

APPENDIX G LUNG BURDEN AND LUNG BIOCHEMISTRY IN MICE

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METHODS

Lung Burden

Lung talc burden was measured to determine the relationship between the exposure concentration and the amount of talc deposited and retained within the pulmonary region of the respiratory tract. The method used for determination of talc in the lungs of rats and mice has been published (Hanson *et al.*, 1985). Lung burdens of talc were determined on the left lung of 4 male and 4 female mice from each exposure group sacrificed at 27, 52, and 79 weeks after the start of exposure. At 103 to 104 weeks, lung burdens were determined on the left lungs of two mice from the biochemistry group. The analysis was based on determination of acid insoluble magnesium in the lung. MRI reported that the value for the magnesium was 19.33% for batch 02, and 19.47% for batch 03. The values reported by MRI and the results of the analysis at LITRI were close to the theoretical value of magnesium for talc (19.22%). Since mice sacrificed at 27, 52, and 79 weeks had been exposed to only batch 02 of talc, 19.33% magnesium was used to calculate quantity of talc for these mice. Since batch 03 was used for the last 4 months of exposure, and lung burdens of mice after 103 to 104 weeks of exposure talc would be expected to contain substantial amounts of batch 03 talc, 19.47% magnesium was used to calculate quantity of talc in lungs for these mice.

All operations in conjunction with the tissue analysis for talc were done with talc-free gloves. Left lung lobes were weighed at necropsy and stored frozen (-20° C) until used. Lungs were homogenized using water and the proteins precipitated with 70% perchloric acid. The individual samples were filtered and washed with 5% trichloroacetic acid (TCA) to remove perchlorates. Washing continued until magnesium levels in the wash were within 10% of levels in the TCA solution (≤ 0.03 ppm magnesium). Filters and tissue residues were placed in 15 mL porcelain crucibles, dried slowly (200° C), and then ashed at 600° C for 1 hour. Ashed samples were transferred to Teflon beakers using 2 mL HCl and evaporated to dryness. Samples were digested in hydrofluoric acid (HF), and the HF evaporated. Additional HF was added and reevaporated. Sulfuric acid was added to remove trace HF, and samples were diluted with distilled water and analyzed for magnesium by atomic absorbance (Perkin Elmer, Model 306, Atomic Absorption Spectrophotometer) with a magnesium hollow cathode lamp and an air acetylene flame (Hanson *et al.*, 1985).

Lung Biochemistry

In this study, bronchoalveolar lavage (BAL) fluid enzyme activity and cell numbers were measured as biochemical and cytological indicators of pulmonary injury from inhalation of talc. Four mice of each sex from each exposure group were sacrificed at 27, 52, and 79 weeks, and all remaining lung toxicology mice were sacrificed at 103 to 104 weeks. Numbers of animals at each sacrifice are shown below.

Mice were anesthetized with halothane and sacrificed by exsanguination from the abdominal aorta or renal artery. The heart and lung block were removed. Mice were given endobronchial saline lavage (3 to 4 mL total volume in four, 0.75 to 1.0 mL washes) and the BAL fluid centrifuged at $300 \times G$ to separate the cells from the supernatant fluid.

At all sacrifices, biochemical analyses were done on lavage fluid from single mice. At the 103 to 104 week terminal sacrifice where lung burden measurements were also performed on the left lung lobes, mouse lavage fluids were paired (from 2 mice) to obtain sufficient cells for the analyses and paired mouse lung tissue samples (from 2 mice) were analyzed to obtain sufficient lung tissue for collagen analyses.

Airway Fluid Enzymes and Cytology

In this study, BAL fluid was analyzed to determine degree of:

- 1) Cell injury as indicated by quantities of BAL fluid lactate dehydrogenase (LDH).
- 2) Chronic inflammatory response as indicated by presence of increased numbers of polymorphonuclear leukocytes (PMN) and pulmonary alveolar macrophages (AM) as well as increased BAL fluid protein and alkaline phosphatase activity.

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- 3) Lysosomal activation as indicated by quantities of BAL fluid β -glucuronidase and acid proteinase. Elevated quantities of these enzymes have been observed in BAL fluid from rodents exposed to particulates. These enzymes may be associated with the breakdown of necrotic tissues.
- 4) Response to oxidant injury as indicated by increased quantities of glutathione reductase and peroxidase activity.

The supernatant fluid was analyzed for the activities of β -glucuronidase, LDH, glucose-6-phosphate dehydrogenase, alkaline phosphatase, glutathione reductase, and glutathione peroxidase by spectrophotometric, kinetic, and enzymatic techniques. Acid proteinase was measured by release of radiolabeled globin from the trichloroacetic acid precipitable protein substrate, and total protein was analyzed colorimetrically (Henderson *et al.*, 1985). β -Glucuronidase was not performed at 27-week interim evaluation, but was performed at all other sacrifice times.

Numbers of total nucleated cells recovered in lavage fluid were determined on each sample using a cell counter (Coulter Electronics, Hialeah, FL) or a hemocytometer. Cyto centrifuge preparations of resuspended cells were made, stained with Wright's stain (Diff-Quik, Curtin Matheson Scientific, Denver, CO) and differential cell counts were determined. At the 27, 52, and 79 week interim sacrifices, analyses were done on individual mice.

Alveolar macrophages (AM) were recovered from BAL fluid of the same mice as described above. Cells (0.5×10^6) in Roswell Park Memorial Institute (RPMI) culture medium were pelleted by centrifugation and the supernatant RPMI removed. Cells were resuspended in 1 mL of a 1% suspension of IgG antibody-sensitized sheep red blood cells (SRBC) in RPMI 1640. The antibody sensitized SRBC were made as previously described (Harmsen and Jeska, 1980). The subagglutinating titer of heat-inactivated rabbit anti-SRBC serum was used to sensitize the SRBC. The AM and SRBC suspensions were incubated at 37° C for 1 hour in a humidified atmosphere of 5% CO₂ in air. The AM and SRBC were sedimented by centrifugation and the supernatant discarded. Unphagocytized SRBC were removed by lysing the RBC with water for 30 seconds. The lysing of unphagocytized SRBC was stopped by the addition of an equal volume of saline and cyto centrifuge preparations were made. The slides were stained with a rapid Wright's stain (Diff-Quik, American Scientific Products, McGaw Park, IL) and the number of AM phagocytizing 0, 1, 2, 3 to 4, and > 4 SRBC was determined by light microscopy. Three fields of 100 cells per preparation were counted. Viability of macrophages was not determined at the 27, 52, and 79 week sacrifices because the small number of cells recovered from these mice lungs precluded the measurement of cell viability. Viability determination of macrophages was made on macrophages obtained at the final sacrifice because sufficient numbers of cells were generally available at this time.

Lung Tissue Collagen and Proteinase

At 27-, 52-, and 79-week sacrifices, collagen content of lungs and lavage fluid was measured. At the 103 to 104 week sacrifice, additional collagen metabolism and protein synthesis measurements were made on survivors from each group. Proteinase activities were measured at all sacrifice times.

The supernatant BAL fluid was analyzed for hydroxyproline and acid proteinase. Lung tissue and bronchoalveolar lavage (BAL) fluid samples were hydrolyzed with 6N HCl at 110° C for approximately 18 hours to convert proteins to their individual amino acids. Collagen quantity was measured and multiplied by 7.46 to convert BAL or lung tissue hydroxyproline content to BAL or lung tissue collagen content, taking into account that collagen is approximately 13% hydroxyproline by weight (Neuman and Logan, 1950).

Additional collagen metabolism measurements were made on the mice sacrificed after 103 to 104 weeks of talc exposure to further define collagen metabolism. Approximately 2 to 3 hours prior to sacrifice, ¹⁴C-proline (0.1 μ Ci/g body weight) was injected intraperitoneally to estimate collagen and protein synthesis. Radioactive proline and hydroxyproline were quantitated in lung hydrolysate. Following this,

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the radioactive proline and hydroxyproline quantities were used to calculate the noncollagenous protein synthesis, and the collagen production.

Noncollagenous protein synthesis was indicated as total ^{14}C -proline incorporation into lung tissue minus the incorporation into lung tissue which was related to collagen synthesis. The radioactive proline in collagen was assumed to be equal to the radioactive hydroxyproline, thus, incorporation into collagen was calculated as twice the radioactive hydroxyproline. Collagen production (% of newly synthesized protein that was collagen) was calculated as the percent incorporation of proline into collagen constituted of the total incorporation of proline into all proteins, and adjusted for the 5.4-fold difference in the content of total amino acids (proline and hydroxyproline) between collagen and noncollagenous protein (Pickrell *et al.*, 1987).

At each sacrifice time, lung tissue proteinase activity was measured as the release of ^{14}C -leucine from prelabeled globin at pH 4.2 and 7.5 (Gregory and Pickrell, 1982; Harkema *et al.*, 1984; Pickrell *et al.*, 1987). Acid proteinase activity was inhibited by leupeptin to indicate either cathepsin B (inhibited) or cathepsin D (not inhibited)-like activity. Neutral proteinase activity was inhibited by 1, 10-phenanthroline to indicate either macrophage elastase (inhibited) or neutrophil elastase-cathepsin G (not inhibited)-like activity.

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Lung Burden and Lung Biochemistry of Mice

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TABLE G1
Number of Mice Evaluated for Lung Talc Burden and Lung Biochemistry

	Male			Female		
	0 mg/m ³	6 mg/m ³	18 mg/m ³	0 mg/m ³	6 mg/m ³	18 mg/m ³
Lung Burden						
6-Month Interim	- ^a	2	4	-	4	4
12-Month Interim	-	4	4	-	4	4
18-Month Interim	-	2	1	-	4	3
24-Month Interim	-	8	6	-	6	5
Lung Biochemistry						
6-Month Interim	4	4	4	4	4	4
12-Month Interim	4	4	4	4	4	4
18-Month Interim	4	4	4	4	4	4
24-Month Interim	9	8	6	7	6	5

^a Lung burden not measured in 0 mg/m³ mice

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TABLE G2
Lung Talc Burden (Normalized to Control Lung Weight) of Mice^a

	6 months	12 months	18 months	24 months
Male				
0 mg/m ³	- ^b	-	-	-
6 mg/m ³	0.415 ± 0.114	1.084 ± 0.130	0.426 ± 0.040	2.973 ± 0.762
18 mg/m ³	1.41 ± 0.29	9.00 ± 1.45	8.36	19.73 ± 4.03
Female				
0 mg/m ³	-	-	-	-
6 mg/m ³	0.524 ± 0.056	0.707 ± 0.170	1.387 ± 0.178	2.667 ± 0.720
18 mg/m ³	1.35 ± 0.24	6.17 ± 1.39	7.83 ± 1.36	20.05 ± 0.98

^a Units are presented as mg talc/g control lung.

^b Not examined

TABLE G3
Lung Talc Burden (Normalized to Exposure Concentration) of Mice^a

	Male		Female	
	6 mg/m ³	18 mg/m ³	6 mg/m ³	18 mg/m ³
6-Month Interim	0.069 ± 0.019	0.078 ± 0.016	0.087 ± 0.009	0.075 ± 0.013
12-Month Interim	0.181 ± 0.022	0.500 ± 0.081 [*]	0.118 ± 0.028	0.343 ± 0.077 [*]
18-Month Interim	0.071 ± 0.007	0.464 ^b	0.231 ± 0.030	0.435 ± 0.075
24-Month Interim	0.496 ± 0.127	1.096 ± 0.224 [*]	0.445 ± 0.120	1.114 ± 0.055 [*]

^{*} Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test

^a Units are presented as mg talc/g control lung/mg/m³

^b n=1; no statistic calculated

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TABLE G4
Bronchoalveolar Lavage Fluid Enzymes of Mice at the 6-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lactate dehydrogenase ^a	1,408 ± 658	1,317 ± 106	2,107 ± 336
Glutathione reductase	148.4 ± 33.8	123.3 ± 28.3	227.2 ± 65.6
Total protein ^b	3.57 ± 0.89	1.92 ± 0.70	6.24 ± 1.23
Female			
Lactate dehydrogenase	1,988 ± 157	2,351 ± 180	1,400 ± 197
Glutathione reductase	206.8 ± 14.7	166.0 ± 21.3	148.5 ± 29.4
Total protein	2.55 ± 0.53	4.43 ± 0.34	6.89 ± 4.29

^a Units are presented as mIU/g control lung.

^b Units are presented as mg/g controls lung.

TABLE G5
Bronchoalveolar Lavage Fluid Enzymes of Mice at the 12-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
β-Glucuronidase ^a	0.188 ± 0.114	0.486 ± 0.346	12.787 ± 3.604*
Lactate dehydrogenase	1,107.6 ± 545	540.2 ± 59.0	1,487.1 ± 456
Glutathione reductase	89.50 ± 11.65	91.67 ± 6.60	302.40 ± 65.15*
Total protein ^b	2.21 ± 0.74	1.56 ± 0.33	6.19 ± 2.63
Female			
β-Glucuronidase	0.073 ± 0.073	0.413 ± 0.251	9.786 ± 2.271**
Lactate dehydrogenase	1,209.7 ± 305	447.5 ± 76.1	1,805.3 ± 285
Glutathione reductase	113.57 ± 19.78	97.93 ± 14.93	198.65 ± 23.44
Total protein	3.54 ± 1.27	3.61 ± 1.38	4.82 ± 2.88

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test

** P≤0.01

^a Units are presented as mIU/g control lung.

^b Units are presented as mg/g control lung.

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TABLE G6
Bronchoalveolar Lavage Fluid Enzymes of Mice at the 18-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
B-Glucuronidase ^a	0.000 ± 0.000	1.344 ± 1.267	9.937 ± 4.196**
Lactate dehydrogenase	434.0 ± 45.7	642.4 ± 119	1,039.9 ± 168**
Glutathione reductase	63.93 ± 14.16	106.38 ± 12.15	217.18 ± 45.29*
Total protein ^b	3.43 ± 0.62	6.23 ± 0.97*	9.45 ± 1.95**
Female			
B-Glucuronidase	4.243 ± 4.203	0.334 ± 0.334	19.064 ± 9.200
Lactate dehydrogenase	501.4 ± 46.9	404.2 ± 97.6	1,217.6 ± 255*
Glutathione reductase	73.19 ± 14.94	71.27 ± 12.11	240.55 ± 44.06*
Total protein	2.96 ± 0.40	3.41 ± 0.92	9.59 ± 1.23*

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test

** P≤0.01

^a Units are presented as mIU/g control lung.

^b Units are presented as mg/g control lung.

TABLE G7
Bronchoalveolar Lavage Fluid Enzymes of Mice at the 24-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
B-Glucuronidase ^a	0.000 ± 0.000	1.811 ± 0.878**	16.571 ± 3.932**
Lactate dehydrogenase	1,769 ± 259	1,439 ± 295	2,965 ± 131*
Glutathione reductase	73.66 ± 9.75	87.55 ± 25.16	229.53 ± 58.46*
Total protein ^b	1.69 ± 0.20	2.34 ± 0.22	4.68 ± 0.70**
Female			
B-Glucuronidase	0.000 ± 0.000	2.624 ± 1.176**	13.778 ± 2.640**
Lactate dehydrogenase	1,082 ± 155	1,596 ± 197*	2,026 ± 279**
Glutathione reductase	68.66 ± 7.42	73.37 ± 13.91	163.46 ± 33.43*
Total protein	1.111 ± 0.310	0.872 ± 0.261	2.228 ± 0.501

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test

** P≤0.01

^a Units are presented as mIU/g control lung.

^b Units are presented as mg/g control lung.

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TABLE G8
Bronchoalveolar Lavage Fluid Cell Populations of Mice at the 6-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Polymorphonucleated cells	0.250 ± 0.250	3.250 ± 1.250	12.000 ± 3.764**
Lymphocytes	0.750 ± 0.750	0.750 ± 0.479	0.000 ± 0.000
Macrophages	92.50 ± 3.23	95.75 ± 1.44	84.75 ± 2.95
Epithelial cells	6.500 ± 3.775	0.250 ± 0.250	3.250 ± 1.250
Female			
Polymorphonuclear cells	0.000 ± 0.000	1.250 ± 0.629*	1.750 ± 0.854**
Lymphocytes	0.000 ± 0.000	1.000 ± 1.000	0.000 ± 0.000
Macrophages	95.00 ± 2.16	94.75 ± 1.44	96.00 ± 1.22
Epithelial cells	5.00 ± 2.16	3.00 ± 1.73	2.25 ± 1.31

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test

** $P \leq 0.01$

^a Units are presented as percent of total cells.

TABLE G9
Bronchoalveolar Lavage Fluid Cell Populations of Mice at the 12-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Polymorphonuclear cells	26.75 ± 15.12	7.50 ± 5.85	15.00 ± 14.01
Lymphocytes	0.750 ± 0.250	2.250 ± 1.436	0.333 ± 0.333
Macrophages	70.50 ± 14.56	83.25 ± 6.91	73.33 ± 12.14
Epithelial cells	2.00 ± 1.41	7.00 ± 2.12	11.33 ± 7.36
Female			
Polymorphonuclear cells	1.33 ± 1.33	34.50 ± 10.27*	2.25 ± 0.85
Lymphocytes	1.000 ± 0.577	3.500 ± 1.500	0.000 ± 0.000
Macrophages	92.67 ± 0.33	58.25 ± 11.65	91.00 ± 2.04
Epithelial cells	5.00 ± 1.53	3.75 ± 1.75	6.75 ± 2.84

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test

^a Units are presented as percent of total cells.

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TABLE G10
Bronchoalveolar Lavage Fluid Cell Populations of Mice at the 18-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Polymorphonuclear cells	0.250 ± 0.250	8.750 ± 4.404	19.000 ± 6.258*
Lymphocytes	0.000 ± 0.000	0.500 ± 0.500	1.000 ± 0.577
Macrophages	89.00 ± 1.22	82.75 ± 5.81	75.75 ± 4.73
Epithelial cells	10.75 ± 1.44	8.00 ± 4.74	4.25 ± 2.39
Female			
Polymorphonuclear cells	0.250 ± 0.250	1.000 ± 0.577	16.000 ± 3.606*
Lymphocytes	0.000 ± 0.000	0.000 ± 0.000	1.333 ± 0.882*
Macrophages	84.50 ± 5.52	92.67 ± 0.88	79.00 ± 3.06
Epithelial cells	15.25 ± 5.54	6.33 ± 0.88	3.67 ± 2.33

- * Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
- ^a Units are presented as percent of total cells.

TABLE G11
Bronchoalveolar Lavage Fluid Cell Populations of Mice at the 24-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Polymorphonuclear cells	0.200 ± 0.200	13.000 ± 2.345*	16.500 ± 1.803**
Lymphocytes	0.000 ± 0.000	0.375 ± 0.239	0.500 ± 0.289
Macrophages	89.10 ± 2.50	78.25 ± 1.61*	80.33 ± 0.60*
Epithelial cells	10.70 ± 2.61	8.38 ± 1.01	2.67 ± 1.59
Female			
Polymorphonuclear cells	0.000 ± 0.000	7.500 ± 1.607*	20.667 ± 5.918**
Lymphocytes	0.000 ± 0.000	0.500 ± 0.500	0.500 ± 0.500
Macrophages	86.38 ± 3.57	87.00 ± 2.08	73.67 ± 8.46
Epithelial cells	13.63 ± 3.57	5.00 ± 1.00	5.17 ± 3.03

- * Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
- ** P≤0.01
- ^a Units are presented as percent of total cells.

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TABLE G12
Phagocytic Activity of Macrophages in Bronchoalveolar Fluid of Mice
at the 12-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Phagocytic Activity	85.50 ± 1.44	56.10 ± 2.23*	16.77 ± 2.98**
Female			
Phagocytic Activity	77.07 ± 9.88	52.10 ± 9.22	17.37 ± 6.17**

* Significantly different (P≤0.05) from the control by Dunn's or Shirley's test

** P≤0.01

^a Units are presented as percent cells phagocytizing sheep erythrocytes.

TABLE G13
Phagocytic Activity of Macrophages in Bronchoalveolar Fluid of Mice
at the 18-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Phagocytic Activity	37.43 ± 8.55	14.10 ± 4.54	11.98 ± 2.22*
Female			
Phagocytic Activity	46.85 ± 11.08	20.03 ± 7.45	6.65 ± 0.35*

* Significantly different (P≤0.05) from the control by Dunn's or Shirley's test

^a Units are presented as percent cells phagocytizing sheep erythrocytes.

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TABLE G14
Viability and Phagocytic Activity of Macrophages in Bronchoalveolar Fluid of Mice
at the 24-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Viability ^a	79.20 ± 3.44	64.60 ± 4.15	83.23 ± 0.87
Phagocytic Activity ^b	37.14 ± 9.80	11.90 ± 4.64	3.56 ± 2.25**
Female			
Viability	60.50 ± 8.80	47.17 ± 2.74	59.77 ± 3.21
Phagocytic Activity	21.57 ± 6.77	13.60 ± 4.71	4.35 ± 2.65*

- * Significantly different (P≤0.05) from the control by Dunn's or Shirley's test
- ** P≤0.01
- ^a Units are presented as percent viable cells.
- ^b Units are presented as percent cells phagocytizing sheep erythrocytes.

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TABLE G15
Measurements of Lung Collagen in Mice at the 6-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lavage Fluid Collagenous Peptides ^a	67.13 ± 9.76	24.83 ± 8.18	79.64 ± 18.03
Total Lung Collagen ^b	7.42 ± 0.48	7.51 ± 1.38	12.27 ± 4.53
Female			
Lavage Fluid Collagenous Peptides	42.92 ± 8.49	70.83 ± 9.09	51.17 ± 5.14
Total Lung Collagen	4.69 ± 0.35	5.85 ± 0.89	11.00 ± 3.88

^a Units are presented as µg/g control lung.

^b Units are presented as mg/g control lung.

TABLE G16
Measurements of Lung Collagen in Mice at the 12-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lavage Fluid Collagenous Peptides ^a	74.23 ± 9.42	68.73 ± 4.11	117.62 ± 11.87*
Total Lung Collagen ^b	11.94 ± 0.47	12.44 ± 0.82	13.30 ± 1.11
Female			
Lavage Fluid Collagenous Peptides	89.88 ± 12.99	73.66 ± 11.58	108.55 ± 7.56
Total Lung Collagen	11.64 ± 0.48	11.84 ± 0.45	13.78 ± 1.09

* Significantly different (P≤0.05) from the control by Dunn's or Shirley's test

^a Units are presented as µg/g control lung.

^b Units are presented as mg/g control lung.

TABLE G17
Measurements of Lung Collagen in Mice at the 18-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lavage Fluid Collagenous Peptides ^a	42.54 ± 2.15	51.18 ± 5.40	70.67 ± 8.41**
Total Lung Collagen ^b	6.60 ± 0.49	7.13 ± 0.30	9.70 ± 0.70**
Female			
Lavage Fluid Collagenous Peptides	54.09 ± 11.27	37.68 ± 6.01	64.88 ± 6.56
Total Lung Collagen	6.16 ± 0.25	6.96 ± 0.31	7.34 ± 0.43

** Significantly different (P≤0.01) from the control by Dunn's or Shirley's test

^a Units are presented as µg/g control lung.

^b Units are presented as mg/g control lung.

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TABLE G18
Lung Collagen Metabolism and Protein Synthesis in Mice at the 24-Month Interim Evaluation

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lavage Fluid Collagenous Peptides ^a	54.39 ± 4.42	65.98 ± 5.01	91.92 ± 4.93**
Total Lung Collagen ^b	8.53 ± 0.71	8.55 ± 0.59	13.71 ± 2.81*
Collagen Production ^c	1.133 ± 0.274	0.779 ± 0.151	1.554 ± 0.291
Non-Collagenous Protein Synthesis ^d	18.73 ± 2.85	16.09 ± 1.15	25.64 ± 2.66
Female			
Lavage Fluid Collagenous Peptides	38.09 ± 4.38	39.26 ± 4.01	62.14 ± 9.04*
Total Lung Collagen	6.04 ± 0.27	6.41 ± 0.36	7.91 ± 0.35*
Collagen Production ^c	1.15 ± 0.33	1.65 ± 0.13	1.33 ± 0.12
Non-Collagenous Protein Synthesis ^d	17.05 ± 2.80	15.45 ± 2.26	27.46 ± 1.57

* Significantly different (P≤0.05) from the control by Dunn's or Shirley's test

** P≤0.01

^a Units are presented as µg/g control lung.

^b Units are presented as mg/g control lung.

^c Units are presented as percent new protein.

^d Units are presented as dpm x 10⁻³/g control lung.

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Lung Burden and Lung Biochemistry of Mice

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TABLE G19
Proteinase Activity in Lavage Fluid and Lung Homogenate Supernatant Fluid of Mice
at the 6-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lavage Fluid			
Acid Proteinase	1.27 ± 0.24	1.65 ± 0.47	2.05 ± 0.23
Cathepsin D	0.078 ± 0.038	0.656 ± 0.321 [*]	0.876 ± 0.107 [*]
Cathepsin B	1.006 ± 0.239	0.992 ± 0.716	0.954 ± 0.010
Homogenate Supernatant Fluid			
Acid Proteinase	5.83 ± 1.07	8.10 ± 0.78	7.45 ± 0.64
Cathepsin D	2.27 ± 0.46	3.30 ± 0.57	- ^b
Cathepsin B	3.56 ± 0.80	4.80 ± 0.58	-
Neutral Proteinase	0.634 ± 0.039	0.360 ± 0.043 [*]	-
PMN Elastase Cathepsin G	0.446 ± 0.014	0.418 ± 0.357	-
Macrophage Elastase Collagenase	0.207 ± 0.058	0.340 ± 0.154	-
Female			
Lavage Fluid			
Acid Proteinase	0.762 ± 0.089	1.595 ± 0.038 ^{**}	1.346 ± 0.097
Cathepsin D	0.457 ± 0.166	0.998 ± 0.016	0.628 ± 0.113
Cathepsin B	0.260 ± 0.068	0.571 ± 0.063	0.718 ± 0.094 [*]
Homogenate Supernatant Fluid			
Acid Proteinase	4.35 ± 0.31	6.95 ± 0.61 [*]	5.77 ± 0.61
Cathepsin D	1.78 ± 0.12	3.89 ± 1.52 [*]	3.12 ± 0.06 [*]
Cathepsin B	2.57 ± 0.22	3.06 ± 1.01	2.65 ± 0.56
Neutral Proteinase	0.522 ± 0.047	0.535 ± 0.039	0.848 ^c
PMN Elastase Cathepsin G	0.416 ± 0.033	0.347 ± 0.066	-
Macrophage Elastase Collagenase	0.106 ± 0.043	0.188 ± 0.058	-

^{*} Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test

^{**} P≤0.01

^a Units are presented as mg/hour/mg control lung.

^b n=0; no data recorded

^c n=1; no statistic calculated

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Talc, NTP TR 421

TABLE G20
Proteinase Activity in Lavage Fluid and Lung Homogenate Supernatant Fluid of Mice
at the 12-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lavage Fluid			
Acid Proteinase	1.65 ± 0.13	2.11 ± 0.82	3.25 ± 0.28
Cathepsin D	0.403 ± 0.163	0.970 ± 0.244	1.796 ± 0.306**
Cathepsin B	1.25 ± 0.10	1.25 ± 0.78	1.46 ± 0.05
Homogenate Supernatant Fluid			
Acid Proteinase	7.21 ± 0.50	9.35 ± 0.07*	16.50 ± 0.95**
Cathepsin D	5.32 ± 0.27	7.71 ± 0.16*	14.32 ± 1.27**
Cathepsin B	1.89 ± 0.48	1.64 ± 0.10	2.18 ± 0.39
Neutral Proteinase	0.386 ± 0.055	1.029 ± 0.416	1.088 ± 0.271*
PMN Elastase Cathepsin G	0.110 ± 0.110	0.005 ± 0.005	0.209 ± 0.148
Macrophage Elastase Collagenase	0.426 ± 0.159	1.127 ± 0.422	0.879 ± 0.162
Female			
Lavage Fluid			
Acid Proteinase	1.94 ± 0.17	1.79 ± 0.35	3.60 ± 0.33*
Cathepsin D	0.526 ± 0.263	0.463 ^b	1.525 ± 0.266*
Cathepsin B	1.50 ± 0.41	2.14 ^b	2.08 ± 0.08
Homogenate Supernatant Fluid			
Acid Proteinase	7.88 ± 0.24	10.48 ± 0.50*	16.92 ± 1.84**
Cathepsin D	6.40 ± 0.70	8.44 ± 0.51	14.76 ± 1.59**
Cathepsin B	1.55 ± 0.54	2.04 ± 0.22	2.16 ± 0.55
Neutral Proteinase	0.423 ± 0.183	0.601 ± 0.108	0.824 ± 0.057
PMN Elastase Cathepsin G	0.215 ± 0.125	0.213 ± 0.213	0.190 ± 0.124
Macrophage Elastase Collagenase	0.280 ± 0.116	0.446 ± 0.127	0.653 ± 0.158

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test

** $P \leq 0.01$

^a Units are presented as mg/hour/mg control lung.

^b n=1; no statistic calculated

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Lung Burden and Lung Biochemistry of Mice

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TABLE G21
Proteinase Activity in Lavage Fluid and Lung Homogenate Supernatant Fluid of Mice
at the 18-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lavage Fluid			
Acid Proteinase	0.264 ± 0.044	0.428 ± 0.120	0.384 ± 0.066
Cathepsin D	0.212 ± 0.046	0.073 ± 0.013*	0.051 ± 0.035*
Cathepsin B	0.069 ± 0.037	0.355 ± 0.127*	0.342 ± 0.057*
Homogenate Supernatant Fluid			
Acid Proteinase	3.29 ± 0.58	4.76 ± 0.49	8.38 ± 0.85**
Cathepsin D	2.71 ± 0.24	4.98 ± 0.63*	8.45 ± 0.63**
Cathepsin B	0.607 ± 0.327	0.053 ± 0.053	0.403 ± 0.270
Neutral Proteinase	0.425 ± 0.079	0.548 ± 0.022	0.528 ± 0.034
PMN Elastase Cathepsin G	0.158 ± 0.066	0.242 ± 0.061	0.254 ± 0.017
Macrophage Elastase Collagenase	0.286 ± 0.093	0.306 ± 0.041	0.275 ± 0.031
Female			
Lavage Fluid			
Acid Proteinase	0.267 ± 0.103	0.561 ± 0.126	0.382 ± 0.040
Cathepsin D	0.219 ± 0.085	0.012 ± 0.012	0.062 ± 0.036
Cathepsin B	0.088 ± 0.034	0.587 ± 0.095*	0.358 ± 0.098*
Homogenate Supernatant Fluid			
Acid Proteinase	3.97 ± 0.41	5.57 ± 0.26*	9.03 ± 0.88**
Cathepsin D	3.28 ± 0.23	5.37 ± 0.16*	9.17 ± 0.75**
Cathepsin B	0.694 ± 0.284	0.232 ± 0.096	0.265 ± 0.265
Neutral Proteinase	0.381 ± 0.041	0.540 ± 0.036*	0.583 ± 0.035*
PMN Elastase Cathepsin G	0.265 ± 0.038	0.391 ± 0.038	0.268 ± 0.041
Macrophage Elastase Collagenase	0.116 ± 0.033	0.149 ± 0.054	0.315 ± 0.045*

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test

** P≤0.01

^a Units are presented as mg/hour/mg control lung.

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Talc, NTP TR 421

TABLE G22
Proteinase Activity in Lavage Fluid and Lung Homogenate Supernatant Fluid of Mice
at the 24-Month Interim Evaluation^a

	0 mg/m ³	6 mg/m ³	18 mg/m ³
Male			
Lavage Fluid			
Acid Proteinase	1.62 ± 0.14	1.92 ± 0.18	3.56 ± 0.67*
Cathepsin D	0.000 ± 0.000	0.260 ± 0.156	1.613 ± 0.632**
Cathepsin B	1.94 ± 0.19	1.72 ± 0.28	1.78 ± 0.29
Homogenate Supernatant Fluid			
Acid Proteinase	9.23 ± 1.16	13.85 ± 1.56	24.34 ± 2.66*
Cathepsin D	6.63 ± 0.96	10.82 ± 0.98*	18.75 ± 1.73**
Cathepsin B	2.60 ± 0.39	3.03 ± 0.78	5.58 ± 1.11*
Neutral Proteinase	0.417 ± 0.072	0.568 ± 0.104	0.862 ± 0.164*
PMN Elastase Cathepsin G	0.251 ± 0.034	0.382 ± 0.093	0.341 ± 0.106
Macrophage Elastase Collagenase	0.166 ± 0.063	0.186 ± 0.040	0.521 ± 0.250
Female			
Lavage Fluid			
Acid Proteinase	0.854 ± 0.077	1.012 ± 0.149	0.998 ± 0.212
Cathepsin D	0.194 ± 0.089	0.114 ± 0.114	0.402 ± 0.146
Cathepsin B	0.708 ± 0.118	1.000 ± 0.365	0.596 ± 0.305
Homogenate Supernatant Fluid			
Acid Proteinase	7.83 ± 1.11	9.76 ± 0.56	22.54 ± 1.29*
Cathepsin D	5.10 ± 0.67	8.04 ± 0.95	17.93 ± 0.55**
Cathepsin B	2.73 ± 0.47	1.71 ± 0.57	4.61 ± 1.00
Neutral Proteinase	0.454 ± 0.096	0.646 ± 0.143	0.922 ± 0.077*
PMN Elastase Cathepsin G	0.172 ± 0.063	0.341 ± 0.082	0.360 ± 0.093
Macrophage Elastase Collagenase	0.421 ± 0.293	0.314 ± 0.162	0.563 ± 0.102

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test

** $P \leq 0.01$

^a Units are presented as mg/hour/mg control lung.

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