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SIGNIFICANCE OF NATURALLY-OCCURRING TUMORS IN EVALUATING THE CARCINOGENICITY OF A TEST COMPOUND: A REVIEW AND AN IMPROVED CARCINOGENICITY BIOASSAY FOR CHEMICALS*

—— PROFILES OF SPONTANEOUS AND INDUCED TUMORS IN ANIMALS AND A NEED FOR A REVISED CARCINOGEN BIOASSAY ——

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Abstract: Naturally-occurring tumors, which generally increase in incidence with age in control rats or mice in 2-year carcinogenicity bioassays, show occasionally an increased or decreased incidence in animals dosed with chemical substances. Analyses of these tumors showing dose-related increases or decreases in their incidence rates in the chronic toxicity and oncogenicity studies of chemicals including 11 tests using F344 rats and 14 tests using B6C3F$_1$ mice carried out in our Center for the past 8 years, revealed that they were exclusively naturally-occurring tumors commonly seen in these animals. These lesions included pituitary tumor, C-cell tumor of the thyroid, and mononuclear cell leukemia in F344 rats and bronchiolar/alveolar adenoma of the lung, hepatocellular adenoma, hepatocellular adenoma plus carcinoma, malignant lymphoma, and forestomach papilloma in B6C3F$_1$ mice.

One approach to understand the changes in the incidence rates of the naturally-occurring tumors is to elucidate the biological effects of the test chemical. Another approach is to identify sets of modifying factors, including nutritional effects, hormonal-imbalances, and other age-associated lesions, as seen in the roles of chronic nephropathy, myeloproliferative changes, and hyperplasias of the various endocrine organs in the development of tumors in corresponding tissues.

The profile approach to induced rodent tumors in the past has revealed several characteristics. Among them, first, induced tumors show early development, mostly within a period less than 52 or 78 weeks of the treatment. Second, the dose levels of each chemical appear likely to have a minimum as well as an upper limit in the amount needed to induce tumors. Consequently, the current carcinogenicity tests using unusually high amounts of chemicals in a pure form will be able to demonstrate tumor induction, if they are really carcinogenic, before the onset of the spontaneous tumors, which are mostly seen mixed with a variety of non-neoplastic lesions in the same groups of animals after 78 weeks of the treatment. This means that the detection of possible chemical carcinogens would be more efficient and accurate by shortening the test period from 104 to 78 weeks in tests using F344 rats and B6C3F$_1$ mice. Addition of a satellite test for interim observation of the pathological findings at 52 weeks, to this new way of testing will give more useful information on early changes and the morphogenesis of tumors induced by chemical substances. (J Toxicol Pathol 3: 1~17, 1990)

Key words: Spontaneous tumor, Carcinogenicity, Induced tumor, Rat, Mouse

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* Dedicated to the late Dr. Elizabeth Cavert Miller, former Associate Director of the McArdle Laboratory for Cancer Research, University of Wisconsin, Medical School, Madison, Wis., U.S.A.

Development of naturally-occurring tumors

There are many reports on the types and incidence rates of naturally-occurring tumors seen in carcinogenicity bioassays using F344 rats and B6C3F$_1$ mice. Extensive data were supplied by the NCI (the National Cancer Institute, U.S.A.) contract studies[1] and the NTP (the National Toxicology Program, U.S.A.) bioassay studies[2]. Tumor types commonly seen in non-treated animals were relatively homogeneous in each strain. However, there were considerable variations in tumor incidence rates within and among the laboratories, as was demonstrated by Taron *et al.* using F344 rats and B6C3F$_1$ mice in NCI contract studies[3] and by Sher *et al.* in the Charles River-CD and F344 rats and B6C3F$_1$ mice[4]. Details of our research on summation and analysis of the historical control findings obtained in 11 tests using F344 rats and 14 tests using B6C3F$_1$ mice were reported in previous reports[5,6]. These tests were conducted in the Biosafety Research Center, Foods, Drugs and Pesticides for over the past 8 years. Our tests have been conducted in compliance with GLP requirements and their protocols were in accordance with the Toxicity Test Guidelines issued from the Government of Japan after 1984. Animals were maintained under a specific pathogen free environment controlled at 23±1°C, 55±5% in humidity, and 225±75 lux 12-hrs in light. They were housed individually in suspended stainless wire-mesh cages. The animals had free access to tap water and to the basal diet, which was manufactured following the modified NIH Open Formula for Rat and Mouse and was sterilized by gamma radiation (Oriental Yeast Co. Ltd.). The basal diet was analyzed 4 times a year and was found to be free from contaminants such as pesticides, benzo(a)pyrene, and aflatoxins.

Four-week-old F344/DuCrj rats were purchased from Charles River Japan Inc., Kanagawa and 4-week-old B6C3F$_1$ (C57BL/6CrSlcxC3H/HeSlc) mice were purchased from JAPAN SLC Inc., Hamamatsu. The significance of the differences in the incidences of neoplastic and non-neoplastic lesions seen in the dosed animals, as compared with the control animals, were evaluated routinely by Fisher's exact probability test, the Chi-square test or, if necessary, by Cochran-Amitage linear trend test.

The tumor incidences in various organs and tissues in the 1,100 control rats and 1,300 control mice used in the 2-year carcinogenicity bioassays in our laboratory were basically similar to those of the spontaneous tumors reported previously in rats and mice[7-11]. The number of tumors, benign and malignant, and the average tumor number per animal are shown in **Table 1**. It is interesting to note that many tumors that developed in these animals were calculated to be 1.8 per male rat, 1.1 per female rat, 1.0 per male mouse, and 0.8 per one female mouse. The cumulative incidences of benign tumors in the rats were around 80 percent and those in mice were around 70 percent of all the

Table 1. Incidence of Spontaneous Tumors per Animal in Control Animals of 109 Weeks in Age

Animals	Sex	No. of animals examined	Total tumors	Total benign tumors	Total malignant tumors
F344 rats	Male	550	1010	897 (88.8%)	113 (11.3%)
		Number of tumors per animal : 1.8			
	Female	550	619	484 (78.2%)	135 (21.8%)
		Number of tumors per animal : 1.1			
B6C3F$_1$ mice	Male	650	637	453 (71.1%)	184 (28.9%)
		number of tumors per animal : 1.0			
	Female	650	523	349 (66.7%)	174 (33.3%)
		Number of tumors per animal : 0.8			

Table 2-A. The Distribution and Incidence(%) of the Commonly Found Tumors in Male Control F344 Rats

Organs	Weeks of scheduled sacrifice / No. of rat examined / Tumors	26 / 10	52 / 99	78 / 38	104 / 446	The earliest onset of tumors in weeks, found in dead or moribund sacrificed rat during 104 wk-study
Testis	Interstitial cell tumor	0.0	0.0	42.1	93.7	72
Lung	Alveolar/bronchiolar adenoma	0.0	1.0	0.0	3.1	78
Spleen/Hematopoiet.*	Mononuclear cell leukemia	0.0	0.0	0.0	5.1	49
Pituitary	Adenoma, pars distalis	0.0	1.0	5.3	28.3	52
Adrenal	Pheochromocytoma	0.0	0.0	5.3	11.2	72
Thyroid	C-cell adenoma	0.0	0.0	5.3	11.4	78
Brain	Glioma	0.0	0.0	0.0	0.2	104

* Hematopoietic organs.

Table 2-B. The Distribution and Incidence(%) of the Commonly Found Tumors in Female Control F344 Rats

Organs	Weeks of scheduled sacrifice / No. of rat examined / Tumors	26 / 10	52 / 99	78 / 38	104 / 446	The earliest onset of tumors in weeks, found in dead or moribund sacrificed rat during 104 wk-study
Uterus	Endometrial stromal polyp	0.0	4.0	5.3	17.0	78
	Endometrial carcinoma	0.0	1.0	0.0	0.2	104
Lung	Alveolar/bronchiolar adenoma	0.0	0.0	0.0	1.1	104
Spleen/Hematopoiet.*	Mononuclear cell leukemia	0.0	0.0	2.6	8.8	59
Pituitary	Adenoma, pars distalis	0.0	0.0	7.9	40.6	65
Adrenal	Pheochromocytoma	0.0	0.0	2.6	2.5	78
Thyroid	C-cell adenoma	0.0	0.0	0.0	9.6	81
Brain	Glioma	0.0	1.0	0.0	0.2	52

* Hematopoietic organs.

tumors developing in these animals.

Death of the control animals seen during the two-year period in the carcinogenicity bioassay was suggested to be mostly caused by tumors, as evidenced in 68% of male and 79% of female rats and 88% of male and 78% of female mice. Malignant tumors of the hematopoietic tissues and pituitary tumors in rats and malignant lymphomas and hepatocellular carcinomas in the mice were the main causes of death. Occasionally, latent tumors were detected microscopically in the dead or moribundly killed animals.

Guidelines of the chronic toxicity and carcinogenicity bioassay for pesticides issued by Ministry of Agriculture, Forestry and Fisheries of Japan in 1984, recommend performing interim observation of the satellite animals at 26, 52, and 78 weeks of study. By adding the data obtained from these interim studies we recorded the incidence and distribution of the representative tumors found in the scheduled to be killed F344 rats and $B6C3F_1$ mice, as shown in **Tables 2** and **3**. Also added to these tables are the rats and mice found dead or moribund with the first onset of each tumor during the 2-year period of carcinogenic bioassays. The incidence of neoplastic lesions like hepatocellular adenoma in male mice exceeded 27% (177 out of 650 cases). One hundred and sixty nine out of 177 male mice with hepatocellular adenoma were killed at the end of 104 weeks of the studies. The remaining 8 male mice died or were moribundly killed during the studies.

The shortest latent period of hepatocellular adenoma was at 44 weeks. In the interim observation period, hepatocellular adenoma was found in 26 out of 115 (22.6%) male mice killed at 78 weeks and in 3 out of 110 (2.7%) male mice killed at 52 weeks. The hepatocellular hyperplastic nodule,

Table 3-A. The Distribution and Incidence(%) of the Commonly Found Tumors in Male Control B6C3F$_1$ Mice

Organs	Weeks of scheduled sacrifice	26	52	78	104	The earliest onset of tumors in weeks, found in dead or moribund sacrificed mouse during 104 wk-study
	No. of rat examined	20	110	115*	527	
	Tumors					
Lymph node	Malignant lymphoma	0.0	0.0	0.0	3.0	63 weeks
Lung	Alveolar/bronchiolar adenoma	0.0	0.0	9.6	12.7	78
	Alveolar/bronchiolar carcinoma	0.0	0.0	1.8	3.0	78
Stomach	Papilloma of forestomach	0.0	0.0	1.8	10.2	57
Liver	Hepatocellular adenoma	0.0	2.7	22.6	24.5	44
	Hepatocellular carcinoma	0.0	0.0	9.6	10.4	55
Pituitary	Adenoma, pars distalis	0.0	0.0	0.9	0.2	104
	Adenocarcinoma	0.0	0.0	0.0	0.0	—
Harderian gland	Adenoma	0.0	0.0	0.9	4.4	78
	Cystadenoma	0.0	0.0	0.0	0.6	104
	Adenocarcinoma	0.0	0.0	0.0	0.2	104

* Adding the data of one test terminated at 78 weks.

Table 3-B. The Distribution and Incidence(%) of the Commonly Found Tumors in Female Control B6C3F$_1$ Mice

Organs	Weeks of scheduled sacrifice	26	52	78	104	The earliest onset of tumors in weeks, found in dead or moribund sacrificed mouse during 104 wk-study
	No. of rat examined	20	110	120*	519	
	Tumors					
Lymph node	Malignant lymphoma	0.0	0.0	4.3	4.6	73 Weeks
Lung	Alveolar/bronchiolar adenoma	0.0	0.0	10.0	4.0	78
	Alveolar/bronchiolar carcinoma	0.0	0.0	1.4	1.2	51
Stomach	Papilloma of forestomach	0.0	0.0	10.0	3.9	76
Liver	Hepatocellular adenoma	0.0	1.8	12.9	11.2	52
	Hepatocellular carcinoma	0.0	0.0	1.4	1.7	91
Pituitary	Adenoma, pars distalis	0.0	0.0	4.3	6.0	76
	Adenocarcinoma	0.0	0.0	1.4	0.4	104
Harderian gland	Adenoma	0.0	0.0	1.4	5.2	94
	Cystadenoma	0.0	0.0	2.9	0.4	104
	Adenocarcinoma	0.0	0.0	1.4	0.2	101

* Adding the data of one test terminated at 78 weeks.

that is the preneoplastic proliferative lesions of the liver cells, were found earlier, even in the mice killed at 26 week[5].

A variety of spontaneous tumors also appeared in rats by 104 weeks. Frequently observed tumors, including mononuclear cell leukemia and tumors of the reproductive and endocrine organs, developed before 78 weeks or appeared in the rats killed at 52 weeks.

Evidence for the age-related occurrence of these naturally-occurring tumors in both rats and mice has already been shown by many pathologists[7-17]. It is interesting to note that rare malignant tumors that usually show a low incidence in animals and humans, like sarcoma of the soft tissue or the visceral organs, appear sporadically and earlier than 78 weeks in the rodents than some of the other common malignant tumors as mentioned above (Fig. 1). This fact suggests that a carcinogenic process similar to that found in humans may play a role in their occurrence in the rodent. In a carcinogenesis study, it may not be possible to rule out that such a rare tumor appearing early in the experiment is attributed to the treatment, as they appeared in the dosed animals and not in the control group[17].

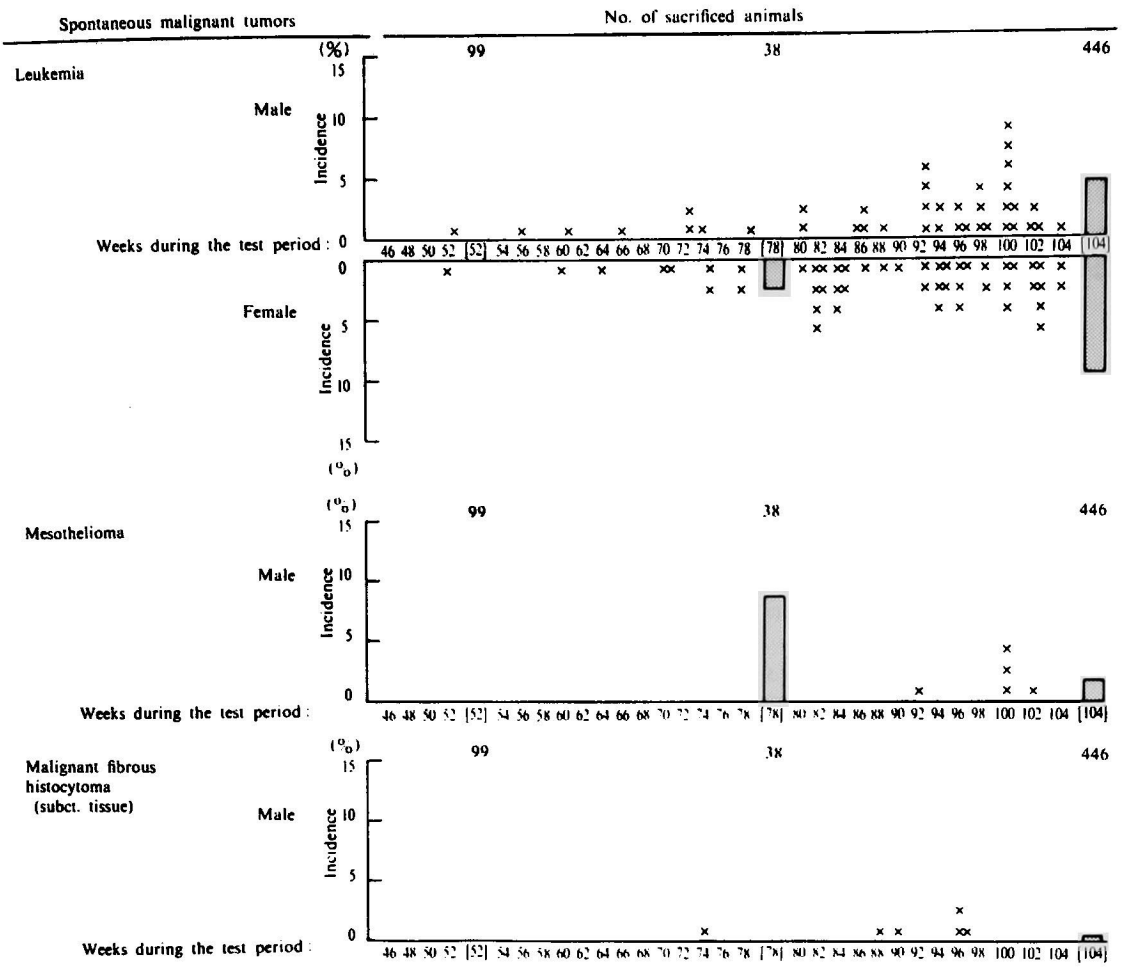

Fig. 1-A-1. See explanation of Fig. 1-A.

Increases or decreases in incidences of naturally-occurring tumors

In bioassays for carcinogenic activity, a carcinogen is defined as an agent that causes an increased incidence of tumors in treated animals as compared to control animals. Treated animals were given three dose levels of the test substance, low, middle, and high. Each dosed group consisted of 50 male and female animals, as did the control group. Results of the carcinogenesis bioassays carried out in our Center as described in the preceding paragraph revealed no increase in the number of tumors of a specific type or no earlier onset of tumors in dosed animals when compared with the control animals. However, several tests showed biologically significant and dose-related increases in the incidences of spontaneous tumors in the treated groups over the concurrent control groups[18]. Among 11 bioassays using rats, an increase in the incidence of pituitary adenoma was observed in the treated male rats in one test. A decrease in the incidence of pituitary adenoma in treated female rats was seen in one test, and a decrease in incidence of C-cell adenoma of the thyroid in treated female rats in another test were also observed. Results of 13 carcinogenesis studies using mice are shown also in **Table 4**.

A parallel increase and decrease in incidences of the same tumor in both male and female animals was found in one test showing a decrease in the incidence rate of leukemia in a rat and in one test showing a decreased incidence rate of malignant lymphoma in a mouse. The difference in incidence rate of these tumors, as compared to corresponding tumors of the controls, was not

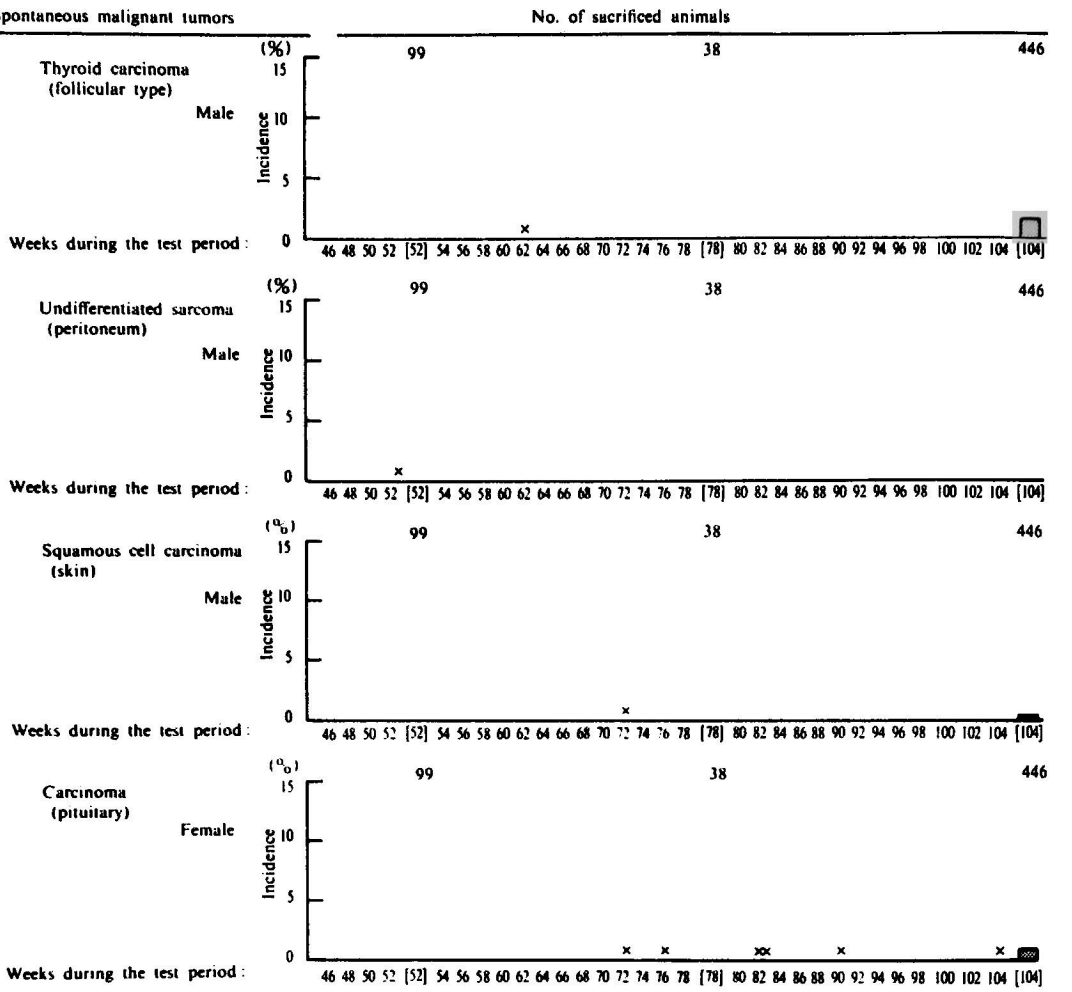

Fig. 1-A-2. See explanation of Fig. 1-A.

Table 4. Number of Tests Showing Significant Increase or Decrease in Tumor Incidences of B6C3F$_1$ Mice (104 wks)

Spontaneous tumor	Male			Female		
	Number of tests showing					
	Increase	No difference	Decrease	Increase	No difference	Decrease
Hepatocellular adenoma		11	2*		13	
Hepatocellular adenoma + carcinoma		11	2**	2*	10	1*
Alveolar/bronchiolar adenoma		12	1*		13	
Forestomach papilloma	1*	11	1***	1***	11	1**
Malignant lymphoma		13			13	2*

Significance of difference, *$p<0.05$, **$p<0.01$ and ***$p<0.001$ in incidence were determined by Chi-Square analysis.

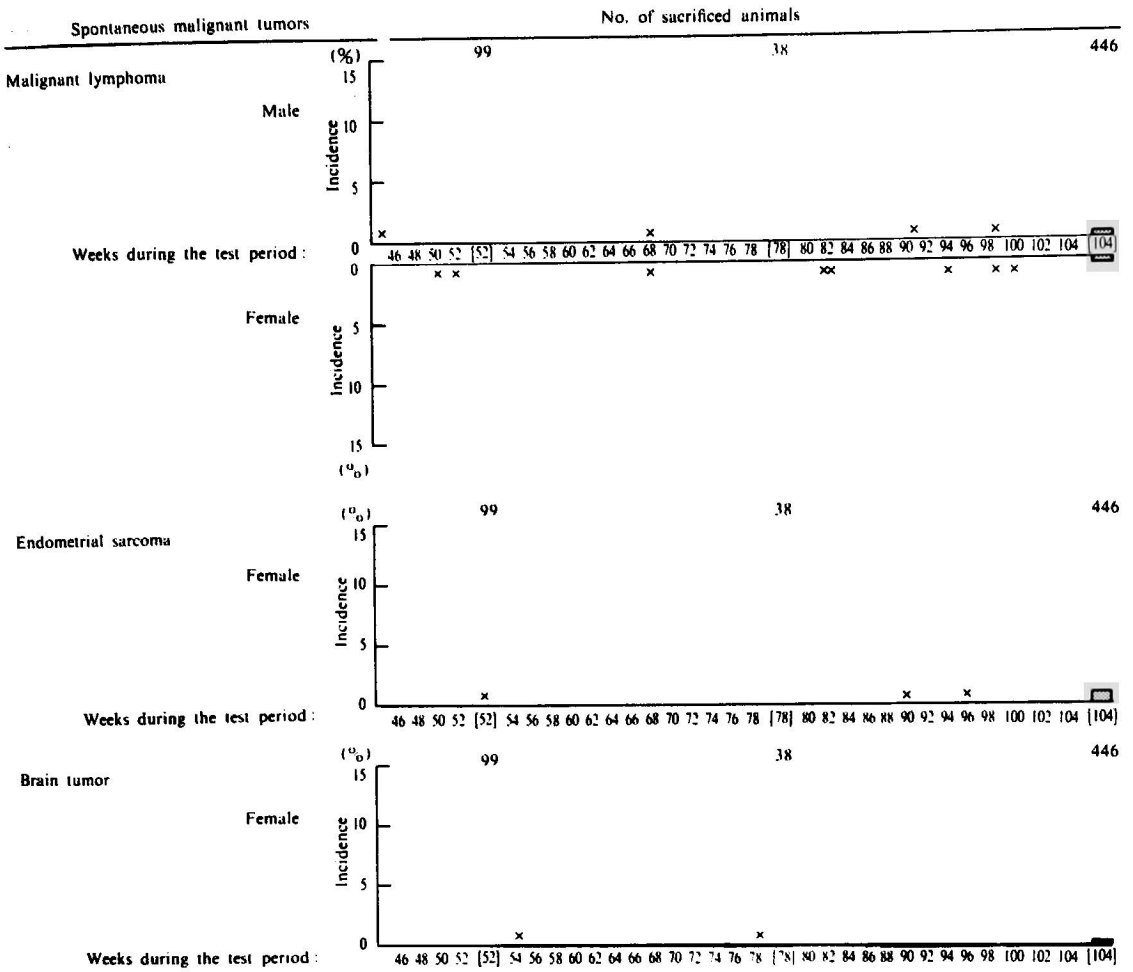

Fig. 1-A-3.

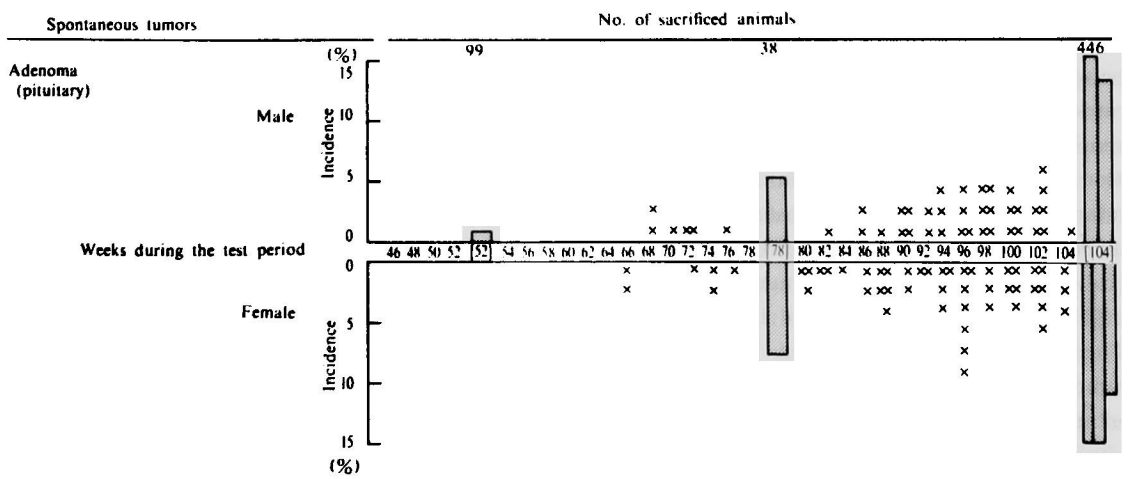

Fig. 1-A-4.

Fig. 1-A. Spontaneously occurring malignant tumors with early onset before 78 weeks and their distribution and incidences during 104 weeks of 11 studies with F344 rats.
X : each one case of animal with tumor.

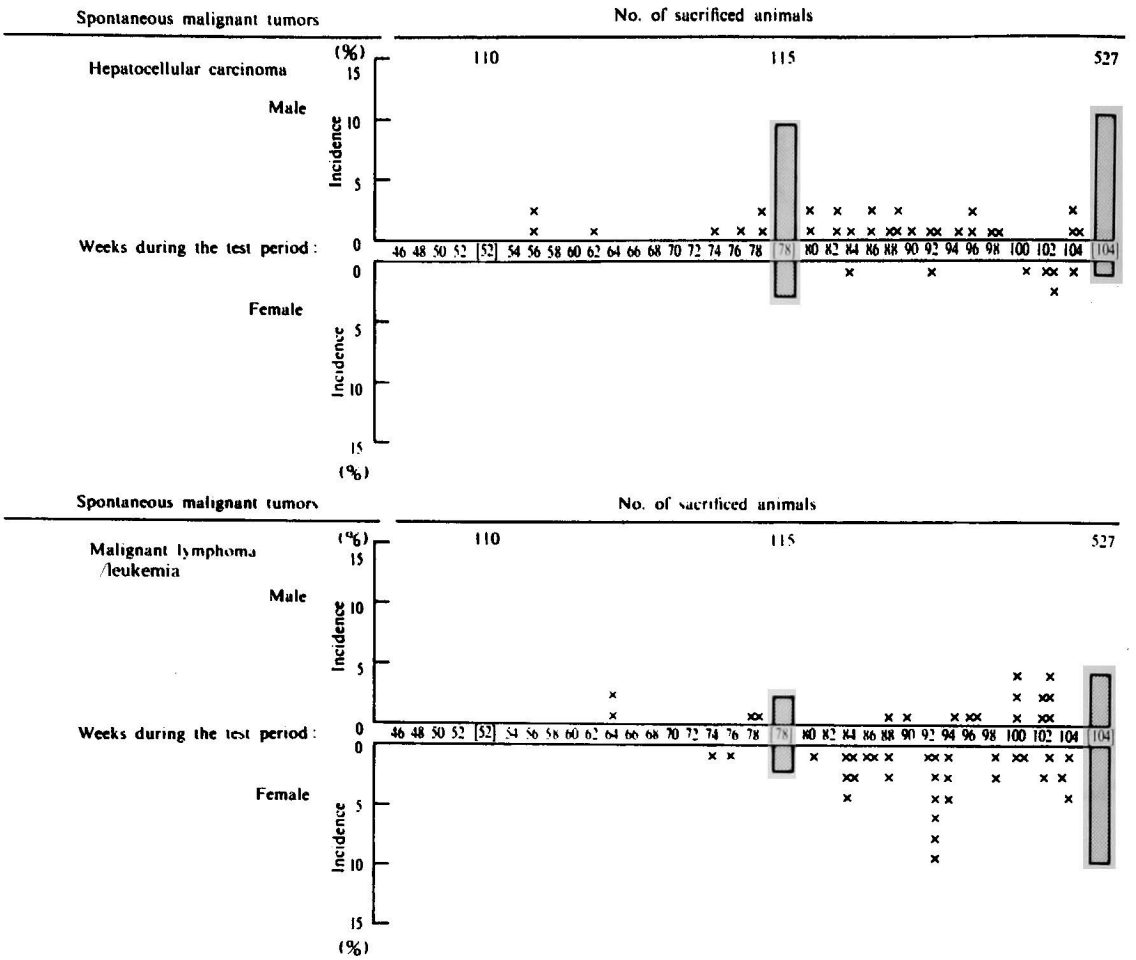

Fig. 1-B-1. See explanation of Fig. 1-B.

significant statistically in these two tests.

In four tests, a contrary effect was seen between male and female rats on the incidence rates of pituitary adenoma. In one mouse test, a similar reverse effect of the incidence rates was seen between male and female mice with hepatocellular adenomas. Five test substances were simultaneously given to both a rat and a mouse. However, no increase in the number of the same organ-specific tumors was seen in these animals. There were also variations in the spontaneous tumors of the control animals among tests conducted at our Center, especially in the incidence rate of pituitary tumors (male; from 16.0 to 48.0%, female; from 22.0 to 48.0%) in rats and hepatocellular adenoma (male; from 10.0 to 48.0%, female; from 4.0 to 24.0%) or hepatocellular adenoma plus carcinoma (male; from 14.0 to 68.0%, female; from 4.0 to 34.0%) in mice[5,6,18].

Profiles of the induced tumors

The distinguishing characteristics between induced tumors and spontaneous ones can be seen in the earlier onset of induced tumors compared to spontaneous ones, as well as in tumor incidences showing dose-related increases due to exposure to chemical substances. Our own experience in observing induced tumors has been obtained by studies on carcinogenesis of synthetic and naturally-occurring chemicals in rats and/or mice[19-31], by extensive comparative carcinogenic studies on metabolically activated or inactivated chemicals including a variety of aromatic amides and 4-nitroquinoline compounds as a collaborator of Miller's group[32-37], and by recent long-term chronic bioassay tests carried out on a variety of

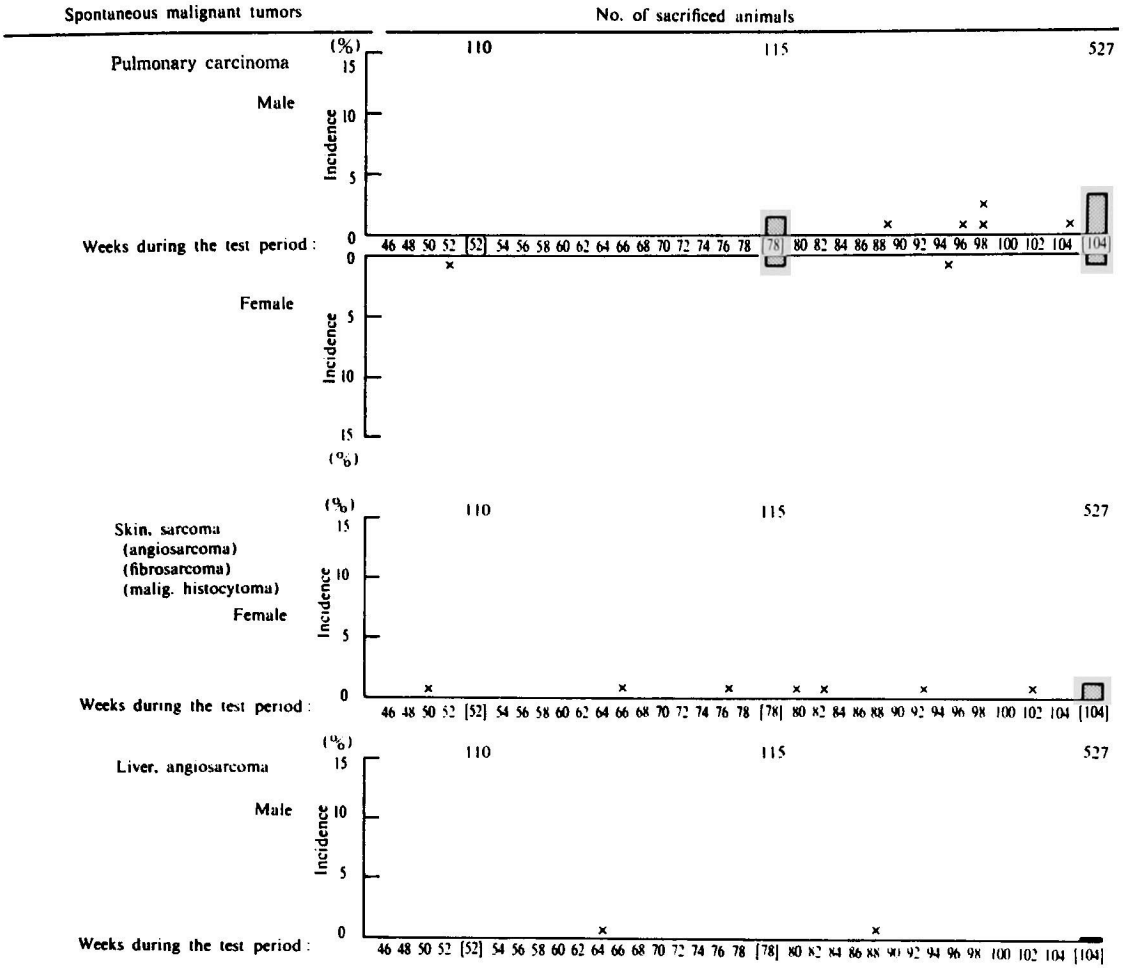

Fig. 1-B-2. See explanation of Fig. 1-B.

chemical substances with the collaborators of the Biosafety Research Center, Foods, Drugs and Pesticides, Shizuoka and the Japan Bioassay Laboratory, Kanagawa[5,6,38,39].

The results of these carcinogenic studies suggest that chemical carcinogens are likely to have both a minimum and upper limit in induction time and even in dose levels, although they may be influenced by a variety of factors such as animal species, strain, sex, age, and target tissues. **Table 5** shows the induction time of tumors observed in these studies, using the representative carcinogens known to induce organ-specific tumors in rat and mouse as a human model. Published data have also been added to those of our own studies.

The induction period showed some differences not only between chemical substances but also in the method of treatment, dose level of chemicals, and administration period. Induction time also varied depending on how the tumors were detected, such as by scheduled killings or by necropsies of dead or moribundly killed animals[14]. However, most of the induced tumors showing progression[40] were found in the period between 3 to 18 months. Both benign and malignant tumors may be detected earlier because of the superficial localization in the body, the injurious effects caused by compression to the brain, and bleeding. The growth speed of the sarcoma may be faster than carcinoma. Undifferentiated types of tumors can be found earlier than differentiated types due to its infiltrative and destructive invasion into the surrounding tissues, as well as of its faster growth speed.

Consequently, it is very difficult to estimate the induction time from the start of the treatment

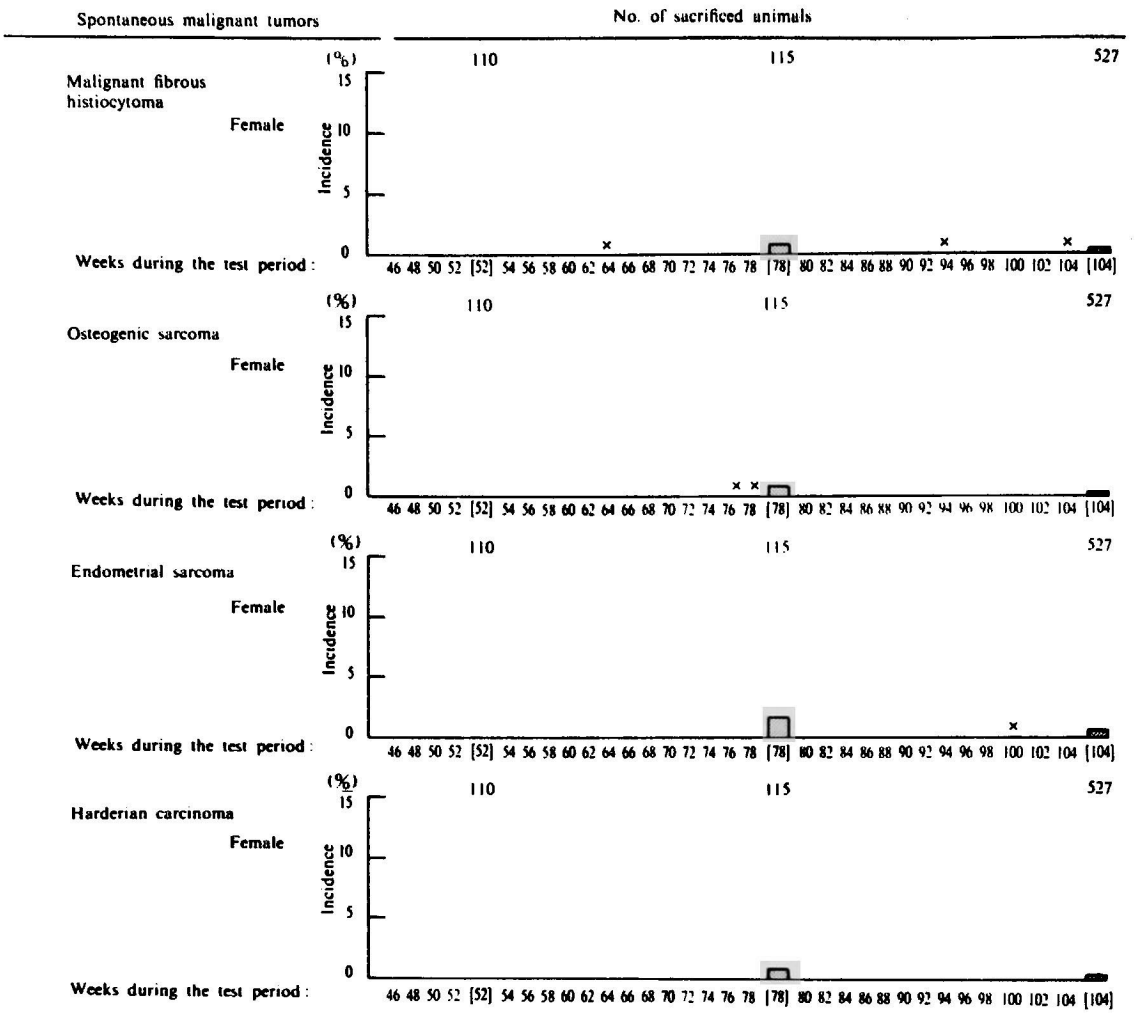

Fig. 1-B-3.

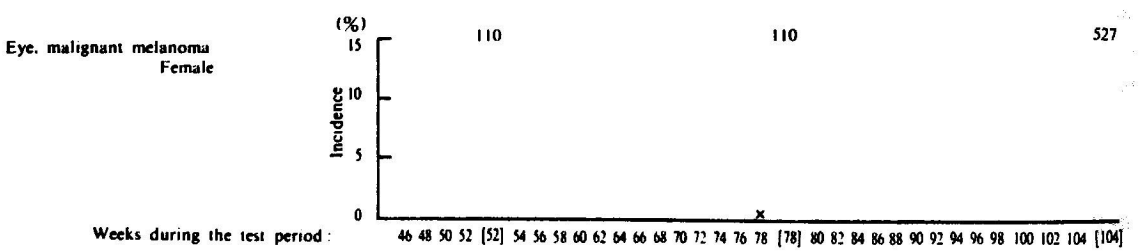

Fig. 1-B-4.

Fig. 1-B. Spontaneouslly occurring malignant tumors with early onset before 78 weeks and their distribution and incidences during 104 weeks of 13 studies with B6C3F$_1$ mice.
X : each one case of animal with tumor.

with a carcinogen to the time of detecting a possible induced-tumor. It is the combined time of the malignant transformation of the cell with time for tumor growth up to the grossly or microscopically visible size. Research work on the time course of radiation-induced carcinogenesis revealed a species difference in the latent period of tumor development[41]. A similar difference in latent period was also shown by an accidental or occupational development of tumors by chemical carcinogens in humans as compared to the experimental induction of tumors in animals[42,43]. The

Table. 5 Time-to-Tumor Occurrence Induced by Chemicals in Rats or Mice

Tumor	Time-to-tumor Weeks	Chemicals
Lung tumor	20-30-52	3Mc, BP, BHP, MNU
Hepatocellulaar adenoma	15-52	AfB1, 2-AAF, Sterig., Luteoskyrin,
Hepatocellular carcinoma	40-52	Safrole, Azo-dye, MAM, DENA, Trp-P-1, ZAMI, Methapyrilene, Choline-devoid diet
Pancreas tumor	57	HAQO, Ethionine + HAQO,
Esophagus tumor	23-40	N-nitrososarcosine ethyl ester
Stomach tumor	26-52	MNNG
Intestinal tumor	26-52	Bracken fern, MAM Dimethylhydrazine
Thymic lymphoma	13-26	BNU
Leukemia	52	ENU
Renal cell carcinoma	36-87	DMN, EHEN, N-nitrosomorpholine
Urinary bladder carcinoma	36-68	BBN
Islet cell tumor	16-45	Streptozotocin, Nicotinamide
Brain tumor	20-80	Nitrosourea-derivatives
Thyroid tumor	52-82	Aminotriazole, Thiourea, DDPM
Pituitary tumor	26	Estrogen
Acoustic duct tumor	32	DMBA, DMBA + DAS

2-AAF ; 2-acetylaminofluorene, AfB1 ; aflatoxin B1, BBN ; 1-butyl-4-hydroxybutylnitrosamine, BHP ; N-nitroso-bis(2-hydroxypropyl)amine, BNU ; N-nitrosobutylurea, BP ; benzo(a)pyrene, DAS ; 4-dimethylaminostilbene, DDPM ; 4-4-diamino-diphenylmethane, DENA ; diethylnitrosamine, DMBA ; 7, 12-dimethylbenzanthracene, DMN : dimethylnitrosamine, EHEN ; N-ethyl-N-hydroxyethylnitrosamine, ENU ; N-nitrosoethylurea, HAQO ; 4-hydroxyaminoquinoline-N-oxide, MAM ; methylazonymethanol, 3Mc ; 3-methylcholanthrene, MNNG ; N-methyl-N-nitro-N'-nitrosoguanidine, MNU ; N-nitrosomethylurea, Sterig. ; sterigmatocystin, Trp-P-1 ; 3-amino-1, 4-dimethyl-5H-pyrido (4, 3-b)-indole, ZAMI ; DL-1-(2-nitro-3-methyl-phenoxy)-3-tert-butylamino-propan-2-ol.

interesting fact thus observed is that induction time of tumor most likely depends on the life span of its host. One approach to estimate the induction time of a tumor is to estimate its growth rate based on the so-called doubling time of a tumor. Doubling time calculated by a change in tumor volume as an index is known to range from one to 10 days ; two to three days on the average in tumors in rats and mice, and from 7 to 2,300 days ; 30 to 120 days[44] on the average in human tumors. It may be emphasized in the induced tumor profile that the induction time of tumors by chemical carcinogens ranges within a determined time frame for each animal species as well as for each tissue. The induction time is estimated by the species-specific doubling time as well as through observation on the time of tumor induction.

The number of large-scale bioassays using known strong carcinogens, like diethylnitrosamine[45-47], 2-acetylaminofluorene[48], aflatoxin B_1[49-51], benzo(a)pyrene[52], N-nitrosomorpholine[53], methylnitrosourea[54], radiation[55,56], and some alkylating agents[52,57] have helped shed light on chemical carcinogenesis from a quantitative stand-point. Method of the current carcinogenesis bioassay was established by referring to such a large-scaled analysis. This method depends primarily on the theoretical base that the same carcinogenic effect can be obtained for small doses as large doses if the time frame is expanded. Consequently, long-term chronic bioassays in which both exposure and observation lasts through all or nearly all of the expected life span of the animals under study has been thought to provide the best evidence showing the negativity of the carcinogenicity of a substance[48-58].

However, careful analysis of the results of these large-scale studies seems to tell us a number of interesting facts. First, the data showed that induction of tumors took place fairly early in the life span, although the tumors were detected late in life. Littlefield *et al*. suggest the initial exposure is very effective within the first 9 months. This conclusion was drawn after observing in their study on 2-acetylaminofluorene carcinogenesis[48] that a cessation of carcinogen administration after 9 months of treatment did not effectively reduce induction of hepatocellular neoplasms. As shown in **Table 5** most of the tumors developed before 52 weeks in the experiments. When analyzing the time-to-tumor period in these studies, lowering the dose levels did not necessarily prolong the time of tumor onset in the same target organ after 78 weeks.

Similar characteristics were shown in the carcinogeneicity of crude materials. Within 52 weeks of the study, hepatocellular adenoma and carcinoma developed in mice fed a diet containing moldy rice infested with *Penicillium islandicum sp*. The dietary concentrations of moldy rice selected for the study were 1, 5, 10, and 30%, all of which were sufficient to induce liver cirrhosis and hepatocellular tumor in mice[20,21,23]. A carcinogenic metabolite, called luteoskyrin, is present in one part per thousand in moldy rice. Consequently, a diet with a 1% concentration of moldy rice was estimated to contain 10 ppm of luteoskyrin, which is near the lowest hepatocarcinogenic dose (2.5 mg/kg body weight/day) possible for mice[51]. A similar early tumor development was demonstrated in the feeding experiment of bracken ferns. When rats were given a diet containing the powdered fronds of young bracken ferns mixed with a basal diet in the proportion by weight of 30 or 33% for 4 months, rats developed multiple ileal tumors 7 to 14 months after the start of the experiment[26,59]. As the carcinogenic ptaquiloside obtained in 0.02% yield from dried powered bracken was demonstrated to induce cancer when given a total dose of 300 mg per rat, the ileal tumors described above [59] were likely to have been caused by this agent in the bracken fern diet. However, contrary to the positive carcinogenicity of the above crude materials, fish meal pyrolysate was not found to be carcinogenic to syrian golden hamsters that were fed on a diet of fish meal pyrolysate mixed with basal diet at a concentration of 5, 10, 20, or 40% for 112 weeks[60]. The reason for the negative results is likely to be due to the low concentration of carcinogenic compounds. Even the highest concentration at 40% of fish meal pyrolysate used in this study was estimated to contain carcinogenic components in amount far lower than their known carcinogenic levels. The carcinogenic daily dose of Trp-P-1, one of the carcinogens isolated from this pyrolysate, is approximately 12 mg/kg body weight for rats. Trp-P-1, however, is reported to be present at only 13 ng/g in boiled sardines[61].

Second, the carcinogenic doses of chemical carcinogens are generally in the dose range between the toxic maximum and the minimum levels of dosage for the observation of tumors induced by these agents. Most carcinogens show a dose-range of less than 100, except for a few strong carcinogens like aflatoxin B_1 or diethylnitrosamine which have a range wider than 100 and closer to 1,000[45-52]. Many carcinogens show ranges less than 10. Also, chemicals requiring high concentration of more than 500 mg/kg body weight/day to reveal their carcinogenicity are not evaluated as possible carcinogens for humans from a practical stand point[62].

Low-dose extrapolation of the data on carcinogens based on the results of long-term or life-span study face difficult problems due to the unpredictable prevalence of naturally occurring lesions, including age-associated spontaneous tumors in rats and mice[63]. It is difficult to determine the low dose carcinogenicity effect of chemicals from an increase in tumor incidence rates that occur later than 78 weeks in the bioassay. We can, however, occasionally find an earlier onset of a spontaneous tumor, like urinary bladder papilloma in the control mouse, as compared to the appearance of the same site-specific tumor in dosed animals in the long-term experiment for low dose extrapolation.

The apparent increase in overall tumor occurrences in the dosed animals, as compared to the control animals, is attributed to the carcinogenicity of the chemical. However, analyses emphasizing overall tumor incidence should be done with care,

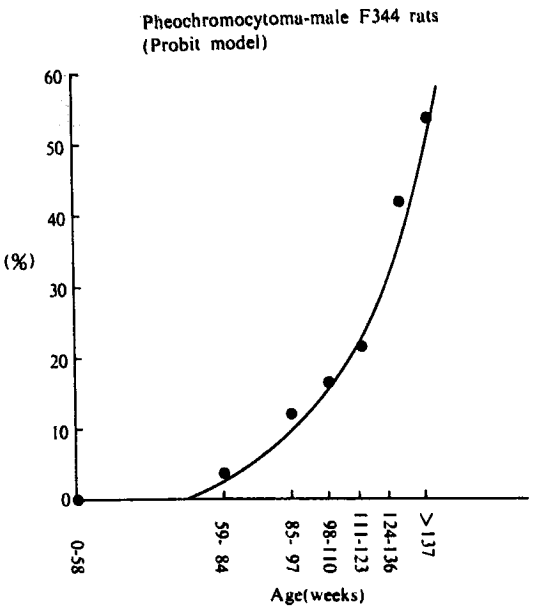

Fig. 2. Age-tumor curve of rats adrenal pheochromocytoma.
(Adapted from Solleveld, Haseman, McConnell, 1984)[13].

as suggested by Haseman *et al*[64]. An increase in the occurrence of multiple tumors appearing after 78 weeks in the experiment may represent an entirely separate process from carcinogenesis of site-specific tumors[54]. Usually, the induction of multiple tumors by strong carcinogens can be seen in a wide range of doses, although they are generally more prevalent at higher doses than at lower doses. This is observed in the simultaneous development of multiple tumors, such as hepatocellular tumors, forestomach tumors, and urinary bladder tumors by the administration of 2-acetylaminofluorene in the mouse[37].

Third, it has been noted that analyses of the experimental results with different carcinogenic agents, that there is no uniformity in the shapes of dose-response curves. Thus, Clayson emphasized in his review discussing the shape of the dose-response curve in relation to a number of factors, that "better risk estimates will be obtained, if more is known about the course of events by which a chemical induces cancer"[56]. Age-associated occurrences of spontaneous tumors is shown by Solleveld and McConnell[14]. Figure 2 shows the incidences of pheochromocytoma of the adrenals observed in F344 rats at various age periods. It is interesting to note that the sloping mode of this age-associated curve of spontaneous tumors is very similar to that of the dose-response curve of induced tumor. Further comparison of this curve pattern of various spontaneous tumors with those of induced tumors may give a clue on patterns of tumorigenesis in general.

Factors influencing the occurrence of naturally-occurring tumors

Evaluation of the significance of spontaneous tumors should be done first by determination of the probable causative agents of each of them. Many attempts are currently being done on the possibility of viral, genetic or hormonal involvement in the development of these tumors, although sufficient evidence has not yet been obtained[12,16,65,66]. At the same time accumulating information on factors influencing the occurrence of naturally-occurring tumors have contributed to the evaluation of long-term bioassay data which show an increase and decrease of the incidence rate of these tumors in the rat or mouse (**Table 6**). Hormonal[12,67] and nutritional factors[12,68-71] are known to be related to tumor promotion and inhibition.

Important facts emerging from these researches are the understanding of the characteristics of non-neoplastic lesions and how they play a role in development of neoplastic lesions as seen, for example, in chronic nephropathy in aged male rats of the F344 strain. The production of α-2u-globulin in this male animal is suggested to be associated with the development of renal cell hyperplasia, adenoma, and carcinoma, although it is low in incidence reference[6,72]. Accelerated preneoplastic and tumorigenic responses in the nephron were also produced by nephrotoxic volatile hydrocarbons, for example, gasoline KJ-5 and JP 10[72,73]. Age-associated increases in the incidence rates of hyperplasia or tumors of the reproductive and endocrine organs in F344 rats are likely to represent hormonal imbalances in these aged animals.

Refinement of the present-day bioassay

Observation of the carcinogenic and/or toxic effects of chemicals on mice and rats in a number of long-term chronic studies has raised the problem

Table 6. Factors Showing Effect on Spontaneous Tumor Incidence

Factors	Effects
Hormones: PRL, ES, GH, TS, ACTH etc.	Tumor promotion or reduction
Nutritional: Dietary orotic acid	Tumor promotion
Deficiency in folic acid	Tumor promotion
Restriction of dietary fat or caloric intake	Tumor reduction
Nonneoplastic lesions: Chronic nephropathy	Renal cell tumor
Hormonal disturbance, like hyperplasia of endocrine glands	Endocrine adenomas
Age-associated metabolic shifts:	Tumor promotion

PRL, prolactin; ES, estrogen; GH, growth hormone; TS, thyroid-stimulating hormone; ACTH, adrenocorticotropin

of evaluating the naturally-occurring tumors as well as the non-neoplastic lesions. The unexpected high incidence of these naturally-occurring tumors and the resemblance of their mode of development to that of human cancer suggests the importance of age-associated or genetically-related occurrence of tumors in the living body.

An improved bioassay providing the maximal opportunity to detect a neoplastic response to chemicals while making a judgment regarding the observed carcinogenicity in a more convincing way than the current bioassay is recommended as follows:

1) The behavior and late occurrence of spontaneous tumors in Fischer 344 rats and B6C3F$_1$ mice in the present-day 104-week carcinogenicity tests strongly suggest that the use of 78-week bioassays of near-maximum tolerated doses of chemicals in these rodents will avoid many false-positive and false-negative risk assessments.
2) Incorporation of a satellite study for interim or serial observation at 52 weeks of study will give more insight into the biological effect of toxic and carcinogenic chemicals. Future development of tumor-marker analyses on blood, urine, or tissue specimens from surviving or necropsied animals will give additional information on carcinogenesis.

The determination of carcinogenic effects of chemicals by this improved bioassay will help us to more accurately eliminate chemical hazards from our occupational and living environments.

Other carcinogenesis bioassays such as those of using fetal, neonatal or infant rodent animals[74-77] or animals like small fish, "medaka" *Orysias lapipes*[78,79], may also be a promising way of detecting carcinogenic chemicals within 52 weeks of experiment. Analysis of a variety of factors or agents causing promotion or progression of tumors is also important[40], but it would require separate approaches from the carcinogenesis bioassay recommended above.

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