Carcinogenicity of Halothane in Swiss/ICR Mice

Jeffrey M. Baden, M.B., B.S.,* Richard I. Mazze, M.D.,† Robert S. Wharton, M.D.,* Susan A. Rice, Ph.D.,‡ Jon C. Kosek, M.D.§

A simplified in-vivo bioassay system was used to test the carcinogenic potential of halothane in Swiss/ICR mice. Halothane was tested only at its maximum tolerated dose, and histologic examination was performed only on tumor masses and other grossly abnormal tissues found at necropsy. Two groups, each of 15 timed pregnant mice, were exposed to either halothane, 500 ppm (0.05 per cent), or compressed air for two hours on days 10–19 of pregnancy. Five days after birth the offspring were similarly exposed, three times weekly, for 78 weeks. After a ten-week, no-treatment, observation period, all remaining mice were examined by necropsy. Mice dying or killed in extremis before final sacrifice at 88 weeks of age also underwent complete gross necropsy unless extensive cannibalism or autolysis precluded examination. The incidences of malignant tumors, hepatomas or nodular hyperplasias, and benign tumors in halothane-treated mice were 7, 6, and 20 per cent, respectively; there were similar incidences of these lesions in control animals. It is concluded that under the conditions of this experiment, lifetime administration of halothane at its maximum tolerated dose is not associated with an increased incidence of neoplasia in Swiss/ICR mice. (Key words: Anesthetics, volatile; halothane. Cancer. Toxicity; carcinogenicity; trace concentrations.)

Malignancies due to chronic inhalation of chemical compounds have been recognized for many years.1-2 It is only in the last few years, however, that epidemiologic surveys have suggested that the operating suite may be an environmentally hazardous location.3-5 A recent study by Corbett resulted in further concern when he reported an increased incidence of hepatic tumors in Swiss/ICR mice exposed to isoflurane.6

The National Cancer Institute (NCI), in its published guidelines for carcinogen bioassay in small rodents,7 recommends that a chronic study be commenced only after preliminary acute and subchronic toxicity studies have been completed. It also suggests that two animal species and at least two dosage levels be employed, and that 50 animals be the minimum group size. To accomplish this, at least 600 animals are needed to study a single drug. Furthermore, the NCI recommends histologic examination of 32 tissues from each animal in the chronic study, a total of more than 18,000 examinations. The space, equipment and personnel needed to accomplish such a project are formidable and are outside the realm of practicality for all but a few commercial laboratories. The estimated cost of testing a single drug is in excess of $600,000.§ Such an enormous expense could hamper the development of new inhalational anesthetic agents and add to the cost of existing agents. We have, therefore, designed and tested a less complicated and more cost-effective protocol than that recommended by the NCI. Halothane, the most commonly used potent inhalational anesthetic, was selected for the initial study.

Materials and Methods

Thirty day-7 timed-pregnant Swiss/ICR mice** were quarantined and observed for three days, then randomly divided into two treatment groups of 15 animals each. Throughout the experiment, mice were bedded on ground corn cob†† and housed in polypropylene plastic cages with zinc-coated lids. They were fed small-animal chow‡‡ and allowed to drink tap water, ad libitum.

Group I mice (control) were exposed to compressed air and Group II mice to halothane,§§ 500 ppm (0.05 per cent; 1/20th MAC), vaporized in air for two hours per day, on days 10–19 of pregnancy. Preliminary subchronic studies had indicated that this was the maximum halothane dosage that did not result in more than 10 per cent body weight loss after 60 days’ exposure. On days 19 and 20, halothane-treated and control mice delivered 129 and 100 offspring, respectively. During the first month of life, approximately 20 mice in each group died, primarily due to gastrointestinal infection. To insure that there would be an adequate number of animals remaining at the end of the study, additional mice, treated exactly as described above, were added to each group two months

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* Assistant Professor of Anesthesiology.
† Professor of Anesthesiology.
‡ Research Associate in Anesthesiology.
§ Professor of Clinical Pathology.

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Address reprint requests to Dr. Baden: Anesthesiology Service (112A), VA Hospital, 3801 Miranda Avenue, Palo Alto, California 94304.

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§ L. Rampy, Ph.D., Dow Chemical Company, Midland, Michigan: Personal communication.
** Hilltop Labs, Chatsworth, California.
§§ Ayerst Laboratories, New York, New York.

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later. Final numbers of halothane-treated and control offspring were 161 and 120, respectively.

Five days after birth, exposure of offspring for two hours per day, three times weekly, commenced. Treatment continued until mice were 78 weeks old, and was followed by a ten-week observation period without treatment before sacrifice.

Exposures were performed in two air-tight, stainless steel and plexiglass chambers, each of 15001 capacity. One chamber was used exclusively for the control group, the other for halothane exposure; both groups were exposed at the same time each day. Animal cages were placed randomly in the chambers. Halothane was vaporized with medical-grade compressed air using a Fluotec Mark II vaporizer and was conducted to the chamber with rubber tubing. An electric fan at each end of the chamber insured uniform distribution of halothane vapor as ascertained by gas chromatographic sampling of the atmosphere from different areas. Food and water were removed from animal cages during the treatment periods.

Halothane concentration in the chamber was determined using a Varian 1440 gas chromatograph and was checked every minute until it reached 500 ppm, usually by the fifth minute. After equilibrium had been achieved, analyses were performed every 15 min. Throughout exposure, halothane concentration, temperature, and humidity were maintained at 500 ± 25 ppm, 25 ± 2 °C, and 50 ± 10 per cent, respectively. At the end of each exposure, the halothane-treatment chamber was exhausted to a fume hood for 15 min before the door was opened and the animals removed. At no time were laboratory personnel exposed to a halothane concentration of more than 0.1 ppm, a value well below the recommended NIOSH standard of 2.0 ppm.8

Litter weights were recorded at the time of birth and weekly weaning. The average weight of mice caged together was determined weekly until the age of 16 weeks, when individual weights were determined until the age of 40 weeks. Biweekly individual weights were determined thereafter until death at the age of 88 weeks. Mice were observed at least once daily for morbidity and mortality. Records of times of onset, incidences, sizes, and locations of tumors, as well as gross signs of systemic toxicity were maintained.

Mice dying or killed in extremis before final sacrifice were subjected to complete gross necropsy except for approximately 5 per cent in which extensive cannibalism or autolysis precluded examination. At 88 weeks surviving animals were sacrificed and examined by necropsy. The following procedures were used: Each mouse was weighed and examined externally. It then was killed by cervical dislocation, the chest cavity was opened, and blood was collected from the left ventricle for a complete blood count. The tissues listed in table 1 were examined in situ, then dissected from the carcass, re-examined, fixed in 10 per cent neutral buffered formalin, and saved. Suspect lesions were removed and prepared for histologic examination. The liver, kidney, testes, and spleen were weighed, then were sectioned at 2–5-mm intervals to detect subsurface lesions. The lungs were inflated with 10 per cent formalin and transilluminated to detect pulmonary adenomas. The trachea, main-stem bronchi, urinary bladder, esophagus, stomach, and rectum were incised and the mucosal surfaces examined using a dissecting microscope. The remainder of the gut was examined externally and palpated, but not incised. The nasopharynx and brain were exposed by removal of the palate and calvarium and were then examined. Bone marrow was obtained from the femur and a smear made for future examination, if needed. The treatment group of mice was not known to the examiner performing the necropsies.

Following fixation in 10 per cent neutral buffered formalin, tumor masses and other suspect lesions were embedded in paraffin. Sections were cut 5μm thick, slide-mounted, and stained with hematoxylin and eosin. Detailed microscopic examination was made by a pathologist experienced in animal histology.

Gross mean values for body and organ weights were compared by the Student t test. The number of tumors observed in the control group was compared
with the number in the halothane-treated group by either chi-square analysis or Fisher's exact test. $P < 0.05$ was considered significant.

**Results**

An early 10 per cent difference in weight gains was observed between halothane-treated and control groups (fig. 1). This weight difference disappeared by the age of 16 weeks and reappeared after the age of 55 weeks. Throughout the study there was no evidence of halothane-related effects with regard to physical appearance or gross behavioral changes. Occasional minor lesions such as ulceration of the skin, nasal and ocular discharges, and alopecia occurred with equal frequencies in the two groups.

The numbers of mice surviving at different times throughout the study are shown in table 2. The first tumor, a malignant thymoma, was discovered at 39 weeks in a control animal. The numbers of animals alive at that time, 115 in the halothane-treated group and 100 in the control group, were used to calculate tumor incidences. Fifty-five per cent of halothane-treated mice and 60 per cent of control mice survived until the 88th week (table 1). The greatest number of deaths occurred during the first two months of the study, after which the death rate remained constant until two months before sacrifice. At that time the death rate increased again, as the mice reached the end of their natural life span. The causes of death among mice dying before sacrifice could not be determined in about half the cases. In the remaining half, causes of death were divided almost equally between infection and trauma secondary to fighting.

There was no significant difference in hematologic values between the groups except that the erythrocyte count of halothane-treated males was significantly lower (6.9 ± 0.4 vs. 7.8 ± 0.3 erythrocytes/mm$^3$ × 10$^6$) and mean corpuscular hemoglobin significantly higher (19.9 ± 0.5 vs. 18.4 ± 0.2 pg) than corresponding values for control males. Splenic weight of halothane-treated females examined by necropsy at 88 weeks was significantly less (0.23 ± 0.05 vs. 0.45 ± 0.11 g) than that of control females. No other significant difference was observed.

**TABLE 2. Survivors**

<table>
<thead>
<tr>
<th></th>
<th>Start</th>
<th>39 Weeks—First Tumor</th>
<th>88 Weeks—Sacrifice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halothane</td>
<td>161</td>
<td>115 (71 per cent)</td>
<td>88 (55 per cent)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73 δ, 42 φ</td>
<td>50 δ, 38 φ</td>
</tr>
<tr>
<td>Control</td>
<td>120</td>
<td>100 (83 per cent)</td>
<td>72 (60 per cent)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>49 δ, 51 φ</td>
<td>33 δ, 39 φ</td>
</tr>
</tbody>
</table>

**FIG. 1.** Mean body weights ± SE for each group of mice during life and at sacrifice (week 88). The halothane-treated animals showed a weight decrease of approximately 10 per cent before age 16 weeks and after age 55 weeks.
A wide variety of nonneoplastic lesions was observed in either group, with no particular relationship to the treatment regimen. These lesions included ulcers of the skin, salivary and thyroid cysts, discolorations of the lungs, pale, mottled livers and kidneys, enlarged granular spleens, and ovarian, uterine, and epididymal cysts. Neoplastic lesions were divided into three groups: malignant tumors, benign tumors, and hepatomas/hepatic nodular hyperplasia. There was no difference between the occurrences of these lesions in halothane-treated and control mice (tables 3–6). The histologic distinctions between focal, nodular hyperplasia and liver-cell adenoma and between adenoma and malignant hepatoma often were not clear, since disorderly bile-ductule proliferation was sometimes present within otherwise typical adenomas, and since some of the neoplastic lesions manifested both aggressive and benign growth patterns and cytologic features. Typical adenomas contained hemorrhage, necrosis and dilated, disordered sinusoidal channels (figs. 2 and 3). Hepatic lesions were often equal to or larger than a normal liver in weight. However, there was no evidence of hepatoma metastases. Since all but one of the hepatic tumors were found at the 88-week sacrifice, this lesion did not appear to shorten the life span of the mice.

**Discussion**

A simplified protocol to evaluate the carcinogenesis of halothane in Swiss/ICR mice was employed in the present study; an increased incidence of tumors was not found. Modifications of the standard NCI protocol were incorporated either to increase the sensitivity of the bioassay or to decrease the cost and resources needed to perform the study without significantly compromising its validity. First, only the maximum tolerated dosage of halothane was studied. We reasoned that if a negative result were obtained at this dosage it would be highly improbable that lower dosages would result in tumor formation. The main reason for studying several dosages is to be able to construct a dose–response relationship when a positive result is found. Elimination of lower dosages, therefore, is a calculated risk that may be worth taking when screening many drugs or when the likelihood of a positive result is small.

A second modification was to start halothane exposure *in utero* rather than at weaning. Prenatal exposure increases offspring susceptibility to the effects of a carcinogen. Not only may tumors occur at a lower dosage than those necessary to produce tumors in the mother,9 but the incidence of tumor formation is higher than when exposure occurs only during adult life.10 This exposure regimen may be particularly relevant for studying inhalational anesthetics, since pregnant operating room personnel may be exposed to trace concentrations of these drugs. Although we have added this feature to the protocol, we recognize that the fetus and newborn differ from the adult in several important respects, including metabolic capabilities, physiologic characteristics, viral susceptibilities, hormonal status and immunologic competence. Therefore, it may not be possible to equate exactly the effects of *in utero* and adult exposure. However, the combination of prenatal and lifetime postnatal exposure is likely to result in the highest incidence of tumor formation.

A third modification was to perform microscopic examination only on lesions detected at gross necropsy. Such an approach differs from that taken by the NCI, which recommends that 32 tissues from each animal be examined.7 This constitutes a major portion

**Table 3. Malignant Tumors**

<table>
<thead>
<tr>
<th></th>
<th>Total 115*</th>
<th>Male 73</th>
<th>Female 42</th>
<th>Total 100*</th>
<th>Male 49</th>
<th>Female 51</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Per Cent</td>
<td>Number</td>
<td>Per Cent</td>
<td>Number</td>
<td>Per Cent</td>
</tr>
<tr>
<td>Halothane</td>
<td>8/115</td>
<td>(7 per cent)</td>
<td>4/73</td>
<td>(6 per cent)</td>
<td>4/42</td>
<td>(10 per cent)</td>
</tr>
<tr>
<td>Control*</td>
<td>8/100</td>
<td>(8 per cent)</td>
<td>2/49</td>
<td>(4 per cent)</td>
<td>6/51</td>
<td>(12 per cent)</td>
</tr>
</tbody>
</table>

* Swiss/ICR mice surviving at least until time of first tumor (9 months); necropsy disclosed the tumors.

**Table 4. Benign Tumors**

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Total 115*</th>
<th>Male 73</th>
<th>Female 42</th>
<th>Total 100*</th>
<th>Male 49</th>
<th>Female 51</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Per Cent</td>
<td>Number</td>
<td>Per Cent</td>
<td>Number</td>
<td>Per Cent</td>
</tr>
<tr>
<td>Pulmonary adenomas</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>10</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Ovarian cysts</td>
<td>12</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>12</td>
<td>29</td>
</tr>
<tr>
<td>Others</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>22</td>
<td>9</td>
<td>12</td>
<td>16</td>
<td>38</td>
</tr>
</tbody>
</table>

* Animals surviving at least until time of first tumor (9 months); necropsy disclosed the tumors.
of the workload involved in carcinogenicity studies and, in effect, restricts the number of chemicals that can be tested. With this mind, Fears and Douglas,¹¹ investigators at the NCI, have proposed two methods for decreasing the number of histologic examinations, using as examples data from five NCI carcinogenicity studies. We have further analyzed the data from these studies and found that 93 per cent (205/220) of malignant tumors in mice and 84 per cent (91/108) of malignant tumors in rats were discovered at gross pathologic examination. Furthermore, no systematic error occurred, since the few tumors discovered only upon histologic analysis did not come from any particular tissue. Extrapolation of this data to other chemicals suggests that histologic examination of tissues that appear normal at the time of necropsy may not be necessary for the accurate evaluation of carcinogenic potential. Thus, we consider that the present protocol was sufficiently rigorous to assess the carcinogenicity of halothane in Swiss/ICR mice. Parenthetically, all tissues from the present investigation have been preserved in case future information makes histologic examination desirable.

The results of this study should be related to other epidemiologic and experimental data linking anesthetic agents to cancer in anesthetists. We believe there is little information to support a causal relationship. In both retrospective and prospective surveys spanning 25 years, Bruce et al.³,¹² were unable to find a statistically significant increase in the death rate due to malignancies among anesthesiologists. Corbett et al.⁴ surveyed a small population, 525 female Michigan nurse-anesthetists, and suggested that there was a higher incidence of cancer among this group. However, statistical probabilities were calculated by comparing data from the Connecticut tumor registry for the years 1966–1969 with the Michigan data taken from a single year, 1971. When Michigan data for the same period are included, there is no statistically significant difference between the two groups. Two other recent epidemiologic surveys were unable to document increased mortality among anesthetists.¹³ The overall death rate among 1,251 male British anesthetists more than 35 years old was 93 per cent, and the death rate due to cancer only 79 per cent, of the expected rates among physicians. The preliminary report of a recently completed American Cancer Society study revealed similar findings. The overall death rate for anesthesiologists was 84.1 per cent, and the death rate due to cancer was the same as that expected among all physicians. In the only report of an increased incidence of cancer among operating room personnel, an ASA Ad Hoc Committee found a higher cancer rate among female members of the ASA and American Association of Nurse Anesthetists as compared with matched controls.⁸ An increased incidence of cancer among male members of these organizations was not demonstrated, nor was a causal relationship between anesthetic exposure and cancer demonstrated among female members.

The experimental evidence suggesting that currently employed inhalational anesthetics are carcinogenic also is open to question. Corbett et al.⁶ suggested that there was an increased incidence of hepatic tumors in Swiss/ICR mice exposed to isoflurane. However, a number of confounding variables make the data difficult to interpret. For example, Corbett*** has found that the livers of his isoflurane-treated mice contained markedly increased levels of polybrominated biphenyls. These compounds are teratogenic¹⁴: their presence may have contributed to the increased incidence of hepatic tumors found in isoflurane-treated animals.

In summary, a simplified version of the standard NCI protocol did not show an increased incidence to tumors in Swiss/ICR mice exposed to the maximum tolerated dosage of halothane. There continues to be no experimental evidence to suggest that anesthetic agents are carcinogenic when they are administered by the inhalational route.

The authors thank J. Topham, Ph.D., for reviewing the manuscript. Halothane was kindly provided by Ayerst Laboratories.

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*Swiss/ICR mice surviving at least until time of first tumor (9 months); necropsy disclosed the hyperplasias.

**N. Greene, M.D., Yale University, New Haven, Connecticut: Personal communication, American Cancer Society report of mortality among A.S.A. members.

***T. Corbett, M.D., University of Michigan, Ann Arbor, Michigan: Personal communication.
Fig. 2. Typical adenoma (A) of liver from mouse (77-B-157). Arrows indicate area with hemorrhage and necrosis at the left, and the dilated sinusoidal channels at the right. There is no fibrosis, and no bile duct proliferation, hematoxylin and eosin (×46).

Fig. 3. In the same lesion, notice the large cells and lipophagic sinusoids of the tumor, compared with the adjacent liver. The inset shows Kupffer cell prominence and variation in hepatocyte nuclear size, which are nonspecific signs of injury. Hematoxylin and eosin (×250).
References


