Mechanism of Skin Tumorigenesis by Contact Sensitizers: The Effect of the Corticosteroid Fluocinolone Acetonide on Inflammation and Tumor Induction by 2,4 Dinitro-1-fluorobenzene in the Skin of the TG.AC (v-Ha-*ras*) Mouse

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The effect of the corticosteroid fluocinolone acetonide (FA) on skin tumor induction and inflammation by the contact sensitizer dinitrofluorobenzene (DNFB) was examined. This study broadly relates to the question of whether contact sensitizers, as electrophilic chemicals that produce protein adduction, may constitute an environmental cancer hazard. The specific aim of this study was to evaluate the extent to which the immunogenic inflammatory response to DNFB, in contrast to DNFB cytotoxicity, might be responsible for tumor induction. Experiments were conducted on a transgenic (TG.AC) mouse, incorporating a mutated *ras* oncogene (v-Ha-*ras*) that responds rapidly and profusely with skin papillomas to tumor promoters as if it were genetically initiated. Various doses and patterns of DNFB and FA were applied to the skin in a 2-week period; DNFB was given four times and FA was given either with the DNFB or daily. The tumor response to DNFB was completed by 8 weeks from the first dose and was consistent with a dose-squared relationship. FA was not tumorigenic alone; when given with DNFB, it caused only a small reduction in inflammation and tumor yield. When given daily, FA increased ulcerative skin damage, inflammation, and the yield of tumors. The results suggest that tumorigenesis by DNFB, in the high-dose short-term regimen used here, is mainly due to its cytotoxicity and not contact sensitization. *Key words*: contact sensitization, corticosteroids, dinitrofluorobenzene, fluocinolone acetonide, TG.AC mouse, tumorigenesis, tumor promotion. *Environ Health Perspect* 104:1062–1068 (1996)

The evidence for the role of environmental factors in human cancer is strong in terms of geographic differences in cancer patterns and the changes in these patterns in migrants (*1*). Yet, with some exceptions, identified carcinogens (even by conservative risk assessments) appear to be responsible for only a small fraction of current cancer deaths (*2*). The role of tumor promoters would seem to be important because there is exposure to large numbers of environmental carcinogens at low dose levels (*3*) that, at a minimum, might be expected to initiate the cancer process. However, the tumor promoters identified in rodent studies are commonly regarded as being too species and strain specific to be of importance to humans (*4*).

There is a class of chemicals to which humans are exposed, namely, the contact sensitizers, that might be of interest because of their possible role as tumor promoters. The large number of such agents is suggested by the fact that conventional dermatological kits for diagnostic patch testing encompass over 350 chemicals (*5*). Of these, 19 are on the Congressionally mandated NIH list of carcinogens (*6*)

Contact sensitizers are chemically related to genotoxic carcinogens; both classes of compounds are electrophiles and cause the adduction of macromolecules (*7*). The contact sensitizers are compounds that cause nucleophilic aromatic substitution reactions, and their macromolecular adduction is limited to protein.

Although there is little direct evidence about the carcinogenic potential of contact sensitizers, suggestive evidence of an overlap with carcinogens comes from structure–activity alerts for contact sensitizers (*8*) and carcinogens (*9*). Of the 41 structural alerts for contact sensitizers, one-third are common to those for carcinogens (D.W. Bristol, personal communication).

In the late 1940s, the concept first emerged that carcinogenesis is a multistage process that can be divided into an initiation stage, which is prompt and irreversible (like the induction of mutations), and a promotion stage in which there is clonal expansion of neoplastically transformed cells into tumors (*10*). The possible connection between contact sensitizers as promoters and classical tumor promoters is that they both cause inflammatory reactions at the site of contact, and inflammation is the hallmark of tumor promotion (*11,12*). The association of chronic inflammation with cancer has been recognized for over a century (*13–16*). How inflammation might cause cancer is unclear. Inflammatory cells produce oxygen free radicals (*17*), which results in oxidized DNA bases (*18*) and DNA damage in the form of single strand breaks, increased sister chromatid exchanges, and chromosome abnormalities (*11*). Activated human leukocytes neoplastically transform 10T1/2 cells in culture, which suggests that inflammatory cells may be carcinogenic; the same effect in this *in vitro* system can be produced by a super oxide generating system (xanthine oxidase and hypoxanthine) (*19*).

There are at least two basic causes of inflammation in the skin: the immune-specific cell-mediated Type IV (delayed-type hypersensitivity) immune process and irritant-based inflammation produced by a wide range of chemical and physical factors. Cell-mediated immunity is a complex two-step process. The first or sensitization step involves the initial skin application of the contact sensitizer, with the formation of haptenes from adducted skin proteins, and activation of T lymphocytes throughout the body. The second or elicitation step involves localization of the activated lymphocytes at the site of the second skin application and the induction of an inflammatory reaction (*20*).

Inflammation in the skin from chemical or physical irritation is due to the release of cytokines by the damaged keratinocytes in the epidermis, which causes mobilization of inflammatory cells and edema (*21*). There is no distinction between immunogenic and irritant inflammation in terms of the types of inflammatory cells. In principle, contact sensitizers could produce inflammation by both immune and irritative (cytotoxic) mechanisms.

The first link between carcinogens and contact sensitization was reported in a study of three polycyclic aromatic hydrocarbon carcinogens (*22*). More recently, an evaluation was made of the relationship of muta-

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genicity, carcinogenesis, and skin sensitization (*7*). The results on 20 agents, including human and rodent carcinogens, clearly supported the high likelihood that mutagens and genotoxic carcinogens are skin contact sensitizers.

Recent studies on the cytokinetic responses of the mouse skin to chronic topical benzo(*a*)pyrene (BaP) exposure indicated that there was an inverse relationship between the dosage level and the degree of latency in the appearance of dermal inflammation and epidermal proliferation (*23*); such an inverse relationship also applies to tumor induction and suggests the possibility that an immune response to BaP might play a role in the promoting component of tumorigenesis. A delay in the appearance of inflammation was also seen with the carcinogen dibenz(*a,l*)pyrene, which was also interpreted as an immune response (E. Cavalieri, personal communication).

The hypothesis that contact sensitizers might be tumor promoters because they produce an immunogenic inflammatory reaction implies that individuals who are allergic hyperreactors to contact-sensitizing chemicals might be at a relatively high risk of cancer and the kinetics of promotion from the promoting component of complete carcinogens or from contact sensitization might be based on the kinetics of immune processes in response to low-level exposure.

The above hypothesis could be tested by determining whether corticosteroids depress the inflammation and tumorigenic action of contact sensitizers. Corticosteroids are commonly used for the treatment of immunological diseases including allergic contact dermatitis, which is a manifestation of T cell-mediated Type IV (delayed-type hypersensitivity) immunity. The mechanisms of action are incompletely understood but inhibition of phospholipase A2, an enzyme that releases arachidonic acid from membrane phospholipids, is considered to be critical; arachidonic acid is converted to inflammatory mediators, prostaglandins, and leukotrienes. Corticosteroids also inhibit the accumulation of inflammatory cells, production of interleukins 1 and 2, and biosynthesis of cyclooxygenase (*24*); they are growth inhibitory and can produce atrophy of the skin. Corticosteroids inhibit tumor promotion by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (*25*), and the degree of inhibition correlates with their anti-inflammatory potency of the phorbol esters (*26*). The tumorigenicity of the complete carcinogens 7-bromomethyl benz(*a*)anthracene (*27*) and methylcholanthrene (*28*) are suppressed in part by the corticosteroid fluocinolone acetonide (FA). However, the reverse has also been reported, namely, that FA can enhance tumor promotion by β-propriolactone (*29*).

To test the hypothesis that the promoting action of contact sensitizers could be due to the induction of an immunogenic inflammatory reaction, we examined the effect of FA on the tumorigenic and inflammatory action of dinitrofluorobenzene (DNFB), a well studied contact sensitizer and tumor promoter. DNFB attacks the terminal amino groups on proteins and has been used to sequence their amino acid structure (*30*). It has been shown to be a tumor promoter for the skin when initiated with 1,2-dimethyl-3,4-benzanthracene (DMBA) in four strains of mice: Swiss, C57BL/6, Balb/C (*31*), and Sencar (*32*). DNFB is not a tumor initiator in the mouse skin (*31*), indicating that it is not genotoxic. DNFB belongs to the protein kinase C-independent class of tumor promoters (*32*). It is nonmutagenic in the V79 cell culture system although it is mutagenic in *Escherichia coli* (*33*). DNFB is not clastogenic nor does it produce sister chromatid exchanges, but it does inhibit metabolic cooperation (*33*); thus, DNFB has many of the usual characteristics of a tumor promoter. Although it is not as potent as TPA on a molar basis, DNFB is similar in that it is less effective in some mouse strains than in others, e.g., C57BL/6 compared to the Swiss (*31*).

The skin tumor promotion model selected for this study was the TG.AC transgenic mouse. This transgenic model incorporates a fetal ζ-globin promoter fused to a v-Ha-*ras* structural gene with mutations at codons 12 and 59, which is linked to a simian virus 40 polyadinylation splice sequence (*34*). The skin of this mouse behaves as if it were genetically preinitiated and has the great advantage of a very rapid and profuse papilloma response to even a few applications of a promoting agent (*35*). We used TPA as a positive control for DNFB.

In the study reported here, we found that FA, when applied together with DNFB, produced only a small reduction in the inflammatory response and no reduction in the tumorigenic response; this is in contrast to the effect of FA on TPA. With daily doses of FA, the damaging effect of DNFB on the skin was worsened and the inflammation and tumor response increased. These results suggest that tumorigenesis, produced by relatively high-level, short-term dosing with DNFB, is due to cytotoxicity and not to immunogenic inflammation.

Materials and Methods

Female homozygous TG.AC mice were obtained from Taconic Farms (Germantown, PA) at 4–5 weeks of age. They were quarantined for 1 week and housed four to a cage in rooms maintained at 70°F with a 12-hr light/dark cycle. The mice were fed Purina Pico Chow 5058 and water *ad libitum*. The mice were shaved with electric clippers on the day of first skin application and as needed. Mice were enrolled in different experiments at ages ranging from 16 to 32 weeks.

Reagant grades DNFB, FA, and TPA were obtained from the Sigma Chemical Company, St. Louis, MO. DNFB was applied in 4:1 acetone/olive oil, and FA and TPA were applied in acetone. Applications of DNFB and TPA to the dorsal skin of the back were made in 100-µl volumes. FA was applied about 30 min before DNFB or TPA, when given close together. FA was administered in a volume of 150 µl to insure that DNFB and TPA were applied to FA-treated skin.

Tumor induction by DNFB or TPA was routinely done by applying two doses per week for 2 consecutive weeks for a total of four applications. Animals were initially observed several times a week for skin changes on the back and then for the appearance of tumors at 1-week intervals. Tumor formation was expressed as the prevalence of tumors per mouse because mortality during the tumor formation period was negligible. The size of treatment groups ranged from 4 to 16 mice each, depending on the experiment.

Contact sensitization was induced by applying four doses of DNFB or TPA to the dorsal skin of the back, as described for tumor induction. The sensitization response was elicited after a 7-day interval by the application of DNFB or TPA at nonirritating concentrations to either the dorsal surface or both surfaces of the ear in 20-µl volumes under fluothane:oxygen anesthesia. Ear thickness was initially measured 24 and 48 hr later using the same anesthetic. Only the 24-hr measurements were made when we ascertained, as did others (*36*), that the maximum response was obtained at 24 hr. The ear thickness measurements were made with a Dyer micrometer (Dyer Corporation, Lancaster, PA). This device opposes two steel disks of 5-mm diameter at a constant pressure and measures the amount of separation in hundredths of a millimeter. Care was taken to apply the disc consistently about 3 mm from the tip of the ear in order to obtain reproducible measurements because the ears in mice are tapered; care was also taken to ensure that the application of the sensitizing agent covered the measured area. The results were expressed as the difference between the ear thickness before and after the application of the eliciting dose to the ear.

Histological sections of the treated skin were obtained for evaluation of the inflammatory reaction in the dermis several days

after the last dose of the agent under study. Two adjacent transverse strips of skin, several millimeters wide, were taken from the treatment area of each mouse and fixed in 10% formalin. Two paraffin-embedded sections, perpendicular to the surface of the skin, were cut from each strip. The sections were 6 µm thick and were stained with Feulgen reagent to highlight the cell nuclei and counterstained with Fast Green. The slides were examined in an image analysis unit using the NIH Image 1.54 program (National Institutes of Health, Bethesda, MD). This permitted measurements of the inflammatory reaction in terms of the number of cell nuclei in the dermis between the hair follicles per unit area. An oil-immersion objective was used to measure cell density, and the resulting image on the monitoring screen was 80 × 60 µm with an enlargement of 340×. The cell density was equal to the number of cell nuclei per 4400 μm^2. Dermal thickness was measured as the distance between the bottom of the epidermis and the top of the hypodermis. A 10-power objective was used to measure dermal thicknesses with a magnification of 34×. Five areas from each slide were measured for cell counts and dermal thickness. Two slides were examined from each of the two skin strips per animal and two animals were used for each treatment group. The product of the cell density and the thickness of the dermis was used as an index of inflammation because it roughly reflected the total number of cells in the dermis. In each treatment group, the means and standard deviations of the inflammation index were determined on the basis of the averaged values for each of the four skin strips from the two mice. The standard error of the mean was taken to be the same as the standard deviation. Statistical comparisons of the effects of FA on the inflammatory response to DNFB, i.e., treatment groups receiving DNFB alone or the same regimen of DNFB together with FA, were again based on the mean inflammation index from the four skin strips from the two mice in each treatment group; the standard deviation of the difference between treatment groups was the square root of half the sum of the squares of the deviations within treatment groups; i.e., deviations of the indices from the two mice in each treatment group from the group mean. The standard error was equal to the standard deviation. The t values were the differences between the means of the treatment groups divided by the relevant standard deviation; the two-tailed p-values were based on the values of t for 2 degrees of freedom. Other statistical calculations were done with EXCEL 5.0 (Microsoft Corporation, Redmond, WA).

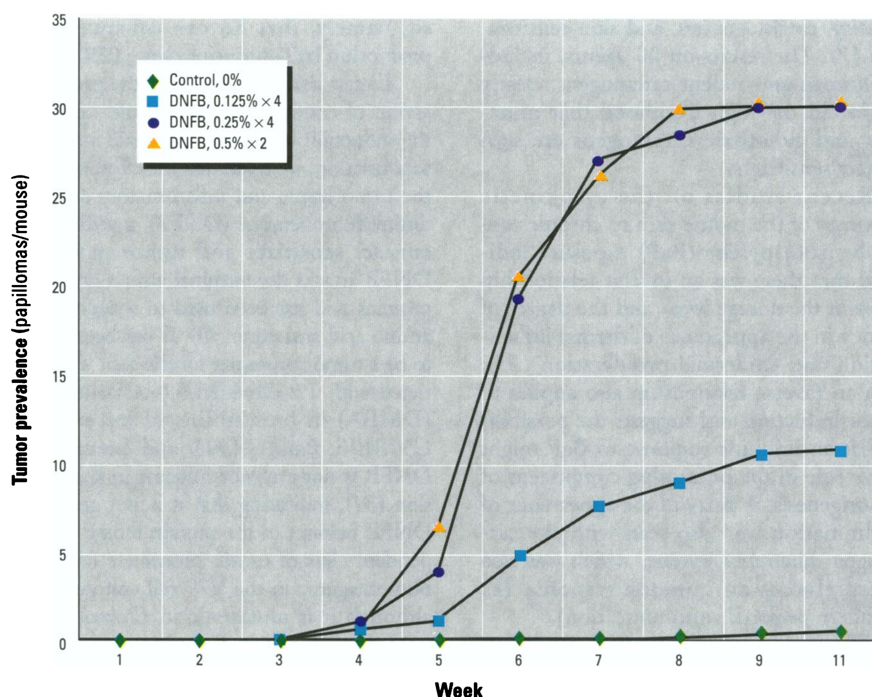

Figure 1. Tumor prevalence (tumors per mouse) at the indicated times after the first exposure to dinitrofluorobenzene (DNFB) shown according to dose.

Results

The gross and histological appearances of the DNFB-induced skin tumors were typical of keratinizing papillomas although one subepidermal keratinizing lesion was found and diagnosed to be a keratoacanthoma. At high doses of DNFB (i.e., 0.25% or 0.5% exposure) where there was extensive skin damage, the tumors forming at the margins of healing ulcers were considerably larger than those on the same mice away from the ulcerated areas or in other mice that were given lower nonulcerogenic doses of DNFB.

Figure 1 shows the temporal pattern of tumor formation in terms of tumor prevalence in relation to the number of weeks after the first exposure. In this experiment the number of starting mice per group ranged from 9 to 15 mice. The acetone controls and DNFB doses of 0.125% and 0.25% were given in four applications. Skin damage at 0.5% DNFB limited the applications to two. Tumor formation began at about 4 weeks and reached a plateau at about 8 weeks. At 8 weeks, the standard error, as a percentage of the tumor prevalence, ranged from 36% at 0.125%, to 9% and 5% at 0.25% and 0.5% DNFB, respectively.

Figure 2 illustrates the rapid rate of tumor formation in terms of the total number of new tumors forming each week in each treated group for several doses of DNFB. The tumor formation rate peaked at about 5.5 weeks, and no new tumors were observed after about 8 weeks. The loss

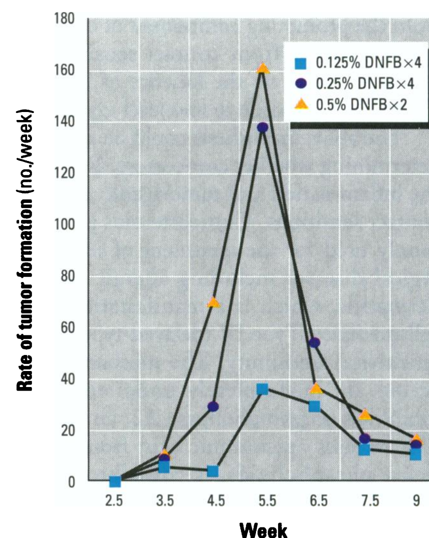

Figure 2. The rate of tumor formation (the number of new tumors forming per week) according to the dose of dinitrofluorobenzene (DNFB) and weeks after initial exposure.

of mice and the regression of tumors was not significant in this brief period of tumor formation so a correction for mortality was not needed. Over a period of months, the tumors shrivelled, darkened, and fell off resulting in a substantial reduction in tumor prevalence.

The dose–response curve for tumor prevalence at 8 weeks after the first exposure for the four-dose regimen is shown in

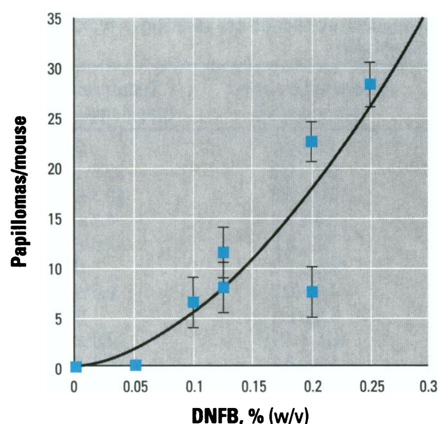

Figure 3. The dose–response curve for tumor yield at 8 weeks after initial exposure to dinitrofluorobenzene (DNFB). A quadratic curve is fitted to the data.

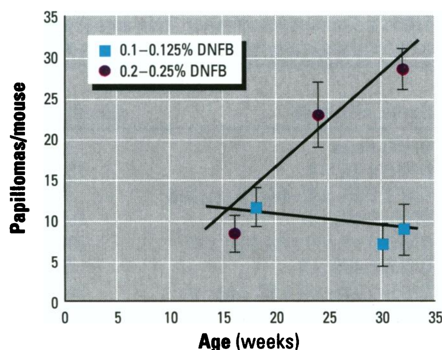

Figure 4. Tumor response to dinitrofluorobenzene (DNFB) at 8 weeks after initial exposure for the indicated ages and dose ranges.

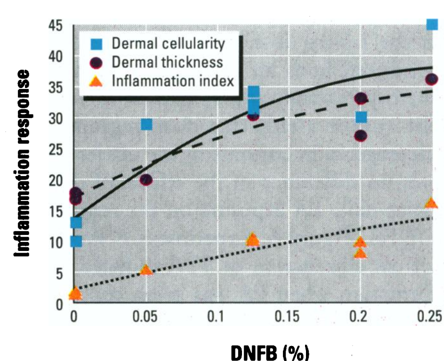

Figure 5. The dinitrofluorobenzene (DNFB) dose–response relationship for inflammation based on dermal cellularity (the number of dermal nuclei/4400 µm²), dermal thickness (the indicated number divided by 100 is the thickness of the dermis in tenths of a millimeter), and the inflammation index (the product of the dermal thickness and cellularity).

Figure 3. The dose–response curve was based on five experiments. There were three control groups with a total of 23 mice. There were seven DNFB dose groups with an average of 7 mice per group (range, 5–10 mice). The dose–response curve with the four dose regimen was relatively steep, with no observed tumors at a concentration of 0.05%. A dose-squared (quadratic) model gave the best fit to the data and took the form 0.3 + 25 (dose) + 286 (dose)^2.

The effect of age at the time of initial dose and the resultant tumor response 8 weeks later is shown in Figure 4 (age range of 16–32 weeks). There was a progressive increase of tumor yield with increasing age at a dose level of 0.2%–0.25% and no effect of age at a dose level of 0.1%–0.125%.

DNFB produced a dose-dependent inflammatory reaction in the dermis manifested by an increase in thickness and cellularity and associated with epidermal hyperplasia. The inflammatory response was predominantly mononuclear except for an occasional focal abscess in hair follicles. A thin scab was frequently seen above the hyperplastic epidermis and, when stained with Feulgen stain for DNA, appeared loaded with fragments of nuclei. Figure 5 shows the monotonic increase in inflammation according to the dose of DNFB in terms of cellularity and dermal thickness. These measurements were made on skin obtained 2–3 days after the fourth dose of DNFB. When the two measures of inflammation were combined as an index of inflammation, the dose response was fairly linear (Fig. 5). At a DNFB dose of 0.05% where there was no observable tumor response, there was a discernable inflammatory reaction.

Experiments were performed to evaluate the effects of FA on the inflammatory and tumorigenic responses to DNFB and TPA. The results are shown in Figure 6. Various dose levels of DNFB and FA were used. DNFB was given in four applications. FA was given with DNFB, about 30 min earlier, or by daily applications with the twice-weekly applications of DNFB. The inflammation index for the experiments are shown in Figure 6A, and the tumor responses are shown in Figure 6B. No tumors were seen when FA was given alone. Concurrent FA doses of 2.5 µg somewhat reduced the inflammation from DNFB at a dose of 0.2% (Fig. 6, Experiment 2), but did not affect the tumor yield. When the doses of both DNFB and FA were lowered to evaluate the possibility that FA might be more effective with smaller and less toxic doses of both agents (Fig. 6, Experiment 3), the inflammatory response to 0.125% DNFB was not affected by concurrent FA doses of 0.5 µg but was significantly reduced by FA at the 1-µg dose level; there were corresponding effects on the tumor yield but not to a level of statistical significance. When a 1-µg dose of FA was given for 3 consecutive days before the first of the four doses of DNFB and, thereafter, FA and DNFB at 0.1% were given concurrently, there were no statistically significant differences in the tumor yields or the inflammatory reactions. The tumor response to 0.2% DNFB was lower in Experiment 2 than in Experiment 4 because of the age effect shown in Figure 4. The tumor response was smaller when the experiment was begun at age 16 weeks (Fig. 6, Experiment 2) than at age 24 weeks (Fig. 6, Experiment 4) at the same 0.2% DNFB dose. The ages at the start of Experiments 2 through 6 are 16, 30, 27, 24, and 24 weeks, respectively. The lack of age standardization was a function of the availability of the TG.AC mice.

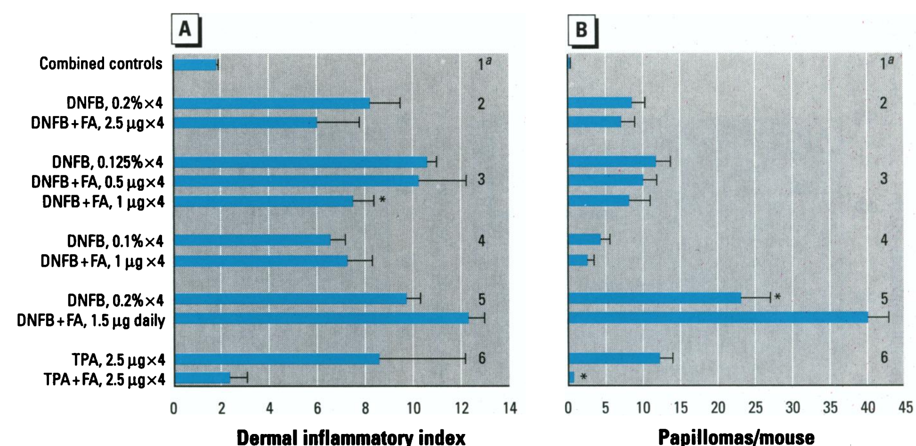

Figure 6. The inflammation index and corresponding tumor yield, at 8 weeks after the initial exposure, for the combined controls and five separate experiments involving exposure to dinitrofluorobenzene (DNFB) alone or the combined exposure to DNFB and fluocinolone acetonide (FA). DNFB was given twice a week for 2 weeks. In the case of the combined DNFB–FA exposures, the dose for DNFB is the same as the exposure to DNFB alone in that experiment. Error bars represent standard errors; asterisks indicate $p < 0.05$.
[a]Experiment number.

Figure 6, Experiment 5 shows that FA at the 1.5-μg dose level, when given daily, produced both an increase in the inflammatory reaction and a marked increase in the tumor yield; this dose regimen was ulcerogenic in contrast to the others. There was no difference in the effects of daily or twice-daily FA exposure on the response to DNFB. Figure 6, Experiment 6 shows the virtual elimination of TPA-induced tumor formation when FA was given concurrently; the inflammatory response to TPA was depressed by FA but not eliminated.

A separate experiment on the FVB mouse, the parent strain of the TG.AC mouse, was done to determine whether the lack of a substantial depressing effect of FA on the inflammatory response to DNFB at dose levels of up to 2.5 μg per application was due to an inadequate dose levels of FA. In this study, individual groups of mice were given FA doses of 0, 4.5, 9, and 18 μg twice weekly for 2 weeks or concurrently with DNFB concentrations of 0%, 0.125%, and 0.25%. The effects on the inflammatory response in the dermis are shown in Figure 7, expressed separately as cellularity and thickness. When DNFB was given without FA, there was a dose-dependent increase in dermal inflammation, both in terms of cellularity and thickness. When FA was given without DNFB, there was little effect on dermal cellularity but the thickness of the dermis progressively diminished with increasing dose. Thinning of the dermis and epidermis and atrophy of the hair follicles reached extremes at the higher two FA doses. When FA and DNFB were given concurrently, there was a depression of inflammation only in association with generalized skin atrophy and in spite of the increasing ulceration caused by the FA.

The induction of contact sensitization by DNFB and TPA and the modification of this sensitization by FA, as measured by the elicitation of ear swelling in TG.AC mice, is shown in Table 1; experiments shown in Figure 6 that did not have ear measurements are omitted. The data across experiments is not strictly comparable because of differences in DNFB concentrations applied to one or two sides of the ear (0.1–0.2%) and the elapsed time since treatment of the dorsal skin; however, the same technique was used within experiments that evaluated the effect of FA on the responses to DNFB and TPA. The irritant effect on the ear swelling by DNFB or TPA, i.e., in the absence of treatment of the dorsal skin, was minor. Contact sensitization was induced by the full range of DNFB dosage applied to the dorsal skin, even at the 0.05% level, which did not induced tumors although it did produce dermal inflammation. The effect of FA on the induction of contact sensitization by DNFB was variable and there was a lack of concordance between the modulation of contact sensitization by FA and the corresponding effects on dermal inflammation and tumor yields. Only TPA showed an FA-induced concordant reduction in contact sensitization, dermal inflammation, and tumor formation. There was a complete lack of correlation between the amount of ear swelling induced by the eliciting doses of DNFB and the tumor yield on individual mice (Fig. 8).

Table 1. Contact sensitization from dorsal skin exposure measured as elicited ear swelling in relationship to the inflammatory and tumor responses induced in the dorsal skin

Dorsal skin dose	Ear swelling[a] (μm)	Inflammation index (dorsal skin)	Tumor response (papillomas/mouse)
DNFB-none	26 ± 5	1.8 ± 0.1	0
DNFB 0.2% × 4	157 ± 40*[b]	9.7 ± 0.3	22.8 ± 4.3
DNFB 0.2% × 4 + FA 2.5 μg daily	70 ± 28	12.3 ± 0.6	40.3 ± 3.0
DNFB 0.1% × 4	180 ± 17*	5.5 ± 0.4	4.3 ± 1.3
DNFB 0.1% × 4 + FA 1 μg × 4[c]	237 ± 21	7.2 ± 0.7	2.6 ± 1.0
DNFB 0.05% × 4	75 ± 11*	5.6 ± 0.2	0
DNFB 0.05% × 4 + FA 2 μg × 4	117 ± 57	4.1 ± 0.3	0.3 ± 0.3
TPA-none	30 ± 6	1.8 ± 0.1	0
TPA 0.003% × 4	87 ± 28[d]	8.5 ± 2.1	12.0 ± 2.2
TPA 003% × 4 + FA 2.5 μg daily	30 ± 6	1.8 ± 0.1	0.3 ± 0.2

[a]Average incremental swelling(treated – nontreated) per ear given as mean ± standard error.
[b]Asterisks refer to treatments that are statistically significant with respect to controls; $p<0.05$.
[c]FA was given for 3 days prior to the four combined exposures.
[d]$p = 0.1$.

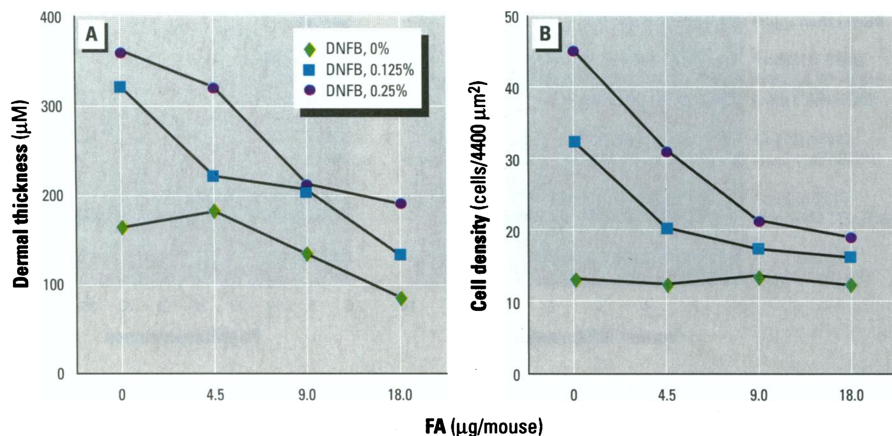

Figure 7. Dermal thickness and cellularity in response to dinitrofluorobenzene (DNFB) and fluocinolone acetonide (FA) in the FVB mouse.

Discussion

This study shows that TG.AC mouse skin responds to DNFB with tumor formation and that the corticosteroid FA, applied twice a week with DNFB, does not appreciably block the inflammatory or tumor response to DNFB as it does with TPA. Both the inflammatory and tumorigenic responses increased with daily exposure to FA, in association with an increase in DNFB-caused skin damage.

Corticosteroids are effective in blocking the elicitation of contact sensitization in the mouse skin (*37*). The failure of FA to substantially reduce the inflammatory response to DNFB in these experiments suggests that, although DNFB is a contact sensitizer, the inflammation at the dose levels administered was due predominantly to cytotoxicity and not an immune-specific response. Whether immunosuppression by FA had any enhancing effect on tumor formation because of impairment of immune surveillance is not known; however, it would not appear to be important because the FA-induced reductions in inflammation that did occur were associated with a decrease in tumor yield. The only instance in which tumorigenesis increased was in association with increased skin damage and an increase in inflammation.

The exacerbation of DNFB-induced skin damage by FA is probably due to the ability of corticosteroids to inhibit cell proliferation and consequently proliferative

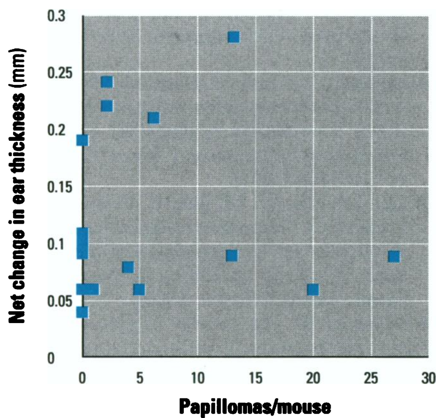

Figure 8. The association between tumor yield and contact sensitization in the TG.AC mouse. Each point represents, for a given mouse, its tumor yield at 8 weeks after the first exposure to dinitrofluorobenzene (DNFB) and the amount of ear swelling at 24 hr after an eliciting dose of DNFB was applied to the ear.

repair (*38*); this effect might be the basis for the observation of Woodworth et al. (*29*) that FA enhanced the promoting action of β-propriolactone in mice initiated by DMBA.

The cytotoxic induction of a tumor response by DNFB may be analogous to physical wounding, which causes tumor promotion in normal (*39*), as well as in TG.AC, mouse skin (J. Spalding, unpublished data). Why physical or chemical wounding causes tumor promotion is not known, but they both involve cell damage and inflammation. Measures that speed the repair of surgical wounding reduce the yield of tumors in the TG.AC mouse (J. Spalding, unpublished data). This may be relevant to the lower tumor response of younger mice at relatively high DNFB doses (Fig. 4) because younger animals tend to heal faster.

DNFB is a tumor promoter for DMBA in four strains of mice other than TG.AC, supporting the idea that the TG.AC mouse behaves as if it were initiated (*34*). The TG.AC model is a curious reversal of the usual initiation–promotion system because, in its conventional form, the rare event is initiation and the generalized effect is promotion. With the TG.AC mouse, initiation is the general event because all of the cells have the transgene; promotion is the uncommon event, which presumably occurs in the stem cells of hair follicles where the tumors arise (*40*).

The question is unanswered as to whether cytotoxicity per se or the inflammation induced by cytotoxicity is responsible for the tumorigenic effect of DNFB. Inflammation, at the tissue level, has two functions: the clean up of cellular debris by protease digestion and the stimulation of proliferative repair by cytokines (*41*). Protease digestion has its intracellular counterpart in terms of removal of adducted proteins (*41*). Cytotoxic inflammatory cells, which function in cell-mediated immunity, kill by injecting proteases into target cells (*41*). Proteases are important in tumor promotion because promotion is blocked by protease inhibitors (*42*). Cytokine stimulation in the injured skin was evidenced by the hyperplastic epidermis in the high-dose DNFB-treated skin and the heightened rate of tumor growth at the margins of healing ulcers. Perhaps the tumorigenic process in the TG.AC mouse skin involves heightened transgene expression in the occasional surviving follicle stem cell that has been injured by protein adducts and is being stimulated by cytokines to proliferate while under protease attack. Interest in the role of protein damage has historical aspects; it was an important concept in the pre-DNA era of carcinogenesis theory (*43*).

The DNFB regimen in these experiments involved relatively acute and high-level exposures; perhaps this favored cytotoxicity as the dominant mode of inflammation. Contact sensitizers, individually, may have two dose responses: one for immunogenic inflammation and the other for cytotoxic inflammation. The relative potencies of cytotoxicity and immunogenicity may differ among different contact sensitizers, and the dose–response curves may not be parallel. Perhaps prolonged low-dose administration of some contact sensitizers, including DNFB, might minimize cytotoxicity and result in predominantly immunogenic inflammation with a relatively long latency period for tumor induction. Contact sensitizers can be tumorigenic in the TG.AC mouse with a longer latency than for DNFB; for example, tripropylene glycol diacrylate, which is a contact sensitizer for both humans and mice, required about 12 weeks of continuous exposure at dose levels that did not induce epidermal hyperplasia to induce skin tumors (*44*).

FA did not block the systemic aspects of contact sensitization because this occurred with all time and dose patterns of DNFB administration. The lack of relevance of the systemic manifestations of contact sensitization to the skin tumor response at the site of DNFB applications was seen in the lack of correlation of the strength of systemic sensitization to the tumor yield in individual mice (Fig. 8).

The dosage of FA required to substantially reduce the DNFB-induced inflammation caused considerable atrophy of the skin and dissolution of the hair follicles; under these circumstances, a suppression of tumorigenesis by FA, even if it did occur, could not be ascribed to reduced inflammation because of the skin atrophy.

In summary, our initial hypothesis that tumor promotion by DNFB might be due to its immunogenically induced inflammation was not supported by the high-dose, short-term experiments reported here. Hence, it is not yet known whether contact sensitizers can be tumor promoters on the basis of their immunogenic properties. However, the induction of cytotoxicity and its inflammatory consequences, presumably from protein adduction by contact sensitizers, may in itself play a role in environmental carcinogenesis.

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