Effect of X-irradiation on Epidermal Immune Function: Decreased Density and Alloantigen-Presenting Capacity of Ia\(^{+}\) Langerhans Cells and Impaired Production of Epidermal Cell-Derived Thymocyte Activating Factor (ETAF)

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The mechanisms involved in the modulation of cutaneous immune responses by UV radiation have been extensively investigated; by contrast, few studies have addressed the effects of x-irradiation on epidermal immune function. We therefore investigated the effect of x-irradiation of mice on: (a) the density of epidermal Ia\(^{+}\) Langerhans cells (LC) in immunofluorescence studies, (b) epidermal cell (EC) allostimulatory capacity in the allogeneic EC-lymphocyte reaction (ELR), and (c) production of epidermal cell-derived thymocyte activating factor (ETAF). C3H/He and BALB/c mice were irradiated with 900, 1,800, 2,700, or 3,600 rad from a \(^{137}\)Cs source, and sacrificed 10 h or 3 days later. X-irradiation of mice 10 h previously only slightly decreased the density of epidermal Ia\(^{+}\) LC and did not affect the capacity of their EC to stimulate allogeneic responder lymphocytes in the ELR. X-irradiation of mice 3 days previously, however, resulted in a dose-dependent decrease in the density of Ia\(^{+}\) LC. This decrease was accompanied by a substantial reduction in EC allostimulatory capacity in the ELR at all doses of x-irradiation. ETAF production by cultured EC from mice x-irradiated 3 days previously was also found to be diminished at all doses of x-irradiation. Trypan blue exclusion studies demonstrated that the observed decreases in EC allostimulatory capacity and ETAF production were not the result of a generalized lethal effect of x-irradiation on EC. The reduction in EC allostimulatory capacity following in vivo x-irradiation could not be reversed by addition of exogenous ETAF or interleukin-1 in the ELR. Taken together, these results indicate that x-irradiation decreases the density of Ia\(^{+}\) LC, impairs LC alloantigen-presenting function, and reduces ETAF production. Thus cutaneous x-irradiation may affect inflammatory and neoplastic processes not only by its antimitotic activity, but also by a direct effect on EC which subserves immunologic functions.

Mammalian epidermis functions as an immunologic organ comprising resident dendritic Ia antigen-bearing (Ia\(^{+}\)) Langerhans cells (LC), which are bone marrow-derived antigen-presenting cells [1-6], and keratinocytes which secrete an interleukin-1 (IL-1)-like cytokine termed epidermal cell-derived thymocyte activating factor (ETAF) [7,8]. The effects of UV radiation on cutaneous immune responses have been extensively investigated. Short-wave UV (UVB) and psoralen plus long-wave UV (PUVA) have been shown to decrease the density of LC expressing cell surface Ia antigen and membrane ATPase [9,10]. UV radiation has been reported to produce a decrease in LC alloantigen-presenting capacity in the allogeneic epidermal-lymphocyte reaction (ELR) which is partially reversible by addition of exogenous EATAF [11-14]. Exposure of mice to UV radiation may result in unresponsiveness to contact allergens and inability to reject highly antigenic UV-induced tumors, associated with the generation of antigen-specific suppressor T cells [15-19].

Studies addressing the effects of x-irradiation on the epidermal immune system have been few in number and have yielded conflicting results. X-irradiation has been reported to cause a decrease in the number of Ia\(^{+}\) and/or ATPase-positive LC in mice, rats, and guinea pigs in some studies [20-22]. In another study in the guinea pig, gamma radiation alone had no effect on LC surface markers, although it did delay the return of LC membrane Ia antigen but not ATPase after animals were treated with corticosteroids [23]. Studies on the function of the epidermal immune system following in vivo x-irradiation have not been reported. During the course of an investigation into the functional aspects of epidermal-lymphocyte interactions in murine acute graft-versus-host disease, we noted impaired LC function in the skin of animals treated only with 900 rad of x-irradiation [24]. We therefore investigated this phenomenon more fully, and report here that in vivo x-irradiation causes a decrease in the density and alloantigen-presenting capacity of Ia\(^{+}\) LC, and reduces EATAF production.

MATERIALS AND METHODS

Animals

Female C3H/HeJ (H-2b) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Female C3H/HeNMTV\(^{+}\) (H-2\(^{b}\)) BALB/cAnN (H-2d) and C57Bl/6N (H-2b) mice were obtained from the Charles River Breeding Laboratories, Wilmington, Massachusetts. Male NZB mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility. Mice were used at 8-16 weeks of age; age-matched controls were used in each experiment. Four- to six-week-old female C3H/HeJ mice were used for the thymocyte proliferation assay.

X-Irradiation

Mice were given 900, 1,800, 2,700, or 3,600 rad of whole-body x-irradiation from a \(^{35}\)Cs source and sacrificed 10 h or 3 days later.

Serologic Reagents

The following murine monoclonal antibodies directed against murine Ia antigen were used: 10.2.16 specific for Ia\(^{b}\) (cells obtained from...
American Type Culture Collection, Rockville, Maryland) and MKD-6 specific for Ia* (Becton Dickinson, Sunnyvale, California). Fluorescein-conjugated IgG fraction of goat antimouse IgG (F(ab')2 fragment specific) was obtained from Cappel Laboratories, Cochranville, Pennsylvania. Complement was obtained from young rabbits.

**Immunofluorescence Staining**

Ia* LC in epidermal sheets obtained from biopsies of trunical skin were stained by an indirect immunofluorescence method as previously described [25]. The mean number of Ia* LC per unit area was determined by counting 5 random fields of 0.0626 mm² per biopsy with the use of an ocular grid.

**Histology and Electron Microscopy**

Biopsy specimens of ears from control and x-irradiated mice were fixed in 10% formalin and processed for light microscopy. Other specimens were fixed in 6% glutaraldehyde, processed for electron microscopy, and examined in a Philips 400 transmission electron microscope.

**Preparation of Cells**

Unfractionated lymph node lymphocytes were obtained by disruption of whole lymph nodes in Hanks’ balanced salt solution (HBSS) and filtration of the resultant suspension through nylon mesh, followed by treatment with ACK lysing buffer (National Institutes of Health Media Unit) to remove erythrocytes. For certain experiments, purified T cells were obtained by passage of suspensions over Sephadex G-10 columns [26] and through nylon wool columns [27], followed by treatment with anti-Ia antibody and young rabbit complement (C). Column-passaged lymph node cells (10⁶/ml) were incubated with diluted antibody for 30 min at 4°C, centrifuged, incubation for 30 min at 37°C with a 1:20 dilution of fresh rabbit serum, and washed in HBSS.

Epidermal cell (EC) suspensions from trypsinized ear skin were obtained as previously described [25]. Cell viability was determined by trypsin blue exclusion. Individual experiments involved the use of pooled EC from at least 5 mice per group of control or x-irradiated animals.

**Preparation of Semipurified ETAF [8]**

Culture supernatants were collected from the murine Pam-212 transformed keratinocyte line cultured in serum-free medium containing RPMI 1640 (Gibco Laboratories, Chagrin Falls, Ohio) with added l-glutamine and penicillin, streptomycin and fungizone. Supernatant and control media were concentrated 50 times using Amicon ultrafiltration cells with PM-10 Dialflo membranes (Amicon Corp., Lexington, Massachusetts). Four milliliters of the concentrates were then placed on Sephadex G 50 superfine columns equilibrated in 50 mM Tris-buffered 100 mM NaCl pH 7.5, using reverse flow. A flow rate of 14 ml/h was used, and 7-ml fractions were collected. Fractions were filter-sterilized (0.22 μm Millex-GS, Millipore Co., Bedford, Massachusetts), aliquots were tested for ETAF activity at a dilution of 1:4 in the thymocyte proliferation assay (below), and active samples eluted from the column loaded with concentrated culture supernatant were identified. Control samples were obtained from material eluted in the identical volume from the column loaded with concentrated medium. The mean Δ cpm of [3H]TdR incorporation in the thymocyte proliferation assay of active compared with control samples was 49,049 cpm. Active and control samples were stored at −70°C until used.

**Allogeneic EC-Lymphocyte Reaction**

ELR assays were carried out as described [11]. Briefly, 2 × 10⁵ unfractionated or purified responder lymph node lymphocytes were cultured with allogeneic stimulator EC from control or x-irradiated animals in 96-well round-bottom microculture plates (Linbro, Flow Labs Inc., McLean, Virginia) for 7 days in humidified air containing 5% CO₂. A range of from 3 × 10⁵ to 2 × 10⁶ EC from each group was added to culture wells in order to obtain an overall profile of the effects of x-irradiation on the ELR, since the concentration of stimulator EC at which peak proliferation in the ELR occurs is variable. Wells with responder lymphocytes alone were used to determine background counts. The culture medium consisted of RPMI 1640 with added 2 mm l-glutamine (Gibco), antibiotics, nonessential amino acids, 10 mm HEPESS buffer (Gibco), 2.5 × 10⁻³ M-mercaptoethanol (complete medium), and 1% heat inactivated male NBS mouse serum. In most experiments, EC suspensions were irradiated with 2,000 rad in vitro from a 137Cs source prior to culture. In certain experiments, human IL-1 (Genzyme, Boston, Massachusetts) (final dilution = 5 U/ml), or semipurified ETAF or control column eluate (final dilution = 1:4) was added to the wells. Cultures were pulsed with 0.5 μCi/well [3H]TdR (Amersham International plc, Amersham, U.K.: sp act 25 Ci/mmol) for the last 18 h, and were then harvested on a MASH harvester (M. A. Bioproducts). [3H]TdR incorporation was assessed by liquid scintillation counting. Results are expressed as means of quadruplicate cultures unless otherwise stated.

**Assessment of ETAF Activity in Skin of X-irradiated and Control Mice**

ETAF activity was assessed as described [7,14]. Briefly, supernatants from EC cultured at 7.5 × 10⁶/ml for 3 days in complete medium were extensively dialyzed against RPMI 1640 and filter-sterilized. Aliquots were then investigated for ETAF/IL-1 activity in a 72-h thymocyte proliferation assay. Culture supernatants were added in varying dilutions to 1.5 × 10⁶ C3H/HeJ thymocytes/well cultured for 72 h in flat-bottom culture plates (Costar, Cambridge, Massachusetts) in complete medium with 10% FBS and 1 μg/ml phytohemagglutinin (Burroughs-Wellcome, Research Triangle Park, North Carolina). Human IL-1 (final dilution = 5 U/ml) served as a positive control. [3H]TdR incorporation was assessed by liquid scintillation counting following an 18-h pulse with 1 μCi/well of [3H]TdR. Results are expressed as means of quadruplicate cultures.

**RESULTS**

**Light and Electron Microscopy**

Light microscopic examination of biopsies of ear skin from x-irradiated animals showed only slight thickening of the epidermis but no necrotic changes even in mice irradiated with 3,600 rad 3 days previously. Electron microscopy of ear skin from mice x-irradiated with 3,600 rad 3 days previously similarly did not reveal degenerative changes in keratinocytes. Quantitation of a population such as LC, which constitute only about 2–4% of all EC, is difficult by electron microscopy, and was not attempted in this study. Occasional morphologically normal LC were, however, detectable on ultrastructural examination of the epidermis of mice x-irradiated with 3,600 rad 3 days previously.

**In Vivo X-irradiation Decreases the Density of Ia* LC**

X-irradiation of C3H/He mice 10 h previously resulted in a variable minor decrease in the density of Ia* LC in epidermal sheets. The density of LC expressed as a percentage of that found in control unirradiated skin at the various doses was as follows (mean ± SEM, n = 5): 900 rad, 99.6 ± 1.2; 1,800 rad, 87.9 ± 1.0; 2,700 rad, 82.9 ± 0.5; 3,600 rad, 91.4 ± 7.8. By contrast, in vivo x-irradiation 3 days previously produced a striking dose-related decrease in the density of Ia* LC (Fig 1). The decrease in the density of Ia* LC following 900 rad in vivo was time-dependent (Fig 2). Remaining Ia* LC in general appeared larger and had longer dendrites than LC in control skin.

**X-irradiation of EC with 2,000 Rad In Vitro Does Not Affect Peak Proliferation in the ELR**

Stimulator EC are routinely treated with 2,000 rad in vitro immediately prior to addition to ELR cultures in our laboratory, to prevent any potential contribution to overall [3H]TdR incorporation by proliferation of the stimulator population. In order to investigate whether such in vitro x-irradiation affects LC alloantigen-presenting capacity, we compared the allostimulatory capacity of control unirradiated EC to that of EC irradiated in vitro with varying doses of x-irradiation. Peak proliferation in the ELR in response to EC treated with 2,000 rad of gamma radiation did not differ from that in response to control unirradiated EC (data not shown). The allostimulatory capacity of EC was not totally abolished even by x-irradiation with 10,000 rad in vitro (mean Δ cpm ± SEM = 11,938 ± 2,186 compared with 76,912 ± 13,677 for unirradiated EC). Stimulator EC were therefore routinely treated with 2,000 rad in vitro x-irradiation immediately prior to addition to ELR cultures in all further experiments.
Fig 1. Density of Ia+ LC in epidermal sheets 3 days after x-irradiation of mice. Results are expressed as mean percentage ± SEM of the density present in control unirradiated skin. The density of Ia+ LC present in control skin was as follows: C3H/HeN (○—○, n = 12): 1,015/mm²; C3H/HeJ (□—□, n = 10): 1,186/mm²; BALB/c (Δ—Δ, n = 5): 1,200/mm².

Fig 2. Variation in density of Ia+ LC in epidermal sheets with time elapsed after x-irradiation of C3H/HeJ mice with 900 rad. Results are expressed as mean percentage ± SEM of the density present in control unirradiated skin (n = 5 mice per time interval).

**X-irradiation of Mice Decreases EC Allostimulatory Capacity in the ELR at 3 Days but Not at 10 Hours after X-irradiation**

X-irradiation of mice 10 h previously did not alter peak proliferation in the ELR at any dose of x-irradiation (data not shown). By contrast, peak proliferation in the ELR induced by both C3H/HeN and BALB/c stimulator EC was substantially decreased at all doses of x-irradiation when EC were harvested from mice x-irradiated 3 days previously (Fig 3). EC allostimulatory capacity was not completely abrogated even after mice were irradiated with 3,600 rad.

**ETAF Production by EC Harvested from Mice X-irradiated 3 Days Previously Is Decreased**

ETAF production as assessed by stimulation in the thymocyte proliferation assay was substantially decreased but not abolished at all doses of x-irradiation given to mice 3 days previously (Fig 4).

The Reduction in EC Allostimulatory Capacity and ETAF Production After X-irradiation of Mice Is Not the Result of a Nonspecific Effect on EC Viability

In order to establish that the observed effects of in vivo x-irradiation 3 days previously on EC allostimulatory capacity
and ETAF production were not the result of a generalized indiscriminate killing of EC, we investigated the viability of EC from control unirradiated and from x-irradiated mice both immediately after harvesting the EC, and after subjecting the EC to a further 3 days in culture. Under normal circumstances an EC suspension culture represents a “dying system” in that the viability of unirradiated control EC decreases rather sharply and progressively with time [11]. In our study, the viability of control EC immediately after harvesting was 79.2 ± 2.6%, and after a further 3 days in culture at a concentration of 7.5 × 10^5/ml in complete medium was 35.2 ± 3.1% (mean ± SEM, n = 5 separate experiments), as measured by trypan blue exclusion. No difference was observed in the overall EC viability immediately after harvesting, or after a further 3 days in culture, between control unirradiated EC and any of the x-irradiated groups (data not shown, n = 5 separate experiments).

The Reduced EC Allostimulatory Capacity After X-irradiation of Mice Cannot Be Restored by Addition of Exogenous IL-1 or ETAF to the ELR

We next investigated the relative contributions of impaired LC alloantigen-presenting capacity and of decreased ETAF production to the reduction in EC allostimulatory capacity observed 3 days after x-irradiation of mice. This was done by determining whether EC allostimulatory capacity could be restored by addition of exogenous IL-1 or ETAF to ELR cultures. Purified T cells were used as responder cells in these experiments. X-irradiation of mice 3 days previously again resulted in decreased EC allostimulatory capacity. Addition of exogenous human IL-1 (Genzyme) or semipurified ETAF to ELR cultures did not restore EC allostimulatory capacity (Tables I and II) and produced only a marginal increase in T-cell proliferation in response to EC from x-irradiated animals. These findings indicate that the decreased EC allostimulatory capacity following in vivo x-irradiation is principally the result of impaired LC alloantigen-presenting capacity.

| X-ray dosage (rad) | Stimulator EC/well* | [3H]dThd Incorporation: Mean cpm ± SEM*
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<td>0</td>
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<td>76 ± 22</td>
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<td></td>
<td>3 × 10^6</td>
<td>42,936 ± 3,239</td>
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<td>900</td>
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<td>7,217 ± 839</td>
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<td>2 × 10^6</td>
<td>8,813 ± 2,295</td>
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<td>1,800</td>
<td>3 × 10^6</td>
<td>4,920 ± 827</td>
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<td></td>
<td>2 × 10^6</td>
<td>5,388 ± 399</td>
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<tr>
<td>2,700</td>
<td>3 × 10^6</td>
<td>4,606 ± 1,327</td>
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<td></td>
<td>2 × 10^6</td>
<td>5,233 ± 1,429</td>
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<tr>
<td>3,600</td>
<td>3 × 10^6</td>
<td>6,258 ± 506</td>
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<td>2 × 10^6</td>
<td>6,278 ± 877</td>
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* BALB/c purified responder T cells (2 × 10^6) were added to each well.

** Results are expressed as the mean ± SEM of triplicate cultures.

*** EC were obtained from C3H/HeN mice irradiated 3 days previously.

** Human IL-1 (Genzyme) was added to a final concentration of 5 U/ml.

** TABLE II. Decreased allostimulatory capacity in the ELR of EC irradiated in vivo is not restored by exogenous ETAF

| X-ray dosage (rad) | Stimulator EC/well* | [3H]dThd Incorporation: mean cpm ± SEM*
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<tr>
<td>0</td>
<td>0</td>
<td>117 ± 33</td>
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<tr>
<td></td>
<td>3 × 10^6</td>
<td>39,659 ± 1,789</td>
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<td></td>
<td>2 × 10^6</td>
<td>36,384 ± 3,026</td>
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<td>900</td>
<td>3 × 10^6</td>
<td>31,396 ± 1,920</td>
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<tr>
<td></td>
<td>2 × 10^6</td>
<td>36,230 ± 751</td>
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<td></td>
<td>1 × 10^6</td>
<td>17,009 ± 1,768</td>
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<td>3 × 10^6</td>
<td>21,669 ± 5,459</td>
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<td></td>
<td>2 × 10^6</td>
<td>27,800 ± 5,942</td>
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<td>18,873 ± 1,690</td>
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<td>15,246 ± 1,597</td>
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<td>2 × 10^6</td>
<td>13,476 ± 2,456</td>
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** BALB/c purified responder T cells (2 × 10^6) were added per well.

** Results are expressed as the mean ± SEM of triplicate cultures.

*** EC were obtained from mice irradiated 3 days previously.

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** DISCUSSION**

The experiments reported here demonstrate that x-irradiation of mice, like UV/PUVA and glucocorticosteroid therapy [9,10,28], results in a dose- and time-dependent decrease in the density of Ia+ LC. This finding is in agreement with the studies of Groh et al [20] and Cole and Lewkowicz [21]. A report by Belsito et al [23] that 750 rad of gamma irradiation had no effect on LC membrane markers in the guinea pig is not explicable on the basis of relative radioresistance in this animal, since Groh et al [20] found decreased numbers of ATPase-positive LC in the guinea pig following irradiation with 800 rad. The decrease in the density of Ia+ LC was not simply the...
result of x-irradiation-induced necrosis of the skin, since light and electron microscopic examination revealed an intact epidermis 3 days after 3,600 rad of x-irradiation, and examination of EC suspensions revealed no adverse effect of x-irradiation on EC viability as determined by trypan blue exclusion.

We next investigated the effects of x-irradiation on epidermal immune function as determined by EC alloantigen-presenting capacity in the ELR. In most laboratories, stimulator EC for use in the ELR are routinely pretreated either with 12.5 μg/ml mitomycin C [11,29] or with 1,000–2,000 rad of gamma radiation [3,14,29–32] to prevent any potential contribution to overall [3H]TdR incorporation by proliferation of the stimulator population. We found that in vitro gamma radiation of stimulator EC with 2,000 rad did not affect peak proliferation in the ELR and accordingly used that dose of radiation in all further experiments. The allostimulatory capacity of EC from mice x-irradiated 10 h previously did not differ from that of EC from control unirradiated mice. By contrast, in vivo x-irradiation resulted in a substantial decrease in EC allostimulatory capacity at all doses when EC were harvested from mice irradiated 3 days previously. EC allostimulatory capacity was, however, not abrogated even after mice were irradiated with 3,600 rad. The fact that there was no difference in viability between EC harvested from unirradiated mice and mice x-irradiated 3 days earlier, either immediately after harvesting the EC or after culturing the EC for a further 3 days, suggests that the decreased EC allostimulatory capacity was not the result of an immediate lethal toxic effect of x-irradiation on all EC.

IL-1 plays a critical role in the activation of T cells following antigen processing and presentation by Ia+ macrophage-like cells [33,34]. Within the epidermis, IL-1 is produced not only by LC [35], but also by keratinocytes in the form of ETAIF [7,8]. We therefore assessed ETAIF production following x-irradiation of mice to determine whether the decrease in EC allostimulatory capacity could be related to a decrease in ETAIF production. We found that ETAIF production was decreased but not abolished 3 days after in vivo x-irradiation at all doses of gamma radiation. Exogenous IL-1 or ETAIF added to the ELR could not, however, restore the x-irradiation-induced defect in EC allostimulatory capacity. IL-1 has previously been reported to be ineffective in restoring the antigen-presenting defect in spleen cells produced by combined UV and gamma radiation [36,37]. The fact that exogenous IL-1/ETAIF could not reverse the EC accessory cell defect also would suggest that the decreased EC allostimulatory capacity following in vivo x-irradiation is the result of a defect in LC alloantigen-presenting capacity rather than a decrease in ETAIF production.

EC harvested from UV-treated skin sites in mice and humans on the third day after UV exposure exhibit enhanced accessory cell function despite a decrease in LC, as a result of an influx of antigen-presenting inflammatory cells into the epidermis [31,32]. The present study has demonstrated that x-irradiation, by contrast, results in a substantial decrease in the accessory cell function of EC from mice irradiated 3 days previously. In the absence of an influx of inflammatory cells, the immune function of the epidermis appears to reflect the dominant inhibitory effect of x-irradiation on LC antigen-presenting capacity. LC alloantigen-presenting capacity was, however, not totally abrogated even after 3,600 rad of in vivo gamma radiation. In comparison with other macrophage-like cells, LC may therefore be relatively radioreistant, since the antigen-presenting capacity of murine spleen cells and peritoneal macrophages is abrogated within 24–48 h of 550–900 rad of whole-body gamma radiation [38,39].

It is not possible to state whether the x-irradiation-induced decrease in the density of Ia+ LC and in LC antigen-presenting capacity seen in this study was the result of altered LC function and expression of membrane markers or of an actual loss of LC, since ultrastructural quantitation was not attempted. It is, however, clear that in vivo x-irradiation profoundly alters the immunologic function of the epidermis.

The authors thank Mr. Jay Linton for excellent technical assistance, Mr. Harry Schaefer for printing the illustrations, and Drs. Ira Green, Thomas Lawley, John Stanley, Kevin Cooper, and Werner Aberer for reviewing this manuscript.

REFERENCES


