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*Hin*dIII Liposomes Suppress Delayed-Type Hypersensitivity Responses In Vivo and Induce Epidermal IL-10 In Vitro[1]

Chikako Nishigori,* Daniel Yarosh,† Adrienne O'Connor,† Vijay K. Shreedhar,* Stephen E. Ullrich,* Patricia Cox,* and Margaret L. Kripke[2]*

Considerable evidence suggests that ultraviolet-B (UV-B) radiation suppresses certain immune responses through the induction of cyclobutane pyrimidine dimers in DNA. To determine whether induction of other forms of DNA damage in the skin mimicked the immunosuppressive effects of UV-B radiation, we produced double-strand breaks in the DNA of epidermal cells with *Hin*dIII restriction endonuclease encapsulated in liposomes. Application of these liposomes, but not liposomes containing inactive *Hin*dIII or an irrelevant endonuclease, to the skin of C3H mice suppressed the induction of delayed-type hypersensitivity responses to *Candida albicans* and alloantigen and induced IL-10 production in the epidermis. Treatment of the Pam212 murine keratinocyte cell line with these liposomes in vitro induced immunosuppressive activity and IL-10 in culture supernatants. Unlike UV-B irradiation, however, *Hin*dIII in liposomes failed to induce suppressor T cell activity in vivo or in vitro. We conclude that double-strand breaks in DNA of epidermal cells can induce immunosuppression and up-regulate the production of immunomodulatory cytokines; however, either DNA damage alone does not account for all the immunosuppressive properties of UV-B irradiation, or cyclobutane pyrimidine dimers differ qualitatively from double-strand breaks in their biologic consequences. These studies raise the possibility that drugs causing DNA damage may induce cytokine dysregulation and immune suppression in addition to cytotoxicity. ***The Journal of Immunology,*** **1998, 161: 2684–2691.**

Ionizing radiation, UV radiation, psoralen plus UV-A[3] radiation (PUVA), and certain carcinogens, such as dimethylbenzanthracene, induce immune suppression (1–4). Whether the same immunosuppressive pathway is activated by all these agents has not been elucidated. Among those agents, UV-B (280–320 nm radiation) has been studied extensively, and much is known about how it modulates immunologic responses. UV-B directly impairs the activity of APCs (5–7) and triggers the influx of dendritic cells with altered Ag-presenting capabilities into the irradiated skin (8). It also up-regulates epidermal cytokines with immunomodulatory activity (9–11) and converts urocanic acid (UCA) from the *trans* to the immunosuppressive *cis* form (12). However, even for UV-B, a complete picture of the molecular steps leading to immune suppression is not yet available.

We previously demonstrated that reducing the number of cyclobutane pyrimidine dimers (CPD) in UV-irradiated skin abrogated the systemic suppression of contact hypersensitivity (CHS) and delayed-type hypersensitivity (DTH) to *Candida albicans* (13, 14). From these results, we inferred that unrepaired DNA damage stimulates keratinocytes to produce cytokines, thereby modifying critical steps in the immunologic pathway and ultimately leading to reduced cell-mediated immune responses and generation of suppressor T cells (Ts) to certain Ags. Other reports support our hypothesis. Hurks et al. (15) reported that the action spectra for UV-induced suppression of the mixed lymphocyte and mixed epidermal cell-lymphocyte reactions closely resemble those for the induction of CPD and 6-4 photoproducts. Action spectra for local and systemic suppression of CHS are also consistent with DNA damage as an initiator of these effects (16, 17). Also, XPA knockout mice, which are deficient in DNA repair, were reported to show increased local and systemic suppression of CHS responses after UV irradiation compared with wild-type mice (18). Recently, we provided the first direct evidence that UV-induced DNA damage in the form of CPD initiates cytokine production, providing further support for the hypothesis that UV-induced DNA damage triggers the production of immunomodulatory epidermal cytokines (19).

Having demonstrated that DNA damage is a primary molecular mechanism for initiation of immune suppression in these models, we hypothesized that DNA damage other than CPD might also cause immune suppression. To test this hypothesis, we used the *Hin*dIII restriction enzyme, which recognizes the 5′-AAGCTT-3′ base sequence in dsDNA and causes double-strand breaks at these sites. This restriction endonuclease is widely used and well characterized and does not directly induce membrane damage. This 6-base cutter averages about 5000 bases between double-strand breaks in purified DNA. Recently, we showed that *Hin*dIII encapsulated in liposomes induces both local and systemic suppression of CHS in vivo (20). In this study, we extended these findings by investigating whether damage caused by the restriction enzyme also suppressed the DTH responses to *C. albicans* and alloantigen and induced Ts induction; we also examined the effect of *Hin*dIII liposomes on the immunosuppressive activity of keratinocyte culture supernatants and on IL-10 production in vivo and in vitro.

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[3] Abbreviations used in this paper: UV-A, 320–400 nm UV radiation; PUVA, psoralen plus UV-A radiation; UV-B, 280–320 nm UV radiation; CPD, cyclobutane pyrimidine dimer(s); CHS, contact hypersensitivity; DTH, delayed-type hypersensitivity; Ts, suppressor T lymphocyte(s); T4N5 liposomes, liposomes containing T4 endonuclease V; UCA, urocanic acid.

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Materials and Methods

Mice

Specific pathogen-free C3H/HeNCrMTV$^-$ and BALB/cAnNCr female mice were obtained from the Frederick Cancer Research Facility Animal Production Area (Frederick, MD). Age-matched mice between 10 and 12 wk of age were housed in filter-protected cages, and ambient lighting was controlled to provide 12-h light/12-h dark cycles. Autoclaved National Institutes of Health open formula mouse chow and water were provided ad libitum. The animal facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International; procedures were approved by the institutional animal care and use committee.

Hind*III endonuclease in liposomes*

*Hind*III restriction enzyme (New England Biolabs, Beverly, MA) was purified as previously described (20). The enzyme was diluted to 10,000 U/ml in the storage buffer (250 mM NaCl, 10 mM Tris-Cl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, and 500 g/ml BSA) and in some cases inactivated by heating at 65°C for 60 min. The enzyme was filtered through a 0.2-μm SFCA filter (Nalgene Division, Sybron, Rochester, NY) and encapsulated in pH-sensitive liposomes, as described previously (20). These liposomes are taken up by cells in culture and pass through the stratum corneum into the epidermal cells of mice, where they release endonucleases intracellularly (21). The encapsulated *Hind*III activity was 400 U/ml, as assayed by the manufacturer's directions, using DNA as substrate and adding 0.1% Triton X-100 to dissolve the liposomes. Control liposomes contained either T4 endonuclease V (T4N5 liposomes, previously described by Kripke et al. (14)) or heat-inactivated *Hind*III. Empty liposomes were prepared identically, except that proteins were omitted. For in vivo use, the liposomes were mixed into 1.5% neutralized Hypan SS201 hydrogel (Lipo Chemicals, Paterson, NJ) in PBS, pH 8, to a final concentration of 40 U/ml and applied to shaved mouse skin (~10 U/mouse) with a moist cotton swab. For in vitro use, the liposome preparation was diluted with MEM (Life Technologies, Grand Island, NY) supplemented with 1% FCS (Life Technologies) to a final concentration of 12 U/ml, followed by filtration through a 0.22-μm filter. Cells were treated with 3 ml of liposomes/100-mm dish (for collecting supernatant) and 1.3 ml/35-mm dish (for immunohistochemistry) for 1 h at 37°C.

Detection of DNA damage

Skin was excised from the mice 4 h after the *Hind*III liposome treatment, and epidermis was separated after the skin was treated with 1.5 U/ml dipase in PBS (Boehringer Mannheim, Indianapolis, IN). DNA was extracted from the epidermis, followed by electrophoresis in a 4% neutral agarose gel. Negatives of the photograph were scanned, and the frequency of double-strand breaks was analyzed as previously described (20). An excess of 13 to 20 double-strand breaks per DNA megabase pair was produced by *Hind*III liposome treatment (not shown), similar to our previous report (20).

Immunohistochemical analysis for IL-10 and p53

IL-10 in the skin and in Pam212 cells was examined by an immunoperoxidase method using monoclonal rat anti-mouse IL-10 (clone JES5-2A5, American Type Culture Collection, Rockville, MD). p53 was examined using mouse polyclonal anti-human p53 Ab Ab-1 (clone 421, Oncogene Science, Uniondale, NY). *Hind*III liposome-treated and UV-irradiated mouse skin was collected 1, 3, 5, 8, and 12 days after treatment, embedded in OTC compound, and frozen in liquid nitrogen; 4-μm cryostat sections were fixed with 2% paraformaldehyde. The slides were placed in a humidified chamber, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide; slides were incubated with protein blocking solution (10% normal horse serum and 1% normal goat serum) for 20 min, followed by treatment with rat anti-mouse IL-10 mAb overnight at 4°C. The slides were washed with PBS and incubated for 30 min with biotin-labeled rabbit anti-rat Ab (Boehringer Mannheim), for 20 min with peroxidase-labeled streptavidin, and for 20 min with diaminobenzidine (Research Genetics, Huntsville, AL). For p53 staining, skin sections were fixed with 2% paraformaldehyde at room temperature for 10 min, followed by methanol at −20°C for 10 min. Endogenous peroxidase activity was again blocked with 3% hydrogen peroxide, and sections were incubated with protein blocking solution for 20 min at 25°C and with Ab-1 Ab (clone 421, Oncogene Science) overnight at 4°C, followed by peroxidase-labeled rat anti-mouse IgG2a. Other procedures were the same as described for IL-10 staining.

For cultured cells, Pam212 cells were grown on coverslips in 35-mm dishes. On the following day, cells were treated with 1.3 ml of *Hind*III liposome solution under the same conditions as those used for supernatant harvest. Twelve and twenty-four hours after UV irradiation, cells were washed with PBS and fixed with 2% paraformaldehyde for 10 min at 25°C; they were rinsed with PBS and stained by the method used for skin sections, except that endogenous peroxidase blocking was omitted.

UV source

The UV source for irradiation of mice was a bank of six FS40 sunlamps (National Biologic, Twinsburg, OH), which emit about 65% of their radiation within the UV-B (280–320 nm) range and have a peak emission at 313 nm. The average irradiance of the source was about 9 watt/m^2, as measured with an IL-1700 radiometer and an SEE 240 UV-B detector equipped with an A127 quartz diffuser (International Light, Newburyport, MA). The dose rate of the incident radiation received by the animals was decreased to 4.5 watt/m^2 by the cage lid screening. Before irradiation, the dorsal fur of the mice was shaved with electric clippers, and the animals were placed in individual compartments in cages located 20 cm below the lights.

A single FS40 sunlamp was used to irradiate keratinocytes. The irradiance of the source averaged 1.43 watt/m^2 at a tube-to-target distance of 23 cm.

DTH responses to Candida *and alloantigen*

Groups of five C3H mice were sensitized by injecting 0.2 ml of 1×10^7 formalin-fixed *C. albicans* cells (14) or 2.5×10^7 allogeneic BALB/c spleen cells s.c. into each flank. Nine (*Candida*) or six (alloantigen) days later, the hind footpad thickness was measured with a spring-loaded micrometer (Mitutoyo, Tokyo, Japan), and the mice were challenged by intradermal injection of 50 μl of *Candida* Ag (Alerchek, Portland, ME) or 1×10^7 BALB/c spleen cells in both hind footpads. Footpad thickness was measured again 24 h later, and the swelling was determined by subtracting prechallenge from postchallenge measurements. Negative controls consisted of mice that were challenged but not sensitized.

Test for Ts activity

Spleens from mice treated with *Hind*III liposomes or UV-B and sensitized with *C. albicans* were removed 10 days after sensitization. Single-cell suspensions were prepared from pooled spleens in HBSS. Clumps were removed by filtration through nylon mesh. The cells were washed once and refiltered, and 1×10^8 viable, nucleated cells were injected i.v. into groups of five syngeneic recipients. Immediately thereafter, the recipients were sensitized with *C. albicans*; the DTH response was measured 9 days later as described above.

Neutralization of IL-10 in vivo

Groups of five C3H mice were treated with *Hind*III liposomes. Four and twenty-four hours later, they were injected i.p. with either 100 μg of monoclonal rat anti-mouse IL-10 (purified from JES5–2A5 supernatants by use of protein A/G columns; Pierce, Rockville, IL) or 100 μg of IgG1 from normal rat serum. The mice were immunized with *Candida*, and the DTH response was measured as described above.

Cell line

A spontaneously transformed, BALB/c keratinocyte cell line, Pam212, obtained originally from Dr. Stuart Yuspa, National Cancer Institute (Bethesda, MD), was used. Cells were maintained in MEM supplemented with 10% FBS, sodium bicarbonate (0.075%), L-glutamine (2 mM), and nonessential amino acids. Cells were incubated at 37°C in 95% air/5% CO_2.

Supernatants from Hind*III liposome-treated keratinocytes*

A total of 4×10^6 Pam212 cells were plated in 100-mm tissue culture dishes in 8 ml of medium. On the following day, the medium was removed, and cells were washed twice with PBS and treated with *Hind*III liposomes (12 U/ml) in MEM containing 1% FCS at 37°C for 1 h, after which the cells were washed twice with PBS and cultured in serum-free medium. Twenty-four hours later, the supernatants were harvested. The protein concentration of the supernatants was determined using bicinchoninic acid (BCA protein assay reagent, Pierce), and supernatants were injected i.v. into mice.

C3H mice were injected with 20 to 25 μg of supernatant protein from *Hind*III liposome-treated keratinocytes. Control supernatants were prepared by treating keratinocytes with control liposomes or UV-B radiation. Five days later, the mice were immunized with allogeneic BALB/c spleen cells and challenged as described above. The interval between supernatant injection and immunization was selected to mimic the protocol used in

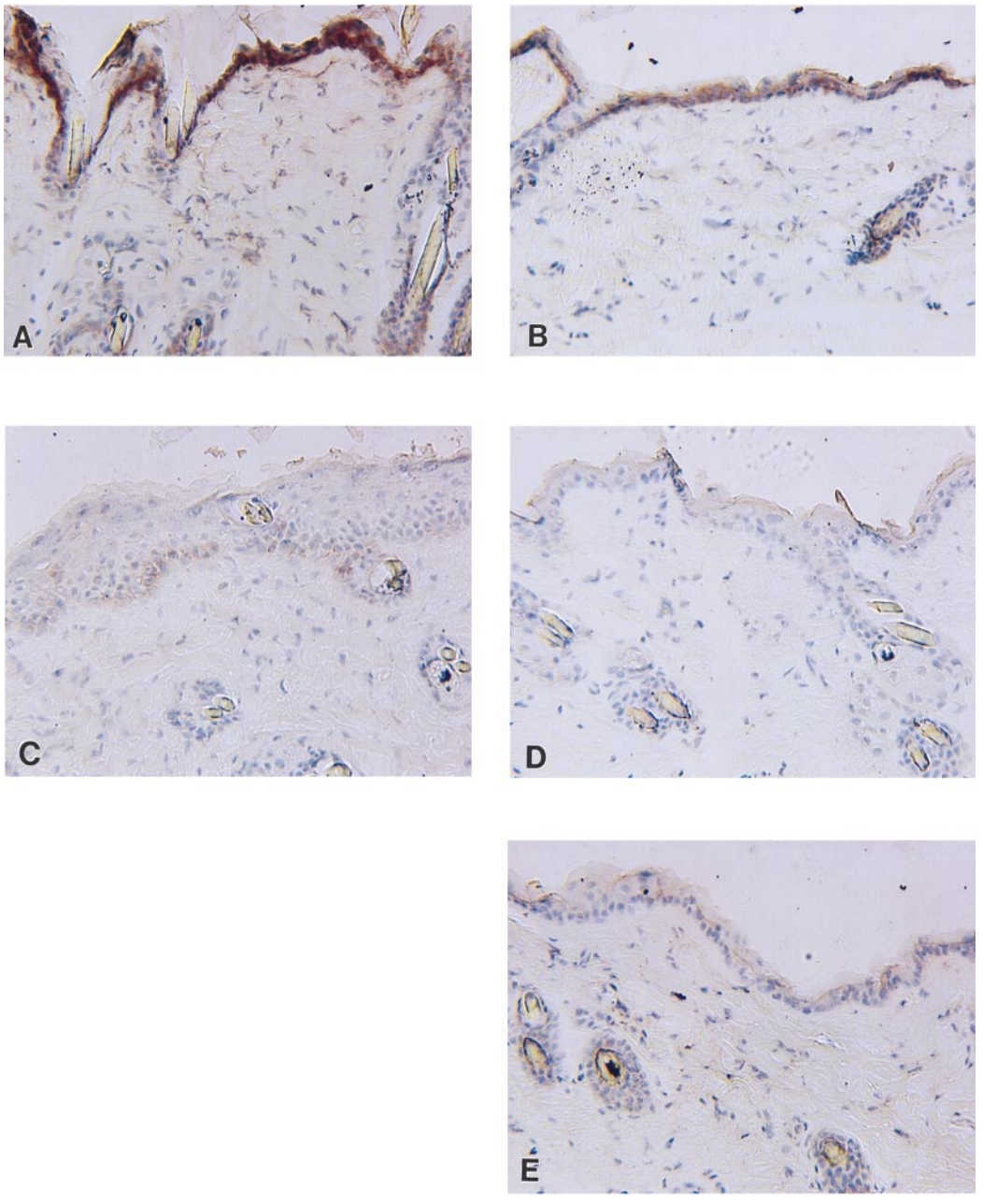

FIGURE 1. *Hin*dIII liposomes induce p53 in mouse skin. Immunoperoxidase staining of C3H mouse skin for p53 on days 1 (*A*) and 3 (*C*) after treatment with 10 kJ/m^2 UV-B and on days 1 (*B*) and 3 (*D*) after treatment with 40 U/ml of *Hin*dIII in liposomes and of untreated skin (*E*).

studies by Rivas and Ullrich (11), which was based on the optimal time of immune suppression following UV irradiation in vivo.

Determination of IL-10 by ELISA

IL-10 release from Pam212 cells was measured by ELISA using the same supernatants that were injected into mice. Rat anti-mouse IL-10 (JES5-2A5) was used as a capture Ab in combination with a biotinylated detection Ab (SXC-1, PharMingen, San Diego, CA). ELISA was conducted according to the manufacturer's procedures, and the IL-10 concentration was determined from the linear portion of a standard curve obtained using rIL-10 (PharMingen).

Statistical analyses

All experiments were performed at least twice and usually three times; results shown are representative of all experiments. Groups of five mice were used for DTH assays, and the significance of differences was determined using Student's *t* test.

Results

Hin*dIII liposomes causes DNA damage*

Application of *Hin*dIII liposomes to murine skin produced double-strand breaks in epidermal DNA, as measured by electrophoresis on neutral agarose gels (18). With the liposome preparation used in these studies, an excess of between 13 and 20 double-strand breaks per DNA megabase pair was produced (not shown). Because p53 protein accumulates in cells in response to DNA damage (22), we examined the p53 content of cells in the skin by immunoperoxidase staining as an additional marker of the in vivo activity of *Hin*dIII liposomes. This approach also demonstrated the ability of *Hin*dIII liposomes to produce DNA damage in vivo, since application of the *Hin*dIII liposome preparation to the skin of mice increased the amount

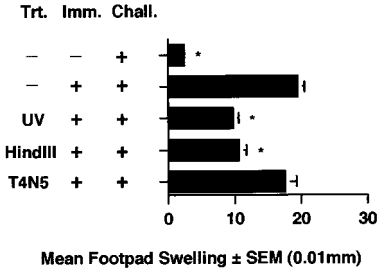

FIGURE 2. *Hind*III liposomes suppress the DTH response to alloantigen. C3H mice were treated (Trt.) with 40 U/ml *Hind*III in liposomes, 0.125 μg T4N5 (control) liposomes, or 10 kJ/m^2 UV-B radiation and immunized (Imm.) 5 days later with 2.5×10^7 BALB/c spleen cells s.c. DTH was assessed by injecting 1×10^7 BALB/c spleen cells into both hind footpads 6 days later and measuring footpad swelling 24 h later (Chall.). * indicates $p < 0.001$ vs mice that were sensitized only (positive control).

of p53 in epidermal cells (Fig. 1). One day after *Hind*III liposome treatment, p53 was present in the epidermal cells, almost to the same extent as after UV irradiation (Fig. 1, *A* and *B*). By day 3, some p53$^+$ cells remained in the basal layer of the epidermis of UV-irradiated mice (Fig. 1*C*), but in *Hind*III liposome-treated skin (Fig. 1*D*), p53 staining had returned to background levels (Fig. 1*E*). There was also considerably more hyperplasia in UV-irradiated skin than in *Hind*III liposome-treated skin (Fig. 1, *C* vs *D*). Liposomes containing heat-inactivated *Hind*III induced no detectable increase in p53 (not shown).

Hind*III liposomes suppress the DTH responses to alloantigen and* C. albicans

To test whether DNA-damaging agents other than UV-B could also suppress DTH responses, we applied *Hind*III liposomes to mouse skin and immunized the animals 5 days later with alloantigen or *C. albicans*. Treatment with *Hind*III liposomes reduced the DTH response to alloantigen to the same extent as UV-B, whereas treatment with control liposomes containing T4 endonuclease V (T4N5 liposomes), an enzyme involved in excision repair of CPD, had no effect on the DTH response in the absence of UV irradiation (Fig. 2). Similarly, the DTH response to *C. albicans* was reduced (Fig. 3*A*). In contrast to the effect of UV-B radiation, however, transfer of spleen cells from mice treated with *Hind*III liposomes failed to impair the induction of DTH to *C. albicans*, suggesting that Ts had not been induced by this treatment (Fig. 3*B*).

Anti-IL-10 Ab inhibits the effect of Hind*III liposomes on DTH to* C. albicans

IL-10 has been reported to play an essential role in UV-induced suppression of DTH responses (11, 23). We asked, therefore, whether IL-10 also plays a role in suppression of DTH by *Hind*III liposome treatment. A neutralizing anti-IL-10 mAb was injected 4 and 24 h after *Hind*III liposome treatment, and the mice were sensitized on day 5 after liposome treatment. As shown in Figure 4, injection of anti-IL-10 Ab prevented the suppression of DTH in response to *C. albicans*, whereas an isotype-matched control Ab had no effect.

Hind*III liposomes induce immunosuppressive activity in keratinocyte culture supernatants*

Previous studies demonstrated that soluble factors secreted by UV-B-irradiated murine keratinocytes can suppress immune responses

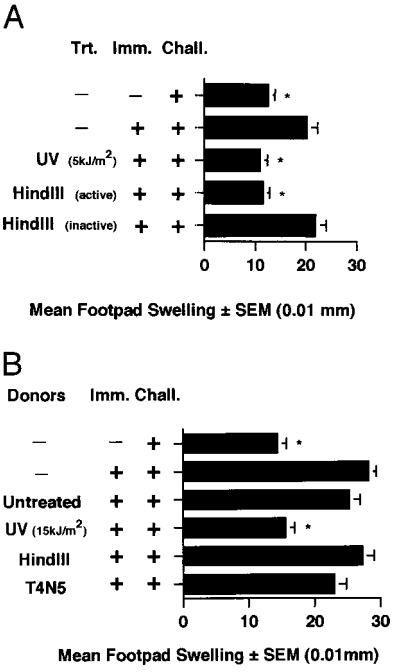

FIGURE 3. *Hind*III liposomes suppress the DTH response to *Candida* but fail to induce Ts. *A*, C3H mice were treated (Trt.) with 40 U/ml *Hind*III in liposomes, heat-inactivated *Hind*III in liposomes, or 10 kJ/m^2 UV-B radiation and immunized (Imm.) 5 days later with 2×10^7 *Candida* cells s.c. DTH was assessed by injecting 50 μl of *Candida* Ag into both hind footpads 6 days later and measuring footpad swelling 24 h later (Chall.). *B*, Spleen cells from these mice (Donors) were injected i.v. into syngeneic mice that were immediately immunized (Imm.) with *Candida* and challenged as described above (Chall.) to assess the DTH response. * indicates $p < 0.001$ vs mice that were sensitized only (positive control).

in vivo (9, 24); one such factor is IL-10 (11, 19, 23). We therefore determined whether *Hind*III liposomes would induce such immunosuppressive activity in vitro also. Cultures of Pam212 cells were treated with *Hind*III liposomes, UV-B (positive control), or T4N5 liposomes (negative control), and supernatants were harvested 24 h later. The supernatants were injected i.v., and 5 days later the mice were immunized with alloantigen, and the DTH response was measured after an additional 6 days. Supernatants from keratinocyte cultures treated with *Hind*III liposomes inhibited the DTH response, as did those from UV-B-irradiated cells, whereas those from cells treated with T4N5 liposomes had no effect on the DTH

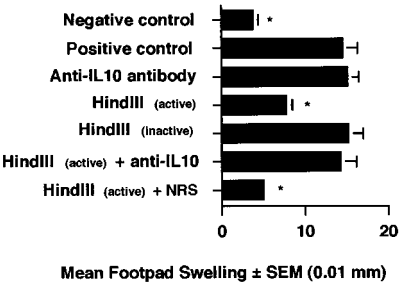

FIGURE 4. Ab against IL-10 abrogates *Hind*III liposome-induced suppression of the DTH response to *Candida*. C3H mice were treated with 40 U/ml *Hind*III in liposomes or with heat-inactivated *Hind*III in liposomes and injected with anti-IL-10 or control Ab (NRS, normal rat serum) 4 and 24 h after treatment. Mice were sensitized with *Candida* 5 days after liposome or UV-B treatment, and DTH was assessed 9 days later as described in Figure 3. * indicates $p < 0.001$ vs *Candida*-immunized mice.

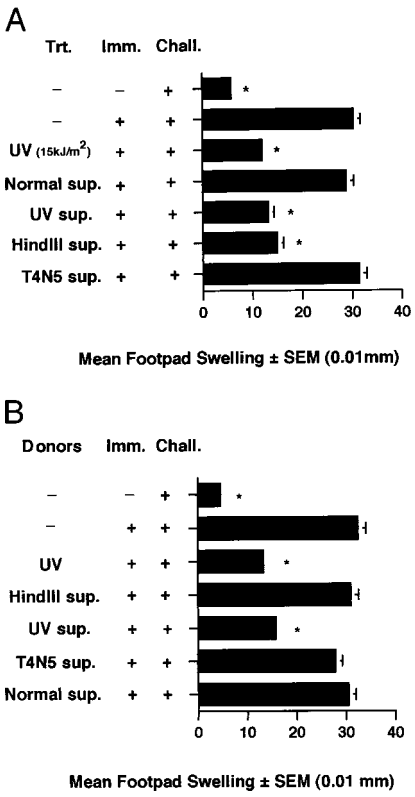

FIGURE 5. Supernatants from *Hin*dIII liposome-treated Pam212 cells suppress the DTH response to alloantigen but do not induce Ts. *A*, Supernatants (Sup.) from untreated Pam212 cells or from Pam212 cells treated (Trt.) with *Hin*dIII in liposomes or T4N5 liposomes were injected i.v. into C3H mice. Five days later the mice were immunized (Imm.) with BALB/c spleen cells to induce DTH, as indicated in Figure 1. *B*, Spleen cells from these mice (Donors) were injected i.v. into syngeneic recipients, which were immediately immunized (Imm.) with BALB/c spleen cells; DTH was assessed on day 6 (Chall.). * indicates $p < 0.001$ vs alloantigen-treated mice.

response (Fig. 5*A*). Spleen cells from these groups of mice were transferred to naive mice, which were then immunized with allogeneic spleen cells to test for Ts activity. As shown in Figure 5*B*, spleen cells from mice receiving supernatants from UV-B-treated keratinocytes contained Ts activity; however, those from mice receiving supernatants from cells treated with *Hin*dIII liposomes or T4N5 liposomes had no Ts activity.

Hin*dIII liposomes induce IL-10 production in keratinocytes in vivo and in vitro*

The ability of *Hin*dIII liposomes to induce IL-10 production in mouse skin in vivo was analyzed by mAb and immunoperoxidase staining of skin sections. IL-10 could be detected in the skin of mice treated with *Hin*dIII liposomes (Fig. 6, *B*, *D*, and *F*) as well as with UV-B (Fig. 6, *A*, *C*, and *E*). Staining was apparent on days 1, 3, and 8 after treatment, but both the intensity of staining and the number of positive cells were greater in the UV-B-irradiated skin than in the *Hin*dIII-liposome-treated skin. Occasional IL-10^+ dendritic cells were evident in the dermis of UV-B-irradiated mice but not in that of *Hin*dIII-liposome-treated mice. Neither skin treated with heat-inactivated *Hin*dIII in liposomes (Fig. 6*G*) nor untreated skin (Fig. 6*H*) showed detectable staining with anti-IL-10 Ab.

IL-10 could also be detected in vitro in keratinocytes and culture supernatants following *Hin*dIII liposome treatment. Figure 7 shows the results of immunoperoxidase staining for IL-10 in Pam212 cells 12 h (Fig. 7*C*) and 24 h (Fig. 7*D*) after treatment with *Hin*dIII in liposomes; untreated cells (Fig. 7, *A* and *B*) and cells treated with heat-inactivated *Hin*dIII liposomes (Fig. 7, *E* and *F*) were only rarely positive for IL-10 staining. IL-10 was also increased in the supernatants of cultures of Pam212 cells collected 24 h after treatment with *Hin*dIII liposomes (940 pg/ml) and UV-B radiation (550 pg/ml), compared with those from cells treated with T4N5 liposomes (<40 pg/ml) or untreated cells (40 pg/ml).

Discussion

UV-B radiation, which induces mainly CPD and (6–4) photoproducts in DNA, and various psoralen compounds plus UV-A radiation, which induce monofunctional adducts and interstrand cross-links, suppress CHS and DTH responses initiated at unexposed sites and induce Ag-specific Ts (2, 14). Although these studies suggest a role for DNA damage as an initiator of immune suppression (3), both types of treatment produce other alterations that could also be involved, such as membrane damage leading to activation of the transcription factors NF-κB and activating protein-1 (25, 26), photoisomerization of UCA (12), and generation of H_2O_2 (27). Using the approaches of direct photoreactivation of CPD (7, 13) and excision repair of CPD by T4N5 liposomes (14, 19), we have provided additional evidence that DNA damage in the form of CPD initiates both local and systemic suppression of CHS and systemic suppression of DTH to *C. albicans*. As an additional test of the role of cutaneous DNA damage as an initiator of immune suppression, we used liposome-encapsulated *Hin*dIII restriction endonuclease to induce double-strand breaks in DNA, an approach unlikely to generate H_2O_2, membrane damage, or *cis*-UCA. In earlier studies, we demonstrated that treatment of mouse skin in vivo with these liposomes suppressed the induction of CHS to haptens applied to either the treated or a distant, untreated site; however, this treatment did not produce tolerance or suppression transferable with spleen cells (20). These effects were due to the DNA cutting ability of the *Hin*dIII enzyme, since heat-inactivated *Hin*dIII in these same liposomes had no effect.

On the other hand, studies using a mAb directed against *cis*-UCA suggested that UV-induced systemic suppression of DTH to alloantigen, sheep erythrocytes, and herpes simplex virus was mediated by *cis*-UCA and therefore was unlikely to involve DNA damage (28, 29). To test more critically the potential role of DNA damage in suppressing DTH responses, we extended our studies of liposomal *Hin*dIII-induced suppression of CHS responses to two models of DTH and an in vitro model for production of immunosuppressive factors, including IL-10. Based on previous studies, we predicted that *Hin*dIII liposomes would suppress DTH to *C. albicans*, which appears to depend on DNA damage (14), but not alloantigen, which appears to depend on *cis*-UCA (29). However, both DTH responses were inhibited by *Hin*dIII liposome treatment, and Ts could not be detected in either model. In addition, although treatment of keratinocytes in vitro with liposomal *Hin*dIII produced immunosuppressive activity and IL-10 in culture supernatants, these supernatants were unable to produce detectable Ts in recipient mice, in contrast to the effect of supernatants induced by UV-B irradiation. These results imply either that DNA damage per se does not account for all of the immunosuppressive properties of UV-B irradiation or that CPD are qualitatively different in their biologic consequences from double-strand breaks in DNA. Furthermore, they provide additional evidence that IL-10 alone is insufficient to reproduce all the immunomodulatory effects of UV-B radiation either in vivo or in vitro.

Previous work demonstrated that IL-10 has an important role in suppressing DTH responses to UV-B irradiation (11, 23) and in

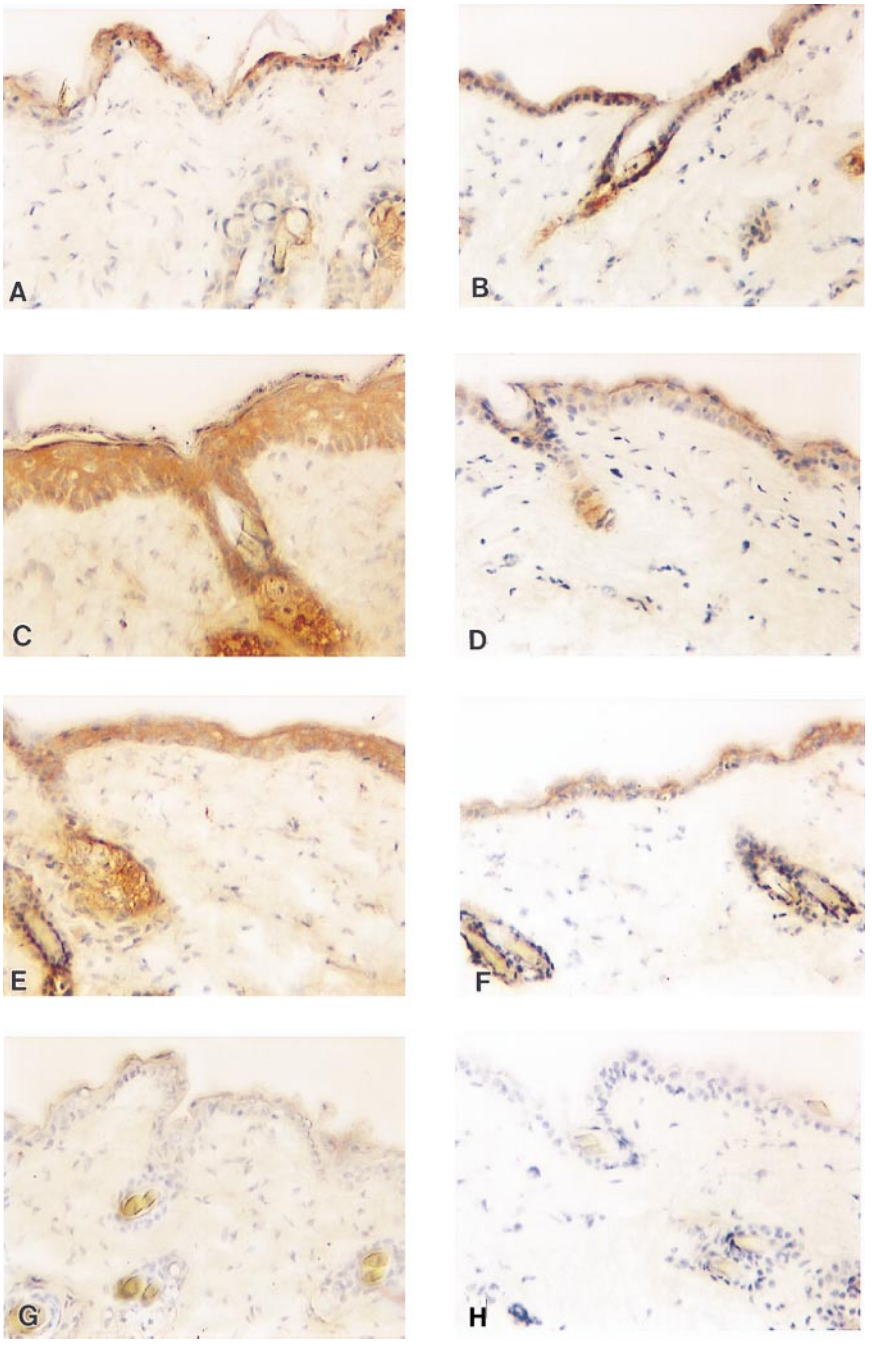

FIGURE 6. *Hin*dIII liposomes induce IL-10 in mouse skin in vivo. Immunohistochemical staining for IL-10 in skin of mice treated 1 (*A*), 3 (*C*), or 8 (*E*) days earlier with 10 kJ/m^2 UV-B radiation; 1 (*B*), 3 (*D*), or 8 (*F*) days earlier with 40 U/ml *Hin*dIII in liposomes; or 1 day earlier with heat-inactivated *Hin*dIII in liposomes (*G*) or given no treatment (*H*).

inducing tolerance to haptens applied to the site of UV-B irradiation (30). Treatment of mice with mAb against IL-10 also restored *Hin*dIII-induced suppression of DTH in response to *C. albicans*, implying that IL-10 is involved in suppression mediated by double-strand breaks in DNA as well. However, supernatants from Pam212 cells treated with *Hin*dIII liposomes failed to induce Ts, even though they contained as much IL-10 as those generated by UV-B radiation. This result implies, as suggested by Rivas and Ullrich (11), that production of IL-10 is necessary for immune suppression by UV-B irradiation, but is insufficient to account for all of its immunosuppressive properties, particularly Ts induction. This contrasts with the CHS model, in which IL-10 appears to play a role in tolerance induction (30) but not in primary immune suppression by UV-B irradiation (31). In our in vivo studies with *Hin*dIII liposomes, we demonstrated increased production of IL-10 in the epidermis; however, it is clear from the immunohistochemical staining for IL-10 that the amount and the timing of IL-10 production in the epidermis and the IL-10 content of dendritic cells in the dermis differed between mice treated with UV-B and *Hin*dIII liposomes. Although these differences may account for some of the discrepancies between the immunosuppressive effects of UV-B and *Hin*dIII liposomes, it seems likely that factors other than IL-10 are crucial contributors to Ts induction in the DTH models. For example, less TNF-α is induced in murine keratinocytes by *Hin*dIII liposomes than UV-B (20), and reactive oxygen species may participate in some UV-B-induced immunosuppressive effects (27).

These and our previous studies (20) demonstrate that *Hin*dIII liposomes also increase the production of both IL-10 and TNF-α in vivo and in vitro. Unlike UV-B radiation, however, this treatment is expected to produce DNA double-strand breaks without the formation of reactive oxygen species, which can cause membrane

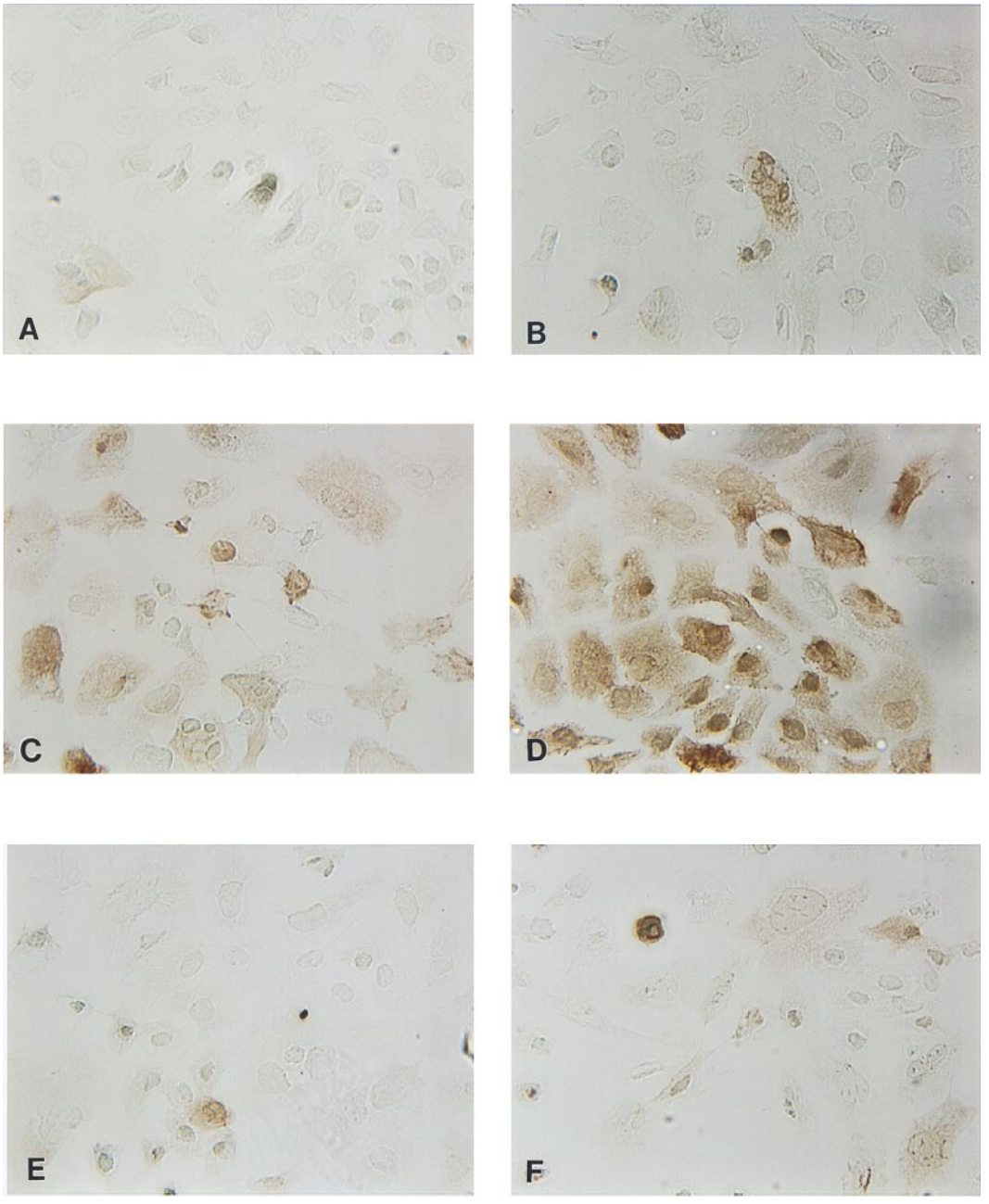

FIGURE 7. *Hin*dIII liposomes induce IL-10 in Pam212 cells in vitro. Immunohistochemical staining for IL-10 in Pam212 cells. *A*, Untreated cells at 12 h; *B*, untreated cells at 24 h; *C*, 12 h after *Hin*dIII liposome treatment; *D*, 24 h after *Hin*dIII liposome treatment; *E*, 12 h after treatment with inactive *Hin*dIII in liposomes; *F*, 24 h after treatment with inactive *Hin*dIII in liposomes.

damage and additional types of DNA damage. These findings reinforce the idea that direct DNA damage can trigger the production of immunomodulatory cytokines, and they have important implications for the use of other DNA-damaging agents, such as chemotherapeutic drugs, which are known to cause leukopenia and immune suppression. Specifically, they raise the possibility that the mechanism of action of such drugs may include alterations in the regulation of cytokines that modify the production or activity of hemopoietic cells as well as direct the cytotoxicity of these cells and their precursors.

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