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Strain Specific Sensitivity to Diethylnitrosamine-induced Carcinogenesis Is Maintained in Hepatocytes of C3H/HeN↔C57BL/6N Chimeric Mice[1]

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ABSTRACT

The C3H/HeN (C3H) and C57BL/6N (C57) mouse strains are known, respectively, for their high and low susceptibility to both spontaneous and chemically induced hepatocarcinogenesis. The present study was aimed at elucidating whether this difference is dependent on intrinsic features of the target hepatocytes or the *in vivo* milieu and associated growth promoting factors to which the cells are exposed. C3H↔C57 chimeric mice were produced and given injections of diethylnitrosamine (20 μg/g body weight) at the age of 15 days. The animals were sacrificed 6 or 9 months thereafter, and the numbers and sizes of altered cell lesions were scored. The clonal growth of both cell types was immunohistochemically confirmed using anti C3H-specific antigen antibodies. Quantitative assessment revealed C3H lesions in the chimera livers to be far larger (5:1) than those of C57 derivation and associated with more frequent malignant progression as was evident histologically. Furthermore, foamy change and hyalin body formation, which have been described as characteristics of C3H and C57BL hepatic tumors, respectively, were also featured as differentiative characteristics in lesions of both cell types in chimera mice. Thus, the results clearly demonstrated that the principal mechanism(s) underlying strain difference in diethylnitrosamine-initiated hepatocarcinogenesis exists in the target cells and is not milieu-dependent.

INTRODUCTION

Striking interstrain differences exist in laboratory mice with respect to liver tumor development (1). In particular, the C3H[3] and C57 strains are well known for their extremely high or low susceptibility, respectively, to both spontaneous and chemically induced hepatocarcinogenesis as well as response to hepatopromoters (2–4).

One fundamental question is whether the strain differences are caused by factors localized in the target cells or are dependent on the milieu including various endogenous and/or exogenous growth modulating factors, to which the target cells are exposed. This question is perhaps most accessible to elucidation by way of chimeric mice derived from susceptible and nonsusceptible strains. Thus, Mintz *et al.* (5) analyzed some years ago the genotype of hepatomas spontaneously developing in C3H↔C57 chimeric mice and came to the conclusion that genetic susceptibility to spontaneous lesion development was cell-localized. Although this was an important achievement, it was not without limitations: because they applied a quantitative isozyme assay for determining genotype, their analysis could only be performed with large tumors. Accordingly, it was not clear whether the overwhelming predominance of C3H cell derived neoplasms (21 of 23) in their results actually reflected numbers or rather only differences in growth rate of initiated or preneoplastic lesions of C3H and C57 types in the chimeric mouse liver.

It has been shown in rodent hepatocarcinogenesis that the effects of initiation and promotion can be reliably and quantitatively estimated by scoring altered cell lesions, which are the proliferated progenies of carcinogen-initiated cells (6–8). For example, previous studies have shown that both the number and size of microscopically detectable lesions induced by DEN in C3H mice are larger than those in C57 mice (4, 9).

Recently, antibodies strictly recognizing CSA were established by Kusakabe *et al.* (10), enabling immunohistochemical discrimination of C3H cells in histological sections of chimera mouse tissues (10). Using this new methodology, we studied: (*a*) strain origin; (*b*) number; (*c*) size; (*d*) histological grade of cancer; and (*e*) strain-associated morphological features, *i.e.*, hyaline body formation in the C57 case (11) and foamy change in the C3H case, of DEN-induced lesions, with the aim of casting light on genetic susceptibility of carcinogenesis.

MATERIALS AND METHODS

Animals. C3H, C57, and ICR mice were purchased from CLEA Japan, Inc., Tokyo. They were fed on basal diet (CE-II; CLEA Japan) and allowed free access to water until use.

Production of Chimeric Mice. C3H↔C57 chimeric mice were produced by an aggregation procedure (12) as described previously (10). Briefly, 8-cell stage embryos of C3H and C57 strains were collected by oviduct flushing, and the zonae pellucidae removed by enzymatic digestion with Pronase (13). A pair of embryos of each genotype were then aggregated in Whittingham (14) medium. After 1 day in culture, the aggregated embryos that had reached the blastocyst stage were surgically transferred into the uterus of pseudopregnant ICR foster mothers. Chimeric animals showing a distinct chimeric pattern of hair color were used in the analysis.

Experimental Protocol. At the age of 15 days, the chimeric mice were given i.p. injections of DEN dissolved in physiological saline at a dose level of 20 μg/g body weight (15). After weaning, the chimeric mice were divided into phenotypically male and female groups and fed on basal diet continuously. Only the male group was used for the present study, because male animals are known generally to be by far more susceptible to carcinogens (1). To establish the karyotypic sexes of C3H and C57 cells in individual chimera, each male chimera mouse was mated with a C57 female, and the hair color of resultant offspring recorded. Since karyotypically female cells are incapable of entering normal spermatogenesis (16), the presence of both agouti and black offspring indicated a chimera containing male karyotype C3H and C57 cells. Experiment 1 was terminated at 6 months of age and experiment 2 at 9 months. Animals were sacrificed by venesection under ether anesthesia, and the livers were immediately resected.

Histology and Immunohistochemistry. The chimera livers were cut into approximately 5-mm slices and fixed initially with microwave irradiation for 5 min at 55°C in sodium phosphate buffer and secondly with ice-cold 95% ethanol plus 1% acetic acid for 2–3 h. The tissues were then embedded in 100% polyester wax after the procedure of Kusakabe *et al.* (10, 17). In every case, testicular tissue was also sampled

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[3] The abbreviations used are: C3H, C3H/HeNJcl^{MTV+}; C57, C57BL/6NJcl; DEN, diethylnitrosamine; CSA, C3H/HeNJcl^{MTV+} mouse-specific antigens; PB, phenobarbital.

and similarly processed for determination of sexual chimerism of C3H and C57 cells.

At least 2 serial or semiserial sections for each liver and testis were made from the polyester wax blocks at a thickness of 4 μm. One serial section per block was stained with hematoxylin and eosin, the other being immunohistochemically stained for CSA using biotinylated mouse anti-CSA antibodies (10) and an avidin-biotin complex kit (Vector Laboratories, Burlingame, CA).

Under routine light microscopy, the number, size, and morphological features of altered cell lesions (18) larger than 319 μm in diameter were recorded in hematoxylin and eosin-stained sections, the strain origin of each lesion being checked in terms of CSA reactivity in serial immunohistochemical sections. When foamy change or hyaline body formation (11) was observed in more than 20% of cells comprising a preneoplastic lesion or a carcinoma, it was recorded positively. Mean total area studied for each liver was about 6 cm^2. CSA staining of spermatogenic cells in testes was also investigated.

Stereological Analysis. Conversion of 2-dimensional data to 3-dimensional values was performed by the method of Enzmann *et al.* (19) with modification of the size classes proposed. The values for lesions were thus obtained as numbers/cm^3 of chimera liver. Because the C3H/C57 hepatocyte ratio varied considerably among individual chimera mice, the number values were then adjusted by dividing by respective C3H/C57 ratios calculated by random sampling of normal portions on CSA-stained liver sections, the C3H/C57 ratio of the adjusted numbers being finally regarded as the C3H/C57 initiation risk ratio.

For statistical comparisons, the Wilcoxon rank-sum test was used.

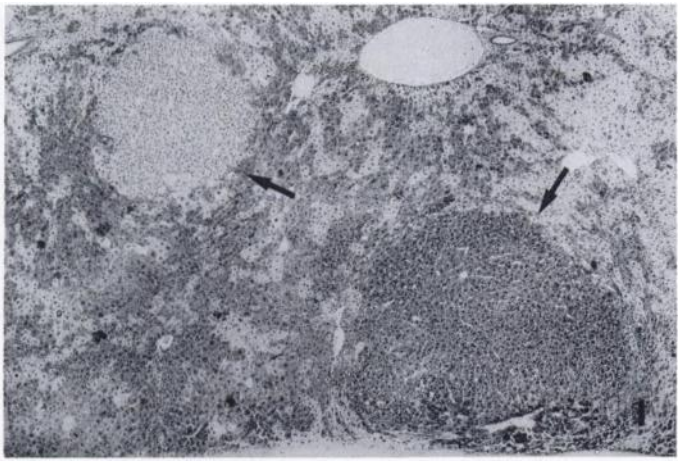

Fig. 1. Typical CSA staining of a DEN-initiated C3H↔C57 chimeric liver. While normal portion shows a mosaic pattern of C3H (*dark*) and C57 (*pale*) hepatocytes, each of two initiated lesions is composed of a single cell type (*arrows*).

RESULTS

CSA staining of the chimeric livers clearly showed that every altered cell focus was composed of a single cell type (C3H or C57) (Fig. 1), indicating the clonality of initiated lesions, in concert with previous investigations (20–22).

The results regarding number and size of C3H and C57 lesions in chimera livers are summarized in Table 1. In all cases except animal 14, the number of C3H lesions per cm^3 chimeric liver was far larger than the corresponding C57 value. After adjustment for genomic proportion of normal liver, it was shown that C3H hepatocytes bear about a 5-fold larger risk on average of becoming initiated than do C57 cells ($P < 0.01$). This value is apparently time-independent because there were no significant differences between experiment 1 and 2 values. The mean volume of C3H lesions was also 3- to 5-fold larger than that of C57 lesions ($P < 0.01$); in both cases, the increase was observed over time. Concerning strain differences in the number and size of the lesions, similar tendencies were observed with all the phenotypically male chimeras, although in some the karyotypic sex of one or the other strain could not be determined either by mating or investigating CSA-stained testes.

The results of accompanying histological features of relatively large C3H and C57 lesions (>0.255 cm in diameter) are summarized in Table 2. Because the number of the lesions observed was small, data for all chimeras were pooled and expressed as number/cm^3 total chimera liver. The results indicated that foamy change (Fig. 2*a*) and malignant features occur much more frequently in C3H lesions in the chimera livers. On the other hand, hyaline bodies were formed almost exclusively in C57 lesions (Fig. 2*b*).

Table 1 *Details of DEN-initiated hepatocellular lesions in C3H↔C57 chimera mice*

Chimera no.	Karyotypic sex		No. of lesions/cm^3 chimera liver		%proportion of C3H hepatocytes in normal liver	Ratio of initiation risk[a] (C3H:C57)	Mean volume of lesions (10^6 μm^3)	
	C3H	C57	C3H	C57			C3H	C57
Experiment 1 (6 mo)								
1	M[b]	M	128.8	20.5	70	2.7:1	4,226	156
2	M	M	139.8	31.5	50	4.4:1	782	173
3	M	?	31.8	21.6	45	1.8:1	12,088	7,439
4	M	M	87.7	14.8	50	5.9:1	564	62
5	M	?	144.6	33.7	65	2.3:1	816	54
6	M	M	173.2	7.0	75	8.3:1	3,624	31
7	M	?	171.6	37.2	55	3.8:1	1,878	165
8	?	M	346.9	9.0	80	9.6:1	532	31
Mean ± SD					61 ± 12	4.9 ± 2.7[c]:1	3,603 ± 3,664[c]	1,014 ± 2,429
Experiment 2 (9 mo)								
9	M	?	105.5	10.2	65	5.6:1	6,936	31
10	?	M	64.9	26.9	40	3.6:1	9,592	92
11	M	?	61.6	33.4	25	5.6:1	7,832	1,040
12	M	M	108.9	13.2	60	5.5:1	8,081	3,749
13	M	?	132.5	17.8	80	1.9:1	6,434	872
14	M	M	28.0	71.3	10	3.5:1	6,891	3,346
15	M	?	88.5	6.9	70	5.5:1	7,630	1,530
16	?	M	31.0	25.7	15	6.8:1	19,807	3,806
Mean ± SD					46 ± 25	4.8 ± 1.5[c]:1	9,150 ± 4,128[c]	1,808 ± 1,490

[a] Risk of hepatocyte being initiated by DEN (see text).
[b] M, male; ?, unknown.
[c] Significantly larger than the corresponding C57 value by Wilcoxon rank-sum test ($P < 0.01$).

Table 2 *Morphological features of DEN-initiated hepatocellular lesions larger than 0.255 cm in diameter*

Experiment no.	Strain	No. of lesions (>0.255 cm)/cm³ pooled chimera liver			
		Total	With foamy change	With hyaline body	With malignant features
1 (6 mo)	C3H	5.57 (100)[a]	3.22 (58)	0.18 (3)	0.19 (3)
	C57	0.37 (100)	0.04 (11)	0.35 (95)	0.00 (0)
2 (9 mo)	C3H	9.80 (100)	2.42 (25)	0.19 (2)	0.62 (6)
	C57	1.12 (100)	0.00 (0)	1.08 (92)	0.03 (3)

[a] Numbers in parentheses, percent.

DISCUSSION

The present analysis of DEN-initiated altered cell lesions in C3H↔C57 chimeric mice demonstrated: (*a*) the monoclonality of altered cell lesions; (*b*) approximately 5 times greater values for C3H lesions, in terms of both number and size, than those of C57, associated with more frequent malignant progression; and (*c*) occurrence of known strain-specific histological features of C3H and C57 liver tumors also in chimeric mouse liver lesions. These findings indicate that the principal mechanism(s) causing strain difference in DEN-initiated hepatocarcinogenesis depend on target cells themselves and not the milieu, C3H hepatocytes being not only more sensitive to initiation by DEN, as evidenced by the larger number of lesions, but also more susceptible to endogenous growth stimuli, as evidenced by the larger volume and more frequent malignant progression, as compared with C57 lesions.

With regard to initiation sensitivity, however, Hanigan *et al.* (9) observed that differences between strains or sexes in numbers of detectable carcinogen-induced foci decreased with increasing animal age, or when "initiation refractory" female B6C3F1 mice were kept under androgenized conditions for a long period (36 weeks) (23). Since lesions smaller than 319 μm in diameter were not included for the sake of reproducible counting, the possibility of underestimation of C57 lesions in the present experiment could not be precluded. The important point is that, whether or not there were more "hidden" initiated C57 hepatocytes, their intrinsic growth ability and/or sensitivity to the environmental growth stimuli in chimeric mice were shown to be quite different from those of C3H lesions.

It is a common understanding that hepatocytes including carcinogen-initiated altered cells grow in response to endogenous or exogenous growth stimulating factors. Candidates as endogenous growth stimulators include androgens, insulin, glucagon, norepinephrine, vasopressin, epidermal growth factor, and transforming growth factor-α (23–26). Concerning androgens, however, Kemp and Drinkwater (27) reported that neither the blood level of testosterone nor the amount or affinity of androgen receptors of hepatocytes correlated with the genetic predisposition to hepatocarcinogenesis in mice. The exogenous growth stimulators include various types of hepatopromoters, typically represented by PB (28).

It should be mentioned that there are occasions during which the strain differences may appear to be caused by differences in the milieu. Thus Diwan *et al.* (3) found that in DBA/2N mice, a strain sensitive to promotion by phenobarbital, the serum level of PB was much higher than that of C57, a PB refractory strain. Between C3H and C57 strains, however, the same investigators found no significant difference in the serum PB level (3). Therefore, although we did not apply any exogenous hepatopromoters in the present study, it is very likely that similar results would have been gained using promoters.

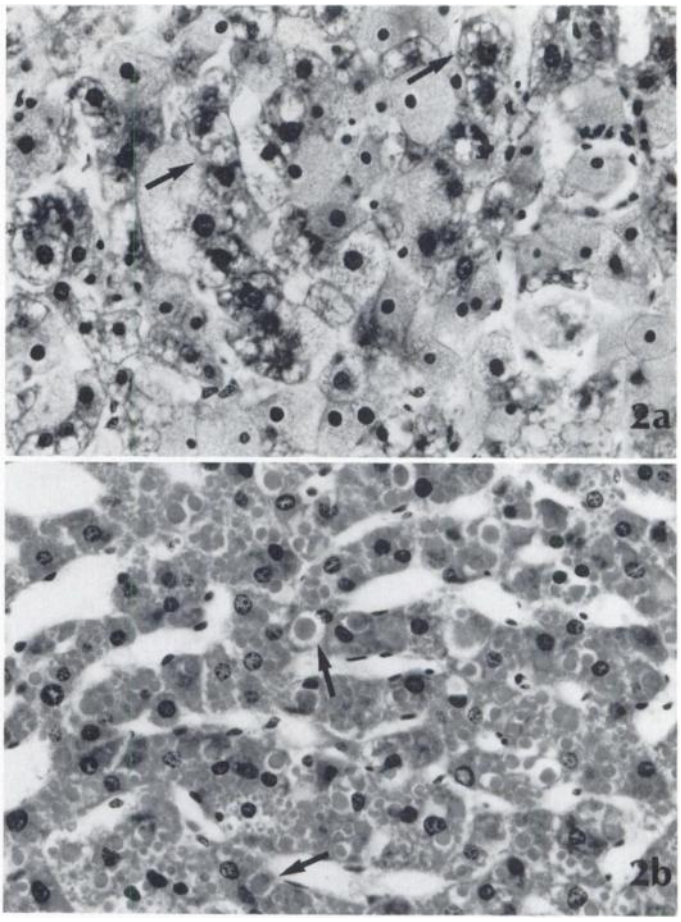

Fig. 2. *a*, Foamy change of hepatocytes with multiple cytoplasmic vacuoles (*arrows*) observed in a C3H lesion; *b*, intracytoplasmic hyaline body formations (*arrows*) in a C57 lesion.

A minor, but nevertheless interesting finding obtained in this investigation concerned the strain-specific cytological features of DEN-initiated lesions in chimeric mice. We earlier noticed that relatively large neoplastic lesions in C3H livers frequently show foamy change.[4] On the other hand, Kakizoe *et al.* (11) reported that more than 90% of hepatic lesions developing in C57 mice had hyaline-like inclusion bodies and that inclusion-positive tumor cells were retarded in growth as compared with inclusion-free cells. Although the biological significance of these phenomena is not exactly known, they do appear to be manifested through cell-localized mechanisms. Since almost all the C57 lesions observed in the present study contained hyaline bodies whereas they were very rare in C3H lesions, the hyaline body can be used as a reliable morphological marker to discriminate between C3H and C57 lesions in chimera livers.

Sexual mosaicism is known to occur frequently in phenotypically male chimera mice (5). In fact, in many cases of our chimera mice, the sexual karyotype of one or the other of the strains couldn't be determined, suggesting that at least some of them were sexually mosaic. This is a point requiring attention in connection with the marked sexual difference described in mouse hepatocarcinogenesis (1). Actually, however, in all of the chimeras, including karyotypically nonmosiac, XY↔XY cases, the results were approximately the same (Table 1). Sexual differences in hepatocarcinogenesis therefore may be controlled by sex-specific microenvironment rather than hepatocyte-local-

[4] G-H. Lee and T. Kitagawa, unpublished observations.

ized factors. This conclusion is in concert with the generally accepted idea that high susceptibility of male relative to female mice is caused by higher blood levels of androgens (23).

Drinkwater and Ginsler (2) found a single locus, designated Hcs, responsible for 85% of the difference in susceptibility to carcinogens between C3H and C57 strain. Since they established that ethylnitrosourea-induced hepatic lesions in C3H male grew 1.7 times faster than those in C57 male, whereas no such difference was evident in female animals, they suggested that the Hcs locus might primarily affect the promotion phase of hepatocarcinogenesis in the male hormonal environment (9). It would therefore be of interest to analyze the phenotypic features of C3H and C57 hepatocyte lesions in female chimeric mice. Our chimeric mouse system offers a useful model for future investigations of the cell-localized mechanisms underlying strain-specific susceptibility to mouse hepatocarcinogenesis.

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