Lung Adenoma Development and NK Activity in Mice Treated with Multiple Carcinogens

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A wide-spectrum initiation model was investigated in mice. Sequential treatments with diethylnitrosamine, urethane and N-methylnitrosourea, with or without a promoter, phenobarbital, resulted in tumor formation in the lungs in 85-90% of animals, but did not produce any tumorous lesions in other organs. The lung tumors were adenomas and the mean number of adenomas was 2.2-2.6 per mouse. Phenobarbital combination had no additive effect on lung tumor incidence and multiplicity. Splenic NK cell activity showed inconsistent increment in the carcinogen plus phenobarbital-treated group during the experiment ($P < 0.05$).

Key Words: Wide-spectrum carcinogenesis, Multiple carcinogens, Lung adenoma, NK cell activity

INTRODUCTION

Since the theory of a 2-stage process was proposed in chemical carcinogenesis using mouse skin (Berenblum and Shubik, 1947), the initiation-promotion process has been recognized as an important step in the carcinogenesis of liver, urinary bladder, mammary gland, thyroid gland, intestine and lung using various chemical carcinogens (Peraino et al., 1971; Weisburger et al., 1975; Cohen et al., 1979; Hall, 1948; Hicks et al., 1982; Reddy et al., 1977; Witchi and Lock, 1978). However, the majority of these studies concerned only one organ system. In general, chemicals show organ specificity in their effects and may exert different effects on different organs. For example, phenobarbital is a well known promoter in the carcinogenesis of the liver (Peraino et al., 1971 and 1980; Diwan et al., 1985; Nakanishi et al., 1982), thyroid (Tsuda et al., 1983), and urinary bladder initiated with N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) (Wang et al., 1983), but has no promoting effect in bladder carcinogenesis initiated with N-butyl-N-(4-hydroxybutyl) nitrosamine

(BBN) in rats (Nakanishi et al. 1982). There have been few reports about multiple organ initiation systems which are useful for the detection of chemical modifiers. Sequential treatment with potent carcinogens, having different wide-spectrum initiating activities, has been introduced in rat models (Ito et al., 1988), but to our knowledge no mouse multi-organ initiation model has been reported. Diethylnitrosamine (DEN) is known to be a strong initiator of hepatocarcinogenesis, whereas N-methylnitrosourea (NMU) initiates the thyroid, urinary bladder and hematopoietic systems. Urethane is carcinogenic in the liver and lung.

We investigated whether a multiple organ initiation with DEN, urethane and NMU was effective in a medium-term multi-organ carcinogenic model in mice, and examined changes of their NK cell activity.

MATERIALS AND METHODS

Experimental Animals

Non-inbred Swiss-Webster mice were obtained from NCI (National Cancer Institute, U.S.A., [NIH]) and bred at random *inter se*. Six-week-old female animals were used in the experiment, consisting of 3 groups; a followed-untreated control group (50 mice), a carcinogen-treated group (20 mice), and a group treated with carcinogen in combination with phenobarbital (50 mice). All the mice were housed in a controlled room and fed solid pellets prescribed by NIH-7-open

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formula *ad libitum*. The body weights of the animals were checked at weeks 3, 5, 10 and 15.

Treatment

In the carcinogen-treated group (Groups 2 and 3), the mice were first given i.p. injections of DEN (Sigma Chemical Co., U.S.A.) in a dose of 90mg/kg B.W. in 0.2ml of normal saline (NS). On the 3rd, 6th, and 9th day after DEN administration, 10mg/kg of urethane in 0.2ml NS was i.p. injected. One week later, NMU (Sigma Chemical Co., U.S.A.) was administered in drinking water at a concentration of 100 ppm for 1 week. In the third group, the mice were given phenobarbital (PB) in drinking water at a concentration of 0.1% for 12 weeks after NMU treatment. Group 1 served as untreated control.

Scoring of Lung Tumors

At the 3rd, 5th and 10th weeks, 10 mice per group from groups 1 and 3 were sacrificed, and at the 15th week, the remaining 20 mice from all the groups were subjected to autopsy. The lungs were removed, weighed, and fixed in Tellyesniczky's solution (70% EtOH 100ml + formalin 3ml + glacial acetic acid 5ml). After 72 hours the number of tumors in each lung was counted grossly. To obtain an index of tumor incidence, the percentage of tumor-bearing mice per total number of mice in each group was calculated. Tumor multiplicity was defined as the average number of tumors per mice, obtained by dividing the total number of tumors by the total number of mice per group, including non-tumor bearing animals. Statistical comparisons were made using the Chi-square test for tumor incidence and Student's *t* (Student's *t*-test) test for multiplicity and organ weights. The null-hypothesis was rejected whenever a *P* value of 0.05 or less was found.

Histo-pathological Analysis

At autopsy at week 15, the major organs, in addition to the lungs, including the esophagus, stomach, liver, kidney and urinary bladder were removed, weighed, and fixed in 10% buffered formalin solution. All organs were embedded in paraffin and stained with hematoxylin and eosin for histologic examination.

Assay of NK Cell Activity

At the 3rd, 5th, 10th and 15th weeks, 5 mice per group from of groups 1 and 3 were sacrificed. The spleens were excised and minced into complete media consisting of RPMI 1640 (GIBCO Laboratories, Grand Island, NY), 100U/ml penicillin and 100ug/ml streptomycin. The lymphocytes were collected by Ficoll-Hypaque method. All cells were resuspended to the desired concentration in complete medium. NK cell cytotoxicity was tested by ^{51}Cr release method using YAC-1 tumor cells as target cells. The effector cells were mixed with Na_2CrO_5 -labeled target cells at a ratio of 100:1 in triplicate. After 4 hours' incubation the supernatants were run with a gamma counter. The NK cell activity equivalent to specific ^{51}Cr release was calculated by the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Average NK activity of each group was compared using Student's *t*-test.

RESULTS

Table 1 shows that the animals tolerated carcinogens or carcinogens+PB treatment well. There was no mortality attributable to the treatment, and overall

Table 1. Mean body weights of mice

Experimental weeks		3	5	10	15
Groups (No. of mice)					
Control	(20)	22.1 ± 2.3*	27.4 ± 2.3	31.0 ± 2.6	33.0 ± 4.5
Carcinogens	(20)	22.7 ± 1.9	27.7 ± 2.1	31.5 ± 2.6	33.7 ± 4.1
Carcinogens + PB	(20)	22.2 ± 1.7	26.2 ± 2.0	29.2 ± 3.1	29.9 ± 2.6**

* : Mean ± SD

** : Significantly different from control group at *P* < 0.02.

Carcinogens: DEN + Urethane + NMU

PB: Phenobarbital

weight gain over a 15 week period was comparable between control and treated animals. Mean relative weights of liver, lung and kidney of each group is shown in Table 2. The carcinogen + PB-treated group had larger liver weights than the control group

($P < 0.001$).

After 15 weeks of treatment, lung tumors occurred in 90.0% and 85.7% of mice given carcinogens only and carcinogens with PB, respectively, with a mean number of 2.6 and 2.2 per mouse, as shown in Table

Table 2. Mean relative organ weights of mice (mg/g B.W.)

Organs Groups (No. of mice)		Lungs	Liver	Kidney	
				R	L
Control	(20)	$8.0 \pm 1.4^*$	44.5 ± 6.4	8.0 ± 1.1	7.8 ± 1.4
Carcinogens	(20)	7.0 ± 1.2	40.4 ± 5.9	7.2 ± 1.1	6.8 ± 1.0
Carcinogens + PB	(20)	8.4 ± 1.5	$63.2 \pm 9.0^{**}$	7.7 ± 1.1	8.6 ± 1.7

* : Mean $\pm$ SD

** : Significantly different from control group at $P < 0.001$.

Carcinogens: DEN + Urethane + NMU

PB: Phenobarbital

Table 3. Incidence and multiplicity of lung adenomas

Groups (No. of mice)		Incidence (%)	Multiplicity
Control	(20)	0	0
Carcinogens	(20)	90.0	$2.6 \pm 1.9^*$
Carcinogens $\pm$ PB	(20)	85.7	2.2 ± 2.0

* : Mean $\pm$ SD

Carcinogens: DEN + Urethane + NMU

PB: Phenobarbital

3. No untreated animal developed lung tumors. Table 4 reveals a sequential increase in the incidence and multiplicity of tumors in mice treated with carcinogens and PB.

On microscopic examination of the major organs, no lesions other than lung tumors were found. The tumors on the lungs consisted histologically of small well-demonstrated round nodules consisting of monotonous cuboidal epithelial cells in papillary configuration. The carcinogens + PB-treated group showed

Table 4. Sequential incidence and multiplicity of lung adenoma

	Experimental weeks Groups (No. of mice)	3	5	10	15
		(10)	(10)	(10)	(20)
Incidence	Control	0	0	10	0
(%)	Carcinogens + PB	20	60	50	86.4
Multiplicity	Control	0	0	0.1 ± 0.3	0
(Mean $\pm$ SD)	Carcinogens + PB	0.3 ± 0.7	0.8 ± 0.8	1.2 ± 1.7	2.2 ± 2.0

Carcinogens: DEN + Urethane + NMU

PB: Phenobarbital

Table 5. Sequential splenic NK activity of mice.

Experimental week	3	5	10	15
Groups (No. of mice)	(5)	(5)	(5)	(5)
Control	$7.2 \pm 2.3^*$	12.9 ± 2.4	26.7 ± 2.7	12.0 ± 1.9
Carcinogens + PB	15.5 ± 9.2	$18.8 \pm 4.0^{**}$	$33.4 \pm 5.5^{**}$	8.9 ± 2.4

* : Mean $\pm$ SD (%)

** : Significantly different from control at $P < 0.05$.

Carcinogens: DEN + Urethane + NMU

PB: Phenobarbital

increased splenic NK cell activity compared to the control group at the 5th and 10th weeks ($P < 0.05$), but not at the end of the experiment (Table 5).

DISCUSSION

The results of the present experiment using DEN, urethane, and NMU as a wide-spectrum initiator were disappointing, since tumors developed only in the lungs. Lung tumor incidence and multiplicity which were 85-90% and 2.2-2.6 respectively with or without promoter, were almost equal to those after a single treatment with 0.5mg/g B.W. of urethane in A/J mouse, the most susceptible species in lung tumorigenesis (Malkinson and Beer, 1983). The high incidence of lung tumors in this model suggested that the mice used in this experiment might have tumor specificity in the lungs. It also should be considered that DEN or NMU might have exerted an additive effect to lung tumorigenesis.

We thought that our experimental period (15 weeks) was too short, and that extension of the period to over 30 weeks might result in wider pathological changes. In our study, phenobarbital did not act as a promoter in lung tumorigenesis nor did it influence other organ carcinogenesis. PB-treated animals showed only reduced body weight gain and increased relative liver weights. Tsuda *et al.* (1983) reported that 0.05% PB treatment for 32 weeks after NMU initiation, showed promoting effects on the thyroid, liver and urinary bladder tumorigenesis in rats. So our short experimental duration could explain the lack of a promoting effect of PB, even if the initiation step had been started.

Ito *et al.* (1988) reported that the wide-spectrum initiation model could be used for the confirmation of site-specific modification potential. And a new design in the type of carcinogens, their dosages and experimental period in consideration of the site-specificity of animals would be necessary to establish a more ideal multi-target carcinogenesis model in mice.

The NK cell activity was reported to be variable according to the kinds and dosages of carcinogens, and generally tended to decrease with carcinogen treatments (Gorelik *et al.*, 1981; Talcott *et al.*, 1984). Our study showed increased NK cell activity in the carcinogens+PB-treated group at the 5th and 10th week, but not at the 15th week, when the NK cell activity began to decrease. This result was concomitant with that of Hong's report (1991) and, a further extended investigation of NK cell activity seemed to be required.

In summary a mouse wide-spectrum carcinogenesis

model will become available when a more defined experimental protocol is established.

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