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Genetic Regulation of Development of Thymic Lymphomas Induced by *N*-Propyl-*N*-nitrosourea in the Rat

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To clarify the linkage between *Hbb* and *Tls-1* (thymic lymphoma susceptible-1) loci and to investigate other loci concerned in thymic lymphomagenesis, the BUF/Mna rat, which is highly sensitive to the lymphomagenic activity of *N*-propyl-*N*-nitrosourea (PNU), the WKY/NCrj rat, reported to be resistant, and their cross offspring were subjected to genetic analysis. F_1 hybrid and backcross generations were raised from the 2 strains, and 6 genetic markers including *Hbb* were analyzed in individuals of the backcross generation. However, no linkage between *Hbb* and *Tls-1* loci could be demonstrated since WKY rats also developed a high incidence of thymic lymphomas in response to PNU. Nevertheless, thymic lymphomas developed more rapidly and reached a larger size in the BUF rats. F_1 rats expressed a rather rapid and large tumor growth phenotype, while the [(WKY × BUF) × WKY] backcross generation consisted of rats with either rapidly growing or slowly growing tumors. It was thus concluded that rapid development of thymic lymphomas is determined by a gene, provisionally designated *Tls-3*. Analysis of the relationship between 6 genetic markers and development of thymic lymphoma in the backcross generation demonstrated that the *Tls-3* locus is loosely linked to the *Gc* locus, suggesting a possible location on rat chromosome 14. *Tls-3* may not be identical with *Tls-1* and other genes known to be relevant to thymic tumors, but its relationship with *Tls-2* remains obscure.

Key words: Chemical carcinogenesis — Thymic lymphoma — Genetic regulation — BUF/Mna rat — Thymic lymphoma susceptible-3 (*Tls-3*) gene

Thymic lymphomas can be readily induced by *N*-propyl-*N*-nitrosourea (PNU) and other *N*-nitrosoureas in F344 and some other strains of rats.[1-4] Shisa *et al.* reported that susceptibility to PNU-induction of such lesions was determined genetically by thymic lymphoma susceptible-1 (*Tls-1*) gene[2,5] and that acceleration of lymphomagenesis was due to another gene, *Tls-2*.[2] The *Tls-1* gene was presumed to be located close to the coat color loci, *c* and *p*, in the order *Tls-1–c–p*, and it was suggested that *Tls-1* may be very closely linked with the *Hbb* locus, though this could not be verified because the F344 rat, which is highly sensitive to the lymphomagenic activity of PNU, and the LES rat, which is resistant to that activity, are identical for *Hbb* type.[2] Further, no report is available on the chromosomal location and/or linkage group of *Tls-2*. The present experiment was conducted to clarify the linkage between *Hbb* and *Tls-1* loci and to investigate other possible loci determining thymic lymphomagenesis.

The BUF/Mna (BUF) rat (*Hbb*^{b}) has a high incidence of spontaneous thymomas originating from thymic epithelial cells,[6] and when continuously treated with PNU for more than 10 weeks in the drinking water, all treated rats of this strain develop thymic lymphomas with a very short survival period, as short as 14 weeks on average.[3] Therefore, the BUF rat is considered particularly sensitive to PNU-lymphomagenesis. On the other hand, the WKY/NCrj (WKY) rat (*Hbb*^{a}) has been reported to be resistant.[4] In the present study, we therefore attempted a genetic analysis of the development of PNU-induced thymic lymphomas using cross offspring of susceptible BUF and resistant WKY strains.

MATERIALS AND METHODS

Rats and diet Inbred WKY/NCrj rats were purchased from Charles River Japan, Inc. (Kanagawa). BUF/Mna rats were established and maintained by sister-brother mating by Matsuyama. Rats of F_1 and backcross generations were prepared by cross-breeding in the animal facility of the Aichi Cancer Center. All animals were maintained on a basal diet CE-2 (CLEA Japan, Inc., Tokyo) and tap water in an air-conditioned animal facility.

Experimental procedures Cross and backcross rats were prepared as follows; F_1 hybrid rats were raised by mating

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WKY females with BUF males, and backcross generations were raised by mating (WKY×BUF)F_1 females with WKY or BUF males.

When rats became 5 weeks old, continuous oral administration of PNU solution as the drinking water was started. PNU (Iwai Kagaku Yakuhin Co., Ltd., Tokyo) was dissolved daily in fresh deionized water at a concentration of 400 ppm, and this solution was given to rats in light-opaque bottles for 6 or 7 weeks. Body weights were determined every week, and PNU consumption was monitored every day. All rats were killed when they became moribund, and full autopsies for histopathological examination were carried out. Thymic lymphomas were diagnosed on the basis of involvement of the thymus and expression of Thy-1.1 antigen on the surface of tumor cells.

Analysis of genetic markers As shown in Table I, 14 genetic markers were tested in BUF, WKY and cross progeny rats: *Akp-1,* alkaline phosphatase-1[7]; *Cs-1,* RBC catalase[7,8]; *Es-1, -2* and *-14* types of esterases[7,9-11]; *Gc,* vitamin D binding protein,[12] which was previously designated *Gl-1*[13]; *Hbb,* hemoglobin beta chain[14]; *Mup-1,* male urinary protein[15,16]; *Pep-3,* pepsidase-3[7,17]; *Pg-1,* urinary pepsinogen-1[18]; *Pgd,* phosphogluconate dehydrogenase[7,19,20]; *Pgm,* phosphoglucomutase[7,20]; *RT1.A,* rat class I major histocompatibility complex[21]; and *Svp-1,* seminal vesicle protein-1.[22] Of these 14 genetic markers, 13 were analyzed biochemically by electrophoresis of the protein in polyacrylamide or starch gels following the methods described by earlier investigators[7-20] with some modifications. The other marker *RT1.A* phenotype was determined by hemolysis assay.[21] Anti-RT1.A^u rat antibody was kindly donated by Dr. Natori, Hokkaido University.

Six markers, *Akp-1, Pep-3, Pg-1, Pgd, Pgm* and *Svp-1,* were identical in both BUF and WKY rats, and the electrophoresis patterns of *Cs-1* and *Es-1* proved indistinguishable between WKY and (WKY×BUF)F_1 phenotypes. Six markers were applied for individuals of the [(WKY×BUF)×WKY] backcross generation. Of these, *Es-14* was detected in females but not in males,[11] and *Mup-1* was effective for males but not females.[15,16]

RESULTS

Incidence of thymic lymphomas Incidences of thymic lymphomas in each group of rats are listed in Table II, BUF rats being most sensitive to induction of thymic lymphomas (100%), followed by [(WKY×BUF)×BUF] backcross rats (95.8%). Although WKY rats were earlier reported to be resistant to induction of thymic lymphomas by PNU, with observed incidences in the literature of less than 10%,[4] the yield was 87.5% in the present experiment. Incidences for (WKY×BUF)F_1 rats and [(WKY×BUF)×WKY] backcross rats were 87.2% and 87.5%, respectively.

Table I. Genetic Markers Examined in BUF and WKY Rats and Their Cross Offspring

Gene symbol	Material	Linkage group (Chr. #)	Strain of rat[a]				
			BUF	WKY	WBF_1	WB×B	WB×W
1. *Akp-1*[b]	Plasma	XI	△	△			
2. *Cs-1*[c]	Erythrocytes	XIII	aa	bb	ab (=bb)	ab/aa	(ab/bb)
3. *Es-1*[d]	Serum	V (19)	bb	aa	ab (=aa)	ab/bb	(ab/aa)
4. *Es-2*	Serum	V (19)	aa	dd	ad	ad/aa	ad/dd
5. *Es-14*[e]	Serum (Female)	V (19)	aa	bb	ab (=aa)	(ab/aa)	ab/bb
6. *Gc*	Serum/Plasma	VI (14)	bb	aa	ab	ab/bb	ab/aa
7. *Hbb*	Erythrocytes	I (1)	bb	aa	ab	ab/bb	ab/aa
8. *Mup-1*	Urine (Male)	II (5)	bb	aa	ab	ab/bb	ab/aa
9. *Pep-3*[b]	Erythrocytes	X (13)	△	△			
10. *Pg-1*[b]	Urine	XII	△	△			
11. *Pgd*[b]	Erythrocytes	— (5)	△	△			
12. *Pgm*[b]	Erythrocytes	— (5)	△	△			
13. *RT1.A*[f]	Erythrocytes	IX (20)	uu	(ll)	ul	(ul/uu)	ul/ll
14. *Svp-1*[b]	Seminal fluid	IV (3)	△	△			

a) WBF_1, (WKY×BUF)F_1 rat; WB×B, [(WKY×BUF)×BUF] backcross rat; and WB×W, [(WKY×BUF)×WKY] backcross rat.
b) △: identical in both strains.
c) aa, fast type; bb, slow type; and ab, slow type (indistinguishable in WB×W backcross rats).
d) aa, positive band; bb, no band; and ab, positive band (indistinguishable in WB×W backcross rats).
e) aa, positive band; bb, no band; and ab, positive band (indistinguishable in WB×B backcross rats).
f) Anti-RT1.A^U-antibody and not anti-RT1.A^l was used for the hemolysis assay.

Table II. Average Latent Period and Average Thymus Weight of Rats with Thymic Lymphomas

Strain	No. of rats examined	No. of rats with thymic lymphomas (%)	Average latent period (weeks)[a]	Average weight of the thymus (g)[a]	No. of rats with lymphoma (%)	
					≧1 g	<1 g
BUF	23	23 (100.0)	17.9±6.1	5.74±2.88	21 (91)	2 (9)
WKY	24	21 (87.5)	23.3±8.3	1.19±1.56	8 (38)	13 (62)
WBF_1	47	41 (87.2)	20.3±4.7	6.31±3.33	35 (85)	6 (15)
WB×B	24	23 (95.8)	17.9±5.3	6.86±2.77	22 (96)	1 (4)
WB×W	48	42 (87.5)	25.6±10.4	3.81±3.30	26 (62)	16 (38)

a) Average±SD.

Average latent period for thymic lymphoma development As listed in Table II, the average latent period was as short as 18 weeks in BUF and [(WKY×BUF)×BUF] backcross rats, and about 20 weeks for (WKY×BUF)F_1 rats. The values for WKY and [(WKY×BUF)×WKY] backcross rats were longest.

Average thymus weights Average weights of the tumor-bearing thymuses were heaviest in BUF, (WKY×BUF) F_1 and [(WKY×BUF)×BUF] backcross rats, lightest in WKY rats and intermediate in [(WKY×BUF)×WKY] backcross rats. Percentages of rats with thymic tumors heavier than 1 g were highest in BUF and [(WKY×BUF)×BUF] backcross rats, followed by (WKY×BUF)F_1 and [(WKY×BUF)×WKY] backcross rats and least in WKY rats.

Thymus weights and survival periods of individual BUF, WKY and [(WKY×BUF)×WKY] backcross rats are plotted in Fig. 1. In general, tumors of BUF rats plotted in the upper left area and those of WKY rats in the lower right area.

Phenotypes of development of thymic lymphomas and other genetic markers Individual data for 6 genetic markers examined in 48 [(WKY×BUF)×WKY] backcross rats (24 males and 24 females) are listed in Table III.

The phenotype of development of thymic lymphomas was determined depending on a combination of the weight of the thymic tumor and the latent period as plotted in Fig. 1. In this figure, lymphomas are divided into BUF and WKY types by curves I or II. With curve I, almost all BUF thymic lymphomas and most of the WKY lesions are located to the left and right sides, respectively, and with curve II, most BUF thymic lymphomas and almost all of the WKY thymic lymphomas are similarly divided. The actual border may exist between the 2 curves, and curves I and II can be thought of as practical borders dividing the BUF and WKY phenotypes. These curves were expressed by the following equations; curve I: $Y=0.095\times2^{(X/6.5)}$ and curve II: $Y=0.095\times2^{(X/4.4)}$, where Y is the weight of the tumor in grams, and X is the experimental week at autopsy.

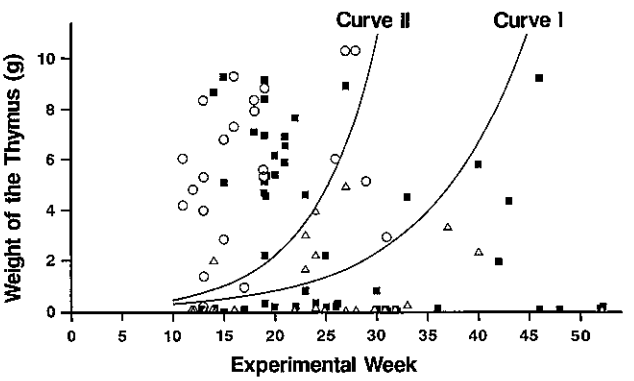

Fig. 1. Distribution of thymic lymphomas induced in BUF (○), WKY (△) or [(WKY×BUF)×WKY] backcross (■) rats. Curve I is expressed as $Y=0.095\times2^{(X/6.5)}$ and curve II as $Y=0.095\times2^{(X/4.4)}$, where Y is the weight of the thymus and X is the experimental week at autopsy.

Thymic lymphomas of the [(WKY×BUF)×WKY] backcross rats were almost evenly divided into 2 types according to the area into which the tumor plotted. This strongly supports the hypothesis that early onset of thymic lymphomas is regulated by one gene, provisionally designated *Tls-3*. The individual *Tls-3* phenotypes in terms of speed of development are also listed in Table III, where B means rapid and large tumor induction as plotted in the left upper area, W means late and/or small tumor development as plotted in the right lower area, and W/B means tumors plotted between curves I and II in Fig. 1. Rats without thymic lymphomas are included in the W phenotype group. Loose linkage between *Gc* and *Tls-3* was demonstrated by χ^2-test analysis [curve I; $\chi^2=2.772$ $(0.1>P>0.05)$, curve II, $\chi^2=4.741$ $(0.05>P>0.02)$]. No other gene was found to be linked to this proposed determinant.

Linkage of *Gc* and *Tls-3* genes A further 39 [(WKY×BUF)×WKY] backcross rats were also treated with PNU to confirm linkage of the *Gc* and *Tls-3* loci, the

Table III. Phenotypes of Thymic Lymphoma Development and Genetic Markers in Individual [(WKY×BUF)×WKY] Backcross Rats

Rat No.	Thymic lymphoma development[a] (*Tls-3*)	Genetic markers[b]					
		Es-2	*Es-14*	*Gc*	*Hbb*	*Mup-1*	*RT1.A*
Male							
1	W/B	W		W	F_1	W	F_1
2	W	W		W	F_1	W	F_1
3	W	F_1		W	W	W	F_1
4	W	F_1		F_1	F_1	W	W
5	W	F_1		F_1	W	W	W
6	B	F_1		F_1	W	W	F_1
7	B	F_1		F_1	W	W	W
8	B	F_1		W	F_1	W	—
9	W	W		W	W	W	F_1
10	W	F_1		F_1	F_1	W	W
11	W	W		W	W	F_1	W
12	B	W		F_1	W	F_1	F_1
13	W	W		W	W	W	—
14	B	W		F_1	F_1	F_1	W
15	B	F_1		F_1	W	W	F_1
16	W	W		W	F_1	W	W
17	B	W		F_1	W	W	F_1
18	W	W		W	W	F_1	—
19	W	F_1		F_1	F_1	F_1	W
20	B	F_1		F_1	F_1	W	F_1
21	W	F_1		W	F_1	F_1	W
22	W	W		F_1	W	F_1	W
23	W	W		W	W	W	F_1
24	B	W		F_1	W	F_1	W
Female							
25	B	W	W	F_1	F_1		—
26	W	F_1	F_1	W	W		F_1
27	B	W	W	W	W		F_1
28	W	F_1	F_1	W	W		F_1
29	W	F_1	F_1	F_1	F_1		F_1
30	W	F_1	F_1	F_1	F_1		F_1
31	W	W	W	W	W		F_1
32	W	W	W	W	F_1		F_1
33	W	W	W	W	F_1		—
34	B	F_1	W	W	F_1		W
35	B	W	W	F_1	W		W
36	B	W	W	W	W		—
37	B	F_1	F_1	F_1	W		F_1
38	W	F_1	F_1	W	F_1		F_1
39	B	F_1	W	F_1	W		F_1
40	W/B	F_1	F_1	W	W		W
41	B	F_1	F_1	W	F_1		F_1
42	B	F_1	F_1	W	W		F_1
43	W	W	F_1	W	W		W
44	W	W	W	W	W		F_1
45	B	F_1	W	W	F_1		F_1
46	B	W	W	W	W		F_1
47	W	F_1	F_1	W	W		F_1
48	W	F_1	F_1	F_1	F_1		F_1

a) B, rapid and large tumor growth type; W, slow and/or small tumor growth type; W/B, tumor growth type plotted between curves I and II in Fig. 1.
b) W, WKY phenotype; F_1, F_1 phenotype.

Table IV. Number of Rats with Each Combination of *Tls-3* and *Gc* Phenotypes

Exp.	Combination of *Tls-3* and *Gc* phenotypes		No. of rats based on	
	Tls-3	*Gc*	Curve I	Curve II
1	W	W	18	20
	W	F_1	8	8
	B	W	10	8
	B	F_1	12	12
2	W	W	8	8
	W	F_1	9	14
	B	W	5	5
	B	F_1	17	12
Total	W	W	26	28
	W	F_1	17	22
	B	W	15	13
	B	F_1	29	24

results being listed in Table IV. The combined results of the 1st and 2nd experiments demonstrated a linkage between the *Gc* and *Tls-3* loci [curve I; $\chi^2=6.071$ $(0.02>P>0.01)$, curve II; $\chi^2=3.715$ $(0.1>P>0.05)$].

DISCUSSION

The initial purpose of the present experiment was to confirm the linkage between *Tls-1* locus, which is considered to regulate incidence of thymic lymphoma, and the *Hbb* locus. Therefore, the BUF rat (Hbb^b), chosen as the most susceptible of various strains regarding induction of thymic lymphomas by PNU,[3] and the WKY rat (Hbb^a), reported to be the most resistant strain,[4] were used for genetic analysis in [(WKY×BUF)×WKY] backcross generation. However, as clearly demonstrated in the present experiment, the WKY rat is in fact not resistant to thymic lymphomagenesis by PNU.

The reason why tumor incidence of WKY rats was high in our experiments and low in the previous report[4] is unclear. In our experiment, development of thymic lymphoma and erythroleukemia was simultaneously observed in many animals (data not shown). In addition, development of thymic lymphomas was slower and tumor size was smaller in WKY rats than in BUF or F344 rats. Therefore, thymic lymphomas were only diagnosed after careful examination of histology and detection of Thy-1.1 antigen on the surfaces of tumor cells. Accordingly, it is probable that small thymic lymphomas were missed because of development of erythroleukemias in the previous experiment.[4] Furthermore, the experimental period might have been too short to detect development of thymic lymphomas in the previous experiment, though this possibility was not mentioned in the report.[4]

In the present experiment, linkage between *Tls-1* and *Hbb* could not be assessed, since the WKY rat is not resistant to thymic lymphomagenesis by PNU. However, development of thymic lymphomas in WKY rats was generally slower by about 5 weeks and tumor weights achieved were less than in BUF rats. Thus, growth rate and/or onset of thymic lymphoma development differ considerably between the strains. This tumor growth regulation appeared to be genetically determined by one gene, provisionally designated *Tls-3*. Tumor growth regulation may be simply expressed by the equation $Y=a\times 2^{(X/b)}$, where Y means tumor weight, X means weeks after starting PNU treatment, and (X/b) is the number of cell cycles. Although it is not clear that this equation is suitable for analysis of tumor development, it is of practical value in the present case.

When BUF and WKY phenotypes in terms of development of thymic lymphomas were divided by curves I or II as shown in Fig. 1, a linkage between *Tls-3* and *Gc* loci (linkage group VI) could be demonstrated. As shown in Table IV, when curve I was adopted as the border between BUF and WKY phenotypes, this linkage in the backcross generation proved to be statistically significant by the χ^2 test [$\chi^2=6.071$ $(0.02>P>0.01)$], while with curve II the correlation was not significant at the 0.05 level [$\chi^2=3.715$ $(0.1>P>0.05)$] although loose linkage was still observed. This result may suggest that the *Tls-3* and *Gc* loci are present within the same linkage group, but are not located close together.

The *Gc* locus is in linkage group VI,[12] in which the *h*, *Alb*, and *Afp* loci are also mapped.[12, 23] Since the *h* gene, which codes coat color hooded, and *Alb* gene, which codes albumin, could not be distinguished in cross progeny of the present strains, the order of the *Tls-3* and *Gc* genes was not determined. The *Alb* and *Gc* genes of linkage group VI have been mapped to chromosome 5 in mice,[24] and the *Gc* gene to chromosome 14 in rats.[25] Therefore, it is likely that the *Tls-3* gene is also located on these chromosomes.

Shisa and Hiai proposed that *Tls-2* accelerates thymic lymphomagenesis,[2] but its linkage with other genes was not assessed in their report. In the present study, *Tls-3* was observed in cross progeny of BUF and WKY strains, while *Tls-2* was found in their experiment using cross offspring of F344 and LES strains, and therefore the question of whether the two might be identical can not yet be answered. This point requires clarification.

Many genes are correlated with induction of hematopoietic neoplasms, such as *Fv-1*,[26, 27] *Fv-2*,[28] *Fv-4*,[29] *Fv-6*,[30] *Rfv-1*, *-2*,[31, 32] *Rgv-1*,[33] *Emv-11*, *-12*,[34] *Rrv-1*,[35] *Ril-1*, *-2*, *-3*,[36] *hr*[37] and *Cxv-1*[38] in mice. Most of these are related to susceptibility to retroviruses or to control of infectious conditions. However, in the case of rat lymphomas, viral factors may not play an important role in induction of thymic lymphomas because murine leukemia virus-related antigens have not been detected in such tumors.[2, 39]

Okumoto *et al.* earlier used recombinant inbred strains derived from two progenitor strains, BALB/c and STS/A, for genetic analysis of radiation-induced thymic lymphomas, and reported that susceptibility is regulated by a locus designated *Lyr* between the *b* and *Ifa* genes on mouse chromosome 4.[40] However, this locus does not appear to be related to chemically induced thymic lymphomagenesis, because PNU induces thymic lymphomas within a very short period and at very high incidence in STS/A mice,[41] which are resistant to radiation thymic lymphomagenesis. Since the *Tls-1* locus is linked to the *Hbb* locus that has been mapped to rat chromosome 1 and mouse chromosome 7, and the *Tls-3* gene is presumably located on rat chromosome 14, corresponding to mouse chromosome 5, both may be different from the *Lyr* gene (mouse chromosome 4). Yamada *et al.* recently reported that early onset of lymphomas in SL/Kh mice is determined by the *Esl-1* gene, and development of folicular center cell lymphomas by the *foc-1* gene.[42] Since these two genes are mapped on mouse chromosomes 17 and 4, respectively, and the *Alb-1* and *Gc* genes are located on mouse chromosome 5, there may be no correspondence with rat *Tls-3*.

Reports of genes regulating development of rat thymic neoplasms are relatively few. Matsuyama *et al.*[6, 43] concluded spontaneous thymomas of the BUF/Mna rat to be controlled by the *Tsr-1* gene and thymus size to be associated with the *Ten-1* gene on rat chromosome 1. They suggested that these genes are allelic. *Ten-1/Tsr-1* genes might be related to the *Tls-1* gene, because induction of thymic lymphomas is regulated by many physiological conditions, given their possible involvement in regulation of growth and/or differentiation of thymocytes. All 3 genes have been mapped to rat chromosome 1, but direct evidence of a link has not been gained so far. *Tls-3* gene may not be identical to *Ten-1/Tsr-1* genes because of the difference in location suggested by the present results.

It may be speculated that *Tls-3* is important for cell growth and tumor promotion/progression, perhaps functioning in one step of multistep carcinogenesis, and not related only to lymphomagenesis but also having a potential role in development of other tumors. Although the locus still requires precise chromosome mapping, this has been facilitated by the recent use of microsatellite/minisatellite analysis. Solution of this problem should help in assignment of the physiological role of the *Tls-3* gene product.

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