

# Preliminary Experimental Study on Carcinogenicity of Arsenic Trioxide in Rat Lung

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To investigate carcinogenic or cocarcinogenic properties, copper ore (Kinkaseki) and flue dust collected from a metal refinery and arsenic trioxide ( $As_2O_3$ ) were administered into the lung of male Wistar-King rats by an intratracheal instillation method. No squamous cell carcinoma of the lung was found among the rats given three arsenical substances, while adenoma or adenocarcinoma was observed. Squamous cell carcinoma of the lung was observed in rats, when copper ore, flue dust, and arsenic trioxide were instilled into the lung together with benzo[a]pyrene (B[a]P). The incidence of squamous cell carcinoma of the lung in rats exposed to Kinkaseki, flue dust, and  $As_2O_3$  in addition to B[a]P was higher than that in rats given B[a]P alone. The results of this study indicate that solid arsenical substances, such as arsenic trioxide, metal ore and flue dust from a metal refinery, seemed to act on the carcinogenicity of B[a]P in a cocarcinogenic manner.

## Introduction

Inorganic arsenical compounds, including arsenic trioxide, have been suspected as human carcinogens in several epidemiological studies on workers in mines and copper smelters (1-3). Many clinical studies, epidemiological, occupational and therapeutic exposure studies have shown the same trend. Recently, Tokudome and Kuratsune (4) reported that the risk of lung cancer was significantly increased in copper smelter workers in a metal refinery in Japan. In spite of the epidemiological events mentioned above, there are essentially negative reports concerning malignant lung tumors in animals following exposure to inorganic arsenical compounds given through the airway into the lung. In the present study, arsenic trioxide, Kinkaseki (copper ore), and flue dust containing some amounts of arsenic collected from the metal refinery reported by Tokudome and Kuratsune (4) were examined for carcinogenicity and cocarcinogenicity with benzo[a]pyrene (B[a]P) in rats.

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These materials were intratracheally instilled into the lung of rats.

## Materials and Methods

Kinkaseki ore and the flue dust were collected from a metal refinery, Oita Prefecture, Japan, in May, 1971. These materials were transferred into a Soxhlet apparatus and were extracted with acetone to remove tarry materials which might contain carcinogenic hydrocarbons. After repeated extraction, it could be ascertained that these materials did not contain B[a]P and did not have any fluorescence. Metals in Kinkaseki and the flue dust were qualitatively determined by emission spectroanalysis; the results obtained are shown in Table 1. Arsenic, nickel, chromium, copper, iron, and manganese were analyzed quantitatively by flameless or flame atomic absorption spectroanalysis, and the results are shown in Table 2. Arsenic concentrations were 3.95% in Kinkaseki and 10.6% in the flue dust.

Arsenic trioxide, analytical grade, from Katayama-Seiyaku Co., Japan, was used in the experiment.

Benzo[a]pyrene used in the experiment was manufactured by Sigma Ltd., Basel, Switzerland.

**Table 1. Qualitative determination of metals in copper ore (Kinkaseki) and flue dust by emission spectroanalysis.<sup>a</sup>**

Element	Sensitivity, ppm	Spectral line, Å	Intensity <sup>b</sup>	
			Copper ore	Flue dust
Ag	1	3280.683 <sup>c</sup>	S	S
Al	3	3092.713	S	S
As	150	2349.840	S	S
Ba	8	4554.042	S	S
Be	15	2348.610	—	—
Bi	30	3067.710	S	SS
Ca	3	4226.728	S	SS
Cd	15	2288.018	WW	S
Co	15	3453.505	M	M
Cr	2	4254.346	M	S
Cu	1	3247.540	SS	SS
Fe	8	3020.489	SS	SS
K	0.1	4047.201	—	—
Mg	3	2795.530	S	SS
Mn	15	2798.271	S	S
Na	1	3302.988	M	SS
Ni	8	3050.819	M	M
Pb	8	2833.069	S	SS
Sb	30	3267.502	—	—
Si	30	2881.578	SS	SS
Sn	15	3175.019	S	SS
Ti	15	3349.035	M	M
V	8	3185.396	WW	W
Zn	150	3345.020	M	SS
Hg	150	2536.519	W	W

<sup>a</sup>Sample: 20 mg + graphite, 10 mg; cupped electrode DCA; 10 AM, 20 μm, 4mm.

<sup>b</sup>Intensity: SS, +++++; S, ++++; M, ++; W, +; WW, ±; —, —.

<sup>c</sup>R. U. (persistent line).

**Table 2. Concentrations of several elements in copper ore (Kinkaseki) and flue dust, used in this experiment.**

Element	Copper ore (Kinkaseki), %	Flue dust, %
As	3.95	10.6
Ni	0.009	0.006
Cr	0.006	0.014
Cu	1.00	0.58
Fe	11.2	0.77
Mn	0.013	0.044

Saline (supplied by the Hospital Dispensary, Kyushu University) was used for the control group in the experiment.

Male Wistar-King albino rats, about 10 weeks old, came from the colony of the Animal Center, Kyushu University. The rats were divided into eight groups according to the following exposure regimen (shown in Table 3): 2.5 mg of Kinkaseki (about 0.1 mg arsenic), 2.0 mg of flue dust (about 0.2 mg arsenic), 0.26 mg of arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) (about 0.2 mg arsenic), 0.4 mg of B[a]P as positive control, 2.5 mg of Kinkaseki with 0.4 mg of B[a]P,

2.0 mg of the flue dust with 0.4 mg B[a]P, 0.2 mg of As<sub>2</sub>O<sub>3</sub> with 0.4 mg B[a]P, and saline as control group.

An intratracheal instillation method was used (5). The rats were given an injection of atropine sulfate subcutaneously and were anesthetized with a mixture of 5% ether and 95% oxygen in a desiccator for 5 min. The anesthetized rat was removed from the desiccator and 0.2 ml of the suspended materials in distilled water or saline was instilled into the lung by a microsyringe with a special metal needle. The needle was carefully inserted into the distal part of the trachea by viewing the slit of the pseudo-vocal cords through magnifying glasses.

Each suspension was prepared as follows. The solid (Kinkaseki, flue dust, As<sub>2</sub>O<sub>3</sub>, B[a]P, Kinkaseki + B[a]P, flue dust + B[a]P, or As<sub>2</sub>O<sub>3</sub> + B[a]P) was suspended in 0.2 ml of distilled water. About 20 ml of each suspension was neutralized with dilute sodium hydroxide solution, and the solutions were homogenized, sterilized, and suspended for 10 min with an ultrasonic wave generator (Taisho Denki Ltd., Japan) under nitrogen gas. The distributions of particle sizes, of Kinkaseki, flue dust, and As<sub>2</sub>O<sub>3</sub> in the suspensions were as follows; 0.45% ≥ 10 μ, 2.00% 10–5 μ, 97.55% < 5 μ for Kinkaseki; 2.72%, 5.35%, and 91.93%, respectively, for flue dust; 1.06%, 1.27%, and 97.67% respectively, for As<sub>2</sub>O<sub>3</sub>. The particle size of B[a]P in the suspension was about 5 μ or less. In all suspensions, especially in flue dust, considerable aggregation of particles were observed.

All rats received 15 exposures in all, once a week for about 4 months. The number of surviving rats among experimental groups after the 15th instillation were as follows; 10 in the group receiving Kinkaseki, 7 in the flue dust group, 8 in As<sub>2</sub>O<sub>3</sub> group, 7 in the B[a]P group, 10 in the Kinkaseki + B[a]P group, 10 in the flue dust + B[a]P group, 7 in the As<sub>2</sub>O<sub>3</sub> + B[a]P group, and 7 in the saline group, as shown in Table 3. They were raised on commercial solid foods (Oriental NMF, Oriental Co. Japan) and were given drinking water *ad libitum*. All surviving rats were observed during their entire life span and allowed to die spontaneously. The animals were autopsied, and main visceral organs and any tumors of tissues or organs were fixed with a 10% formalin/water solution. The respiratory organs were especially carefully examined with magnifying glasses. The lung was inflated and fixed by filling 10% formalin/water solution with a syringe through trachea. For microscopical histopathological examination, sections (about 6 μ) were prepared and stained with hematoxylin-eosin. For precise examination, sections were prepared from two to six blocks from each of the five lobules of the lung.

**Table 3. Malignant Lung Tumors Induced in Rats by Copper Ore (Kinkaseki), Flue Dust, As<sub>2</sub>O<sub>3</sub>, B[a]P, Copper Ore + B[a]P, Flue Dust + B[a]P, As<sub>2</sub>O<sub>3</sub> + B[a]P, and Saline.**

Group	Sex	Number of rats surviving after 15 instillations	Survival after instillation, days		Number of malignant tumor-bearing rats	Incidence rates, %	
			Average	Range			
Copper ore (2.5 mg × 15)	M	10/14	430	132-752	0	0.	
Flue dust (2.0 mg × 15)	M	7/23	487	182-708	1 <sup>b</sup>	14.3	4.0 <sup>c</sup>
As <sub>2</sub> O <sub>3</sub> (0.26 mg × 15)	M	8/14	395	192-643	0	0.	
B[a]P (0.4 mg × 15)	M	7/21	837	196-1050	1	14.3	14.3
Copper ore + B[a]P [(2.5 mg + 0.4 mg) × 15]	M	10/20	372	121-632	2	20.0	
Flue dust + B[a]P [(2.0 mg + 0.4 mg) × 15]	M	10/25	394	106-619	3	30.0	29.6 <sup>c</sup>
As <sub>2</sub> O <sub>3</sub> + B[a]P [(0.26 mg + 0.4 mg) × 15]	M	7/21	670	416-850	3	42.9	
Saline	M	7/23	475	134-678	0	0.	0.

<sup>a</sup>No. of rats surviving of number at the beginning of instillation.

<sup>b</sup>Adenocarcinoma.

<sup>c</sup>Incidence rates between two groups are significantly different at 4% level by Fischer's direct method.

## Results

As shown in Table 3, in the eight experimental groups, the average survival after 15 instillations ranged from 372 days in the (copper ore + B[a]P) group to 837 days in the B[a]P group. In three groups, Kinkaseki (copper ore), flue dust, and As<sub>2</sub>O<sub>3</sub>, induction of squamous cell carcinoma was not observed. However, adenoma of the lung was

observed in 1 of 10 survivors in the Kinkaseki group and 1 out of 8 survivors in the As<sub>2</sub>O<sub>3</sub> group. In the flue dust group, adenocarcinoma was observed in 1 out of 7 survivors. In histopathological examination, all rats of the above mentioned three groups had squamous cell metaplasia in the airway or osteometaplasia in the alveolus of the lung as shown in Table 4 (Figs. 1 and 2).

In the four groups given B[a]P, some rats bearing

**Table 4. Histological classification of lung tumors and other pathological changes.**

Group	Sex	No. of rats	Malignant tumors (A)			Benign tumors (B). adenoma	All tumors (A+B) (tumor incidence rates, %)	Squamous cell metaplasia	Osteo-metaplasia
			Squamous cell carcinoma	Adeno-carcinoma	Sub-total				
Copper ore (Kinkaseki) (2.5 mg × 15)	M	10	0	0	0 (1) <sup>a</sup>	1	1 (10.0)	5	2
Flue dust (2.0 mg × 15)	M	7	0	1	1	0	1 (14.3) 3 (12.0)	5	1
As <sub>2</sub> O <sub>3</sub> (0.26 mg × 15)	M	8	0	0	0	1	1 (12.5)	3	1
B[a]P (0.4 mg × 15)	M	7	1	0	1	0	1 (14.3) 1 (14.3)	2	2
Copper ore + B[a]P [(2.5 mg + 0.4 mg) × 15]	M	10	2	(1) <sup>b</sup>	2 (1)	0	2 (20.0)	2	2
Flue dust + B[a]P [(2.0 mg + 0.4 mg) × 15]	M	10	3	0	3	0	3 (30.0) 8 (29.6)	4	1
As <sub>2</sub> O <sub>3</sub> + B[a]P [(0.26 mg + 0.4 mg) × 15]	M	7	3	0	3	0	3 (42.9)	2	0
Saline	M	7	0	0	0	0	0 (0.) 0 (0.)	1	1

<sup>a</sup>Lung metastasis of osteosarcoma from the femur.

<sup>b</sup>Coexistence of squamous cell carcinoma and adenocarcinoma.

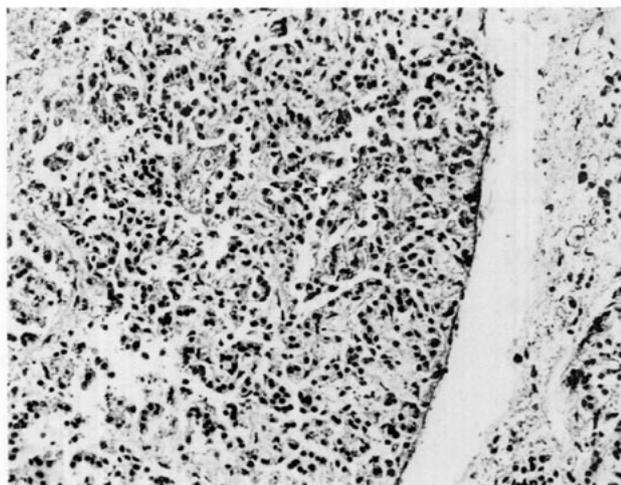


FIGURE 1. Male rat of the  $As_2O_3$  group dying 581 days after the beginning of the intratracheal instillation. There is adenoma in the right lower lobule of the lung. H. E. stain,  $\times 200$ .

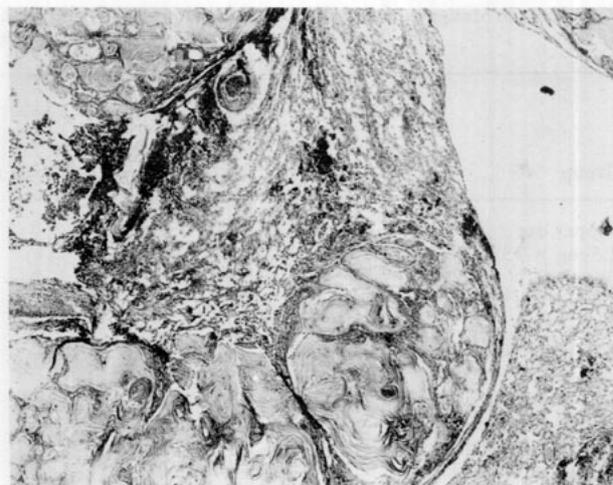


FIGURE 3. Male rat of the Kinkaseki + B[a]P group dying 390 days after the beginning of the intratracheal instillation. There is squamous cell carcinoma with pearl formation in the right lower lobule of the lung. H. E. stain,  $\times 26$ .

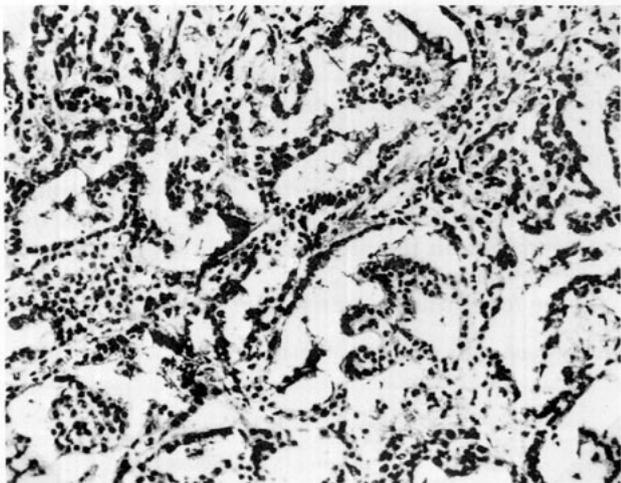


FIGURE 2. Male rat of the flue dust group dying 512 days after the beginning of the intratracheal instillation. There is an adenocarcinoma in the subcardial lobule of the lung. H. E. stain,  $\times 200$ .

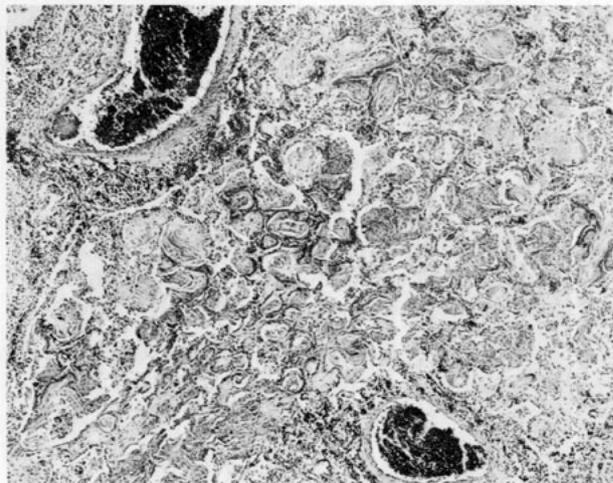


FIGURE 4. Male rat of the flue dust + B[a]P group dying 569 days after the beginning of the intratracheal instillation. There is squamous cell carcinoma with pearl formation in the right lower lobule of the lung. H. E. stain  $\times 59$ .

squamous cell carcinoma of the lung were observed. The incidence of lung carcinoma was 1 out of 7 rats (14.3% incidence rate) in the B[a]P group, 2 of 10 rats (20.0%) in the Kinkaseki plus B[a]P group, 3 of 10 rats (30.0%) in the flue dust plus B[a]P group, and 3 out of 7 rats (42.9%) in the  $As_2O_3$  plus B[a]P group (Figs. 3-5). Furthermore, squamous cell metaplasia in the lining cells of the airway and osteometaplasia in the alveolar cells of the lung were observed in almost all rats as shown in Table 4 (Fig. 6). No incidence of malignant or benign tumors existed in rats of the saline group, and other histopathological changes in these rats were much less than those of other groups.

The incidence rates of malignant lung tumors of rats in the groups; the combined group of rats which received the different arsenical substances, the B[a]P group, the combined group of rats receiving arsenical substances plus B[a]P, and the saline group, were statistically compared with each other. There was a significant difference of incidence rate only between the combined group of three arsenical substances (1 out of 25 rats, 4.0%) and the combined group of three arsenical substances plus B[a]P (8 out of 27 rats, 29.6%) as shown in Table 3. The tumor incidence rate in each group (malignant tumors plus benign tumors) is shown in Table 4, and the tumor incidence rate of the lung in the combined

group of three arsenical substances was 12%, that is 3 out of 25 rats.

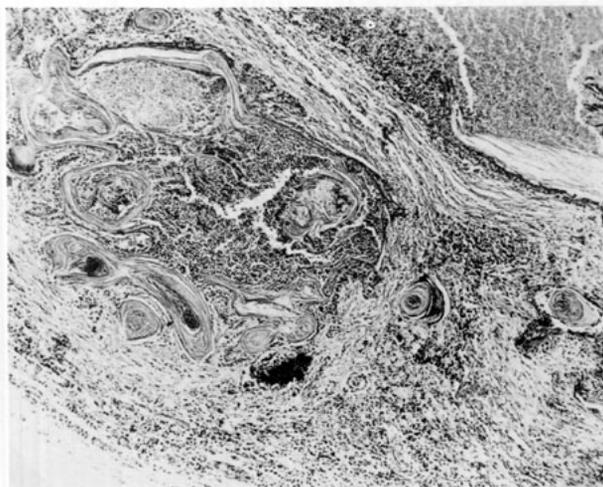


FIGURE 5. Male rat of the  $As_2O_3 + B[a]P$  group dying 657 days after the beginning of the intratracheal instillation. There is squamous cell carcinoma with pearl formation in the right lower lobule of the lung. H. E. stain,  $\times 59$ .

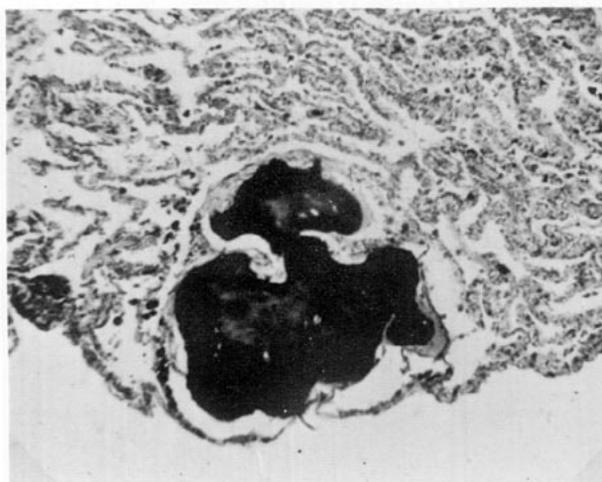


FIGURE 6. Male rat of the Kinkaseki +  $B[a]P$  group dying 462 days after the beginning of the intratracheal instillation. There is osteometaplasia in the left lobule of the lung. H. E. stain,  $\times 66$ .

## Discussion

The number of rats surviving in each group after 15 instillations in this experiment was not adequate to confirm carcinogenicity of arsenic to the lung. Squamous cell carcinoma of the lung in Wistar-King rats could not be induced by intratracheal instillation of arsenical substances. Spontaneous lung tumors are rare in Wistar-King rats; however, adenoma and adenocarcinoma of the lung were ob-

served in the rats among three groups exposed intratracheally to arsenical substances. Taking account of the tumorigenesis mentioned above, carcinogenicity of arsenic itself to the lung of animal cannot be absolutely denied in the field of animal experiment.

The results of this study indicate that solid arsenical substances such as arsenical trioxide, metal ore, and flue dust, containing some amount of arsenic, from a metal refinery seemed to act on the carcinogenicity of  $B[a]P$  in a cocarcinogenic manner. The incidence of squamous cell carcinoma of the lung in rats exposed to Kinkaseki (copper ore), flue dust, and  $As_2O_3$  plus  $B[a]P$  was higher than that of  $B[a]P$  alone. The cocarcinogenic action of other solid substances as well as arsenicals to inducing lung tumor in animals were reported by Saffiotti et al. (6) and Feron (7). In epidemiological consideration of high risk of lung cancer among workers in copper smelters, Lee and Fraumeni (1) and also Tokudome and Kuratsune (4) suggested an association for carcinogenicity between arsenic trioxide and other carcinogens or/and cocarcinogens, such as polycyclic aromatic hydrocarbons and sulfur dioxide. In conclusion, this experiment indicates that carcinogenicity of inorganic arsenical compounds, particularly arsenic trioxide, for animals, cannot be absolutely ruled out by the method of intratracheal administration. Further animal experiments by intratracheal instillation technique on larger numbers of animals and with higher dosages of inorganic arsenicals are necessary.

## Summary

Carcinogenicity to the lung of Kinkaseki (copper ore), the flue dust collected from a metal refinery in Japan, and arsenic trioxide ( $As_2O_3$ ) was studied in male Wistar-King rats by an intratracheal instillation method. Simultaneously carcinogenicity to the lung of  $B[a]P$ , Kinkaseki plus  $B[a]P$ , flue dust plus  $B[a]P$ ,  $As_2O_3$  plus  $B[a]P$  also was examined in the rats by the same method. In the experiment, 2.5 mg of Kinkaseki, 2.0 mg of flue dust, and 0.26 mg of  $As_2O_3$  were instilled intratracheally into the lung once a week for 15 weeks. No squamous cell lung cancer was observed. One adenoma out of 10 rats in the Kinkaseki group, one adenocarcinoma out of 7 rats in the flue dust group, and one adenoma out of 8 rats in the  $As_2O_3$  group were observed.

When the same method of instillation was used on other groups of rats, 0.4 mg of  $B[a]P$  induced one squamous cell lung cancer out of 7 rats, 2.5 mg Kinkaseki plus 0.4 mg  $B[a]P$  induced 2 lung cancers out of 10 rats, 2.0 mg flue dust plus 0.4 mg  $B[a]P$  induced 3 lung cancers out of 10 rats, and 0.26 mg

As<sub>2</sub>O<sub>3</sub> plus 0.4 mg B[a]P induced 3 lung cancers out of 7 rats. Neither malignant nor benign tumors were found in the control group of rats receiving instillations of saline. Carcinogenicity of inorganic arsenical compounds, especially arsenic trioxide, for animals cannot be absolutely ruled out on the basis of intratracheal instillation experiments. Arsenic trioxide seems to act as a cocarcinogen with B[a]P in inducing lung cancer in animals.

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