THE TIME OF APPEARANCE OF LUNG TUMOURS IN MICE INJECTED WHEN NEWLY BORN WITH 9,10-DIMETHYL-1,2-BENZANTHRACENE (DMBA)

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THE neonatal injection of carcinogenic polycyclic hydrocarbons or urethane gives rise to a high incidence of pulmonary adenomas in many strains of mice (Pietra, Spencer and Shubik, 1959 ; Pietra, Rappaport and Shubik, 1961 ; Kelly and O'Gara, 1961 ; Roe, Rowson and Salaman, 1961 ; and De Benedictis *et al.*, 1962). An experiment has been carried out in an attempt to establish whether the appearance of lung tumours is confined to a definite period or continued throughout life.

MATERIALS AND METHODS

Chemical agents.—A suspension of 9,10-dimethyl-1,2-benzanthracene (DMBA) (Roche Products Ltd.) in 3% aqueous gelatine (British Drug Houses) was prepared by adding an acetone solution of the carcinogen to aqueous gelatine warmed to 56° C. The acetone was driven off in a stream of nitrogen. Doses of 15 μg. or 30 μg. DMBA were contained in 0·02 ml. aqueous gelatine.

Mice.—A breeding nucleus of " 101 " strain mice was obtained from Dr. M. F. Lyon, Radiobiological Research Unit, Harwell, Didcot, Berks., in 1961. They were bred by brother-sister mating and mice from the resulting colony were used in both experiments. A cubed diet (Diet 86, Messrs. Dixon and Sons, Ware, Herts.) and water were administered *ad libitum*, and the mice were housed in metal cages. At 6 weeks of age the mice in Experiment 2 were vaccinated with sheep lymph as a precaution against ectromelia.

Experiment 1

Newly born mice from 53 litters were injected, subcutaneously, when less than 24 hours old with 30 μg. DMBA. Nineteen of these litters were killed by the mothers. From the survivors 10 mice, 5 males and 5 females picked at random, were killed with ether 24 hours, 48 hours, 72 hours, 1 week, 2 weeks, 4 weeks, 8 weeks and 16 weeks after injection. A control group of untreated mice was killed at 8 weeks of age. Post-mortem examination was carried out on every animal. Before the thorax was opened a suture was tied tightly around the trachea to prevent pulmonary collapse. The trachea, lungs, heart and thymus were removed intact and fixed in aqueous Bouin's solution, together with the kidneys and pieces of liver and skin. The stomachs were distended with formol saline. Sections were stained routinely with haematoxylin and eosin. In addition, some sections of the lungs of each mouse were stained by Gordon and Sweet's stain for reticulin. The lungs of 2 males and 2 females in each age group were examined in serial sections.

No neoplastic or preneoplastic changes were observed in the heart, thymus, kidneys, liver, stomach or skin of any mouse. There were no lung adenomas in DMBA-treated mice less than 8 weeks of age, nor in the 8 week-old untreated controls. Quite frequently, however, groups of alveolar cells, usually situated just beneath the pleura, were seen to be cuboidal in shape and generally similar in appearance to those of which alveologenic adenomas are composed. Such lesions were often associated with areas of atelactasis. Two mice (one male and one female) killed at 8 weeks each had one small subpleural adenoma, about 0·25 mm. diameter. Another male had a nest of closely-packed alveolar cells, thought to be an early adenoma, near the centre of one lobe. The cells partially filled the alveolar lumen and lined the septa of several adjacent alveoli. Eight of the 10 mice killed at 16 weeks had between 1 and 4 lung adenomas each. The largest of these was 2 mm. in diameter, but the majority were less than 1 mm.

Experiment 2

15 μg. DMBA in 3% aqueous gelatine was injected subcutaneously into mice from 99 litters. Many treated mice were lost during the first week of life owing to cannibalism. At 4 weeks the mice were weaned, sexed, numbered on the ears and housed 4–6 to a cage. As far as possible a representative of each litter was killed at 30, 40, 50, 60 and 70 weeks. The resulting groups consisted of 35 to 40 mice with approximately equal numbers of males and females. Post-mortem examination was carried out on each mouse and the adenomas on the surface of the lungs were counted. The diameter of the largest tumour was measured and all tumours larger than 3 mm., and a sample of the smaller adenomas were fixed and sectioned. A histological classification of lung tumours, described in an accompanying paper (Walters, 1966) was employed. Lesions from other organs which were definitely, or possibly, neoplastic, were also taken for microscopic examination.

A further 30 litters were injected with aqueous gelatine alone. Groups of about 40 mice were killed at 40 and 60 weeks and the same post-mortem procedure was followed as for the treated mice.

Sick mice, which were killed, or mice which died during the experiment were autopsied. Table I shows the number of deaths up to 60 weeks and the tumours which occurred in treated and control mice. The 15 μg. dose of DMBA induced a variety of tumours, including pulmonary adenomas, injection-site sarcomas, malignant lymphomas, hepatomas, haemangiomas and skin papillomas. Sixteen mice, including both males and females and DMBA-treated and control animals, developed unilateral or bilateral parotid gland tumours of the type attributable to polyoma virus infection. Rowe (1961) reported that antibodies to polyoma virus have been detected in 0–75% of uninoculated mice in laboratory and breeding colonies, though the occurrence of tumours attributable to the virus in untreated mice is rare. Parotid tumours appeared in mice from litters which were the first of the day's batch to be injected when a sterile needle and syringe were used. Therefore, the possibility that the tumours were caused by the transfer of polyoma virus from one litter, which had passive immunity, to another which had not, is ruled out. Since there were approximately equal numbers of tumours in DMBA-treated mice and in the aqueous gelatine controls, synergism between the virus and DMBA, *per se* (Rowson *et al.*, 1961) is unlikely. It is possible that " 101 " strain

TABLE I.—*Induction of Tumours by the Neonatal Injection of 15 μg. DMBA in "101" Strain Mice: Mice which Died During the Experiment*

Treatment	Sex	Number of deaths at (weeks) 0–4	−8	−12	−16	−20	−24	−28	−32	−36	−40	−44	−48	−52	−56	−60
15 μg. DMBA < 24 hours	♀	0	0	2(PA,Ly) (Hm)	1(Hm)	1(PG)	4(O) (PA,S) (PA,H) (PA,PG)	0	1(PA,PG)	5(O) (O) (PA) (PA) (PA)	6(O) (PA) (PA) (PA) (PA) (PA,PG)	4(O) (O) (PA,Ly) (PA,Ly)	3(O) (PA,Ly) (PA,Hm)	2(PA) (PA,Pap)	1(PA)	4(PA) (PA) (PAm) (PAm)
	♂	2(O) (O)	0	0	3(O) (PA) (Ly)	5(O) (PA) (PA,Ly) (PA,S) (PG)	5(O) (PA,S) (PA,PG) (PA,PG) (PG)	1(PA)	2(PA) (PA)	3(O) (PA) (PA)	2(PA) (PA)	0	4(PA) (PA) (PA,Ly) (PA,Hp)	5(PA) (PA) (PA) (PA,Hp) (PAm)	2(PA) (PAm)	1(PAm)
3% AG < 24 hours	♀	0	0	0	2(PG) (PG)	1(O)	0	0	0	0	0	0	0	0	0	0
	♂	0	0	0	2(PG) (PG)	1(PG)	1(O)	1(O)	2(O) (PG)	1(PG)	1(PG)	0	0	0	0	0

AG = Aqueous gelatine
O = No tumours
H = Haemangioma (subcutaneous)
Hm = Malignant haemangioma
Hp = Hepatoma
Ly = Malignant lymphoma
PA = Pulmonary adenoma
PAm = Pulmonary adenoma (malignant)
Pap = Squamous papilloma of skin
PG = Parotid gland tumour
S = Sarcoma at injection site

TABLE II.—*Induction of Tumours by the Neonatal Injection of DMBA in "101" Strain Mice: The Effect of the Length of the Induction Period*

Group	Treatment	Age at death (weeks)	No. of mice	No. of mice with lung tumours	% mice with lung tumours	Mean No. lung tumours per mouse	Mean size of largest lung tumour (m.m.)	No. mice with tumours (by classes of malignancy) 1 2 3 4 5	Other tumours
Females									
1	15 μg. DMBA 24 hours	30	16	13	81·25	3·2	1·2	13 – – – –	—
2	,,	40	18	18	100	12·9	1·8	18 – – – –	—
3	,,	50	17	16	94·1	13·5	2·1	16 – – – –	—
4	,,	60	14	13	92·8	16·7	3·3	12 1 – – –	1 melanocytoma 1 benign haemangioma (subcutaneous)
5	,,	70	11	11	100	16·1	3·8	10 1 – – –	1 malignant lymphoma
6	3% AG 24 hours	40	19	0	0	0	—	– – – – –	—
7	,,	60	22	9	40·9	0·4	1·3	9 – – – –	—
Males									
1	15 μg. DMBA 24 hours	30	19	18	94·7	2·8	1·2	18 – – – –	—
2	,,	40	17	17	100	6·9	1·5	17 – – – –	1 skin papilloma
3	,,	50	18	18	100	9·9	2·7	17 1 – – –	1 spindle cell sarcoma at injection site
4	,,	60	22	22	100	16·7	3·3	20 – 2 – –	—
5	,,	70	27	26	96·3	16·7	3·6	21 2 3 – –	8 hepatomas 1 adenocarcinoma of the lachrymal gland 1 sebaceous adenoma
6	3% AG 24 hours	40	13	1	7·6	0·07	2	1 – – – –	—
7	,,	60	17	2	11·8	0·18	1	2 – – – –	—

AG = Aqueous gelatine.

mice infected with polyoma virus are more prone to develop parotid gland tumours than are other strains.

The incidence of lung tumours and other neoplasms in treated and control mice killed at 30, 40, 50, 60 or 70 mice is shown in Table II. Fig. 1 and 2 show that the mean number of lung tumours and the mean size of the largest tumour increase

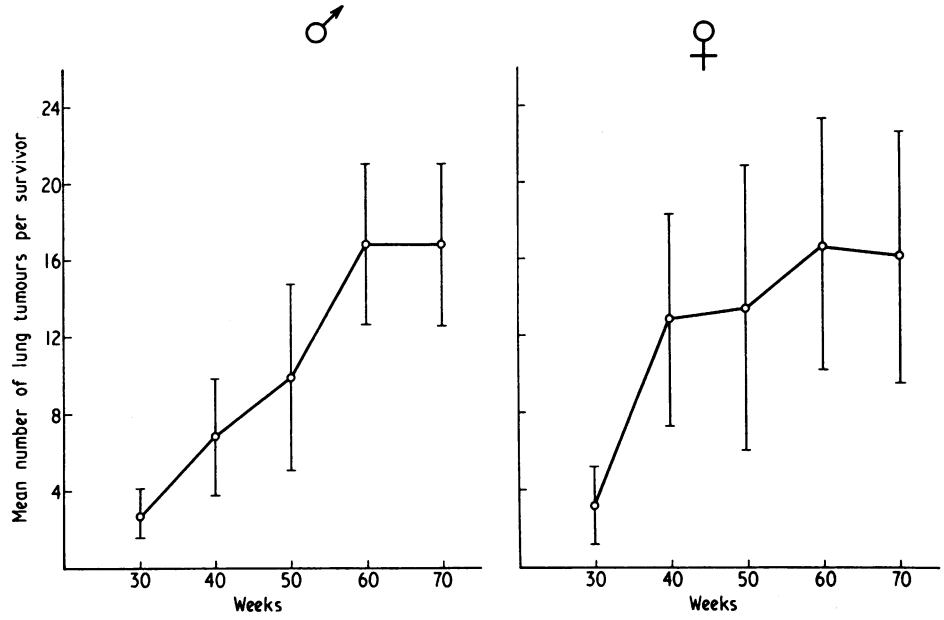

FIG. 1.—Number of lung tumours induced in mice by 15 μg. DMBA.

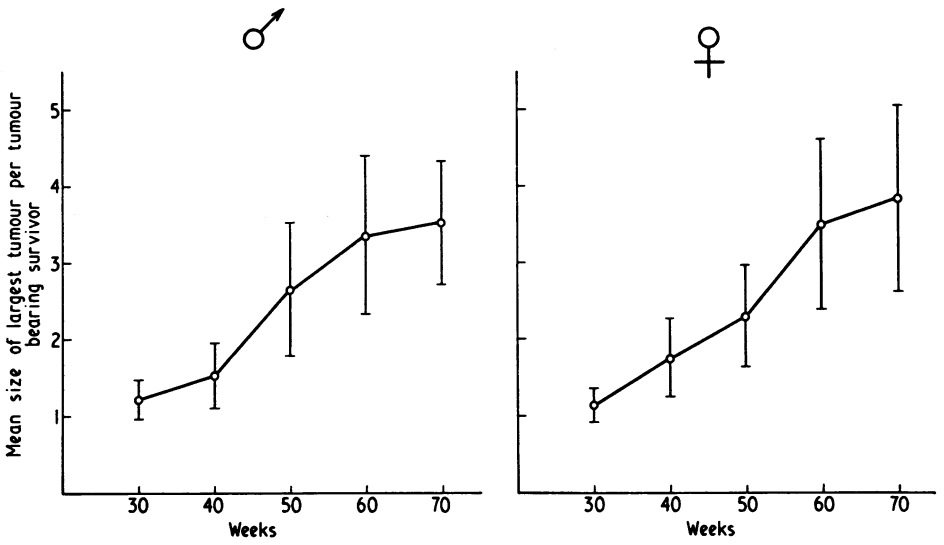

FIG. 2.—Size of lung tumours induced in mice by 15 μg. DMBA.

with the length of induction period from 30 to 60 weeks. The curve flattened from 60 to 70 weeks. There were too few survivors to include a group at 80 weeks. Many deaths were caused by papillonephritis, a disease to which " 101 " strain mice are prone. 50–100% of male mice over 30 weeks were affected. The highest incidence in females, however, was 27% at 40 weeks.

Malignant lung tumours (Class 3) (Walters, 1966) which had metastasised throughout the lung occurred in five males, two of which were killed at 60 weeks and three at 70 weeks. Tumours which showed intrabronchial spread but no metastases (Class 2) were seen in one male killed at 50 weeks and two killed at 70 weeks.

DISCUSSION

Many workers have found that both the incidence and the multiplicity of pulmonary adenomas in mice increase with the length of the induction period. Shimkin (1940) reported that the mean number of tumours in A strain mice injected intravenously with methylcholanthrene rose from 5 at 4 weeks to 47 at 20 weeks. For C strain mice given the same treatment a mean nodule count of 4 was recorded at 20 weeks and this rose to 40 at 32 weeks. The incidence and multiplicity of lung adenomas in A strain mice following the injection of dibenzanthracene, dibenzocarbazole, benzopyrene, benzodehydrocholanthrene and dibenzacridine rose between the 8th and 14th weeks (Andervont and Shimkin, 1940). There was, however, only a slight further increase in the mean number of tumours per mouse between the 14th and 20th weeks. When a comparison was made between groups of mice which received a range of doses of methylcholanthrene, a significant difference was found between mice kept for 8 weeks and those kept for 13 or 18 weeks, when the dose was greater than 0·125 mg. (Shimkin and McClelland, 1949).

Thirty per cent of newborn A strain mice injected with methylcholanthrene developed lung adenomas by 4 weeks (Kimura and Senra, 1964). Methylcholanthrene and dibenzanthracene gave a 50% yield of lung adenomas at 8 weeks after being injected into newborn albino mice (Kelly and O'Gara, 1961). Tumour incidence rose to 96% at 24 weeks. The mean nodule count after methylcholanthrene was 6 at 8 weeks, 26 at 16 weeks and 23 at 24 weeks : after dibenzanthracene it was 3 at 8 weeks, 11 at 16 weeks and 24 at 24 weeks. De Benedictis *et al.* (1962) found that the neonatal injection of urethane induced lung adenomas in 20% Swiss mice at 3 weeks. The average number of tumours was 1. One hundred per cent of the mice had tumours by 13 weeks when the mean nodule count was 5. The mean count rose further to 17 at 30 weeks.

Strains differ in their susceptibility to the induction of pulmonary adenomas and in the time of appearance of the earliest tumour. Kelly and O'Gara (1961), De Benedictis *et al.* (1962) and Kimura and Senra (1964) describe the appearance of lung adenomas in albino, Swiss and A strain mice only 3 or 4 weeks after the neonatal injection of a carcinogen, but in " 101 " strain mice no tumours were seen in mice younger than 8 weeks. Of 10 mice killed at 8 weeks, 2 had definite adenomas and one had a lesion which was probably an early adenoma.

Lung tumour incidence was high, between 80 and 100%, in all DMBA-treated mice killed at 30 weeks or more. The mean number of tumours per mouse and the mean size of the largest tumour increased between the 30th and 60th week, but the curve for the mean number of tumours per mouse levelled out between

60 and 70 weeks. This suggests that the rate of appearance of new tumours fell off at about 60 weeks, though the tumours already present continued to increase in size. Unfortunately, because of small numbers due to poor survival, and because of wide mouse to mouse variation, the figure shown (Fig. 1) for mean number of tumours at 70 weeks, is not significantly different from the number which would have been expected if new tumours had continued to appear between the 60th and 70th weeks at the same rate as they were appearing between the 30th and 60th weeks.

Clearly in the case of " 101 " mice injected neonatally with DMBA, there is wide variation in the time of appearance of macroscopically visible tumours, since new tumours were still appearing between the 50th and 60th weeks. It remains uncertain whether there is a point in the life span of the mouse after which lung tumours attributable to exposure to carcinogens during the neonatal period do not occur.

SUMMARY

1. " 101 " Strain mice injected neonatally with 30 μg. 9,10-dimethyl-1,2-benzanthracene (DMBA) in 3% aqueous gelatine were killed at 1 day, 2 days, 3 days, 1 week, 2 weeks, 4 weeks and 8 weeks of age. No neoplastic or preneoplastic lesions were seen in any organ except the lung : 2 out of 10 mice had 1 adenoma at 8 weeks and another had an early adenoma. Eight out of 10 mice had adenomas at 16 weeks.

2. In mice injected with 15 μg. DMBA at birth, the average number of tumours per mouse and the size of the largest tumour increased with age from 30 to 60 weeks. The mean nodule count at 70 weeks was similar to that at 60 weeks, but it is not certain that the appearance of new tumours stopped or even slowed down at 60 weeks. Tumour size continued to increase between the 60th and 70th weeks.

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