高LET放射線で誘発した(C57BL/6N x C3H/HeN)F1マウス肝腫瘍の経時的生存率

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Comparison of the Effectiveness of High and Low LET Radiations for the Proportion of Survivals with Liver Tumors at Every Age in (C57BL/6N × C3H/HeN) F_1 Mice

Yumiko NITTA[1], Kenichi SATOH[2], Shinji SUGA[3], Satoru ENDO[4] and Kohsaku NITTA[5]

[1]*International Radiation Information Center,* [2]*Division of Bio-Medical Informatics,* [3]*Radiation Research Center for Frontier Science Facilities for Radiation Experiments,* [4]*Division of Radiation Biophysics, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8553 and* [5]*Senogawa Hospital, Senogawa Medical Corporation, 4–11–13, Nakanohigashi, Aki-ku, Hiroshima 739–0323, Japan*

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ABSTRACT. To investigate the late effects of neutrons at the energy below 1 MeV on the liver carcinogenesis as a function of age, one-week old mice were exposed to 1.0 Gy monoenergetic neutrons (0.317, 0.525 and 1.026 MeV) or ^{137}Cs gamma rays. Survival and carcinogenesis were examined by 18 months of age. Following radiation, tumor incidences in liver, Harderian gland, lung, ovary and pituitary gland were compared. The proportion of the lifespan with liver tumors exposed to neutrons to that exposed to gamma rays was calculated as a function of age. Survival rates among the three groups exposed to neutrons of different energies were not significantly different from one another but shorter than those treated with gamma rays for both sexes. With regard to liver tumor incidence evaluated at 18 months of age, the effectiveness of neutrons to gamma rays was 2.54 for females, and 2.08 for males by the factor. Levels of estrogen in the serum were similar between mice bearing liver tumors and those devoid of tumors. In conclusion, all three energies of neutrons induced similar effectiveness with respect to liver carcinogenicity. Proportions of the lifespan with liver tumors of neutron-exposed to gamma-exposed were shorter in females than males along with ages over 12 months. To obtain this factor at every age contributed for the evaluation of the biological effectiveness of radiations with the parameter of tumor incidence and latency simultaneously.
KEY WORDS: lifespan, liver tumor, mouse high LET radiation neutron.

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The biological effectiveness of every radiation differs depending on the linear energy transfer (LET). Firstly because, the pattern of energy deposition is different between the low and high LET-radiations. The low LET radiation attack the genome by single track to cause single strand breaks, on the other hand, the high LET radiation attack it by multiple tracks to cause double strand breaks followed by the apoptotic cell death. Therefore, long-term effects of high LET radiations were limited by the probability of the single-track-survival in the traversed cell [8]. Secondary, the low and high LET-radiations induced different forms of genomic instability. The former induced transgenerational destabilization of the genome detected by a length change mutation at a minisatellite locus in mice [26]. The latter induced chromosomal instability following the exposure to lymphocytes in mice and humans [11, 12]. However, the relationship between the genomic instability and the specific disease has not been proved clearly, yet. Thirdly, the high LET radiations but not the low LET radiations caused morphological changes in the inner ear of mice when exposed to during the specific stage of the development [25]. Fourthly, animals subjected to high and low LET radiations displayed difference in the tumor spectrum. The low LET-radiations induced granulosa cell tumors; on the other hand, the high LET-radiations induced tubular adenomas instead in the ovary [22].

The energy spectrum is an important factor for the evaluation of biological effectiveness of high LET radiations. Higher incidences of teratogenic [2, 31], toxic [15] and carcinogenic [29, 36] disorders among the atomic bomb survivors of Hiroshima than those of at Nagasaki [14] had suggested the stronger effectiveness of neutrons at the energy range of 10^{-2}~1.0 MeV than >1.0 MeV [37]. These facts were confirmed experimentally using the fission neutrons form ^{252}Cf [17, 30, 34] and monoenergetic neutrons from the Hiroshima University Radio Reactor (HIRRAC) [16, 19, 20, 35]. However, limited reports are available on the late effects including carcinogenesis of neutrons to evaluate the probability of the single-track-survival or the genomic instability of the neutron-traversed cell [8, 21, 22].

The neutron, one of high LET-radiations has been used clinically for the radiotherapy which offers both local control rates and survival rates in patients with tumors that are not candidates for surgical resection [1, 33]. The main disadvantage of neutrons has been the toxicity to the normal tissue. When neutrons were irradiated to the tumor mass at the salivary gland, not only the acute toxicity but late effects were observed; optic neuritis, soft tissue necrosis or ulceration, cervical meylopathy, progressive sensory loss of the trigeminal nerve and necrosis of the lobe of the brain [5].

This study was performed to evaluate late effects of neutrons in the mouse experimental model for offering the information to contribute toward the development of radiotherapy equipments safe and high-performance for human use. The indicator of late effects in this experiment was the liver tumorigenicity. The energy of the neutron used was below 1.0 MeV, where the range of LET was between 63 and 77 keV/μm [7, 16, 19, 20, 34]. The (C57BL/6N × C3H/He) F_1 mouse was used because of its' genetic background susceptible to radiations as well as the high incidence of

spontaneous liver tumors [21–23]. The proportion of the lifespan with liver tumors as a function of age was compared between groups exposed to neutrons and those to gamma rays.

MATERIALS AND METHODS

Radiation: Monoenergetic neutrons were generated by a ^{7}Li[p,n]^{7}Be reaction at the Hiroshima University Radiobiological Reactor (HIRRAC) [6]. A ^{3}He gas proportional counter measured spectra of neutrons from HIRRAC. As the thickness of the ^{7}Li target used was 15 micron, the values of neutron energies in Table 1 were calculated after the substitution of the average energetic loss in the target, which was estimated as 100 keV. Values of the LET were 66.0, 71.3 and 67.5 keV/μm for 0.317, 0.525 and 1.026 MeV of the neutron energy, respectively [7]. Gamma rays were generated by the ^{137}Cs source (Shimadzu biotech, Japan).

Mice: Female C57BL/6N and male C3H/HeN mice were purchased from Charles River Japan Inc. to generate (C57BL/6N × C3H/He) F_1. The mice were maintained in an animal facility at 22 ± 2°C under a 12-hr light/dark cycle. Food and tap water were provided *ad libitum*. All procedures for treating mice were performed in accordance with instructional guidelines of the Institute of Laboratory Animal Science, Hiroshima University.

Radiation carcinogenesis experiment: One-week-old mice were whole body-exposed to neutrons or gamma rays (Table 1) [22, 25]. Mice were killed when naturally moribund, and subjected to autopsy. Alternatively, animals were sacrificed at 18 months of age. A complete study of occurring tumors was conducted, and the actual number of tumors observed was recorded. Macroscopic lesions and main organs were processed for the preparation of tissue sections. The numbers and sizes of liver tumors were measured at autopsy.

Statistical model for the estimation of effectiveness of radiations on lifetime with liver tumors: The survival of mice bearing liver tumors in the three neutron-irradiated groups was treated as one group for the comparison of effectiveness between the two high and low LET-radiations. The proportion of the survival of neutrons to gamma rays [F] at time t is defined by

$$F[t/b] = \{1-S[t, b]\}/\{1-S[t,0]\},\ S[t,b] = S_o[t]\exp[bx],$$

where $S[t]$ is the survival function on the Cox Proportional Hazard model, $S_o[t]$ is the baseline survival function, x is the dose of neutrons, especially x=0 shows gamma rays, and b is the coefficient. From the partial likelihood method, the estimator b of b can be obtained, and then the variance of F is given by

$$Var[F[t/b]] = 1/\{[1-S\ [t]]2\}\ Var[S[t, b]].$$

Let $b\hat{}$ has mean b and variance σ^2. According to a version of the Talor's theorem, $S[b\hat{}] \sim S[b^*] + S[1][b^*][b\hat{}-b^*]$ with $S[1][b] = dS[b]/db = dS[b]\ ln[s[b]]x$. This leads to an approximation of the variance of survival function,

$Var\ [S[b\hat{}]] \sim \{S[b^*]\ ln[S[b^*]]x\}^2\sigma^{2*}$. In this manuscript, $S_o[t]$ was estimated by Weibul Hazard Model through Cox Proportional Hazard Model with estimated coefficient $b\hat{}$. Thus the variance of F is approximated and 95% confidence interval is given by $F[t/b\hat{}] + 1.96Var[F[t/b\hat{}]]^{1/2}$.

Radioimmunoassay (RIA): Blood was extracted from mice at autopsy. After centrifugation at 3000 rpm, serum was stored at –20°C. The concentration of 17-β-estradiol (E2) was measured by RIA using an E2 detection kit (Japan D.P.C. Ltd., Tokyo, Japan). Duplicate serum samples extracted with diethyl ether were incubated with the E2 antibody and ^{3}H-labeled E2 overnight at 4°C. Next, samples were incubated with charcoal solution and centrifuged. Radioactivity was measured from the supernatant in a scintillation counter (Aloka Ltd., Mitaka, Japan).

Statistical analysis: Kaplan-Meier analysis was performed for the data of radiation carcinogenesis [13]. The survival proportion of mice with liver tumors and survival curves were compared by the Log-Rank, Wilcoxon and Cox-Mantel tests [28].

RESULTS

Radiation carcinogenesis experiment: Experimental groups were summarized in Table 1. Survival rates were depicted in Fig. 1-A and B. The survival proportion was very low in all of the three neutron-exposed groups at 18 months of age, while more than 60% of mice were alive in the gamma-irradiated group.

Data on the pathology of neoplastic lesions were presented in Table 2. Tumors in the liver, Harderian gland and lung were found developing from nine months of age for both sexes in the neutron-irradiated groups (Fig. 2). Pitu-

Table 1. Experimental groups, radiaiton quality, dose and dose rate used for the experiment

Groups	*Number of mice*		Radiation			Neutron or γ-ray component (cGy)		*Dose rate (cGy/min)*
	Female	Male	Neutron energy (MeV)		Dose (Gy)	Neutron (mean ± SE)	γ-ray	(mean ± SE)
1	20	20	None	–	0	0	0	0
2	25	16	Neutron	0.317	1.0	93.09 ± 1.45	6.91	1.09 ± 0.25
3	20	27	Neutron	0.525	1.0	96.89 ± 1.32	3.11	4.38 ± 0.74
4	24	20	Neutron	1.026	1.0	95.48 ± 0.90	4.52	2.94 ± 1.25
5	31	19	Cs-137	–	1.0	0	100	3.63

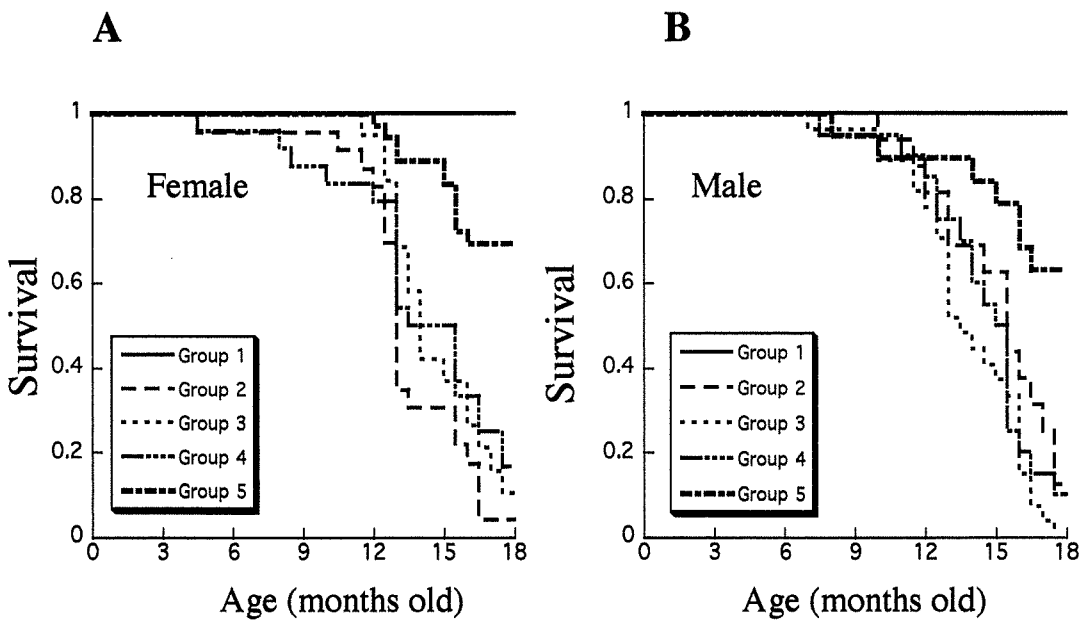

Fig. 1. Survival proportion of (C57BL/6N × C3H/He) F_1 mice following the exposure to neutrons or gamma rays. Kaplan-Meier analysis was performed as a function of age.

Table 2. Pathology data of the (C57BL/6N × C3H/He)F_1 mouse whole body-irradiated with 1 Gy of neutrons or gamma rays

Groups		Radiation	Number of	Number of mice (%) bearing tumors at sacrifice					
Number	Sex		mice	Liver	Pituitary	Harderian gland	Ovary	Lung	Others[a)]
1	Female	None	20	0	0	0	0	0	0
2	Female	Neutron	25	15 (60.0)	12	1	5	2	4
3	Female	Neutron	20	11 (55.0)	9	4	6	2	4
4	Female	Neutron	24	15 (62.5)	9	8	7	2	4
5	Female	Cs-137	31	6 (19.4)	1	1	12	2	3
1	Male	None	20	0	0	0	–	0	0
2	Male	Neutron	16	15 (93.8)	0	3	–	1	4
3	Male	Neutron	27	16 (59.3)	1	5	–	4	3
4	Male	Neutron	20	18 (90.0)	1	2	–	4	2
5	Male	Cs-137	19	7 (36.8)	0	1	–	1	2

a) osteosarcoma, thymic lymphoma, non-thymic lymphoma, adrenal gland tumor, thyroid tumor, mammary gland tumor and skin tumors.

itary tumors were observed mainly in females.

When the proportions of survivals were compared among the neutron irradiated- groups by the Cox-Mantel test, there was no energy dependency (Table 3). The proportions of mice bearing four types of tumors at autopsy were tested individually for incidence and latency by the Cox-Mantel test (Table 3). Statistical differences in the tumor development between the two radiations were significant for liver tumorigenesis in all the three neutron-irradiated groups of both sexes ($p<0.01$ for all groups).

Analysis of liver tumorigenesis: In order to examine the sex difference on liver tumorigenesis, three neutron-irradiated groups were included into one group for each sex. Then, the proportion of survivals with liver tumors of the neutron-irradiated group to the gamma-irradiated group was calculated as a function of age in Fig. 3. Factors were 5.22 and 5.58 at the age of 12 months, and then 2.54 and 2.08 at 18 months of age for females and males, respectively.

To make sure the physiological condition of females with or without liver tumors, the serum E2 level was measured (Fig. 4). The average E2 levels of mice with and without liver tumors were compared. No statistically significant difference in the serum E2 level was observed.

Table 4 depicted the number and size of liver tumors. The tumor number per mouse among neutron-irradiated females increased most significantly in the Group 2 ($P=0.00064$, compared to the Group 5). The group 2 developed large tumors with high frequency ($P<0.01$ for <20 mm, compared to the Group 5). The number of tumors per mouse increased most significantly in the Group 4 in males

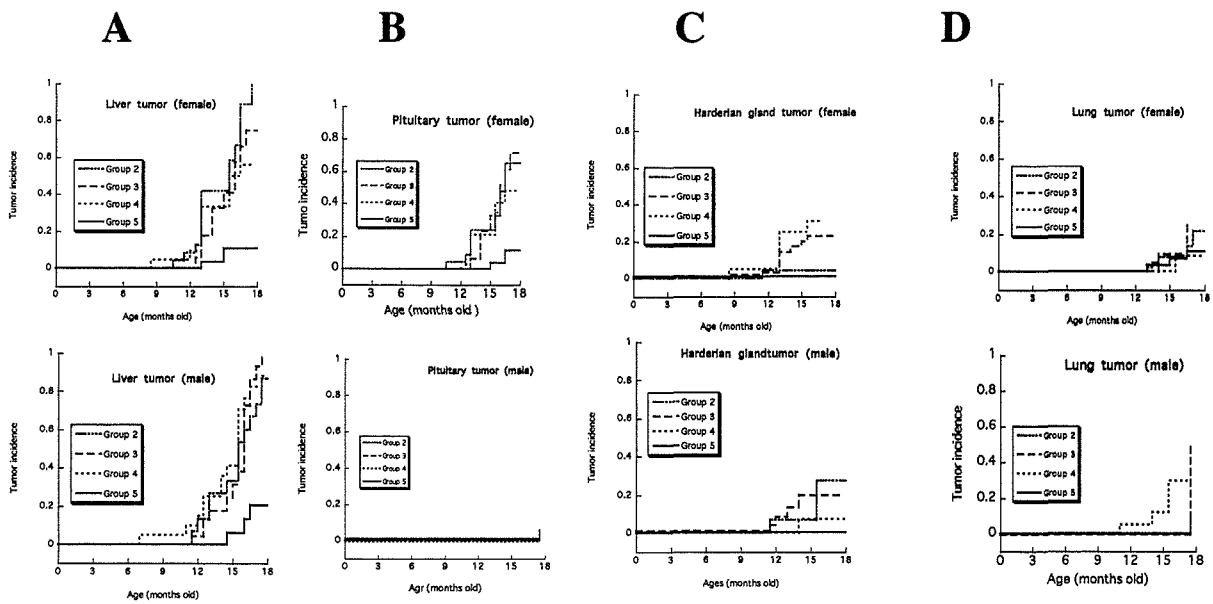

Fig. 2. The development of tumors in the (C57BL/6N × C3H/He) F_1 mice after the exposure to neutrons or gamma rays. Tumors in the liver (A), pituitary (B), Harderian gland (C) and lung (D) increased with age.

Table 3. P-values of the Cox-Mantel test on the raw number of neutron-irradiated mice with tumors at autopsy compared to gamma-irradiated mice

Groups			P-values of the Cox-Mantel test			
Number	Sex	Life span	Liver tumor	Pituitary tumor	Harderian tumor	Lung tumor
2	Female	0.00001	0.00000[a)]	0.00002	0.45917	0.60131
3	Female	0.00317	0.00006	0.00002	0.00224	0.17215
4	Female	0.07480	0.00062	0.00042	0.00380	0.77005
5	Female	1	1	1	1	1
2	Male	0.00494	0.00001	–	0.03744	0.13301
3	Male	0.00000[a)]	0.00000[a)]	–	0.01041	0.00528
4	Male	0.00253	0.00001	–	0.01910	0.45501
5	Male	1	1	–	1	1

a) P<0.00001.

(P=0.00871, compared to Group 5). Sizes of tumors of the male group 4 were small (<5 mm for P<0.01, 5~10 mm for P<0.01 and 10~20 mm for P<0.05 compared to Group 5). The size of liver tumors did not correlate with the life shortening neither the serum E2 level.

DISCUSSION

The estimated relative biological effectiveness (RBE) of neutrons to gamma rays was about 6 for all solid tumors [9]. The radiation-weighting factor for neutrons was between 5 and 20, dependent on the energy [10]. For the estimation of RBE, more than three doses should be set up for any experiment, the doses of which are within the range of liner dose-effect relationship. Considering the long latencies for the induction of solid tumors by radiation in mice [3, 20–24], it is not practical to set up such large scales of experiments to obtain the factor of RBE for tumorigenesis. Above two factors are obtained from the comparison of the end point-phenomenon and this is enough for the evaluation of acute effectiveness of radiation. However, the latency is important for the evaluation of late effectiveness of radiations. The figure, which indicates the daily RBE, must make the deficit good.

We here showed the factor for the comparison of effectiveness of the neutron for the survival with liver tumors as a function of age. This factor is useful to indicate the biological effectiveness of high- to low-LET radiations. Because, neutrons showed the bell-shape curve for the dose-effect relationship, the peak of which is around 1 Gy when exposed at ages of young ages for the examination of late effects [21, 22]. On the other hand for gamma rays, doses less than 1Gy were in the range of threshold for the induction of late effects [21, 22]. Therefore, the dose, 1 Gy was

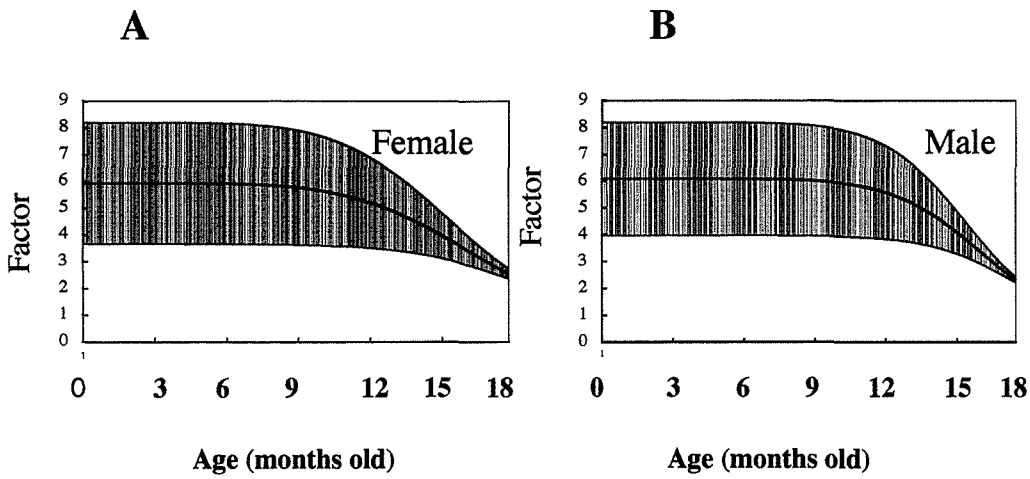

Fig. 3. Proportion of survivals with liver tumors in female (A) and male (B) (C57BL/6N × C3H/He) F_1 mice after exposure to neutrons as a function of age. The gray area shows the upper and lower 95% confidence limits. The ^{137}Cs gamma rays were used for the reference radiation. In order to obtain the Factor in y-axis, the statistical models are explained in the MATERIALS AND METHODS.

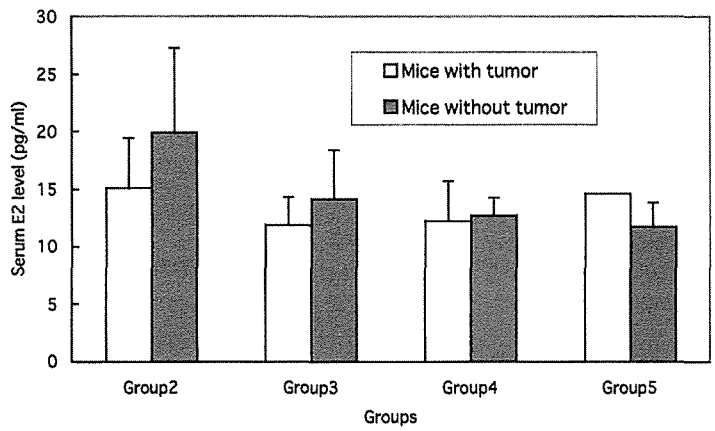

Fig. 4. Serum E_2 levels of female (C57BL/6N × C3H/He) F_1 mice with or without liver tumors, measured at autopsy after exposure to neutrons. The column and error bars represent mean ± standard error of data from three mice. The E_2 values from three mice were used for each bar.

maximum for neutrons and minimum for gamma rays. Actually, the liver tumor started to be observed at 12 months of age and continued to the 18 months of age in the monoenergetic neutron-irradiated groups. On the other hand in the gamma-irradiated group, the development of liver tumors has not reached to the maximum rate at 18 months of age, yet. We could show the incidence as well as the shortening of the latency simultaneously by the one-dose-experiment. The factor, we have presented in this article indicates the possibility not only to substitute the RBE for the high LET radiations, but also to apply for any agent which influences on the natural lifespan of experimental animals and humans.

Sex differences exist in the tumorigenesis in mice [4]. Males developed liver tumors with higher incidences than females [18, 34]. Two indicators showed the high susceptibility of female livers to neutrons in the present experiment, the incidence at the end point and the factor of the proportion of lifespan throughout. The latter parameter indicated the stronger effectiveness of neutrons for shortening the tumor latency in females than in males. Furthermore, it showed the time to start and the duration diminishing the tumor latency, especially in their later lives. In the cohort study of atomic bomb survivors, there was no sex difference in the risk of life shortening by hepatocarcinogenesis [27].

The energy dependency was observed for neutron-induced mutations at both the *hprt* and *tk* loci in a rodent fibroblast cell line [38] and at the *HPRT* locus in a human cell line [32]. To date, there has been no report on the relationship between the energy dependency and the experimental tumorigenesis. Our result showed no energy

Table 4. Numbers and sizes of the liver tumors of (C57BL/6N × C3H/He)F_1 mice

Groups		Number of	Number of		Number of tumors/mouse				Tumor size (mm in diameter)			
	Sex	mice	tumors	P value[a)]	(mean	±	SD)	P value[b)]	<5	5~10	10~20	20<
2	Female	25	42	0.00028	1.68	±	1.29	0.00073	11*	8*	4	14**
3	Female	20	38	0.00110	1.90	±	1.70	0.00064	19**	9*	4	4
4	Female	24	31	0.00406	1.29	±	1.09	0.00975	12**	7	6	6
5	Female	31	10	1	0.32	±	0.54	1	2	2	4	2
2	Male	16	37	0.03700	2.18	±	1.19	0.00184	11	8*	5	11
3	Male	27	37	0.02499	1.37	±	1.33	0.06416	6	12**	12*	8
4	Male	20	47	0.00871	2.35	±	1.09	0.00001	15**	12**	15*	5
5	Male	19	13	1	0.57	±	0.79	1	7	2	4	0

a) A test of the difference between two proportions (number of tumors/number of mice).
b) A test of the difference between two values (number of tumors/mouse, mean ± SD).
*: P<0.05 when compared to the value of Group 5 by a.
**: P<0.01 when compared to the value of Group 5 by a.

dependency on the liver tumorigenesis in the energy range from 0,317 to 1.026 MeV. Their values of LET were between 66.0 and 67.5 keV/μm, and their values of RBE were between 5.0 and 6.5 by the *in vitro* test of survivals using the mouse lymphoma cells [7]. Since the purpose of our present experiment has been the detection of the late effect of neutrons caused by the single-track-survival or the genomic instability of the neutron-traversed cell [8, 21, 22], it is not suitable to discuss concerning to the values of high LETs.

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