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Significance of Strain and Sex Differences in the Development of ^{252}Cf Neutron-induced Liver Tumors in Mice

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Mouse liver tumors occurring in C3H/HeN, C57BL/6N and C3B6F1 hybrid (C3H × C57BL) were studied following ^{252}Cf fission neutron irradiation. Three strains of mice of both sexes (about 30 mice/group) were irradiated once with ^{252}Cf at doses of 0, 12.5, 50 and 200 cGy. The groups were observed for 13 months after irradiation. The incidence of liver tumors in the non-irradiated controls was 0% in both sexes of C57BL/6N, 11.7% in males and 0% in females of C3B6F1 and 39.5% in males and 11.4% in females of C3H/HeN mice. In the four strains of mice thus far studied, including B6C3F1 hybrid (C57BL × C3H) which was previously studied, ^{252}Cf irradiation has increased the tumor incidence dose-dependently in males and in females, but less effectively in females. The mean number and size of liver tumors were clearly correlated with tumor incidence. The incidence was always highest in C3H/HeN mice of both sexes, followed by B6C3F1, C3B6F1 and C57BL/6N mice. The influence of sex hormones was studied in B6C3F1 mice of both sexes after 200 cGy of ^{252}Cf irradiation. In males, the incidence of liver tumors was significantly decreased from 55.2% to 23.3% and 25.9% after orchidectomy, and in females it was slightly decreased from 27.6% to 14.8% and 18.8% after ovariectomy. Supplementation of testosterone in orchidectomized mice did not restore the occurrence of liver tumors.

Key words: Liver tumor — Neutron — Strain difference — Sex difference — Mice

Liver tumors appearing in B6C3F1 mice have served as a good marker to evaluate the toxicities of environmental chemicals such as food additives,[1] cosmetics and medicine,[2, 3] and for the assessment of biological effect of radiation.[4, 5] B6C3F1 mice are also suitable for life-long study because this strain is quite resistant to various types of tumors except for liver tumor and is also resistant to infectious diseases. Spontaneous occurrrence of liver tumors has been observed in C3H/HeN[6] with the prevalence being higher in males than in females. Although the precise mechanism of this unique property is not known, it is genetically inherited and amplified by radiation.[5] On the other hand, C57BL/6N strain can develop spontaneous lymphomas in later life,[7] but is resistant to liver tumors. We have also found unique immunologic disorders characterized by hair loss, mesenteric or systemic lymphoadenopathies, hepatosplenomegaly with predominant infiltration of plasma cells associated with accumulation of collagenous tissues and sclerotic kidney (A. Ito *et al.*, unpublished data).

In this series of radiation tumorigenesis studies with ^{252}Cf irradiation,[4, 5] the influence of strain and sex differences in the development of liver tumors was examined.

MATERIALS AND METHODS

Animals B6C3F1, C3B6F1, C57BL/6J and C3H/HeN mice of both sexes were purchased from Charles River Japan, Inc. (Kanagawa). About 6–8 mice were housed together in autoclaved cages with sterilized wood chips and were kept in a room with controlled temperature (24±2°C) and humidity (55±10%) under a regular light-dark (12 h-12 h) cycle. All the mice were given a normal diet (Oriental Co., Ltd., Tokyo) and tap water *ad libitum*. Mice were maintained under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" by Hiroshima University.

Gonadectomy and testosterone Orchidectomy or ovariectomy were performed under light ether anesthesia. Testosterone containing cholesterol pellets were prepared by melting testosterone (Sigma Chemical No. T-1750) with cholesterol powder by heating until the mixture fused. Each pellet was individually weighed and cut into pellets containing 0.1 mg of testosterone. This was implanted subcutaneously and renewed every month until the animals were killed.

^{252}Cf neutron irradiation A ^{252}Cf source was used for neutron irradiation. The radiation facility and irradiation conditions employed have been described in detail previously.[5] Six-week-old mice were exposed to whole-body irradiation of 0 to 200 cGy at a dose rate of 0.6–0.8 cGy/

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min. Individual mice were placed in acrylate mesh containers that allowed free movement within the space.

Experimental groups Two experiments were carried out in the present study. Experiment 1 was to observe the effect of strain difference and experiment 2 was to analyze the effect of sex on the development of tumors. Three strains of mice were studied in this experiment. In experiment 1, both sexes of C57BL/6N, C3B6F1 and C3H/HeN mice at 6 weeks of age were irradiated once with 0, 12.5, 50 and 200 cGy of ^{252}Cf neutrons. In the case of C3B6F1 mice only 0 and 200 cGy groups were studied. In experiment 2, both sexes of B6C3F1 mice were divided into the following groups; group 1: male mice, group 2: orchidectomy at one week after irradiation, group 3: orchidectomy at six months after irradiation, group 4: orchidectomy at two weeks before irradiation and supplemented with testosterone, group 5: normal female mice, group 6: ovariectomy at one week after irradiation, group 7: ovariectomy at six months after irradiation.

Pathology Experimental animals were examined daily and weighed once a month throughout the experimental periods. The observation period was limited to 13 months after irradiation, since any period longer than this may increase the occurrence of spontaneous liver tumors. All the mice were killed under ether anesthesia and their body and liver weights were measured. The number and size of the liver tumors were also scored. Liver tumors were routinely stained with hematoxylin and eosin (H & E) and some mice received subcutaneous injection of iron dextran as described previously.[5] Neoplastic liver nodules were devoid of iron-positive Kupffer cells, thus permitting the differentiation of neoplastic nodules from non-neoplastic foci.

Statistical analysis Chi-square test and Student's t test were used for data analysis.

Table I. Experimental Protocol, Organ Weights and Incidence of Tumors in C57BL/6N Mice

Cf dose (cGy)	Effective no. of mice	Survival rate (%)	Body weight (g±SD)	Liver weight (g±SD)	Liver wt. as mean % of body wt.	Incidence of tumors (%)								Multiplicity	
						Total	Liver tumor			Lymphoma	Adrenal	Ovary	Others	Double	Triple
							Incidence (%)	No./ mouse	Size (mm)						
Male															
0	23	82	33.8±6.9	1.62±0.55	4.91±1.90	4.3	0	0	0	0	0	—	4.3[a]	0	0
12.5	32	100	36.9±5.6	1.60±0.72	4.45±2.48	15.6	6.3	0.09	0.22	9.4	0	—	0	0	0
50	31	97	35.2±6.3	1.31±0.21	3.80±0.72	16.1	3.2	0.03	0.08	3.2	0	—	12.8[b]	0	0
200	31	91	33.1±6.0	1.33±0.26	4.07±0.74	25.8	9.7	0.16	0.32	16.1	3.2	—	0	1	0
Female															
0	25	89	26.9±4.0	1.23±0.34	4.70±1.65	20.0	0	0	0	16.0	0	12.0	0	2	0
12.5	30	94	33.0±6.5	1.17±0.30	3.67±1.19	33.3	3.3	0.10	0.10	13.3	0	20.0	9.9[c]	2	1
50	31	97	32.7±6.8	1.23±0.45	4.17±2.21	32.3	0	0	0	19.4	3.2	9.7	6.5[d]	2	0
200	26	81	32.8±5.1	1.07±0.16	3.26±0.46	30.8	3.8	0.04	0.08	15.4	11.5	0	0	0	0

a) 1 forestomach papilloma. *b*) 1 pituitary, 1 urinary bladder and 1 lung tumors. *c*) 1 pituitary, 1 forestomach and 1 skin tumors. *d*) 2 forestomach tumors.

Table II. Experimental Protocol, Organ Weights and Incidence of Tumors in C3B6F1 Mice

Cf dose (cGy)	Effective no. of mice	Survival rate (%)	Body weight (g±SD)	Liver weight (g±SD)	Liver wt. as mean % of body wt.	Incidence of tumor (%)								Multiplicity	
						Total	Liver tumor			Lymphoma	Adrenal	Ovary	Others	Double	Triple
							Incidence (%)	No./ mouse	Size (mm)						
Male															
0	34	100	48.8±5.7	2.28±0.35	4.66±0.53	14.7	11.7	0.15	1.06	0	0	—	2.9[a]	0	0
200	31	97	38.6±3.9	2.14±0.56	5.10±1.62	61.3	54.8	1.00	5.00	3.2	0	—	6.9[a]	1	0
Female															
0	33	97	36.4±4.8	1.37±0.16	3.69±0.41	9.1	0	0	0	0	0	6.1	3.0[b]	0	0
200	27	84	41.5±4.2	1.57±0.47	3.82±1.22	51.9	18.5	0.26	2.11	3.7	29.6	0	0	1	0

a) 1 or 2 lung tumors. *b*) 1 uterine tumor.

Table III. Experimental Protocol, Organ Weights and Incidence of Tumors in C3H/HeN Mice

Cf dose (cGy)	Effective no. of mice	Survival rate (%)	Body weight (g±SD)	Liver weight (g±SD)	Liver wt. as mean % of body wt.	Incidence of tumor (%)								Multiplicity	
						Total	Liver tumor			Lymphoma	Adrenal	Ovary	Others	Double	Triple
							Incidence (%)	No./ mouse	Size (mm)						
Male															
0	43	78	36.6±4.5	1.79±0.34	4.90±0.78	44.2	39.5	0.72	3.02	2.3	2.3	—	2.3[a]	1	0
12.5	28	88	38.9±3.2	2.02±0.42	5.27±1.19	64.2	60.7	1.36	7.57	0	3.6	—	0	0	0
50	37	95	37.3±5.9	1.95±0.53	5.12±1.07	78.4	70.3	1.78	9.00	13.5	18.9	—	4.2[b]	11	0
200	48	79	33.1±4.5	1.69±0.34	5.16±0.82	75.0	70.8	2.95	11.35	6.3	4.2	—	8.3[c]	6	1
Female															
0	35	100	32.7±4.8	1.33±0.17	4.12±0.64	74.3	11.4	0.11	0.51	2.9	0	65.9	0	2	0
12.5	29	91	33.7±6.7	1.58±0.23	4.80±0.80	34.5	0	0	0	0	0	34.5	0	0	0
50	40	100	34.2±6.0	1.34±0.22	3.89±0.75	100	17.5	0.18	1.50	12.5	0	93.8	17.5[d]	10	4
200	35	79	31.6±4.9	1.31±0.20	4.22±0.78	94.1	31.4	0.37	2.07	2.9	14.7	85.3	11.4[e]	14	5

a) 1 skin tumor. *b*) 1 lung and 1 skin tumors. *c*) 1 lung, 1 skin, 1 kidney and 1 salivary gland tumors. *d*) 3 Harderian gland, 1 lung, 1 skin, 1 thyroid and 1 bone tumors. *e*) 1 pituitary, 2 breast and 1 skin tumors.

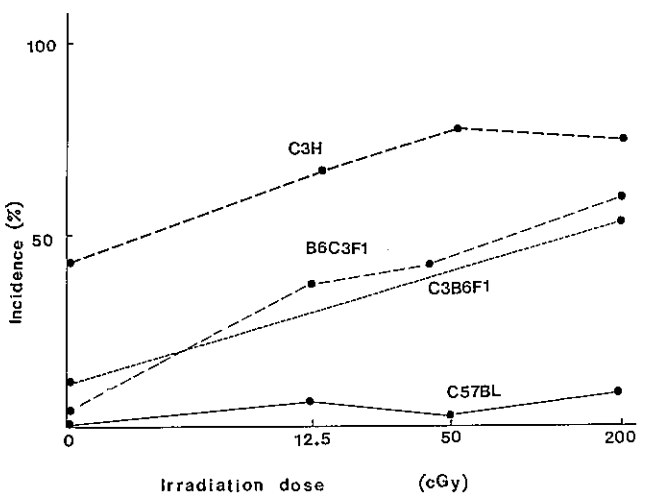

Fig. 1. Incidence of liver tumors in male C3H/HeN, B6C3F1, C3B6F1 and C57BL/6N mice is correlated with the administered dose of ^{252}Cf neutrons.

RESULTS

Strain difference In our previous study, a dose-response increase of liver tumors in both sexes of B6C3F1 mice exposed to ^{252}Cf neutron was established.[4] In this study, we examined the occurrence of tumors in the parental strains of B6C3F1 mice such as C57BL/6N and C3H/HeN and in the reversed strain of C3B6F1 mice of both sexes. The total tumor incidence was high in C3H/HeN, moderate in B6C3F1 and C3B6F1 and low in C57BL/6N mice. This was because the tumors induced with the highest frequency by ^{252}Cf neutron irradiation were liver tumors in males and ovarian tumors in females.

The occurrence of liver tumors was always more prominent in males than in females in any strain. Multiplicity and average tumor size were consistent with tumor incidence (Tables I and II). Specifically, the highest incidence was at 200 cGy: 9.7% in C57BL/6N males and 3.8% in females (Table I), 54.8% in C3B6F1 males and 18.5% in females (Table II) and 70.8% in C3H/HeN males and 31.4% in females (Table III). Including the data on B6C3F1 mice, the incidence of liver tumors in the three strains of male mice demonstrated a good correlation with the administered dose of ^{252}Cf neutrons (Fig. 1). C57BL mice responded to ^{252}Cf neutron irradiation with minimum efficiency for the development of liver tumors. The incidence of ovarian tumors was most frequent in C3H/HeN followed by B6C3F1, C3B6F1 and C57BL/6N, but was not correlated to the administered dose of radiation. B6C3F1, C57BL/6N and C3H/HeN were most sensitive at lower doses around 50 cGy. The development of lymphomas showed the highest frequency in C57BL mice (Table I). Although it was greater in females than in males, no dose-response relationship was observed between the incidence of lymphoma and the administered dose of ^{252}Cf. Most of the lymphomas which developed in C57BL/6N were observed in the mesenteric lymph nodes, spleen and liver, and the thymus was rarely involved. Infiltration of lymphoma cells into the relevant organs was mainly composed of IgG-producing plasma cells. Different from C57BL/6N mice, the incidence of lymphomas was low in C3H/HeN and C3B6F1 mice (Tables II and III). The incidence of adrenal tumors seemed to be increased by ^{252}Cf neutron irradiation. They were found mainly in the cortical zones and were steroid-producers, since accumulation of steroid hormones was observed as yellowish nodules.

Table IV. Experimental Protocol, Organ Weights and Incidence of Tumors in ^{252}Cf Neutron-irradiated (200 cGy) and Gonadectomized B6C3F1 Mice

Exp No.	Treatment[a]	Effective no. of mice	Body weight (g±SD)	Liver weight (g±SD)	Liver wt. as mean % of body wt.	Incidence of tumors (%)							Multiplicity	
						Total	Liver tumor		Lymphoma	Adrenal	Ovary	Others	Double	Triple
							Incidence (%)	No./ mouse						
1	♂	29	38.6±5.0	1.91±0.45	4.89±1.16[b]	62.1	55.2	1.21	3.4	0	—	17.2[e]	2	0
2	♂ −1 wk	27	42.2±3.7[c]	2.23±0.41[c]	5.29±0.87	37.0	25.9*	0.33	0	11.1	—	7.4[f]	2	0
3	♂ +6 mo	30	40.0±3.1	2.25±0.58[c]	5.53±1.34	43.3	23.3	0.27	3.3	20.0	—	16.7[g]	2	0
4	♂ +T, 6 mo	29	42.0±3.7[c]	2.18±0.58	5.10±1.17	48.3	27.6	0.31	13.8	3.4	—	3.4[h]	1	0
5	♀	29	38.0±9.2	1.73±0.47	4.37±0.74	34.5	27.6	0.34	0	20.7	0	24.1[i]	1	0
6	♀ −1 wk	32	43.9±2.4[d]	2.27±0.39[d]	5.19±0.91[d]	34.5	18.8	0.22	0	34.4	—	12.5[j]	2	0
7	♀ +6 mo	27	41.7±2.2[d]	1.77±0.26[d]	4.26±0.52	29.6	14.8	0.19	14.8	7.4	—	7.4[k]	2	0

a) Gonadectomy was done 1 week before or 6 months after irradiation. Testosterone (T) was given 6 months after irradiation.
b) Means±SD. *c*) Significantly different from group 1. *d*) Significantly different from group 5. *e*) 2 skin, 1 forestomach and 1 kidney tumors. *f*) 1 Harderian gland and 1 lung tumors. *g*) 3 lung, 1 Harderian gland and 1 thyroid tumors. *h*) 1 pituitary tumor. *i*) 1 lung, 2 skin, 1 forestomach, 2 Harderian gland and 1 urinary bladder tumors. *j*) 1 lung, 2 Harderian gland and 1 pituitary tumors. *k*) 2 lung tumors.
* Significantly lower than group 1 by $P<0.05$.

Role of sex hormones in the development of liver tumors
In spontaneous or radiation-induced liver tumors in B6C3F1 mice, the incidence is always higher in males than in females. In this experiment, the role of the gonads in the development of liver tumors was examined. In groups 1 to 4 (Table IV), male mice were irradiated and received gonadectomy before or after irradiation. Their average body weights increased after gonadectomy, but the incidence of liver tumors was significantly decreased by gonadectomy (group 2) and the incidence was not restored by supplementation of testosterone (group 4) in this study. Ovariectomy showed a slight inhibitory effect in females (groups 6 and 7).

DISCUSSION

It has previously been reported that the sensitivity of C57BL/6N and C3H/HeN mice to chemical carcinogens, such as nitroso compounds, for liver tumor development has a pattern similar to that of the natural occurrence of liver tumors: C3H/HeN mice are sensitive and C57BL/6N mice are resistant.[8] This is one of a series of studies to analyze radiation-induced liver tumors. Previously we presented a report on a dose-response study in B6C3F1 mice exposed to ^{252}Cf neutrons. Although there is no clear-cut evidence to explain the development of hepatic tumors in C3H/HeN male mice, genetic background seems to play a critical role in the tumor development. In this respect, it is interesting to note that C3B6F1 mice and B6C3F1 mice produced similar incidences of liver tumors after 200 cGy of ^{252}Cf neutron irradiation.[4] This finding indicates that susceptibility can be inherited through both sexes of parents and it will be significantly amplified in the male. The high frequency of liver tumors occurring in C3H/HeN mice is genetically transmitted and its genetic constituents have been named hepato-carcinogen sensitive locus.[9] Recently, Lee *et al.* have demonstrated that diethylnitrosamine-induced chimeric liver lesions between C3H/HeN and C57Bl/6N were predominantly composed of C3H/HeN constituent and those of C57BL/6N were relatively small.[10] Our experimental results obtained by ^{252}Cf neutron irradiation showed a definite increase in the liver tumor incidence in a dose-dependent manner in C3H/HeN and B6C3F1 male mice. Thus, it is essential for the occurrence of liver tumors to have C3H/HeN traits in either sex. In contrast, C57BL/6N mice did not respond to ^{252}Cf neutron irradiation. It has been reported that mutant C3H/HeN males lacking androgen receptor showed a marked decrease in tumor incidence,[11] but plasma testosterone levels and androgen receptor bindings were not well correlated with the occurrence of liver tumors.[12] The presence of testosterone is essential for the development of liver tumors, but the direct growth regulator may be epidermal growth factor.[13] Further studies are in progress to explain the preferential occurrence of liver tumors in males.

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