DOSSIER ON TITANIUM DIOXIDE
- GENERAL ANNEXES -
ANNEX 20

Series on the Safety of Manufactured Nanomaterials
No. 54

This document is only available in PDF format.
Final report

Intratracheal administration study of titanium oxide nanoparticles into rats

Study No. 1028

April 13, 2011

Correspondence:
Kohei Mizuno
National Institute of Advanced Industrial Science and Technology
Central 3, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8563, Japan
Fax: +81-29-861-4070.  E-mail address: k-mizuno@aist.go.jp
Table of contents

Table of contents ............................................................................................................. 1

Information on the study ................................................................................................. 3

Preparation of the final report .......................................................................................... 3

1 Overview ...................................................................................................................... 4

2 Introduction .................................................................................................................. 5

3 Material and methods ................................................................................................... 5

3.1 Test substance ........................................................................................................ 5

3.2 Positive control substance ..................................................................................... 6

3.3 Laboratory animals ................................................................................................. 6

3.4 Rationale for the experimental system selected for this study ................................ 6

3.5 Management of laboratory animals ....................................................................... 6

3.5.1 Rearing conditions ........................................................................................... 6

3.5.2 Animal cage and bedding ................................................................................. 7

3.5.3 Feed and method of feeding ............................................................................ 7

3.5.4 Drinking water and method of providing water to animals ............................ 7

3.5.5 Identification of individual animals ................................................................... 8

4 Test methods ................................................................................................................ 8

4.1 Treatment period and method of administration ................................................... 8

4.2 Isoflurane anesthesia ............................................................................................. 9

4.3 Dose levels and treatment groups ......................................................................... 9

4.5 Rationale for selection of the route and method of administration ..................... 10

4.6 Observation and measurements ............................................................................ 10

4.6.1 General status ................................................................................................ 10

4.6.2 Body weight ..................................................................................................... 10

4.6.3 Serum samples ................................................................................................. 10

4.6.4 Tissue samples ................................................................................................. 11

4.6.5 Resection of the lung and preparation of BALF ............................................. 11

4.6.6 BALF analysis ................................................................................................. 11

4.6.7 Organ weight ................................................................................................. 11

4.6.8 Cryopreservation of tissues ............................................................................. 12
4.6.9 Histopathological examination .................................................................................................................................. 12
4.6.10 Delivery method for specimens .................................................................................................................................. 12
4.7 Statistical analyses ............................................................................................................................................................. 12
4.8 Preservation of study-related materials .................................................................................................................................. 12
5 Results .................................................................................................................................................................................. 12
5.1 Survival rate and general status .............................................................................................................................................. 12
5.2 Body weight .......................................................................................................................................................................... 13
5.3 BALF analysis ......................................................................................................................................................................... 13
5.3.1 Cell fractions ..................................................................................................................................................................... 13
5.3.2 Biochemical tests .................................................................................................................................................................. 14
5.4 Pathological examinations ....................................................................................................................................................... 15
5.4.1 Macroscopic pathological examination .................................................................................................................................. 15
5.4.2 Lung weight and lung-to-body weight ratio .......................................................................................................................... 15
5.4.3 Histopathological examinations ........................................................................................................................................... 15
5.4.3.1 Lung ............................................................................................................................................................................... 15
5.4.3.2 Other tissues/organs ....................................................................................................................................................... 17
6 Summary of results and discussion ........................................................................................................................................... 18
7
Information on the study

Title : Intratracheal administration study of titanium oxide nanoparticles into rats

Study No. : 1028

Objectives : A single dose of titanium oxide nanoparticles was intratracheally administered into rats, and the toxic effects of the substance, mainly on the lung, was investigated for 3 months after administration.

Classification of the study : This study was conducted as a non-GLP study. Animal husbandry, various procedures, data handling, etc., were carried out in accordance with in-house SOPs established by DIMS Institute of Medical Science, Inc.

Animal welfare : The study was carried out in compliance with the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No. 88 of the Ministry of Environment dated April 28, 2006), as well as with the DIMS Institute of Medical Science policies for laboratory animals (dated September 1, 2010).

Testing facility : DIMS Institute of Medical Science, Inc.
Postal code: 491-0113
64 Nishi-asai-gouura, Asai, Ichinomiya, Aich, Japan

Study initiation date : November 5, 2011

Study dates
Receipt date of lab. animals : November 11, 2010
Date of treatment allocation : November 18, 2010
Date first animal was dosed (Experimental start date) : November 19, 2010
Date of autopsy (Day 3) : November 22, 2010
Date of autopsy (Week 1) : November 26, 2010
Date of autopsy (Week 4) : December 17, 2010
Date of autopsy (Week 13) : February 18, 2011
Experimental end date : April 5, 2011

Study completion date : April XX, 2011
1 Overview

A single high or low dose of titanium oxide nanoparticles (substances A-D and AEROXIDE® P25 as a positive control) was intratracheally administered to Crl:CD (SD) rats under isoflurane anesthesia. After test substance administration, 5 rats from each treatment group were killed at Day 3, Week 1, Week 4, and Week 13. Bronchoalveolar lavage fluids (BALF) were analyzed to classify the cells contained in the fluid, and subjected to biochemical tests. Moreover, major organs/tissues, including the lung, were subjected to histopathological examination.

Analysis of the collected data showed that none of the four titanium oxide nanoparticles affected the general status or body weight of the rats.

From Day 3, changes in WBC counts and cell fractions of the BALF, suggestive of an inflammatory response, were observed in all treatment groups. Notably, the values obtained from rats treated with a high dose of substance C were comparable to values obtained from rats treated with the positive control substance. However, 13 weeks later, these values returned to a level almost equivalent to that of the vehicle control group. In the substance A and substance D groups, the values of the inflammatory parameters were less marked than the values observed in the positive control group, whereas in the substance B group, only mild changes were observed at Day 3 and Week 1 in the rats administered a high dose. These mild changes returned to a level comparable to the values observed in the vehicle control group at Week 4. Moreover, in the rats administered a high dose of substance A, B, or C, high levels of micro-proteins were detected at Day 3, as compared with the vehicle control group. The level was particularly high in the substance C group, consistent with the results of the BALF cell fraction analysis.

In macroscopic pathological examinations, one to many white spots/areas were found in the lungs of rats treated with any of the test substances. However, no differences in the areas or numbers of these spots were determined among the test substances, and no relationship was found between the white spots/areas and other parameters.

An increase in the absolute or relative weight of the lung was observed at Day 3 and Week 1 in rats treated with a high dose of substance A or C. However, these values returned to a level comparable to the values observed in the vehicle control group at 4 Weeks.

In histopathological examination, inflammatory changes were observed at Day 3 in rats from all substance groups. In both the low- and high-dose groups, a clear relationship was noted between the dose and the results of histopathological examination. The inflammatory response was not considered strong because no granulation or fibril formation was observed in any rat, but a relatively strong response was observed in the rats treated with a high dose of substance C, as compared with the other substance groups. This is consistent with the data of other parameters, including BALF analysis and biochemical tests. Moreover, although the severity of inflammation was comparable to that of the positive control group, it had a tendency to resolve faster than the inflammation observed in the positive control group. The inflammatory changes observed in rats treated with a high dose of substance A or D were similar. Comparable inflammatory changes were observed in rats treated with a high dose of substance B and those
treated with the vehicle. The severity of the inflammatory changes observed in rats treated with a low dose of any of the test substances was nearly comparable to that of the vehicle control group. However, alveolar macrophages were frequently observed in substance groups, other than substance A at Week 13.

In the positive control group, inflammatory changes started to appear from Day 3, and the severity gradually decreased over time thereafter. Moreover, no clear relationship was observed in the WBC count, cell fractions, and micro-proteins in BALF.

In conclusion, intratracheal administration of a high dose of substance C in rats caused an inflammatory response in the lung, with a severity of inflammation comparable to that of the positive control substance. The severity, however, gradually decreased over time. The inflammatory changes observed in rats treated with a high dose of substance A or D were milder than those observed in the rats treated with a high dose of substance C. The inflammatory changes observed in rats treated with a high dose of substance B were nearly comparable to those of the vehicle control group. In rats treated with a low dose of any of the test substances, the severity of the inflammation was comparable to that of the vehicle control group; however, alveolar macrophages were observed even at 13 weeks after test substance administration.

2 Introduction

A single high or low dose of titanium oxide nanoparticles (substance A, B, C, or D) was intratracheally administered to rats, and the toxic effects of the substance, primarily on the lung, were investigated for 3 months after administration.

3. Material and methods

3.1 Test substance

Four substances A-D were obtained from Tayca corp., Japan. Substances A and B were rutile-type nanoparticles of large and small particle diameter, respectively. Substances C and D were modifications of A; substance C was coated with fatty acid group to achieve hydrophobic surface (a typical cosmetic product). Substance D was functionalized with isobutyl moiety as a product for external additives of toner.

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Size</th>
<th>SA</th>
<th>Surface modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MT-150AW</td>
<td>15 nm</td>
<td>28.2</td>
<td>none (hydrophilic)</td>
</tr>
<tr>
<td>B</td>
<td>MP-1133</td>
<td>250 nm</td>
<td>2.4</td>
<td>none (hydrophilic)</td>
</tr>
<tr>
<td>C</td>
<td>MT-100TV</td>
<td>15 nm</td>
<td>12.9</td>
<td>fatty acid (hydrophobic)</td>
</tr>
<tr>
<td>D</td>
<td>JMT-150IB</td>
<td>15 nm</td>
<td>17.4</td>
<td>isobutyl moiety (hydrophobic)</td>
</tr>
</tbody>
</table>

* Information from Tayca corp.

** Specific surface area (m2/g) measured by BET method
3.2 Positive control substance

AEROXIDE® P25 (principal material for the OECD sponsorship program, lot no. 4168112198) was employed for a positive control.

3.3 Laboratory animals

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Rats</td>
</tr>
<tr>
<td>Strain</td>
<td>Crl:CD (SD) (SPF animal)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
</tr>
<tr>
<td>Age (weeks) at the time of delivery</td>
<td>7 weeks old</td>
</tr>
<tr>
<td>Age (weeks) at the start of experiment</td>
<td>8 weeks old (body weight at the start of experiment: 296–351 g)</td>
</tr>
<tr>
<td>The number of animals purchased (the number of animals used for the experiment)</td>
<td>227 animals (200 animals)</td>
</tr>
<tr>
<td>Supply source</td>
<td>Charles River Laboratories Japan Inc.</td>
</tr>
<tr>
<td>Address</td>
<td>735 Shimo-kamiduki, Hino-machi, Gamo-gun, Shiga, 529-1633, Japan</td>
</tr>
<tr>
<td>Quarantine/acclimatization period</td>
<td>8 days</td>
</tr>
<tr>
<td>Examinations during the quarantine/acclimatization period</td>
<td>General status (observation was made twice daily) Body weight measurements (on the day following the delivery, and at the time of treatment group assignment)</td>
</tr>
<tr>
<td>Treatment of the unused animals after treatment group assignment</td>
<td>Two animals were maintained in the same animal housing room for microbiological monitoring, the remaining 25 animals were removed from the experimental system.</td>
</tr>
<tr>
<td>The results of animal monitoring</td>
<td>No abnormality was found in the body weight or the general status over the monitoring period. Moreover, no abnormality was observed when the animals were killed at scheduled time points.</td>
</tr>
</tbody>
</table>

3.4 Rationale for the experimental system selected for this study

The experimental system was designed on the basis of Kobayashi et al. (Toxicology 264, 110-118, 2009) and private communication with the authors.

3.5 Management of laboratory animals

3.5.1 Rearing conditions

<table>
<thead>
<tr>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal housing room</td>
<td>Room No. 7 (The animals were kept in room No. 7 during the quarantine/acclimatization period)</td>
</tr>
</tbody>
</table>
Temperature : Actual values; 20.0–24.0°C (Control temperature; 22 ± 3°C)
Relative humidity : Actual values; 47%–66% (Control range; 55% ± 15%)
Lighting : 12 hours/day (7:00–19:00)
Frequency of ventilation : ≥10 times/hour
Rearing : 1–3 animals/cage (1–3 animals/cage during the quarantine/acclimatization period)
Frequency of changing the cage (bedding) : Twice/week
Frequency of changing the water bottle : Twice/week

### 3.5.2 Animal cage and bedding

<table>
<thead>
<tr>
<th>Cage</th>
<th>Plastic cage (W257 × L426 × H200 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cage cover</td>
<td>Stainless cover</td>
</tr>
<tr>
<td>Method of disinfection</td>
<td>Plastic cages were disinfected using a normal-pressure steam sterilizer. Cage covers were disinfected using a high-pressure steam sterilizer.</td>
</tr>
<tr>
<td>Bedding</td>
<td>Soft chips (Yugen Kaisha, Hara Shoten)</td>
</tr>
<tr>
<td>Method of disinfection</td>
<td>High-pressure steam sterilization</td>
</tr>
<tr>
<td>Environmental pollutants</td>
<td>The results of the analysis were obtained from Chubu Kagaku Shizai. We confirmed that the concentrations were below the upper limit of acceptance ranges established by the DIMS Institute of Medical Science, Inc. (9 Attachment 1)</td>
</tr>
</tbody>
</table>

### 3.5.3 Feed and method of feeding

<table>
<thead>
<tr>
<th>Feed</th>
<th>Solid feed MF (Oriental Yeast Co., Ltd.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot Numbers</td>
<td>101013, 101109, 100702, 100903</td>
</tr>
<tr>
<td>Method of feeding</td>
<td>The feed was placed in a stainless feeder inside the cage. Animals were allowed free access to food.</td>
</tr>
<tr>
<td>Method of disinfection for the feeder</td>
<td>High-pressure steam sterilization</td>
</tr>
<tr>
<td>Contaminants in the feed</td>
<td>The results of the analysis of each lot were obtained from Oriental Yeast Co., Ltd. We confirmed that the concentrations were below the upper limit of acceptance ranges established by the DIMS Institute of Medical Science, Inc. (9 Attachment 2)</td>
</tr>
</tbody>
</table>

### 3.5.4 Drinking water and method of providing water to animals

<table>
<thead>
<tr>
<th>Drinking water</th>
<th>Tap water taken from Ichinomiya-shi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method of providing water to animals</td>
<td>Water was supplied in a clear water feeding bottle. Animals were allowed free access to water.</td>
</tr>
</tbody>
</table>
Method of disinfection for the water feeder: Water feeding bottles and the tap were disinfected with soft oxidizing water.

Contaminants in water: Water was analyzed by the Environmental Science Laboratory. We confirmed (twice per year) that the quality of water met the acceptance criteria established by the DIMS Institute of Medical Science, Inc. (9 Attachment 3)

Environmental Science Laboratory
152 Wakaduru-cho, Kita-ku Nagoya-shi

3.5.5 Identification of individual animals

Quarantine/acclimatization period: Study number, tentative cage ID number, tentative animal ID number, and name of the person responsible for management of laboratory animals were written on the cage label. Individual animals were identified using the aforementioned information in combination with the in-cage animal ID number (written using an oil-based marker pen).

After treatment group assignment: Study number, sex, treatment group, cage ID number, name and dose level (dose groups were distinguished by different colors) of test substance, animal ID number, experimental start date, date of autopsy, and name of the study director were written on the cage label. Individual animals were identified using the aforementioned information in combination with the in-cage animal ID number (an ear punch and an oil-based marker pen).

4 Test methods

4.1 Treatment period and method of administration

For the intratracheal instillation, test solution was prepared by suspending the test substance in 30 mM phosphate buffer solution containing 2 mg/mL of Tween®80 (MP Biomedicals) in concentrations of 1 and 5 mg/mL for a higher and lower doses, respectively. The test solution was sonicated with ultrasonic bath (Elmasonic S30H, 37kHz, 280W) for 30 minutes to achieve homogeneous dispersion. Suspension of the positive substance control was also prepared with the same procedure in the concentration of 5 mg/mL. The pH of suspensions after the sonication were 6.0-6.2. Particle size distribution of aggregates/agglomerates of the test substance suspended in the test solution was measured with a dynamic light scattering instrument (Microtrac UPA-EX150) soon after the sonication and just before the instillation as summarized in the following figures.
Volume-based cumulative size distribution of aggregates/agglomerates of the test substances suspended in the test solution represented by 10th (D10), 25th (D25), 50th (D50), 75th (D75), and 90th (D90) percentiles. Soon after the preparation (A) and just before the instillation (B). A, B, C, D, and P on the horizontal axis denotes the test substances A, B, C, D, and positive control, respectively.

For the instillation, a disposable syringe and a DIMS intratracheal administration tube (R-1) were connected using a Luer bulb. Under isoflurane (Escain®, Mylan Inc.) anesthesia, 1.0 mL/kg of the test solution or vehicle (30 mM phosphate buffer solution containing 2 mg/mL of Tween®80) without substances (i.e. negative control) was intratracheally administered to rats. Test solution was administered only once at the start of the experiment.

4.2 Isoflurane anesthesia

Using NARCOBIT-E (KN1070, NATSUME SEISAKUSHO CO. LTD.), a simple inhalation anesthesia system for small laboratory animals, rats were anesthetized in the induction box.

4.3 Dose levels and treatment groups

Two hundred and twenty-seven rats were purchased, and in accordance with their body weights by computer-based randomized block design, 200 out of 227 were selected and assigned to 10 treatment groups (20 rats per group) on the experimental start date as shown in the following Table. Using the F-test (Bartlett method, significance level = 5%) and Tukey’s test (two-tailed test, significance level = 5%), it was confirmed that the baseline (at the time of randomization) body weights were comparable, with no statistical differences among the treatment groups. The baseline body weights of the rats were in the range of 296–351 g, and were contained within ±20% of the mean body weight. Unused animals were removed from the experimental system after randomization.
<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Test substance</th>
<th>Doses</th>
<th>Number of animals</th>
<th>Animal ID number a</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Sample A</td>
<td>Low dose</td>
<td>20</td>
<td>041–045 046–050 051–055 056–060</td>
</tr>
<tr>
<td>4</td>
<td>Sample A</td>
<td>High dose</td>
<td>20</td>
<td>061–065 066–070 071–075 076–080</td>
</tr>
<tr>
<td>5</td>
<td>Sample B</td>
<td>Low dose</td>
<td>20</td>
<td>081–085 086–090 091–095 096–100</td>
</tr>
<tr>
<td>7</td>
<td>Sample C</td>
<td>Low dose</td>
<td>20</td>
<td>121–125 126–130 131–135 136–140</td>
</tr>
<tr>
<td>8</td>
<td>Sample C</td>
<td>High dose</td>
<td>20</td>
<td>141–145 146–150 151–155 156–160</td>
</tr>
<tr>
<td>9</td>
<td>Sample D</td>
<td>Low dose</td>
<td>20</td>
<td>161–165 166–170 171–175 176–180</td>
</tr>
</tbody>
</table>

a: From the rat with the smallest ID number (in ascending order), 5 rats were killed at each of Day 3, Week 1, Week 4, and Week 13.

b. Vehicle substance was administered.

4.4 Rationale for selection of the route and method of administration

Intratracheal administration was selected as the route of administration to analyze the biokinetics of the test substances after exposure by inhalation. A simple intratracheal administration method was selected.

4.6 Observation and measurements

4.6.1 General status

After the start of the experiment, the general status, toxicity symptoms, and life/death status of each animal were evaluated and recorded twice daily (in the morning and in the evening).

4.6.2 Body weight

Using an electronic balance (LA4200, SARTORIUS K.K.), the body weight of each animal was measured at the start of the experiment, and thereafter at 1-week intervals. In addition, when rats were killed at scheduled time points, the body weight was measured for each animal after fasting overnight (approx.16 hours) (body weight on the day of autopsy).

4.6.3 Serum samples

Starting from the evening (approx. 16:00) on the day before the autopsy, feed was removed from the cage (bedding was changed), and animals were fasted overnight. The following day, the animals underwent laparotomy under isoflurane anesthesia. A 3-mL sample of blood drawn from the abdominal aorta was transferred to a Vacutainer® tube. The tube was allowed to stand for approximately 30 minutes to 1 hour at
room temperature, and then centrifuged (4°C, 2500 rpm, 10 minutes) to extract the serum. The serum sample was stored in a frozen state.

To reduce bias, blood was first collected from the animal with the smallest ID number in the group No. 1, and then from the animal with the smallest ID number in the group No. 2, and so on. After the animal with the smallest ID number in the last group was killed, the procedure was repeated in the animal with the next smallest ID number in group No. 1.

4.6.4 Tissue samples

The lungs (right and left), liver, spleen, cerebrum (including the olfactory bulb), and kidneys (right and left) were collected from the animals bled to death (see Section 4.6.3) at the scheduled time points.

4.6.5 Resection of the lung and preparation of BALF

The trachea on the side closest to the left lobe of the lung was ligated using a cotton thread; a cannula was inserted into the trachea, and 3 mL of EDTA-2Na/PBS (at room temperature) was injected through the cannula into the right lung. The right lung was washed by injecting and drawing the fluid 3 times. After the BALF was collected, the right lung, including the trachea, was resected through a part of the trachea. The lung was dissected in RNA storage solution into the anterior lobe, intermediate lobe, posterior lobe, and accessory lobe. The posterior lobe was further diced to 1–2 mm in size in RNA storage solution, left to stand overnight at 4°C, and then stored at −20°C. The remaining lobes were placed in individual containers and immediately stored at −20°C.

4.6.6 BALF analysis

The following test was performed using BALF samples.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell fractions</td>
<td>Automated Hematology Analyzer XT-2000i (SYSMEX CORPORATION)</td>
</tr>
</tbody>
</table>

The remaining BALF samples were centrifuged (1000 rpm for 5 minutes), and the supernatant was subjected to the following tests.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Method</th>
<th>Equipment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate dehydrogenase (LDH)</td>
<td>JSCC-recommended method</td>
<td>Hitach 7070 automated analyzer</td>
</tr>
<tr>
<td>Micro-proteins (µTP)</td>
<td>Pyrogallol red method</td>
<td>Hitach 7070 automated analyzer</td>
</tr>
</tbody>
</table>

4.6.7 Organ weight

The weight of the left lung was measured using an electronic balance CP323S (Sartorius K.K.).
4.6.8 Histopathological examination

The left lung, liver, spleen, cerebrum (including the olfactory bulb), and kidney (left) were fixed with 10% neutral buffered formalin fixative, embedded in paraffin, sliced into sections, and stained with hematoxylin and eosin. The stained sections were then subjected to histopathological examination.

4.7 Statistical analyses

The control groups (Group Nos. 1 and 2) and the test substance groups (Group No. 1 vs. Group Nos. 3/4, Group No. 1 vs. Group Nos. 5/6, Group No. 1 vs. Group Nos. 7/8, and Group No. 1 vs. Group Nos. 9/10) were compared to determine whether differences between them were statistically significant. Results were interpreted using a significance level of 5% (P < 0.05) or 1% (P < 0.001). F-tests were performed using the Bartlett method at a significance level of 5% on the mean body weight, mean organ weight, and mean values for the BALF parameters. If the variance was homogeneous, a two-tailed test using the parametric Dunnett’s method was performed. If the variance was not homogeneous, a two-tailed test using the non-parametric Steel method was performed. F-tests were performed to compare body weight, organ weight, and the values of BALF parameters between Group Nos. 1 and 2. If homogeneity of variance was confirmed, a two-tailed Student’s t test was performed. If homogeneity of variance was not confirmed, a two-tailed Welch test was performed. To compare differences in the frequency of macroscopic pathological findings or histopathological changes, each substance group was compared against the control group using Fisher’s exact probability test (one-tailed). Comparisons of the severity of graded lesions was performed using the Wilcoxon test (two-tailed). No statistical analysis was performed on observations of the general status.

4.8 Preservation of study-related materials

The study protocol (original), study-related documents, raw data, wet specimens, and final study report will be preserved for 3 years after the final report has been submitted. These study-related materials will be managed by the DIM Institute of Medical Science, Inc., and will be stored at a GLP-compliant facility. After the 3-year preservation period, the sponsor and the CRO will discuss the matter to decide how the study-related materials will be disposed of or stored.

5 Results

5.1 Survival rate and general status

TABLE 1 presents the number of surviving animals, and TABLE 2 summarizes the observation of general status. APPENDIX A shows the observation of individual animals. No deaths occurred in any group throughout the duration of the study. Crepitus was noted in all animals following test substance administration, but it disappeared on the next day. Thereafter, no significant changes were observed.
5.2 Body weight

TABLE 3 presents mean body weights and standard deviations by group, and APPENDIX B shows the data of individual animals. A significant increase in body weight was observed at Week 12 and Week 13 in rats treated with a high dose of substance D (Group No. 10), as compared with the vehicle control group (Group No. 1). No statistically significant difference was observed in body weight between the vehicle control group (Group No. 1) and any of the other test substance groups.

5.3 BALF analysis

5.3.1 Cell fractions

TABLE 4 presents the measured values by group, and APPENDIX C shows the data of individual animals.

[Positive control group (Group No. 2)]

At Day 3, significant increases in the WBC count, neutrophil count ratio, and eosinophil count ratio, and a significant decrease in the monocyte count ratio were observed in the positive control group (Group No. 2) as compared with the vehicle control group (Group No. 1). At Week 1, significant increases in the WBC count, neutrophil count ratio, and lymphocyte count ratio, and a significant decrease in the monocyte count ratio were observed. At Weeks 4 and 13, no statistically significant differences were observed between the vehicle control group and the positive control group.

[Substance A (Group Nos. 3 and 4)]

At Day 3, significant increases in the WBC count, neutrophil count ratio, and eosinophil count ratio and a significant decrease in the monocyte count ratio were observed in the high-dose substance A group (Group No. 4) as compared with the vehicle control group (Group No. 1). At Weeks 1 and 13, the only parameter significantly increased in this group was the WBC count, but at Week 4, no significant difference was observed. No statistically significant difference was observed between the vehicle control group and the low-dose group (Group No. 3).

[Substance B (Group Nos. 5 and 6)]

At Day 3, significant increases in the neutrophil count ratio and eosinophil count ratio, and a significant decrease in the monocyte count ratio were observed in the high-dose substance B group (Group No. 6) as compared with the vehicle control group (Group No. 1). At Week 1, a significant increase was observed only in the neutrophil count ratio, and at Weeks 4 and 13, no statistically significant difference was observed between the vehicle control group and the high-dose substance B group. No statistically significant difference was observed between the vehicle control group and the low-dose group (Group No. 5).

[Substance C (Group Nos. 7 and 8)]

At Day 3, significant increases in the WBC count, neutrophil count ratio, and eosinophil count ratio, and a significant decrease in the monocyte count ratio were observed in the high-dose substance C group (Group No. 8), as compared with the vehicle control group (Group No. 1). At Week 1, a significant increase
in the WBC count and neutrophil count ratio and a significant decrease in the monocyte count ratio were observed. At Week 4, the WBC count was significantly increased in the high-dose group, but at Week 13, no significant difference was observed between the vehicle control and high-dose groups. No statistically significant difference was observed between the vehicle control group and the low-dose group (Group No. 7).

[Substance D (Group Nos. 9 and 10)]

At Day 3, significant increases in the WBC count, neutrophil count ratio, and eosinophil count ratio, and a significant decrease in the monocyte count ratio were observed in the high-dose substance D group (Group No. 10), as compared with the vehicle control group (Group No. 1). At Week 1, significant increases in the WBC count and neutrophil count ratio were observed. At Week 4, significant increases in the neutrophil count ratio and eosinophil count ratio, and a significant decrease in the monocyte count ratio were observed. At Week 13, a significant increase in the WBC count was observed. No statistically significant difference was observed between the vehicle control group and the low-dose group (Group No. 9).

5.3.2 Biochemical tests

TABLE 5 presents measured values by group, and APPENDIX D shows the data of individual animals.

[Positive control group (Group No. 2)]

At Day 3, micro-proteins (µTP) were significantly increased in the positive control group as compared with the vehicle control group (Group No. 1), but at Weeks 1, 4, and 13, no significant difference was observed. Furthermore, no statistically significant difference in lactate dehydrogenase (LDH) was observed.

[Substance A (Group Nos. 3 and 4)]

At Day 3, a significant increase in µTP was observed in the high-dose group (Group No. 4), as compared with the vehicle control group (Group No. 1), but was not found at Weeks 1, 4, and 13. Furthermore, no statistically significant difference in LDH was observed between the vehicle control group and either of the substance A groups.

[Substance B (Group Nos. 5 and 6)]

After Day 3, no statistically significant difference was observed in µTP or LDH between the vehicle control group (Group No. 1) and either of the substance B groups.

[Substance C (Group Nos. 7 and 8)]

At Day 3 and Week 4, a significant increase in µTP was observed in the high-dose group (Group No. 8), as compared with the vehicle control group (Group No. 1). However, no statistically significant difference was observed in LDH between the vehicle control group and either of the substance C groups.

[Substance D (Group Nos. 9 and 10)]

At Day 3, a significant increase in µTP was observed in the high-dose group (Group No. 10) as compared with the vehicle control group (Group No. 1), but at Weeks 1, 4, and 13, no significant difference
was observed. Moreover, no statistically significant difference in LDH was observed between the vehicle control group and either of the substance D groups.

5.4 Pathological examinations

5.4.1 Macroscopic pathological examination

TABLE 6 presents the macroscopic findings obtained from the pathological examination by group, and APPENDIX E shows the data of individual animals.

At Day 3 and Week 1, one to many white spots/areas were observed in the lungs of animals in the positive control group (Group No. 2), the high-dose substance A group (Group No. 4), the high- and low-dose substance B groups (Group Nos. 5 and 6), the high-dose substance C group (Group No. 8), and the high-dose substance D group (Group No. 10). The number/area of these spots was statistically significant as compared with the vehicle control group (Group No. 1). At Week 4, a significant change was observed in the positive control group and the high-dose substance A, B, C, and D groups. However, these gradually resolved, so that by Week 13, no statistically significant difference was observed.

5.4.2 Lung weight and lung-to-body weight ratio

TABLE 7 presents the lung weights (absolute weight) and the lung-to-body weight ratios (relative weight) by group, and APPENDIX F shows the data of individual animals.

Both the absolute and relative lung weights were significantly higher in the positive control group (Group No. 2) than in the vehicle control group (Group No. 1) at Week 1. Compared with the vehicle control group, the relative lung weight was significantly higher at Day 3, and both the absolute and relative lung weights were significantly higher at Week 1 in the high-dose substance A group (Group No. 4). Both the absolute and relative lung weights were significantly higher in the high-dose substance C group (Group No. 8) than in the vehicle control group at Day 3. No statistically significant difference was observed between the vehicle control group and either of the other test substance groups.

5.4.3 Histopathological examinations

TABLE 8 presents the histopathological findings by group, TABLE 9 presents histopathological findings by test substance, and APPENDIX G shows the data of individual animals. Photographs of typical histopathological findings are presented in FIGURES 1–8.

5.4.3.1 Lung

[Vehicle control group (Group No. 1)]

In the vehicle control group (Group No. 1), scarce appearance of macrophages in the alveolar space, localized scarce or moderately scarce appearance of forming macrophage foam cells, scarce to moderately scarce infiltration of perivascular mononuclear and inflammatory cells, and mild thickening of the alveolar and bronchiolar epithelia were observed at Day 3. At Week 1, scarce appearance of macrophages in the alveolar space, infiltration of perivascular mononuclear and inflammatory cells, bleeding, and thickening of
the alveolar epithelium were observed. At Week 4, similar observations were made, but the appearance of macrophages in the alveolar space and thickening of alveolar epithelium were observed less frequently than in Week 1. At Week 13, localized appearance of forming macrophage foam cells and infiltration of perivascular mononuclear cells were observed.

[Positive control group (Group No. 2)]

In the positive control group (Group No. 2), scarce to moderately scarce appearance of macrophages in the alveolar space, localized scarce appearance of forming macrophage foam cells, scarce to moderately scarce infiltration of perivascular mononuclear and inflammatory cells, mild granulation, scarce appearance of intra-alveolar foreign bodies, mild bleeding, scarce hemoglobin crystals, and mild thickening of the alveolar and bronchiolar epithelia were observed. Among these findings, the appearance of macrophages in the alveolar space was significant from Day 3 to Week 13, as compared with the vehicle control group (Group No. 1). Moreover, perivascular inflammatory cell infiltration, appearance of intra-alveolar foreign bodies, and thickening of the bronchiolar epithelium were also significant at Day 3 and Week 1. Finally, thickening of the alveolar epithelium was significant at Day 3 and Week 13. At Week 13, however, the localized appearance of forming macrophage foam cells was significantly less frequent.

[Substance A (Group Nos. 3 and 4)]

In the low- and high-dose substance A groups (Group Nos. 3 and 4), scarce to moderately scarce appearance of macrophages in the alveolar space, localized scarce appearance of forming macrophage foam cells, scarce infiltration of perivascular mononuclear cells or scarce to moderately scarce infiltration of perivascular inflammatory cells, scarce appearance of intra-alveolar foreign bodies, mild bleeding, and mild thickening of the alveolar and bronchiolar epithelia were observed. Among these findings, the appearance of macrophages in the alveolar space was significant at Week 13 in the high-dose group as compared with the vehicle control group (Group No. 1). Moreover, thickening of the alveolar epithelium was also significant at Day 3 and Week 13. At Day 3, perivascular inflammatory cell infiltration, appearance of intra-alveolar foreign bodies, and thickening of the bronchiolar epithelium were significant as compared with the vehicle control group. On the other hand, perivascular mononuclear cell infiltration was significantly less frequent at Day 3. No statistically significant difference was observed between the vehicle control group and the low-dose group.

[Substance B (Group No. 5 and 6)]

In the low- and high-dose substance B groups (Group Nos. 5 and 6), scarce to moderately scarce appearance of macrophages in the alveolar space, localized scarce appearance of forming macrophage foam cells, scarce to moderately scarce infiltration of perivascular mononuclear cells, scarce infiltration of perivascular inflammatory cells, scarce hemoglobin crystals, and thickening of the alveolar epithelium were observed. Among these findings, the appearance of macrophages in the alveolar space was significant at Week 13 in the high- and low-dose groups. Moreover, thickening of the alveolar epithelium was significant at Week 13 in the high-dose group as compared with the vehicle control group (Group No. 1). In the low-dose group, perivascular mononuclear cell infiltration was significantly less frequent at Day 3.
[Substance C (Group Nos. 7 and 8)]

In the low- and high-dose substance C groups (Group Nos. 7 and 8), scarce to moderately scarce appearance of macrophages in the alveolar space, localized scarce appearance of forming macrophage foam cells, scarce infiltration of perivascular mononuclear cells, scarce to moderately scarce infiltration of perivascular inflammatory cells, mild granulation, scarce appearance of intra-alveolar foreign bodies, mild bleeding, mild to moderate thickening of the alveolar epithelium, and mild thickening of the bronchiolar epithelium were observed. At Day 3 and Week 13, the appearance of macrophages in the alveolar space was significantly higher in the high-dose group as compared with the vehicle control group (Group No. 1). At Week 13, the appearance of macrophages in the alveolar space was significant in both the high- and low-dose groups as compared with the vehicle control group. At Day 3, infiltration of perivascular inflammatory cells, appearance of intra-alveolar foreign bodies, thickening of the alveolar and bronchiolar epithelia were significantly increased in the high-dose group. At Week 1, the bronchiolar epithelium was significantly thicker in the high-dose group. At Week 13, the alveolar epithelium was significantly thicker in the high-dose group. At Day 3, perivascular mononuclear cell infiltration was significantly less frequent in the low-dose group, and at Week 13, the localized appearance of forming macrophage foam cells was significantly less frequent in the high-dose group.

[Substance D (Group Nos. 9 and 10)]

In the low- and high-dose substance D groups (Group Nos. 9 and 10), scarce to moderately scarce appearance of macrophages in the alveolar space, localized scarce appearance of forming macrophage foam cells, scarce infiltration of perivascular mononuclear or inflammatory cells, scarce appearance of intra-alveolar foreign bodies, mild bleeding, and mild thickening of the alveolar bronchiolar epithelia were observed. The appearance of macrophages in the alveolar space was significantly increased from Day 3 to Week 4 in the high-dose group as compared with the vehicle control group (Group No. 1). At Week 13, the appearance of macrophages in the alveolar space was significantly increased in both the low- and high-dose groups as compared with the vehicle control group. At Day 3, perivascular inflammatory cell infiltration, appearance of intra-alveolar foreign bodies, and thickening of the bronchiolar epithelium were significantly increased in the high-dose group. At Day 3 and Week 13, the alveolar epithelium was significantly thicker in the high-dose group. At Day 3, perivascular mononuclear cell infiltration was significantly less frequent in the high-dose group, and at Week 13, the localized appearance of forming macrophage foam cells was significantly less frequent in the high- and low-dose groups.

5.4.3.2 Other tissues/organs

Localized splenic hyperplasia was observed in 1 animal in each of the high-dose substance B group (Group No. 6) at Week 1, the positive control group (Group No. 2) at Week 4, and the high-dose substance A group (Group No. 4) at Week 4.

Mild cyst formation was observed in the kidney of 1 animal in each of the high-dose substance C group (Group No. 8) at Week 1, and the low-dose substance C group (Group No. 7) at Week 4.
No histopathological changes were observed in the liver or the brain.
Since only sporadic changes were observed in the spleen and the kidney, these changes were considered unrelated to the test substance.

6 Summary of results and discussion

A single high or low dose of 1 of 4 titanium oxide species (substance A, substance B, substance C, or substance D) was intratracheally administered to Crl:CD (SD) rats. After administration of the test substances, 5 rats from each treatment group were killed at Day 3, Week 1, Week 4, and Week 13. Bronchoalveolar lavage fluids were analyzed to determine WBC counts, to classify the cells contained in the fluid, and to establish biochemical parameters. Moreover, major organs/tissues, including the lung, were subjected to histopathological examination.

No changes in the animal’s general status, other than crepitus (a normal outcome after intratracheal administration) were observed in any of the animals in any of the treatment groups.

A significant increase in body weight was observed in the high-dose substance D group at Week 12 and Week 13; however, these changes were not related to the dose level, and thus considered spontaneous changes.

From Day 3, a significant increase in the neutrophil count ratio and eosinophil count ratio, and a significant decrease in the monocyte count ratio, was observed in the analyses of WBC counts and cell fractions of the BALF in all treatment groups. In the substance C group, changes suggestive of an inflammatory response were more evident as compared with other substance groups. These changes were comparable to the changes found in the rats administered the positive control substance. However, 13 weeks later the WBC count and cell fraction values returned to a level nearly equivalent to that of the vehicle control group. Compared with the positive control group, the changes in the inflammatory parameters were relatively small in substance A and D groups. Moreover, resolution of the increased WBC count seemed slow, since a significant increase in the WBC count was observed at Week 13, and a notable increase in the WBC count was also observed at Week 4 were observed. However, this tendency was not observed in other parameters or histopathological findings. In the substance B groups, only mild changes were observed at Day 3 and Week 1 in the rats given a high dose, and these returned to levels comparable to those observed in the vehicle control group and Weeks 4 and 13. Furthermore, in rats given a high dose of substance A, B, or C, the results of biochemical tests showed a high value of micro-proteins at Day 3 as compared with the vehicle control group. The value was particularly high in the substance C group, which was consistent with the results of the BALF cell fraction analysis.

White spots/areas were frequently observed in the macroscopic pathological evaluation of rats administered high-dose substance A, C, or D, and high- or low-dose substance B from Day 3 to Week 4. Substance B was unique in being the only material provoking a change when administered at a low dose. However, in the BALF analysis and biochemical tests, rats administered substance B group had rather
smaller changes compared with the other substance groups; thus, no clear relationship was observed between the result of macroscopic pathological examination and the values of other parameters.

In rats treated with a high dose of substance A or C, an increase in the absolute or relative weight of the lung was observed at Day 3 and Week 1. Four weeks after test substance administration, these values returned to a level comparable to the values observed in the vehicle control group.

In histopathological examination, inflammatory changes were observed at Day 3 in all groups. In both low- and high-dose groups, a relationship was noted between the dose level and the result of histopathological examination. Although infiltration of perivascular inflammatory cells or thickening of the alveolar epithelium were observed, the inflammatory response was not considered to be strong because the incidence of granulation was low, and no fibril formation was observed in any of the substance groups. When the appearance of alveolar macrophage and infiltration of perivascular inflammatory cells were compared between the substance groups, a relatively strong response was observed in rats treated with a high dose of substance C, as compared with the other substance groups. This is consistent with the data of other parameters, including BALF analysis and biochemical tests. Moreover, although the severity of inflammation was comparable to that of the positive control group, it had a tendency to resolve faster than the inflammation observed in the positive control group. The inflammatory changes observed in rats treated with a high dose of substance A or D were comparable. The inflammatory changes observed in rats treated with a high dose of substance B were comparable (with no significant difference) to that of the vehicle control group at Day 3. No intra-alveolar foreign bodies were found in either of these 2 groups. Inflammatory changes were observed in rats treated with a low dose of any of the test substances; however, the severity was considered comparable to that of the vehicle control group. Alveolar macrophages were frequently observed in rats treated with substances other than substance A at Week 13.

In the positive control group, inflammatory changes started to appear from Day 3, but the severity of these reactions gradually decreased over time. Moreover, a clear relationship was observed in the WBC count, cell fractions, and micro-proteins in the BALF.

In conclusion, a high dose of intratracheally administered substance C caused the strongest inflammatory response in the lung with a severity of inflammation comparable to that of the positive control substance. The severity of the change gradually decreased over time. The inflammatory changes observed in rats treated with a high dose of substance A or D were milder than the inflammation observed in the rats treated with a high dose of substance C. The inflammatory changes observed in rats treated with a high or low dose of substance B was nearly comparable to that of the vehicle control group. In rats treated with a low dose of any of the test substances, the severity of the inflammation was comparable to that of the vehicle control group; nonetheless, alveolar macrophages were observed even at 13 weeks after test substance administration.