

A Comparison of the Initiating and Promoting Actions of 9,10-Dimethyl-1,2-benzanthracene and 1,2,5,6-Dibenzanthracene in Skin Tumorigenesis*

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SUMMARY

The skin of Swiss albino mice was exposed once to 0.16 μg . of 9,10-dimethyl-1,2-benzanthracene (DMBA) or 1,2,5,6-dibenzanthracene (DBA), followed by continuous treatment with croton oil. Other mice were exposed to 0.02 μg . of the same carcinogens. Control mice were treated once with acetone followed by continuous treatment with croton oil.

A higher incidence of skin tumors was observed for all the carcinogen-treated groups as compared with the controls. However, the increase was statistically significant only for the mice exposed to 0.16 μg . DBA. A significantly greater number of skin tumors per mouse was obtained among the mice treated with 0.16 μg . DMBA ($P < 0.05$), 0.16 μg . DBA ($P < 0.005$), and 0.02 μg . DBA ($P < 0.05$), as compared with the acetone-treated controls. It is apparent that the carcinogen DBA contributes a greater initiating stimulus in skin tumorigenesis than DMBA at the low dosages employed.

Strain DBA mice were exposed once on the skin to an initiating dose of DMBA followed 1 month later by monthly applications of 0.15 per cent DMBA or 0.3 per cent DBA. Fifty per cent of the mice given monthly treatments of DMBA developed visible skin tumors after a latent period of 226 days. On the other hand, a latent period of 339 days was observed in the case of the mice treated with DBA, despite the higher concentration employed. This indicates that DMBA is more potent than DBA in the promotion of skin tumors.

In investigating the relative carcinogenic potency of 20-methylcholanthrene (MCA), 3,4-benzpyrene (BP), and 1,2,5,6-dibenzanthracene (DBA) following subcutaneous injection into mice, Bryan and Shimkin (3) observed that these carcinogens ranked in the order given when potency was evaluated on the basis of average latent period. If, however, minimum dose response was used as a basis for evaluation, the relative position of the carcinogens was changed to DBA, MCA, and BP. In more recent investigations in which these and related carcinogens, as well as croton oil, were applied to the skin of mice, Berenblum and Shubik (1, 2) suggested that skin tumorigenesis

consisted of two sequential stages: (a) a conversion of some of the cells in the treated skin to "latent tumor cells" (initiation) and (b) the development of these "initiated" cells to the visible tumor stage (promotion). It appeared to these investigators that minimum dose response and average latent period were reliable indicators of initiating and promoting action, respectively. Also, if carcinogens were active both as initiators and as promoters, this introduced the distinct possibility that a carcinogen might be potent as an initiator and, at the same time, be a weak promoter. Berenblum and Shubik (2) concluded that, if the carcinogen DBA was such an agent (i.e., a potent initiator and a weak promoter), this would help explain the anomalous finding of Bryan and Shimkin (3) referred to above. This possibility was investigated in the present study by comparing the

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initiating and promoting activities of DBA with those of 9,10-dimethyl-1,2-benzanthracene (DMBA), a related carcinogenic hydrocarbon noted for its high potency both as an initiator and as a promoter in skin tumorigenesis.

MATERIALS AND METHODS

Two- to 3-month-old Swiss albino and strain DBA/2 Jax male mice were employed. Mice were housed in metal cages in air-conditioned quarters, and were provided with a continuous supply of Purina Laboratory Chow pellets and tap water. Prior to the time of treatment, the nape of the neck, or the entire back, was clipped free of hair, and different solutions were applied as evenly as possible to the denuded area with the aid of a 1-ml. tuberculin syringe. When solutions containing minute amounts of carcinogen were required, these were prepared by serial dilution, and concentrations were checked with a Beckmann Model DU spectrophotometer. Precautions were taken to minimize changes due to evaporation or light by storing all solutions in rubber-stoppered, amber-colored serum bottles.

The mice were examined once weekly and all grossly visible tumors recorded. However, only those tumors which persisted for a minimum of 1 month and which measured 1 mm. or more in diameter were included in the data. Latent period of tumorigenesis was defined as the time required for 50 per cent of the mice in any group to develop one or more visible skin tumors. Tumors were excised at autopsy, fixed in Tellyesniczky's fluid (20 parts 70 per cent ethyl alcohol, 2 parts formalin, and 1 part glacial acetic), prepared for routine sectioning, and stained with hematoxylin and eosin. Tumor diagnoses were confirmed by microscopic examination.

The investigation consisted of two separate experiments, run concurrently as follows:

Experiment 1.—Five groups of Swiss albino mice were treated once over the entire back with 0.2 ml. of a 0.00008 per cent or 0.00001 per cent solution of DMBA¹ or DBA¹ in acetone (Table 1, Groups 1-4), or with acetone alone (Group 5). Beginning 2 weeks thereafter, the backs of all mice were treated once weekly, a total of 25 times, with a 1 per cent solution of croton oil² in acetone. The mice were sacrificed 4-6 weeks following the end of treatment, for a total observation period of 210-224 days.

Experiment 2.—Four groups of strain DBA/2

¹ Obtained from Eastman Kodak Company, Rochester, N.Y.

² Obtained from Fisher Scientific Company, New York, N.Y.

Jax mice were exposed once on the nape of the neck to 0.05 ml. of a 0.15 per cent solution of DMBA in acetone. Beginning 1 month thereafter, the same site was exposed once monthly in the first three groups (Table 2) to 0.05 ml. of (a) a 0.15 per cent solution of DMBA in acetone, for a total of eight applications (Group 1), (b) a 0.3 per cent solution of DBA in acetone, for a total of eleven applications (Group 2), or (c) 100 per cent acetone for a total of eleven applications (Group 3). In Group 4, the skin was exposed twice weekly to 0.05 ml. of a 1 per cent solution of croton oil in acetone for a total of 50 applications. Experiment 2 was terminated 2 months following the last treatment in Groups 1-3 and 2 weeks following treatment in Group 4. The total observation period was 300 days for Group 1, 390 days for Groups 2 and 3, and 219 days for Group 4.

RESULTS AND DISCUSSION

Experiment 1 (Table 1).—For evaluating the statistical significance of the observed differences in tumor incidence and in average number of tumors per mouse, the χ^2 and Student's "t" test, respectively, were employed.

A skin tumor incidence of 26 per cent and an average of 0.5 tumors per mouse were observed among the Swiss albino mice which had been exposed once to 0.16 μ g. DMBA followed by continuous treatment with croton oil (Group 1). In the case of the control mice in Group 5 which had been treated with acetone and croton oil only, 13 per cent of the mice bore skin tumors, with an average of 0.1 tumors per mouse. These results correspond with those obtained in an earlier experiment in which Swiss albino mice were similarly exposed to 0.16 μ g. of DMBA (8). The increased tumor incidence in Group 1 over that in Group 5 did not prove to be significant statistically at the 0.05 level. However, analysis of the data on average number of tumors per mouse showed a significant increase in Group 1 over Group 5 ($P < 0.05$). Thus, the initiating influence of a 0.16- μ g. dose of DMBA appears to be equivocal. In a recent publication, Hadler *et al.* (4) observed that a 0.1- μ g. dose of DMBA applied once to the skin of Swiss albino mice elicited a marginal initiating response in skin tumorigenesis. It is possible that a more clear-cut initiating effect would have been obtained for DMBA at this low dose in the present study with a larger group of mice or a longer observation period. In Group 3, a still lower dose of DMBA (0.02 μ g.) was tested for initiating action. Again, more tumors were produced in this group than among the controls in Group 5 (20 vs.

13 per cent). This difference did not prove to be significant at the 0.05 level.

Among the mice treated with 0.16 μg . DBA (Group 2), 38 per cent developed skin tumors, with an average yield of 0.9 tumors per mouse. The increased tumor incidence in this group compared with that in Group 5 is statistically significant ($P < 0.05$ -Yates correction). Also, a comparison of average number of tumors per mouse for these two groups showed significantly more tumors in Group 2 than in Group 5 ($P < 0.005$). These data demonstrate that one application of 0.16 μg . of DBA is more effective than a single dose of 0.16 μg . DMBA in initiating skin tumorigenesis. Application to the skin of 0.02 μg . of DBA in Group 4 also resulted in a higher tumor incidence and a greater tumor multiplicity than in the control mice (Group 5). However, the dif-

ference in tumor incidence (33 vs. 13 per cent) did not prove to be significant at the 0.05 level. On the other hand, the difference in the average number of tumors per mouse (0.5 vs. 0.1) was statistically significant ($P < 0.05$). Initiation of skin tumorigenesis with a dose of DBA as low as 0.16 μg . demonstrates that this agent is indeed a potent initiator. Berenblum and Shubik (2) anticipated from their studies on the mechanism of skin tumorigenesis that the anomalous behavior of DBA in subcutaneous tumorigenesis described by Bryan and Shimkin (3) might be accounted for if DBA is a potent initiator and a weak promoter. The results with this carcinogen in the present study substantiate this suggestion.

Experiment 2 (Table 2).—In this experiment, the mice were all treated at the outset with the same initiating dose of DMBA while the difference

TABLE 1

INITIATING INFLUENCE OF 9,10-DIMETHYL-1,2-BENZANTHRACENE (DMBA) AND 1,2,5,6-DIBENZANTHRACENE (DBA) APPLIED ONCE TO THE SKIN OF SWISS ALBINO MALE MICE

GROUP NO.	EFFECTIVE* TOTAL NO.	TREATMENT			MICE WITH TUMORS		TUMORS PER MOUSE (AV. NO.)	OBSERVATION PERIOD† (AV. DAY)	
		Primary agent	Conc. (per cent)	Dose (μg .)	Secondary agent†	No.			Per cent
1	42	DMBA	8×10^{-5}	0.16	croton oil, 25×	11	26	0.5	210
2	37	DBA				14	38	0.9	214
3	45	DMBA	1×10^{-5}	0.02	oil, 25×	9	20	0.3	221
4	30	DBA				10	33	0.5	218
5	32	acetone 100				4	13	0.1	210

* Mice alive at time of appearance of first tumor in the group.

† 1 per cent croton oil in acetone applied once weekly beginning 2 weeks following start of experiment.

‡ Based on effective totals in column 2.

TABLE 2

PROMOTING INFLUENCE OF 9,10-DIMETHYL-1,2-BENZANTHRACENE (DMBA) AND 1,2,5,6-DIBENZANTHRACENE (DBA) IN SKIN TUMORIGENESIS IN STRAIN DBA MALE MICE

GROUP NO.	EFFECTIVE* TOTAL NO.	TREATMENT		MICE WITH TUMORS			
		Primary	Secondary†	Tumor incidence		Tumors/mouse Av. no.	Latent period‡ (days)
				No.	Per cent		
1	26	1×, 0.15% DMBA	0.15% DMBA, 8×	25	96	5.4	226
2	27		0.3% DBA, 11×	15	56	1.4	339
3	33		100% acetone, 11×	0	0	—	—
4	35		1.0% croton oil, 50×	27	77	2.5	122

* Mice alive at time of appearance of first tumor in group.

† Agents applied once monthly (Groups 1-3), or twice weekly (Group 4), to nape of neck beginning 1 month following primary treatment.

‡ Time required for 50 per cent of mice to bear one (or more) skin tumors.

in promoting potency between DMBA and DBA was compared. It was assumed that visible skin tumors would not appear in the present experiment following one application of 0.15 per cent DMBA alone, since one application of 1.5 per cent DMBA to the backs of strain DBA/Jax male mice was shown previously to be ineffective under similar conditions during an average observation period of 237 days (7). Secondary treatment was begun 1 month following the primary treatment and was continued at monthly intervals thereafter in the case of DMBA, DBA, and acetone (Table 2). It was considered that little, if any, of the carcinogen applied initially was present in the skin at the start of secondary treatment. Since DMBA and DBA, in themselves, are capable of producing multiple visible skin tumors upon repeated application, it is evident that, besides promoting tumorigenesis in the present experiment, secondary treatment with these agents in Groups 1 and 2 also resulted in the production of additional centers of initiation. This added initiation probably did not affect the latent period significantly in Experiment 2 for the following reasons: (a) The time of appearance of the first tumor only in each mouse was used in calculating latent period. (b) Latent period was based on the development of a 50 per cent tumor incidence, and the primary dose of DMBA employed in initiation was sufficient to initiate skin tumorigenesis in well over 50 per cent of the mice as shown by subsequent treatment with croton oil (Group 4). In an earlier experiment with strain DBA male mice (6), no visible skin tumors were observed following repeated application (av., 90X) to the skin of 5 per cent croton oil alone during a mean observation period of 252 days. (c) It is considered likely that initiation is a rapid, more or less instantaneous response (2), and it is reasonable to assume that tumors initiated during the period of primary treatment with DMBA would be promoted to the visible tumor stage sooner than those initiated 1 month or more later, i.e., during the period of secondary treatment.

It was anticipated from previous work in this laboratory³ that a skin tumor incidence greater than 50 per cent would be obtained in strain DBA/Jax male mice by the use of repeated monthly application to the skin of a 0.15 per cent solution of DMBA in acetone but not with 0.15 per cent DBA. With this in mind, and because a tumor incidence of 50 per cent was employed as the basis for measuring latent period, the concentration of DBA was increased to 0.3 per cent in

³ M. Klein, unpublished data.

Group 2. Examination of the data in Table 2 shows that 50 per cent of the mice exposed to repeated monthly doses of DMBA developed visible skin tumors by the 226th day (Group 1). The latent period of tumorigenesis was considerably extended, however, in the case of those mice which had been treated with DBA (339 days, Group 2). Based on the assumption that a shorter latent period is indicative of a greater degree of promoting action (2), these findings demonstrate that DMBA is more active than DBA in tumor promotion. This conclusion is strengthened in view of the fact that the concentration of DMBA applied during the course of secondary treatment was only half that employed in the case of DBA; also, the mice treated with DBA received eleven applications as compared with eight for DMBA. Berenblum and Shubik (2) suggested in their investigation in skin tumorigenesis that, if DBA is an especially effective initiator and at the same time a relatively weak promoter, this would help to explain the anomalous findings of Bryan and Shimkin (3) referred to earlier. The results in the present experiment and in Experiment 1 provide evidence in support of this suggestion.

The demonstration that an agent, e.g., DBA, which is considered generally to be relatively weak in its over-all carcinogenic action on the skin of the mouse (5), possesses, nevertheless, considerable activity in tumor initiation emphasizes the need for a more thorough testing program of suspected carcinogens, e.g., one in which each agent is examined for initiating and promoting activity as well as for over-all carcinogenic activity. It is entirely possible that some of the hydrocarbons now considered to possess little or no carcinogenic activity will show a significant amount either of initiating or promoting activity when re-examined in this light.

The mice in Group 4 were exposed to a series of twice-weekly treatments with croton oil following a primary, initiating dose of DMBA. Tumors appeared more rapidly in this group than in the other three groups, so that 50 per cent of the mice already had one or more skin tumors by the 122d day (Table 2). It is probable that most, if not all, of these tumors were initiated by the primary treatment with DMBA, since repeated application of croton oil alone to the skin of strain DBA male mice was shown previously to be ineffective in the production of visible skin tumors (6). It is evident from the data in Group 4 that treatment twice weekly with croton oil provided a more effective promoting stimulus than monthly treatments with 0.15 per cent DMBA or 0.3 per cent DBA.

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