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Inhibitory Effect of Toluene on Tumor Promotion in Mouse Skin (42240)

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Abstract. In the two-stage mouse model for skin tumorigenesis with phorbol-12-myristate-13-acetate (PMA) as promoter, topical application of 40 μl of toluene 2×/week at the initiation/promotion site (the back) reduced the average number of tumors/mouse (ANT/M) to approximately one-fourth that of controls. Control procedure involved initiation of C3H mice with benzo[*a*]pyrene (BaP) and CD-1 mice with 7,12-dimethylbenz[*a*]anthracene (DMBA) followed by promotion with from 1 to 5 μg PMA in 40 μl acetone 2×/week. Forty microliters of toluene 2×/week per se was a weak promoter (6–13% of control ANT/M), and produced mild skin irritation at the application site but behavior and body weights were normal. The toluene inhibition of tumorigenesis was not a direct chemical action on PMA since similar effects occurred whether toluene was the vehicle for PMA or whether it was applied up to 1 day before PMA (i.e., prepromotion). Prepromotion with acetone had no effect on tumorigenesis, substantiating its use as control vehicle and suggesting that the toluene inhibition was a specific tissue reaction. The inhibitory effect appeared to be on PMA promotion rather than on initiation since toluene and acetone produced similar numbers of tumors when used as the vehicle for BaP or DMBA in two-stage or BaP in single-stage trials. The inhibition was not permanent since tumorigenesis returned to control rates 2–3 weeks after prepromotion with toluene ceased but promotion with PMA in acetone continued. Toluene may be unique among reported promotion inhibitors in that it is a widely used commercial chemical which sometimes serves as a vehicle in cancer-screening trials. Since its metabolism is reasonably well defined, it may be of value in exploring further the process of tumor promotion.

In continuation of single-stage mouse skin tumor trials in which the carcinogen was dissolved in toluene (1, 2), we proceeded to use toluene as the vehicle for both initiator and promoter in two-stage experiments. With toluene as the solvent for the potent promoter, phorbol-12-myristate-13-acetate (PMA), tumorigenesis was considerably less than expected on the basis of data in the literature in which acetone was the usual vehicle (3). This led us to consider further the possibility that toluene was inhibiting PMA promotion.

Although some skin tumor enhancing activity has been attributed to toluene (4), we knew of no reports on its possible role as an inhibitor. The subject seemed worthy of pursuit for several reasons. First, the multistage model is apparently of increasing importance among theories of chemical carcinogenesis (see, e.g., 3, 5, 6). Second, although many inhibitors of tumor promotion have been reported, toluene does not appear on the list (3). Third, although acetone is the usual vehicle for PMA, toluene is sometimes used in this capacity (7, 8) and could lead to erroneous results in cancer-screening trials. Fourth, toluene is a widely used commercial solvent (4, 9) and thus could be an environmental factor affecting tumorigenesis. Last, since the metabolic fate of toluene is well defined (4, 9), its possible role as an inhibitor might be useful in further deciphering the still evolving understanding of the mechanism of tumor promotion (3, 5, 6).

Materials and Methods. The two-stage skin tumorigenesis procedure was as follows: Mice (C3H, Jackson Labs or CD-1, Charles River) were obtained at 5–7 weeks of age and housed three to four each in shoe-box plastic cages on ground corn cob litter with food (Purina Lab Chow) and water *ad libitum*. Initiation gen-

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erally followed within 2–5 days on the clipped back with either benzo[*a*]pyrene (BaP, Aldrich) or 7,12-dimethylbenz[*a*]anthracene (DMBA, Sigma), carried in 40 to 80 μl of either acetone or toluene. In some trials, however, 2 weeks of acclimation to cool (10–13°C), normal (23°C), or warm (29–31°C) temperature preceded initiation (1, 2).

Promotion with PMA (Chemicals for Cancer Research Inc.), dissolved in either acetone (considered the control) or toluene, began at the initiation site 2 days to 2 weeks following initiation. The PMA dose varied from 1 to 5 μg in 40 μl of solvent 2×/week. In some trials, additional solvent was applied to the initiation/promotion site either 1–2 min, 1 hr, or 1 day before promotion (referred to as prepromotion). Controls for these mice were promoted with PMA in acetone without prepromotion. In single-stage trials, BaP in 40 μl of either acetone or toluene, was applied repetitively 2×/week to the clipped back. All applications were accomplished by micropipet in multiples of 20 μl.

Tumors were counted once a week and evaluated in terms of both incidence (% mice with tumors) and average number tumors/mouse (ANT/M ± standard eror; SE). Body weights were measured approximately every 4 weeks. Toluene effects are reported primarily in terms of ANT/M at specific times after the start of a trial, but in some cases comparisons are based on slopes of the lines fitted by linear regression to the ANT/M data (10).

In any one trial, the initial size of the solvent treatment groups varied from 10 to 25 mice. Multiple trials were used to bring the size of the solvent groups into the range of 30–70 mice. Two-way analysis of variance (ANOVA) was used to separate solvent effects from trial effects in these multiple trials (10).

Results. Fig. 1 is an example of the marked difference in tumorigenesis resulting from the use of toluene vs acetone as the vehicle for PMA in two-stage trials. Both incidence (A) and ANT/M (B) were clearly reduced by toluene compared to acetone over the full course of the trial, although latency appeared little affected. Linear regression indicates that straight lines fit the ANT/M data well, with correlations of 0.9 or better (see legend to Fig. 1). Comparison of the slopes of the regression lines suggests that the rate of tumorigenesis

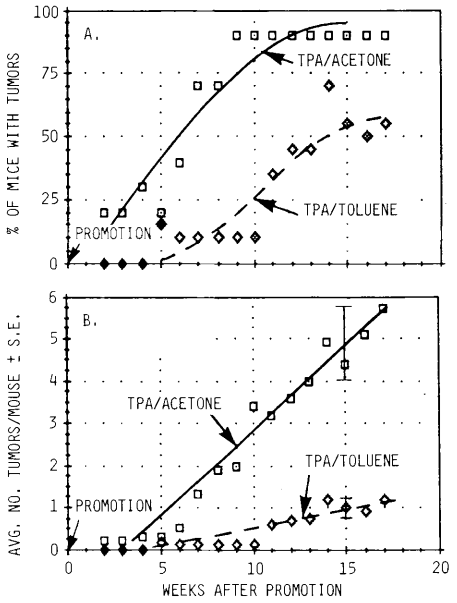

FIG. 1. Example of toluene vs acetone as vehicle for the promoter, PMA, in a two-stage carcinogenesis trial with CD-1 male mice living in a 13°C environment. Initiation: 25 μg DMBA in 80 μl toluene; promotion: 1 μg TPA in 40 μl vehicle 2×/week. Incidence curves (A) fitted by eye. Average number tumors/mouse/week curves (B) fitted by linear regression: toluene: (◇) $Y = 0.1X - 0.4$ ($n = 16$, $S_b = \pm 0.01$, $r = 0.91$); (□) acetone control: $Y = 0.4X - 1.3$ ($n = 16$, $S_b = \pm 0.02$, $r = 0.98$).

with toluene is about one-fourth that with acetone.

Table I presents a summation of the eight solvent comparisons which comprise the bulk of this paper. Columns 1, 2, and 3 describe the treatment used in each comparison category, generally in terms of initiation, promotion and prepromotion. The major comparison categories are in rows labeled A through E with subcategories within them labeled 1 or 2. References in the text to pertinent portions of the table are by row designations for each category and subcategory. The result of each comparison between solvents shown in Table I is based on a 2 × 3 ANOVA (two solvent treatments replicated three times) in order to limit extraneous effects likely to follow from simple averaging of multiple trials carried out at different times or conditions. However, only the solvent comparisons are shown in the table in terms of numbers, means, pooled standard error of mean, and significance level (columns

TABLE I. SOLVENT EFFECTS ON AVERAGE NUMBER OF TUMORS/MOUSE (ANT/M) BASED ON TWO-WAY ANALYSIS OF VARIANCE OF THREE TRIALS PER CATEGORY

Initiation (1)	Promotion (PMA, 2×/week) (2)	Prepromotion (3)	No. mice/treatment (mouse strain) (4)	Av. No. tumors/mouse[a] Control (5)	Av. No. tumors/mouse[a] Solvent (6)	Solvent as % of control (7)
A. Toluene (solvent) vs acetone (control) as vehicle for promoter (Week 15)						
(1) 1 mg BaP in 40 μl toluene	2 μg in 40 μl (toluene or acetone)	None	53 (C_3H)	2.2 (±0.23)	0.6*	28
(2) 25 μg DMBA in 80 μl toluene	1 μg in 40 μl (toluene or acetone)	None	45 (CD-1)	2.4 (±0.34)	0.8*	33
B. Toluene prepromotion (solvent) vs no prepromotion (control) (Week 10)						
(1) 25–100 μg DMBA in 80 μl acetone	1–5 μg in 40 μl acetone	40 μl toluene 1–2 hr before PMA	32 (CD-1)	4.0 (±0.48)	0.7*	17
(2) 100 μg DMBA in 80 μl acetone	5 μg in 40 μl acetone	20–80 μl toluene 1 hr or 1 day before PMA	28 (CD-1)	3.7 (±0.45)	0.9*	25
C. Toluene (40 μl 2 ×/week) as promoter per se (solvent) vs PMA (control) (Week 15)						
(1) 1 mg BaP in 40 μl toluene	*Control:* 2 μg in 40 μl acetone	None	54 (C_3H)	2.2 (±0.13)	0.3*	13
(2) 25 μg DMBA in 80 μl toluene	*Control:* 2 μg in 40 μl toluene	None	30 (CD-1)	2.4 (±0.23)	0.1*	6
D. Toluene (solvent) vs acetone (control) as vehicle, single stage (Week 20)						
125 μg BaP in 40 μl toluene or acetone 2×/week			72 (C_3H)	1.3 (±0.18)	1.9	144
E. Acetone prepromotion (solvent) vs no prepromotion (control) (Week 10)						
100 μg DMBA in 80 μl acetone	5 μg in 40 μl acetone	40 μl acetone, 1 min, 1 hr, or 1 day before PMA	28 (CD-1)	5.2 (±0.65)	4.9	96

* P value of difference from control ≤ 0.005.

Note. Time of the analysis is weeks after start of promotion in the two-stage carcinogenesis trials on male mice.

[a] Mean ± pooled SEM.

4, 5, and 6). Finally in the last column of Table I the results are reduced to the solvent effect as a percentage of control.

The trial shown in Fig. 1, which was carried out in cool acclimated CD-1 mice, is one of the three trials analyzed by two-way ANOVA in Table IA2. Inhibition with toluene also occurred in the other two trials, one on mice living in normal room temperature (23°C) and one in warm temperature (31°C). The average effect was a highly significant reduction in ANT/M at Week 15 after the start of promotion to 33% of the acetone control, as shown in column 7, Table IA2. In these as well as all other trials in this study the inhibition of incidence by toluene was qualitatively the same as for ANT/M.

Similar highly significant inhibition of tumorigenesis with toluene as the vehicle for PMA was seen with the inbred C3H mouse as with the outbred CD-1 mouse. As shown in Table IA1, the average ANT/M for three trials

with toluene was reduced to a highly significant 28% of the acetone controls. These three trials also involved mice living at cool, normal, and warm temperature, all of which responded equivalently to the toluene effect. It may be noted also that the toluene inhibition occurred with different initiators, BaP for the C3H mice, DMBA for the CD-1 mice.

To test whether the toluene effect depended on it serving as the solvent for PMA, toluene was applied at the initiation/promotion site before promotion with PMA in acetone (i.e., prepromotion treatment). In the first series of these trials shown in Table IB1 the toluene dose was 40 μl 2×/week, the same as when it served as the vehicle for PMA (Table IA). The interval between prepromotion and promotion was 1–2 min. The inhibition of ANT/M, to a highly significant 17% of controls (not prepromoted) was as great if not greater than when toluene was the vehicle for PMA (Table IA). It may be noted too that the solvent for the initiator here was acetone compared to toluene in the vehicle substitution trials (Table IA). Also, the three trials in this comparison were all at normal room temperature but run at different times. (Note: the first 10 weeks of data shown in Fig. 2 is from one of the trials in this series.)

A second series of trials involving prepromotion with toluene included increases in the interval before PMA from 1 to 2 min to 1 hr (two trials) and 1 day (one trial), but also variations in toluene dose down to 40 μl 1×/week (one trial) and up to 80 μl 2×/week (1 trial) (Table IB2). Inhibition of tumorigenesis was observed in all trials with an average reduction in ANT/M to a highly significant 25% of controls. That these particular changes in time and dose of toluene had little effect on the pattern of inhibition tended to be confirmed by a nonsignificant interaction term in the ANOVA. It was noted, however, that the 80 μl toluene 2×/week produced signs of hyperactivity and depressed body weight by 10–15% at the 10th week, although survival was normal. These were the only adverse effects on behavior or body weight seen in any of the treatments used in this study.

The four sets of three trials each summarized in Table IA and B, involving application of toluene to the back of the mouse either as the vehicle for PMA or preceding PMA, presented a consistent picture of inhibition of tumorigenesis. The overall average for ANT/M for these four sets was (4) 26 ± 3.3% of controls.

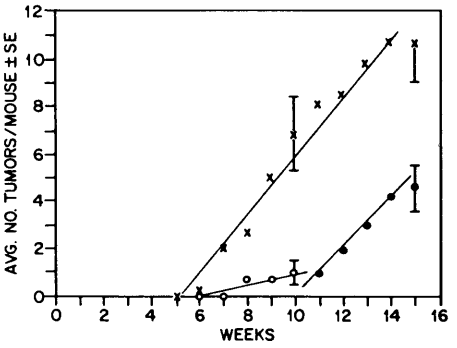

FIG. 2. Effect of prepromotion with toluene and its discontinuance on skin tumorigenesis in CD-1 male mice living at normal room temperature (23°C). Average number tumors/mouse evaluated by linear regression. All mice initiated on the clipped back with 100 μg DMBA in 80 μl acetone at Week 0 and promoted starting 1 week later with 5 μg PMA in 40 μl acetone 2×/week. (○) Toluene: prepromoted at the initiation site with 40 μl toluene 2×/week 1–2 min before promotion; $Y = 0.2X - 1.2$ ($n = 5$, $S_b = \pm 0.05$, $r = 0.92$). (●) toluene discontinuance: prepromotion with toluene stopped at Week 10; $Y = 1.0X - 9.7$ ($n = 5$, $S_b = \pm 0.08$, $r = 0.99$). (×) Control: no prepromotion; $Y = 1.2X = 6.3$ ($n = 11$, $S_b = \pm 0.07$, $r = 0.98$).

The possible role of toluene as a promoter per se was tested in two sets of three trials each, Table IC. In C3H mice with BaP as initiator and in CD-1 mice and with DMBA as initiator, promotion with 40 μl toluene 2×/week alone without PMA, produced some tumors with ANT/M in the range of 6–13% of control. Within the experimental periods used in this study, toluene alone as a complete carcinogen (i.e., initiator and promoter) at 40 μl 2×/week caused no tumors, nor did the various doses of either BaP or DMBA alone, without promotion. However, all the toluene treatments caused mild skin irritation at the site of treatment.

Toluene was also compared to acetone as the vehicle for BaP in single-stage carcinogenesis of mouse skin (Table ID). The trend was for more rather than fewer tumors with toluene but the average difference was not significant.

The specificity of inhibition by toluene was tested by using acetone as a prepromoter. In

three trials (Table IE), 40 μl of acetone was applied 2×/week either 1–2 min, 1 hr, or 1 day before PMA in acetone. There was no statistically significant effect on ANT/M (avg = 96% of controls). Furthermore there was no interaction between trial and solvent in the two-way ANOVA and therefore no reason to suspect any difference in response due to time of application of acetone.

Permanency of the toluene inhibition was examined in two trials. In one (Fig. 2) prepromotion with toluene was stopped after 10 weeks but standard promotion with PMA in acetone continued. Between Weeks 5 and 10, the typical depression of tumorigenesis by toluene was seen compared to controls without prepromotion treatment. However, 2–3 weeks after the end of toluene prepromotion, rate of tumorigenesis in this group rose markedly and approached that of controls. If one uses the slopes of the linear regression lines to compare treatments, for toluene prepromotion in the period between Weeks 5 and 10, the slope is 17% of controls whereas for Weeks 11 to 15, after stopping toluene, it is 83% of controls. (The reliability of the linear regression analysis in this type of comparison is supported by the small SE of the regression coefficients (S_b) and the high correlation coefficients (r) for the straight lines, as shown in the legend to Fig. 2.) Recovery from the depressed tumorigenesis due to toluene also occurred in a second trial with CD-1 mice (not shown). Inhibition to one-third of acetone controls had been produced initially by 17 weeks of promotion with PMA in toluene, but by 10 weeks after switching to PMA in acetone, the rate of tumorigenesis had returned to that of controls promoted throughout only with PMA in acetone.

Discussion. These data show that topical treatment with the common solvent toluene can markedly inhibit skin tumorigenesis in the two-stage mouse model in which promotion is accomplished with the potent phorbol ester, PMA. The depression in tumorigenesis was observed in C3H mice initiated with BaP and CD-1 mice initiated with DMBA and with PMA used in doses from 1 μg to the optimal range of 5 μg 2×/week (3). Inhibition of tumorigenesis followed whether toluene was used simultaneously with or preceding PMA by up to 1 day (i.e., as prepromoter), and is thus not likely due to a direct chemical effect on the promoter. For controls, promotion was accomplished with PMA dissolved in acetone without any prepromotion.

When toluene (40 μl 2×/week) was substituted for acetone as the vehicle for PMA (six trials, Table IA) or toluene (40 μl 1×/week to 80 μl 2×/week) was applied 1 min or 1 day preceding the PMA (six trials, Table IB), ANT/M was depressed significantly to approximately one-fourth of the acetone controls ((4) 26 ± 3.3% of control); the depression in incidence was qualitatively similar. Some part of this residual tumorigenesis can probably be attributed to the intrinsic promotional activity of toluene per se (Table IC), as has been reported (4). Toluene produced mild skin irritation at the application site, probably related to epithelial hyperplasia (1). However, within the experimental time periods of up to 22 weeks, survival, behavior, and body weight were normal, except for one trial at 80 μl of toluene 2×/week where the mice appeared hyperactive and a 10–15% weight depression was observed.

In using PMA in acetone as the control for toluene treatment, it had been tacitly assumed that acetone per se is without effect on tumorigenesis. Evidence in support of acetone being neutral in two-stage carcinogenesis is indicated by the data in Table IE, where in three trials, prepromotion application had no significant effect on tumorigenesis. Absence of an effect with acetone prepromotion suggests that the inhibition following toluene treatment is most likely due to specific tissue reactivity rather than a generalized response to a lipid solvent on the skin. It remains to be determined, however, whether the toluene inhibition involves circulating metabolites. Although toluene applied topically is reportedly to be poorly absorbed (4), it can at the doses used here produce systemic effects such as changes in oxygen uptake of liver homogenates (11).

The toluene effect shows some persistence since similar inhibition was seen whether toluene was applied simultaneously with or preceded PMA by 1–2 min, 1 hr, or 1 day (Table IA vs IB). Persistence of the inhibitory effect of toluene after its application is also suggested by the two trials in which several weeks elapsed before tumorigenesis returned to acetone control rates when prepromotion with toluene ceased (see, e.g., Fig. 2). The fact that tumor-

igenesis returned to control levels after discontinuance of toluene at the initiation/promotion site shows that the initiated cell was not permanently altered but remained capable of responding normally, although after some delay, to the full promotional action of PMA.

The similar tumorigenesis whether toluene or acetone was used in conjunction with either BaP or DMBA in two-stage trials indicates that these solvents have little differential effect on the initiation process (Table IA and B). Further, the nonsignificant changes in ANT/M in single-stage trials (Table ID) indicate that toluene does not inhibit the function of BaP as a complete carcinogen. Since PMA was the only promoter used in these trials, the inhibitory effect of toluene may be limited to PMA and need not extend to promotion in general.

Toluene may be unique among reported promotion inhibitors (3, 5, 6) in that it is a widely used commercial chemical (4, 9) and therefore could play some role in the balance among environmental factors influencing tumorigenesis (6). Although acetone is the usual choice of solvent for PMA, toluene is sometimes used as a vehicle in cancer-screening trials (7, 8) and might thus lead to erroneous data on carcinogenicity of test materials. Also the fact that the metabolic pathways and products of toluene in the body are reasonably well defined (4, 9) may be of value in exploring further the generally obscure process of tumor promotion. Among the possibilities for consideration is whether the toluene inhibition relates to competition by a weak promoter for a PMA receptor on the cell surface; or, since toluene is likely very soluble in cell membranes, whether it interferes at some step within the membrane or in the intracellular cascade between the membrane and the nucleus.

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