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Carcinogen Bioassay of Enflurane in Mice

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A carcinogen bioassay of enflurane was performed in Swiss/ICR mice. Two groups of weanling mice, each of 125 males and 125 females, were exposed to either a maximum tolerated dose of enflurane, 3000 ppm (0.3 per cent v/v) or compressed air for 4 hs per day, 5 days per week. After 52 weeks exposure, 25 males and 25 females from each group were killed. After 78 weeks exposure, a 4-week period without treatment was allowed before the remaining mice were killed. Mice killed at the scheduled periods and those killed or dying at other times throughout the study, underwent extensive gross and histologic examinations unless autolysis or cannibalism precluded examination. After 78 weeks exposure, male mice in the enflurane-treated group had a 36 per cent incidence of liver tumors compared with 24 per cent in the control group; however, the difference was not statistically significant. The incidence of lung tumors was about 20 per cent in all groups. Other neoplastic lesions occurred in small numbers and were unrelated to treatment. It was concluded that under the conditions of the present experiment, chronic administration of enflurane at its maximum tolerated dose did not lead to an increased incidence of neoplasia in Swiss/ICR mice. (Key words: Anesthetics, volatile: enflurane. Cancer. Toxicity: carcinogenecity; subanesthetic concentrations.)

ENFLURANE, introduced into clinical practice in the United States in 1974, is now the most frequently used potent inhalational anesthetic agent. As with other such drugs, there is concern that trace concentrations of enflurane pose a carcinogenic risk for operating room personnel. Because of the long latency period, often 20 years or more between exposure to a carcinogen and the appearance of tumors, epidemiologic surveys at this time would be unlikely to suggest a relationship between enflurane exposure and tumor formation, even if one were to exist. In lieu of reliable human data, animal studies provide the most definitive means of establishing a carcinogenic hazard. An accepted protocol for conducting *in vivo* studies is to expose animals for most of their life span to the test agent using a route of administration similar to that experienced by humans.[1] In the present study, such a protocol was used to examine the carcinogenic potential of enflurane in mice.

Materials and Methods

Two hundred and fifty male and 250 female, 3-week-old Swiss/ICR mice (Hilltop Labs, Chatsworth, California) were kept in quarantine for 1 week. They were individually ear tagged and divided randomly into two groups, each containing 125 males and 125 females. Throughout the experiment, they were housed not more than four to a cage and were bedded on ground corn cob (San-I-Cell®; Paxton Processing Company, Paxton, Illinois) in polypropylene plastic cages with zinc-coated lids. They were fed small animal chow (Wayne Lab Blox®, Allied Mills, Inc., Chicago, Illinois) and allowed to drink tap water *ad libitum* except during the daily treatment period when food and water were removed. There was a fixed diurnal cycle of 12 h light and 12 h darkness, with exposure during the light phase. All mice were inspected daily for disease and were weighed at least every 4 weeks. Group 1 (control group) was exposed to compressed air and group 2 to 0.3 per cent (3000 ppm v/v) enflurane for 4 h daily, 5 days per week. Both groups were exposed at the same time each day. This regimen was established in preliminary studies as one that provided the maximum tolerated dose (MTD) of enflurane, *i.e.*, it did not cause greater than a 10 per cent loss of body weight or early death.

Animal cages were placed randomly in the chamber. Enflurane was vaporized in a copper kettle with medical grade compressed air and was delivered at a flow of 10 l/min through rubber tubing. Uniform vapor concentration was maintained by a high volume recirculation fan. Enflurane concentration was determined at 5–15-min intervals using a Varian 1440 Gas Chromatograph® and was maintained within 10 per cent of the desired value. Chamber temperature was maintained at 25 ± 2°C, humidity at 50 ± 10 per cent and carbon dioxide concentration at <0.3 per cent. Air flow and chamber conditions were kept the same for both chambers. After 52 weeks of exposure, 25 males and 25 females selected randomly from each group were killed. After 78 weeks of exposure, a 4-week period without treatment was allowed following which the remaining animals were killed by carbon dioxide exposure.

All animals killed at the scheduled time or dying or killed in extremis at other times, were subjected to complete autopsy examinations. The only exceptions were eight mice in which cannibalism or advanced autolysis precluded examination. The procedures used for the gross autopsy are the same as those recommended by the National Cancer Institute[2] and have previously been described.[3] More than 40 tissues were examined *in situ*, then dissected from the carcass, incised and reexamined before being fixed in 10 per cent buffered formalin. Liver, spleen, and kidneys were weighed fresh. After fixation,

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TABLE 1. Number of Animals in the Cancer Study

	Start	Unscheduled Deaths	Exposed 52 weeks	Exposed 78 weeks
Control				
Male	124	25	25	74
Female	124	20	25	79
Enflurane-treated				
Male	123	20	25	78
Female	124	13	25	86

tissues were again examined grossly and 32 routine sections from different organs and all abnormal tissues were obtained for microscopic examination. Because of the overall high incidence of liver tumors in males found during autopsy examinations, an independent observer outside of the team of investigators and experienced in the examination of mouse liver tumors, was asked to reexamine the livers of male mice. Microscopic examinations were performed by a pathologist experienced in mouse histology. Examiners were unaware of the treatment groups of the mice at the time of all examinations. Consultation was sought from other pathologists when a histopathologic diagnosis was in doubt. In particular, several sections were sent to the Armed Forces Institute of Pathology (AFIP) for examination.

Intergroup comparisons were made using Student's t test, chi-square analysis, or Fishers' exact test as appropriate; $P < 0.05$ was considered statistically significant.

Results

Mean body weights of enflurane-treated mice were slightly but significantly lower than corresponding controls throughout the study (fig. 1). The difference for both males and females was about 5 per cent until animals were 55 weeks old, then it slowly increased to about 10 per cent by the end of the study (86 weeks old). The appearance and behavior of enflurane-treated mice were generally the same as control mice. A low incidence of eye and nasal discharge, skin ulcers, and alopecia was noted in all groups, and increased as the animals aged.

The numbers of animals dying or killed in extremis or killed at the scheduled times are shown in table 1. Five mice, two from the control and three from the enflurane group, failed to thrive during the first month of exposure and were eliminated from the study. There were a number of unscheduled deaths. The main reason for killing animals at times other than that scheduled was extensive skin infection, usually secondary either to trauma from fighting or from mites. Other animals dying prematurely suffered most often from overwhelming infection with terminal pneumonia. The numbers of unscheduled deaths in the two groups were not statistically different.

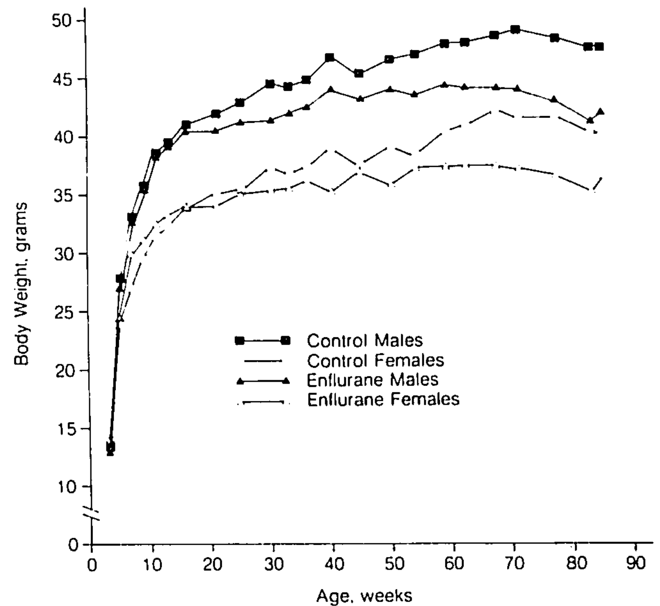

FIG. 1. Enflurane carcinogenicity: Mean body weights for each group of mice throughout the experiment. The enflurane-treated animals weighed about 5 per cent less than control group until animals were 55 weeks old; the difference increased to about 10 per cent by the end of the study (86 weeks old).

Generally, organ weights from enflurane-treated mice, recorded at the time of killing, were slightly less than those from control mice (table 2). When comparisons were made using organ/body ratios, only spleen weights from males exposed for 52 weeks remained significantly less than control mice.

Gross and microscopic examination of tissues revealed a variety of non-neoplastic lesions. These included small

TABLE 2. Weights in Grams (Mean ± SEM)

	Exposed 52 weeks				Exposed 78 weeks			
	Enflurane (male)	Control (male)	Enflurane (female)	Control (female)	Enflurane (male)	Control (male)	Enflurane (female)	Control (female)
Body	44.5 ± 0.6†	48.2 ± 0.8	38.3 ± 0.8	38.9 ± 0.9	42.0 ± 0.5†	47.5 ± 0.7	36.7 ± 0.4*	39.8 ± 0.5
Liver	2.6 ± 0.1	2.7 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.0 ± 0.01	2.2 ± 0.01
Spleen	0.12 ± 0.01†	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.20 ± 0.03	0.21 ± 0.03	0.25 ± 0.02
Right kidney	0.45 ± 0.01*	0.49 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.45 ± 0.01†	0.51 ± 0.02	0.30 ± 0.02	0.33 ± 0.01
Left kidney	0.47 ± 0.02	0.48 ± 0.02	0.30 ± 0.01	0.30 ± 0.01	0.46 ± 0.03	0.50 ± 0.02	0.29 ± 0.02	0.32 ± 0.01

* Significantly less than control, $P < 0.05$. † Significantly less than control, $P < 0.01$.

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TABLE 3. Number of Mice with Tumors

	Unscheduled Deaths*				Exposed 52 weeks				Exposed 78 weeks			
	M-Con (n = 22)	M-Enf (n = 18)	F-Con (n = 19)	F-Enf (n = 11)	M-Con (n = 25)	M-Enf (n = 25)	F-Con (n = 25)	F-Enf (n = 25)	M-Con (n = 74)	M-Enf (n = 78)	F-Con (n = 79)	F-Enf (n = 86)
Pulmonary adenomas	1	1		2	5	2	2	4	18	20	13	19
Liver tumors						2			18	28	2	
Lymphosarcomas	1		2	1						2	2	2
Other malignant tumors		2		2					1	1	2	3
Other benign tumors								1	1			

Abbreviations: M-Con = male control; M-Enf = male enflurane; F-Con = female control; F-Enf = female enflurane.

* n for unscheduled deaths is based on the number of animals that underwent autopsy examinations.

ovarian cysts, lymphadenopathy, large mottled spleens, bladder stones, and myocarditis; none was related to enflurane treatment. The first neoplastic lesion, a lymphosarcoma in a control female mouse, was observed 41 weeks after the start of the experiment. Most neoplasms recorded subsequently were either lung adenomas or liver tumors (table 3). Lung adenomas were distributed equally between groups, were often visible grossly as subpleural nodules, and were of alveolar cell origin (fig. 2). Occasionally, smaller adenomas were located deeper in the lung parenchyma. By the morphologic classification of Frith and Ward,[4] most liver tumors were basophilic hepatocellular adenomas (fig. 3). The hepatic architecture was disrupted by a nodular monomorphous proliferation of cells that had a strong resemblance to normal hepatocytes. In general, the tumor cells were smaller, more basophilic, and contained more cytoplasmic vacuoles than normal hepatocytes. Mitotic figures were easily identified but the nuclei were round and regular and did not show pleomorphism. A reliable finding was the sharp line of demarcation between the tumor cells and the surrounding compressed hepatic parenchyma. Although enflurane-treated male mice exposed for 78 weeks had a slightly higher incidence of liver tumors (36 per cent) compared with control mice (24 per cent), the difference was not statistically significant ($P = 0.19$). The incidences of liver tumors in all enflurane-treated and control male mice autopsied throughout the study were 25 per cent and 15 per cent, respectively; again, the difference was not statistically significant ($P = 0.08$). The presence of small numbers of benign and malignant tumors in other tissues was not related to enflurane treatment.

Discussion

The carcinogenic potential of enflurane has been examined in only one other animal study. In 1978, Eger *et al.*[5] reported that Swiss/ICR mice exposed to enflurane in concentrations up to 1 per cent v/v did not have

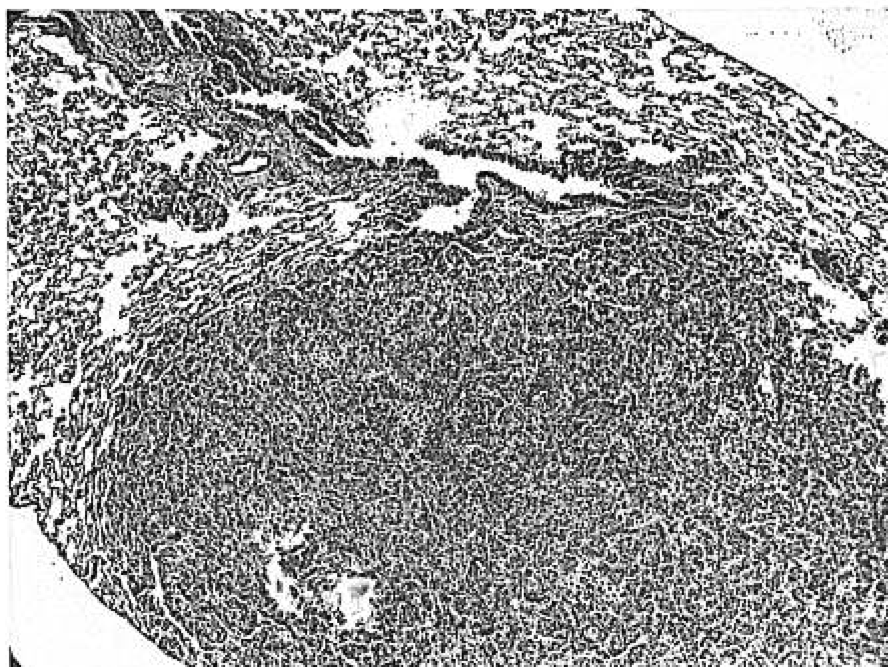

FIG. 2. Typical pulmonary adenoma. This subpleural tumor, which is compressing the surrounding normal lung, is of alveolar cell origin.

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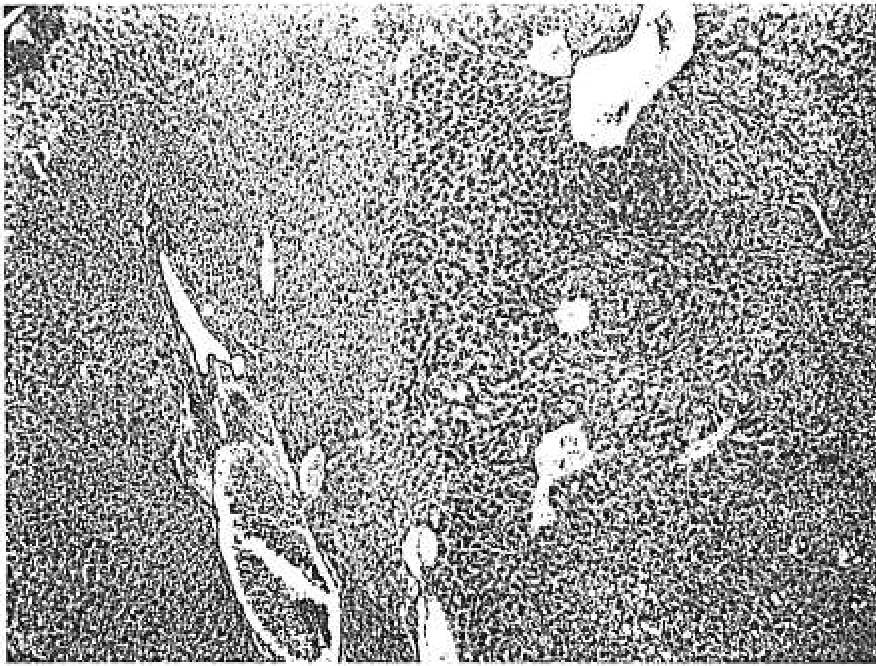

FIG. 3. Typical adenoma of the liver. The tumor is seen in the darker half of the photomicrograph. It contains small basophilic cells, many of which contain more cytoplasmic vacuoles than normal hepatocytes. There is a sharp line of demarcation between the tumor cells and the surrounding compressed hepatic parenchyma.

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an increased tumor incidence compared with unexposed control mice. Animals were exposed for 2 h per day, four times while *in utero* and 24 times during the first 3 months of life. They were left unexposed for the next year. At 15 months of age, the mice were killed, autopsied, and examined for neoplastic lesions.

Our results are in agreement with those of Eger *et al.* There are several reasons, however, why the design of the present study leads to greater confidence in the negative results we obtained. First, the cumulative dose of enflurane we delivered to animals was much greater, 234 MAC hours compared with 28 MAC hours. In fact, our exposure regimen provided the maximum tolerated dose of enflurane for Swiss/ICR mice since larger doses could not be given without risk of serious toxicity. Second, our animals were exposed to enflurane for most of their lives. Such long-term exposure is necessary to accurately assess the risk of contracting some types of tumors. For example, the induction of bladder tumors in mice chronically fed 2-acetylaminoflurene (2-AAF) is a continuous process throughout the animals life span.[6] Thus, if tumor induction has not occurred by the time that 2-AAF is discontinued, it probably will not occur. If enflurane induces tumor formation in a similar way, administering it only during the early part of an animal's life would be an insensitive method for detecting its carcinogenic potential. A third reason for the increased sensitivity in our study is that mice underwent autopsy examinations at 20 rather than 15 months of age. In the 2-AAF study, serial sacrifices established that liver tumors appeared very late in life, although induction took place early in life.[7] That is, unlike the case with bladder tumors, removal of the carcinogen following the initial period of exposure did not greatly reduce the incidence of hepatocellular neoplasms. Most liver tumors occurred after 18 months of age, so that only data from animals autopsied after that time showed 2-AAF to be a hepatocarcinogen.

Finally, a comparatively large number of mice were included in each group (250 *vs.* about 100) with at least 153 remaining in each group exposed for 78 weeks. Use of greater numbers increases the statistical power of a study, *i.e.*, the probability of detecting a treatment related difference. Power also is increased when the background incidence of a particular tumor is low. For example, in the present study, we would expect to confidently detect small treatment-related increases in the numbers of lymphosarcomas and carcinomas of the breast, because their overall background incidence was low. Unexpectedly, the background incidence of pulmonary adenomas and liver tumors was 24 per cent for males exposed for 78 weeks. Under these circumstances, it is calculated that an increase in tumor incidence to about 50 per cent would be needed before we could be 80 per cent confident of detecting an enflurane-related effect.[8] Prior calculations of the power of the present study were based on a 10 per cent incidence of lung and liver tumors in control Swiss/ICR mice observed in previous studies.[5] The present finding that the background incidence of these tumors was much higher reemphasizes the importance of including concurrent controls during animal carcinogenicity studies.

There have been three other controlled carcinogenicity studies of modern anesthetics administered by the inhalation route. The first by Eger *et al.* has already been described in relation to the carcinogenic potential of enflurane.[5] In that same study, isoflurane, halothane, methoxyflurane, and nitrous oxide also were examined. Under the limited conditions of that experiment, they were not carcinogenic. Coate *et al.*[9] reported on the tumor incidence in Fischer-344 rats following prolonged exposure to low concentrations of combinations of halothane and nitrous oxide. Rats were exposed either to halothane, 1 ppm and nitrous oxide, 50 ppm, or to halothane, 10 ppm, and nitrous oxide, 500 ppm, for 7 h per day, 5 days per week for 104 weeks; there was no enhancement of the spontaneous tumor rate in animals exposed to the anesthetics. Finally, Baden *et al.*[3] showed that the tumor incidence in Swiss/ICR mice exposed for 18 months to a maximum tolerated dose of halothane was not increased above control. Thus, at the present time, there is no convincing experimental evidence that modern inhalation anesthetics are carcinogenic.

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