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Action Site of the Gene Determining Susceptibility to Propylnitrosourea-induced Thymic Lymphomas in F344 Rats

Hayase Shisa[1,2] and Michiyo Suzuki[2]

[1]*Department of Pathology and* [2]*Section of Animal Experimentation, Saitama Cancer Center Research Institute, Ina, Kitaadachi-gun, Saitama 362*

To clarify whether the determining effect of the thymic lymphoma susceptible-1 (*Tls-1*) gene is on putative N-propyl-N-nitrosourea (PNU) target cells among T-lineage cells or on other host factors, we investigated the PNU-induced lymphomagenesis in transplantation chimeras between susceptible F344 and resistant LES strain rats. Administration of PNU to lethally irradiated (F344×LES)F1 rats reconstituted with bone marrow cells from either F344 or LES parental rats invariably led to development of donor-origin thymic lymphomas. On the other hand, thymic lymphomas were induced in thymectomized F1 rats grafted with neonatal LES thymus, of which 4 out of 8 were of the donor origin. These observations indicate that the target cells of thymic lymphomagenesis of F344 and LES rats are equally susceptible to PNU provided they are in susceptible hosts and the LES thymus seems capable of supporting thymic lymphomagenesis, although this capability wanes with aging of the thymus. The effect of the *Tls-1* gene, therefore, is neither on PNU susceptibility of the target cells nor on the capability of the thymus to support lymphomagenesis, but on other host factors either in or out of the thymus.

Key words: Genetic susceptibility — Thymic lymphomagenesis — Rat — Propylnitrosourea — Target cells

Administration of N-propyl-N-nitrosourea (PNU) to the rat induces a variety of hemo-lymphatic malignancies including lymphomas, erythroleukemias and myeloid leukemias at a high incidence.[1,2] Our previous study showed that some strains of rats exhibit a remarkable preference for thymic lymphoma induction over other forms of leukemias.[3] Fischer 344 strain rats (F344) exhibit a particularly higher incidence of PNU-induced lymphomas (98%) than Long-Evans (LES) rats (10%). Genetic analysis of the crosses between F344 and LES strains has demonstrated that the higher susceptibility to thymic lymphomas of F344 rats is determined by a single autosomal dominant allele, *Tls-1* (thymic lymphoma susceptible-1). This gene is linked to the coat color loci, *p* and *c* in the linkage group I, in the order *Tls-1–c–p*.[3] Our previous studies have shown that thymus is absolutely required for thymic lymphomagenesis and the target cells of PNU lymphomagenesis are presumably localized in the thymus.[4,5]

In this study, we analyzed whether the step affected by *Tls-1* is the susceptibility of target cells among T-lineage cells to PNU. Radiation as well as transplantation chimeras between susceptible F344 and resistant LES strains were used here to address this problem. This study provides evidence that the LES cells with *Tls-1* resistant genotype are susceptible to thymic lymphoma induction when exposed to PNU in susceptible hosts. The action of *Tls-1*, therefore, has to be sought in host factors other than the target cell susceptibility.

The abbreviations used are: PNU, N-propyl-N-nitrosourea; *Tls*, thymic lymphoma susceptible; LES, Long-Evans/Stm pink-eyed dilution; AGT, O^6-alkylguanine DNA alkyltransferase.

MATERIALS AND METHODS

Animals Inbred Fischer 344/DuCrj (F344) rats were purchased from Charles River Japan, Inc. (Kanagawa). Long-Evans/Stm pink-eyed dilution (LES) rats were maintained by sister-brother mating in our animal facility and used at their 45th or 46th generation.[3] F1 hybrids were raised by mating F344 female to LES male in our laboratory.

PNU administration PNU (Iwai Kagaku Yakuhin Co., Ltd., Tokyo) was dissolved in deionized water at a concentration of 400 ppm immediately before use. All the rats were given PNU in drinking water *ad libitum* for a period of 90 days. Thereafter, they were given PNU-free water.

Irradiation Fifty-day-old rats were exposed to X-rays at a sublethal dose of 900 R delivered from a Shimadzu Shin-Ai 250 X-ray machine, operated under the following conditions; 250 kVp, 16 mA, with filters of 0.5 mm Cu plus 1 mm Al, HVL 1.2 mm Cu, average exposure rate 95–100 R per min, at a distance of 50 cm from the focus of the X-ray tube.

Chimeric rats and experimental design Radiation chimeras: The 50-day-old (F344×LES)F1 hybrid rats were irradiated at 900 R. Within 3 h after irradiation, F1 rats were injected with 5×10^6 BM cells from one of the

parental strain rats. Bone marrow (BM) cells from a pool of 5–6 donors of 40–50 days of age were washed and resuspended in tissue culture medium 199 (Gibco, Grand Island, N.Y.). Three weeks after irradiation, they were exposed to PNU in drinking water for a period of three months.

Transplantation chimeras: Thymectomy was carried out at the age of 35 days. In the experimental groups A and C, LES and F344 rats were thymectomized and grafted with a syngeneic neonatal thymus under the kidney capsule at 40 days of age. In groups B and D, F1 rats were thymectomized and grafted with a neonatal thymus from one of the parental strains of rats under the kidney capsule at the same age. In all experimental groups, PNU administration was started at the age of 45 days.

Effect of inherent age of the thymus To investigate possible change in susceptibility of the thymus to lymphomagenesis with the age of the organ, PNU was given at various times after thymus grafting. (F344×LES)F1 rats were thymectomized at 35 days of age and grafted with a whole thymus from a newborn LES rat under the left kidney capsule at 37 days of age. PNU administration was started at 40, 58 or 79 days of age. The first day of PNU exposure corresponded to the 3rd, 21st or 42nd day of age of the thymus, respectively. All the rats were killed when they became moribund or at the age of 12 months and full autopsy including histological examination was carried out. Thymic lymphomas were diagnosed on the basis of involvement of the thymus and expression of a T-cell marker *Thy-1.1* antigen on lymphoma cells in a cytotoxicity test as described previously.[6] Donor or recipient origin of lymphoma cells was determined by transplantability in F1 and parental strains of rats.

RESULTS

Radiation chimeras Our preliminary data showed that the thymuses of lethally irradiated and BM cell-reconstituted rats were totally repopulated with donor cells after 3 weeks. In our protocol of radiation chimeras, administration of PNU was started 3 weeks after cell inoculation, and thus virtually all the lymphocytes in thymuses that were exposed to PNU were donor-derived cells. If the resistance of LES rats to thymic lymphomas is solely determined by the refractoriness of the BM T-precursors or thymic lymphocytes, F1 rats reconstituted with LES BM cells should not develop thymic lymphomas on exposure to PNU.

Table I shows the incidence, mean latent period and cell origin of the lymphomas thus induced in F1 rats. Out of 40 radiation chimeras, 37 developed thymic lymphoma. Contrary to the prediction above, the F1 rats reconstituted with BM cells from resistant LES strain developed thymic lymphomas at an incidence as high as in other chimeras reconstituted with BM cells from susceptible F344 and F1 rats. In transplantation bioassay, all 12 thymic lymphomas developed in F1 rats reconstituted with LES BM cells were of donor origin.

Transplantation chimeras The above observation indicated that the LES BM cells or their progeny were fully susceptible to thymic lymphoma induction by PNU in an F1 host. Subsequently, to address the question of whether the thymus itself provided any essential function

Table I. Incidence and Cell Origin of Lymphomas Induced by PNU in Lethally Irradiated F1 Rats Restored with Bone Marrow Cells[a]

Donors[b]	No. of rats used	Thymic lymphomas				Origin of lymphoma cells
		No.	%	Latent days	*Thy-1.1* positive	
LES	18	16	89	142	8/8	12/12 Donor
F1	11	11	100	124	6/6	—
F344	11	10	91	134	3/3	4/4 Donor

a) (F344×LES)F1 rats were irradiated with 900 R at 50 days of age and restored by injection of 5×10^6 bone marrow cells intravenously.
b) Donors: 40–50 days of age.

Table II. Effects of Thymus Graft on PNU Leukemogenesis in Thymectomized F344, LES and F1 Rats

Groups	Treatments[a]		No. of rats used	Leukemias			Origin of lymphoma cells
	Hosts	Donors		No.	%	Thymic lymphomas (%)	
A	LES	LES	16	13	81	0	NT
B	F1	LES	20	15	75	8 (53)	4/8 Donor
C	F344	F344	16	14	87	13 (92)	NT
D	F1	F344	14	12	86	10 (83)	4/10 Donor

a) Host rats were thymectomized at 35 days of age and grafted with neonatal thymus at 37 days of age.
NT; Not tested.

for thymic lymphomagenesis, thymectomized rats were grafted with neonatal thymuses with different susceptibility to PNU-induced thymic lymphomas and exposed to PNU.

As shown in Table II, leukemias developed at a high incidence in all four groups. Thymic lymphomas were diagnosed based on the observation of enormously enlarged thymus grafts as well as positive *Thy-1.1* antigen on neoplastic cells. In group A (thymectomized LES hosts with LES thymus grafts), however, none of the leukemias induced was of the thymic type. In contrast, out of 20 F1 rats thymectomized and grafted with LES thymus (group B), 15 (75%) developed leukemias, of which 8 (53%) were thymic lymphomas. Out of these 8 thymic lymphomas, 4 were of donor origin as determined by the transplantation bioassay.

In groups C and D, combinations of susceptible strain hosts and grafts, almost all tumors were thymic lymphomas. Lymphoma cells in 4 out of 10 cases in group D were derived from donor thymus cells.

From this finding, the thymus of LES rats homozygous for the resistant allele at *Tls-1* locus, although less effective than thymuses of F344 and F1 rats, is capable of supporting PNU lymphomagenesis.

Effect of the age of the thymus It is well known that the thymus has its inherent age even after transplantation to a secondary host. To study if lower susceptibility of LES rats to PNU-induced thymic lymphomagenesis is due to its premature loss of capability to support lymphomagenesis, (F344×LES)F1 rats were thymectomized at 35 days and were grafted with neonatal thymus of LES rats at 37 days of age. PNU administration was started at 40, 58 or 77 days of age of the host, corresponding to 3, 21 or 42 days of age of the grafted thymus, respectively.

As shown in Table III, when PNU administration was started at 3 days of the age of the thymus, thymic lymphomas developed at 53% incidence, but when PNU was started at 21 days and 42 days of the age of thymus, thymic lymphomas developed at incidences of 31%, and 11%, respectively. This observation indicates that the LES thymus rapidly loses the ability to support thymic lymphomagenesis with aging. According to our regular protocol, administration of PNU is started at 45 days of age *in vivo*. Age-dependent decrease in the capability of LES thymus to support lymphomagenesis may be relevant to the remarkable strain difference.

Table III. Effects of the Age of Grafted Thymus on PNU Lymphomagenesis in F1 Rats[a]

Age of thymus (LES)	No. of rats used	Leukemias			Origin of lymphoma cells
		No.	%	Thymic lymphomas (%)	
3 days	20	15	75	8 (53)	4/8 Donor
21 days	25	16	64	5 (31)	5/5 Host
42 days	25	9	36	1 (11)	1/1 Host

a) (F344×LES)F1 were thymectomized at 35 days and grafted with neonatal thymus of LES at 37 days of age.

DISCUSSION

Chemical leukemogenesis is a complicated process with multiple steps, many of which are affected by host genes.[7] Such steps include activation and metabolism of chemical carcinogens, their access to the target tissues, susceptibility of the target cells, pathophysiological characteristics of the hemato-lymphatic system, immunity to transformants, and so on. PNU induced a variety of hemato-lymphatic tumors in rats. However, F344 and several other rat strains show a preference for thymic lymphoma, that was found to be under genetic control.[3] In this study, we explored the determining step in the susceptibility of the target tissues. In thymic lymphomagenesis, it has been controversial whether the transformation of the target cells takes place in BM precursor cells or their progeny in the thymus.[8–14] As for PNU lymphomagenesis in the rat, our previous study as well as others indicated that the direct target cells are localized in the thymus.[4, 5] Another problem addressed herein is whether there is any difference in the capacity of thymuses to support lymphomagenesis between susceptible and resistant strain rats. Strict dependence of thymic lymphomagenesis on the presence of the thymus[4] may be partly explained because early thymic lymphoma cells require cell interaction with thymic stromal microenvironments that normally support thymic lymphopoiesis.[15, 16]

From the experiments with radiation chimeras, BM cells of resistant LES rats were found to be fully susceptible to PNU lymphomagenesis in F1 host. According to our protocol, both thymus cells and BM cells containing their T-precursors exposed to PNU were of LES origin. Therefore, whichever was the actual stage of cell differentiation at which transformation occurred, T-lineage cells of LES rats seemed to be susceptible to PNU-induced transformation. Also, the LES thymus, when implanted in thymectomized F1 host, was not absolutely incapable of supporting thymic lymphomagenesis. It is therefore likely that the resistance of LES strain rats to thymic lymphomagenesis is not due to either refractoriness of the target cells to PNU or profound failure of the thymus organ to support lymphomagenesis.

Metabolism of a carcinogen may be a determining factor of disease type. Skin painting of 3-methylcholanthrene induced thymic lymphomas in certain mouse strains but in another strain, it induced local skin tumors.[17] By reciprocal BM cell transfer between thymoma-resistant

and susceptible strains, Ishizuka and Lilly demonstrated that the resistance is a phenotype of BM cells.[18] In this model, susceptibility is determined by an allele of aryl hydrocarbon hydroxylase (AHH), which is involved in the activation of the carcinogen.

PNU is an active carcinogen with a very short life time *in vivo*. Morimoto *et al.* studied the metabolism of PNU and alkylation of thymic DNA with special reference to the high susceptibility of F344 rats to thymic lymphoma induction. Shortly after administration of radiolabeled PNU, it accumulates in the thymus at the highest concentration, about 6-fold higher than in blood.[19] DNA of PNU-treated F344 rats is highly alkylated and AGT (O^6-alkylguanine DNA alkyltransferase) activity in the thymus of F344 rats is about half of that of Long-Evans rats. They suggested that the high level of alkylation by PNU of DNA combined with the low activity of AGT in the thymus may contribute to the high incidence of thymic lymphoma induced by PNU.[19]

The evident susceptibility of LES target cells to PNU thymic lymphomagenesis shown in this study, however, gives rise to several questions concerning the interpretation of the biochemical data. Is AGT activity in the thymus a genetically determined trait that is sufficient to explain differences in thymic lymphoma incidence among rat strains? Also, are PNU accumulation in the thymus and high-grade DNA alkylation of thymus cells specific to thymic lymphoma-prone rats? In respect of the latter point, no comparison between F344 and Long-Evans rats is given in Morimoto's paper.[19]

In the present study, there are two other important clues for determination of the disease type. Firstly, LES cells are susceptible to PNU thymic lymphomagenesis, but only when they are grafted in susceptible F1. Certain host environments inherent to the susceptible strain might operate to facilitate transformation of the LES T-lineage cells. Secondly, a remarkable age-dependent decrease in thymic lymphoma susceptibility of the LES thymus was observed. In virus- or carcinogen-induced thymic lymphomagenesis in the mouse, newborns frequently show much higher susceptibility than adults,[20,21] because they are immunologically immature or their thymuses contain a larger number of replicating cells more susceptible to carcinogens.[22,23] In PNU lymphomagenesis in normal rats, thymuses are exposed to PNU as young adults. The LES strain thymus, even *in situ*, may have lost some function to maintain the susceptibility to PNU-induced thymic lymphomagenesis during early postnatal development. Our preliminary observation showed that after PNU administration in young adults, recovery of thymus organ weight and its cell number are much less in LES rats than in F344 and F1 rats. It is also possible that in certain strains the high toxicity of the carcinogen may eradicate the local cell renewal system that is essential for the cells with DNA damage to resume replication.

In conclusion, the present data argue against the hypothesis that the function of dominant *Tls-1* gene is to dictate the thymic lymphoma susceptibility at the level of target cells but indicate that an as-yet-unidentified host factor, either in or out of the thymus, plays a determining role.

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