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Tumor Promotion by 1-Fluoro-2,4-dinitrobenzene, a Potent Skin Sensitizer[1]

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SUMMARY

1-Fluoro-2,4-dinitrobenzene (DNFB) is a potent tumor-promoting agent. After topical application with 125 μg of 7,12-dimethylbenz[α]anthracene followed by 5 paintings a week with 0.1% DNFB in acetone, mice developed tumors in as short a time as 4 weeks. After 38 weeks, about two-thirds of the animals had tumors. Treatment with DNFB alone does not produce tumors, nor is the lysine conjugate of DNFB a tumor promoter. These results suggest a relationship between tumor promotion and disturbance of the immune system in sensitive animals.

INTRODUCTION

In recent years, growing attention has been given to the relationship between chemical carcinogens and the immunologic system in experimental animals. Stjernsward (11, 12) reported an immunodepressive effect of polycyclic hydrocarbons which reduced reaction against weak antigenic homografts. Rubin (10) showed that in certain situations intensive treatment of mice with carcinogenic benz[α]anthracene derivatives abolished the rejection of tumors that differ from the host, even at the strongly antigenic H-2 locus. He suggested that this involved "an active immunologic process initiated by carcinogen treatment." Other skin carcinogens with different molecular structures had the same effect (5). Prehn (8) provided a scheme by which positive selection favoring immunologically different malignant cell lines, together with suppression of the immune response of the host, might account for chemical carcinogenesis.

A similarity in the immunologic properties of polycyclic carcinogens, croton oil, and 1-fluoro-2,4-dinitrobenzene (DNFB) was recently reported by Fjelde and Turk (4). It had been shown that topical application of DNFB to the skin of guinea pigs was followed by the appearance of large pyroninophilic cells in draining lymph nodes 4 days later, one day before the animals developed contact sensitivity (13). Fjelde and Turk obtained a similar result when guinea pigs or any of 5 strains of mice were treated with either of the potent carcinogens benzo[α]pyrene and 7,12-dimethylbenz[α]anthracene (DMBA). Croton oil, which is primarily a tumor promoter (3), produced a similar result in the single strain of mice tested. In contrast, the noncarcinogenic hydrocarbon anthracene was inactive in both mice and guinea pigs.

This similarity among the complete carcinogens, the tumor promoter croton oil, and the immunologically active DNFB, together with the observed effects of carcinogens in homograft rejection, led us to determine whether DNFB might act as a carcinogen or tumor promoter in the mouse skin system. In an earlier study, Fjelde *et al.*[2] obtained no tumors when DNFB was applied in croton oil to mice under conditions providing severe immunologic response. DNFB thus did not seem to be an effective tumor initiator. The present report describes the tumor-promoting activity of this interesting compound.

MATERIALS AND METHODS

DMBA and DNFB were obtained from Distillation Products Industries, Rochester, N. Y. N^6-(2,4-Dinitrophenyl)-L-lysine hydrochloride (DNPL) was obtained from Mann Research Laboratories, New York, N. Y. All compounds were used without further purification. The concentration of the DMBA solutions used in the study was checked with a spectrophotometer. Solutions of DNFB and DNPL in acetone were prepared fresh daily.

To test DNFB as a tumor-initiating agent, 60 female ICR Swiss mice were treated at 55 and 62 days of age with 0.25 ml of a 1% solution of DNFB in a 1:1 mixture of acetone and sesame oil. Of the 60 mice, 22 died following the second treatment. After 7 days, the remaining 38 mice were treated 5 times a week for 30 weeks with 0.25 ml of 0.03% croton oil in acetone. A group of 30 mice, treated with the croton oil solution alone, served as negative controls.

To determine whether DNFB was a promoting agent, groups of 30 female ICR Swiss mice were painted at 55 days of age with 125 μg of DMBA in 0.25 ml of acetone. After 21 days, the mice were treated 5 times a week with 0.25 ml of acetone

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[2]A. Fjelde, E. Sorkin, and T. Strauli. Immunological Factors and Tumor Development. I. Studies of Leukocytes from Fluoro-2,4-dinitrobenzene Treated Mice Challenged in Tissue Culture with Dinitrophenyl-lysine. Submitted for publication.

solutions of DNFB ranging in concentration from 0.03% to 3%. One group of 30 mice, treated with DMBA followed by acetone, served as negative controls; another group, treated with DMBA followed by paintings 5 times a week with 0.03% croton oil in acetone, served as positive controls. The experiment was continued for 32 weeks, after which the animals were used for other studies (7).

A second experiment was designed to repeat the first, and to determine whether DNFB might have acted through nonspecific conjugates formed with amino acids, peptides, or proteins. Groups of 50 female ICR Swiss mice were treated with DMBA as before. After 3 weeks, one group was treated with 0.1% DNFB in 0.25 ml of acetone 5 times a week. A second group was treated with 0.5% of DNPL, the lysine conjugate of DNFB, in a 3:1 mixture of acetone and water 5 times a week. The third group consisted of 50 mice that were not treated with DMBA but were treated only with 0.1% DNFB 5 times a week throughout the experiment. Two control groups consisted of mice treated with DMBA followed by either acetone or 0.03% croton oil in acetone as before. A second series consisted of groups of 30 C57BL/6 mice, 15 of each sex, which were treated with DMBA followed by acetone, croton oil, or 0.1% DNFB as in the other studies. A third experiment involved 12 male and 18 female BALB/c mice treated with DMBA followed by acetone, 0.03% croton oil, or 0.1% DNFB.

The mice were examined at weekly intervals, and the distribution of skin tumors was noted. In all, the three experiments covered a time span of 13 months.

The apparent toxic effects of DNFB were more pronounced in the mice treated with DMBA. To determine whether this observation was real, 7 groups of mice were painted with various combinations of DMBA and DNFB (Table 1). The schedule was arranged so that, for each group, the DNFB treatment commenced on the same day and followed the last DMBA treatment by 7 days. To provide objective evidence of toxicity, a 0.2% as well as a 0.1% solution of DNFB was employed.

RESULTS

DNFB proved to be quite toxic under the experimental conditions. Animals painted with either a 3% or a 1% solution of this compound in acetone died after a single application. Of 30 mice, 6 died after 8 applications of 0.3% DNFB in acetone.

Table 1

Group	DMBA (μg)	DNFB[a] (%)	Total no. of mice	No. dead
1	3 X 125[b]	0.1	10	1
2	125[c]	0.1	10	1
3	0	0.1	10	0
4	125[c]	0.2	10	6
5	0	0.2	10	0
6	125[c]	0	8	0
7	0	0	8	0

Potentiation of acute 1-fluoro-2,4-dinitrobenzene (DNFB) toxicity by 7,12-dimethylbenz[α]anthracene (DMBA).

[a] 0.25 ml of acetone solution applied 5 times a week for 4 weeks beginning at 82 days of age.

[b] 0.25 ml of acetone solution applied at 61, 68, and 75 days of age.

[c] 0.25 ml of acetone solution applied at 75 days of age.

The Swiss and C57BL/6 mice treated with either 0.1% or 0.03% DNFB in acetone appeared to tolerate the compound, but the BALB/c mice were more sensitive. The experiment with BALB/c mice was terminated after 14 weeks with only 14 mice surviving out of the original 30. Indications that combined treatment with DMBA plus DNFB was more toxic than DNFB alone were confirmed. The lethal effects of 0.2% DNFB were significantly greater when the mice were first treated with DMBA (Table 1).

DNFB also proved to be a very potent tumor-promoting agent (Table 2). Indeed, the first tumors produced by this agent appeared after 4 weeks of painting, whereas the first tumors produced by croton oil were seen only after 6 weeks. In each of the four experimental series, the first tumors appeared in the DNFB-treated mice. On the other hand, many more tumors were finally produced by croton oil treatment. Of 80 ICR Swiss mice treated with DMBA and 0.1% DNFB for 32 weeks, 51 developed papillomas. In the same time, 59 of 80 mice treated with DMBA and 0.03% croton oil developed tumors. The number of tumors per tumor-bearing mouse, however, was 5.7 with croton oil, but only 1.5 with DNFB. This difference in effects was less marked in the C57BL/6 mice (Table 3).

Although 0.1% DNFB was very toxic to the BALB/c mice, they developed tumors rapidly. Of 20 mice that survived 6 weeks, 5 had tumors by that time. The C57BL/6 mice developed fewer tumors than either the Swiss or the BALB/c mice;

Table 2

Initiating stimulus	Promoting stimulus[a]	No. of mice at risk[b]	Mice with tumors		Total no. of tumors	No. of mice in which tumors regressed
			No.	%		
None	0.03% croton oil	56	1	2	1	1
2 X 2500 μg DNFB	0.03% croton oil	38[d]	1	3	1	1
125 μg DMBA	None	30	0	0	0	0
	0.03% croton oil	30	15	50	79	0
	0.3% DNFB[c]	24	6	25	8	2
	0.1% DNFB	30	21	70	41	1
	0.03% DNFB	21	9	38	11	0

Tumor promotion by 1-fluoro-2,4-dinitrobenzene (DNFB).

[a] 0.25 ml of acetone solution 5 times a week for 32 weeks.

[b] ICR Swiss mice surviving at least 2 weeks of promoting stimulus.

[c] 6 of 30 mice died after only 8 applications of 0.3% DNFB; further treatment was discontinued.

[d] 22 of 60 mice died after the second DNFB treatment.

Table 3

Strain of mouse	Promoting stimulus[a]	Duration of promotion (weeks)	No. of mice at risk	Mice with tumors		Total no. of tumors	Tumors per tumor-bearing mouse
				No.	%		
Swiss	0.1% DNFB[b]	50	50	35	70	55	1.6
C57BL/6		50	30	6	20	8	1.3
BALB/c		14	30	5	17	7	1.4
Swiss	Acetone only	50	50	2	4	2	1.0
C57BL/6		50	30	0	0	0	
BALB/c		14	30	0	0	0	
Swiss	0.03% croton oil	50	50	49	98	346	7.1
C57BL/6		50	30	20	67	35	1.8
BALB/c		14	30	5	17	8	1.6
Swiss	0.5% dinitrophenyllysine hydrochloride	34	50	0	0	0	

Tumor promotion by 1-fluoro-2,4-dinitrobenzene (DNFB) in various stocks of mice.

[a] 0.25 ml of acetone solutions, applied 5 times a week beginning 21 days after a single application of 125 μg of DMBA in 0.25 ml of acetone.

[b] 50 Swiss mice treated with 0.1% DNFB, but not with DMBA, developed no tumors in 38 weeks.

the first tumors appeared in the C57BL/6 mice after 10 weeks. The C57BL/6 mice were also less sensitive to croton oil.

The effect of DNFB showed a clear dose-response pattern. After only 8 applications, 0.3% DNFB produced tumors in 6 of 24 surviving animals. Although the final number of tumors produced by 0.03% DNFB was more than a third of that produced by 0.1% DNFB, most of the tumors produced by the dilute solution appeared very late in the experiment. After 26 weeks, only two tumors appeared in 2 mice painted with the dilute 0.03% solution, whereas 31 tumors appeared in 21 mice painted with 0.1% solution for 26 weeks.

A single tumor appeared in mice treated 2 times with 1% DNFB followed by croton oil for 32 weeks. A tumor also appeared in the croton oil controls. Thus, in this experiment, DNFB was not a tumor initiator. Furthermore, treatment with DNFB alone produced no tumors.

The lysine conjugate of DNFB produced no tumors in DMBA-treated mice even when applied at 0.5% (14 mM) concentration. In contrast, 0.03% DNFB (1.6 mM) produced tumors in 38% of DMBA-treated mice.

DISCUSSION

DNFB acted as a tumor promoter in the three stocks of mice that were studied. The compound did not demonstrate tumor-initiating activity when mice were painted twice with this material followed by repetitive application of croton oil. Likewise, treatment with DNFB alone did not produce tumors, as would be expected with a potent tumor promoter that also exhibited initiating activity. Failure to find initiating activity is in agreement with the earlier observation by Fjelde *et al.*[2] that DNFB dissolved in croton oil did not produce tumors in Rudiger mice.

None of the tumors promoted by DNFB grew to a large size during the experimental period. In this respect, the agent appears much more like croton oil than like the carcinogenic polycyclic hydrocarbons, which, if applied in doses producing tumors in 4 weeks, would produce a large number of malignant tumors well within a 30-week period. On the other hand, a comparison of the total numbers of tumors that developed shows a striking difference between croton oil and DNFB treatment. Although tumors appeared earlier with DNFB, many more tumors finally appeared after croton oil treatment.

To some extent, DNFB resembles anthralin, which is a very potent tumor promoter (2, 3). In an earlier study, 50% of 30 Swiss mice treated with 125 μg of DMBA followed by 0.033% anthralin in acetone developed tumors in 32 weeks (1). In the present study, 38% of 21 mice treated with 0.03% DNFB developed tumors in the same period. Aside from the phorbol esters, the active agents of croton oil (6, 14), DNFB is one of the most potent tumor-promoting agents available.

DNFB may owe its tumor-promoting activity to direct chem-

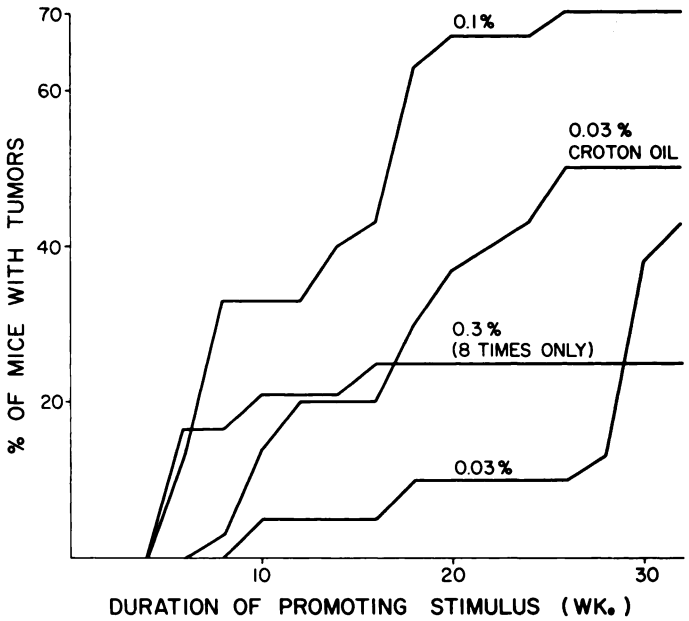

Chart 1. Dose-time response of tumor induction by 1-fluoro-2,4-dinitrobenzene (DNFB). Note in Table 2 that more tumors were induced by 0.03% croton oil than by 0.1% DNFB in spite of the fact that DNFB produced tumors earlier and in more mice than did croton oil. Mice bearing multiple tumors were rare in groups painted throughout the study with 0.03% DNFB or with 0.3% DNFB for eight applications only.

ical reaction with a specific active site. Conversely, its activity may be due to formation of a nonspecific conjugate with amino acids, peptides, or proteins, the conjugate being more directly involved in the expression of activity. The lysine conjugate of DNFB, N^6-(2,4-dinitrophenyl)-L-lysine, is immunologically active in some systems (4). In the present experiments, the lysine conjugate was inactive. This inactivity, however, does not preclude consideration of such conjugates as proximal promoting agents. The conjugate has substantially different solubility properties and might be inactive after skin application because of nonpenetration into the target areas. Lipid solubility is of great importance in skin penetration (1).

In these experiments, the DNFB was applied to the same site as the DMBA, and the tumors arose in the treated area. It might be considered whether the DNFB could exhibit systemic effects and promote tumors in initiated cells remote from the site of DNFB application. This would be the case if the progression from initiated cell to gross tumor depended on generalized sensitization to DNFB. Studies of this possibility are currently underway.

DNFB and 1-chloro-2,4-dinitrobenzene are well-known causes of allergic dermatitis. Many skin carcinogens of diverse structure alter the immune mechanisms of mice (4, 9–11; Footnote 2). Presumably they serve as both initiators and promoters. For several polycyclic hydrocarbons, initiating action can be distinguished from promoting action (8, 15). It is interesting to speculate that alterations in the immune system are a necessary feature of tumor-promoting activity. Two active tumor promoters, croton oil and DNFB, do mobilize pyroninophilic cells, a process which is immunologically specific in the latter case at least.

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