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Synergistic induction of tumours in NMRI mice by combined foetal X-irradiation with low doses and ethylnitrosourea administered to juvenile offspring

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Mice were X-irradiated on day 15 of gestation with 0.2, 0.4, 0.8 or 1.6 Gy. Offspring were reared by their mothers and divided into two subgroups at an age of 21 days, one subgroup receiving a single dose (45 mg/kg) of ethylnitrosourea (ENU). All animals were kept ultimately until 22 months to register the long-term tumour pattern. The carcinogenic effects of ENU alone were also studied in two separate experiments. Prenatal X-irradiation with 1.6 Gy mostly abolished the carcinogenic late effects of ENU, with the exception of an almost constant leucosis incidence and an unchanged lung tumour frequency. Lowering the prenatal X-ray dose to 0.8 Gy resulted in a significantly increased rate of liver tumours and ovary tumours. Synergistic effects on various tissues were observed after both 0.4- and 0.2-Gy foetal X-irradiation treatment in combination with postnatal application of ENU. These effects mainly involved a significant increase in the frequency of leucosis and of tumours of the liver, intestine, uterus and ovaries. The greater-than-additive effect in the case of these tumours suggests that low-level prenatal X-irradiation leads to a lasting sensitivity of some tissues towards a subsequent carcinogenic stimulus.

Introduction

In recent studies we examined the susceptibility to tumour induction in prenatally X-irradiated animals. This was tested by means of a two-stage carcinogenesis model. X-irradiation of mice *in utero* was regarded as an initiating stimulus, the promoting treatment then being given either by ethylnitrosourea (ENU*) during the foetal stage (1) or by TPA phorbol-ester between 12 and 26 weeks of postnatal life (2). After prenatal X-irradiation of NMRI mice either with 2.0 Gy on gestation day (g.d.) 12 or with three daily doses of 0.8 Gy between g.d. 11 and g.d. 13, TPA treatment did not result in a higher tumour yield than that observed after X-irradiation alone (2). It was only after lowering the prenatal X-ray dose on g.d. 12 to 1.0 Gy that postnatal TPA treatment resulted in an increased tumour incidence in the lungs, ovaries and liver (3).

The present study described another experimental set-up using ENU as an effective carcinogen in the role of the postnatal stimulus after prenatal X-irradiation, instead of the promoter substance TPA.

Previous studies in our laboratory (4), as well as studies by others (5–8) who tested either prenatal X-irradiation or diaplacental ENU application for the induction of carcinogenic long-term effects in mice, provided evidence of an increasing sensitivity of the foetus between gestation days 14 and 16. We therefore concentrated our studies on X-irradiation on gestation day 15. Application of four X-ray doses at this developmental stage is combined with a single ENU treatment on postnatal day 21. The purpose is to analyse the dose/effect relationships upon the tumour spectrum for a period of 22 months.

*Abbreviations: ENU, ethylnitrosourea; DES, diethylstilbestrol; g.d., gestation day.

Materials and methods

Female, virgin NMRI mice from our own breeding colony were maintained in a temperature- and humidity-controlled animal facility with a 12-h light/dark cycle. All animals were given Altromin standard diet and water *ad libitum*. Males and females were placed together between 8 a.m. and 10 a.m. for breeding. The zygote of the pregnant animals was considered to be 0 days old at 1 p.m. when the plug control was done. The next morning was considered to be gestation day (g.d.) 1.

All irradiation procedures were completed using a Müller Roentgen (X-ray) unit operating at 180 kV and 10 mA. Five mice were placed in a round flat Plexiglass wheel for irradiation. The beam was filtered with a 0.3-mm copper plate. The average target–object distance was 40 cm, the dose rate was 0.01 Gy/s. Calibrations were completed using a Victoreen chamber.

The experiments were performed during two different periods: series I started in February 1984 and ended in 1986, series II started in May 1985 and lasted until spring 1987. Each series consisted of five groups, including controls and ENU-treated animals, as well as one irradiation experiment using the lowest dose also applied in the combination studies. The 1984 series (I) tested the combined effects of 0.8 Gy and 1.6 Gy X-irradiation respectively, with subsequent ENU application. The second series tested the 0.4 Gy and 0.2 Gy X-irradiation doses respectively, for combination studies with ENU.

The 115 pregnant animals from series I and the 137 dams from series II were randomly assigned to one of the 10 groups (Tables I and II). X-irradiation was performed on gestation day 15 at 10 a.m.

ENU was administered (45 mg/kg) to prenatally untreated or X-irradiated mice on postnatal day 21. The ENU solution (Sigma-Chemie München) was freshly prepared by dissolving 10 mg/ml in a sterile acetic buffer at a pH of 4.5. Each animal (mean weight 35 g) received a standard amount of 0.15 ml of the ENU solution by i.p. route, equivalent to 45 mg/kg body weight. This was done at 10 a.m. within 30 min after dissolving ENU in the buffer. Control animals received the buffer solution only.

Postnatal examinations

To evaluate the early postnatal effects on both treated and untreated animals the number of live offspring and the number of stillbirths were scored and the animals

Table I. Treatment scheme and fetal data of mice in this study

Group		Sample size	Litters		Mean litter size ± SD
			Live born	Dead born[a]	
Experiment I					
1	Controls I	31	388	1 (0.0)	12.5 ± 2.3
2	0.8 Gy, g.d. 15	22	296	3 (0.01)	13.4 ± 2.1
3	ENU I (45 mg/kg) postnatal day 21	21	280	0 (0.0)	13.3 ± 1.9
4	0.8 Gy + ENU	20	268	7 (0.02)	13.8 ± 2.3
5	1.6 Gy + ENU	21	262	2 (0.01)	13.2 ± 2.4
Experiment II					
6	Controls II	25	355	0 (0.0)	13.0 ± 2.4
7	0.2 Gy, g.d. 15	26	358	2 (0.01)	12.5 ± 2.8
8	ENU II (45 mg/kg) postnatal day 21	28	316	2 (0.01)	11.4 ± 3.3
9	0.2 Gy + ENU	30	379	0 (0.0)	12.6 ± 2.5
10	0.4 Gy + ENU	28	334	5 (0.01)	12.1 ± 3.0

[a]Numbers in parentheses are percentages of dead births.

Table II. Early and late mortality data for mice X-irradiated *in utero* and treated postnatally with ENU

Group	Cumulative mortalities (%) until					No. of animals		
	1 month	3 months	6 months	9 months	12 months	Alive beyond 9 months	Lost by autolysis (%)	With diagnoses up to 22 months
1	30 (7.7)	30 (7.7)	31 (8.0)	34 (8.7)	37 (9.5)	354	14 (3.9)	340
2	21 (7.1)	24 (8.1)	26 (8.8)	30 (10.1)	32 (10.8)	266	11 (4.1)	255
3	38 (13.6)	40 (14.3)	43 (15.3)	51 (18.2)	55 (19.6)	229	8 (3.5)	221
4	51 (19.0)	56 (20.9)	69 (25.7)	77 (28.7)	86 (32.1)	191	19 (9.9)	172
5	36 (13.7)	45 (17.2)	66 (25.2)	74 (28.2)	81 (30.9)	188	17 (9.0)	171
6	24 (6.8)	29 (8.2)	32 (9.0)	32 (9.0)	34 (9.6)	323	12 (3.7)	311
7	17 (4.7)	24 (6.7)	30 (8.4)	37 (10.3)	44 (12.3)	321	19 (5.9)	302
8	26 (8.2)	28 (8.9)	42 (13.3)	48 (15.2)	62 (19.6)	268	26 (9.7)	242
9	23 (6.1)	25 (6.6)	30 (7.9)	41 (10.8)	49 (12.9)	338	30 (8.9)	308
10	19 (5.7)	19 (5.7)	39 (11.7)	51 (15.3)	57 (17.1)	283	15 (5.3)	268

Table III. Tumour incidences (%) in male NMRI mice after prenatal X-irradiation, after postnatal ENU application or after both treatments in combination

	Group									
	1	2	3	4	5	6	7	8	9	10
Autopsied animals	148	139	129	98	97	159	171	127	180	152
Leucosis	(6) 4.1	(1) 0.7	(29) 22.5	(27) 27.6	(23) 23.7	(6) 3.8	(5) 2.9	(34) 26.8	(100) 55.6	(53) 34.9
Lung tumours	(18) 12.2	(45) 32.4	(126) 97.7	(96) 98.0	(96) 98.9	(42) 26.4	(41) 24.0	(120) 94.5	(162) 90.0	(144) 94.7
Liver tumours	(2) 1.3	(1) 0.7	(30) 23.3	(32) 32.6	(7) 7.2	(3) 1.9	(6) 3.5	(27) 21.3	(102) 56.7	(77) 50.6
Intestinal tumours	(0) –	(0) –	(3) 2.3	(0) –	(0) –	(0) –	(0) –	(3) 2.4	(22) 12.2	(8) 5.3
Ano-genital tumours	(0) –	(0) –	(3) 2.3	(2) 2.0	(1) 1.0	(1) 0.6	(0) –	(3) 2.4	(6) 3.3	(8) 5.3
Renal tumours	(1) 0.7	(0) –	(1) 0.8	(0) –	(1) 1.0	(0) –	(0) –	(8) 6.3	(11) 6.1	(3) 2.0
Tumours of testes and appendices	(0) –	(0) –	(4) 3.1	(2) 2.0	(0) –	(0) –	(0) –	(2) 1.6	(3) 1.7	(11) 7.2
Other tumours	(3) 2.1	(1) 0.7	(3) 2.4	(2) 2.0	(2) 1.9	(1) 0.7	(1) 0.4	(4) 3.6	(8) 4.4	(6) 4.0

per litter determined (Table I).

Postnatal development of the offspring was observed by controlling the mortality rates over 22 months, the first 12 months of which are listed in Table II. Until ~9 months of age mortality in all groups was largely caused by malformation-related functional disorders, degenerative diseases and bacterial infections. The main diagnoses were diffuse oedema, haemorrhagic necrosis of liver parenchyma, hypoplasia of the brain, the kidneys and the dermis (purulent infections). Severe undernutrition was caused by abscesses at the jaws and teeth. Wastage of unknown origin also occurred and a few animals (~0.5%) were also lost by advanced autolysis. These observations of non-neoplastic diseases within the first 9 months were consequently not included into the final statistical evaluation.

The number of all animals still alive beyond 9 months which were to be pathologically evaluated with respect to tumours is shown at the end of Table II. These numbers were diminished by loss of some animals mostly due to advanced autolysis. The last column compiles the number of remaining animals which were used for analysis of the long-term effects.

Animals which appeared to be severely ill were killed under ether anesthesia. These animals were autopsied, as were the spontaneously dying ones, and were then examined histologically whenever no severe autolysis was apparent. The remaining mice were killed in the course of the 22nd month when mortality rate significantly increased and this would have been in conflict with a holiday period (see experiment I).

The animals were sectioned and when the general inspection was doubtful about the origin of certain tumours, also examined histologically by preparing one or two sections from the relevant tissues or organs. This procedure aimed to differentiate between primary tumours and metastatic nodules (e.g. in lungs or liver). If these histological observations led to additional tumour diagnoses, these were not included in the final analysis because of their only sporadic occurrence. The statistical evaluation is therefore based exclusively on the pathological anatomy at the time of autopsy. Further classification of tumours – as mentioned in the Results – was only a secondary aim without indicating any significant shifts within the subclasses of a given tumour type.

Statistics

The significance of the difference between the number of tumours or the number of tumour diagnoses per animal examined in two experimental groups was determined using the Kruskal–Wallis test and the *t*-test. To recognize a significant difference between two relative incidences of some tumour diagnoses, a contingency table analysis was performed. The probability of a significant difference was investigated by the χ^2-test.

Calculation of the interaction factor

In order to interpret correctly the tumour frequency in the animal groups with combined treatment, we calculated the interaction factors for the main diagnoses. This was done according to the proposals made by the United Nations Scientific Committee on the Effects of Atomic Radiation (9).

Results

Early postnatal effects

At the expected time of birth the litters were controlled every hour in order to collect stillbirths at an optimum rate. The number of stillbirths varies from zero to seven in the various groups (Table I), but did not show any relation to a definite treatment scheme. The mean litter sizes were all in roughly the same range and did not differ on account of the experiments.

The cumulative mortality rates of the live offspring revealed marked differences between the groups (Table II): X-irradiation on g.d. 15 alone did not change the mortality rates significantly from the controls, not even in the course of 12 months. In each series the ENU-treated animals (groups 3 and 8) showed a mortality rate of 19.6% within 12 months, which significantly differs from the control groups (1 and 6). The initial rise of the mortality incidence in group 3 to 13.6% even 1 week after ENU application remained obscure. ENU group 8, however, revealed a significant difference from the controls only after 6 months or more.

Combined treatment led to an immediate rise of mortality to

Table IV. Tumour incidences (%) in female NMRI mice after prenatal X-irradiation, after postnatal ENU application or after both treatments in combination

	Group									
	1	2	3	4	5	6	7	8	9	10
Autopsied animals	192	116	92	74	74	152	143	115	128	116
Leucosis	(16) 8.3	(0) –	(17) 18.5	(17) 23.0	(12) 16.2	(17) 11.2	(5) 3.5	(33) 28.7	(67) 52.3	(52) 44.8
Lung tumours	(29) 15.1	(30) 25.9	(85) 92.4	(73) 98.6	(74) 100.0	(30) 19.7	(25) 17.5	(93) 80.9	(108) 84.4	(110) 94.8
Liver tumours	(1) 0.5	(0) –	(13) 14.1	(16) 21.6	(4) 5.4	(1) 0.6	(2) 1.4	(18) 15.7	(48) 37.5	(46) 39.6
Intestinal tumours	(0) –	(0) –	(1) 1.1	(0) –	(0) –	(0) –	(0) –	(1) 0.9	(10) 7.8	(9) 7.8
Ano-genital tumours	(0) –	(0) –	(2) 2.2	(1) 1.3	(0) –	(0) –	(0) –	(0) –	(3) 2.3	(5) 4.3
Renal tumours	(0) –	(0) –	(0) –	(1) 1.3	(1) 1.3	(0) –	(0) –	(4) 3.5	(3) 2.3	(2) 1.7
Tumours of the uterus	(35) 18.2	(22) 19.0	(23) 25.0	(6) 8.1	(5) 6.8	(35) 23.0	(31) 21.7	(33) 28.7	(72) 56.3	(63) 54.3
Ovary tumours	(19) 9.9	(11) 9.5	(26) 28.3	(24) 32.4	(10) 13.5	(17) 11.2	(15) 10.5	(28) 24.3	(40) 31.3	(39) 33.6
Other tumours	(2) 1.0	(3) 2.6	(7) 8.0	(4) 5.2	(4) 5.4	(2) 1.2	(2) 1.4	(8) 7.0	(10) 8.5	(8) 6.8

19.0% and 13.7% after prenatal X-irradiation with 0.8 Gy and 1.6 Gy respectively (groups 4 and 5). Within 12 months 32.1% and 30.9% respectively of the offspring died. In contrast, pretreatment with 0.2 Gy or 0.4 Gy (groups 9 and 10) at no time led to a mortality rate above those animals with only X-irradiation (group 7) or with ENU treatment (group 8).

Tumour incidences within the 22-month observation period (Tables III and IV)

Leucosis. This disease complex comprises mainly cases of lymphatic leukaemia, but also some myeloid leukaemias and reticulosarcomas. The incidence was significantly ($P < 0.01$) decreased in both sexes after prenatal X-irradiation with 0.8 Gy (group 2) and after 0.2 Gy (group 7) in the female offspring only. In both series, ENU proved to be a potent inducer of leucosis with no apparent preference for one sex. The frequency was almost the same in those animals with a prenatal X-ray dose of 1.6 Gy (group 5), and increased insignificantly after pretreatment with 0.8 Gy (group 4). Further subdivision of the X-ray dose to 0.4 Gy resulted in a drastic increase in the leucosis rate to 34.9% in males and 44.8% in females ($P < 0.001$). These values increased once again after 0.2 Gy X-irradiation pretreatment, resulting in 55.6% incidence in males ($P < 0.01$ versus group 10) and 52.3% ($P = 0.5$) in females. Analysis of the interaction factors revealed the presence of synergistic effects not only in the combination 0.8 Gy plus ENU (group 4) but even more so after 0.2 Gy plus ENU (group 9, Table V). The latter factors were 2.34 for male and 4.19 for female offspring.

Lung tumours. These comprise mainly alveologenic adenomas. The lung tumour frequency is apparently not constant in our stock. In the earlier series 12.2% of the males and 15.1% of the females revealed an involvement of the lungs. In the series starting 1 year later, however, 26.4% of the males and 19.7% of the females were found to be lung tumour bearers. Accordingly, an increase to 24.0% in males and 17.5% in females after 0.2 Gy X-irradiation, as well as to 32.4% (male) and 25.9% (female) after 0.8 Gy, cannot be regarded as a significant result in groups 2 and 7. In contrast to this, the ENU effect upon the lungs, amounting to 97.7% and 94.5% in males and 92.4% and 80.9% in females, differs significantly ($P < 0.001$) from the controls and X-irradiation experiments. Combination of both types of treatment did not change the lung tumour frequency markedly, irrespective of the X-irradiation dose (groups 4, 5, 9 and 10) and without any synergistic effect (Table V).

Liver tumours. Most of the tumours of the liver have been hepatocellular adenomas and hemangiomas. Prenatal X-irradiation (0.2 Gy) nearly doubled the liver tumour rate from the controls

Table V. Interaction factors for the late effects after combined treatments with prenatal X-irradiation and postnatal ENU application

	0.8 Gy plus ENU		0.2 Gy plus ENU	
	Male	Female	Male	Female
Leucosis	1.57	1.44	2.34	4.19
Lung tumours	0.81	0.95	0.97	1.10
Liver tumours	1.46	1.61	2.60	2.32
Intestinal tumours	NA	NA	5.18	8.67
Ano-genital tumours	0.86	0.59	1.55	NA
Renal tumours	NA	NA	0.96	0.65
Tumours of testes	0.65	–	1.06	–
Uterus tumours + hyperplasias	–	0.05	–	7.57
Ovary tumours	–	1.25	–	1.63
Other tumours	0.10	0.49	1.42	1.22

NA = not applicable.

in both sexes to 3.5% and 1.4% respectively. This was significant at a level of $P = 0.2$. ENU-induced liver tumours occurred at a rate of 21.3–23.3% in male and 14.1–15.7% in females ($P < 0.001$). In the combination experiments 1.6 Gy X-irradiation led to a significant depression of the hepatoma frequency to 7.2% in males and 6.5% in females ($P < 0.001$). In contrast, 0.8 Gy X-irradiation of the foetuses led to a significant increase to 32.6% (male; $P < 0.05$) and 14.1% (female; $P < 0.1$). Halving the irradiation dose (group 10) had a remarkable effect, the hepatoma rate increasing to 50.6% in the males ($P < 0.01$ versus group 4) and 39.6% in the females ($P < 0.001$ versus group 4). Lowering the prenatal X-ray dose to 0.2 Gy (group 9) further increased the hepatoma rate in the ENU-treated male offspring (56.7%), whereas the liver tumour incidence in the female counterparts remained almost constant (37.5%). The interaction factor for these synergistic effects was 2.60 for males and 2.32 for females respectively (Table V).

Gastro-intestinal tumours. These consisted mainly of squamous cell carcinomas, differentiated and anaplastic adenocarcinomas, reticulum cell sarcomas, as well as unclassified sarcomas. Gastrointestinal tumours were found only in some experimental groups. This condition was seen neither in the controls nor after prenatal X-irradiation alone. ENU treatment of the offspring at 21 days led to an incidence of 2.3–2.4% in the males and 0.9–1.1% in the females. Within the first experimental series no gastrointestinal tumours were found in combination groups 4 and 5. However, in series II pretreatment of the mice with 0.4 Gy (group 10) led to a tumour frequency of 5.3% (male) and 7.8% (female) respectively. After 0.2 Gy prenatal X-irradiation this frequency

remained constant in the females (7.8%), whereas a significant ($P < 0.01$) rise was seen in male animals to 12.2%. The interaction factors for the combined effect of 0.2 Gy X-irradiation plus ENU were calculated to be 5.18 in males and 8.67 in females (Table V).

Ano-genital papillomas. By this term we compiled observations of squamous cell carcinomas, anaplastic carcinomas, papillary adenomas and adenocarcinomas, as well as muco-epidermoid carcinomas. ENU treatment induced ano-rectal tumours in 2.3% of the male animals. Another two cases (= 2.2%) of squamous cell carcinoma were seen in female mice after ENU treatment. Prenatal X-irradiation with 0.4 Gy plus postnatal ENU induced these tumours in 5.3% of all males and 4.3% of all females ($P < 0.05$). After 0.2 Gy X-irradiation pretreatment these tumours occurred in only 2.3% and 3.3% of male and female animals respectively. The interaction factor (Table V) for male mice was thus 1.55, while for females this analysis was not applicable.

Renal tumours. We observed cystic papillary adenomas, solid epithelial tumors and renal sarcomas. ENU alone induced eight renal tumours in males (= 6.3%, group 8) and four tumours (= 3.5%) in females. In the combination experiments the frequency of renal tumours was significantly decreased to 2.0% (male) and 1.7% (female) ($P < 0.05$) in those animals with an X-irradiation dose of 0.4 Gy (group 10). An irradiation dose of 0.2 Gy (group 9) did not change the potency of ENU alone (6.1% in males and 2.3% in females).

Tumours of the male testes. These tumours mainly derived from the Leydig interstitial cells. ENU induced four Leydig tumours in group 3 (= 3.1%) and two in group 8 (= 1.6%). Prenatal X-irradiation with 0.8 Gy or with 1.6 Gy plus ENU proved to be of no significant effect. When the dose was reduced to 0.4 Gy in these combination experiments (group 10), 7.2% of the males had Leydig tumours and seminomas at autopsy ($P < 0.001$). However, those animals treated with an X-ray dose of 0.2 Gy plus ENU revealed testicular tumours at a low incidence of 1.7%, which was within the control level.

Tumours of the uterus. We observed endometrial hyperplasias and/or adenomas, some cases of adenocarcinomas, as well as leiomyomas and sarcomas. Uterus tumours occurred in 18.2–23.0% of all controls, depending on the experimental period. This finding was not altered by X-irradiation (0.2 Gy or 0.8 Gy) and was increased only insignificantly by ENU to 25.0% and 28.7% respectively. Prenatal X-irradiation with 1.6 Gy or 0.8 Gy plus subsequent ENU application drastically reduced the frequency of this finding to 6.8% ($P < 0.001$) and 8.1% ($P < 0.001$) respectively. In contrast, lowering the X-ray dose to 0.4 Gy (group 10) or to 0.2 Gy (group 9) led to a nearly 2-fold increase (54.3% to 56.3% respectively; $P < 0.001$). Providing an interaction factor of 7.57, the latter result proved to be a genuine synergistic effect (Table V).

Ovary tumours. Herein we compiled all cases of granulosa cell tumours, luteomas, cystadenomas, tubular adenomas and angiomas. In control animals these tumours occurred at an incidence of 9.9–11.2%. Prenatal X-irradiation alone irrespective of the dose showed no intensifying effects in this case. However, ENU induced tumours at a significant rate of 24.3–28.3% ($P < 0.001$). Prenatal X-irradiation with 1.6 Gy prior to ENU treatment had negative effects upon ovary tumour development, which occurred only in 13.5% of all females ($P < 0.01$). The tumour incidence rates of the other three doses in combination with ENU (0.8 Gy, 0.4 Gy and 0.2 Gy) are in the range 31.3–33.6%. This increase is of only minor significance ($P < 0.05$ versus group 8; $P < 0.1$ versus group 3). The interaction factors were nevertheless calculated at 1.25 (group 4) and at 1.63 (group 9), which is indicative of a mild synergistic effect (Table V).

Other tumours. These comprise cases of skin papilloma, hemangiosarcoma, osteosarcoma, mammary carcinosarcoma, Harderian gland adenoma, myosarcoma and Schwann cell tumour. Tumours of these types amounted to 0.7–2.1% in male controls and 1.0–1.2% in the females. X-irradiation with 0.2 Gy (group 7) or 0.8 Gy (group 2) apparently had no significant influence. ENU application alone increased the frequency of these tumours to 2.4–3.6% ($P < 0.1$) in male animals and to as much as 7.0–8.0% ($P < 0.01$) in the females. In combined treatment with 0.8 Gy or 1.6 Gy the tumour rate of the males remained unchanged, whereas that of the females was lower than after ENU alone (5.2% and 5.4%, respectively). In both sexes the interaction factors were significantly below 1.0 (Table V). By lowering the X-ray doses to 0.2 Gy and 0.4 Gy respectively the tumour outcome is moderately increased in males to a consistent 4.4% ($P < 0.5$) and in females to 8.5% and 6.8% with each of the two doses ($P < 0.5$). These results reflect a moderate synergism with interaction factors of 1.42 for males and 1.22 for females (Table V).

Discussion

In view of our aim of observing a sufficient number of mice–thus avoiding statistical problems–some difficulties arose in performing these experiments. The size of the study was in contrast to our animal facilities. We therefore started the experiments with prenatal X-irradiation doses of 0.8 Gy. This is sufficient to prevent overkill phenomena in perinatal carcinogenesis studies, as have been described in some reports (2,3,10) and were confirmed again in the present experiments by combining prenatal treatment with 1.6 Gy and postnatal ENU application.

After an increase in leucosis frequency in the animals with 0.8 Gy plus ENU treatment (group 4) had become evident at the beginning of 1985 we decided to carry out a second series of experiments. Treatment with 0.4 Gy plus ENU as well as 0.2 Gy plus ENU was performed to outline the lower limits of possible synergistic effects. To ensure a suitable standard of accuracy another series of control animals and ENU treatment only seemed necessary. With respect to the capacity of the animal housing facilities we dispensed with testing the late effects of prenatal X-irradiation with only 0.4 Gy. This was justified by the recently reported negative results on solid tumour incidence in NMRI mice (3,4) and C57 × C3H mice after X-irradiation *in utero* on g.d. 16 (according to our pregnancy staging) with doses between 0.3 Gy and 0.9 Gy (11).

Our study reveals some opposite effects of prenatal X-irradiation as regards the carcinogenic response of the juvenile animal to ENU. In general, pretreatment of mice with 1.6 Gy on g.d. 15 significantly depresses ENU effects throughout the whole tumour spectrum, with the exception of leucosis incidence and lung tumour frequency. Similarly, earlier studies (2,3) showed that the latter tumour spectrum remained at a constant rate irrespective of the application of 3 × 0.8 Gy X-irradiation at late organogenesis stage alone, or in combination with phorbol ester treatment of the offspring. This consistency clearly contradicts other observations on synergism in the leukaemogenesis of radiation and various chemicals (12). However, as the latter synergistic effects are exclusively described

in rather low irradiation dose ranges, we assume that overkill effects are responsible (10) for the constant leucosis rate and lung tumour rate in our experiments with 1.6 Gy.

Reduction of the prenatally applied X-irradiation doses to 0.4 Gy and 0.2 Gy effectively augments the tumour response in female offspring after postnatal treatment with ENU. This response includes leucosis incidence, intestinal tumours, as well as tumours of the ovaries and uterus. With regard to leucosis and the intestinal tumours, an increased tumour frequency can also be observed in male offspring as a result of the combined treatment, albeit at a markedly lower level. The only exception is the increased liver adenoma frequency in both sexes. This preference of tumour induction in females is surprising. An analogous sex dependency in tumour development has so far been described only in one study (10), using DMBA as the prenatal initiation stimulus and TPA as the postnatal promoting agent within a two-stage carcinogenesis experiment.

The only experiments comparable with the present ones have been conducted by Nomura (13). He applied single prenatal X-irradiation (0.36 Gy) on various gestation days to ICR mice and treated the offspring with urethane at 21 days of age. Consequently, his study only examined lung tumour development. Although the number of animals observed by Nomura was quite small, he nevertheless found a marked decrease of lung sensitivity to postnatally induced carcinogenesis when X-irradiation was performed after g.d. 14. This observation is confirmed by the present study with X-irradiation of g.d. 15. The lungs proved to be rather hyposensitive to the postnatal ENU action. This is reflected by an interaction factor invariably ranging around 1, but with a tendency towards values <1. In contrast to this effect, other tumour responses such as those of the gastro-intestinal tract were significantly augmented. Induction of these tumours by ENU itself either by pre- or postnatal application is a rare event in rodents (5,6), but has been reported to occur at a rather high rate in B10A mice due to their specific genetic background (14). The fact that we observed a marked increase in gastro-intestinal cancers only in the second series of experiments—which included ENU-treated groups identical with the first series—definitely outlines a synergistic action of X-ray doses between 0.2 and 0.4 Gy. The question also arises as to the general conditions under which the mice were used within the second series, especially if we consider the occurrence of ano-genital tumours. Such tumours are known to show a distinct correlation with an immunodeficient status of animals and man (15), as well as with the presence of papillomaviruses (16). Both conditions can be induced or aggravated by prenatal X-irradiation (17) or by perinatal exposure to chemicals (18), either separately or in mutual interdependency (19). Therefore it cannot be excluded that the mice from the 1985 series latently carried viruses, which after radiation-related activation (20) co-operated with the ENU action towards a process of syncarcinogenesis. In principle, this argument may also explain the outstanding frequency of hyperplasias and neoplasias of the uterus in the combined treatment of series II. NMRI mice are apparently predisposed for this pathological condition of the uterus (1,21). Their spontaneous uterine tumour rate is not altered by foetal X-irradiation (4,22,23) and only marginally increased by ENU application to infantile mice (5,6). Verdeal *et al.* (24) recently found endometrial adenomatous hyperplasia and carcinoma as a late effect of methylnitrosourea application to juvenile rats. A similar pathological picture was also produced in BALB/c mice by neonatal treatment with diethylstilbestrol (DES) (18). Interestingly, those authors describe a strictly parallel pattern between the DES-induced immune suppression in young adults and the subsequent occurrence of preneoplastic and neoplastic uterus lesions. It is striking to note that some lesions were induced in our experiments with low-dose X-irradiation plus ENU treatment. Endometrial pathological conditions are described (24) to involve frequently ovary tumours. In fact, their incidence was also significantly increased in the combination experiments in series II.

In conclusion, our long-term study revealed an outstanding sensitivity of several organs and tissues to neoplastic transformation subsequent to low-dose X-irradiation during the fetal period. In terms of their magnitude, the present results are comparable with the inducibility of neoplasms in beagle dogs by low γ-irradiation doses alone (0.16–0.83 Gy) during the perinatal stage (25). In this study irradiation itself proved to be an effective carcinogen in various organ systems such as the lymphohaemopoietic tissues. A similar conclusion was drawn by Shiono *et al.* (26) for human childhood neoplasia incidence after analysing data from 56 000 mothers and their children in relation to postconception X-ray exposure. Our investigation, however, did not show a neoplasm-inducing effect of X-irradiation alone in the low-dose range. In agreement with several other studies (1–4,7,11,22,23) it appears that the mouse system is too insensitive for demonstrating whole-body irradiation effects similar to those in dogs (25) in a dose range <0.5 Gy (3). However, under environmental and medical aspects it seems more important to us to outline a persisting disposition throughout postnatal life for effectively reacting carcinogens. Thus, the present experiments are to be regarded as an effect-amplifying system of lesions which are otherwise hardly demonstrable.

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