TUMOUR PROMOTION BY THE NEUTRAL FRACTION OF CIGARETTE SMOKE

G. R. CLEMO AND E. W. MILLER

From The Laboratory, Cherryburn, Mickley-on-Tyne, and Cancer Research Laboratory, Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne

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IT is known that the incidence of lung cancer amongst heavy cigarette smokers is higher in urban than in rural districts. While this might be attributed solely to exposure to the carcinogenic substances present in the urban atmosphere (e.g. particularly smoke from coal fires, also diesel fumes and car exhaust fumes (Kotin, 1956)), there is also the possibility of a co-carcinogenic action between cigarette smoke and city smoke. The following series of experiments was designed to test this hypothesis. Since the experiments were begun three years ago, Gellhorn (1958), Roe, Salaman and Cohen (1959) and Wynder and Hoffman (1960) have confirmed the presence of incomplete carcinogens in cigarette smoke as foreshadowed by Hamer and Woodhouse (1956) and Gwynn and Salaman (1956).

MATERIAL AND METHODS

One hundred and fifty-six C57BL mice (68 females, 88 males) were divided into 7 groups for the purposes of treatment. Aged between 3 and 6 weeks at the beginning of the experiment, the mice were allowed to live to the end of their normal lives or, in the case of those with skin tumours, they were killed when the tumours became large or frankly malignant. All skin tumours except the smallest papillomata were sectioned and examined histologically. A tumour was judged to be malignant when the panniculus carnosus was invaded. Tumours were classed as " probably malignant " when the tumour cells had not yet reached the muscle although other signs of malignancy were present.

The test substances in solution were applied with two strokes of a No. 4 paint-brush to the skin in the interscapular region. There were three substances: fraction " C " from city smoke, a known carcinogenic material (Clemo, Miller and Pybus, 1955), applied as a 1·0 per cent solution in benzene ; croton oil, a known tumour promoter, applied as a 0·5 per cent solution in acetone ; and the neutral fraction of cigarette smoke applied as a 10 per cent solution in benzene. This last fraction was extracted from the whole tar from cigarette smoke as described by Clemo (1958) and approximately 6·4 mg. was applied to each mouse at each painting.

The 7 groups in the experiment received the following treatments :

Group I.—Twenty-one mice (9 females, 12 males) were painted 3 times a week, for 2 weeks only, with fraction " C ". They received no further treatment.

Group II.—Twenty-one mice (6 females, 15 males) were painted with fraction " C" as in Group I. After an interval of 3 weeks they were painted with neutral fraction 3 times a week till death.

Group III.—Twenty-four mice (15 females, 9 males) were painted with fraction " C " as in Group I. After an interval of 3 weeks they were painted with croton oil twice weekly until death.

Group IV.—Twenty-two mice (8 females, 14 males) were painted 3 times a week, for 2 weeks only, with neutral fraction. They received no further treatment.

Group V.—Twenty-one mice (6 females, 15 males) were painted with neutral fraction as in Group IV. After an interval of 3 weeks they were painted with fraction " C " 3 times a week until death.

Group VI.—Twenty-five mice (11 females, 14 males) were painted with neutral fraction as in Group IV. After an interval of 3 weeks, they were painted with croton oil twice weekly until death.

Group VII.—Twenty-three mice (13 females, 10 males) were painted with croton oil twice weekly until death.

RESULTS

The results are summarised in Table I.

Group I.—The dose and duration of painting with fraction " C " were arbitrary. It was known to produce many skin tumours in C57BL mice when applied throughout life (Clemo and Miller, 1957). From the present experiment it was evident that a minimal carcinogenic dose had been given. Two males (aged 19 and 29·5 months) developed 3 small papillomata, 2 of which, at the site of painting, were superficial, while in the third (slightly larger and on the abdominal surface) the epithelium had not yet reached the panniculus carnosus. One female developed a rapidly growing spindle-celled subcutaneous sarcoma over the left scapula, at the age of 20 months. The non-tumour mice all lived well into tumour age, 4 dying with leukaemia, 2 with hepatomata and one with leukaemia plus hepatoma.

Group II.—Three males developed epitheliomata (one per mouse) at the site of painting, all growing steadily so that the mice had to be killed 3 to 4 months after the first appearance of the lesions ; 7 papillomata appeared in 5 other males, all at the site of painting. In 4 females there were 8 skin tumours (one mouse had 4 and in another mouse 2 coalesced to form one) ; of these, 5 were innocent papillomata but 3 were classified as " probably malignant ". Non-tumour mice lived well into tumour age ; one female died at 11 months of leukaemia, the other at 15·5 months of pneumonia, while gross kidney disease caused the deaths of the 7 non-tumour males.

Group III.—The maximum total number of skin tumours in the 9 tumour females was 14, but 4 disappeared before death, leaving a final total of 10, the greatest individual number being 4 ; of these 10, one grew slowly but steadily to become " probably malignant " at the time of death 8 months later, the rest remaining small. The three tumours in males (one each) were all small. The non-tumour mice were all of tumour age and the majority died with grossly diseased kidneys.

Group IV.—In this group 2 male mice each developed one small skin papilloma ; one of these, in the lumbar region, disappeared before death ; the second was in the centre of the abdominal surface. The remaining mice died at tumour age without skin tumours but 3 had leukaemia, one a very large lung tumour (rare in this strain), one had haemangioma of the spleen, one a hepatoma and the rest had diseased kidneys.

TABLE I.—*The Tumour-promoting Effect of the Neutral Fraction of Cigarette Smoke, after Tumour Initiation by Fraction " C " from City Smoke*

Group (1)	Treatment (2)	Number of mice at risk (3)	Tumour mice: Number (4)	Tumour mice: Per cent (5)	Number of skin tumours: Total: Maximum (6)	Number of skin tumours: Total: Final (7)	Number of skin tumours: Probably malignant (8)	Number of skin tumours: Malignant (9)	Age of mice in months: Tumour: At tumour appearance: Av. Range (10)	Age of mice in months: Tumour: At death: Av. Range (11)	Age of mice in months: Non-tumour At death: Av. Range (12)
I	C + *nil*	21	3	14·3	4	4	0	1	22·8 (19·0–29·5)	26·5 (31·0–30·5)	25·6 (21·0–31·0)
II	C + neutral	21	12	57·1	18[1]	17	3	3	20·2 (9·5–28·0)	21·9 (13·5–28·5)	20·3 (11·0–28·0)
III	C + croton oil	24	12	50·0	16[2]	12	0	1	13·8 (7·0–21·5)	18·6 (12·0–23·5)	17·4 (8·0–22·5)
IV	Neutral + *nil*	22	2	9·1	2[3]	1	0	0	25·5 (25·0–26·0)	26·0 (25·5–26·5)	26·3 (17·0–33·0)
V	Neutral + C	21	21	100·0	103[4]	68	8	19	10·1 (7·5–12·5)	14·5 (11·5–17·5)	—
VI	Neutral + croton oil	25	2	8·0	2[3]	1	0	0	15·5 (14·0–17·0)	17·0 (16·0–18·0)	16·1 (12·0–23·0)
VII	Croton oil only	23	1	4·4	2[3]	1	0	1	21·0	22·5	16·4 (12·5–22·0)

1 = 2 tumours coalesced.
2 = 4 papillomata regressed.
3 = 1 papilloma regressed.
4 = many tumours coalesced as they grew ; none regressed.

The first substance named in the treatment (column 2) of each group was the initiator, the second the promoter. The great majority of skin tumours were benign papillomata and the number attained its maximum before the end of treatment (column 6), being reduced thereafter either by complete regression or by coalescence into fewer larger tumours (column 7), a small number of which continued to develop to malignancy (i.e. the numbers in columns 8 and 9 are included in the numbers in column 7).

TABLE II.—*Tumour Production in Male and Female Mice by Fraction " C " from City Smoke, in Group V*

	Number of mice (1)	Total number of tumours: Maximum (2)	Total number of tumours: Final (3)	Number of tumours per mouse: Maximum (4)	Number of tumours per mouse: Maximum average (5)	Number of tumours per mouse: Final average (6)	Tumour incidence: Probably malignant: Number (7)	Tumour incidence: Probably malignant: Percent of total (8)	Tumour incidence: Malignant: Number (9)	Tumour incidence: Malignant: Percent of total (10)	Percentage which was malignant and probably malignant (11)
Males	15	79	55	15	5·3	3·7	2	3·6 (of 55)	16	29·1 (of 55)	32·7
Females	6	24	13	7	4·0	2·2	6	46·2 (of 13)	3	23·1 (0f 13)	69·2

Group V.—The majority of the mice, otherwise healthy, were killed when their tumours became large and malignant or " probably malignant ", but two had kidney disease and died before their tumours had grown to any size. Every mouse had multiple skin tumours. The tumour incidence in males and females is given in Table II. Although the males had on an average more tumours per mouse than the females, the difference was not significant either for the maximum average numbers (column 5) (d = 1·3, 2 × S.E. = 2·1) or for the final average numbers (column 6) after tumours had coalesced (d = 1·5, 2 × S.E. = 1·9). But when the proportions of tumours which became malignant or " probably malignant " were compared (column 11), the difference between the sexes was significant (d = 36·5, 2 × S.E. = 28·6). The average age of the females at tumour appearance was 10·8 months (range = 9·0–12·5 months) and that of the males was 9·8 months (7·5–11·5 months) ; the average age of the females at death was 14·2 months (12·0–17·0 months) and of the males was 14·5 months (11·5–17·5). Thus although the latent period was longer in the females, the tumours became malignant more rapidly than in the males, and a greater proportion of the tumours became malignant (and " probably malignant ") in the females.

Group VI.—The only mice to produce skin tumours were 2 males, each having one small papilloma, one at the age of 14 months (on the forehead) and the other at 17 months (interscapular) ; the latter had disappeared at the time of death 2 months later. The mice in this group, although of tumour age, died sooner than those of other groups, most of them with diseased kidneys.

Group VII.—There was only one tumour mouse, a female with a skin papilloma on the left flank at the age of 21 months. That tumour disappeared before death 7 weeks later, but meanwhile another papilloma, which had appeared in the dorsal region a week or two after the first, had developed into a small epithelioma. The remaining mice died tumour-free at a similar age to those in Group VI and all but one had grossly diseased kidneys.

DISCUSSION

It is clear from these experiments that, under the conditions of treatment described, the neutral fraction of cigarette smoke can act as a definite tumour promoter. Some evidence has already appeared in the literature that whole tar from cigarette smoke may have tumour-promoting properties when applied after a known carcinogen such as 3 : 4-benzopyrene (Hamer and Woodhouse, 1956 ; Gwynn and Salaman, 1956) ; these workers found no proof of tumour-initiation. Gellhorn (1958) demonstrated convincingly the tumour-promoting activity of whole tobacco tar after the preliminary application of 3 : 4-benzopyrene ; he found also that although croton oil promoted a higher incidence of carcinomata, tobacco tar increased the " conversion rate " of carcinomata from papillomata compared with croton oil. More recently Roe, Salaman and Cohen (1959) have shown that the phenolic fraction is a tumour promoter, the initiator in this case being 9,10-dimethyl-1,2-benzanthracene ; they proved also that the neutral fraction is carcinogenic. Wynder and Hoffman (1960) mention the tumour-promoting properties of the phenol fraction and of the nicotine-free basic fraction.

As far as is known, the present series of experiments is the first in which the initiating substance has been, not one of the well-known carcinogens such as

3 : 4-benzopyrene, but a mixture (of proved carcinogenic power) obtained direct from city smoke. Although its components are still not wholly identified, fraction " C " was chosen deliberately in order to simulate more closely the actual conditions of daily life. That it is a strong carcinogen was shown once again by the mice of Group V, to which it was applied until death and in which the tumour incidence was 100 per cent ; this result was the same as had been obtained in previous experiments with fraction " C " alone and any hypothetical promoting effect due to the preliminary treatment with neutral fraction would be quite obscured by the potency of fraction " C ". That a minimal initiating dose was given to the mice in Group I was shown by the very low tumour incidence in that group.

The crucial results are given by Groups II and III ; in these, fraction " C " applied in minimal dose as initiator was followed in Group II by neutral fraction and in Group III by croton oil as promoters. Croton oil is a well-known tumour promoter ; it has also been shown to be a weak carcinogen, producing a number of papillomata which usually regress when treatment ceases, but Roe (1956) reported 7 malignant tumours in 20 mice which had been treated for from 55 to 72 weeks. In the present instance, applied alone throughout life (up to 21 months' treatment), it produced in Group VII only one malignant tumour and one papilloma which regressed in 23 mice living over one year.

In Group III 12 of the 24 mice developed a total of 16 skin tumours, of which 4 regressed and only one (which took 6 months to grow and was the only tumour of any size in that group) became malignant ; the final tumour incidence was 33 per cent.

In Group II 12 of the 21 mice produced a total of 18 skin tumours, of which none regressed (but 2 coalesced as they increased in size) and 6 became malignant or probably malignant, 5 of them attaining quite a large size in from 2 to 4 months. Thus although the latent period (see Table I) was much longer with neutral fraction as promoter, there was a much shorter time between tumour appearance and death than with croton oil as promoter. While the differences in tumour incidence are not statistically significant, a comparison of the charts of tumour growth was most convincing and showed that under the conditions of the experiment a 10 per cent solution of neutral fraction applied three times a week was a more powerful tumour promoter than a 0·5 per cent solution of croton oil applied twice weekly.

The present experiments provide less certain evidence of tumour intiation by neutral fraction ; with what was intended to be a minimal dose (Group IV) papillomata appeared in 2 mice out of 22, but one regressed and the other was far from the site of painting. When, in Group VI, after this preliminary treatment with neutral fraction, croton oil was applied as in Group II as a promoter, again only 2 mice developed skin papillomata, no more than might be expected from the use of croton oil alone. While this might mean that the original dose was too small to give tumours, in previous experiments in which a 10 per cent solution of neutral fraction was applied throughout life to mice of the A and C57BL strains no skin tumours were observed. On the other hand Roe, Salaman and Cohen (1959) obtained proof of complete carcinogenesis with neutral fraction when they produced papillomata and two skin carcinomata in 5 mice living up to 60 weeks (from 37 survivors at 30 weeks), painting being stopped after 47 weeks ; they applied neutral fraction three times weekly at a dose of 40 mg. at each painting.

In the present work the dose of neutral fraction, forming about 18 per cent by weight of the whole original tar (Clemo, 1958), was approximately 6·4 mg. per mouse at each painting. The neutral fraction used by Roe, Salaman and Cohen (1959) which apparently contained the ester fraction removed from our neutral fraction, formed approximately 56 per cent by weight of the original tar. It is thus not possible to compare strictly the individual doses used by ourselves and by Roe *et al.* (1959). Gellhorn (1958) gave approximately 50 to 60 mg. of tar per week to each mouse ; our dose of 19·2 mg. neutral fraction per week probably represents about twice as much neutral fraction as would be present in his dose. Although Gellhorn used the same strength of croton oil, he was giving three times as much per week as in our experiments ; he obtained far more tumours with croton oil than he did with the tar (as promoters), and given the differences in dosage the present results are not inconsistent with his.

It was noticed in the present experiments that mice receiving croton oil throughout their lives (Groups III, VI and VII) died much earlier than those in other groups (except Group V where they all developed tumours) and the majority had severe kidney disease.

SUMMARY

Experiments are described in which a definite tumour-promoting effect was observed when the neutral fraction from cigarette smoke was applied to C57BL mice after an initiating dose of fraction " C " from city smoke. Eighteen skin tumours, of which 6 became malignant or " probably malignant " were produced in 12 out of 21 mice painted in the interscapular region throughout their lives (up to 28 months). Similar treatment with croton oil after fraction " C " resulted in 16 skin tumours, of which 4 regressed and one became malignant, in 12 out of 24 mice.

There was little evidence of either a tumour-initiating or a complete carcinogenic effect with a small dose of neutral fraction. No more tumours were produced with croton oil applied after neutral fraction than were produced by croton oil alone.

When fraction " C " was applied throughout life, the latent period was longer in females than in males, but tumour growth was more rapid in the females and a significantly greater percentage of tumours in females developed to malignancy.

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