufficient to cause the epidermal surface to glisten. Epidermal sheets prepared from tape stripped skin and stained with fluorescent tagged anti-Ia antibodies failed to display any Ia+ cells under fluorescent microscopy.

Presentation of Data Each experiment was performed a minimum of three times. Results of representative experiments are presented in each Figure.

Statistical Analysis The statistical significance of differences in the means of each experimental group was calculated with Students t test. Mean differences were considered to be significant when $p < 0.05$.

EXPERIMENTS AND RESULTS

Effect of Tape Stripping on CH Induction in UVB-resistant Mice UVB treatment of body wall skin of BALB/c and C3H/HeJ mice has no important effect on the capacity of that skin to support the induction of CH to DNFB [10]. We refer to mice that develop vigorous CH, despite the fact that the cutaneous surface has been UVB irradiated, as "UVB-resistant". To determine whether a similar result would occur if skin of UVB-resistant mice was first tape stripped, ventral body wall skin of BALB/c and C3H/HeJ mice was razor-shaved. The surface was then stripped with repeated applications of cellophane tape. We have previously reported that this procedure effectively removes all Ia+ cells from the epidermis [8]. Depletion of LC by tape-stripping was verified in these experiments (data not shown). After 15 applications of tape, the surface glistened. Immediately thereafter, 125 μg DNCB in carrier (50 μl) was carefully applied to the stripped surface and allowed to dry. Control mice received hapten on shaved, but non-stripped epidermis. When these mice were ear challenged 6 d later (See Fig 1), both tape-stripped and non-stripped mice displayed ear swelling responses that were vigorous and comparable at 24 and 48 h. This indicates that an epidermal surface, depleted of detectable LC, can readily support the induction of contact hypersensitivity. Because BALB/c mice are UVB resistant and therefore may possess extra-epidermal cells capable of antigen presentation, we next examined the CH responses of UVB susceptible mice after tape stripping. The prediction from the UVB experiments is that UVB susceptible mice are deficient in extra-epidermal antigen-presenting cells.

Effect of Tape Stripping on CH Induction in UVB-susceptible Mice Both C57BL/6 and C3H/HeN mice develop only feeble CH when DNFB is placed on body wall skin that has first been exposed to UVB (Ref 10 and unpublished observations). We refer to mice such as these as "UVB-susceptible." To determine whether tape stripping could produce a similar effect, panels of normal mice of these genetic strains were razor shaved and the epidermis tape stripped. Immediately thereafter, DNFB was applied. When the

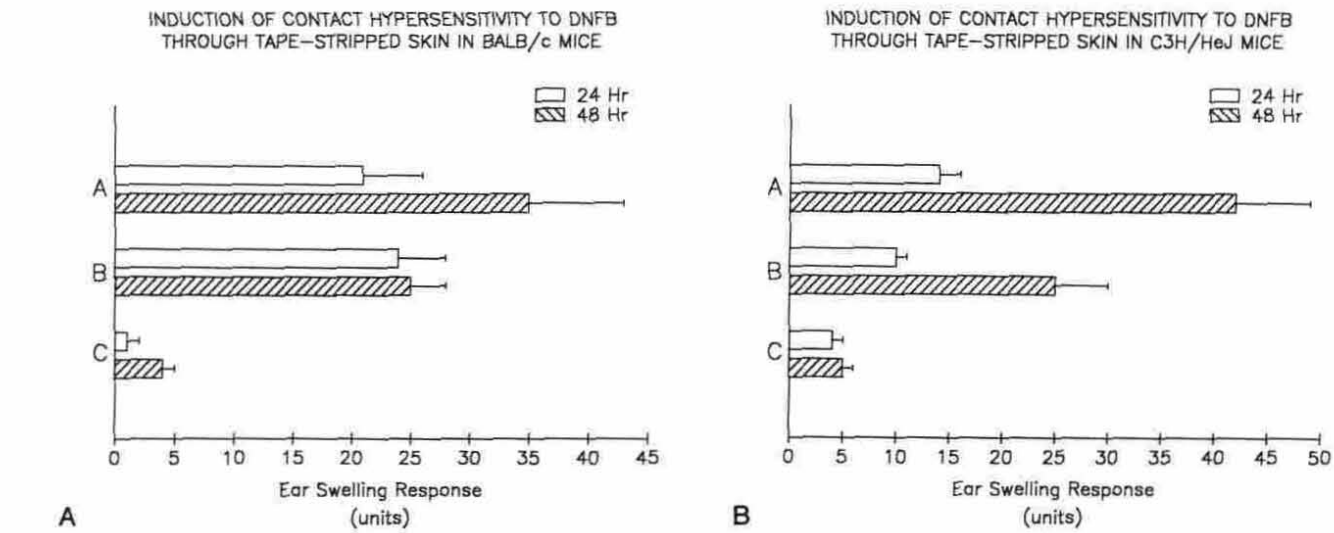

Figure 1. Effect of tape stripping on induction of contact hypersensitivity in UVB-resistant mice. Panels of five mice each received 125 μg DNFB in carrier on tape-stripped *(A)* or intact *(B)* abdominal skin. *Bars* represent mean (± one standard error of the mean-SEM) ear swelling responses after painting external surface of pinna with 40 μg DNFB 6 d later. Negative controls *(C)* were only ear challenged. Responses of groups *A* and *B* are not significantly different from each other but are significantly greater than negative controls (1 unit: 10^{-4} inches).

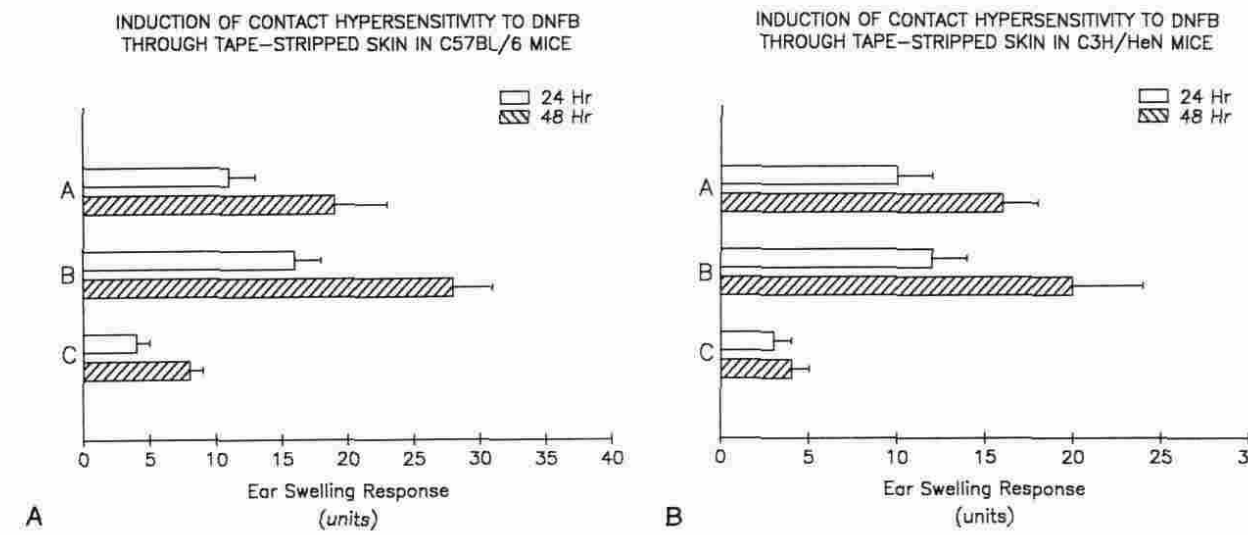

Figure 2. Effect of tape stripping on induction of contact hypersensitivity in UVB-susceptible mice. Panels of five mice each received DNFB on abdominal skin and ear challenge with DNFB as described in legend to Fig 1. Responses of groups *A* and *B* are not significantly different from each other but are significantly greater than negative controls.

ears of these animals were challenged 6 d later, all animals responded with intense CH responses, equivalent to non-stripped and painted control mice (Fig 2). This result was surprising because we had anticipated that in the absence of epidermal LC (caused by tape stripping) no, or only feeble, sensitization to hapten would take place, having assumed (incorrectly) that these mice lacked extraepidermal antigen-presenting cells. Therefore, we were forced to conclude that both UVB susceptible and UVB resistant mice can utilize non-LC dependent pathways of antigen presentation for the induction of CH to haptens. We sought next to determine where the putative extraepidermal antigen-presenting cells might reside.

Effect of Epidermal/Dermal Excision on CH Induction Because tape-stripping effectively removes all Ia+ cells acutely from the epidermis [8], we expected that the location of alternative antigen-presenting cells would be in the dermis. We reasoned that by excising both epidermis and dermis from a cutaneous area prior to epicutaneous application of hapten, no sensitization would be expected to take place. Normal murine skin is comprised of a thin epidermis, a relatively thick papillary dermis, and a reticular dermis that is separated by loose areolar tissue from a pancutaneous skeletal muscle layer called the panniculus carnosis. By careful dissection, it is possible to remove the epidermis and dermis in their entirety, leaving the surface of the panniculus carnosis intact. Histologic evaluation of the tissue that remains following excision revealed that the muscle layer is covered only by loose areolar tissue of variable thickness in which very few nucleated cells can be found (data not shown). Raw wounds such as these were prepared on the abdominal walls of panels of BALB/c and C57BL/6 mice. Immediately thereafter, DNFB in the usual dose was applied and allowed to dry. The wound was then covered with a protective dressing. When the ears of these mice were challenged 6 d later, intense CH was observed, as the data presented in Fig 3 indicate. Although this result (which confirms our previously reported findings [16]) implies that antigen-presenting cells reside outside both epidermis and upper dermis, an alternative possibility was considered.

During the course of these experiments, it was observed that when the hapten containing solution was placed on the freshly

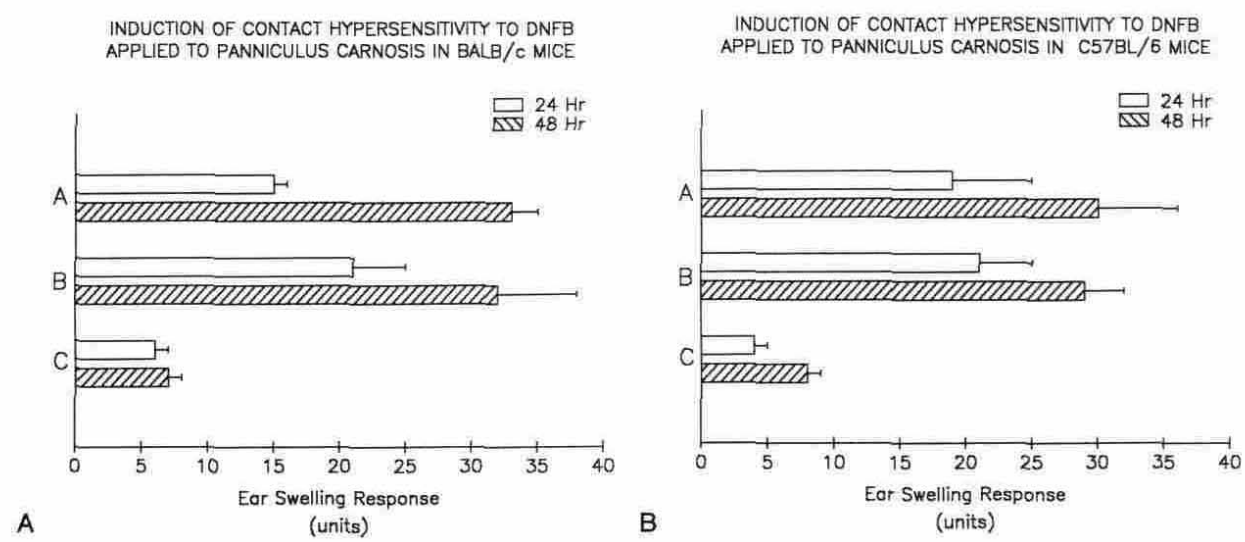

Figure 3. Effect of excision of epidermis and dermis on induction of contact hypersensitivity in UVB-resistant (BALB/c) and UVB-susceptible (C57BL/6) mice. DNFB (125 μg in carrier) was applied to panniculus carnosis of abdominal walls of five mice each *(A)*. Positive control mice *(B)* received 125 μg DNFB epicutaneously. Responses of groups *A* and *B* to ear challenge with 40 μg DNFB are not significantly different from each other, but are significantly greater than negative controls *(C)*.

excised cutaneous surface, the solution quickly wicked across the surface to the edges of the bed, adjacent to the cut surface of dermis and epidermis. This raised the possibility that hapten might be sensitizing these animals by reaching LC and dermal Ia+ cells at the periphery of the wound. To address this issue and to attempt to rule out this trivial explanation, epidermis and dermis of BALB/c mice was excised as before. A ring of silicon gel was then placed at the periphery of the wound, near to, but not touching the raw edges of cut epidermis and dermis. DNFB was then placed carefully within the ring and allowed to dry. The yellow colored hapten solution did not penetrate into, or wick over, the silicon. A protective dressing was then applied. When the ears of these animals were challenged 6 d later, vigorous CH was observed, comparable in intensity to that achieved without the use of a silicon ring (data not shown). Thus, it appears that CH can be induced in mice by application of hapten to cutaneous surfaces not only devoid of epidermal cells, but with little if any contribution from cells of the upper dermis.

Although sensitization was achieved in these experiments by placing hapten directly on the panniculus carnosis, it is not known whether hapten painted on intact, normal skin ever reaches this muscle layer in significant amounts, i.e., in amounts sufficient to lead to sensitization. The next experiments were designed to address this issue.

Panels of BALB/c mice received an epicutaneous application of DNFB (125 μg) according to our conventional immunizing regimen. One hour later these mice were reanesthetized and the epidermis and upper dermis that had been painted with hapten were surgically excised, leaving the panniculous carnosis intact. Control mice were similarly painted with hapten, and a segment of epidermis and dermis (equivalent in size to that removed from experimental mice) was excised from skin to which hapten had not been applied. Six days later the ears of these mice were challenged with DNFB. As the results displayed in Fig 4*a* indicate, significantly less intense CH developed in the mice from which hapten-derivatized skin was excised, compared with the positive controls. To reveal the effect of skin excision more dramatically, additional panels of BALB/c mice were skin painted with much lower amounts of DNFB (50 μl of 0.1% DNFB-50 μg). We have previously reported that 50 μg DNFB applied epicutaneously is sufficient to sensitize adult BALB/c mice [16]. In the present experiments, skin was painted with the dilute sensitizing dose (50 μg) of hapten, and the painted area was excised 1 h later. Positive controls were painted with the dilute hapten solution, but, as before, a comparable area of non-painted skin was excised. The ear swelling responses of these mice when challenged 6 d later are presented in Fig 4*b*. No evidence of sensitization was found in mice from whom skin painted with dilute hapten was excised. By contrast, readily detectable CH was measured in the ears of the positive controls. These results indicate that the induction of CH can be aborted by early excision of hapten-painted skin, a finding similar to that first reported by Macher and Chase in 1969 [17]. Moreover, these results reveal that the relevant antigen-presenting events leading to the induction of CH following epicutaneous painting with hapten take place *within the epidermis and upper dermis*. Thus, induction of CH through intact skin depends upon cells present within the epidermis and upper dermis but not upon cells within the panniculous carnosis and deeper layers. Although these experiments do not preclude the possibility that penetration of hapten into the panniculus carnosis after epicutaneous painting takes place, the results do reveal that any putative antigen-presenting events that might take place within and beyond this muscle later are probably irrelevant to CH induction through the epidermis. Therefore, we conclude that there are at least two cutaneous sites in which antigen-presenting cells reside, cells that are sufficient to induce contact hypersensitivity through intact skin: the epidermis and the dermis. Langerhans cells are undoubtedly the relevant cells located within the epidermis, whereas the identity of the cells within the upper dermis have yet to be precisely defined.

Induction of CH with Subcutaneous Injections of DNFB

The ability of DNFB painted directly on the panniculus carnosis to induce CH raises the possibility that antigen-presenting cells beyond the skin are capable of promoting the induction of CH. To verify this possibility formally, BALB/c mice received a single subcutaneous injection of DNFB (125 μg) in oil, placed beneath the panniculus carnosis. Six days later, their ears were challenged with DNFB. As revealed in Fig 5, these mice displayed vigorous CH, equivalent to that observed in positive controls that were immunized epicutaneously. Thus, extra-cutaneous cells can present hapten successfully for the induction of CH.

DISCUSSION

Our goal in conducting the series of experiments described in this report was to test with tape stripping of mouse epidermis the hypothesis that UVB resistant mice utilize extraepidermally located antigen-presenting cells for CH induction when their epidermal LC have been severely damaged by exposure to UVB. In a narrow sense, the validity of the hypothesis was confirmed because a) tape stripped skin of UVB-resistant BALB/c mice did support the induc-

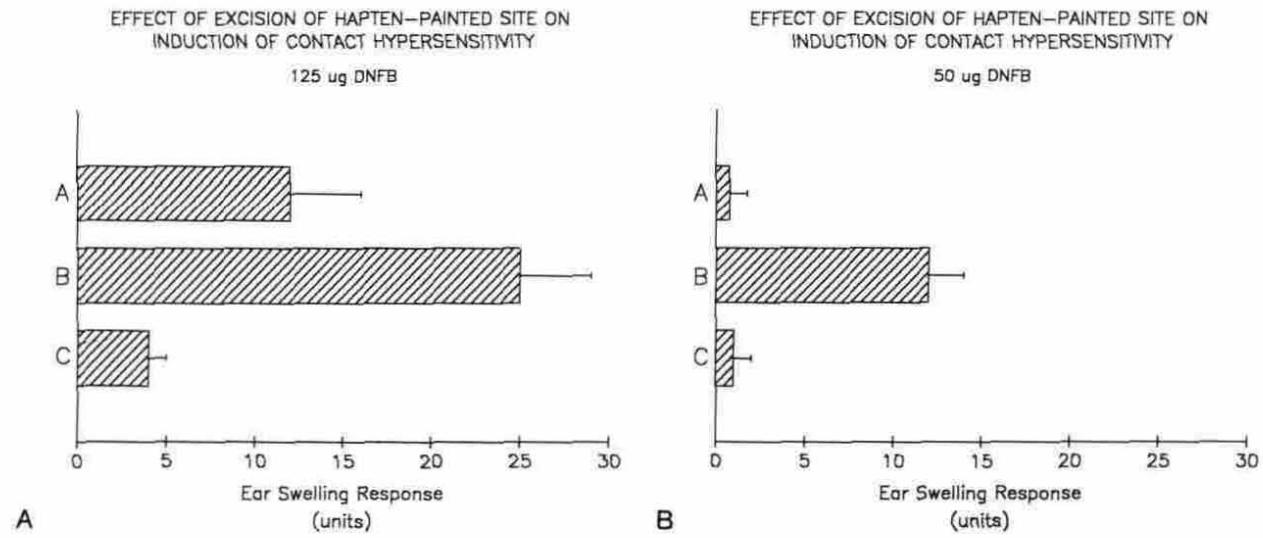

Figure 4. Effect of excision of hapten-painted skin on induction of contact hypersensitivity. One hour after epicutaneously application of 125 or 50 μg DNFB in carrier, the painted area was excised *(A)*. Positive control mice *(B)* received 125 or 50 μg DNFB epicutaneously; an unpainted segment of skin was excised. Responses of *A* to ear challenge are significantly less than *B*. Response of *A* after 50 μg are statistically indistinguishable from negative control *(C)*.

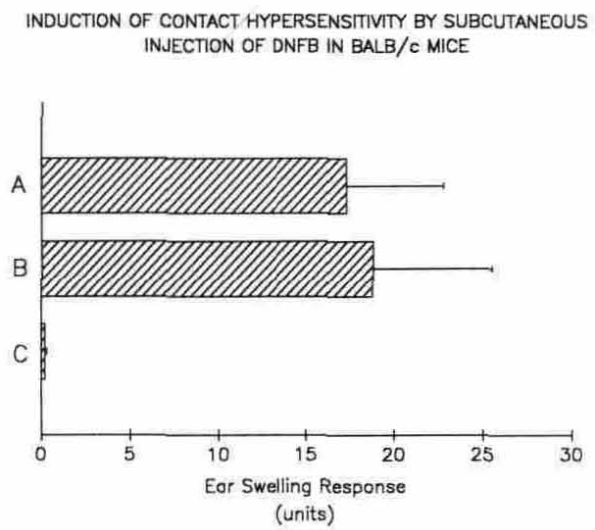

Figure 5. Induction of CH by subcutaneous injection of 125 μg DNFB *(A)*, compared with positive controls *(B)* and negative controls *(C)*. *Bars* represent ear swelling responses ± SEM at 48 h. *A* and *B* are indistinguishable statistically from each other, and both are significantly greater ($p < 0.02$) than *C*.

tion of hapten-induced CH in the absence of epidermal LC, and b) surgical excision of the hapten-painted skin within 1 h of hapten application aborted the induction of CH. However, tape stripped skin of UVB-susceptible mice also supported the induction of CH. This finding is consistent with the hypothesis that cells other than LC can be important in the induction of CH but fails to illuminate the reason why these mice are susceptible to UVB irradiation. Moreover, our finding that a cutaneous surface from which epidermis and superficial dermis have been surgically excised also supports the induction of CH, points to the existence of additional antigen-presenting cells that do not even reside within these two cutaneous compartments. This last observation may not be as improbable as it first appears. It has been reported that subcutaneous injections of hapten readily induce vigorous delayed hypersensitivity [18]. Injections of this type place the antigenic inoculum beneath the panniculus carnosis, a site that is far removed from the Ia+ antigen-presenting cells known to be present within the upper dermis and epidermis, and a site that is relatively deficient in resident Ia+ cells. Because the hapten-containing inoculum itself can induce a vigorous, local inflammatory reaction comprised in part of recruited and activated macrophages, it is reasonable to expect that this injection site rapidly acquires an ad hoc capacity for antigen processing and presentation. To that end, our experiments demonstrate that subcutaneous injection of hapten is sufficient to induce specific, systemic CH. We believe that application of hapten to excised wounds such as we employed may have the same significance as a subcutaneous injection of hapten. In this sense, the positive result we obtained is probably irrelevant to our original concern about the putative effects of UVB radiation on the antigen-presenting capabilities of cells within dermis and epidermis. In addition, the fact that surgical excision of hapten-painted epidermis and upper dermis impairs or even prevents the induction of CH strongly implies that the antigen-presenting cells of relevance to epicutaneous application of hapten are located in the upper dermis and epidermis.

Evidence that supports the contention that the antigen-presenting events relevant to induction of CH occur within the epidermis and/or dermis also comes from our previous studies using hapten-derivatized skin grafts as immunogens [16]. The critical results were that H-2 allogeneic skin that has been derivatized with dilute amounts of epicutaneous hapten (50 ug DNFB, as in these experiments) is incapable of sensitizing recipient mice, whereas hapten-derivatized syngeneic skin grafts readily induce CH. These results, which confirm that MHC molecules restrict the specificity of the T cells that effect CH, require that hapten processing and presentation takes place only on cells *within the skin graft*. Because the grafts contain epidermis and upper dermis, but lack subdermis and panniculus carnosis, the relevant cells must be Langerhans cells or dermal antigen-presenting cells of the type revealed by our current experiments.

The major paradox created by our findings is that a strategy (tape stripping) that selectively removes epidermal, but not dermal, Ia+ cells from skin produces a completely different effect on induction of CH from that achieved with our low-dose UVB regimen, even though the two procedures both deplete the epidermis of normal LC. These diametrically opposing effects have made us question some of the conclusions and hypotheses we have drawn from our own data as well as that of others over the past few years. First, we had deduced that LC are the primary antigen-presenting cells of normal mouse skin. However, because tape stripped skin, which is devoid of Langerhans cells, provides a suitable substrate for induction of contact hypersensitivity, we must conclude that cells other than LC (presumably within the dermis) are similarly equipped to provide antigen-presenting function in skin. Second, we have hypothesized that UVB resistant animals retain the capacity to respond to epicutaneously applied hapten because they possess a "second" pathway of antigen presentation (presumably dependent upon dermal Ia+ cells); we also proposed that UVB susceptible mice lack this pathway and these cells [13]. However, because hapten applied to tape stripped skin of UVB susceptible animals induced intense CH, these animals must also possess this "second" pathway; therefore, the reason for their failure to develop CH after UVB treatment remains unexplained. Third, we concluded that the capacity of UVB to prevent induction of CH in certain genetically defined strains of mice was mediated through the effects of UVB on epidermal LC. However, because LC are equally damaged by UVB in resistant and susceptible strains of mice and because both types of mice develop CH when hapten is painted on tape stripped (LC depleted) skin, we are forced to re-examine the epidermis for another UVB-induced perturbation, other than the damage caused to LC.

Among the possible target cells within the epidermis, keratinocytes and Thy 1+ dendritic epidermal cells (Thy 1+ DEC) [19,20] represent attractive candidates, although for different reasons. Previously, Sauder et al [21] demonstrated that epidermal cell suspensions prepared enzymatically from normal skin and then hapten derivatized were able to immunize mice in vivo. Moreover, if such hapten-derivatized cells were exposed to UVB in vitro prior to injection, they failed to sensitize and induced unresponsiveness. Although those experiments were performed prior to the discovery of the Thy 1 DEC, the original interpretation given by the authors remains an attractive one: epidermal cells, deprived of the positive immunogenic influence of normal LC, are poorly able to sensitize and can even promote unresponsiveness. The tolerogenic influence was suspected as being the property of UVB-damaged keratinocytes and/or LC. Very recently, Cruz et al [22] claimed that purified and hapten-derivatized LC exposed to UVB in vitro are tolerogenic when injected in vivo into syngeneic recipient mice. Thus, it is possible that UVB damaged LC themselves function as a tolerogenic stimulus. Unfortunately, the known effects of UVB on LC in UVB susceptible and resistant strains fail to reveal any difference, and therefore, it is difficult to see how this property of LC could account for the genetic polymorphism revealed by the results of our previous UVB susceptibility/resistant experiments. With regard to the Thy 1 DEC, it has recently been demonstrated that purified, hapten-derivatized Thy 1 DEC can induce hapten-specific unresponsiveness when injected into syngeneic recipients [23]. Bergstresser has reported that, compared to Langerhans cells, Thy 1 DEC are more resistant to the damaging effects of UVB [10]. Alternatively, Aberer et al [24] found LC and Thy 1 DEC to be comparably damaged by UVB, although these investigators used ear skin, rather than shaved body wall skin. To our knowledge, no study of

the relative sensitivity to UVB of Thy 1+ DEC from UVB-resistant and UVB-susceptible strains has yet been reported.

It has been proposed that the skin, and even the epidermis, contains cellular elements within it that are responsible for delivering immunogenic and tolerogenic signals to the systemic immune apparatus [25]. In light of the results presented here, it would now appear that strong, immunogenic signals actually come from two important cutaneous sources: epidermal LC and Ia+ dermal macrophages/dendritic cells. The data imply that either cell source is sufficient to lead to successful induction of CH. However, we can offer no explanation for the failure of UVB susceptible mice to develop CH because penetration of UVB into mouse dermis is thought to be insignificant, and therefore the dermal Ia+ cells should be spared. No formal, quantitative study of UVB radiation reaching the dermis has been reported for mouse skin, and so the possibility remains that the assumption that "the dermis is spared" may actually not be correct. Moreover, there could be genetic differences that would differentially limit UVB penetration in mouse skin. The fact that black and agouti mice are UVB susceptible seems to rule out a genetic effect acting through pigmentation. We favor the hypothesis that UVB susceptibility results from the "sparing" of the cellular source of putative tolerogenic signals from the skin, although at present we have no direct supporting data.

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