Duangrudee Cherdwongcharoensuk

Changes in the Respiratory System Caused by Exposure of Mice to Selenium or its Derivative

Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto
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Thesis Submitted in Accordance with the Requirements of the University of Porto for the Degree of Doctor in Biomedical Sciences

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I wish to express my thanks to my Parent and my Family from the depths of my heart for their love and support throughout my life.
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resumo</td>
<td>I</td>
</tr>
<tr>
<td>Abstract</td>
<td>III</td>
</tr>
<tr>
<td>บทความย่อ</td>
<td>V</td>
</tr>
<tr>
<td>Status Thesis</td>
<td>VIII</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>IX</td>
</tr>
<tr>
<td>Chapter 1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 2. Objectives</td>
<td>23</td>
</tr>
<tr>
<td>Chapter 3. Experimental Results and Articles</td>
<td>26</td>
</tr>
<tr>
<td>3.1. Pathology of Respiratory Epithelia Caused by Selenium Inhalation</td>
<td>27</td>
</tr>
<tr>
<td>3.2. Respiratory Inflammation Caused by Dimethyl Selenide</td>
<td>35</td>
</tr>
<tr>
<td>3.3. <em>In Vivo</em> Ingestion of Heavy Metal Particles by Murine Macrophages</td>
<td>60</td>
</tr>
<tr>
<td>3.4. Lung Collagens after Selenide Inhalation</td>
<td>68</td>
</tr>
<tr>
<td>3.5. Acute Respiratory Inflammation Induced by Selenium Particles</td>
<td>86</td>
</tr>
<tr>
<td>Chapter 4. General Discussion</td>
<td>108</td>
</tr>
<tr>
<td>Chapter 5. Bibliography</td>
<td>121</td>
</tr>
</tbody>
</table>
Os efeitos nocivos nos tecidos respiratórios devido à inalação de selênio (Se) e a um seu derivado, o dimetilSe (DMSe), foram investigados nesta tese com particular atenção às alterações morfológicas e inflamatórias causadas por estas substâncias no ratinho. A presente dissertação inclui ainda o estudo da remoção das partículas de Se pelos macrófagos alveolares e pelos granulócitos e das alterações na deposição de colágeno nos pulmões de ratinhos tratados com Se ou DMSe.

As alterações histopatológicas do epitélio traqueal e dos pulmões provocadas por uma única instilação intratraqueal de duas doses diferentes de DMSe (0.05 e 0.1 mg Se/Kg BW) foram estudadas após 1, 7, 14 e 28 dias após o tratamento. O epitélio traqueal mostrou diminuição do número de cílios e necrose aguda e, em algumas amostras, mesmo uma transformação metaplastica. Edema e lesão alveolar difusa foram observados nos pulmões. As lesões respiratórias causadas pelo Se foram correlacionadas com a dose de DMSe administrado. Em contraste com as lesões traqueais agudas, inflamação crónica assim como o aumento da espessura do septo alveolar ocorrem nos pulmões dos mesmos animais, sem evidência de recuperação das lesões 4 semanas após a instilação inicial.

O estudo da cinética dos biomarcadores inflamatórios do líquido broncoalveolar incluiu o número de leucócitos, e o doseamento de lactato desidrogenase (LDH) e das proteínas totais. A investigação foi feita com vista a caracterizar-se a resposta inflamatória dos pulmões dos animais a uma única instilação intratraqueal de DMSe. Os neutrófilos foram os leucócitos mais numerosos encontrados no período inicial da inflamação causada por DMSe. O número de macrófagos aumentou apenas moderadamente, enquanto que os linfócitos não evidenciaram aumento significativo após a instilação de Se. Verificou-se um ligeiro aumento de proteína total no exsudado alveolar e, mais
tardiamente, um aumento de LDH nos animais a que foi administrada a dose mais elevada de DMSe. Esta dose de DMSe (0.1 mg Se/kg BW) causou um aumento sustentado do número de neutrófilos e macrófagos durante as 4 semanas de estudo, enquanto a dose mais baixa (0.05 mg Se/Kg) resultou numa reacção inflamatória observada apenas durante 2 semanas. Pode-se concluir que o DMSe origina uma resposta inflamatória que é dependente da dose administrada e que se reflecte em alterações do epitélio respiratório.

Os colagénios I, II e IV, foram estudados nos pulmões dos animais tratados com DMSe, verificando-se que o colagénio do tipo I sofreu um aumento em septos alveolares 7 e 14 dias após a administração de DMSe. O colagénio do tipo II sofreu apenas um aumento moderado, mas o colagénio IV não sofreu qualquer alteração durante o tratamento. Pode-se concluir que a instilação por DMSe causa um transitório aumento em colagénio I provocando a sua acumulação na parede alveolar, sem que hajam alterações do colagénio das membranas basais (tipo IV). O aumento de colagénio I foi temporário indicando que a fibrogénese pulmonar causada por uma única instilação de DMSe é um processo reversível.

A microscopia de luz e a microscopia electrónica de varrimento acoplada à microanálise elementar de raio-X (SEM-XRM) foram usadas para investigar a remoção das partículas de Se do pulmão do ratinho. Foi observado que praticamente todos os macrófagos alveolares continham partículas de Se. Apenas menos de metade dos neutrófilos mostravam ingestão de partículas de Se. Observou-se que os macrófagos alveolares com Se migram para o espaço intersticial onde são capturados 72 horas após a instilação das micropartículas. Conclui-se que a resposta inflamatória aguda do pulmão às partículas de Se é dominada pelos neutrófilos, mas que a remoção do metal é realizada majoritariamente pelos macrófagos alveolares.

Esta dissertação oferece a definição morfológica e inflamatória das alterações agudas da traqueia e do pulmão à instilação de Se ou de DMSe.
ABSTRACT

The harmful effects on respiratory tissues due to inhalation of selenium (Se) and of one of its derivatives dimethyl selenide (DMSe) were investigated in this thesis. The focus was on the morphological and inflammatory changes that were experimentally induced in mice by Se or DMSe. This effort includes the study of removal of Se particulates by alveolar macrophages and granulocytes, and the modifications in collagen deposition triggered in the lungs by Se or DMSe.

The histopathological alterations of murine tracheal epithelium and lungs caused by a single intratracheal instillation of two different doses of DMSe (0.05 and 0.1 mg Se/kg BW) were defined 1, 7, 14 and 28 days after the DMSe treatment. The tracheal epithelium showed loss of cilia and acute necrosis and, in some instances, metaplastic transformation. Edema and diffuse alveolar damage was observed in the lungs. Moreover, the respiratory lesions caused by Se were found to be dose dependent since the higher DMSe dose triggered more serious respiratory lesions than the lower one. In contrast with the transient nature of tracheal lesions, chronic inflammation and increased thickness of alveolar septa occurred in the lungs with no amelioration of lesions after 4 weeks of Se the initial instillation.

The kinetic of inflammatory biomarkers in bronchoalveolar lavage (BAL) samples, including leukocytes, lactate dehydrogenase (LDH) and total protein, were used to characterize the inflammatory response of the mouse lungs induced by a single intratracheal instillation of DMSe. Neutrophils were the most numerous leukocytes during the early inflammatory influx. Macrophages increased moderately and lymphocytes showed no increase after the Se instillation. An early increase in total protein of BAL, and late enhancement in LDH was observed in mice treated with the high DMSe dose. Furthermore, the higher dose of DMSe triggered a sustained enhancement in
the number of neutrophils and macrophages during the 4 weeks of the study, while the lower dose of DMSe resulted in an inflammation reaction that lasted only for 2 weeks. In addition, DMSe led to a dose-dependent inflammatory reaction in the mouse airways that resembles the kinetics of damage of respiratory epithelia that occurred upon DMSe inhalation.

Staining of collagen I, II and IV was investigated in the lungs of DMSe treated mice, I was found that collagen type I was enhanced in alveolar septa, at day 7. Collagen II was moderately increased but collagen IV was not significantly changed by the treatment. It can be concluded that DMSe instillation caused an increase in collagen I fibrils with no changes in basement-membrane collagen (type IV), and also that the enhancement in lung collagen was transient, indicating that lung fibrosis triggered by a single airway instillation of DMSe is a reversible event.

Light microscopy and scanning electron microscopy coupled with X-ray elemental microanalysis (SEM-XEM) were used to investigate the removal of inhaled Se particle in the mouse lung. It was documented that virtually all BAL macrophages contained Se particles of different size. Only less than half of the neutrophils showed ingested Se particles. There was evidence that the Se-loaded alveolar macrophages migrated into the interstitial space of the alveoli where they were often captured 72 hrs after inhalation of the microparticulate. The acute inflammatory response in the lung to Se particles was dominated by neutrophils but the removal of Se was done mostly by alveolar macrophages.

Taken together, the experimental data of this thesis define the early structural changes of respiratory tissues in response to the instillation of Se particles or of a Se derivative.
บทคัดย่อ

วิทยานิพนธ์นี้ ท้าการศึกษาถึงผลกระทบอันร้ายแรงต่อเนื้อเยื่อระบบหายใจจากการสูดสารซิลิเนียม และอนุมูลส์ โดยมีกลิ่น ซิลิเนียม การทดลองนี้เน้นถึงการเปลี่ยนแปลงโครงสร้างและการอักเสบอันเนื่องมาจากผลของการสูดสารซิลิเนียมหรือโสมมิลิ่งซิลิเนียมในหนู นอกจากนี้ได้ทำการศึกษาถึงการเคลื่อนย้ายอนุภาคของสารซิลิเนียม โดย alveolar macrophages และ granulocytes ตลอดจนการเปลี่ยนแปลงของคลอลาเจนในโพล อันเนื่องมาจากได้รับสารซิลิเนียมหรือโสมมิลิ่งซิลิเนียม.

ได้ทำการตรวจสอบพ่อกำพันของเนื้อเยื่อหลอดคอและโพล ในวันที่ 1, 7, 14 และ 28หลังจากการสูดสารโดยมีกลิ่น ซิลิเนียมที่มีความเข้มข้นแตกต่างกัน 2 ระดับ คือ 0.05 และ 0.1มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัว. พบว่ามีการสูญหายของ cilia ในเนื้อเยื่อหลอดคอ, พบเซลล์ตาย และมีการเปลี่ยนแปลงรูปร่างของเซลล์ (metaplastic transformation). ลักษณะพบความผิดปกติในส่วนของเนื้อเยื่อโพล คือ มี edema และ diffuse alveolar damage. นอกจากนี้ยังพบว่าความร้ายแรงของพ่อกำพันของระบบหายใจขึ้นอยู่กับความเข้มข้นของสารซิลิเนียม โดยซิลิเนียมที่มีความเข้มข้นสูงจะมีผลทำลายระบบหายใจมากกว่าที่มีความเข้มข้นต่ำ. การอักเสบและการหัวด้วีของผนัง alveolar septa เกิดขึ้นที่เนื้อเยื่อโพลด์ 4สัปดาห์หลังจากการสูดสารซิลิเนียม แต่การอักเสบของเนื้อเยื่อหลอดคอเป็นแบบไม่กว้าง.

ผลของการอักเสบของโพลด์จากการได้รับสารโดยมีกลิ่น ซิลิเนียม ศึกษาได้โดยศูนย์การเปลี่ยนแปลงของ inflammatory biomarkers ในตัวอย่าง bronchoalveolar lavage.
(BAL) ซึ่งประกอบด้วย เม็ดเลือดขาว, lactate dehydrogenase (LDH) และ total protein. สำหรับ neutrophils นั้นพบว่าเป็นเม็ดเลือดขาวที่พบมากที่สุดของการอักเสบในระยะแรก. สำหรับ macrophages พบว่ามีจำนวนมากขึ้น แต่ไม่พบการเพิ่มจำนวนของ lymphocytes หลังการอักเสบซีกเนียม. ระดับของ LDH และ total protein ใน BAL เพิ่มขึ้น เม็ดเลือดขาวได้แมทิล ซิลิโนซินที่มีความเข้มข้นสูง. นอกจากนี้สารได้แมทิล ซิลิโนซินที่มีความเข้มข้นสูงก็ให้เกิดการเพิ่มจำนวนของ neutrophils และ macrophages ลดลง 4 สัปดาห์ของการศึกษา แต่สารได้แมทิล ซิลิโนซินที่มีความเข้มข้นต่ำนั้นก็ให้เกิดการอักเสบเฉพาะในช่วง 2 สัปดาห์แรกของการศึกษา. การอักเสบเฉพาะมาจากสารได้แมทิล ซิลิโนซินจะขึ้นอยู่กับความเข้มข้นของสาร ซึ่งตรงกับสภาพการเปลี่ยนแปลงทางกายที่ความร้ายแรงนั้นขึ้นอยู่กับความเข้มข้นของสารได้แมทิล ซิลิโนซินที่สูงสุดเข้าไป.

เมื่อนำเนื้อเยื่อปอดของทุนมาเยื่อคู่ตลอดเลجانชนิดที่ 1, 2 และ 4 พบว่าตลอดเลานชนิดที่ 1 เป็นชนิดที่เพิ่มจำนวนใน alveolar septa โดยเฉพาะในวันที่ 7. สำหรับตลอดเลานชนิดที่ 2 เพิ่มจำนวนมากขึ้น แต่จำนวนของตลอดเลานชนิดที่ 4 ไม่พบว่ามีการเปลี่ยนแปลงหลังอักเสบ. แต่แน่นอนถูกสรุปได้ว่าการเปลี่ยนแปลงตลอดเลานของโรคหรือ lung fibrosis เนื่องจากการอักเสบได้แมทิล ซิลิโนซินเพียง 1 ครั้งเป็นแบบไม่ยาว โดยตลอดเลานชนิดที่ 1 มีการเพิ่มจำนวน ตามตลอดเลาน ชนิดที่ 4 ใน basement-membrane ไม่พบการเปลี่ยนแปลงจำนวนแต่อย่างใด.

ได้ศึกษาเพิ่มเติมถึงการเคลื่อนย้ายอนุภาคของซิลิโนซินในปอดของทุน ด้วยกล้องจุดทรัพนแบบส่องสว่าง และกล้องจุดทรัพนอีเล็กตรอนแบบส่องกระดาษ ร่วมกับ X-ray
ผลการทดลองจากการศึกษานี้แสดงถึงการเริ่มแรกของการเปลี่ยนแปลงโครงสร้างของเนื้อเยื่อระบบท่อ气 ซึ่งเป็นผลเนื่องมาจากการตกค้างของอนุภาคซิลิเนียมหรืออนุภาคซิลิเนียม.
The results of the work of this thesis have been published, accepted or submitted for publication, as follows:


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACGIH</td>
<td>American Conference of Government Industrial Hygienists</td>
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<tr>
<td>ARB</td>
<td>Air Resources Board</td>
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<tr>
<td>ATSDR</td>
<td>Agency for Toxic Substance and Disease Registry</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BEI</td>
<td>Backscattered electron image</td>
</tr>
<tr>
<td>BOOP</td>
<td>Bronchiolitis obliterans-organizing pneumonia</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAPCOA</td>
<td>California Air Pollution Control Officers Association</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DAD</td>
<td>Diffuse alveolar damage</td>
</tr>
<tr>
<td>DMDS{sub}e</td>
<td>Dimethyl diselenide</td>
</tr>
<tr>
<td>DMS{sub}e</td>
<td>Dimethyl selenide</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy dispersive spectrometry</td>
</tr>
<tr>
<td>EFP</td>
<td>Emission Factor Program</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>HAPs</td>
<td>Hazardous air pollutants</td>
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<tr>
<td>HE</td>
<td>Haematoxylin-eosin</td>
</tr>
<tr>
<td>IDLH</td>
<td>Immediately dangerous to life or health</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>NIOSH</td>
<td>National Institute for Occupational Safety and Health</td>
</tr>
<tr>
<td>NOES</td>
<td>National Occupational Exposure Survey</td>
</tr>
<tr>
<td>OSHA</td>
<td>Occupational Safety and Health Administration</td>
</tr>
<tr>
<td>PEL</td>
<td>Permissible exposure limit</td>
</tr>
<tr>
<td>REL</td>
<td>Reference exposure level</td>
</tr>
<tr>
<td>SEI</td>
<td>Secondary electron image</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SEM-XRM</td>
<td>Scanning electron microscopy coupled with X-ray elemental microanalysis</td>
</tr>
<tr>
<td>TLV</td>
<td>Threshold limit value</td>
</tr>
<tr>
<td>TMSe+</td>
<td>Trimethyl Se ion</td>
</tr>
<tr>
<td>TWA</td>
<td>Time-weighted average</td>
</tr>
<tr>
<td>USEPA</td>
<td>United State Environmental Protection Agency</td>
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Chapter 1

Introduction
Selenium (Se) is found in nature as a trace element that is classified in the group VI-A of the periodic table. The Swedish scientist Jakob Berzelius has discovered this chemical element in 1817, when he and his colleague Johann Gottlieb Gahn were investigating the oxidation process of sulphur dioxide from copper pyrites, which was used in the production of sulphuric acid in lead cameras (this historical information is referred in detail in a publication by the National Research Council, 1983). The residual substance found on the bottom of these cameras was first interpreted by Berzelius to be tellurium, a chemical element that had been identified before by Klaproth. After several analytical approaches, Barzelius concluded that the residues corresponded to an element other than tellurium. Because he first thought that the element was tellurium (meaning from the Earth in Greek), Barzelius decided to name the new element selenium (meaning from the Moon in Latin): he saw Se as the sister element of tellurium (Krogt, 2003).

The first reports on the toxicity of Se are dated from the early 1930s, when lameness, followed in some cases by death, was observed in livestock grazing certain range of plants that were contaminated by Se in the states of North and South Dakota and Wyoming of the USA (Franke, 1934). Later on, the important nutritional benefits of Se, at very low doses, was identified in experimental studies that established Se as an essential trace nutrient in the diet of humans and animals (Schwarz and Foltz, 1957). In fact, Se participates in many physiological processes, e.g., it is a component of the mitochondrial electron transport system, it regulates ion fluxes across membranes, it participates in the integrity of cytoskeletal keratins, it stimulates antibody synthesis, and, last but not the least, it activates glutathione peroxidase, a key enzyme in the prevention of oxidative damage of cells and tissues (Doull et al., 1980; Opresko, 1993; Barceloux, 1999; Huel et al., 2000; ATSDR, 2001).
Se has been extensively employed in a number of modern industrial processes. Because of this increasing industrial use of Se, more and more of this trace element is getting into the environment; this has enhanced human exposure to Se. Humans can be exposed to Se having different origins that range from food, water, soil and air. The most frequent route for human pathology caused by Se is inhalation in industrial environments.

The potential toxicity of inhaled Se has only recently been thoroughly considered; this has led USA authorities to consider Se and its compounds as federal hazardous air pollutants since these substances were identified as air contaminants of a number of industrial environments (California ARB, 1997). Furthermore, Se is one of 129 priority pollutants that have been listed (CAS number 7782-49-2) by the Environmental Protection Agency of the USA (Keith and Telliard, 1979). The number of experimental studies devoted to the adverse health effects of inhaled Se is, however, still small.

**Physical and Chemical Properties of Se**

Se depicts both metallic and non-metallic properties; this led to its classification as a metalloid element in group VI-A of the periodic table that is located between the metals tellurium and polonium and the non-metals oxygen and sulphur by group, and between the metal arsenic and the non-metal bromine by period (National Research Council, 1983).

In nature, Se occurs in three major allotropic forms. The lustrous grey hexagonal form is the most stable of the three. The other two forms are monoclinic and amorphous Se that exists in black and red colours. Black amorphous is vitreous, but red amorphous is colloidal (Irwin et al., 1997; California ARB, 1997; ATSDR, 2001). In their native forms, Se is odourless and insoluble in water and alcohol; it is soluble in chloroform, methyl iodide, benzene, quinoline, nitric acid, sulphuric acid, ether, carbon disulfide, aqueous
potassium cyanide and potassium sulphate solutions (California ARB, 1997). Se shows photovoltaic properties where light is converted directly to electricity and photoconductive features where electrical resistance decreases with increased illumination. These physical features of Se led to its early employment in the electrical industry. The physical properties of Se are summarized in Table 1.

**Table 1.** Physical and chemical properties of Se. Adapted from the references: Irwin *et al.*, 1997; California ARB, 1997; ATSDR, 2001.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atomic number</td>
<td>34</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>78.96</td>
</tr>
<tr>
<td>Oxidative states</td>
<td>-2, 0, +4, +6</td>
</tr>
<tr>
<td>Colour</td>
<td>red, grey or black</td>
</tr>
<tr>
<td>Physical state</td>
<td>dark red brown to bluish-black solid; dark red transparent crystals;</td>
</tr>
<tr>
<td></td>
<td>lustrous grey to black hexagonal crystal</td>
</tr>
<tr>
<td>Melting point</td>
<td>221 °C (red); 220.5 °C (grey); 180 °C (black)</td>
</tr>
<tr>
<td>Boiling point</td>
<td>685 °C</td>
</tr>
<tr>
<td>Density/specific gravity (g/cm³)</td>
<td>4.39 (red); 4.81 (grey); 4.28 (black)</td>
</tr>
<tr>
<td>Odour</td>
<td>Odourless; upon combustion, smells like rotten horseradish</td>
</tr>
<tr>
<td>Solubility:</td>
<td></td>
</tr>
<tr>
<td>Water (g/100 ml)</td>
<td>insoluble</td>
</tr>
<tr>
<td>Organic solvents</td>
<td>insoluble in alcohol, slight soluble in carbon disulfide (2 mg/100 ml room temperature), soluble in ether, potassium cyanide, potassium sulphate, caustic alkaline, methyl iodide, benzene, chloroform, nitric acid, sulphuric acid and quinoline</td>
</tr>
<tr>
<td>Vapour Pressure</td>
<td>0.001 mmHg at 20 °C</td>
</tr>
<tr>
<td>Properties</td>
<td>photovoltaic and photoconductive action</td>
</tr>
</tbody>
</table>
The four natural occurring valance states of Se are -2 (hydrogen selenide, sodium selenide, dimethyl selenium, trimethyl selenium, and selenoamino acids such as selenomethionine); 0 (elemental Se); +4 (Se dioxide, selenious acid and sodium selenite); and +6 (selenic acid and sodium selenate) (Opresko, 1993; Barceloux, 1999). The different forms of Se can be classified either into inorganic or in organic Se compounds.

Elemental Se can be oxidized to +4 or +6, and these are the most important forms of inorganic Se. Inorganic compounds also include the -2 state. In the -2 reduced states, Se exists as hydrogen selenide and inorganic metal selenide. Hydrogen selenide (H₂Se) is a highly toxic gas produced in industrial factories by hydrolysis of metal selenides or by heating (400 °C) elemental Se into the air. Metal selenides are formed with cadmium, copper and mercury.

In the +4 state, Se exists as Se dioxide, selenious acid and sodium selenite. Se dioxide can be formed by oxidation of elemental Se with concentrated nitric acid or combustion of fossil fuels. Selenious acid is weakly dibasic and often acts as an oxidizing agent; it occurs upon oxidation of amorphous Se in water. Selenites are formed under slightly less oxidized conditions and can be reduced to elemental Se at low pH by mild reducing agents, as ascorbic acid or sulphur dioxide. In the +6, Se occurs as selenic acid (H₂SO₄) or selenate (SeO₄²⁻) salts.

Selenic acid is a strong acid that can be obtained by oxidizing Se or selenious acid with strong agents as NaBrO₃ in NaHCO₃ or Br₂, Cl₂ or H₂O₂ in water or sulphuric acid. Selenate salts are more soluble than selenite compounds, and are usually found in water and soil. Dissolved inorganic compounds can be found in natural water as selenide, colloidal elemental Se, selenite and selenate (National Research Council, 1983; Irwin et al., 1997; Pyrzyńska, 1998).
The organoselenium compounds may make up to 30-60% of the Se detected in fresh and marine waters. These compounds include: (1) amino acid (seleno-aminoacid, selenocysteine (C₃H₇NO₂Se), selenocystine (C₆H₁₂N₂O₄Se₂) and methylated volatile Se forms (dimethyl selenide ((CH₃)₂Se) (DMSe) and dimethyl diselenide ((CH₃)₂Se₂) (DMDSe); (2) selenomethionine (C₅H₁₁NO₂Se). In most cases, the organoselenium compounds can be originated from amorphous Se by addition reactions: from H₂Se or alkali selenides by addition or nucleophilic displacement reactions, from potassium selenocyanate by nucleophilic displacement or electrophilic substitution reactions, from phosphorus pentaselenide by reactions with primary alcohols, and from Se oxides by substitution reactions at carbon atoms or by electrophilic substitution reactions (National Research Council, 1983). Selenomethionine may be the dominant form of Se in plant tissues whereas selenocysteine may dominate in animal tissues (Irwin et al., 1997).

Inorganic compounds can be transformed into volatile organic compounds such as DMSe and DMDSe through microbial methylation of fungi, plants and animals (Pyrzyńska, 1998). Both of them exist in the atmosphere in vapour phase, whereas elemental Se and other Se compounds are present in the atmosphere as particulates (California ARB, 1997).

The end products of Se metabolism in animals and humans are DMSe, molecules which have a distinctive garlic-like odor, and are secreted in sweat and saliva, as well as exhaled, and also trimethyl Se ion (TMSe⁺), the major form of Se secreted in the urine (Harbison, 1998). The biomethylation processes are considered to be detoxification steps because DMSe and TMSe⁺ are less toxic than other Se compounds (Pyrzyńska, 1998).

Due to its role as end product in Se metabolism, and to its relatively low toxicity when compared with other Se compounds that occur in the atmosphere, DMSe was chosen, instead Se, in the herein studies of this thesis that are
devoted to the structural changes of cells and tissues of the respiratory system of rodents that are caused by DMSe instillation in the tracheal airway.

**Emission of Se and Se Compounds**

Se is a rare component of the planet earth that makes up just 0.09 parts per million (ppm) of the crust of our planet. Se occurs naturally in water, rocks, soil, volcanic materials, sulphide ores of heavy metals and in fossil fuels, for instance, in coal, crude oil, oil shale, coal conversion material (liquefaction oils and synthetic gases), and their waste by-products (California ARB, 1997; National Research Council, 1983). Se is a component of approximately 40 minerals, with highest Se levels being found in clausthalite, naumanite, tiemannite and berzelianite; it is also a minor component of 37 other minerals, mainly in sulphides (National Research Council, 1983).

Most of the environmental deposits of Se are seen in igneous rocks, where the metal occurs as selenite minerals, in sedimentary rocks, such as shales, sandstones, lime stones, in phosphorus rocks, in chalcopyrite, in bornite, in pyrite minerals, in sulphides as isomorphous with sulphur, in hydrothermal deposits that are associated epithermally with antimony, silver, gold and mercury, and in massive sulphide and porphyry copper where Se is present in small concentration but in large quantities (National Research Council, 1983). Most of the Se that is used in industry is originated from Se recovery from anode mud of electrolytic copper refineries, from roasting mud with soda or sulphuric acid, or by smelting it with soda and nitré (Clayton and Clayton, 1994; ATSDR, 2001).

Natural emission of Se into the atmosphere can occur during plant or bacterial biomethylation processes and during volcanic explosions. Other Se emissions may result from mining and milling operations, metal or Se smelting and refining, burning of fossil fuel, and waste of Se by-products in various
industries. In the USA, up to 90 percent of Se is emitted into the air during the burning of fossil fuels (Clayton and Clayton, 1994; California ARB, 1997). Most of the atmospheric Se is bound to fly ash (1.4 – 11 µg/g) and to suspended particles (1-110 µg/g). The world-wide atmosphere emission of Se from natural sources is 10,000 tons/year and it exceeds the emission from anthropogenic sources (5,100 tons). In addition, 4,100 tons of Se are emitted yearly into aquatic ecosystems (Clayton and Clayton, 1994).

Air pollution concentrations average from 0.38 ng/m$^3$ in remote areas to 13 ng/m$^3$ in urban areas (Clayton and Clayton, 1994; ATSDR, 2001). Se was detected in the atmosphere of seven areas of United Kingdom at concentrations ranging from 0.1 to 42.3 ng/m$^3$ (Lee et al., 1994). The concentration of suspended Se in the atmosphere was found to be high in industrial cities such as Niles, Michigan (2.5 ng/m$^3$) and Buffalo, New York (3.7-9.7 ng/m$^3$), and low in rural areas such as Porker Flats, Alaska (0.035-0.067 ng/m$^3$) (Dams et al., 1970; Pillay et al., 1971; Sturges and Shaw, 1993).

In 1973, total world production of Se compounds was 1.1 million kg, with Japan, USA and Canada as the leading producers (National Research Council, 1983). USA production of Se was 373 and 379 metric tons in 1995 and 1996, respectively, but no production data was reported for the years 1997-1999. USA exports of Se were 270, 322, 127, 191 and 240 metric tons for 1995, 1996, 1997, 1998 and 1999 (ATSDR, 2001). The import volume of Se was 324, 428, 346, 339 and 320 metric tons for 1995, 1996, 1997, 1998 and 1999, respectively (ATSDR, 2001). The distribution of consumer industries of Se compounds in the USA was as follows in 1995: glass 35%, electrical 30%, pigments 10%, metallurgy 10%, agricultural/biological 5% and miscellaneous 10% (ATSDR, 2001). Moreover, the Emission Factor Program (EFP) of the United State Environmental Protection Agency (USEPA) estimated the total emissions of
hazardous air pollutants (HAPs) from fossil-fuel-fired electric utility steam generating unit in 1990, 1994, as well as in 2010 (USEPA, 1998). This is as shown in Table 2 and 3. It was documented that Se is one of the hazardous air pollutants that comes from coal- and oil-fired units. The coal-fired unit emission increased from 153.83 to 213.21 tons/years, but the oil-fired unit emission decreased from 1.65 to 0.84 tons/years. In addition, the Se emission of around $4.7 \times 10^{-5} - 4.6 \times 10^{-4}$ lb/ton of brick producing was also found in the process of brick and structural clay product manufacturing, based on the data reported by the USEPA (1997).

**Table 2.** The median emission factors determined from the test report data, and the total emissions in 1990, 1994, as well as 2010 estimated values from emission factor program for inorganic HAPs from coal-fired units. Reproduced from USEPA (1998).

<table>
<thead>
<tr>
<th>Coal-fired units: inorganic HAPs</th>
<th>Estimated total 1990 emissions (tons)</th>
<th>Estimated total 1994 emissions (tons)</th>
<th>Estimated total 2010 emissions (tons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimony</td>
<td>7.95</td>
<td>7.98</td>
<td>9.93</td>
</tr>
<tr>
<td>Arsenic</td>
<td>60.93</td>
<td>55.81</td>
<td>70.61</td>
</tr>
<tr>
<td>Beryllium</td>
<td>7.13</td>
<td>7.93</td>
<td>8.20</td>
</tr>
<tr>
<td>Hydrogen chloride</td>
<td>143,000</td>
<td>134,000</td>
<td>155,000</td>
</tr>
<tr>
<td>Hydrogen cyanide</td>
<td>240.66</td>
<td>250.8</td>
<td>318.31</td>
</tr>
<tr>
<td>Hydrogen fluoride</td>
<td>19,500</td>
<td>23,100</td>
<td>25,700</td>
</tr>
<tr>
<td>Cadmium</td>
<td>3.33</td>
<td>3.15</td>
<td>3.82</td>
</tr>
<tr>
<td>Chromium</td>
<td>73.27</td>
<td>61.60</td>
<td>87.43</td>
</tr>
<tr>
<td>Cobalt</td>
<td>21.21</td>
<td>22.67</td>
<td>27.08</td>
</tr>
<tr>
<td>Lead</td>
<td>75.47</td>
<td>61.77</td>
<td>86.89</td>
</tr>
<tr>
<td>Manganese</td>
<td>163.97</td>
<td>167.72</td>
<td>219.02</td>
</tr>
<tr>
<td></td>
<td>Estimated total 1990 emissions (tons)</td>
<td>Estimated total 1994 emissions (tons)</td>
<td>Estimated total 2010 emissions (tons)</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------------</td>
<td>---------------------------------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>Arsenic</td>
<td>5.02</td>
<td>3.51</td>
<td>2.54</td>
</tr>
<tr>
<td>Beryllium</td>
<td>0.46</td>
<td>0.40</td>
<td>0.23</td>
</tr>
<tr>
<td>Cadmium</td>
<td>1.71</td>
<td>1.09</td>
<td>0.86</td>
</tr>
<tr>
<td>Chromium</td>
<td>4.74</td>
<td>3.91</td>
<td>2.4</td>
</tr>
<tr>
<td>Cobalt</td>
<td>20.41</td>
<td>15.84</td>
<td>10.31</td>
</tr>
<tr>
<td>Hydrogen chloride</td>
<td>2860</td>
<td>2100</td>
<td>1450</td>
</tr>
<tr>
<td>Hydrogen fluoride</td>
<td>143</td>
<td>284</td>
<td>73</td>
</tr>
<tr>
<td>Lead</td>
<td>10.58</td>
<td>8.92</td>
<td>5.35</td>
</tr>
<tr>
<td>Manganese</td>
<td>9.28</td>
<td>7.30</td>
<td>4.70</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.25</td>
<td>0.19</td>
<td>0.13</td>
</tr>
<tr>
<td>Nickel</td>
<td>392.83</td>
<td>322.37</td>
<td>198.17</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>67.25</td>
<td>50.89</td>
<td>34.10</td>
</tr>
<tr>
<td>Selenium</td>
<td><strong>1.65</strong></td>
<td><strong>1.42</strong></td>
<td><strong>0.84</strong></td>
</tr>
</tbody>
</table>

**Table 3.** The median emission factors determined from the test report data, and the total emissions in 1990, 1994, as well as estimated 2010 values from emission factor program for inorganic HAPs from oil-fired units. Reproduced from USEPA (1998).
The total emission of Se and its compounds from stationary sources in California was estimated to be at least 8617 kg/year. At least 5896 kg were contributed by Se, 2177 kg were Se sulphide, and 181 kg were scattered by other Se compounds. The primary stationary sources that have been reported concerning the emission of Se sulphide are metal mining sites, but the primary stationary sources of Se and other Se compounds are electrical services and manufactures of flat or blown glass (California ARB, 1997).

Industrial surveys have shown that Se is an important element used in several manufactures. There is an ongoing increase in Se-using industrial activity that has resulted in gradual redistribution of Se from the crust of the Earth into the environment and this has enhanced the chance of human exposure to Se. Two decades ago, NIOSH (NOES Survey 1981-1983) has estimated the number of US workers that could be potentially exposed to Se compounds. These numbers are presented in Table 4.

**Table 4.** Rank-ordered of Se and its compounds estimated by NIOSH (NOES survey 1981-1983) with regards to the total number of employees potentially exposed to various agents. Reproduced from the site: http://www.cdc.gov/noes/default.html

<table>
<thead>
<tr>
<th>Rank Order</th>
<th>Total Employees (Male &amp; Female)</th>
<th>Total Female Employees</th>
<th>Agent Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,806</td>
<td>27,208</td>
<td>9,632</td>
<td>Selenium</td>
</tr>
<tr>
<td>3,253</td>
<td>10,544</td>
<td>3,736</td>
<td>Sodium selenite</td>
</tr>
<tr>
<td>4,717</td>
<td>5,207</td>
<td>823</td>
<td>Selenium oxide</td>
</tr>
<tr>
<td>5,680</td>
<td>3,434</td>
<td>849</td>
<td>Selenium, isotope of mass 75</td>
</tr>
<tr>
<td>5,962</td>
<td>2,965</td>
<td>2,491</td>
<td>Selenium sulfide</td>
</tr>
<tr>
<td>11,691</td>
<td>105</td>
<td>-</td>
<td>Selenium powdered</td>
</tr>
<tr>
<td>12,407</td>
<td>33</td>
<td>-</td>
<td>Selenium sulfate</td>
</tr>
</tbody>
</table>
Emission of Se and Se compounds during combustion is a potential health hazard to humans by inhalation of its vapours. The reagents that are most likely to occur in the air are Se, Se dioxide dust and hydrogen selenide gas. Other volatile Se compounds that might be present are DMSe and DMDSe (Opresko, 1998). DMSe and DMDSe exist in the atmosphere in the vapour phase, while other Se compounds occur in the particulate phase (California ARB, 1997). Hence, Se and its compounds were considered to be federal hazardous air pollutants in the USA and were identified as toxic air contaminants in 1993 (California ARB, 1997). Several USA institutes have developed standards and guidelines to regulate exposure to Se in the environmental atmosphere, and to protect individuals from this possible health hazard, as it is presented in Table 5.


<table>
<thead>
<tr>
<th>Reference</th>
<th>Source</th>
<th>Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACGIH</td>
<td>Selenium and compounds</td>
<td>TLV = 0.2 mg/m³ (8-hour TWA)</td>
</tr>
<tr>
<td></td>
<td>Selenium hexafluoride</td>
<td>TLV = 0.16 mg/m³ (8-hour TWA)</td>
</tr>
<tr>
<td>EPA</td>
<td>Hazard rank under section 112 (g) of the Clean Air Act Amendments: Selenium and compounds</td>
<td>42 out of 1-100, with 100 being the most toxic</td>
</tr>
<tr>
<td></td>
<td>Reference air concentration</td>
<td>3.0 µg/m³</td>
</tr>
</tbody>
</table>
### OSHA

<table>
<thead>
<tr>
<th>General industry</th>
<th>Selenium and compounds</th>
<th>PEL: 0.2 μg/m$^3$ (TWA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Selenium hexafluoride</td>
<td>PEL: 0.4 μg/m$^3$ (TWA)</td>
</tr>
<tr>
<td></td>
<td>Hydrogen selenide</td>
<td>PEL: 0.2 μg/m$^3$ (TWA)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Construction industry</th>
<th>Selenium and compounds</th>
<th>PEL: 0.2 μg/m$^3$ (TWA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Selenium hexafluoride</td>
<td>PEL: 0.16 μg/m$^3$ (TWA)</td>
</tr>
</tbody>
</table>

### CAPCOA

<table>
<thead>
<tr>
<th>Selenium</th>
<th>An acute REL = 2 μg/m$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenium and compounds</td>
<td>A chronic-non-cancer REL = 0.5 μg/m$^3$</td>
</tr>
</tbody>
</table>

### NIOSH

<table>
<thead>
<tr>
<th>Selenium and compounds except Selenium hexafluoride</th>
<th>REL: 0.2 μg/m$^3$ (TWA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenium and compounds</td>
<td>IDLH = 1 μg/m$^3$</td>
</tr>
</tbody>
</table>

**Note:**

- **TLV** = threshold limit value
- **PEL** = permissible exposure limit
- **REL** = reference exposure level
- **TWA** = time-weighted average
- **IDLH** = immediately dangerous to life or health
- **ACGIH** = American Conference of Government Industrial Hygienists
- **EPA** = Environmental Protection Agency
- **OSHA** = Occupational Safety and Health Administration
- **CAPCOA** = California Air Pollution Control Officers Association
- **NIOSH** = National Institute for Occupational Safety and Health
- **NOES** = National Occupational Exposure Survey
**The Usage of Se and its Compounds**

A decolorizing agent and pigments, Se and its compounds are used in the plastic, glass, ceramic and porcelain industries. According to its photoelectrical properties and semiconducting characteristics, Se is used in the making of electrodes for arc light, electrical instruments, photo cells, solar batteries, and semiconductor fusion mixtures. Other manufactures use Se as a part of photographic emulsions, and as vulcanizing agents in the process of rubber and metal alloys. It is also employed as antibacterial agent and in polymers. Se is added to increase the stability of stainless steel and copper alloys, and to enhance the wear resistance and elasticity of rubber. In addition, Se is added to improve the machinability of copper or lead or steel alloys, and to displace lead in the plumbing process.

The uses and sources of Se are summarized in Table 6 and the industrial applications of some Se compounds are shown in Table 7.


<table>
<thead>
<tr>
<th>Sources</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electronic industries</td>
<td>Photoelectrical devices and rectifiers (to convert alternately current to direct current), photo cells, solar batteries, semiconductor fusion mixtures, electrodes for arc light, and electrical instruments.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elemental selenium</td>
<td>In rectifiers, photoelectric cells, blasting caps, xerography, stainless steel; as dehydrogenation-catalyst</td>
</tr>
<tr>
<td>Sodium selenate (Na$_2$SeO$_4$)</td>
<td>As insecticide; in glass manufacture; in medicinal to control animal diseases</td>
</tr>
<tr>
<td>Compound</td>
<td>Use</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>-----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Sodium selenite ($\text{Na}_2\text{SeO}_3$)</td>
<td>In glass manufacture; as soil additive for selenium-deficient areas</td>
</tr>
<tr>
<td>Selenium diethyldithiocarbamate</td>
<td>Fungicide; vulcanizing agent</td>
</tr>
<tr>
<td>Selenium disulphide ($\text{SeS}_2$)</td>
<td>In antidandruff agents and fungicides</td>
</tr>
<tr>
<td>Selenium sulphide ($\text{SeS}$)</td>
<td>In fungicides for animals and people</td>
</tr>
<tr>
<td>Selenium dioxide ($\text{SeO}_2$)</td>
<td>Catalyst for oxidation, hydrogenations or dehydrogenation of organic compounds</td>
</tr>
<tr>
<td>Selenium hexafluoride ($\text{SeF}_6$)</td>
<td>As gaseous electric insulator</td>
</tr>
<tr>
<td>Selenium oxychloride ($\text{SeOCl}_2$)</td>
<td>Solvent for sulphur, selenium, tellurium, rubber, bakelite, gums, resins, glue, asphalt and other materials</td>
</tr>
<tr>
<td>Aluminium selenide ($\text{Al}_2\text{Se}_3$)</td>
<td>Preparation of hydrogen selenide for semiconductors</td>
</tr>
<tr>
<td>Aluminium selenite [(NH$_4$)$_2\text{SeO}_3$]</td>
<td>Manufacture of red glass</td>
</tr>
<tr>
<td>Arsenic hemiselenide ($\text{As}_2\text{Se}$)</td>
<td>Manufacture of glass</td>
</tr>
<tr>
<td>Bismuth selenide ($\text{Bi}_2\text{Se}_3$)</td>
<td>Semiconductor research</td>
</tr>
<tr>
<td>Cadmium selenide ($\text{CaSe}$)</td>
<td>Photoconductor, photoelectric cells, rectifier</td>
</tr>
<tr>
<td>Calcium selenide ($\text{CaSe}$)</td>
<td>In electron emitters</td>
</tr>
<tr>
<td>Cupric selenate ($\text{CuSeO}_4$)</td>
<td>In coloring Cu or Cu alloys black</td>
</tr>
<tr>
<td>Dimethyl selenide ($\text{CH}_3\text{Se}$)</td>
<td>In process for producing compound semiconductor using amorphous nucleation site; in process for producing semiconductor device using compound semiconductor obtained; as a magnetic recording medium</td>
</tr>
<tr>
<td>Indium selenide ($\text{InSe}$)</td>
<td>In semiconductor research</td>
</tr>
<tr>
<td>Tungsten diselenide ($\text{WSe}_2$)</td>
<td>In lubricants</td>
</tr>
</tbody>
</table>
Toxicity and Exposure to Se

Accidental and Suicidal Cases

Se is a useful element only in a very narrow spectrum of dosage; in excess, Se has harmful actions (Uminska, 1990). Acute toxicity of Se poisoning in humans has been reported in several accidental or suicidal cases.

A 3-year-old boy died 1.5 hours after he ingested an undetermined amount of selenious acid (Carter 1966), a 44-year-old man industrial worker died 1.5 hours after being exposed to 450 liters of neutralizing selenic acid and caustic soda mixture that erupted in a factory hall (Schellmann et al., 1986), and two men survived after ingesting either a 30 g/L of selenious acid or 1.7 g of sodium selenite (Gasmi et al., 1997).

Other cases were described as acute poisoning of Gun Blue solution that contained 4% selenious acid and 2.5% cupric sulfate in HCl. The same Gun Blue solution was ingested by a 2-year-old boy (15 ml) (Nantel et al., 1985), a 2-year-old girl (11 ml) (Lombeck et al., 1987), and two suicidal adult patients (Matoba et al., 1986; Pentel et al., 1985). Three of these cases of the Gun Blue ingestion resulted in death but the girl survived and recovered. In addition, excess oral intake of Se dioxide (Koppel et al., 1986) resulted in the death of a 17-year-old boy.

Furthermore, acutely inhaled hydrogen selenide was reported to cause dyspnea and pneumomediastinum in a healthy young man (Schecter et al., 1980). It also irritated the mucous membranes of nose, eyes and upper respiratory tract and induced gastrointestinal complaints, dental caries, conjunctivitis, nail deformities, and garlicky breath in a young adult woman (Alderman and Bergin, 1986). Clinical overall signs of acute Se toxicity include excessive salivation, tachycardia, nausea, vomiting, abdominal pain, abnormal liver function, diarrhea, restlessness, garlic odor in the breath, shallow breathing,
muscle aches, muscle spasm and pain, irritability, chill, and tremors (Carter, 1966; Koppel et al., 1986; Lombeck et al., 1987).

Se-induced death is due to hypotension as a consequence of both vasodilation and low cardiac output, respiratory distress syndrome, and severe myopathy that contributes to respiratory failure (Nantel et al., 1985). Findings in autopsy include burn of both esophagus and stomach, intestinal distension, congestion of lung and kidney, and pulmonary edema (Gasmi et al., 1997; Motaba et al., 1986). In addition, the highest concentrations of Se in the tissues of the patients were observed in the lungs, kidney and stomach (Schellmann et al., 1986; Koppel et al., 1986). According to these data, it seems that the lungs are target organs in Se exposure in man.

**General Public and Industrial Exposure to Se**

Se is extensively used in several industries. Because Se at high concentration is known to be toxic, there should be a greater understanding of its toxicity as well as its of beneficial effects. Both the general public and industrial workers may be exposed to Se by inhalation at worksites or in the atmosphere, or by ingestion in food (Medinsky et al., 1985). Inhaled Se is, of course, the route used by Se to cause harmful effects in the respiratory system. Combustion of domestic coal may also caused Se poisoning and may have adverse effects on the health of millions of people worldwide, particularly for the hundred million people in China that burn Se-rich raw coal in unvented stoves in their homes (Finkelman, 1999).

Humans are usually not exposed to large amounts of Se in the air, unless Se is present at the workplace. Fossil fuel combustion, smelting and refining processes can generate Se in the air and increase the possibility of Se inhalation. Se is one of toxic substances that workers are exposed to in smelter, refinery
industries (Brune et al., 1980; Gerhardsson et al., 1985, 1986, and 1988; Gerhardsson and Nordberg 1993; Holness et al., 1989; Wester et al., 1981), and also glass bangle workers (Rastogi et al., 1991). Neutron activation analysis and atomic absorption spectrophotometry was used to analyze the concentration of elemental Se in lung tissue of deceased smelter workers. Significantly higher levels of Se were found in the lungs of the smelter workers compared with rural controls (Gerhardsson et al., 1988; Gerhardsson and Nordberg, 1993). The levels of 23 elements, including Se, in the lungs, liver and kidneys of autopsy specimens from exposed workers in North Sweden were 2-16 times higher than those of control group (Brune et al., 1980). In addition, Diskin et al. (1979) reported an autopsy of a man, who had been employed in a Se refinery, exposed with red elemental Se and died with congestive heart failure, and found numerous perivascular granulomas and fibrosis in lung and high concentrations of Se in the peribronchial lymph nodes and lung.

Workers from copper industries that were exposed to Se compounds have reported an increased frequency of complaints of nose and eye irritation, as well as indigestion, stomach pain and fatigue (Holness et al., 1989). Additionally, glass bangle workers exposed to the salts of several heavy metals, including Se, showed a significantly higher prevalence of respiratory impairment in comparison to that observed in controls (Rastogi et al., 1991). Moreover, dust of elemental Se and Se dioxide can irritate the mucous membranes of the nose and throat and cause coughing, nosebleed, loss of sense of smell, dyspnea, bronchial spasms, bronchitis, and chemical pneumonia (Clinton, 1947). Concentrations of 0.007 - 0.05 mg Se/m³ have produced tracheobronchitis in 9 of 62 rectifier workers (Kinnigkeit, 1962). In addition, a drum manufacture worker came down with alopecia aereta which later deteriorated to alopecia universalis, after working 6 months in photocopy machines involving the use of a Se alloy (Srivastava et al., 1995).
Experimental Investigations on Inhaled Se

The experimental studies that have been published on the toxicity of inhaled Se, I describe now the main information coming from these previous studies are not many. Most of these articles are case reports of humans accidentally exposed to Se inhalation.

Early research by Hall et al. (1951) revealed that rats exposed to Se dust at levels of 33 mg Se/m³ showed severe respiratory distress, including hemorrhage and edema of the lungs; histopathological examination revealed chronic interstitial pneumonitis. It also resulted in mild interstitial pneumonitis or congestion, vascular lymphocytic infiltration, intra-alveolar foci of large macrophages, and slight emphysema in both guinea pigs and rats (Hall et al., 1951). Exposure by acute inhaled hydrogen selenide of guinea pigs to amounts of 0.002 to 0.57 mg/L (10, 30 and 60 min) resulted in slight thickening of the alveolar wall, and congestion of alveolar capillaries (Dudley and Miller, 1937), and higher amounts (8 mg Se/m³) used during (4 hrs) produced diffuse bronchopneumonia, and pneumonitis (Dudley and Miller, 1941).

In contrast, 1-hour exposure of rats to 25,958 mg Se/m³ as dimethyl selenide produced only minor effects (increased weight of lungs and liver); these changes disappeared after 7 days (Al-Bayati, 1992). In addition, intratracheal injection of Se dioxide, at a dose of 0.06 mg Se/100 g wt, caused pulmonary dysfunction in guinea pigs (Nonavinakere et al., 1999). Intratracheal injection of male guinea pig with sodium selenite (0.06 or 0.3 mg Se per animal) caused leukocytic infiltration in the lung that triggered local edema and injury (Bell et al., 2000).

Rhoads and Sanders (1985) studied lung clearance of several metal oxides, and found that the time used to remove 50% of Se (44 min) from the
lungs was longer than for arsenic (31 min), but shorter than cadmium (8 hours), cobalt (15 days), or beryllium (405 days). In addition, arsenic, cadmium, lead, vanadium and Se were soluble in the lung, and around 1-20% fraction remained in the lung for long time. Medinsky et al. (1981b) concluded that inhaled selenious acid is absorbed into blood faster than elemental Se. This is consistent with the findings of Weissman et al. (1983) reporting that inhaled selenious acid was more rapidly absorbed to blood circulation than aerosol Se, these metal substances were then distributed to liver, kidney, spleen and heart. The continuous inhalation of Se at threshold limit value, leads to Se accumulation in the lung, liver, and blood with the values of 22,000, 1,200 and 440 ng S/g, respectively (Medinsky et al., 1985). The burden of Se in the body was found to come more from dietary sources than from inhalation intake (Bennett, 1983).

Se or selenious acid was shown to decreased the numbers of viable alveolar macrophages *in vitro* (Medinsky et al., 1981a). Gregson et al. (1982) found that alveolar macrophages and Type-II pneumonocytes are the predominant phagocytes that take up horseradish peroxidase antigen after its intratracheal instillation. Medinsky and his co-workers (1981a) reported that Se and selenious acid (0.001 to 0.5 mMSe) caused cell lysis of rabbit alveolar macrophages. Gabor et al. (1985) have compared the cytotoxicity of Se (as sodium selenite), quartz, and quartz+selenite in guinea pig peritoneal macrophages. They found that quartz decreased cell viability, adhesiveness and migration and increased lipid peroxidase, whereas selenite was shown to be a nontoxic agent in all of these cytotoxic parameters. This protective role of selenide was also observed with selenomethionine that caused a significant enhancement of the survival of cultured peritoneal macrophages, and also with sodium selenite that delayed the toxicity of mercury (Christesen et al., 1989).

The acute effects of selenium-enriched spiruline (SESP) treatment was expressed by severe lung injury that included hyperemia, hemorrhage, exudation
and thickness of alveolar well; hydroxyproline and type III collagen mRNA were, however, decreased (Bai et al., 1998). Enhanced elastin and decreased collagen were observed in the lungs of rats treated with high amounts of sodium selenite (0.3 mg/kg BW) 10 weeks earlier (Kucharz and Olczyk, 1993).

The relative scarcity of published studies on the toxicity of inhaled Se indicates that this area of experimental toxicology needs further research. This is the reason for performing the experiments reported in this thesis.
Chapter 2

Objectives
OVERVIEW OF THE AIMS OF THIS THESIS

The main goal of this thesis was to investigate the inflammatory and morphological responses of the murine respiratory system that are caused by intratracheal instillation of Se and one of its derivatives. Several components can be distinguished as partial aims of this dissertation:

1. To investigate the early pathology of respiratory tissues caused by Se inhalation (Chapter 3.1).

2. To evaluate the cellular kinetics of inflammation detected in BAL samples after Se instillation to mice (Chapter 3.2).

3. To identify Se particles in situ and at high resolution in pulmonary phagocytes and in the lung tissue (Chapters 3.3 and 3.5).

4. To search for changes in collagen in the deep lung after a single Se intratracheal instillation (Chapter 3.4).

The initial reason to start these investigations came from the few publications that are currently available on the changes of the respiratory tract after experimental exposure to Se or its derivatives. The research work was focused on the kinetics of the pathological alterations of the respiratory epithelia that are caused by Se inhalation (See Chapter 3.1). For that, we have characterized inflammation and cell injury of the deep lung (see Chapter 3.2); this was done by the analysis of the BAL fluid, namely its content in leukocytes, serum proteins (marker of increased permeability of the alveolar-capillary barrier), and lactate dehydrogenase (marker of cytotoxicity).

Because of the frequent fibrotic transformation of the lung in response to toxic chemicals, we have studied the changes in the staining of different collagen types in the lung triggered by inhalation of a Se derivative. This was based on the reports of inflammation-related alterations in the amount and distribution of collagens I and IV that are known to lead to fibrotic lungs. We
have, thus, decided to investigate the distribution of collagen type I, II and IV in
the lung after instillation of a Se derivative to mice (see chapter 3.4 of thesis).

Because epithelial cells and resident macrophages of the respiratory are
the first to interact with inhaled toxic chemicals, we have studied these cells
after Se instillation. For that, we have used high-resolution methods both to
identify Se inside alveolar macrophages and to define the sorting of Se particles
in the lung (see chapter 3.3 and 3.5 of the thesis).
Chapter 3

Experimental Results and Articles
Chapter 3.1

Pathology of Respiratory Epithelia
Caused by Selenium Inhalation
Pathology of respiratory epithelia caused by selenium inhalation

Toxic effects of selenium inhalation: acute damage of the respiratory system of mice

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2Department of Medical Science, Faculty of Science, Burapha University, Thailand;
3Department of Pathology, Portuguese Oncology Institute, Porto, Portugal

Accidental inhalation of selenium by humans has been associated with damage of respiratory tissues that is lacking a detailed histological definition. We have investigated the natural history of injury to the tracheal epithelium and lungs induced by a single intratracheal instillation of CD-1 mice with two different doses of dimethyl selenide (0.05 and 0.1 mg Se/kg of body weight). The animals were sacrificed 1, 7, 14, and 28 days after the single selenium treatment. Samples of the trachea and lungs were studied by light microscopy. The tracheal epithelium showed loss of cilia and acute necrosis that was followed by metaplastic transformation. Edema and diffuse alveolar damage was observed in the lungs. Our data suggest that: i) severity of respiratory lesions caused by selenium is dose dependent; ii) selenium causes transient metaplastic transformation of the tracheal epithelium; iii) chronic inflammation and increased thickness of alveolar septa occur in the lungs; iv) 4 weeks after selenium treatment, mice recover from the tracheal injury, whereas no amelioration of pulmonary lesions was observed. Human & Experimental Toxicology (2003) 22, 551–557

Key words: inflammation; lung alveoli; lung edema; metaplasia

Introduction

Selenium (Se) participates in many physiological processes, e.g., the biosynthesis of coenzyme Q (a component of mitochondrial electron transport systems), regulation of ion movement across membranes, maintenance of keratin integrity, stimulation of antibody synthesis, and activation of glutathione peroxidase, an enzyme involved in the prevention of oxidative damage.1,2 Physiological levels of selenium in the blood and in the tissues have to be tightly regulated because excess of selenium, either through inhalation or oral ingestion, is toxic for mammals.

Accidental ingestion of selenium by humans of toxic amounts of selenium induces acute vomiting, dyspnea, pulmonary congestion, bleeding, convulsions, generalized edema, and it may lead to death due to respiratory failure.1–9 Necropsies of human fatalities caused by selenium also showed congestion and bleeding of liver and kidney.9,10,11 In contrast with the comprehensive information available on the toxicity of selenium after oral ingestion, scarce data are available on the effects of accidental inhalation of selenium. This is due to the relative rarity of human exposure to airborne selenium. This health hazard may, nevertheless, occur accidentally in a number of industries. In fact, selenium is widely used in glass, ceramic, and porcelain plants as a decolorizing agent and pigment, and also in the production of electrodes for arc light, electrical instruments, photo cells, solar batteries, and semiconductor fusion mixtures. In addition, selenium is employed as a photographic emulsion, as a vulcanizing agent in the process of rubberization, and also in metal alloys.12,13

In order to advance current understanding of respiratory lesions caused by selenium, we have investigated the changes that occur during 4 weeks in the trachea and lungs of mice submitted to a single intratracheal instillation of dimethyl selenide used at two different doses.
Pathology of respiratory epithelia caused by selenium inhalation

Materials and methods

**Selenium**
Dimethyl selenide ($\text{C}_2\text{H}_5\text{Se}$) in liquid form, at analytical grade, was purchased from Fluka Chemical Co. (ref. no. 41572) with a purity greater than 99%.

**Animals**
For histological studies, we have used 64 one-month-old CD-1 mice (Charles River strain) of both sexes (32 males and 32 females). Additional mice were employed to determine mortality rates. An equal number of males and females was used in all experimental groups for histological investigation. Distribution of animals for experimental groups is shown in Table 1. The mice weighed about 20 g, were kept under standard housing conditions, and had unrestricted access to food (commercial chow) and water. They were treated in accordance with the European Union law on animal protection (directive 86/609/EC). Two groups of 24 mice each were exposed to intratracheal instillation of dimethyl selenide, each group receiving one of two different amounts: 0.05 or 0.1 mg Se/kg of body weight (BW). Additional 16 mice were used as controls.

**Intratracheal instillation of dimethyl selenide**
The CD-1 mice were anesthetized by intramuscular injection of 4.0–6.0 mg/kg BW of ketamine (Ketalar, Parke-Davis Co., Barcelona, Spain), and of 0.8–1.6 mg/kg BW of xilazine (Rompun, Bayer Co., Amares, Portugal). The anesthesia was 15–20 min long. Anesthetized mice were placed on a surgical board, and their neck exposed by surgical dissection until reaching the tracheal wall. Dimethyl selenide was delivered to the lungs through the tracheal wall with the use of a Hamilton microsyringe. The total volume of dimethyl selenide instilled in tracheas of the mice was 2 μL. In control mice equal volume of 0.9% saline was injected in the tracheal airway. After the instillation, mice were held up during 1 min, the surgical wound was closed, and the animals returned to their cages. The mice were observed twice daily for signs of toxicity. Six mice for each selenium dose were sacrificed at 1, 7, 14 and 28 days after the treatment. Controls were also sacrificed 1, 7, 14 and 28 days after the intratracheal injection. Tracheas and lungs of the sacrificed mice were rapidly removed and fixed by immersing in Bouin’s solution. The tissue samples were processed for paraffin embedding by standard methods. Transverse sections of tracheas (3-μm thick) and sagittal sections of lungs were cut in a Leica RM 2125 RT microtome. They were stained with haematoxylin-eosin and Masson’s trichrome and viewed by light microscopy.

**Results**

**Mortality**
We found that the selenium-induced acute mortality of mice (i.e., death of animals up to 1 hour after treatment) depended on the dose of dimethyl selenide that was instilled in the tracheal airway. Of the 31 mice treated with the low dose (0.05 mg Se/kg BW) only one died, whereas nine out of 60 mice (i.e., 15%) treated with the high dose (0.1 mg Se/kg BW) died within 1 hour (Table 2). Total mortality (i.e., death of animals during the overall 28 days period of this investigation) of mice submitted to the low dose of selenium was lower (3/31, i.e., 9.7%) than that of mice treated with the high dose of the metal (23/60, i.e., 38.3%).

**Clinical symptoms**
The earliest clinical change observed in mice after selenium instillation was tachypnea (i.e., increase frequency of respiratory movements) that started within 2–3 min of the treatment and continued for at least 10–15 min. In most of the mice treated with the high dose of selenium, there was evidence of dyspnea throughout the first two weeks of the study. In addition, some of these mice showed tremor, loss of mobility, and decreased food intake. Necropsies of mice that died from the selenium treatments were

<table>
<thead>
<tr>
<th>Dose (mg Se/kg BW)</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>Treat</td>
<td>Control</td>
<td>Treat</td>
<td>Control</td>
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<td>( \downarrow = 3 )</td>
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</table>

Table 1: Total number of mice used in this study

- 29 -
Table 2  Lethal effect of a single intratracheal instillation of dimethyl selenide to 1-month-old CD-1 mice

<table>
<thead>
<tr>
<th>Dose (mg Se/kg wt)</th>
<th>Group</th>
<th>Sex</th>
<th>N</th>
<th>Mortality (Days)</th>
<th>Total</th>
<th>Percent</th>
<th>Total percent</th>
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<td>♂</td>
<td>4</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>treated</td>
<td>♂</td>
<td>15</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>0.1</td>
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<td>♂</td>
<td>27</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>treated</td>
<td>♂</td>
<td>35</td>
<td>8</td>
<td>-</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

not conclusive in the identification of cause of death.

Pathology induced by low dose of dimethyl selenide (0.05 mg Se/kg BW)

Trachea  The major alterations observed in the trachee of mice treated with 0.05 mg Se/kg BW were, initially, the transformation of the epithelium from the pseudostratified ciliated type into low columnar (Figure 1), and, later on, its progression into stratified squamous metaplasia (Figures 4 and 6). The tracheal lining also showed an early loss of cilia. There was evidence of acute damage of the epithelium involving necrosis of the lining cells that was observed in four out of six animals after 1 day of the selenium instillation (Figure 2A and B). Epithelial cell death was no more evident in tracheas collected at later timings. In those samples there was progressive metaplastic transformation of the epithelium. This was particularly exuberant in one of the mice that was sacrificed at day 14; this animal presented a large cushion of squamous metaplastic cells growing into the tracheal lumen (Figure 3). Morphology of tracheas of mice sacrificed 28 days after metal instillation suggested that there was an almost complete recovery of the earlier lesions caused by selenium on the tracheal epithelium.

Lungs  Lung samples from these mice showed either the multiple morphological abnormalities that are commonly described as diffuse alveolar damage (DAD) (Figure 5A and B), or they just presented edema. DAD encompassed, in addition to edema, inflammatory infiltration with enlarged thickness of alveolar septa, hyperplasia of the alveolar lining, blood vessel congestion, and increased cellularity of the lung. Edema alone was seen in two of the six mice 1 day after treatment, it increased to four/six at day 7, and was absent at days 14 and 28. In contrast, DAD was the dominant lung pathology both at day 1 (four/six) and at day 28 (five/six). Increased cellularity of the alveoli was particularly evident 14 days after the selenium instillation. One of the mice sacrificed 28 days after the selenium treatment showed a lung morphology that was comparable to that of controls.

Table 3  Pathology induced by high dose of dimethyl selenide (0.1 mg Se/kg BW)

Pathology induced by high dose of dimethyl selenide (0.1 mg Se/kg BW)

Trachea  The instillation of a higher dose of selenium was associated with increased severity of lesions observed in the tracheal lining. In fact, evidence of epithelial necrosis was found in four of six animals after 1 day of treatment. Metaplastic transformation of the low columnar lining was also detected earlier than after the administration of the low dose of selenium. Also, two of the six mice sacrificed after 7 days of selenium instillation presented large cushions of metaplastic cells, that had been seen after the low dose of selenium in just one mouse after 14 days of treatment. Increased thickness of mucous layer covering the tracheal lining was also observed in some of the mice undergoing epithelial necrosis 1 day after treatment. Resembling what was found in mice submitted to the low dose of selenium, most animals (five/six) showed signs of recovery of tracheal lesions 28 days after the initial treatment.

Lungs  The most striking morphological feature of the lungs of these mice was that all animals presented edema and DAD, or more severe pathology, such as lung hemorrhage or bronchiolitis obliterans-organizing pneumonia (Figure 7A, B). Therefore, lung injury caused by the high dose of selenium appears to evolve rapidly into the several tissue lesions that characterize DAD. This fast progression into DAD contrasts with what was observed in mice treated with the low dose of selenium. We present a summary of our data in Table 3 where we compare the morphological changes of mouse tracheas and lung after each of the two instillations of dimethyl selenide.
Figure 1  Light micrographs of paraffin sections of trachea and lung of CD-1 mice submitted to a single instillation of the low dose selenium (0.05 mg Se/kg BW). Section of trachea showing low columnar epithelium at day 1. HE staining (x 400).

Figure 2  Light micrographs of paraffin sections of trachea and lung of CD-1 mice submitted to a single instillation of the low dose selenium (0.05 mg Se/kg BW). (A) Section of trachea showing epithelial necrosis and inflammatory cells infiltrating of several layers of the tracheal wall, 1 day after instillation of selenium. HE staining (x 100). (B) Higher magnification showing infiltrating inflammatory cells (x 400).

Figure 3  Light micrographs of paraffin sections of trachea and lung of CD-1 mice submitted to a single instillation of the low dose selenium (0.05 mg Se/kg BW). Section of trachea 14 days after selenium instillation showing a cushion of squamous metaplastic cells protruding into the lumen of the airway. Masson's trichrome staining (x 400).

Figure 4  Light micrographs of paraffin sections of trachea and lung of CD-1 mice submitted to a single instillation of the low dose selenium (0.05 mg Se/kg BW). Section of tracheal 28 days after selenium administration showing squamous metaplasia of the epithelium. HE staining (x 400).

Figure 5  Light micrographs of paraffin sections of trachea and lung of CD-1 mice submitted to a single instillation of the low dose selenium (0.05 mg Se/kg BW). (A) Section of lung 28 days after selenium administration illustrating diffuse alveolar damage with increased thickness of the septa. HE staining (x 100). (B) Higher magnification showing increased cellularity of the lung (x 400).
Discussion

The herein study reports on the pathology induced in the tracheal epithelium and in the lungs of mice by intratracheal instillation of two different doses of dimethyl selenide. The major contributions of this investigation are: i) the degree of severity of selenium-induced respiratory lesions depends on the dose of selenium that is inhaled; ii) selenium causes transient metaplastic transformation of the tracheal epithelium; iii) edema and diffuse alveolar damage are the major alterations observed in the lungs of selenium-treated animals; iv) 4 weeks after selenium treatment, the tracheal lining appears recovered from the earlier damage, whereas no amelioration of lung lesions is seen.

A few reports have addressed before the changes of the respiratory tract induced by intratracheal administration of selenium. These studies have not aimed at establishing, as we have done here, a comprehensive kinetics of tissue damage that occurs along the several weeks following the initial instillation of selenium. Our work is, therefore, the first to offer detailed information on the natural history of tissue pathology attending a single instillation of selenium into the respiratory airway. Bell and co-workers have investigated the histology of lungs after a single intratracheal injection of selenium in guinea pigs. Their investigation was limited to animals sacrificed 24 hours after the selenium instillation, and they reported moderate leukocytic infiltration and edema of the lung. Nonavinkere et al. showed that intratracheal instillation of selenium dioxide caused severe impairment of the respiratory physiology of guinea pigs. Rhoads and Sanders compared lung clearance of different metal oxides, and found that the time needed to remove 50% of the initial burden of selenium from the lungs was relatively short (44 min), i.e., comparable with the clearance of arsenic (31 min), but much shorter than the clearance time for cadmium (8 hours), cobalt (15 days), or beryllium (405 days). Morgan et al. used rats to study the effects of intratracheal injection of new compounds used in semiconductor industries that are made of selenium, gallium and copper. The inflammatory alterations that they have observed in the lungs of these animals can not however be ascribed solely to the toxicity of selenium.

Accidental inhalation of selenium by workers has been reported during extraction, purification, and pyrolysis processes of copper refining, in metal rectifying, and in selenium-recovery process, and in other industrial activities involving the metal. It caused irritation and injury of the respiratory tract.

Figure 6 Light micrographs of paraffin sections of trachea and lung of CD-1 mice submitted to a single instillation of the high dose selenium (0.1 mg Se/kg BW). Section of trachea 7 days after the selenium instillation depicting epithelial metaplasia. HE staining (×400).

Figure 7 Light micrographs of paraffin sections of trachea and lung of CD-1 mice submitted to a single instillation of the high dose selenium (0.3 mg Se/kg BW). (A) Section of lung illustrating bronchiolitis obliterans-organizing pneumonia, 28 days after selenium administration. Masson's trichrome staining (×100). (B) Higher magnification showing inflammatory and alveolar cells (×400).
Pathology of respiratory epithelia caused by selenium inhalation

D Cherdwongchareonsuk et al.

Table 3 Morphological changes of mouse trachea and lung after single intratracheal instillation of dimethyl selenide

<table>
<thead>
<tr>
<th>Days after instillation</th>
<th>Trachea</th>
<th>Lung</th>
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<tr>
<td>0.05 mg Se/kg BW</td>
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<tr>
<td>1 - low columnar</td>
<td>- lung edema</td>
<td>- low columnar</td>
</tr>
<tr>
<td>(δ = 2)</td>
<td>(γ = 2)</td>
<td>(δ = 1)</td>
</tr>
<tr>
<td>- necrosis</td>
<td>- DAD</td>
<td>- DAD</td>
</tr>
<tr>
<td>(δ = 1, γ = 3)</td>
<td>(δ = 3, γ = 1)</td>
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<tr>
<td>7 - low columnar</td>
<td>- lung edema</td>
<td>- low columnar</td>
</tr>
<tr>
<td>(δ = 1)</td>
<td>(δ = 2, γ = 2)</td>
<td>(δ = 3, γ = 3)</td>
</tr>
<tr>
<td>- stratified squamous</td>
<td>- DAD</td>
<td>- DAD</td>
</tr>
<tr>
<td>(δ = 1, γ = 3)</td>
<td>(δ = 3, γ = 1)</td>
<td></td>
</tr>
<tr>
<td>14 - squamous metaplasia</td>
<td>- increased cellularity</td>
<td>- normal</td>
</tr>
<tr>
<td>(δ = 3, γ = 3)</td>
<td>(δ = 2, γ = 2)</td>
<td>(δ = 1)</td>
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<td>- large cushion of squamous metaplasia</td>
<td>- squamous metaplasia</td>
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</tr>
<tr>
<td>(δ = 1, γ = 1)</td>
<td>(δ = 1)</td>
<td></td>
</tr>
<tr>
<td>- squamous metaplasia</td>
<td>- DAD</td>
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<tr>
<td>(δ = 1, γ = 3)</td>
<td>(δ = 3, γ = 1)</td>
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</tr>
<tr>
<td>28 - normal</td>
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</tr>
<tr>
<td>(δ = 2)</td>
<td>(γ = 3)</td>
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<tr>
<td>- squamous metaplasia</td>
<td>- DAD</td>
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</tr>
<tr>
<td>(δ = 2)</td>
<td>(δ = 3, γ = 2)</td>
<td></td>
</tr>
</tbody>
</table>

Note: DAD = diffuse alveolar damage; BOOP = bronchiolitis obliterans-organizing pneumonia

that was expressed by coughing, nose bleeding, loss of the sense of smell, dyspnea, bronchial spasms, bronchitis, chemical pneumonia and tracheobronchitis. Investigations on acute inhalation of selenium dust have documented interstitial pneumonitis, lung hemorrhage, and edema. Nevertheless, the detailed damage induced by selenium in the respiratory system, and its natural history, has not been well established.

Our experimental data offer the structural setting for the respiratory symptoms of humans exposed to airborne selenium, and we also extend the information of previous reports on experimental pathology induced by selenium. The four week follow up of morphological changes of trachea and lung of selenium-treated mice demonstrated the acute necrotic effect of the metal on the tracheal lining, with subsequent metaplastic transformation of the epithelium, and it pointed to a more severe and durable damage induced by selenium on the alveolar septa of the lung than on the trachea.

Acknowledgements

The authors are very grateful to Mr Antonio Costa e Silva, Mr Emanuel Monteiro, Dr Madalena Costa, and Mrs Alexandrina Ribeiro for technical assistance. This work was supported in part by grants from Fundação Oriente, FCT and IDICT, Portugal.

References

Pathology of respiratory epithelia caused by selenium inhalation


Chapter 3.2

Respiratory Inflammation Caused by Dimethyl Selenide
Respiratory Inflammation Caused by Dimethyl Selenide
Cherdwongcharoensuk et al.

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Respiratory inflammation caused by dimethyl selenide

Changes in Bronchoalveolar Lavage Cells after Intratracheal Instillation of Dimethyl Selenide in Mice

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Respiratory inflammation caused by dimethyl selenide

**Key words:** Selenium, Neutrophil, Macrophage, Inflammation, Leukocyte, Lymphocyte, Lung.
## Respiratory inflammation caused by dimethyl selenide

### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BAL</td>
<td>bronchoalveolar</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
</tr>
<tr>
<td>Co</td>
<td>company</td>
</tr>
<tr>
<td>DMSe</td>
<td>dimethyl selenide</td>
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<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>Se</td>
<td>selenium</td>
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Respiratory inflammation caused by dimethyl selenide

Abstract

CD-1 mice were submitted to a single intratracheal instillation of either one of two doses of dimethyl selenide (DMSe) (0.025 or 0.075 mg Se/kg wt). They were studied during 4 weeks to define the cellular inflammatory response of the airways to DMSe. Bronchoalveolar (BAL) lavage was used to collect the DMSe-induced inflammatory exudates. The DMSe instillation resulted in phlogistic responses that had the neutrophil as the main leukocyte; they were present in BAL samples, mostly at days 1 and 7. Macrophages were also increased during DMSe-induced inflammation. The lower DMSe dose resulted in an inflammatory reaction lasting for 2 weeks. Mice treated with the higher DMSe dose still showed elevated numbers of neutrophils and macrophages 4 weeks after instillation. DMSe did not change the number of lymphocytes harvested from the airways. An early increase in total protein of BAL, and late enhancement in lactate dehydrogenase was observed in mice treated with the high DMSe dose. We conclude that inhalation of DMSe triggers a moderate and dose-dependent inflammatory reaction in the mouse airways, and that this phlogistic reaction is likely to participate in the damage of respiratory epithelia that occurs upon DMSe inhalation.
Respiratory inflammation caused by dimethyl selenide

Introduction

Selenium (Se) is, at low concentration, a key element in a number of essential pathways of human metabolism (Oldfield, 1987; Bedwal et al., 1993; Barceloux, 1999). Because of its antioxidative activity, it may also play a preventive role against cancer (Gerhardsoon, 1985; Wilkinson and Chodak, 2003). Above physiologic levels, Se becomes harmful to humans (Vinceti et al., 2001). Most studies on the toxicology of Se have dealt with the pathology caused by excessive oral intake of Se (Nantel et al., 1985; Pentel et al., 1985; Köppel et al., 1986; Matoba et al., 1986; Gasmi et al., 1997). Few investigations have been devoted to the effects of inhaled Se. Bell and co-workers (1997 and 1998) have documented alterations caused by Se on the guinea pig lung after a single intratracheal injection performed 24 hours earlier; they showed that Se modified Bronchoalveolar lavage (BAL) samples by increasing the activity of lactate dehydrogenase (LDH), beta-glucuronidase, alkaline-phosphatase, and also the protein content. Enhanced prostaglandin concentration and decreased thromboxane and leukotriene in the lung were also reported by the same authors after Se instillation. These biochemical changes were associated with constriction of the large airways and impairment of lung function. In addition, Nonavinakere and co-workers (1999) described that intratracheal instillation of Se in guinea pigs resulted in pulmonary dysfunction that involved lung resistance and alteration of the respiratory rate.

We have recently documented structural changes of respiratory epithelia of mice after intratracheal instillation of dimethyl selenide (DMSe) (Cherdwongcharoensuk et al., in press). Because we had observed infiltrates of inflammatory cells in association with DMSe-induced lesions of trachea and lung, we decided to investigate now the cellular composition of the local inflammatory reaction caused by DMSe instillation in the respiratory airways of mice. We report here on the cytology and biochemistry of BAL samples during
Respiratory inflammation caused by dimethyl selenide

the 4 weeks that followed treatment of mice with a single intratracheal instillation with either one of two different doses of DMSe.
Materials and Methods

Dimethyl selenide (DMSe)

Dimethyl selenide (C$_2$H$_6$Se) in liquid form, at analytical grade, was purchased from Fluka Chemical Co (ref. no. 41572) with a purity grade greater than 99%.

Animals

72 one-month-old CD-1 mice (Charles River strain) of both sexes (36 males and 36 females) were purchased to a commercial breeder (Charles River Laboratories SA, Spain). Equal numbers of males and females were employed in all experimental groups. The mice weighed 20 g, were kept under standard housing conditions, and had unrestricted access to food (commercial chow) and water. They were treated in accordance with the European Union law on animal protection (directive 86/609/EC). Mice were exposed to intratracheal instillation of either one of two different amounts of Se: 0.025 or 0.075 mg/kg of weight (wt) of DMSe in 10 µl of saline. Control mice received an intratracheal instillation of 10 µl of saline.

Intratracheal Instillation of Se

The CD-1 mice were anesthetized by intramuscular injection of 4.0-8.0 mg/kg BW of ketamine (Ketalar, Parke-Davis Co., Barcelona, Spain), and of 0.8-1.6 mg/kg BW of xilazine (Rompun, Bayer Co., Amadora, Portugal). The anesthesia was 15-20 min long. Anesthetized mice were placed on a surgical board, and their necks exposed by surgical dissection until reaching the tracheal wall. DMSe was delivered to the lungs through the tracheal wall with the use of a Hamilton microliter syringe. In control mice, an equal volume of 0.9% saline was injected in the tracheal airway. After the instillation, mice were held up during 1 min, the surgical wound was closed, and the animals returned to their cages. The mice were observed twice daily for signs of toxicity. Six mice for
Respiratory inflammation caused by dimethyl selenide

each Se dose were sacrificed at 1, 7, 14 and 28 days after the single instillation. Controls were also sacrificed 1, 7, 14 and 28 days after the intratracheal injection.

**Bronchoalveolar Lavage (BAL)**

Mice were anesthetized by intramuscular injection of 4.0–8.0 mg/kg BW of ketamine (Ketalar, Parke-Davis Co., Barcelona, Spain), and of 0.8–1.6 mg/kg BW of xilazine (Rompun, Bayer Co., Amadora, Portugal). This was followed by exsanguination by cutting the abdominal aorta and by securing a blunt 21-gauge needle into the trachea. Lungs were lavaged five times with 0.25 ml PBS, and 1 ml of pooled lavage fluid was recovered. Total white blood cell counts were determined using SEAC cell counters 2013, and 100 μl of fluid cytospun (5 min, 1000 rpm) onto slides was stained with Giemsa for cellular differentials. More than one hundred cells per sample were identified by light microscopy and counted under oil immersion. BAL fluid was also analyzed for total protein and LDH by Roche Cobas Integra system.

**Statistical Analysis**

Numerical data is expressed as average ± standard deviation. Statistical comparison between experimental groups was performed using nonparametric approach, followed by two relate sample comparison using the Wilcoxon test. Statistical significance was considered at p<0.05.
Results

The overall mortality rate of CD-1 mice to intratracheal instillation of dimethyl selenide (DMSe) was 14% for the lower dose (0.025 mg/kg wt) and 39% for the higher dose (0.075 mg/kg wt). Death of mice occurred only during the first 3 days that followed the DMSe instillation (Table 1).

We found that the intratracheal instillations of DMSe resulted in a statistically significant (p<0.05) increase in the number of white blood cells harvested by BAL from the respiratory airways of the mice (Fig. 1). The increase in BAL leukocytes caused by the instillation of the lower dose of DMSe was restricted to the first 2 weeks that followed the treatment. The higher dose of DMSe triggered higher numbers of BAL leukocytes than the lower one; these elevations were seen during the whole 4 weeks of follow-up of the DMSe effects in the mice (Fig. 1).

The neutrophil was the more frequent leukocyte that was found in BAL samples of mice after intratracheal instillation of DMSe (Fig. 2A). The higher dose of DMSe caused the harvesting of a significant larger number of neutrophils in the mouse airways than the lower DMSe dose. Furthermore, the neutrophilic inflammation caused by the higher dose lasted longer than the one induced by the lower dose of DMSe which was absent from the second week on after. A second peak of granulocytes was detected 4 weeks after the treatment of the animals with the higher dose of DMSe (Fig. 2A).

Interestingly, the acute inflammatory reaction observed after 1 day of instillation was different between the two doses of DMSe: neutrophils were in significant higher numbers in BAL samples of mice treated with the higher dose (Fig. 2A), whereas the number of macrophages was higher after the treatment of mice with the lower dose (Fig. 2B). Elevated numbers of macrophages were observed in BAL samples throughout the whole 4-week period of the experiments with both doses of DMSe (Fig. 2B).
No significant changes in the number of BAL lymphocytes was caused by DMSe throughout the 4 weeks course of this study (Fig. 3). Values for LDH and total protein measured in the BAL fluid were generally not modified by the DMSe treatments, with the exception of a significant elevation observed 4 weeks after treatment of the mice with the high dose of DMSe (Figs. 4 and 5). Treatment of mice with the high DMSe dose caused an early increase in the protein content of BAL samples that was detected only 1 day after the instillation.
Discussion

We have characterized here the inflammatory response of the respiratory airways of the mouse to intratracheal instillation of dimethyl selenide (DMSe). Most of the studies on the toxicology of selenium (Se) have addressed its oral ingestion (Nantel et al., 1985; Pentel et al., 1985; Köppel et al., 1986; Matoba et al., 1986; Gasmi et al., 1997). Only a few investigations have documented that Se inhalation is harmful for the respiratory airways and for the lungs (Bell et al., 1997 and 1998; Nonavinakere et al., 1999 Cherdwongcharoensuk et al., in press). Human exposure to airborne Se may occur accidentally in several industrial activities. Se is employed in glass, ceramic, and porcelain plants as a decolorizing agent and pigment, and also in the production of electrodes for arc light, electrical instruments, photocells, solar batteries, and semiconductor fusion mixtures. Se is also used as a photographic emulsion, as a vulcanizing agent in the process of rubberization, and also in metal alloys (Glover, 1970; Holness et al., 1989).

The herein data reveals that DMSe caused a neutrophilic inflammation of the respiratory airways, and that this change was present for several weeks after a single instillation of DMSe. Interestingly, lymphocytes were not found to be attracted in significant numbers to the airways in response to the presence of DMSe, suggesting that the immunogenicity of Se is probably low for the respiratory system.

We found that the number of leukocytes and the duration of local inflammation depended on the dose of inhaled DMSe: the higher dose (3 times the lower dose) induced about the double of white blood cells in the airway than the lower dose. In addition, the inflammation lasted longer. This indicates that the local phlogistic reaction triggered by DMSe inhalation is a dose-dependent phenomenon. We observed a second elevation in the number of neutrophils 4 weeks after the instillation of higher dose of DMSe in mice. This event recalls
similar secondary peaks of granulocytes that we have described in a previous study on the inflammatory response of the respiratory tract to the presence of exogenous particles of tungsten (Peão et al., 1993).

In a recent study, we have characterized by light microscopy the DMSemi-induced lesions observed in trachea and lungs of treated mice (Cherdwongcharoensuk et al., in press). The current investigation offers a rationale for the pathology that we and others have reported in respiratory tissues after DMSemi instillation (Bell et al., 1997 and 1998; Nonavinakere et al., 1999, Cherdwongcharoensuk et al., in press). In fact, the herein observations indicate that inflammatory neutrophils and macrophages are likely mediators of the alterations caused in respiratory tissues by the presence of Se in the airways. In conclusion, we have established here the cellular kinetics of the inflammatory response of the respiratory tract to the presence of inhaled Se, and we propose that the neutrophilic response is a key cellular mediator of the previously described pathology of respiratory tissues during the first weeks that follow Se inhalation.
Acknowledgments

The authors are very grateful to Mr. António Costa e Silva, Mr. Emanuel Monteiro, Dr. Madalena Costa, Mrs. Alexandrina Ribeiro, Mrs. Maria Julia da Silva Reis, and Mrs. Maria Isabel Matos Garrido for technical assistance. This work was supported in part by grants from Fundação Oriente and IDICT, Portugal.
Respiratory inflammation caused by dimethyl selenide

References


Respiratory inflammation caused by dimethyl selenide

Legends of the figures

Table 1. Mortality of CD-1 mice to intratracheal instillation of DMSe at two different doses. The higher dose of DMSe caused higher mortality (39%) than the lower one (14%).

Figure 1. Kinetics of white blood cells present in BAL samples of mice submitted to a single intratracheal instillation of DMSe. With the exception of values at 2 weeks, the number of leukocytes induced by the higher dose of DMSe were significantly higher (p<0.05) than those caused by the lower DMSe dose. Four weeks after the Se instillation, leukocytes induced by lower DMSe dose were in similar numbers than those induced in control experiments. * Significant difference (p<0.05) between experimental and control groups. ▲ significant difference (p<0.05) between higher and lower Se treatments.

Figure 2A and B. Kinetics of BAL neutrophils (A) and macrophages (B) after intratracheal instillation of mice with higher and lower doses of DMSe. The neutrophil was the most numerous leukocyte presented in BAL sample after DMSe treatment. The high DMSe dose induced neutrophils for a longer period of time than the lower DMSe dose. Elevated numbers of granulocytes were seen only at day 1 after treatment of mice with the lower DMSe dose. A consistent number of macrophages was seen in BAL samples after DMSe instillation of either the low or the high dose of the metal. The values of DMSe-treated mice were statistically different (p<0.05) from those of controls. * Significant difference (p<0.05) between experimental and control groups. ▲
significant difference (p<0.05) between higher and lower Se treatments.

**Figure 3.** Kinetics of BAL lymphocytes after intratracheal instillation with high and low dose of DMSe. No significant changes were caused by the DMSe instillation in the number of BAL lymphocytes.

**Figure 4.** Values for LDH in BAL samples after airway instillation of DMSe. The DMSe treatments caused no significant changes in LDH values, with the exception of significant elevation of the values measured after 4 weeks of treatment of mice with the higher dose of DMSe. * Significant difference (p<0.05) between experimental and control groups. ▲ significant difference (p<0.05) between higher and lower DMSe treatments.

**Figure 5.** Values for total protein present in the BAL samples after DMSe intratracheal instillation. No significant changes were observed as consequence of the DMSe treatments, with the exception of elevation after 1 day of treatment with the higher dose of DMSe (p=0.028).
## Table 1.

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<th>Dose (mg Se/kg wt)</th>
<th>Sex</th>
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<th>Percent</th>
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Respiratory inflammation caused by dimethyl selenide

Fig. 1

![Graph showing white blood cells over days with different conditions and significance stars.](image-url)
Respiratory inflammation caused by dimethyl selenide

Fig. 2A

Fig. 2B
Respiratory inflammation caused by dimethyl selenide

Fig. 3

[Bar graph showing lymphocytes (x1000) over days 1, 7, 14, and 28 for control, Se 0.025, and Se 0.075 conditions.]

Fig. 4

[Bar graph showing LDH (U/L) over days 1, 7, 14, and 28 for control, Se 0.025, and Se 0.075 conditions.]
Respiratory inflammation caused by dimethyl selenide

Fig. 5

![Graph showing changes in total protein (g/L) over days for different treatments: Control, Se 0.025, Se 0.075. The graph indicates a decrease in total protein levels over time, with significant differences (*).](image-url)
Chapter 3.3

*In Vivo* Ingestion of Heavy Metal Particles by Murine Macrophages
**In vivo** ingestion of heavy metal particles by murine macrophages

*Toxicology and Industrial Health* 2004; (in press)

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**In vivo** ingestion of heavy metal particles of Se, Hg and W by murine macrophages. A study using scanning electron microscopy coupled with X-ray microanalysis

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Several heavy metals that are currently employed in industry may become polluters of work and natural environments. As particulate matter, heavy metals are suitable for entering the human body through the respiratory and digestive systems. They often end up inside phagocytes; the size of the microscopic particles modulates both their phagocytosis, and the physiology of macrophages. We have adopted here an experimental model to investigate the ingestion of particles of three industrial heavy metals (Se, Hg, W) by murine peritoneal macrophages in vivo. The phagocytes were studied by scanning electron microscopy coupled with X-ray elemental microanalysis (SEM-XRM), a method that allows specific identification of Se, W and Hg in cells at high resolution. We found that Hg that was taken up by macrophages was organized into small, round particles (0.31 ± 0.14 μm). This was in contrast with the larger size of intracellular particles of Se (2.37 ± 1.84 μm) or W (1.75 ± 1.34 μm). Ingested particles of Se and W, but not Hg, often caused bulging of the cell surface of macrophages. We conclude that particulate matters of Se, W and Hg are organized in particles of different size inside macrophages. This size difference is likely to be associated with distinct phlogistic activities of these heavy metals, Se and W causing a milder inflammatory reaction than Hg. *Toxicology and Industrial Health* 2007; 00(1): 7.

**Key words:** cell membrane; peritoneal cells; phagocytes; ultrastructure

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**Introduction**

Microscopic particles of heavy metals are potential workplace contaminants in several industries; some of these elements are used in a large number of manufacturing activities (Linnainmaa *et al.*, 1996; Alvim-Ferraz and Afonso, 2003; Horvat *et al.*, 2003). Particulate matter of heavy metals may enter the body by inhalation, by ingestion of tainted food, or through the skin (Cheng, 2003). Phagocytes are usually the cells that first take up and accumulate exogenous particles of microscopic size. The potential damage to biological systems caused by ingestion and storage of heavy metal particles depends on a number of factors, such as particle...
size, rate of biotransformation, binding form, etc. (Burbure et al., 2003). Recent studies have revealed that the size that particulate matters undertake inside macrophages is a key parameter to predict the severity of the inflammatory reaction that they will trigger (Fach et al., 2002; Becker et al., 2003).

We have investigated here the phagocytosis of different heavy metals, that is, Se, Hg and W, by murine macrophages in vivo. These three heavy metals are currently used in a number of industries, and have been identified as having the ability to cause disease in humans (Cugell, 1992; Goldman and Shannon, 2001). Based on the different size of the macrophage-ingested particles, our data offer a prediction on the relative inflammatory reaction induced by the uptake of particulate matter of Se, Hg and W.

Materials and methods

Animals

Twelve 1-month-old female mice (purchased from Charles River Laboratories Spain, SA), weighing about 20 g, were kept under standard housing conditions, and had unrestricted access to food (commercial chow) and water. They were treated in accordance with the European Union law on animal protection (directive 86/609/EC).

Heavy metal particulates

Selenium metal (Se, MW 78.96), and mercuric chloride (HgCl₂) were purchased from Sigma Chemical Co. (St. Louis, MO), and calcium tungstate (CaWO₄) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Se, Hg and W suspensions were prepared in 0.9% saline (100 mg/mL). All suspensions were sonicated and resuspended vigorously before injection in the peritoneal cavity of mice.

Induction of inflammatory macrophages in mice

The peritoneal cavity of CD-1 mice was first primed with 500 μl BSA, in order to attract inflammatory cells to this serosal space. Three days later, 500 μl of the suspensions of Se, Hg and W were injected in the same cavity of the animals. Mice were sacrificed three hours later, and the cell exudates were harvested by washing of the peritoneal cavity with 3 mL of PBS. The exudates were cytospun (5 minutes, 1000 rpm) onto slides by using Shandon Cytospin 3. Cells in the cytospin preparations were used for SEM-XRM (see below), or stained with Giemsa to be observed by light microscopy.

Scanning electron microscopy coupled to X-ray elemental microanalysis (SEM-XRM)

Cell samples attached to cover slips were fixed at room temperature in an aldehyde mixture with 4% formaldehyde, 1.25% glutaraldehyde, and 10 mM CaCl₂ in 0.05 M cacodylate buffer, pH 7.2. The samples were dehydrated in serial ethanol and critical-point dried in a Balzers apparatus using CO₂ as the transitional fluid. The preparations were mounted on metal stubs and coated by carbon under vacuum and examined in a JEOL JSM-6301F SEM that was coupled to a Norav Voyager XRM with an energy dispersive spectrometry (EDS) detection system. The electron micrographs were derived from secondary (SEI) or backscattered (BEI) electron imaging modes, the latter mode being used to detect Se, Hg and W in situ by X-ray microanalysis (see elemental spectrum of intracellular inclusion illustrated as Fig. 1).

Figure 1. Spectrum of X-ray microanalysis revealing that an intracellular particle observed by scanning electron microscopy contains Se.
In vivo ingestion of heavy metal particles by murine macrophages

Quantification and statistics

Macrophages containing Se, Hg and W inclusions were photographed at random by SEM-XRM. At least 100 cells for each heavy metal were photographed. The maximum diameter of each mouse treated with intracellular inclusion of heavy metal was measured. Average values for maximum diameter and standard deviation were determined for the intracellular inclusions of heavy metal. Statistical comparison between Se, Hg and W inclusions, with regards to maximum diameter of intracellular particles, was made using the Student's t test; differences of $P < 0.05$ were considered to identify two statistically different populations.

Results

We have induced here in vivo ingestion of particulate matter of three heavy metals (Se, Hg and W) by mouse phagocytes, in order to investigate how the phagocytosed particles are stored inside macrophages. For that, we have attracted mononuclear cells to the peritoneal cavity of CD-1 mice by in situ injection of BSA. Three days later, we confirmed that the inflammatory exudates present in this serosal cavity were made up mostly of macrophages (Fig. 2). Heavy metal particles were then injected in the mouse peritoneal cavity, and three hours later a complement of the peritoneal macrophages was found to contain intracellular particles of Se, Hg and W.

Metal particles of Se, Hg and W were specifically identified at the ultrastructural level by SEM-XRM (Fig. 3). In these electron microscopy preparations, it was possible to measure the average maximum diameter of each metal particle taken up by the murine macrophages (Fig. 4). We found that intracellular Hg was made up of particles of small size ($0.31 \pm 0.41 \mu m$). In contrast, macrophages showed significantly larger particles of Se ($2.37 \pm 1.84 \mu m$) or W ($1.75 \pm 1.34 \mu m$). Hg particles also stood out because of their constant round shape (Fig. 5), whereas intracellular particulates of Se and W presented irregular outlines and a wide variation in size (Fig. 4).

Upon their ingestion, the large particles of Se or W caused morphological alterations on the cell surface of the macrophages (Figs. 6 and 7). We have also studied the morphology of Se, Hg and W inclusions by light microscopy. In Figure 8 we illustrate macrophages containing Se, Hg and W, as they are seen by light microscopy. Both Se and Hg inclusions showed up as black inclusions, whereas W appeared as yellow-green particles.

Discussion

We have defined here the fine structure of macrophage-ingested particulate matter of three heavy metals (Se, Hg, W) that are currently used in industry. The rationale of our investigation can be found in the following statements: i) Se, Hg and W are potential environmental pollutants, at least in some regions of the world (Alvim-Ferraz and Afonso, 2003; Cheng 2003); ii) nondegradable microscopic particles, such as heavy-metal particu-
In vivo ingestion of heavy metal particles by murine macrophages

late matter, are accumulated in living systems mostly inside macrophages (Tao et al., 2003); iii) the size of microparticles has been recently shown to modulate their uptake by phagocytes and, upon ingestion, to alter the physiology of macrophages, namely with regards to the phlogistic activity that is mediated by these phagocytes (Kleinman et al., 2003).

The use of Se, Hg and W in industry is diverse. Se is employed as a decolourizing agent and as a pigment in glass; it is used in ceramic and porcelain industries, as well as in semiconductor fusion mixtures, in electrodes for arc light, and in photographic emulsion (Glover, 1970; Holness et al., 1989). Hg is part of a number of instruments and chemical processes, namely in the purification of gold-containing minerals. There is evidence of wide contamination of the seas with Hg, leading US authorities to issue recent warnings against frequent eating of tuna fish (FDA, 1999; Ginsberg and Toal, 2000; Pless and Risher, 2000; Goldman and Shannon, 2001). W is employed in the manufacture of filaments for electrical lamps, fluorescent lighting, television tubes, glass-to-metal seals, chemical and tanning industries. Chronic inhalation of airborne particles of W can cause a pneumoconiosis known as hard metal lung disease (Rizzato et al., 1986; van Sprundel, 1990; Cugell, 1992).
In vivo ingestion of heavy metal particles by murine macrophages

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In vivo ingestion of heavy metal particles by murine macrophages
D Cherdwongcharoensuk et al.

found to further stimulate phagocytosis, namely of other metal particles such as cobalt that are involved in hard metal disease, an occupational disorder of industrial environments where airborne microparticles of W are present (Lison and Lauwerys, 1992).

We have documented here that Hg is organized inside macrophages as small round particles, in contrast with internalized Se and W that are detected as coarse particulate matter of irregular shape. The size of microparticles has been shown to be an important parameter in the modulation of both phagocytosis and of macrophage function. In fact, a recent investigation by Becker et al. (2003) has demonstrated that ingestion by macrophages of coarse particles (with similar sizes of the herein studied Se and W particles) inhibits the oxidative burst, and impairs further phagocytosis of exogenous particles. This phenomenon was also associated with absence of surface expression of CD11b on the plasma membrane of macrophages. The same phenomenon was reported by Fach et al. (2002) on studying the phagocytosis of zeolite-based aluminosilicate particulates of different sizes: they found that the oxidative burst of macrophages increased with the decreasing size of the particulates, and also that this event was independent of variations in the chemical composition of zeolite. The ingestion of large particles was also reported by Lundborg et al. (2001) to impair the capacity of attachment of macrophages. The size limit for microparticles to be suitable for ingestion by macrophages was calculated by Morhenn et al. (2002) to be around 20 micra.

Taken together, these recent investigations on the modulatory role of particle size on macrophage physiology have indicated that ingestion of large particles causes significantly milder inflammatory reactions than the uptake of small particulate matter. In fact, upon the uptake of large particles, macrophages will enter a dormant state, since they will not be able to activate the oxidative burst, or to ingest infectious agents, or to attach themselves to inflammatory cells or surfaces (Becker et al., 2003; Kleinman et al., 2003). Also, large particles will not be targets for digestion by the enzyme machinery of the macrophage, and, thus, will remain undisturbed inside the macrophage, i.e., also failing to induce a

Figure 7. Light micrographs of intracellular particles of Se (large black inclusions) and W (green smaller dots) in mouse macrophages ( x 1000).
significant inflammatory reaction. In contrast, ingested ultrafine particles are the target of the intense chemical attack of the oxidative burst, and of the numerous enzymes of the macrophage (Fach et al., 2002), and this is likely to modify the chemical composition of the metal particulate. Intracellular Hg falls into this size category and chemical composition of the metal particulate. Changes inside living systems from the inorganic to of the numerous enzymes of the macrophage (Fach intense chemical attack of the oxidative burst, and ingested depicting higher biological activity and, in the long run, greater toxicity for animals and humans (Harris et al., 2003).

Acknowledgements

The authors are very grateful to Professor Carlos M Sa, director of CEMUP, for the use of his facilities and for his expertise. We thank Dr Daniela Silva, Mr Antonio Costa e Silva, Mr Emanuel Monteiro, Ms Madalena Costa and Mrs Alexandra Ribeiro for technical assistance. This work was supported in part by grants from Fundação Oriente and FCT, Portugal.

References


In vivo ingestion of heavy metal particles by murine macrophages


Chapter 3.4

Lung Collagens after Selenide Inhalation
Transient Changes in Lung Collagens Induced by a Single Intratracheal Instillation of Mice with Dimethyl Selenide

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Lung collagens after selenide inhalation

**Key words:** Immunocytochemistry, Fibrogenesis, Inflammation, Lung Alveolus, Light Microscopy, Collagen I, II and IV.
Abstract

Immunocytochemistry for collagen I, II and IV was used to investigate early fibrogenic activity induced in the mouse lung by a single intratracheal instillation of dimethyl selenide (DMSe) at two different doses (0.025 or 0.075 mg Se/kg wt). Semiquantitative comparison of collagen staining was performed up to 4 weeks following the DMSe treatments. Collagen I was transiently enhanced after the DMSe instillation: a marked staining was seen in alveolar septa 7 days after the treatment, and it decreased thereafter. Collagen II staining was slightly increased in the treated mice. DMSe instillation did not change collagen-IV labelling of the lung. The higher dose of DMSe appeared to cause a greater enhancement in lung collagen I and II than the lower dose. No difference in collagen staining was seen between DMSe-treated and control mice 4 weeks after the instillations. We conclude that: (i) DMSe instillation triggers early enhancement in collagen I in the alveolar domain of the lung; (ii) no changes in basement-membrane collagen (type IV) occur in the lung after DMSe treatment; (iii) the enhancement in lung collagens caused by DMSe is a transient and dose-dependent phenomenon.
Introduction

Selenium (Se) and its derivatives are employed in a number of industries where accidental inhalation of these chemicals may occur; fossil fuel combustion also involves the emission of Se into the air (Clayton and Clayton, 1994). Early experimental investigations have documented that Se inhalation causes edema, hemorrhage and pneumonitis in rats or pigs (Dudley and Miller, 1941; Hall et al., 1951). In humans, inflammatory symptoms and pathology of the respiratory system have been reported after inhalation of industrial Se derivatives (Clinton, 1947; Kinnigkeit, 1962; Holness et al., 1989; Rastogi et al., 1991). We have recently shown that experimental intratracheal instillation of dimethyl selenide (DMSe) in mice also causes diffuse alveolar damage and bronchiolitis obliterans-organizing pneumonia, as well as increase in the number of leukocytes, of total protein and of lactate dehydrogenase in the lung (Cherdwongcharoensuk et al., 2003 and 2004a and b).

It is well established that inflammation triggered by inhalation of harmful chemicals may lead to enhanced fibrogenic activity in the lung and this can lead to fibrotic transformation of the lung (Bonner et al., 2000; Porter et al., 2001; Chapman, 2004). Taking into account previous evidence on the phlogistic activity of inhaled DMSe, we have investigated now whether a single intratracheal of DMSe is capable of increasing collagen labelling of the lung. For that, we have monitored the staining of type I, II and IV collagens up to 4 weeks after the mice were submitted to intratracheal instillation of DMSe.
Materials and Methods

Dimethyl selenide (DMSe)

Dimethyl selenide (C₂H₆Se) in liquid form, at analytical grade, was purchased from Fluka Chemical Co (ref. no. 41572) with a purity grade greater than 99%.

Animals

Seventy two 1-month-old CD-1 mice (Charles River strain) of both sexes (36 males and 36 females) were purchased to a commercial breeder (Charles River Laboratories SA, Spain). Equal numbers of males and females were employed in all experimental groups. The mice weighed 20 g, were kept under standard housing conditions, and had unrestricted access to food (commercial chow) and water. They were treated in accordance with the European Union law on animal protection (directive 86/609/EC). Mice were exposed to intratracheal instillation of either one of two different amounts of Se: 0.025 or 0.075 mg/kg of weight (wt) of DMSe in 10 μl of saline. Control mice received an intratracheal instillation of 10 μl of saline.

Intratracheal Instillation of Se

The CD-1 mice were anesthetized by intramuscular injection of 4.0-8.0 mg/kg BW of ketamine (Ketalar, Parke-Davis Co., Barcelona, Spain), and of 0.8–1.6 mg/kg BW of xilazine (Rompun, Bayer Co., Amadora, Portugal). The anesthesia was 15-20 min long. Anesthetized mice were placed on a surgical board, and their necks exposed by surgical dissection until reaching the tracheal wall. DMSe was delivered to the lungs through the tracheal lumen with the use of a Hamilton microliter syringe. In control mice, an equal volume of 0.9% saline was injected in the tracheal airway. After the instillation, mice were held up during 1 min, the surgical wound was closed, and the animals were returned to their cages. Six mice for each DMSe dose were sacrificed 7, 14 and 28 days...
after the single instillation. Controls were also sacrificed 7, 14 and 28 days after the intratracheal injection of saline. Lungs of the sacrificed mice were rapidly removed and fixed by immersing in formalin. The tissue samples were processed for paraffin embedding by standard methods. Sagittal sections of lungs were cut in a Leica RM 2125 RT microtome. Some of the tissue sections were stained with haematoxylin-eosin and Masson’s trichrome and viewed by light microscopy.

**Collagen Immunocytochemistry**

Lung sections were deparaffinized, hydrated and underwent an antigen retrieval procedure. Intrinsic peroxidase activity and non-specific binding were blocked by incubation with 30% H$_2$O$_2$ in 10% methanol. The tissue was then sequentially incubated with primary antibodies for type I collagen (1:20) (Biogenisis Inc., UK) or types II (1:15) or IV collagens (1:100) (Novocastra Laboratories Ltd., UK.), followed by biotinylated secondary antibody, strepavidin conjugated to horseradish peroxidase, and DAB substrate, sequentially. The slides were counterstained with haematoxylin-eosin and examined by light microscopy. A semiquantitative evaluation of collagen staining was performed. The staining was evaluated with regards to area and density of positive staining. A score of 0 to 5+ was ascribed to the collagen staining observed in each sample.
Results

Collagen Immunocytochemistry

Comparison of the 3 tested collagen types revealed that collagen I was the one that showed a clear increase in area and intensity of staining in lungs of DMSe treated mice. The staining of collagen II was slightly elevated with regards to controls; collagen IV was comparable to controls. Our semiquantitative data is summarized in Table 1.

Day 7 after DMSe Treatments

The inflammation was organized in focal areas of leukocyte infiltration of the lung parenchyma. The staining was increased for collagen I (Fig. 1A); collagen II labelling was moderately enhanced, and collagen IV staining was comparable to controls (Fig. 2A). There was small variation among the different mice regarding the scoring of collagen labelling. The increase in labelling for both collagen I and II was more pronounced in mice treated with the higher dose of DMSe.

Days 14 and 28 after DMSe Treatments

Collagen staining decreased drastically in DMSe-treated mice 14 and 28 days after the instillation. Increase in collagen I labelling was still observed 14 days of the single instillation of DMSe (Fig. 1B). At 4 weeks, no increase in any collagen staining was seen in samples from DMSe-treated mice in comparison with controls.
Lung collagens after selenide inhalation

Discussion

We have documented here the transient increase in the labelling of type I and II collagens of the mouse lung in response to a single intratracheal instillation of DMSe. Our data show that this enhancement in lung collagen is more evident for type I than type II and not observed for type IV collagen.

In the deep lung of control mice, small areas of type I collagen were labelled in the interstitium of alveolar septa, whereas type II collagen was usually located at the walls of the trachea and bronchi and in association with cartilaginous tissue. These observations are in agreement with previous reports on the distribution of collagen in the normal lung (Bateman, et al., 1981, Madri 1980, Green, 2002). Type IV collagen was unchanged by the DMSe treatment; it is detected in the normal lung in basement membranes of alveolar and endothelial cells (Raghu, et al. 1985). Similarly to our results, but in reference to the fibrotic human lung, Madri (1980) found increased staining of type I collagen, whereas type IV collagen remained unchanged. Several authors have reported that large deposition of type I collagen in the alveolar domain occurs during the advanced stages of human lung fibrosis (Kuwano et al., 2001; White et al., 2003). Here, we report that a transient enhancement of the same type of collagen may occur during the inflammatory response to DMSe. Because lung labelling of DMSe-treated mice became comparable to that of controls after 4 weeks of the initial treatment, it can be concluded that the long-term fibrogenic potential of a single accidental inhalation of DMSe is low. The labelling for collagen IV in the lung did not change much in response to the intratracheal instillation of DMSe. This finding is accordance with the view that the enhanced width of basement membranes is a change that occurs only at the very late stages of fibrotic transformation of the lung (Raghu et al., 1985).

Taken together with our recent reports on the inflammatory changes caused in the mouse lung by selenium derivatives (Cherdwongcharoensuk et al.,
2003 and 2004a), the herein data add on the response of the connective tissue of the lung to DMSe, and indicate that, albeit triggering an increase in type I and II collagens, a single inhalation of DMSe is associated only with a transient alteration in collagen content of the lung.
References


Lung collagens after selenide inhalation


Legends of Figures

Table 1. Semiquantitative evaluation of collagen staining of the mouse lung after DMSe treatment with a high (0.075 mg Se) and low (0.025 mg Se) dose of DMSe.

Figure 1. Light micrograph of immunocytochemical labeling of collagen I in the mouse lung 7 (A) and 14 (B) days after DMSe instillation; C = control mouse. (x 400).

Figure 2. Light micrograph of immunocytochemical labeling of collagen II in the mouse lung 7 (A) days after DMSe instillation. B = control mouse. (x 400).
Lung collagens after selenide inhalation

Table 1.

*Low DMSe dose*

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*High DMSe dose*

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<th>Days</th>
<th>Collagen I</th>
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Lung collagens after selenide inhalation

Fig. 1A

Fig. 1B
Lung collagens after selenide inhalation

Fig. 1C
Lung collagens after selenide inhalation

Fig. 2A

Fig. 2B
Chapter 3.5

Acute Respiratory Inflammation Induced by Selenium Particles
Acute Pulmonary Inflammation Induced by Selenium Microparticulate: Leukocyte Response and In Situ Detection of Selenium in the Lungs

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Short Title: Inflammation Induced by Selenium in the Lung
Acute respiratory inflammation induced by selenium particles

Abstract

The kinetics of the acute inflammatory response of the lung was triggered in CD-1 mice by a single intratracheal instillation of a large amount of Se (10 mg); it was studied by quantitative cytology of bronchoalveolar lavage samples (BAL), light microscopy, and scanning electron microscopy coupled with X-ray elemental microanalysis (SEM-XEM). BAL leukocytes were mostly neutrophils and increased from 12 to 24 hrs of Se treatment and decreased at 72 hrs. Only less than half of the granulocytes showed ingested Se particles; in contrast, virtually all BAL macrophages contained Se particles. SEM-XRM revealed that the intracellular Se particles were heterogenous in size (diameters from 0.4 and up to 14 μ), and that Se inclusions were sometimes accumulated at a pole of the cell. At 72 hrs after inhalation of the microparticulate, Se-loaded alveolar macrophages were captured in the interstitial space of the alveoli. Se-positive regions had a focal distribution in the lung; accumulation of inflammatory cells erased the alveolar architecture of these areas of the deep lung. We conclude that: (i) Se particles trigger an acute inflammatory response in the lung that is dominated by neutrophils; (ii) the early removal of Se is done mostly by alveolar macrophages, and (iii) there is formation of focal areas of invasion of the lung parenchyma by inflammatory infiltrates.

Key words: neutrophil, alveolar macrophage, phagocyte, particles.
Introduction

Accidental inhalation of selenium (Se) may cause harmful effects on the respiratory system of workers of industrial environments where Se particulates are present (Kinnigkiet, 1962; Glover and Chir, 1970; Medinsky et al., 1985). Epithelial cells of the respiratory tract and resident macrophages of alveoli are the first cells to interact with inhaled particles (Camner et al., 2002; Geiser, 2002). We have recently reported on the injury of respiratory epithelia caused by a Se derivative (Cherdwongcharoensuk et al., 2003 and 2004). We address now the early response of lung alveoli to Se particles.

Regarding the alveolar macrophage, a few studies have reported that the phagocyte can prevent lesions caused by inhaled Se particles. For instance, Gabor et al. (1985) have compared the in vitro cytotoxicity of Se (as sodium selenite), quartz, and quartz plus selenite in guinea pig peritoneal macrophages. They found that quartz decreased cell viability, adhesiveness, and migration, whereas selenite was not harmful for macrophages in the several cytotoxicity parameters. Furthermore, the toxic effects of quartz were reduced when macrophages were treated with both quartz and selenide (Gabor et al., 1985). They concluded that Se stabilized the membrane damage that was caused by quartz on macrophages. In addition, Christensen et al. (1989) have documented the protective effects of Se in mercury toxicity caused in mouse peritoneal macrophages by exposure to 4 μM of mercuric chloride. They found that selenomethionine enhanced the survival of the phagocytes, and also that sodium selenite delayed the toxicity caused by mercury.

Notwithstanding these reports, it must be also recalled that Se at a 0.5 mM concentration was shown to induce lysis of alveolar macrophages in vitro (Medinsky et al., 1981). We have, therefore, decided to investigate the effects on the deep lung of inhalation of Se particles. Both the inflammatory response of
Acute respiratory inflammation induced by selenium particles

the alveolar space and the sorting of Se particles by phagocytes were studied with several cytological methods, namely by in situ detection of Se by X-ray elemental microanalysis coupled to scanning electron microscopy (XRM-SEM).
Material and Methods

Animals

Twenty 1-month-old female mice (Charles River Laboratories, Spain), weighing 20 g were kept under standard housing conditions, and had unrestricted access to food (commercial chow) and water. They were treated in accordance with the European Union law on animal protection (directive 86/609/EC).

Selenium Particulate

Selenium (Se) in powder form, at analytical grade, was purchased from Sigma-Aldrich (ref. no. 20965-1) with a degree of purity greater than 99.5%. Se powder (100 mg) was suspended in 1 ml of 0.9% saline. Each mouse was instilled with 100 μl of this suspension, thus receiving 10 mg of Se. Immediately before the intratracheal injection of the mice, the suspension was sonicated and vigorously resuspended.

Intratracheal Instillation of Selenium

The CD-1 mice were anesthetized by intramuscular injection of 4.0–8.0 mg/kg BW of ketamine (Ketalar, Parke-Davis Co., Barcelona, Spain), and of 0.8–1.6 mg/kg BW of xilazine (Rompun, Bayer Co., Amadora, Portugal). The anesthesia was 15-20 min long. Anesthetized mice were placed on a surgical board, and their neck exposed by surgical dissection until reaching the tracheal wall. The 100 μl of the suspension was delivered into the airway through the tracheal wall with the use of an insulin syringe with a 26 gauge needle. In control mice 100 μl of 0.9% saline were similarly injected into the respiratory airway. After the instillation, mice were held up during 1 minute, the surgical wound was closed, and the animals returned to their cages. The mice were
observed twice daily for signs of toxicity and sacrificed at 12, 24 and 72 hours after the treatment. Controls were also sacrificed 12, 24 and 72 hours after the intratracheal injection of 100 μl saline.

**Bronchoalveolar Lavage Procedure**

Mice were anesthetized by intramuscular injection of ketamine and xilazine, placed on a surgical board, the abdominal wall was exposed, and the animals were sacrificed by cutting the abdominal aorta. The neck was dissected until the tracheal wall, a cannula was introduced in the trachea, and kept in place by a ligature. BAL was performed by infusion and gentle aspiration of 250 μl of phosphate-buffered saline (PBS), pH 7.4. This was done 5 times, and allowed the recovery of around 1 ml of pooled lavage fluid. The BAL samples were kept on ice until they were centrifuged.

**Leukocyte Counts**

Total white blood cell counts of BAL samples were determined using a SEAC cell counter 2013. A portion of the BAL sample was also cytopspun onto a glass slide using a cytopsin apparatus (Shandon Southern Products Ltd, Cheshire, UK) at 1000 rpm for 5 min. These slides were stained with Giemsa to determine the number of each leukocyte cell type; at least 100 cells per sample were identified and counted under oil immersion. In addition, BAL samples were cytopspun onto the slides coated with cover slips and were fixed and processed for scanning electron microscopy.

**Light Microscopy**

Sacrificed mice were perfused with saline through the heart, the lungs were removed, and right lung was fixed by immersing in 10% formal solution. The
tissues were processed for paraffin embedding by standard methods. Sagittal sections of lungs were cut in a Leica RM 2125 RT microtome, and stained with haematoxylin-eosin and Masson’s trichrome to be viewed by light microscopy.

**Scanning Electron Microscopy (SEM)**

Sections of left lung and BAL cells attached to cover slips were fixed at room temperature in an aldehyde mixture of 4% formaldehyde, 1.25% glutaraldehyde, and 10 mM CaCl₂ in 0.05 M cacodylate buffer, pH 7.2 (Águas et al., 1991). The samples were dehydrated in a serial of ethanols and critical point dried in a Balzers apparatus using CO₂ as the transitional fluid. The preparations were mounted on metal stubs and coated by carbon under vacuum, and examined in a JOEL JSM-6301F scanning electron microscope (SEM) that was coupled to a Norav Voyager x-ray elemental micro-analyser (XRM) with EDS (Energy Dispersive Spectrometry) detection system. The electron micrographs were derived from secondary (SEI) and backscattered (BEI) electron imaging modes, the latter mode being used to detect Se in situ by X-ray microanalysis, as described before (Cunha et al., 2003).

**Statistical Analysis**

Statistical comparison between data from experimental groups and controls was performed using 2-way ANOVA. Statistical significance was considered for p<0.05. Numerical data are expressed in the figures as average ± standard deviation.
Results

We have characterized here the acute inflammatory response of the deep lung to tracheal instillation of Se microparticles. Several methods were used to address: (i) the kinetics of leukocytes present in BAL; (ii) the topography of Se-positive areas of the lung; (iii) the fine cytology of Se-positive cells.

In Situ Leukocyte Response to Se in the Lung

BAL were used to quantify the number of leukocytes present in the bronchoalveolar space of Se-treated mice, 12, 24 and 72 hours after the intratracheal instillation of the microparticulate (Fig. 1). We found that the number of treated total leukocytes and granulocytes were significant increased (p<0.05) after instillation. The highest number of leukocytes was seen 24 hours after the Se instillation (Fig. 1A). This enhancement in inflammatory cells was derived from the migration of granulocytes, mostly neutrophils, into the alveolar space of the lung (Fig. 1B). Less than half (45%) of these granulocytes showed intracellular particles of Se at 24 hours of treatment; the complement of Se-positive neutrophils was lower (27%) in BAL collected 72 hours after the Se deposition (Fig. 1B). BAL macrophages did not show significant changes in number during the 3-day course of this study (Fig. 1A and C). Virtually all of the BAL macrophages showed intracellular particles of Se, thus indicating that these macrophages were differentiated cells, rather than monocyte-derived phagocytes entering the alveolar space in response to the phlogistic stimuli triggered by Se (Fig. 1C).

Topography of Se in the Lung

Both SEM-XRM and light microscopy were used to identify Se-positive cells, to determine their cytological features and also the topography of Se in the
different histological domains of the lung. SEM-XRM allowed the specific detection of Se throughout large areas of sectioned lung. These samples showed that Se was often accumulated in a focal pattern: the Se-positive regions were usually seen at the walls of alveolar sacs surrounding a respiratory bronchiolus (Fig. 2).

Light microscopy illustrated that numerous alveolar macrophages were already loaded with Se particles 12 hours after the intratracheal instillation of Se (Fig. 3). Later on, at 72 hrs, some of these Se-positive phagocytes were seen in the interstitial space of alveolar septa (Fig. 3). In areas of heavy accumulation of Se, the lung tissue showed high density of inflammatory cells that erased the alveolar organization of the deep lung (Fig. 3B).

SEM-XRM of Se-positive macrophages revealed that the metal inclusions were made up of particles of heterogenous size and having diameters as small as 0.38 \( \mu \) and up to 14 \( \mu \) (Fig. 4). The Se particles were sometimes accumulated at a pole of the cytoplasm of the phagocytes (Fig. 4). This polarity of Se-deposition inside macrophages was also observed in some of the cells seen by LM (Fig. 3).
Acute respiratory inflammation induced by selenium particles

Discussion

We have characterized here the early phlogistic activity of Se microparticles on the deep lung, and we document that: (i) the acute inflammatory reaction of the lung to Se is dominated by neutrophils; (ii) these granulocytes have a lesser role in the removal of Se than macrophages; (iii) some of the Se-loaded alveolar macrophages migrate into the interstitial space of the alveolar septa where lymphatic capillaries are located.

Se Induced Neutrophilic Inflammation of the Lung

We found that neutrophils made up the majority of leukocytes found in the alveolar space of the mice submitted to a single Se instillation that was performed up to 3 days before sacrifice of the animals. That granulocytes were predominant in BAL is a common finding of the acute phase of the inflammatory response of the alveolar space to exogenous particles (Li et al., 1997; Elder et al., 2000). In spite of being the more numerous cell types, most neutrophils were not, however, involved in the ingestion of the Se particles. This excess of exudate granulocytes probably contributed to the formation of focal areas of the deep lung where the alveolar architecture of the tissue was erased by inflammatory infiltrates (Abraham, 2003; Burns et al., 2003).

Alveolar Macrophages Remove Most of the Se Particles

Our data showed that most of the instilled Se particles were ingested in situ by alveolar macrophages. In fact, large amounts of intracellular Se were detected inside virtually all of the large phagocytes. Taken together with other reports (Camner et al., 2002; Geiser, 2002; Medinsky et al., 1985), it can be concluded that alveolar macrophages depict high avidity for Se particles. This
Acute respiratory inflammation induced by selenium particles

Conclusion was confirmed by SEM-XRM that documented Se particles of variable size inside macrophages and also by the observation that Se particles could accumulate at a pole of the phagocyte, a phenomenon that suggests intracellular sorting of Se into residual bodies (Kagan et al., 2003).

Our light microscopy data indicated that macrophages sorted out the Se particles from the airway into the interstitial space of alveolar septa. This is part of the sorting pathway of alveolar macrophages in the deep lung: upon activation of their phagocytotic function, macrophages usually migrate in the direction of lymphatic capillaries located near alveolar septa, and then to regional lymph nodes, as we and others have illustrated before (Peão et al., 1993; Laskin et al., 2001; Iles and Forman, 2002).

In conclusion, the herein study offers the definition the early response of the lung to airway instillation of Se, it documents the neutrophilic response of acute inflammation of the deep lung to Se, and it reveals that a large amount of Se microparticulate is fastly taken up by alveolar macrophages that initiate migration into regional lymphatics. These data establish the initial structural setting of the lung response to Se inhalation and provide the foundation for further investigations on the long-term effects on the respiratory system of inhaled Se particles.
Acknowledgements

The authors are very grateful to Professor Carlos M. Sá, director of CEMUP for the use of his facilities and for his expertise. We thank Dr. Daniela Silva, Mr. Antonio Costa e Silva, Mr. Emanuel Monteiro, Ms. Madalena Costa, and Mrs. Alexandrina Ribeiro for technical assistance. This work was supported in part by grants from Fundação Oriente and FCT, Portugal.
Acute respiratory inflammation induced by selenium particles

References

Acute respiratory inflammation induced by selenium particles


Acute respiratory inflammation induced by selenium particles

**Legend of the figures**

Figure 1. Kinetics of inflammatory cells in the alveolar space of mice 12, 24 and 72 hrs after Se instillation. A: Changes in total leukocytes, macrophages and granulocytes. * Significant differences (p<0.05) were found between experimental and control groups regarding total leukocytes and granulocytes B: Comparison between total and Se-positive granulocytes. C: Comparison between total and Se-positive macrophages.

Figure 2. SEM-XRM micrograph of section of mouse lung 72 hrs after Se instillation. The white dots correspond to Se particles; they follow a focal distribution in the lung tissue. Bar 270 μ.

Figure 3. Light micrographs of sections of deep lung of mice submitted to intratracheal instillation with Se particles. A: Alveolar macrophages loaded with Se (x1000). B: Erasement of the alveolar architecture of an area of the deep lung caused by inflammatory cells, some of them loaded with Se (x 200).

Figure 4. SEM of alveolar sac showing a macrophage in the center of the micrograph (A in the figure) that contains several particles of Se as revealed by SEM-XRM (white dots in B in the figure). Bar 2 μ.
Acute respiratory inflammation induced by selenium particles

Fig. 1A

Fig. 1B

Fig. 1C
Acute respiratory inflammation induced by selenium particles

Fig. 3A

Fig. 3B
Acute respiratory inflammation induced by selenium particles

Fig. 4A

Fig. 4B
Chapter 4

General Discussion
GENERAL DISCUSSION

This dissertation follows the European model favoring that original research articles are to be included in the thesis and in publication form. The five previous chapters of this book are, therefore, either the reproduction of published research reports or manuscripts that have been submitted for publication. All of them follow the organization of the scientific article and, thus, include a specific section of "Discussion" of the experimental results.

For this reason, it would be repetitive to discuss here again, and in detail, the experimental data. In alternative, in the following paragraphs I present the main conclusions of the research work of this thesis, and I discuss in brief their foundations and scientific significance.

Severity of Respiratory Lesions Caused by Se is Dose Dependent

This conclusion came from the results of experiments using DMSe and reported in chapter 3.1. Two different doses of DMSe 0.05 mg (the lower Se dose) or 0.1 mg (the higher Se dose) Se/kg BW were intratracheally injected to one-month-old CD-1 mice and the tissue lesions were investigated 1, 7, 14 and 28 days after the treatment. Lung edema, increased cellularity and DAD were the dominant pathologies that were caused by the DMSe instillation.

The higher dose of DMSe triggered more severe pathology than the lower one, such as lung hemorrhage or bronchiolitis obliterans-organizing pneumonia. In addition, early tracheal metaplastic transformation with the formation of a large cushion of metaplastic cells was seen only after the administration of the higher DMSe dose to the mice. Furthermore, the higher dose of DMSe induced an enhanced number of inflammatory neutrophils and macrophages in BAL
samples of the mice and during 4 weeks upon DMSe instillation, while the lower dose of DMSe resulted in an inflammatory reaction that lasted only for 2 weeks.

Other authors have reported this dose-dependent action of Se derivatives. For instance, increasing concentrations of elemental Se or selenious acid was reported to cause decreasing numbers of viable alveolar macrophages \textit{in vitro} (Medinsky \textit{et al.}, 1981a). Additionally, Bell and co-workers (2000) documented that a high dose of Se (0.8 mg Se/kg) as sodium selenite induced lung edema and injury but a lower dose of Se (0.15 mg Se/kg) failed to cause respiratory lesions in the guinea pig. Hydrogen selenide inhalation by guinea pigs (0.002 to 0.57 mg/litter) induced a mild lung injury showing slight thickening of the alveolar wall and congestion of alveolar capillaries (Dudley and Miller, 1937), whereas higher amounts (8 mg Se/m$^3$) produced diffuse bronchopneumonia, and pneumonitis (Dudley and Miller, 1941).

Our own data on lung collagen presented in chapter 3.4 have also documented a higher intensity of labelling of collagen I and II after treatment of mice with the higher DMSe dose than with the lower one. The same phenomenon of increased collagen deposition was seen in the rat lung treated with ultrafine Kevlar aramid synthetic fibers: a high dose caused it but not a low one (Lee \textit{et al.}, 1983). High density of collagen was also found in the rat lung instilled with a high amount of Phosgene agent but not with a low one (Kodavanti \textit{et al.}, 1997).
Se Causes Different Changes in Tracheal and Alveolar Epithelia

The respiratory airways are presumably the most important exposure route for Se present in the atmosphere. Experiments have been done concerning the effect of inhaled toxic agents on the respiratory system, by either aerosol inhalation or intratracheal instillation. The latter has considered to be sufficiently close to inhalation exposure; it has been used to treat animals with both soluble and insoluble particles (Henderson et al., 1995; Nonavinakere et al., 1999; Bell et al., 2000). This method allows the definition of the exact amount of the agent and also decreases the chance of contamination from other the external agents (Bell et al., 2000). In addition, it must be stated that particle deposition and distribution patterns are slightly different from that of the aerosol method (Pritchard et al., 1985). Experiments of instillation of Se by both routes have shown that the respiratory lesion is similar.

In my experiments I have found that a single intratracheal instillation of two different doses of Se as DMSe (0.05 or 0.1 mg Se/kg) caused the damage to respiratory epithelia. The tracheal injury induced by Se consisted in extensive loss of cilia, transformation of the epithelium from pseudostratified columnar to squamous, and, in some samples, progression of the lesions into metaplasia.

There is no consistent published data on the pathology of trachea caused by Se instillation. There is an old report of irritation caused by Se in humans and regarding the mucous membranes of the throat with coughing (Clinton, 1947). Pathological changes of tracheal epithelium resembling those of Se derivatives have been induced by other toxic substances: Konradova and Bencko (1976) found that inhaled inert pyrite dust resulted in damage of the cilia and apical region of rabbit tracheal epithelial cells. Alteration from pseudostratified columnar to squamous epithelium in rabbit trachea was reported after inhaled pine wood smoke (Bhattacharyya et al., 1998). Proximal tracheal cells injury, loss of epithelial cells, increasing of alveolar macrophages, and dysfunction of
the mucociliary blanket of rabbits' lungs caused by wood smoke inhalation was described by Loke \emph{et al}. (1984).

Squamous metaplasia was observed in murine trachea, 2 and 4 weeks after dichlorosilane inhalation (Nakashima \emph{et al}., 1996). It, thus, appears that the tracheal injuries induced by Se are not specific of this substance but rather quite similar to those caused by inhalation of other toxic agents.

Intratracheal instillation of Se triggered lung injury, resulting edema, hemorrhage, diffuse alveolar damage and bronchiolitis obliterans-organizing pneumonia. Leukocytic infiltration and edema were previous described in the lungs of guinea pigs at 24 hours after sodium selenite intratracheal instillation (Bell \emph{et al}., 2000). Se dust inhalation caused edema, mild or chronic interstitial pneumonitis, congestion, vascular lymphocytic infiltration, intra-alveolar foci of large macrophages, and slight emphysema in the lungs of both guinea pigs and rats (Hall \emph{et al}., 1951). Inhaled hydrogen selenide induced thickening of the alveolar wall in guinea pigs, and also congestion of alveolar capillaries (Dudley and Miller, 1937); in higher amounts, it triggered diffuse bronchopneumonia, and pneumonitis (Dudley and Miller, 1941). In contrast, 1-hour exposure of rats to 25,958 mg Se/m$^3$ as DMSe produced only minor effects (increased weight of lungs and liver); these changes disappeared after 7 days (Al-Bayati, 1992).

Accidental inhalation of Se by workers has also resulted in respiratory lesions expressed by dyspnea, bronchial spasms, bronchitis, chemical pneumonia, interstitial pneumonitis, lung hemorrhage and tracheobronchitis (Glover, 1970; Wester \emph{et al}., 1981; Holness \emph{et al}., 1989; Rastogi \emph{et al}., 1991; Gerhardsson \emph{et al}., 1988). Loss of epithelial cells, increasing of alveolar macrophages, and dysfunction of the mucociliary blanket of rabbits' lungs caused by wood smoke inhalation was described by Loke \emph{et al}. (1984).

Nevertheless, the overall inflammatory alterations reports of the respiratory system induced by Se can not, however, be ascribed solely to the
toxicity of Se. This is because it was not possible to show: (i) clearly amount of Se that reached to the lung (ii) the progression of the respiratory lesions because of the short period of time that was studied. Although, the data clearly support the toxic effects of Se onto the respiratory system. The 4-week follow-up of morphological changes of trachea and lung of Se-treated mice demonstrated the acute necrotic effect of the metal on the tracheal lining, with subsequent metaplastic transformation of the epithelium, and it pointed to a more severe and durable damage induced by Se on the alveolar septa of the lung than on the trachea.

Clinical data on accidentally inhaled Se by humans have been reported by several investigators. Acute inhaled hydrogen selenide caused dyspnea and pneumomediastinum and irritated the mucous membranes of nose, eyes and upper respiratory tract (Alderman and Bergin, 1986; Schecter et al., 1980). Nose and eye irritation were also found in the workers from copper industries (Holness et al., 1989). In addition, respiratory impairment was seen in the glass bangle workers (Rastogi et al., 1991). Also, Clinton (1947) reported that dust of elemental Se and Se dioxide can irritate the mucous membranes of the nose and throat and cause coughing, nosebleed, loss of sense of smell, dyspnea, bronchial spasms, bronchitis, and chemical pneumonia. Finally, Nantel et al. (1985) concluded that Se-induced death is due to hypotension as a consequence of vasodilatation and low cardiac output, respiratory distress syndrome, and severe myopathy that contributes to respiratory failure.

In our experimental studies we also offer evidence of clinical change observed in mice after Se instillation. Treated animals showed tachypnea that started within 2-3 minutes of the treatment and continued for at least 10-15 minutes. In most of the mice treated with the high dose of selenium, there was evidence of dyspnea throughout the first two weeks of the study. In addition, some of these mice showed tremor, loss of mobility, and decreased food intake.
All of the above reported symptoms can presumably be useful to compare with Se exposure in human.

**Neutrophils are the Major Inflammatory Cells of Se-induced Acute Response of the Lungs, and Macrophages Remove the Particles**

**Biomarkers of Inflammation or Injury**

Se provoked inflammatory changes in the lung as assessed by biochemical and cell profile analysis in bronchoalveolar fluid (BAL) and these responses can used to confirm with the histopathological analysis. The BAL method is a rapid screen assay of lung injury that is detected by the alterations of inflammatory cells including neutrophils, macrophages and lymphocytes, enzymes and total protein (Henderson, 1984; Henderson *et al.*, 1985; Henderson, *et al.*, 1987).

**Neutrophils**

Se-induced inflammation was investigated using a nonmicroparticulate Se, that is DMSe (Chapter 3.2), and also microparticulate Se (Chapter 3.3, 3.5). Neutrophils were the dominant inflammatory cells found in BAL samples, mostly at day 1 and 7 after the instillation of Se particles. Neutrophils are known to be the main inflammatory cells in response to releasing of cytoplasmic enzymes from death or injury of lung epithelia. Li *et al.* (1997) described increased influx of BAL neutrophils (up to 15% of total BAL cells) in the alveolar space after intratracheal instillation of fine particles (size less than 10 mm in diameter (PM10)). Predominant number of neutrophils in BAL samples is also commonly found in the acute phase of the inflammatory response to exogenous particles (Peão *et al.*, 1993; Elder *et al.*, 2000). Thus, that neutrophils
are the main inflammatory cells seen in the lung after toxic inhalations not surprising.

The recruitment of neutrophils from blood vessels to the injury area is known to be induced by the cytoplasmic enzymes released from the death or injury of lung epithelia or macrophages (National Research Council, 1989). In addition, neutrophils can secrete several substances, such as lipids (leukotrienes, platelet activating factor, thromboxane A$_2$ (TXA$_2$)), cytokines (interleukin (IL)-1$\beta$, IL-6, IL-8, tumor necrosis factor alpha (TNF$\alpha$)), proteases (elastase, collagenase), microbicidal products (lactoferrin, myeloperoxidase, lysozyme), reactive oxygen species and nitric oxide (Sampson, 2000). Excess numbers of neutrophils, as it occurs in most inflammations can cause lung damage because of its secretory proteolytic enzymes, such elastases, that are released during phagocytosis; these enzymes can strip the bronchial epithelium, stimulate mucus secretion, inhibit the function of cilia, impair phagocytic microbial clearance and activate chemokine production (Lomas et al., 1995; Stockley, 1995; Sampson, 2000). These effects may retard Se clearance and also cause excess neutrophil recruitment into the lung. Moreover, phagocytosis of apoptotic neutrophils generates important signals to down regulate the pro-inflammatory cytokine production of macrophages (Sampsom, 2000).

The herein data showed that the higher dose of Se causes lesion of lung tissue in mice at least during 28 days after the initial treatment. Furthermore, Se also induced the biphasic kinetics of neutrophils that has been previously documented by other authors, namely Peão et al. (1993) who studied the effects of tungstate particles in the murine lung. The first peak of neutrophils induced by Se was detected 24 hrs after treatment; this is a similar timing as the one of the first peak of tungstate treated mice. The second peak occurred 28 days after Se instillation. It is possible that the first influx neutrophils corresponded to a
non-specific inflammatory response and the second one may correspond to the specific immune response of the lungs to the Se agent.

*Macrophages*

Macrophages are the major phagocytic cells of the lung. They also produce a number of cytokines; for instance, TNFα, IL1, IL6, IL8, IL-12, IL-18, interferons, defencins and nitric oxide (Semenzato et al., 2000). In addition, macrophages can secrete several other products such as enzymes (e.g. lysozyme and collagenases), biologically active lipids (e.g. TXA₂, prostaglandins and leukocytes), antiproteases and other inhibitors, fibronectin, complements, binding proteins (e.g. transferring), free fatty acids, antioxidants (glutathione), and coagulation factors (National Research Council, 1989; Sibille and Reynolds, 1990). The lung contains alveolar, interstitial, intravascular and airway macrophages (Sibille and Reynolds, 1990). Nevertheless, the alveolar macrophage is the main phagocyte of the lung in the defense of the organ with regards to inhaled toxic particles (Gregson et al., 1982; Camner et al., 002, Geiser, 2002). We also found that BAL macrophages increased at moderate levels, clearly at a lower level than neutrophils. Light and SEM-XEM micrographs identified Se and confirmed that the Se particles were loaded in macrophages and neutrophils. The percentage of macrophages loaded with Se was higher than neutrophils. The intracellular Se particles were heterogenous in size and had diameters as small as 0.4 and up to 14 μ. The average diameter of Se particles was around 2.37±1.84 μ, that is bigger than those of W and Hg particles. Hg was organized inside macrophages as small round particles, in contrast with internalized Se and W that were detected as coarse particulate matter of irregular shape. Apart from the size of particles, it seems that ingestion of large particles causes significantly milder inflammatory reactions than the uptake of small particulate matter (Becker et al., 2003; Fach et al., 2002;
Lundborg et al., 2001). In fact, upon the uptake of large particles, macrophages will enter a dormant state, since they will not be able to activate the oxidative burst, or to ingest infectious agents, or to attach themselves to inflammatory cells or surfaces (Becker et al., 2003, Kleinman et al., 2003). Also, large particles will not be targets for digestion by the enzyme machinery of the macrophage, and, thus, will remain undisturbed inside the macrophage, i.e., also failing to induce a significant inflammatory reaction. In contrast, ingested ultrafine particles are the target of the intense chemical attack of the oxidative burst, and of the numerous enzymes of the macrophage (Fach et al., 2002), and this is likely to modify the chemical composition of the metal particulate. Additionally, Oberdorster (1996) studied inhaled ultrafine particles of TiO₂ in rat lung and found that these particles migrated from alveolar space into interstitial areas. Alveolar macrophage transport particles to submucosal and tracheobronchial lymph nodes where lymphocytes and tissue macrophages may mount an immune response (National Research Council, 1989). In addition, Gregson et al. (1982) found that alveolar macrophages and Type-II pneumonocytes are the predominantly phagocytes that take up the intratracheally antigen. Conceivably, alveolar macrophages carry particles from the luminal surface across the bronchiolar epithelia via the apical cytoplasm and the intracellular space into the basement membrane, after that, they penetrate the basement membrane into the bronchoalveolar lymphoid tissues to present the antigen to lymphoid cells. Clearance of Se particles from the alveolar space was performed by alveolar macrophages that sorted out the particles from the airway into the interstitial space of alveolar septa, and from there to lymphatic vessels and regional lymph nodes, as illustrated in this thesis and before by others (Peão et al., 1993; Laskin et al., 2001; Iles and Forman, 2002). Interestingly, Diskin et al. (1979) reported data of autopsy of an employer in a Se refinery, who was
exposed to red elemental Se and died of congestive heart failure, and found high concentrations of Se in the peribronchial lymph nodes and in the lung.

**Lymphocytes**

Lymphocytes are the key cells in the initiation and development of an immune response. The current investigations evaluated lymphocytes as one of the biomarkers of changes in the respiratory system induced by Se. It was found that the number of BAL lymphocytes showed no significant change with the Se instillation. Kaltreider *et al.* (1977) also reported that antigen deposited in the dog lung is rapidly translocated to draining lymph nodes where the immune response may occur. In fact, proliferation of antigen-specific B cells was demonstrated to occur in the lymph nodes (Bice *et al*., 1980). Thus, the absence of changes in BAL lymphocytes may reflect this phenomenon.

**Mild Increase of Total Protein and LDH was Observed in BAL Samples of Se-treated Mice**

**Lactate Dehydrogenase (LDH)**

It is well known that LDH is a cytoplasmic enzyme and that it is commonly used as indicator of cell damage or inflammation (Drent *et al*., 1996). LDH shows increase in patients with several pulmonary disorders (Cobben *et al*., 1999) and in toxic instillations (Li *et al*., 1997). Cobben *et al.* (1999) studied LDH levels in BAL samples obtained from patients with several pulmonary disorders; they found that the LDH levels of a group samples with numerous polymorphonuclear neutrophils were higher than a group with mainly alveolar macrophages; and they concluded that LDH appears to be useful monitoring of pulmonary inflammation that is driven by neutrophils. Li *et al.* (1997) also
General Discussion

reported the increasing of BAL LDH after intratracheal instillation of mice with PM10. We found that Se provoked mildly increased levels of LDH in BAL samples after treatment, especially after the higher Se dose. This observation provided additional information that Se causes cell injury in pulmonary epithelia.

**Total Protein**

BAL total protein is usually evaluated as marker of increased permeability of the alveolar-capillary barrier (National Research Council, 1989). Se induced a slight increase in total protein level in BAL samples after treatment. This raise in total protein may be associated with influx of neutrophils and alveolar macrophages to the inflammatory areas where Se is present. Increased total protein in BAL samples has previously been reported in lung inflammation studies (Bignon et al., 1975; Oberdorster et al., 1992, Li et al., 1997). In addition, Bell and Hook (1979) found that the elevated BAL protein levels observed in pulmonary alveolar proteinosis patients did not result from leakage of blood-air barrier damage but resulted from plasma transferred through alveolar-capillary barrier. Immunoglobulin G (IgG) and IgA were the main component of total protein in BAL in contrast with serum of smokers (Bell et al., 1981).

LDH and total protein levels of BAL samples of Se-treated mice were slightly increased after 1 day of Se exposure. This suggests that Se causes only a low toxic response.
Se Instillation Causes Transient Increase in Lung Collagen I but not Collagen IV

Collagen labelling was also applied in this study to define the progression of lung injury caused by Se. The connective tissue of the deep lung is made up of several types of collagen molecules. Type I and III collagen are commonly found in the interstitium of alveolar septa, and type II collagen is usually located at trachea and bronchus in association with cartilaginous tissue (Madri, 1980; Bateman et al., 1981). Type IV and V are predominately presented at epithelial and endothelial basement membranes (Raghu et al., 1985). Collagen types I, II and IV were evaluated here in order to investigate lung injury caused by Se.

Type I collagen was increased in the alveolar septa after 7 days of treatment; collagen type IV was not changed during treatment. The increase in lung collagen type I, and the no significant changes of collagen type IV, were also found before in patients with fibrotic lung disease (Madri, 1980). Raghu and his co-workers (1985) found that accumulation of type I collagen appeared during advanced stages of human lung fibrosis. The transient enhancement in labelling of collagen I, with a pattern that is similar to control samples after 4 weeks of Se inhalation, suggests that the fibrogenic potential of a single instillation of Se is low. In addition, the more clear increase in labelling of collagen I and II was seen in the lung of mice that were treated with the higher DMSe dose. Enhanced collagen deposition in the rat lung treated with ultrafine Kevlar aramid synthetic fibers was also seen only after the high dose was used (Lee et al., 1983). High density of collagen was found in rat lung instilled with high amount of Phosgene agent (Kodavanti et al., 1997). Concentration or dose of the treatment seems to be the main trigger of pathological alteration of the lung. That the fibrogenic potential of a single instillation of Se was low is concluded from the normal labelling for collagen I and II that was seen after 4 weeks of treatment.
A


B


**C**


D


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H


I


K


L


**M**


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N


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**P**


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W

