Interleukin-1α Up-Regulation *in Vivo* by a Potent Carcinogen 7,12-Dimethylbenz(*a*)anthracene (DMBA) and Control of DMBA-induced Inflammatory Responses[1]

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ABSTRACT

Tumor-initiating properties of complete carcinogens such as 7,12-dimethylbenz(*a*)anthracene (DMBA) are well known but not the mechanism of DMBA-mediated tumor promotion. Our hypothesis is that interleukin (IL)-1α, an early proinflammatory cytokine that initiates a cascade of other cytokines and growth factors, is up-regulated by DMBA and contributes to inflammation and carcinogenesis. We found that topical exposure of SENCAR mice to a carcinogenic DMBA dose indeed triggers significant increases in mouse skin IL-1α and IL-1α mRNA. Five DMBA applications (200 nmol each) caused a statistically significant ($P = 0.02$) increase in serum IL-1α, comparable with that induced by 12-*O*-tetradecanoylphorbol-13-acetate, a potent tumor promoter. IL-1α increase in serum was evident 24 h after the first DMBA application, whereas that in skin required five DMBA doses and became statistically significant ($P < 0.0003$) 48 h later. Skin IL-1α enhancement was preceded by a 6-fold up-regulation of IL-1α mRNA. A pretreatment with antimurine IL-1α antibody (Ab) nearly abolished DMBA-induced IL-1α mRNA ($P = 0.0001$) in skin and substantially decreased IL-1α in serum. Infiltration of polymorphonuclear leukocytes into skin was elevated 6-fold ($P = 0.002$) and >10-fold ($P = 0.001$) 24 h and 48 h after the fifth DMBA exposure, respectively. A pretreatment with anti-IL-1α Ab decreased polymorphonuclear leukocyte infiltration by >65% ($P < 0.02$), which suggests that this process is at least 65% under IL-1α control. Anti-IL-1α antibodies had no effect on edema, thus dissociating the two inflammation markers. Injecting anti-IL-1α Ab before DMBA applications significantly ($P < 0.04$) decreased the volume of carcinomas (CAs) in comparison with CAs that arose in mouse skin injected with a nonspecific serum. These results prove that IL-1α is induced by a carcinogenic DMBA dose and contributes to DMBA-induced inflammation and volume of CAs, hallmarks of tumor promotion and progression.

INTRODUCTION

Although much is known about tumor-initiating properties (1–12), it is not clear what the crucial processes are that contribute to tumor promotion and progression by complete carcinogens, such as DMBA.[3] To understand the mechanism of action by complete carcinogens, it is important to establish whether they induce factors indispensable to tumor promotion. IL-1α, known to be up-regulated by tumor promoters, can act as an autocrine growth stimulant (13). The precursor protein gives rise to the mature IL-1α by calcium-dependent proteolytic splitting of the COOH-terminal and to the 16-kDa NH$_2$-terminal, a transforming nuclear oncoprotein (13). IL-1α is an early proinflammatory cytokine, which causes a rapid up-regulation of other cytokines, chemokines, and inflammatory factors, as well as oxidative stress and inflammation (14–24). It has been shown that transgenic mice overexpressing IL-1α in basal keratinocytes suffer from an inflammatory skin disease (25), whereas chronic inflammation and oxidative stress contribute to experimental and human carcinogenesis (23, 26–33).

The two-stage carcinogenesis model stipulates initiating this process by a subcarcinogenic dose (*i.e.,* DMBA) followed by multiple, long-term applications of a tumor promoter, such as TPA (34, 35). This type of Tx causes predominant formation of benign tumors (papillomas) and also of some CAs. In contrast, a complete carcinogenesis model requires multiple topical applications of DMBA to mouse skin, which leads to the development of a much higher proportion of CAs than the two-stage model. There are great differences in responses of various mouse strains to carcinogens and tumor promoters (34, 36–38). For example, TPA evokes oxidative stress in SENCAR mice at much lower doses and to a greater extent than in C57BL/6J mice (39). This oxidative stress is manifested by the formation of higher levels of H$_2$O$_2$ and oxidized bases HMdU and 8-OHdG in SENCAR mouse skin DNA. Differences in oxidative stress correlate well with the susceptibility to TPA-mediated tumor promotion in these mouse strains. Interestingly, multiple topical DMBA applications to SENCAR mice also induce high HMdU and 8-OHdG, levels that are comparable with those evoked by TPA (40). In contrast with TPA, DMBA-induced oxidized bases HMdU and 8-OHdG persisted longer in mouse skin and were significantly higher 5 weeks after the last (10th) DMBA exposure, which resulted in tumor development in 40–50% of DMBA-treated animals. Similarly, inflammation assessed by skin edema and PMN infiltration also persisted longer and at higher levels in DMBA-treated mice in comparison with those treated by TPA. These results indicate that although some of the processes induced by tumor promoters and complete carcinogens are the same, an increased CA yield may depend on the persistence of inflammatory responses and oxidative DNA base modification.

As shown using congenic strains of mice, one of the reasons for differences in the carcinogenic outcome is because of immune responses to carcinogen exposures (41). Although polycyclic aromatic hydrocarbons evoke inflammation during a carcinogenic process (6, 42), there has been no indication that a low DMBA dose induces IL-1α in mouse skin (17). Unmetabolized DMBA and other polycyclic aromatic hydrocarbons have no effect on PMN activation,[4] whereas TPA rapidly stimulates both murine and human PMNs to undergo oxidative burst (43–45). We postulated that similar to TPA, DMBA induces IL-1α formation in SENCAR mice but that IL-1α production may be evident at a different time after DMBA application than that found for TPA. The difference in the induction time may be

attributable to the fact that DMBA requires time for the metabolic activation before its effectiveness as a carcinogen or immune activator manifests itself (41, 42).

In this study, we unequivocally proved that topical DMBA Tx of SENCAR mice mediates IL-1α formation in mouse skin and blood plasma, which is accompanied by IL-1α mRNA up-regulation and increased PMN infiltration into the mouse skin. All of these outcomes were greatly inhibited by a pretreatment with antimurine IL-1α Abs injected 2 h before DMBA application. These results suggest that IL-1α production and IL-1α-mediated inflammatory responses, which lead to oxidative stress and oxidative DNA damage, may participate in the process of DMBA carcinogenesis. This conclusion is strengthened by our recent data showing that anti-IL-1α Abs cause a statistically significant decrease in the volume of CAs in DMBA-treated mice in comparison with DMBA-treated mice injected with nonspecific IgG.

MATERIALS AND METHODS

Mice. SENCAR mice were purchased from Biological Testing, National Cancer Institute (Frederick, MD).

Chemicals. DMBA (95% purity), TPA, MPO, HPLC grade solvents and buffers, SDS, ethidium bromide, and protein determination kits were obtained from Sigma Chemical Co. (St. Louis, MO). Affinity-purified goat antimurine IL-1α polyclonal Ab and nonimmune goat IgG were from R&D Systems, Inc. (Minneapolis, MN), and ELISA IL-1α detection kits were from Endogen (Woburn, MA). According to the manufacturers, the Abs used for IL-1α detection or preinjection were highly specific for their intended targets. Taq DNA polymerase, reverse transcriptase, and nucleotide triphosphates were from Perkin-Elmer (Boston, MA); TRIZOL and a standard mix of oligonucleotides were from Life Technologies, Inc. (Rockville, MD). Forward and reverse primers for PCR amplification of the reverse-transcribed IL-1α mRNA and β-actin mRNA were synthesized by the New York University School of Medicine and Kaplan Comprehensive Cancer Center's Shared Resource.

Tx of Animals. Protocols were approved by New York University School of Medicine Institutional Animal Care and Use Committee. SENCAR mice (6–7-week-old females) were acclimated at the Animal Facility and kept under standard conditions with food and water *ad libitum* (40). The dorsal hair was shaved with surgical clippers 48 h before Tx; only those animals at the hair follicle resting phase were used for the experiments. Initially, three groups of mice were injected via tail vein with nonimmune serum (IgG), whereas the other three groups, with antimurine IL-1α Ab (50 μg in 200 μl PBS/injection). After 2 h, dorsal skin was topically treated with 200 μl of acetone, 3.2 nmol TPA, or 200 nmol DMBA in acetone, and applications repeated on 5 consecutive days. Preinjection with a nonspecific serum or anti-IL-1α Ab was carried out on days 1, 3, and 5. Mice were sacrificed under light anesthesia with pentobarbital 24 h after the last exposure. Blood serum or plasma was collected by heart puncture and aliquoted, whereas skin was detached, rapidly frozen in liquid nitrogen, and stored at −80°C or placed in RNA Later (Ambion, Inc., Austin, TX) and stored at −20°C until use for biochemical determinations. In other experiments, mice were treated with acetone or DMBA for 1, 3, 4, or 5 days, and sacrificed 24 or 48 h after the last exposure. We found previously that DMBA (200 nmol) Tx on 5 consecutive days induces formation of papillomas and CAs within 8 weeks. Tumor levels (46) were comparable with those evoked by a typical carcinogenic Tx with 100 nmol DMBA, twice per week, for 5 weeks (40).

In the carcinogenesis studies, mice were treated with 200 nmol DMBA or acetone on 5 consecutive days. Anti-IL-1α Ab or normal IgG were injected on days 1, 3, and 5, 2 h before the DMBA or acetone Tx. Mice were observed for tumor development for 7–8 weeks and CAs measured before sacrifice. Blood and skin were collected as described above for the short-term experiments.

MPO Quantitation. PMN infiltration into mouse skin was quantitated in whole skin punches using MPO as a surrogate measure (47) with some modifications. Briefly, skin punches were minced in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide homogenized with a polytron homogenizer (3 times, 10 s each) keeping samples in an ice-water bath, and centrifuged at 13,800 × g at 4°C. Superna-

tants were filtered through 40–50-μm filters (Fisher Disposable Filter Columns) and stored at −80°C. MPO was measured in supernatants using 4-aminoantipyrine/phenol as the substrate for MPO-mediated oxidation by H_2O_2, and changes in absorbance at A_{510} were compared with standard curves. One unit of MPO activity is defined as degrading 1 μmol H_2O_2/min at 25°C. Data are presented as mean values using several mice/point (each mouse analyzed separately) and expressed in MPO units/cm² of mouse skin (or units/mg protein) ± SE. We found that MPO units/cm² are the most reliable measure of PMN infiltration (39, 47–49), because the area of a punch is not significantly changed by exposures. Effects of such exposures on protein content within the punch are more variable depending on the extent of hyperplastic responses.

IL-1α, Edema, and Protein Determination. IL-1α was measured in mouse skin extracts and in sera (plasma) by ELISA according to the manufacturer's instructions, and quantitated from standard curves using a microplate reader (Anthos). Serum (50 μl) and skin extracts (5 μl) were used for IL-1α and protein (50 μl) determinations. Protein was measured using a commercial kit (Sigma Chemical Co.). Edema was assessed by weight of skin punches.

RT-PCR Analysis of IL-1α mRNA and β-Actin mRNA in Mouse Skin (According to Ref. 50). Skin samples were powdered in liquid nitrogen with mortar and pestle, and RNA was isolated by direct lysis with TRIZOL (100 mg tissue/ml; Life Technologies, Inc.). Lysates were incubated for 10 min, extracted with phenol-chloroform (0.2 ml/ml TRIZOL), and RNA was precipitated with isopropanol (0.5 ml/ml TRIZOL) from the aqueous layer. The pellets were washed with 75% ethanol, air-dried (not completely), and dissolved in RNase-free water or 0.5% SDS at 55–60°C for 10 min.

The following primers (17, 51) were used for quantitative RT-PCR with β-actin as the internal control. IL-1α, forward primer: 5′-T GCC ATT GAC CAT CTC TCT CTG-3′; and reverse primer: 5′-TGG CAA CTC CTT CAG CAA CAC G-3′. β-Actin, forward primer: 5′-CAA CCG TGA AAA GAT GAC CCA G-3′; and reverse primer: 5′-CAC ACA GAG TAC TTG CGC TCA G-3′.

cDNA was synthesized from IL-1α or β-actin mRNA using a standard mix containing dATP, dTTP, dCTP, and dGTP (800 μM each) in 20 μl of PCR buffer (Perkin-Elmer), reverse primer (0.75 μM), 5 mM $MgCl_2$, and reverse transcriptase (2.5 units/μl). For IL-1α determination, this mix (15 μl) was added to a microfuge tube (500 μl) containing 800 ng of total mRNA in 5 μl of RNase-free water. Only 0.8–2 ng of total mRNA were used to quantitate β-actin. We found that up to 1:1000 ratio of RNAs is needed to obtain comparable signals for β-actin and IL-1α mRNAs. Fig. 1 shows the results of RT-PCR of β-actin mRNA quantitation and its linearity between 0.8 ng and 25 ng ($r = 0.989$), and plateau at higher mRNA levels. Tubes were incubated in a Thermal Cycler (Omni Hybaid) using an optimal temperature profile for reverse transcription of IL-1α and β-actin mRNAs (42°C for 25 min, 99°C for 6 min, and 4°C for 5 min).

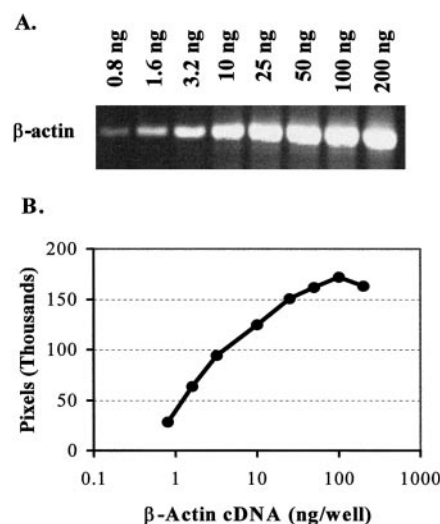The master mix (79 μl) containing 2.5 mM $MgCl_2$ and 2.5 units of Taq DNA

Fig. 1. Linearity of β-actin cDNA using gel electrophoresis: need to use low amount of β-actin mRNA (0.8 ng) for RT-PCR. *A,* representative gel; *B,* linearity of detection.
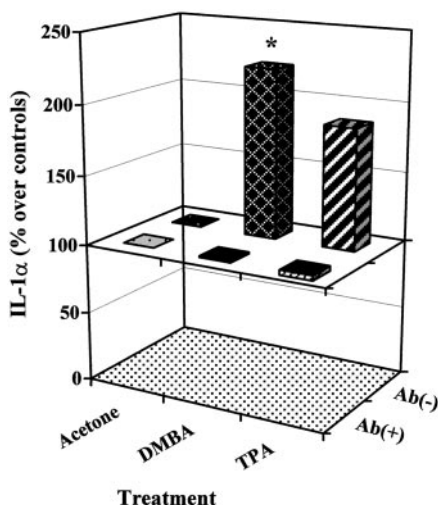
Fig. 2. IL-1α induction in SENCAR mouse serum: DMBA *versus* TPA. Mice were topically treated five times with acetone, DMBA, or TPA in acetone (six/group). Half of the mice from each of the three Tx groups were preinjected with three doses of antimurine IL-1α Ab [Ab(+)], the other half, with nonimmune IgG [Ab(−)]. IL-1α was quantitated by ELISA in sera obtained 24 h after the fifth dose. Results are presented as percentage changes relative to acetone controls. Under these conditions, both TPA and DMBA increased IL-1α levels, with those induced by DMBA being significantly (∗, P = 0.02) elevated over those mediated by acetone alone. A pretreatment with anti-IL-1α Abs decreased DMBA- and TPA-induced IL-1α to the control levels, thus proving that cytokine quantitated by ELISA was in fact IL-1α.

polymerase was added to 20 μl of cDNA synthesized in the step above followed by the addition of 0.15 μM forward primer (100 μl final total volume) and incubated in the thermal cycler. Before use, the number of cycles was optimized to 26 for both IL-1α and β-actin to obtain amplified cDNA within a linear portion (*i.e.,* Fig. 1). cDNA amplification was performed at 95°C, 1 min 45 s for the first cycle (hot start PCR), then 10 cycles, each 15 s at 95°C and 1 min 45 s at 62°C, then 10 cycles, each 15 s at 95°C and 2 min 05 s at 62°C, followed by 6 cycles, each 15 s at 95°C and 2 min 40 s at 62°C. Amplified products were quantitated by digital measurements of fluorescent staining of agarose gel and/or by HPLC.

Gel Electrophoresis. Amplified products were quantitated by gel electrophoresis on agarose at 5 V/cm for 1 h in 0.5 × Tris-borate EDTA buffer [89 mM Tris borate, 2 mM mEDTA (pH 8.3)] using a 100-bp ladder (100 ng) as a marker in a separate well. The respective products of IL-1α cDNA and β-actin cDNA amplification are 564 bp and 678 bp. Gels were stained with 0.5 μg/ml ethidium bromide in water (25 min) then destained with water (25 min), and bands were visualized under UV transilluminator (254 nm) with photographs taken at 1-s exposures. Images were analyzed using Digital Imaging Software (Un-Scan-it; Silk Scientific Inc., Orem, UT), and the intensity of fluorescent signal given by each DNA band scanned was quantitated by the peak area. The results are expressed as [IL-1α cDNA peak area divided by β-actin cDNA peak area (× dilution from 800 ng)] × 100.

HPLC Analysis of cDNA. Initially, samples of the amplified cDNA were analyzed by HPLC on a 2.5-μm nonporous DEAE NPR TSK gel anion exchange column (4.5 × 75 mm) at 40°C [Ref. (52) with modifications] and cDNA quantitated by peak height. The results were normalized for β-actin and expressed as (IL-1α cDNA/β-actin cDNA) × 100. Products of gene amplification were separated on an HPLC column at 0.7 ml/min with 25 mM Tris-HCl (pH 9.0) as a mobile phase A, and 25 mM Tris-HCl/1 M NaCl (pH 9.0) as a phase B. Starting at 75:25% A:B, B content was increased to 45% in 0.1 min, to 50% in 2.9 min, to 55% in 5 min, and to 66% in 20.9 min. Then, B was decreased back to 25% in 0.1 min. These conditions provided a baseline separation of IL-1α and β-actin cDNAs. Our analysis of DMBA-treated mouse skin for IL-1α mRNA has shown that both gel electrophoresis and HPLC give comparable results with a linear dose response when β-actin mRNA is amplified using up to 10³-fold lower levels than those of IL-1α mRNA. Initially, using these two methods of cDNA separation and analysis, we proved that the products of amplification were IL-1α and β-actin cDNAs. Therefore, subsequent analyses were performed using only gel electrophoresis, because it is

much faster than the HPLC method and is amenable to multiple analyses at a time.

Statistical Evaluation. Statistical significance of differences between controls and experimental treatments was determined by *t* test using the actual (Fig. 2) or ln-transformed (Figs. 3–8) data to assure normal distribution. When acetone controls from different groups of the same experiment did not significantly differ, they were combined into one control ("composite") group for that experiment, which is noted in the figure legends.

RESULTS

DMBA Increases IL-1α in Sera of SENCAR Mice. To test the hypothesis that exposure to DMBA, a complete carcinogen requiring oxidative metabolism for its activity, evokes IL-1α as does tumor-promoter TPA, SENCAR mice were topically treated with five doses of DMBA, TPA, or acetone, and analyzed 24 h after the last applications. Both DMBA and TPA induced increases in IL-1α levels in sera of treated mice in comparison with acetone-treated controls. Fig. 2 clearly shows that DMBA Tx was at least as potent as that with TPA and caused a statistically significant (P = 0.02) rise in IL-1α levels over those present in acetone controls. Pretreatment with murine anti-IL-1α Ab suppressed DMBA- and TPA-mediated increases in IL-1α, with significance of the inhibition being P < 0.01 for each. These results proved that the cytokine induced by DMBA and measured in sera of treated mice was indeed IL-1α.

DMBA Up-Regulates IL-1α and IL-1α mRNA in Mouse Skin. Fig. 3 shows that DMBA has a very pronounced effect on the transcription of the *IL-1α* gene, because IL-1α mRNA was strongly up-regulated in the treated mouse skin of the same animals that exhibited significant elevation in serum IL-1α. DMBA mediated a 5-fold increase in IL-1α mRNA in comparison with acetone controls. Interestingly, acetone itself caused a statistically significant increase (P < 0.0004) in IL-1α mRNA relative to the nontreated animals. Preapplication of anti-IL-1α Ab diminished acetone-mediated transcription of this gene and inhibited by >95% that induced by DMBA (P < 0.0001 for each). Another experiment corroborated these results showing that five DMBA doses caused a 5.7-fold increase of IL-1α
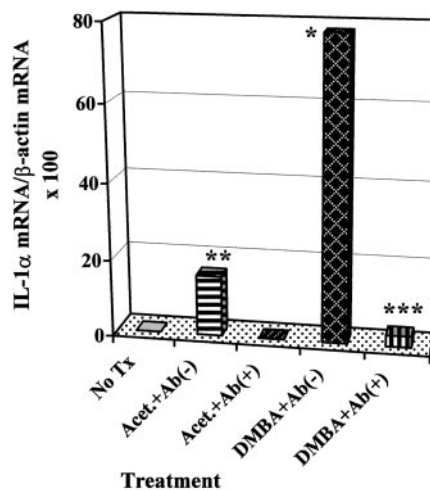

Fig. 3. DMBA up-regulates IL-1α mRNA in SENCAR mouse skin. mRNA was isolated from the skin of the same mice as in Fig. 2, reverse-transcribed, and the amplified cDNA was separated by HPLC. The results were normalized for β-actin, which was baseline separated from IL-1α under HPLC conditions used ("Materials and Methods"). Even acetone Tx of mouse skin significantly increased IL-1α mRNA levels (∗∗, P = 0.0004 acetone *versus* nontreated skin). However, DMBA application additionally enhanced (5-fold) IL-1α mRNA, a highly significant (∗, P < 0.002) increase over the acetone controls. A pretreatment with anti-IL-1α Ab abolished acetone-mediated IL-1α mRNA increase and decreased by >95% that induced by DMBA (∗∗∗, P < 0.0001 for both).
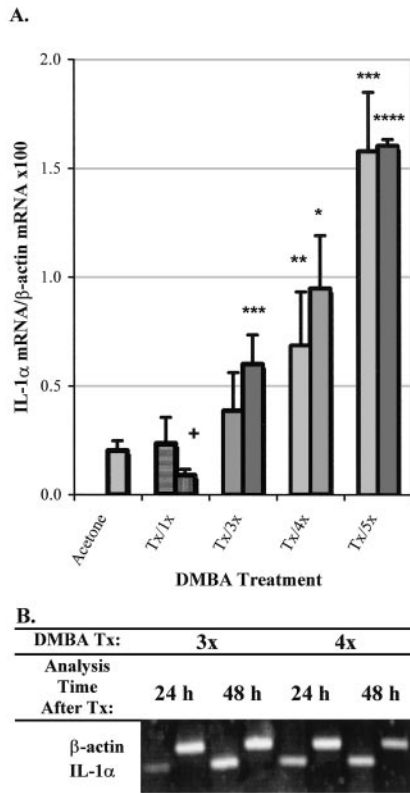
A.

DMBA Treatment

B.

Fig. 4. Effects of DMBA dose and time of analysis on IL-1α mRNA. *A,* histogram, bars ± SE (standard error); *B,* representative gel. SENCAR mice were topically treated one, three, or four times (five/group), or five times (10/group) with acetone or DMBA, and mRNA analyzed 24 or 48 h after last exposures. After RT-PCR, the resultant IL-1α cDNA was analyzed by gel electrophoresis and visualized with ethidium bromide. The results were normalized for β-actin cDNA, which was derived from 0.8–2 ng β-actin mRNA used for RT-PCR *versus* 800 ng for IL-1α mRNA. Because there were no significant differences in acetone controls applied one, three, or five times, these controls were combined into one group. Mice painted three times did not show differences in IL-1α mRNA levels from those treated only once when analyzed 24 h after the third DMBA exposure but were significantly different (***, $P = 0.02$) from acetone controls when analyzed 48 h after the third Tx. Four DMBA applications caused significant increases in IL-1α mRNA 24 h (**, $P = 0.05$) and 48 h (*, $P < 0.06$) later. Five DMBA applications caused ~5-fold increase in IL-1α mRNA measured in skin samples 24 h after the fifth DMBA dose (***, $P = 0.02$), which remained elevated for the subsequent 24 h (****, $P < 0.0001$).

mRNA when analyzed 24 h after the last DMBA Tx (Fig. 4*A*). An extensive up-regulation of IL-1α mRNA was also evident 48 h after the fifth DMBA dose (6.8-fold). The up-regulation of IL-1α mRNA was gradual, with the first increase being significantly different (3.0-fold; $P = 0.02$) 48 h after the third DMBA application (Fig. 4, *A* and *B*). The fourth DMBA Tx resulted in a 4-fold increase ($P = 0.051$ and $P = 0.058$, 24 h and 48 h after the fourth Tx, respectively). Interestingly, IL-1α mRNA was significantly decreased from the acetone controls ($P < 0.03$) 48 h after the first DMBA application. Overall, the increase in IL-1α mRNA became first evident 48 h after three DMBA applications and gradually increased after four and five exposures to DMBA (Fig. 4, *A* and *B*). Under the same conditions, cytokine itself was elevated in mouse skin extracts 24 and 48 h after the fifth DMBA application and reached statistical significance at 48 h ($P < 0.0003$ DMBA *versus* acetone; $P = 0.03$ 48 h versus 24 h after five DMBA exposures; Fig. 5). There were no increases in IL-1α in mouse skin treated once (Fig. 5) or three times (not shown). In contrast, IL-1α increased dose-dependently in plasma of the same mice analyzed 24 h after the last DMBA exposure, with P *versus* acetone being 0.025, 0.1, and 0.03 in plasma of mice treated one, three, and five times, respectively (Fig. 6). Pretreatment with anti-

IL-1α Ab significantly decreased DMBA-induced IL-1α in plasma (overall $P = 0.003$).

IL-1α Formation in Mouse Skin and PMN Infiltration Are Dependent on DMBA Dose and Time of the Analysis. PMN infiltration [quantitated by MPO (units/cm²)] was first evident 48 h after the fourth DMBA application (not shown), a near 2-fold increase over acetone controls ($P < 0.03$) and was highly significant 24 h after five DMBA doses ($P < 0.002$ *versus* acetone) being 6.2-fold higher than its acetone control (Fig. 5). MPO nearly doubled in the next 24 h ($P < 0.001$ versus acetone and $P = 0.002$ 48 versus 24 h after five
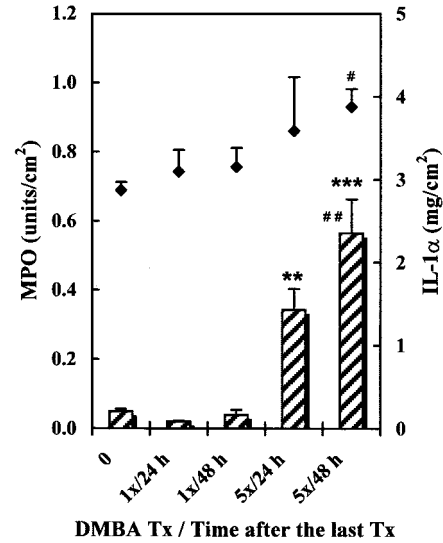

DMBA Tx / Time after the last Tx

Fig. 5. Relationship between DMBA-induced IL-1α (♦) in mouse skin and PMN infiltration (▨). Skin punches were obtained from mice treated as described in Fig. 4 legend, homogenized, and analyzed for MPO and IL-1α 24 or 48 h after one or five acetone or DMBA applications. Results are presented as MPO units/cm² of the skin. **, $P = 0.002$; *** , $P = 0.001$, DMBA *versus* combined acetone controls; ##, $P = 0.002$, 24 h *versus* 48 h. IL-1α first increased 24 h after five DMBA applications and significantly (#, $P = 0.0003$) within 48 h; bars ± SE.

Treatment (Tx)
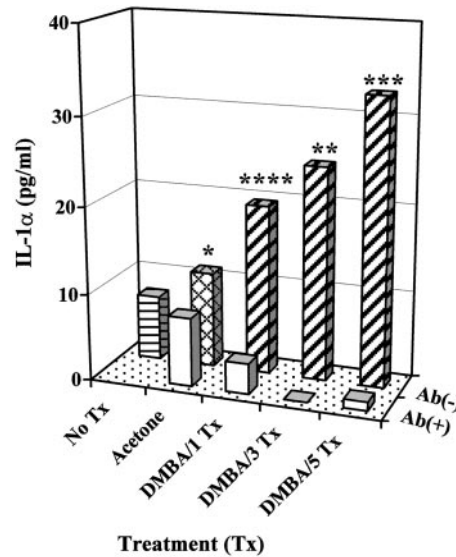
Fig. 6. Effects of DMBA on the systemic IL-1α release 24 h after the last exposure. Half of the mice were pretreated with nonimmune serum before first, third, and fifth applications of acetone or DMBA [Ab(−)], the other half were pretreated with anti-IL-1α Ab [Ab(+)]. *, $P < 0.025$, one DMBA dose *versus* combined acetone controls in the absence of anti-IL-1α Ab. A pretreatment with anti-IL-1α Ab caused significant declines in IL-1α induced by one, three, or five DMBA doses (****, $P < 0.0001$; **, $P = 0.02$; ***, $P = 0.01$, respectively). There was a significantly greater ($P = 0.0002$) decline in DMBA-induced IL-1α because of the Ab pretreatment than in that induced by acetone.

DMBA doses), a 10.2-fold increase. Interestingly, 24 h after a single ($P = 0.033$) or triple (not shown) DMBA exposure, MPO decreased in comparison to the respective acetone controls. Analysis of the same skin extracts for IL-1α (Fig. 5) indicated that increases in IL-1α in the DMBA-treated mouse skin occur at the earliest 24 h after five DMBA applications. Those changes differed significantly after 48 h ($P = 0.0003$ for IL-1α and $P < 0.001$ for MPO) in comparison to the combined (one, three, and five acetone treatments) controls.

PMN Infiltration into Mouse Skin but not Edema Is Mediated through DMBA-induced IL-1α. To assess whether IL-1α or IL-1α-induced inflammatory processes are responsible for DMBA-mediated edema and PMN infiltration into mouse skin, which we observed before (40), SENCAR mice were treated five times either with acetone or DMBA in acetone, with or without pretreatment with anti-IL-1α Ab. MPO levels (units) per mg protein of treated mouse skin was used as a quantitative measure of PMN infiltration. DMBA increased MPO in mouse skin 24 h after five DMBA doses by ~7-fold in comparison to acetone controls ($P = 0.002$; Fig. 7). DMBA-induced MPO increase was inhibited >65% by the pretreatment with anti-IL-1α Ab ($P < 0.02$) but was still significantly higher than MPO present in the Ab-pretreated acetone controls ($P = 0.02$). DMBA also increased edema and protein content of the same extracts, but anti-IL-1α Ab did not counteract those increases (Tables 1 and 2). These results strongly suggest that DMBA-induced IL-1α and/or
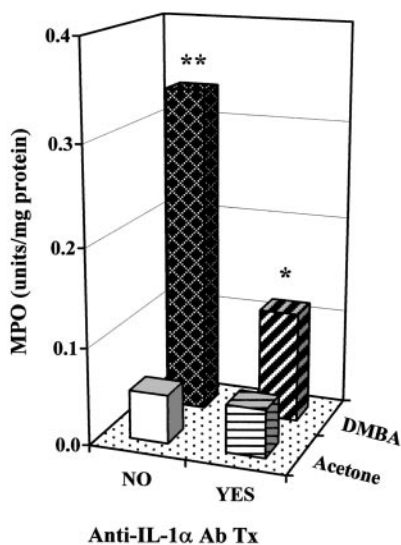

Fig. 7. Role of DMBA-induced IL-1α in PMN infiltration into mouse skin. Half of the mice treated daily for 5 days with acetone or DMBA (four/group) were pretreated with nonimmune IgG [Ab(−)], the other half (four/group) with anti-IL-1α Ab [Ab(+)] on days 1, 3, and 5. PMN infiltration was assessed by quantitating MPO activity/mg protein in skin homogenates 24 h after the fifth acetone or DMBA application. DMBA increased MPO >6.5-fold over that induced by acetone alone (∗∗, $P = 0.002$). A pretreatment with anti-IL-1α Ab inhibited that increase in PMN infiltration by >65% (∗, $P < 0.02$), which indicates that this inflammatory response is (at least in 65%) under control of DMBA-induced IL-1α.

Table 1 *Effects of DMBA-induced IL-1α on inflammatory responses in mouse skin (mean ± SE)[a]*

Endpoint measured (per cm²)	Acetone		DMBA	
	Ab(−)	Ab(+)	Ab(−)	Ab(+)
Edema (mg)	47.55 ± 4.71	39.66 ± 2.10	61.64 ± 3.30	54.26 ± 3.50
Protein (mg)	2.08 ± 0.19	1.81 ± 0.10	2.35 ± 0.10	2.50 ± 0.25
MPO (units)	0.102 ± 0.004	0.089 ± 0.004	0.743 ± 0.175	0.277 ± 0.066

[a] Edema, protein, and MPO were measured in the same skin punches. Values are derived from samples treated five times with acetone or DMBA and pretreated with nonimmune IgG [Ab(−)] or with anti-IL-1α Ab [Ab(+)].

Table 2 *Anti-IL-1α Ab-mediated changes in DMBA-induced inflammatory responses in mouse skin (% differences using values from Table 1)*

	Acet(+) vs. Acet(−)[a]	DMBA(+) vs. DMBA(−)	DMBA(−) vs. Acet(−)	DMBA(+) vs. Acet(+)
Edema	7.89 (83.4%)	7.38 (88.0%)	14.09 (129.6%)[b]	14.60 (136.8%)[c]
Protein	0.27 (87.0%)	0.12 (106.4%)	0.27 (113.0%)	0.69 (138.1%)[d]
MPO	0.013 (87.7%)	0.466 (37.2%)[e]	0.641 (730.6%)[f]	0.175 (310.1%)[g]

[a] Acet, acetone treatment; (+), Pretreatment with anti-IL-1α Ab; (−), Pretreatment with nonimmune IgG.
[b] $P = 0.03$.
[c] $P = 0.008$.
[d] $P = 0.03$.
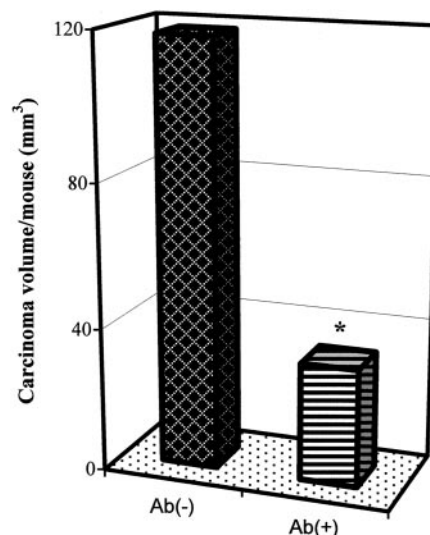[e] $P = 0.012$.
[f] $P = 0.002$.
[g] $P = 0.009$.

Fig. 8. Effects of anti-IL-1α Ab [Ab(+)] injected into SENCAR mice ($n = 31$) on days 1, 3, and 5 before DMBA applications on five consecutive days on volume of CAs in comparison to normal IgG [Ab(−)]-preinjected mice ($n = 34$). ∗, $P = 0.039$: CA volume per mouse with CAs.

factors induced by IL-1α significantly enhance infiltration of ≥65% of PMNs but do not affect edema or protein levels.

Effects of DMBA-induced IL-1α on Skin Carcinogenesis. Five topical DMBA applications caused the appearance of CAs and papillomas in the skin of ~50% SENCAR mice within 8 weeks. The greatest anti-IL-1α Ab-mediated changes occurred in the volume of CAs (Fig. 8). Mice injected with this Ab had a significantly ($P = 0.039$) lower volume of CAs than the volume of CAs in mice, which received a nonspecific serum.

DISCUSSION

Similarities of Responses Induced by DMBA and TPA in SENCAR Mice. Many laboratories have extensively studied processes evoked by tumor promoters. Although much has been learned, there is still no consensus as to the mechanism of tumor promotion. However, it has become evident that inflammatory responses induced by tumor promoters play a major role in the development of tumors. If that is really the case, then complete carcinogens should also evoke some of the same inflammatory processes as tumor promoters do. Results presented in this paper strongly support the idea that a complete carcinogen DMBA is as capable of IL-1α induction in the treated mouse skin and systemically (Figs. 2–6) as is TPA, an archetypal tumor promoter. DMBA also causes PMN infiltration into the treated mouse skin (Figs. 5 and 7), again akin to that mediated by TPA. Moreover, PMN infiltration induced by DMBA is strongly dependent

on IL-1α, because that infiltration decreased significantly in mice pretreated with anti-IL-1α Ab (Fig. 7).

Differences between DMBA-induced Target Tissue IL-1α and Systemic Levels. It is puzzling that increases in systemic IL-1α levels become evident within 24 h after a single DMBA application (Fig. 6), whereas elevation in skin IL-1α becomes evident 24 h after the fifth Tx and statistically significant increase occurs within 48 h (Fig. 5). Perhaps even low levels of metabolites formed after a single DMBA Tx are sufficient to initiate a rapid release of IL-1α stored in suprabasal keratinocytes into the circulation (17, 18) and/or that produced by infiltrating phagocytic cells. Another possibility is that DMBA metabolite(s) stimulate translation of the basal IL-1α mRNA. These ideas are strengthened by the fact that a pretreatment with anti-IL-1α Ab potently decreased DMBA-induced IL-1α in plasma (Fig. 6) by perhaps forming Ab-IL-1α complexes. An Ab-mediated decline in systemic IL-1α probably provides a signal to decrease IL-1α mRNA synthesis that would normally occur after five DMBA doses (Fig. 3). The statistically significant increase in tissue IL-1α, a likely measure of intracellular stores, seems to follow a substantial (>5-fold) IL-1α mRNA up-regulation requiring five DMBA doses and increasing 5.7- and 6.8-fold 24 and 48 h after the last Tx (Fig. 4A). At this point, the exact cell type(s) of the mouse skin producing IL-1α in response to DMBA exposure is not known; it will be important to determine this in the future.

IL-1α Induced by DMBA Is Necessary for PMN Infiltration but not for Edema. In comparison to acetone Tx, five DMBA doses increased edema and protein content, as well as PMN infiltration, as established using the same skin punches. A pretreatment with anti-IL-1α Ab inhibited DMBA-mediated PMN infiltration by 65% (Fig. 7; Table 2; $P < 0.02$), as assessed by MPO levels. However, anti-IL-1α Ab had virtually no effect on DMBA-mediated edema (as measured by weight) and, surprisingly, actually increased protein levels within the same area of the skin (Tables 1 and 2). The >250% difference between the net protein values [DMBA(−) − Acetone(−) *versus* DMBA(+) − Acetone(+), where (−) and (+) refer to the pretreatment with anti-IL-1α Ab] was statistically significant ($P = 0.03$). An apparent decline in the DMBA/acetone-induced edema caused by anti-IL-1α Ab pretreatment can be explained by a decrease in the acetone-induced edema, which was sensitive to Ab Tx. Thus, when only a net increase in DMBA-induced skin weight is considered, there is no difference in those weights between groups of mice pretreated either with nonspecific serum or with anti-IL-1α Ab.

These results are in agreement with our previous observation dissociating effects on PMN infiltration from those on edema. In those experiments, caffeic acid phenethyl ester, an anti-inflammatory antioxidant and antitumor promoter, was orders of magnitude more potent as an inhibitor of PMN infiltration than of edema (48).

Interestingly, work of Casale *et al.* (42) has shown that DMBA-induced edema is one of the measures of contact hypersensitivity evoked by this carcinogen. DMBA-mediated response was much weaker than that induced by dibenzo[*a,l*]pyrene, a much more potent carcinogen and more effective inducer of hypersensitivity in SENCAR mouse skin. Similarly, lower levels of IL-1β, tumor necrosis factor-α, and IFN-γ, cytokines up-regulated by allergens, were induced by DMBA than by dibenzo[*a,l*]pyrene. In contrast, PMN infiltration was comparable in mice treated with either carcinogen. Although PMN infiltration *per se* cannot be directly related to tumor promotion efficacy, PMN activation with the ensuing generation of oxidants and oxidative DNA base damage, which occur when those PMNs are stimulated, are predictive of promotion (29, 53). Thus, although carcinogens of different potencies cause comparable PMN infiltration into the target tissue, their metabolites may exhibit different PMN-activating potencies.

Together, these results suggest that complete carcinogens are capable of evoking a complex array of immune responses that differ in magnitude and that some of them correlate with the carcinogenic potency. Certain of those responses may be protective, whereas others might override such protection and enhance tumorigenesis. Contact hypersensitivity, which is mediated by cytokines IL-1β, tumor necrosis factor-α, and IFN-γ, may be one of those tumor-enhancing responses; we are in the process of comparing contributions of IL-1α and tumor necrosis factor-α to DMBA-induced carcinogenic process. Another aspect of the immune response to a carcinogen Tx is the induction of inflammatory processes mediated by cytokines, such as IL-1α, which appear to be important in controlling PMN infiltration through chemotactic factors. IL-1α was not determined in the dibenzo[*a,l*]pyrene- or DMBA-treated mouse skin (42). However, our recent data (Fig. 8) indicate that DMBA-induced processes controlled by the IL-1α pathways enhance malignancy, as assessed by a statistically significant decline in the volume of CAs in anti-IL-1α Ab pretreated mice. Thus, it is likely that a higher degree of hypersensitivity induced by dibenzo[*a,l*]pyrene in comparison to DMBA compounds the severity of the resulting hyperplasia and carcinogenesis. This heightened allergic response is probably the cause of the intense toxicity exhibited by dibenzo[*a,l*]pyrene (54).

In summary, our results showed that DMBA-induced IL-1α contributes to malignancy, because anti-IL-1α antibodies significantly decreased CA volume in mice exposed to DMBA in comparison to the volume of CAs arising in mice that were treated with nonspecific IgG. Early in the carcinogenic process levels of IL-1α, IL-1α mRNA, and PMN infiltration were substantially increased in the mouse skin 24 h and 48 h after the fifth DMBA exposure. It appears that IL-1α mRNA was up-regulated first being significantly induced by ∼3-fold 48 h after the third exposure to DMBA, 4-fold 24 and 48 h after the fourth DMBA Tx, and 6-fold 24 h and 48 h after the fifth application of the carcinogen. PMN infiltration was first evident 48 h after the fourth DMBA Tx, then significantly, 24 h after the last DMBA exposure, and it has nearly doubled in the subsequent 24 h. Similarly, IL-1α in the mouse skin was first enhanced 24 h after the fifth DMBA Tx and became significantly elevated in the following 24 h. It is likely that IL-1α and/or PMN infiltration have remained elevated after the five DMBA doses. Our previous study, in which SENCAR mice were topically treated with the same overall DMBA dose over a period of five weeks (100 nmol, twice per week), showed that PMN infiltration remained high in the subsequent weeks and was still significantly elevated after tumors appeared (40). At the same time, HMdU and 8-OHdG levels were also increased and remained elevated during the time of tumor development. We intend to determine whether there is a relationship between IL-1α induction by DMBA and oxidative DNA base damage formation in the same tissue. Both of these oxidized bases are mutagenic (55–58) and affect methylation patterns (59, 60), processes important to carcinogenesis.

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