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Precancerous Mammary Hyperplastic Alveolar Nodules in Four Strains of Virgin Mice with Different Mammary Tumor Potentials: Influence of Chronic Caffeine Ingestion*

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Abstract—*Based on the stimulating effects of caffeine on the formation of precancerous mammary hyperplastic alveolar nodules (HAN) in mice, the onset time of HAN was estimated by chronic caffeine ingestion in four strains of virgin mice with varying mammary tumor potentials (SHN, SLN, GR/A and C3H/He). Beginning at* 21 *days of age at weaning, mice were given tap water with* (0.05%) *or without caffeine and were killed at* 40, 60, 90, 120 *and/or* 150 *days of age. In the caffeine-treated groups, HAN appeared at* 60, 120, 60 *and* 90 *days of age in SHN, SLN, GR/A and C3H/He, respectively, while no such changes developed in the controls at the respective ages. These are the consequence of stimulation by caffeine of the growth of very early foci of HAN and indicate that HAN appear after* 40, 90, 40 *and* 60 *days of age in SHN, SLN, GR/A and C3H/He, respectively. The onset time of HAN is associated with mammary tumor potential of virgin mice. Caffeine did not affect estrous cycle, plasma levels of prolactin and growth hormone and endocrine organ weights, suggesting that promotion by caffeine of HAN growth is minimally mediated by the endocrine system.*

INTRODUCTION

MINTON *et al.* [1] first reported the effects of caffeine on 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors in rats. The study was extended by Welsch *et al.* [2], who found that mammary tumor incidence was increased when caffeine ingestion was initiated 3 days after DMBA treatment, but not when the treatment was initiated prior to or during carcinogen administration. Also a higher tumor yield was observed when caffeine treatment was initiated 20 weeks after DMBA administration [2]. From these studies it was concluded that caffeine acts as a promoter of this type of tumor. We [3] have observed that caffeine stimulates the growth of precancerous mammary hyperplastic alveolar nodules (HAN) in virgin mice of the SLN strain, whose mammary tumor incidence is low (Fig. 1). Because it is of importance for mammary tumor etiology to determine the onset time of HAN [4], the relationship between caffeine ingestion and the incidence of HAN in four strains of mice with varying mammary tumor potentials were studied in this paper.

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MATERIALS AND METHODS

Animals and treatments

Four inbred strains of virgin mice (SHN/Mei, SLN/Mei, GR/AMei and C3H/HeMei) maintained in our laboratory by brother × sister mating were used. The animals were kept in plastic cages (16 × 28 × 13 cm) with sawdust, 4–6 each, maintained in an animal room air-conditioned (20–22°C and 55-75% relative humidity) and artificially illuminated (14 hours of light from 5:00 a.m. to 7:00 p.m.) and had free access to a commercial diet (Lab MR Breeder, Nihon Nosan Kogyo KK, Yokohama, Japan). At the time of weaning (21 days *post partum*), half of each female litter was given tap water containing caffeine (Sigma Co., St Louis, MO, U.S.A.) at a concentration of 0.05% (500 mg/l) *ad libitum*. The other half received tapwater only. Mice of both experimental and control groups were weighed every 7 days and vaginal smears were taken every morning. They were killed at 40, 60, 90, 120 and/or 150 days of age (Table 1).

Table 1. *Effects of caffeine on normal and preneoplastic mammary gland growth in four strains of virgin mice*

Strain	Group and treatment*	Age in days: 40 HAN	40 Mammary rating	60 HAN	60 Mammary rating	90 HAN	90 Mammary rating	120 HAN	120 Mammary rating	150 HAN	150 Mammary rating
SHN	Experimental	0/6†	1.0 ± 0.0‡	1/10	1.8 ± 0.3	1/7	2.6 ± 0.1				
		—		2§		5					
	Control	0/6	1.1 ± 0.1	0/9	2.1 ± 0.3	2/7	2.8 ± 0.1				
		—		—		3.5					
SLN	Experimental					0/7	1.1 ± 0.1	1/8	1.3 ± 0.4	2/7	1.6 ± 0.7
						—		4		6	
	Control					0/6	1.0 ± 0.0	0/8	1.0 ± 0.0	0/7	1.0 ± 0.0
						—		—		—	
GR/A	Experimental	0/5	1.0 ± 0.0	2/9	2.5 ± 0.3	3/4	2.7 ± 0.1				
		—		1.5		3.0 ± 0.2					
	Control	0/4	1.2 ± 0.2	0/9	2.2 ± 0.3	2/5	2.7 ± 0.5				
		—		—		1					
C3H/ He	Experimental			0/6	1.5 ± 0.2	5/12	2.5 ± 0.3‖	6/6	3.5 ± 0.4‖		
								17.3 ± 9			
				—		3.4 ± 0.2		.0‖			
	Control			0/6	1.4 ± 0.2	0/11	1.7 ± 0.2	4/6	2.2 ± 0.4		
								3.3 ± 0.8			
				—		—					

*Experimental mice were given tap water containing caffeine (500 mg/l tap water) beginning 21 days of age at weaning. The controls received tap water with no caffeine.
†Number of mice with HAN/total number of mice examined.
‡Means ± S.E.M.
§Number of HAN per HAN-bearing mouse.
‖Significantly different between groups at $P < 0.05$.

Evaluation of normal and preneoplastic mammary gland growth

At autopsy, the bilateral third thoracic mammary glands were mounted for preparations and examined under 10-fold magnification. The degree of the formation of normal end-buds was rated from 1 to 7 in increments of 1 [5]. The mean of the values for the bilateral glands represents the rating in the individual. The number of HAN, i.e. larger than 0.5 mm dia., was also counted. The sum of the values for the bilateral glands was employed as the value in the individual for the number of HAN.

Endocrine studies

Just before autopsy, some mice were bled by orbital puncture under light ether anesthesia. Blood was centrifuged at 1000 ***g*** for 20 min and plasma was immediately frozen and kept at −20°C. Plasma levels of prolactin and growth hormone were assayed by homologous radioimmunoassay using the kit donated by Dr. Parlow, Torrance, CA, U.S.A. Only the data at ages when HAN appeared in the experimental mice, but not in the control, are cited in the Results in all strains.

At autopsy, anterior pituitary, adrenals and ovaries were removed and weighed.

Determination of water intake

Water intake was determined every 10 days by the difference in weights of water bottle during 3 days in some cages of each group.

Statistics

Statistical significance of difference in parameters between groups was evaluated by Student's *t*-test.

RESULTS

Normal and preneoplastic mammary gland growth (Table 1)

HAN were not found at 40, 90, 40 and 60 days of age in either experimental or control mice of any strain. Only the caffeine-treated mice developed HAN at 60, 120, 60 and 90 days in SHN, SLN, GR/A and C3H/He, respectively. This is the consequence of stimulation of the growth of early foci of HAN and, therefore, HAN can be estimated to develop after 40, 90, 40 or 60 days in each strain.

The number of HAN increased in the experimental groups of all strains compared to the controls. However, because of the small number of individual HAN formation, the differences between experimental and control mice were not statistically significant

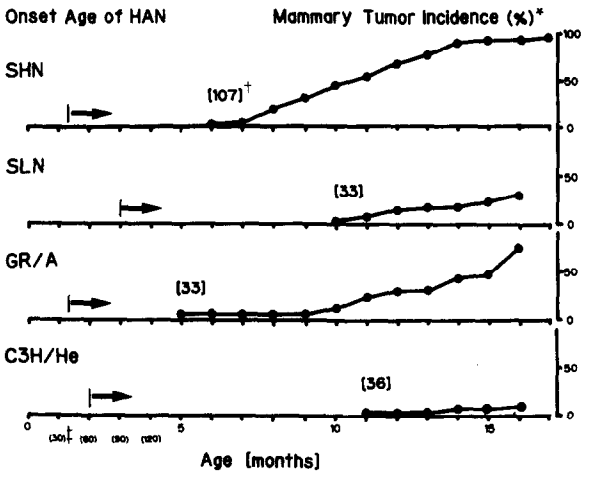

*Fig. 1. Relationship between the onset time of HAN and mammary tumor incidence in four strains of virgin mice. *Data from mice at the same generations as used in the present experiment. †Number of mice examined. ‡Age in days.*

in any strains except C3H/He, in which the number of HAN was significantly greater in the experimental mice.

Mammary rating was also significantly higher in caffeine-treated mice than in the control at both 90 and 120 days of age only in C3H/He.

Relationship between the onset time of HAN and mammary tumor potential (Fig. 1)

The age of onset of HAN paralleled mammary tumor potential in all strains except C3H/He. SHN developed HAN earlier than SLN and C3H/He was associated with earlier and higher mammary tumor appearance. A similar trend was also seen in GR/A. Meanwhile, C3H/He had earlier HAN development, but lower mammary tumor incidence than SLN.

Plasma levels of prolactin and growth hormone (Table 2)

In all strains, plasma levels of prolactin or growth hormone at the time when HAN were found only in the experimental mice did not differ between caffeine-treated and untreated animals.

Growth (Fig. 2)

Growth, as determined by body weight change, was retarded by caffeine in all strains, especially in SLN.

Water intake (Fig. 3)

Daily water intake was lower in caffeine-treated mice than in the controls at any time examined in SHN. On the contrary, in C3H/He, water intake was higher in the experimental group than in the control. There was a slight difference between groups except at the early stages in SLN and GR/A. Nevertheless, daily water intake per 30 g body wt of the experimental mice was 4.5–5 g in all strains.

Estrous cycle

The patterns of estrous cycle, which are characteristic of strains, were markedly different between strains; SHN and GR/A had longer diestrous stages than SLN and C3H/He. However, the patterns were not affected by caffeine in all strains (data not shown).

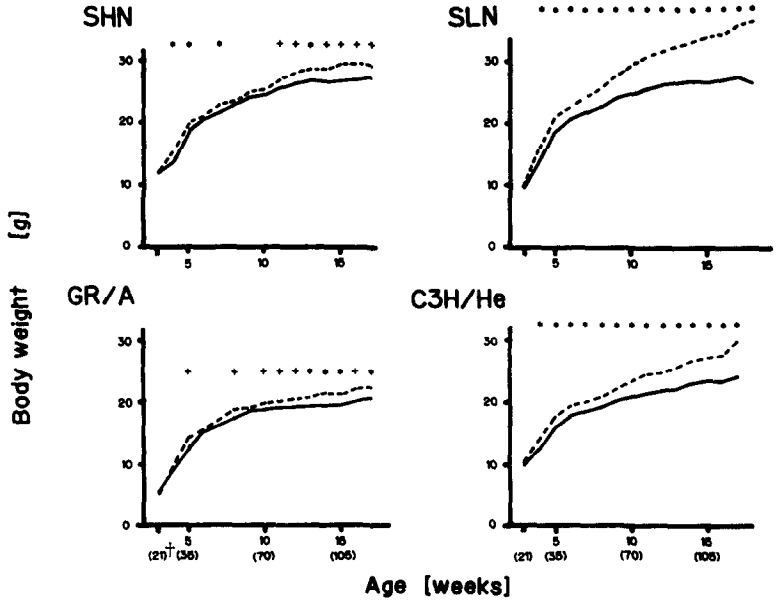

*Fig. 2. Growth curves of the experimental (——) and the control (----) groups in four strains. Number of mice in each group is from 30 to six, which decreases with age. †Age in days. * or + Significantly different between grups at* $P < 0.05$ *or* 0.01.

Table 2. Plasma levels of prolactin and growth hormone in the experimental and the control mice of four strains (means ± S.E.M.)

Hormone	Group and treatment*	Strain SHN (60)†	SLN (120)	GR/A (60)	C3H/He (90)
Prolactin (ng/ml)	Experimental	1029 ± 252 (10)‡	800 ± 131 (14)	280 ± 95 (9)	627 ± 187 (12)
	Control	835 ± 181 (9)	611 ± 141 (14)	283 ± 108 (9)	453 ± 131 (11)
Growth hormone (ng/ml)	Experimental	23 ± 7 (10)	10 ± 1 (13)	18 ± 3 (9)	17 ± 3 (12)
	Control	30 ± 4 (9)	14 ± 3 (14)	17 ± 4 (9)	11 ± 1 (11)

*See Table 1 for details of treatments.
†Age in days at the time when HAN was found only in the experimental mice.
‡Number of samples.

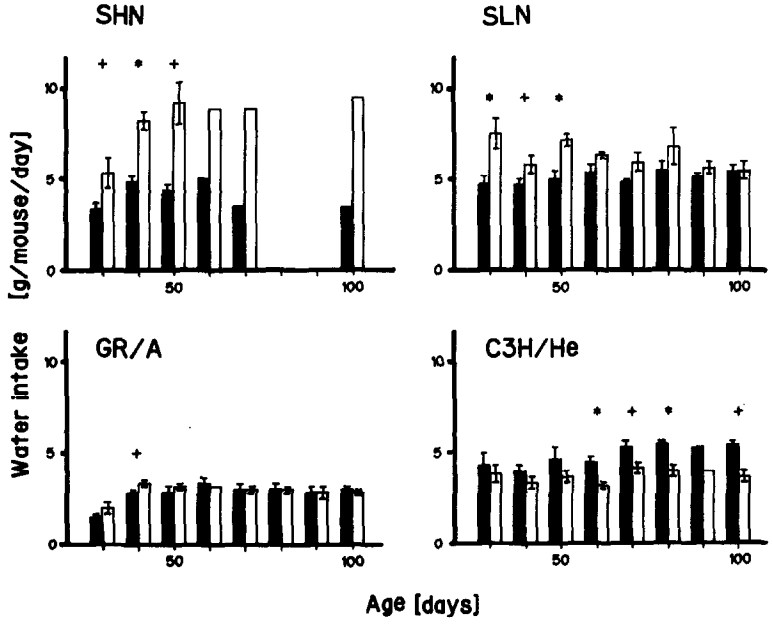

*Fig. 3. Water intake in the experimental (●) and the control (○) groups in four strains (means ± S.E.M.). Number of estimates in each group is from two (with no S.E.M.) to 10. * or + Significantly different between groups at* $P < 0.05$ *or* 0.01.

Endocrine organ weights

No significant difference was observed between caffeine-treated and untreated mice at all ages examined in the weight of either anterior pituitary, adrenals or ovaries in any strain (data not shown).

DISCUSSION

The results of this study confirm our previous findings that chronic caffeine ingestion enhances HAN appearance in mice [3]. Furthermore, they show the apparent strain-related difference in the onset time of HAN; 40, 90, 40 and 60 days in SHN, SLN, GR/A and C3H/He, respectively. These onset ages paralleled mammary tumor potentials except in C3H/He, in which the onset age of HAN was intermediate between SHN or GR/A and SLN; however, this strain's mammary tumor potential was the lowest. This behavior is unique because in the C3H/He strain mammary tumor potential was high more than 10 years ago and declined thereafter with advancing generations [6] despite retaining the high mammary tumor activity and normal mammary growth potential [7]. Also in this study normal and preneoplastic mammary glands of this strain showed a high response to caffeine. HAN formation, their growth and malignant transformation and tumor growth are complex processes and controlled by many factors. This C3H/He strain may be different from others in the HAN-tumor process.

The mechanism(s) by which caffeine stimulates HAN growth has (have) not been defined [2, 8]. Nevertheless, since our endocrine studies did not

show any difference between experimental and control groups in all strains, the observed effects of caffeine on mammary glands are seemingly not modulated by changes of activities of mammotropic hormones.

Body weight was significantly lower in caffeine treated mice in all strains. The lipolytic effect of caffeine [9, 10] may play a role in this process, since the effect of caffeine on body weight was most marked in SLN, which are apt to become obese with advancing age [11].

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