

Original Article

## Acute Phase Pulmonary Responses to a Single Intratracheal Spray Instillation of Magnetite (Fe<sub>3</sub>O<sub>4</sub>) Nanoparticles in Fischer 344 Rats

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**Abstract:** Iron nanomaterials are of considerable interest for application to nanotechnology-related fields including environmental catalysis, biomedical imaging, drug delivery and hyperthermia, because of their superparamagnetic characteristics and high catalytic abilities. However, information about potential risks of iron nanomaterials is limited. The present study assessed pulmonary responses to a single intratracheal spray instillation of triiron tetraoxide nanoparticles (magnetite) in rats. Ten-week-old male and female Fischer 344 rats (n=5/group) were exposed to a single intratracheal spray instillation of 0 (vehicle), 5.0, 15.0 or 45.0 mg/kg body weight (BW) of magnetite. After 14 days, the rats were sacrificed, and biological consequences were investigated. The lung weights of the 15.0 and 45.0 mg/kg BW male and female groups were significantly higher than those of the control groups. The lungs of treated rats showed enlargement and black patches originating from the color of magnetite. The typical histopathological changes in the lungs of the treated rats included infiltration of macrophages phagocytosing magnetite, inflammatory cell infiltration, granuloma formation and an increase of goblet cells in the bronchial epithelium. The results clearly show that instilled magnetite causes foreign body inflammatory and granulating lesions in the lung. These pulmonary responses occur in a dose-dependent manner in association with the increase in lung weight. (DOI: 10.1293/tox.25.233; J Toxicol Pathol 2012; 25: 233–239)

**Key words:** magnetite, Fe<sub>3</sub>O<sub>4</sub>, nanoparticles, lung, intratracheal spray instillation, Fischer 344 rat

### Introduction

Nanomaterials are defined as having a size of 100 nanometers or less in at least one dimension. Nanotechnology -the creation, manipulation and application of nanomaterials- involves the ability to engineer, control and exploit the unique chemical, physical and electrical properties that emerge from infinitesimally tiny man-made particles<sup>1</sup>. Engineered nanoparticles, the surface volume increases, can result in having unique photonic and catalytic properties that display great differences from those of over-nanoscaled materials with the same composition. The superb biological and environmental reactivities of nanoparticles have led to their wide and considerable use in disease treatment, pollutant degradation and so forth<sup>1,2</sup>. Among them, iron nanomaterials are of considerable interest for application to nano-

technology-related fields including environmental catalysis, magnetic storage, biomedical imaging<sup>1</sup>, magnetic target drug delivery<sup>3,4</sup> and hyperthermia<sup>5–8</sup> because of their superparamagnetic characteristics and high catalytic abilities.

Acute toxic reactions of nano-magnetic ferrofluid have been evaluated, and half lethal doses (LD<sub>50</sub>) of >2104.8, >438.50 and >1578.6 mg/kg were found in cases of oral, intravenous and intraperitoneal administrations, respectively, where no apparent pathological changes were observed<sup>9</sup>. It was shown *in vitro* that triiron tetraoxide, Fe<sub>3</sub>O<sub>4</sub>, causes a decrease in mitochondrial function and lactate dehydrogenase leakage in Neuro-2A cells only with concentrations reaching greater than 200 µg/mL<sup>10</sup>. Magnetic nanoparticles of Fe<sub>3</sub>O<sub>4</sub> affect the ICR mice immune system such that Fe<sub>3</sub>O<sub>4</sub> nanoparticles enhance the production of interleukin (IL)-2, interferon-γ and IL-10, but not IL-4, in the peripheral blood<sup>11</sup>. A high amount of magnetite, 15 mg × 15 intratracheal instillations, led to an unexpected lung tumor in the female Wistar rat<sup>12</sup>, while chronic exposure to iron oxide has been shown not to increase the incidence of pulmonary tumors<sup>13,14</sup>. Iron is a transition metal that is considered to play a pivotal role in modulating oxidative stress and other biological responses<sup>15,16</sup>, which is speculated to be the critical mechanism in eliciting the adverse effects of iron

Received: 12 March 2012, Accepted: 19 June 2012

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particulate matter exposure. Despite the above information, the risk data for iron nanomaterials are limited. The present study assessed pulmonary responses to a single intratracheal spray instillation of  $\text{Fe}_3\text{O}_4$  nanoparticles (magnetite) in male and female Fischer 344 rats.

## Materials and Methods

### *Ethical considerations*

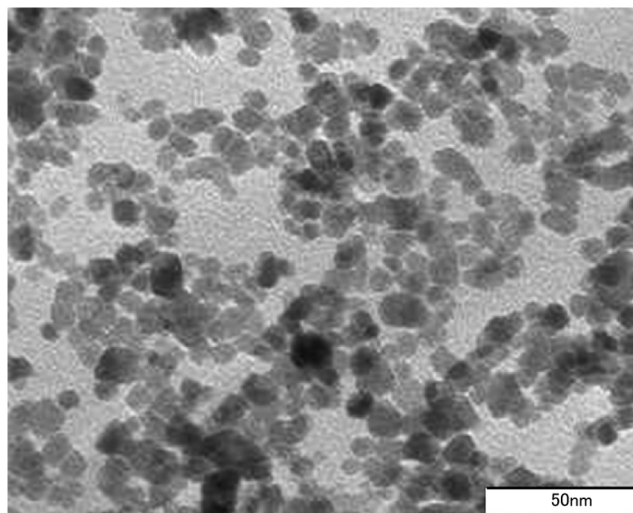
The current study was performed principally in conformity with the Guidelines for the Toxicity Testing of Pharmaceuticals released by the MHLW (Ministry of Health, Labour and Welfare) of Japan ([http://www.pmda.go.jp/ich/s/s4\\_93\\_8\\_10.pdf](http://www.pmda.go.jp/ich/s/s4_93_8_10.pdf)). The experimental protocol was approved by the Experiments Regulation Committee and Animal Experiment Committee of the Tokyo Metropolitan Institute of Public Health prior to its execution. All the animals were handled in accordance with the Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals and the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science<sup>17</sup>.

### *Animals*

A total of 42 male and female specific pathogen-free Fischer 344 (F344/DuCrI/CrIj) rats were purchased at 8 weeks of age from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The rats were housed individually in stainless steel cages and were kept under controlled conditions of temperature (22–24°C), relative humidity (50–60%) and ventilation (more than 10 times/hour) with a 12-hour light/dark cycle; they were allowed free access to pelleted chow CE-2 (CLEA Japan, Inc., Tokyo, Japan) and drinking water throughout both the acclimation and experimental periods. After confirming normal health status at the end of the 2-week acclimation period, 20 rats of each sex were selected for use and randomly allocated to 4 groups of 5 rats. The rats were observed twice daily, and clinical signs and mortality were recorded.

### *Test chemical and animal treatments*

The magnetite slurry ( $\text{Fe}_3\text{O}_4$  nanoparticle suspension; lot number, 90828) was generously supplied by Toda Kogyo Corp. (Otake, Hiroshima, Japan). A representative transmission electron microscopic (TEM) view of magnetite particles is shown in Fig. 1. The estimated primary particle size of the prepared sample is 5–15 nm in diameter (TEM measurement). The purity of the test chemical was determined by an energy dispersive X-ray spectrometer, and only iron and oxygen were detected. Prior to the toxicity study, the optimal way to disperse magnetite was preliminarily examined. Magnetite was dispersed in physiological saline, physiological saline+0.05% tween 80, 0.1 M Tris buffer (pH 8.0), 0.5% carboxymethyl cellulose, 0.1 M phosphate buffer (pH 8.1) or ultrapure water (Milli-Q water, 18.2 M $\Omega$ ). Observation of dispersed particles was carried out under a light microscope and judged on the basis of Brownian motion. Among



**Fig. 1.** Representative transmission electron microscopic view of magnetite nanoparticles. The estimated primary particle size is about 5–15 nm in diameter.

these test dispersion vehicles, Milli-Q water was the best vehicle to obtain the most homogeneous suspension. Magnetite slurry was thus diluted with sterile Milli-Q water and adjusted to about pH 7.4 with 0.1 N hydrochloric acid. The intratracheal instillation technique was performed according to the recommendations of Driscoll *et al.*<sup>18</sup>. Before the intratracheal spray instillation, the rats were anesthetized by diethyl ether and placed in a supine position on an angled board with their necks extended. Magnetite suspension was placed in an ultrasonication bath (SONOREX RK31, BANDELIN electronic, Berlin, Germany) and then instilled into the trachea by a sterile stainless steel tube (IA-1B Micro-Sprayer, Penn-Century, Inc., Wyndmoor, PA, USA) at the concentrations of 0 (control), 5.0 (low), 15.0 (middle) and 45.0 (high) mg/1 mL/kg body weight, which was followed by the insufflation of 0.2 mL of air.

### *Animal sacrifice and assessments*

Two weeks after instillation, all rats were deprived of food (but not water) overnight. The rats were then lightly anesthetized by diethyl ether and sacrificed by exsanguination after collecting blood samples via the abdominal aorta. The blood for hematology was collected into tubes treated with dipotassium ethylenediaminetetraacetate (EDTA-2K). The hematological examination was carried out using an automatic analyzer (Sysmex KX-21NV; Sysmex Corporation, Kobe, Hyogo, Japan) to determine the red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit level (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC) and platelet count (PLT). Differential counts of leukocytes were made by a light microscopic observation of smeared specimens stained with a routine May-Grünwald-Giemsa protocol. A serum biochemistry analysis was performed with an automatic analyzer (TBA-120FR; Toshiba Medical Systems

**Table 1.** Initial and Final Body Weights and Lung Weights in Fischer 344 Rats Treated with a Single Intratracheal Administration of Magnetite on Day 14

Item	Dose of magnetite (mg/kg body weight)			
	0 (control)	5.0	15.0	45.0
<b>Males</b>				
Initial number of rats	5	5	5	5
Initial body weight (g)	213.6 ± 3.9 <sup>a</sup>	211.7 ± 5.5	214.9 ± 4.6	212.9 ± 4.0
Final effective number of rats	5	5	5	5
Final body weight (g)	239.4 ± 8.6	239.1 ± 7.4	239.1 ± 10.3	229.0 ± 2.8
Absolute lung weight (mg)	722.4 ± 27.4	823.8 ± 58.0	923.4 ± 91.5*	1105.1 ± 94.5*
Relative lung weight (mg/100 g BW)	301.9 ± 9.5	344.4 ± 19.4	386.4 ± 38.1*	482.5 ± 41.0*
<b>Females</b>				
Number of rats	5	5	5	5
Initial body weight (g)	138.9 ± 7.1	138.0 ± 5.8	138.6 ± 7.7	141.8 ± 9.3
Final effective number of rats	5	5	4	4
Final body weight (g)	151.0 ± 6.7	148.7 ± 6.1	149.3 ± 6.6	145.2 ± 12.4
Absolute lung weight (mg)	556.7 ± 77.2	642.1 ± 55.5	724.9 ± 88.1*	821.7 ± 68.0*
Relative lung weight (mg/100 g BW)	368.3 ± 46.6	431.3 ± 23.5	485.6 ± 57.7*	566.7 ± 32.6*

<sup>a</sup> Values are means ± standard deviations. \*Significantly different from the corresponding control values ( $P < 0.05$ , Dunnett's test).

Corporation, Tokyo, Japan) to determine the levels of total protein (TP), albumin (ALB), albumin/globulin ratio (A/G), glucose (GLU), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-CHO), triglyceride (TG), total bilirubin (T-BIL), blood urea nitrogen (BUN), creatinine (CRE) and uric acid (UA). Upon sacrifice, the rats were macroscopically examined and subjected to a full autopsy. The brain, heart, lung (including bronchi, fixed by inflation with fixative), spleen, liver, kidneys, testes and ovaries were weighed and then fixed in 10% neutrally buffered formalin. Paraffin-embedded sections were routinely prepared and histopathologically examined after being stained with hematoxylin and eosin (HE), Azan Mallory and Berlin blue procedures.

### Statistical analysis

For numerical data such as body and organ weights and hematological and serological outcomes, equality of means between the values of the control group and those of each treated group was assessed by Bartlett's test. Homogeneity of variance was then analyzed by one-way analysis of variance, and finally, differences between the values of the control group and those of each treated group were evaluated by Dunnett's test. If the Bartlett's test was significant, the data were subjected to the Kruskal-Wallis test and Dunnett's-type rank sum test. For contingent data such as incidences of histopathological lesions, differences between the values of the control group and those of each treated group were evaluated by Fisher's exact probability test<sup>19</sup>. Statistical processing was conducted using the StatLight software (Yukms Co., Ltd., Tokyo, Japan). Intergroup differences were considered statistically significant when P-values less than 0.05 were obtained.

## Results

### General findings

All male rats survived throughout the experimental period. In the females, one rat in each of the middle- and high-dose groups died within 1 hour from deep anesthesia following intratracheal spray instillation. After instillation, the male and female rats in the high-dose group were slightly less active than the control rats but recovered later.

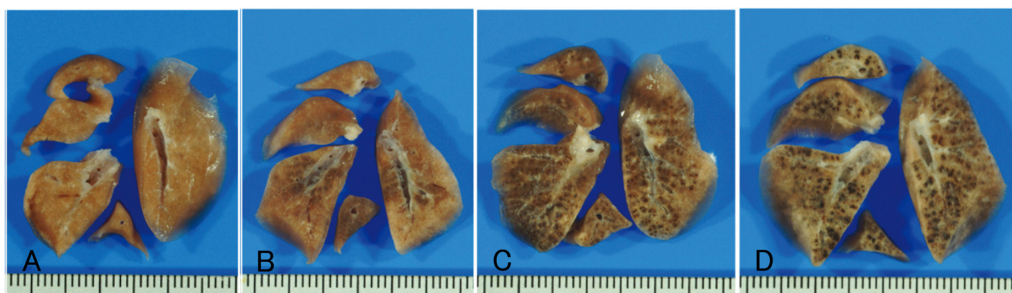
### Hematology and serum biochemistry

In the hematology, the HGB of the high-dose group males was significantly but slightly higher than that of the male control group. In the serum biochemistry, the A/G of the high-dose group females was significantly lower than that of the female control group. There were no significant differences in other items of hematology or biochemistry between the control and treated rats at any doses. Morphological findings and differential counts of leukocytes showed no significant effects in any of the treated groups.

### Pathology

There were no significant differences in the initial or final body weights between the control and treated groups of either sex. Absolute and relative lung weights of the treated groups were higher than those of the control groups, and the differences were statistically significant for the middle- and high-dose groups (Table 1). In the male rats, the absolute and relative testes weights of the low-dose group were significantly higher than those of the control group. There were no significant differences in other organ weights between the control and treated groups for both sexes.

At necropsy, the lungs of the magnetite-treated groups of both sexes were enlarged, and scattered dark brown patches were observed in almost every lobe in all treated rats (Fig. 2). These changes were more marked in the middle- and high-dose groups than in the low-dose groups.



**Fig. 2.** Representative gross views of cross-sections of the formalin-fixed lungs from Fischer 344 rats treated with a single intratracheal spray instillation dose of 0 (control, A), 5.0 (B), 15.0 (C) or 45.0 (D) mg/kg body weight of magnetite on day 14. The enlargement and a large number of scattered dark brown patches are marked in almost every lobe at the middle and high doses.

**Table 2.** Histological Findings of the Lungs and Parathymic Lymph Nodes in Fischer 344 Rats Treated with a Single Intratracheal Administration of Magnetite on Day 14

Item	Dose of magnetite (mg/kg body weight)			
	0 (control)	5.0	15.0	45.0
<b>Males</b>				
Effective number of rats	5	5	5	5
<b>Lung</b>				
Inflammatory cell infiltration	0 <sup>a</sup>	4*	4*	5*
Infiltration of macrophages phagocytosing magnetite	0	5*	5*	5*
Granuloma	0	1	3	5*
Increase of goblet cells in the bronchial epithelium	0	0	0	5*
<b>Parathymic lymph nodes</b>				
Infiltration of macrophages phagocytosing magnetite	0	5*	5*	5*
Deposit of magnetite	0	5*	5*	5*
<b>Females</b>				
Effective number of rats	5	5	4	4
<b>Lung</b>				
Inflammatory cell infiltration	0	3	4*	4*
Infiltration of macrophages phagocytosing magnetite	0	5*	4*	4*
Granuloma	0	0	1	4*
Increase of goblet cells in the bronchial epithelium	0	0	0	3
<b>Parathymic lymph nodes</b>				
Infiltration of macrophages phagocytosing magnetite	0	5*	4*	4*
Deposit of magnetite	0	5*	4*	4*

<sup>a</sup> Number of rats with the lesion. \*Significantly different from the corresponding control values ( $P < 0.05$ , Fisher's exact test). No apparent histological changes were observed in the other organs, when compared with the control rats.

Additionally, dark brown patches were also observed in the parathymic lymph nodes of male and female rats in all treated groups.

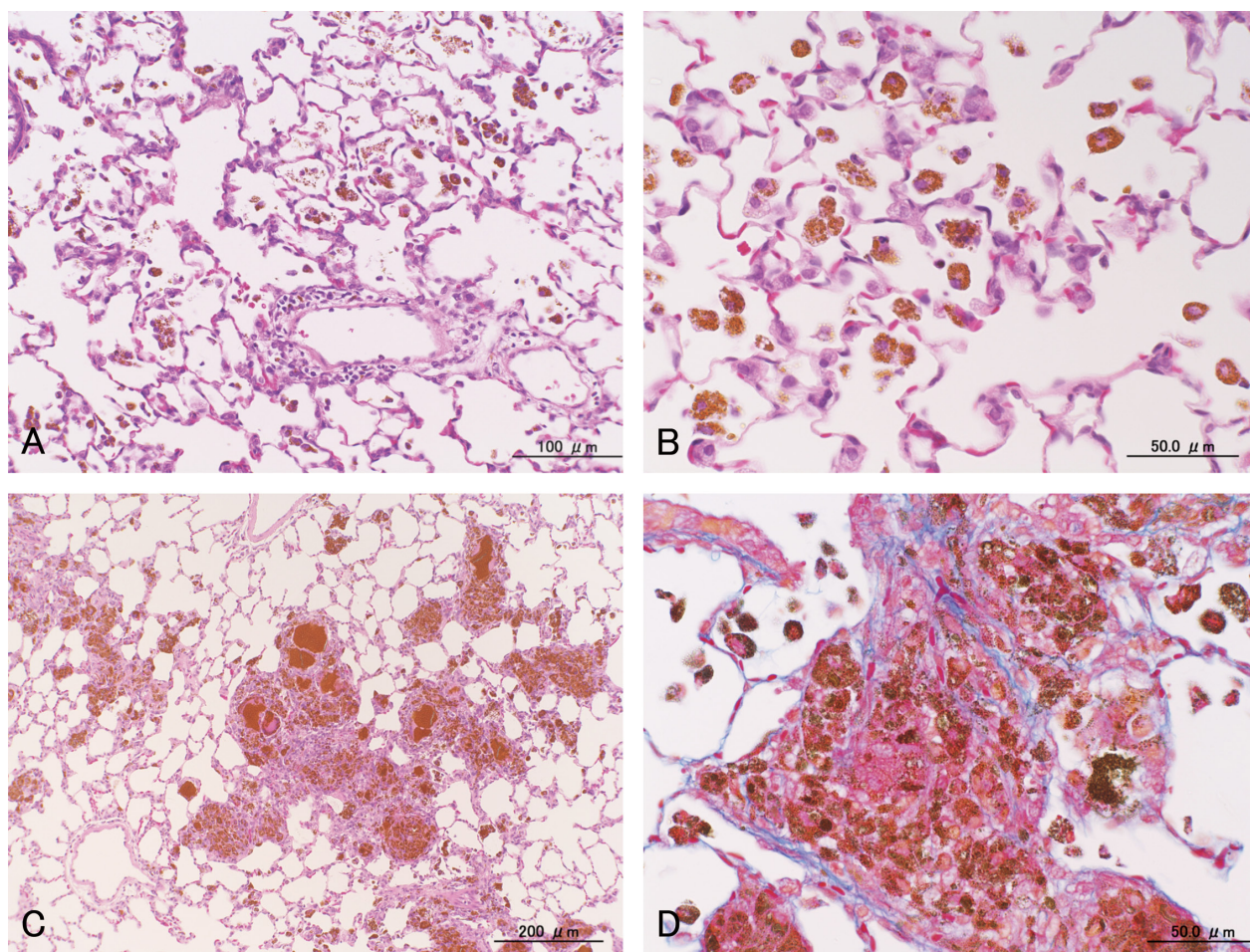
The histopathological changes in the lungs and parathymic lymph nodes are summarized in Table 2. In the lungs of most of the rats in all the treated groups, typically observed changes included the infiltration of multinucleated cells and lymphocytes (Fig. 3A) and the infiltration of dark-brownish pigmented macrophages phagocytosing magnetite in the alveolar walls and spaces (Fig. 3B). In addition, granulomas were observed in 1, 3 and 5 of the low-, middle- and high-dose males and in 1 and 4 of the middle- and high-dose females, respectively. The granulomas were characterized by the aggregation of macrophages phagocytosing magnetite, inflammatory cell infiltration and proliferation of collagenous fiber (Fig. 3C and D). Reactive changes in alveolar and bronchial epithelia were observed in most of

the rats of all treated groups and in the high-dose groups, respectively. An increase of goblet cells in the bronchial epithelium was observed in 5 and 3 of the high-dose males and females, respectively (Fig. 4). In the parathymic lymph nodes, infiltration of macrophages phagocytosing magnetite and deposits of magnetite particles were observed in male and female rats of all treated groups (Fig. 5). Dark-brownish pigmented macrophages were observed at the center or end of the lymph node. No treatment-related changes were observed in other organs of either sex, whereas sporadic spontaneous lesions were observed identically in the control and treated rats.

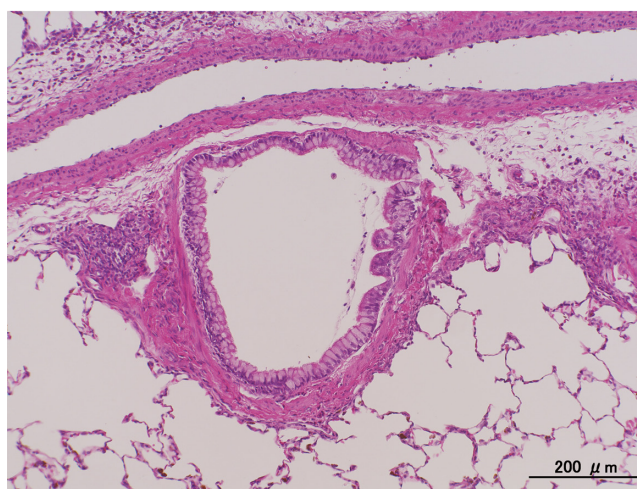
## Discussion

In the present study, rats were exposed to the magnetite nanoparticles by a single intratracheal spray instillation





**Fig. 3.** Representative histologies of the lungs from Fischer 344 rats treated with a single intratracheal spray instillation dose of 5.0 (A, B) or 45.0 (C, D) mg/kg body weight of magnetite on day 14 (A–C, hematoxylin and eosin; D, Azan-Mallory). Infiltration of inflammatory cells (A) and alveolar macrophages phagocytosing magnetite particles (B) are evident in rats given the low dose. Granulomas consisting of macrophages with magnetite particles, infiltrating inflammatory cells and proliferative collagenous fiber (C and D) are evident in rats given the high dose.

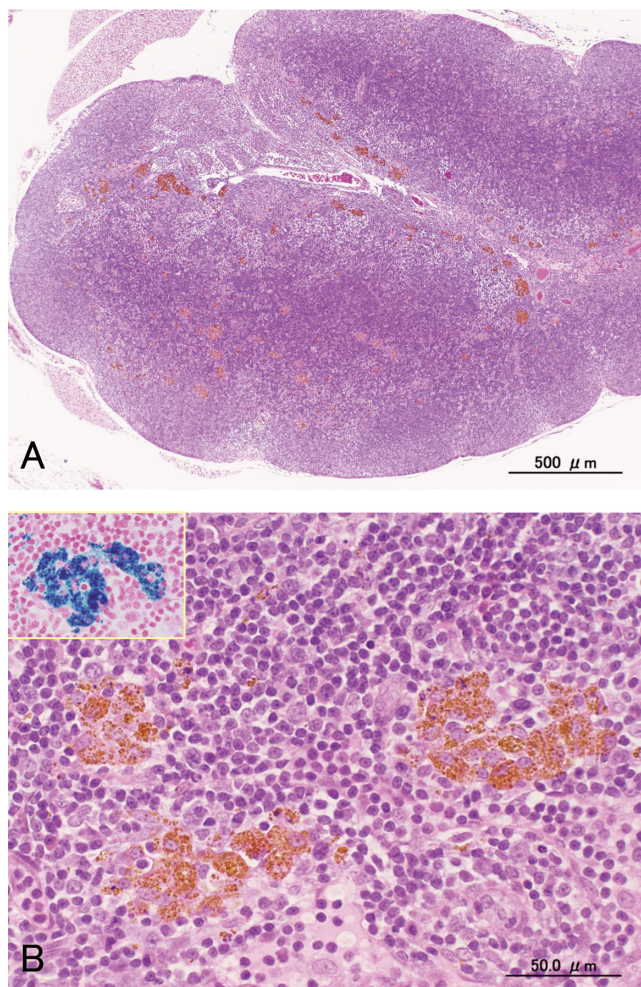


**Fig. 4.** Representative histology of the lung from Fischer 344 rat treated with a single intratracheal spray instillation dose of 45.0 mg/kg body weight of magnetite on day 14 (hematoxylin and eosin). An increase in goblet cells in the bronchial epithelium is evident in a rat given the high dose.

at the doses of 0, 5.0, 15.0 and 45.0 mg/kg body weight. Macroscopically, enlargement of the lung was marked at middle and high doses. Black patches were scattered in almost every lobe of the lungs and the lung-associated lymph nodes of all treated rats. Two weeks after instillation, a large amount of the administered magnetite remained in the lung, and some of the magnetite was distributed into the regional lymph nodes. Histopathologically, infiltration of macrophages phagocytosing magnetite, multinucleated cells and lymphocytes were observed in the lungs of the treated rats. In addition, granulomas were observed in the lungs of the treated rats. Infiltration of multinucleated cells and lymphocytes which supposed to be macrophages, generally indicates chronic change. From the present result, it was not clear whether the macrophages died because of frustrated phagocytosis.

In an inhalation (nose-only) toxicity test using pigment-sized  $\text{Fe}_3\text{O}_4$  for Wistar rats, subchronic responses have been reported. Pulmonary inflammation was evidenced by bronchoalveolar lavage (BAL) fluid analysis, histopathology, particle deposition and increased lung and lung-asso-





**Fig. 5.** Representative histologies of the parathyroid lymph node (hematoxylin and eosin; inset, Berlin blue) from Fischer 344 rat treated with a single intratracheal spray instillation dose of 15.0 mg/kg body weight of magnetite on day 14. Infiltration of macrophages phagocytosing magnetite and deposits of magnetite particles are evident in rats given the middle dose.

ciated lymph node weights at the inhalation doses of 16.6 and 52.1 mg/m<sup>3</sup><sup>20</sup>. Another report described data from an inhalation study in which male and female Han Wistar rats were exposed to a photocopying toner with an average particle size of 5.1 μm that contained 45–50% magnetite by inhalation for 6 hours/day, 5 days/week, for a total of 13 or 104 weeks<sup>14</sup>. The microscopic findings indicated a mild inflammatory response and infiltration of black-pigmented macrophages in the lungs and the tracheobronchial and mediastinal lymph nodes after 104 weeks of exposure at an inhalation dose of 16 mg/m<sup>3</sup>. It is thus suggested that a longer time is required to develop apparent adverse effects, while the responses are generally less severe in the case of administration by inhalation than in the case of administration by instillation. Osier *et al.* compared the response of rats exposed by intratracheal inhalation to titanium dioxide particles with that of rats exposed to similar doses by intratracheal instillation<sup>21</sup>. Animals receiving particles through inhalation showed a decreased pulmonary response, measured

by bronchoalveolar lavage parameters, in both severity and persistence when compared with those receiving particles through instillation. These results demonstrate a difference in pulmonary response to an inhaled vs an instilled dose, which may be due to differences in dose rate, particle distribution or altered clearance between the two methods. Several experiments have been performed in rats using quartz as a typical lung toxic particle in order to establish an appropriate bioassay for detection of lung damage after particle instillation<sup>22</sup>. The results of those experiments indicated that an intratracheal instillation bioassay system for detection of lung toxicity appeared to be suitable for rapid hazard identification. Even though inhalation is more similar to the exposure route of magnetite in humans than intratracheal instillation, studies using the latter route are still important.

Generally, the acute pulmonary response to foreign bodies is characterized by the inflammation to remove such substances and the resultant cellular debris. If this process is successfully accomplished, complete resolution can occur. On the other hand, if the injury caused by foreign bodies and the subsequent inflammation are severe and sustained due to the persistence of the substances, irreversible pulmonary damage may occur<sup>23</sup>. It is well known that, if the lung is not overloaded with dust, dust-laden macrophages on the alveolar surface migrate upward and are carried by the mucociliary “escalator” system up to the trachea to be cleared into the esophagus. However, when dust enters into the interstitial or subepithelial space of the lung, it becomes very difficult to clear from the lung<sup>24</sup>. Ultrafine particles that penetrate into the lung interstitium make contact with interstitial macrophages and other sensitive cell populations, which is likely to have powerful inflammatory effects that underlie the development of subsequent disease<sup>25</sup>. In the present study, magnetite-laden macrophages were seen not only in the alveolar space but also in the alveolar septa or interstitial space, even in the low-dose group, which indicates the possibility of severe injury resulting from intratracheal administration of magnetite.

In conclusion, these results show that intratracheally administered magnetite nanoparticles cause foreign body inflammatory and granulating lesions in the lung. Magnetite particles are accumulated mainly in the lung and partly translocated to the regional lymph nodes. These pulmonary responses occur in a dose-dependent manner in association with the increase in lung weight.

**Acknowledgments:** We would like to express our gratitude to Dr. Yukari Totsuka (Cancer Prevention Basic Research Project, National Cancer Center Research Institute, Tokyo, Japan) for arrangement of the supply of magnetite and helpful suggestions. We also thank Mr. Toshiyuki Hakata and the staff of Toda Kogyo Corp. (Otake, Hiroshima, Japan) for their generous supply of magnetite and its technical information. This work was supported in part by a Health and Labour Sciences Research Grant (awarded to Dai Nakae) from the Ministry of Health, Labour and Welfare (MHLW) of Japan.

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