Respiratory effects of air pollutants: experimental studies in humans

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ABSTRACT: Epidemiological and environmental chamber studies in man, and toxicological studies in animals, have provided valuable insights into the biological effects, the mechanisms of action, and the dose-response characteristics of some major air pollutants. This review describes the information currently available on air pollutant effects in man, as the result of experimental studies. There are certain advantages, as well as some limitations, in human chamber exposure studies, but if carefully designed and based upon relevant background data they may give information that is valuable for understanding the effects of air pollutants in man. Reversible effects on the airway mechanics, the responsiveness of the airways to methacholine and allergen have been shown to be caused by air pollutants. Furthermore, significant changes have been demonstrated in airway permeability, bronchoalveolar lavage, nasal lavage, and peripheral blood cells and inflammatory markers. Currently, human toxicology to air pollutants is a progressive research area.


Air pollutant research began with investigations based upon clinical suspicions that polluted air could be the reason for symptoms in the airways. Epidemiological tools were used, that at first were rough but gradually became more advanced. The epidemiological evidence that air pollutants may indeed be the cause of airway symptoms in humans is reviewed in the European Respiratory Journal by Lebowitz [1]. In order to investigate air pollutant effects in the lungs, animal models were developed and have, over the years, gradually become more advanced, as reviewed by Chitano et al. [2].

The present review is intended to reflect the current understanding of the effects of air pollutant in the airways of humans, based upon experimental studies. Some background data are given on chemical properties, sources of exposure and deposition. The effects in the lungs have been explored with tests of airway mechanics, measurements of airways responsiveness, bronchoalveolar and nasal lavage, and measurements of soluble markers in lavage fluids and blood. Occasionally, measurements of mucociliary clearance and airway permeability have been used. Data are presented for all the common gaseous air pollutants, sulphur dioxide (SO₂), nitrogen dioxide (NO₂), and ozone (O₃), as well as acid aerosols. All factors mentioned may act together with other pollutants, such as suspended particles from combustion and other sources contributing to produce air pollution that may cause adverse effects in the airways.

Since man encounters a number of potentially harmful factors, both in indoor and outdoor environments, it can be very difficult to characterize the effects of a specific pollutant. The use of specially designed challenges with a single air pollutant in exposure chambers has, therefore, become much favoured in studies of air pollutants. Exposure chambers have provided an opportunity to create controlled and standardized atmospheres, and the exposures have also been standardised to selected workload, breathing pattern, duration and number of exposures. Experimental protocols have been based on examination of exposure conditions in indoor and outdoor environments. This is important, in order to mimic the conditions for certain exposure situations in the environment. Otherwise, the results obtained cannot be correctly interpreted, and, consequently, cannot be applied in judgement of the hazards of pollutant exposure.

Exposure studies in humans have certain limitations as well as advantages. Firstly, it is evidently beneficial to conduct studies in the species that the exposure limits actually concerns. Since both inter- and intraspecies differences have been demonstrated, as reviewed elsewhere [3], data obtained in humans are expected to be more reliable. This probably does not concern very basic effects upon primitive molecules and functions that have not changed during evolution. More complex systems and reactions may, however, not respond consistently in different species. An example of this is the complex immunoregulatory function in the airways. Data from animal models must, therefore, be carefully interpreted and the adequacy of an animal model should, preferably, be confirmed by parallel human studies.

Another important issue is that selected populations with potentially different susceptibility to air pollutant
effects may be investigated individually, and different groups compared. Important groups are atopics, asthmatics, smokers, and chronic obstructive lung disorder (COPD) patients, as well as healthy normals of different ages, from children to elderly people.

The drawback of controlled human exposure studies is that for ethical reasons there is a certain limit upon what investigations may be performed. Fibreoptic bronchoscopies with bronchoalveolar lavage (BAL) and forced biopsies from the airway mucosa in the more central airways have proved safe. There is, however, no easy way to obtain sufficient biopsy material from the small airways and alveolar regions. Transbronchial lung biopsies are associated with certain risks of pneumothorax and bleeding, and multiple biopsies would be needed in order to describe the morphology accurately. In animal models, these ethical constraints do not apply; whole lungs may be fixed, cut and stained for morphological evaluation. In humans, there are also limitations upon concentrations that can be used in exposure studies. In general, it can be said that concentrations that have been found to be frequent in work situations are generally approved by Human Ethics Committees. There are also limitations upon how frequently and to what cumulative concentration exposures can be conducted in the same individual. Furthermore, the investigations that can be performed in more diseased subjects are limited, because of the risks involved in the experiment.

An obvious advantage of experimental exposures in general is that the effects of a single pollutant may be identified; whereas, in ambient or workroom air, there is a mixture of pollutants. Once the effects are well characterized in man, more or less complex combination studies with different air pollutants may be undertaken. However, both in animals and man there is still a shortage of complex combination studies that can explore what role each pollutant may have in the mixed exposures of real life. Effects in the concentration ranges both for ambient air and workplace environments are of importance to explore; ambient air as it concerns the general population, and workroom concentrations for the safety of workers. In Europe, the trend has been to cover all aspects of exposure. In several experimental series, workroom concentrations have been investigated and parameters and markers of exposure effects documented, before the research has moved on towards ambient levels. The reason for this has been to adjust the registration or processing of samples, in order to increase sensitivity in detecting air pollution effects.

In conclusion, there are certain advantages, as well as limitations, in human chamber exposure studies, but if carefully designed and based upon relevant background data, they may provide information that is invaluable in understanding the effects of air pollutants.

Ozone

Chemical properties

Ozone is generated from oxygen by electrical discharge or photochemical reactions in ultraviolet irradiation with a wavelength of 185–210 nm. Ozone ($O_3$) is a highly potent oxidant. It is not a free radical, but by interaction with molecules free radicals are frequently produced.

Solubility and deposition

In contrast to the highly water soluble sulphuric oxides and partly soluble nitric oxides, $O_3$ is virtually insoluble in water. Therefore, it is deposited from the proximal airways down to the most peripheral airspaces. Intrathoracic uptake of $O_3$ has been estimated to be around 90%, depending upon the inhaled volumes [4, 5]. Furthermore, it has been estimated that the deposition of the highest local doses in the airways occurs in the respiratory acini, both in humans and rodents [6, 7]. Further data from humans are expected.

Toxicological mechanisms

Ozone is the strongest oxidizing gas in air pollution. The $O_3$ toxicity on cell membranes involves oxidation of amino acids and unsaturated fatty acids [9, 10]. The peroxides produced cause toxic effects and also free radicals affect enzymes, structural proteins, fatty acids and numerous other molecules [11–13]. The cascade of products produced may be as reactive as $O_3$ itself. Alpha1-antitrypsin ($\alpha_1$-proteinase inhibitor) can be oxidized and deactivated by $O_3$ in vitro, which may potentially lead to emphysema [14]. During recent years, knowledge about formation of lipid hydroperoxides and glutathione oxidation has vastly improved [15, 16].

Protein and a number of nonenzyme antioxidants are of enormous importance for preventing or limiting toxic effects of oxidants, such as $O_3$ and nitric oxides. Superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GSH-Px), and catalase are well-known in this context. Interestingly, vitamin $E$ may protect against $O_3$ initiated oxidation and peroxidation, and can limit the toxic effects. Vitamin $C$ may also be of some importance [17–19]. Increasing antioxidant capacity following or during oxidant exposures represents one of the most important protection mechanisms.

Sources and exposure

Ozone may be produced in workplaces due to welding and electrical processes. The majority of $O_3$ is, however, produced in the atmosphere. The $O_3$ generated in the stratosphere adds substantial amounts to the lower
atmosphere levels during air exchange. This is particularly pronounced during springtime. Complex photochemical reactions take place in polluted air, where radiation, nitrogen oxides, organic vapours and other agents interact in the generation of O₃ [20]. The latter is the most important source of O₃. Due to the fact that O₃ is produced mainly in reactions driven by radiation and other air pollutants, it may be considered to be a secondary air pollutant. During hot summers, with periods of stagnant air, ground concentrations of ozone may increase from around 0.025 parts per million (ppm) (25 parts per billion (ppb)) to 0.1 ppm (100 ppb), and sometimes above 0.2 ppm in the UK and Central Europe [21] (tables 1 and 2). Similar conditions have been reported from other areas, such as southern California.

Lung function studies

It has been shown, in a variety of species, that O₃ exposure causes alterations in the breathing pattern that are unique. During exposure, the ability to perform deep inspiration is limited through reflex mechanisms [24]. Cough and irritation occur when deep inspiration manoeuvres are tried. Furthermore, breathing becomes shallow, with increased frequency. This leads to a decreased vital capacity [25, 26]. The reflex mechanisms appear to be aimed at protecting the deeper airways of the host against further noxious inhalation. Atropine has been shown to counteract the increase in airway resistance caused by O₃ exposure, indicating that a parasympathetic reflex is involved [27]. However, atropine does not affect the altered vital capacity. In contrast, lidocaine, an anaesthetic, decreases the response when applied to the upper airway [26]. Nonsteroidal anti-inflammatory drugs (NSAIDs), inhibiting cyclo-oxygenase products, have also been found to reduce the O₃ effects on lung function and symptoms [28].

Numerous studies have been performed on the effects of O₃ on lung function. They have indicated that concentrations of O₃ occurring in ambient air may affect lung function. The early studies used a wide range of exposure concentrations and protocols. Recently, studies have been more fine-tuned with quite similar set-ups in a series of studies. This vastly increases the possibility of adequately interpreting the data and of obtaining more precise impression of, e.g. dose-effect relationships.

When we look at the lung function studies performed so far, we can observe that the majority of studies have

| Table 1. – Conversion of units (ppm and mg·m⁻³) at 25°C, 101.3 kPa |
| O₃: 1 mg·m⁻³ = 0.5 ppm |
| NO₂: 1 mg·m⁻³ = 0.56 ppm |
| SO₂: 1 mg·m⁻³ = 0.38 ppm |

O₃: ozone; NO₂: nitrogen dioxide; SO₂: sulphur dioxide.

<p>| Table 2. – Air quality standards |</p>
<table>
<thead>
<tr>
<th>Mean Reference period</th>
<th>SO₂ ppm</th>
<th>MP10</th>
<th>Black smoke μg·m⁻³</th>
<th>NO₂ mg·m⁻³</th>
<th>O₃ ppm</th>
<th>O₃ mg·m⁻³</th>
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<td>WHO Europe</td>
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<td>1 h</td>
<td>0.122</td>
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<td>24 h</td>
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<td>USA</td>
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<td>0.36</td>
<td>150</td>
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<td>Peak exposure limit/short-term exposure limit</td>
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<td>USA</td>
<td>8 h</td>
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<td>Peak exposure limit/short-term exposure limit</td>
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WHO: World Health Organization; PM₁₀: particulate matter with an aerodynamic diameter ≤10 µm. *: total suspended particles (TSP); **: proposed new limits, 8 h 0.05 ppm, threshold limit value 0.2 ppm. (Modified after Wardlaw AJ. The role of air pollution in asthma. Clin Exp Allergy 1993; 23: 81–96).
been carried out on healthy nonsmoking volunteers. This has been the approach following studies that indicated that asthma and atopic status do not regularly evoke any enhanced susceptibility to O₃ in laboratory studies [29–31]. This is a property that O₂ does not share with the other common air pollutants, NO₂ and SO₂, which have elicited lung function effects at lower concentrations in asthmatics than healthy subjects in the laboratory.

Of certain interest is the fact that it has been demonstrated that a subpopulation of 10–20% of subjects, asthmatics or not, are sensitive to developing a significant fall in forced expiratory volume in one second (FEV₁) after O₃ exposure, whilst others are nonsensitive [32, 33]. At present, it is not fully understood whether there is a graded response in the population or two distinct groups, i.e. O₃ responders vs nonresponders. This requires large studies for elucidation and is one of the more important issues for estimating the population at risk of developing unwanted effects of O₃ inhalation. The fact that most O₃ studies have been carried out with unknown relationships between responders and nonresponders makes it difficult to draw definite conclusions from them.

At the present time, some groups are known to be less prone to react to O₂ exposure. For instance elderly people are less sensitive to developing lung function decrements following O₃ exposure [34]. The reason for this is unknown. It is also not known how the responder/nonresponder ratio in the population changes during aging.

Another group of interest is COPD patients, who have been the subject of several studies. COPD patients were exposed to 0.2 ppm O₃ for 2 h without any significant decrease in lung function. This is a concentration that healthy subjects frequently respond to. In the COPD patients a small significant decrease in oxygen saturation was demonstrated by pulse oximetry [35, 36]. The reason why COPD patients do not respond with measurable lung function reduction is unknown, as with healthy elderly people. The COPD patients were not compared with an age-matched control group. On the other hand, it could be due to consequences of smoking. Cigarette smokers have been shown to experience reduced effects on lung function compared with nonsmokers [37]. This has been confirmed in a recent, elegantly designed study by Esmons and Foster [38]. They investigated 10 smokers, before and 6 month after smoking cessation. During smoking no effects were seen on lung function, whereas forced mid-expiratory flow (FEF₂₅–₇₅) increased during 6 months of smoking cessation, but then decreased after 2 h of 400 ppb ozone exposure. The modified effects of O₃ in smokers could be due to the induction of enhanced antioxidant capacity in the cells of the airways. Age per se can also be a factor; a recent paper showed a decreasing response with increasing age [39].

As mentioned above, most of the knowledge about changes in lung function parameters is based on studies in healthy nonsmoking volunteers. It has been known since a pivotal study in the early 1970s by Bates et al. [40] that when exercise is added to the protocol, mimicking exercise in outdoor polluted air, bronchoconstriction becomes far more pronounced compared with during rest. The elevated minute ventilation during work gives a far higher accumulated inhalation dose of O₃ compared with the resting state. This was first demonstrated in studies in the concentration interval 0.3–0.5 ppm [41–44]. Usually, higher concentrations have been needed to cause effects during rest, although occasionally lung function decrements has been found at 0.3 ppm O₃ even during rest [45]. Folsinsee et al. [46] demonstrated that a lower concentration than previously investigated, 0.21 ppm O₃ for 1 h, also caused bronchoconstriction when the subjects underwent heavy exercise for one hour. The exposure protocol in their study was designed to mimic outdoor exposure in subjects who were undertaking more severe training or heavy outdoor work. Since there were substantial indications that the exposure limits for ambient air contained no safety margin, thought to be due to the effects of cumulative exposure, several studies have been undertaken with even lower concentration but with prolonged exposure, lasting several hours [47].

Folsinsee et al. [46] performed a study in which 10 healthy volunteers were exposed to 120 ppb for 6.6 h, including 5 h of moderate exercise, in a similar fashion to outdoor labour [42]. The decrease in lung function progressed during each hour of exposure. This study confirmed a report by Spector et al. [48], who had found similar lung function decrements in children attending a summer camp and exposed to similar O₃ concentration in the ambient air. The data were also supported by some earlier studies of smaller design [49, 50].

The findings of Folsinsee et al. [46] were subsequently confirmed in a large scale study [33]. Apart from following the previous exposure protocol, this study not only investigated the effect of 120 ppb O₃ but also 100 and 80 ppb. At the two lower concentrations, smaller changes in FEV₁ were seen, but the effects progressed for each hour of exposure. The data were later re-evaluated using multiple linear regression on the hourly responses, and it was concluded that the exposure time was as important as the exposure concentration [51]. This conclusion only concerns the investigated interval of ambient air O₃ of 80–120 ppb. The applicability at higher concentrations remains to be proven.

Using the same protocol as Horstman et al. [33], McDonnell et al. [52] confirmed the lung function decline after 80 ppb O₃, a mean of -8% in FEV₁. These relatively large scale studies clearly demonstrated that O₃ can cause an accumulating decline in lung function over at least 6 h exposure. The accumulation of lung function reduction increases by the hour, but is not linear. Hazucha et al. [53] showed that effects after O₃ were maximal up to 2 h later. They also showed that at low concentrations, the key determinant of lung function changes appears to be the averaged exposure rate.

A study by McDonnell et al. [52] demonstrated that concentrations as low as 80 ppb (0.08 ppm O₃) may cause bronchoconstriction in exercising subjects. The distribution of lung function decline within the subjects is of interest. Despite a relatively moderate mean decline
whereas FOLINSBEE and co-workers [42] were unable to in San Francisco reported evidence for correlation, regarding adaptation of lung function decrements to O₃ other studies have produced the same conclusions re-

an almost total attenuation by day 3–5 [55]. Several the most pronounced decreases in lung function, with have been conducted daily, the second day has caused when exposures have been conducted daily, the second day has caused an attenuation to O₃ exposure has also been shown in other studies. When exposures have been conducted daily, the second day has caused an almost total attenuation by day 3–5 [55]. Several other studies have produced the same conclusions regarding adaptation of lung function decrements to O₃ exposure [56–58].

The ability of O₃ to increase airway responsiveness has been less commonly investigated than alterations purely in lung function parameters. In earlier studies, the subjects at most underwent moderately severe work during relatively short-term exposure. FOLINSBEE and HAZUCHA [59] reported one hour exposure to 350 ppb to cause an increase in methacholine responsiveness. In other studies, effects of airway hyperresponsiveness were demonstrated at higher concentrations [31, 60].

A more recent series of experiments at the US Environmental Protecting Agency (EPA), have to a large extent clarified the dose-response relationship for ozone-induced hyperreactivity at low doses with high workload over longer time. This has been investigated with methacholine tests, and FOLINSBEE and co-workers [42] demonstrated significant hyperresponsiveness following 6.6 h exposure to 120 ppb O₃. In another study by this group [33], also using the same 6.6 h exposure, 80, 100 and 120 ppb ozone exposures were investigated in healthy subjects. The exposures were found to cause 56, 89 and 121% increases in methacholine responsiveness, respectively [33]. For short-term exposure, MCDONNELL and co-workers [61] demonstrated increased histamine responsiveness in allergic rhinitis subjects after 180 ppb during 2 h.

Some controversy exists regarding possible correlation between the response to hyperreactivity tests and reduction in FEV₁ or forced vital capacity (FVC). ARIŞ et al. [62] in San Francisco reported evidence for correlation, whereas FOLINSBEE and co-workers [42] were unable to detect any correlation. Methodological differences could possibly explain some of the differences in these results, with ARIŞ et al. [62] using carbachol, whereas FOLINSBEE and co-workers [42] used methacholine. There were also several differences in exposure protocol. It is, however, a question that is so far unresolved.

Another issue of controversy is the question of whether repeated exposure may cause attenuation of response to methacholine and histamine or not. In some early studies with O₃ concentrations of 0.3–0.4 ppm, attenuation was identified after 2–3 days [63, 64]. More recently, a considerably lower concentration, 0.12 ppm O₃, has been demonstrated to cause persistent elevation of airway responsiveness. This is an important issue to clarify for regulatory purposes. The mechanisms causing airway responsiveness are still unsolved. Bronchoalveo-

lar lavage (BAL) studies showing a cascade of reactive and inflammatory responses to O₃ may provide some explanation. However, there are at present only temporal similarities between the findings and no proof for these mechanisms. This will have to be investigated in studies specifically designed to explore this question and requires pharmacological intervention.

**Bronchoalveolar lavage studies**

BAL has only in recent years been employed in investiga-
tions of the effects of noxious gaseous pollutants in man. The combination of controlled chamber exposure and BAL provides unique possibilities for assessing intrapulmonary effects in man. The drawback of BAL is that information is only gained regarding changes in the airspaces. By analyses of proximal and peripheral BAL liquid portions, some information about effects at different levels can be gained. However, the peripheral airways and the alveolar spaces cannot be satisfactorily separated. Events in cells within the walls of the bronchi, bronchioli and the alveoli and blood vessels are largely inaccessible, unless specific products can be measured in the bronchoalveolar lavage fluid (BALF), indirectly reflecting effects. Despite the drawbacks of the "key-

hole view" of events that are occurring in the lungs, BAL may provide useful information for understanding the human toxicology of air pollutants.

The inflammatory effects of O₃ have been investigated in a number of animal studies [65]. Not until 1986, was the first BAL study exploring the effects of O₃ expos-

ure conducted in humans by SELZER et al. [60] in San Francisco. This group studied the effects of 2 h exposure of 0.4 and 0.6 ppm O₃, equivalent to a stage III smog alert. The authors demonstrated bronchoalveo-

lar neutrophilia, but also increases in prostaglandin E₂ and F₂ (PGE₂ and PGF₂ₐ) and thromboxane B₂ (TxB₂).

The US EPA conducted a pivotal series of studies on ozone-induced inflammation, not only using BAL for studying the lower airways for studying the nose, but also using nasal lavage (NAL). The studies were largely matched with previous and current studies of respira-
tory function. KOREN and co-workers [66] exposed healthy subjects to 0.4 ppm O₃ for 2 h, with BAL 18 h later. Gross changes in inflammatory markers were demonstrated in this thorough study. In addition to confirming bronchoalveolar neutrophilia, there were also increases in BAL albumin, protein, immunoglobulin G (IgG), fibronectin, elastase, urokinase plasminogen activator (U-PA), tissue factor, factor VII, C3a, PGE₂, and neutrophil elastase in the BAL cell pellet. Leukotriene B₄ (LTB₄) was unaffected and did not provide an explanation for the neutrophil recruitment, whilst interleukin-8 (IL-8) is a yet unproven but likely candidate.
Koren and co-workers [67] later investigated the effects of low dose ozone, 0.08 and 0.10 ppm for 6.6 h, with BAL 18 h after exposure. Both exposure protocols produced enhanced numbers of neutrophils, lactate dehydrogenase (LDH), PGE2, and interleukin-6 (IL-6). Only the higher concentration, however, elevated the total concentration of BALF proteins and fibronectin. Alpha1-antitrypsin tended to be elevated at 0.10 ppm, but was only significantly elevated after 0.08 ppm. Again, LT-B4 was unaffected, but on this occasion leukotrienes C4–E4 (LTC4–LTE4) were increased. Interestingly, macrophage phagocytosis by the complement receptor was depressed, but not IgG-mediated or unopsonized phagocytosis.

In a follow-up study Koren and co-workers [68], using an identical protocol to the 1989 study [66], elucidated the earlier response with BAL 1 h following a 0.4 ppm exposure for 2 h. Surprisingly, ozone was found to be capable of causing a very early inflammatory cell response and cell recruitment. In fact neutrophils, IL-6 and PGE2, were higher 1 h than 18 h after exposure. In contrast, fibronectin and plasminogen activator were higher at 18 h in the previous study.

Using cells obtained from a BAL study at the EPA, McGee et al. [69] used northern blot and dot blot analysis to evaluate whether genes for clotting factors would be produced by human alveolar macrophages obtained in a previously presented BAL study. Indeed, messenger ribonucleic acid (mRNA) concentrations were elevated for tissue factor and factor VII. This was accompanied by morphometric evidence of increased numbers of immature alveolar macrophages, despite an ability to demonstrate significantly increased macrophage numbers.

**Nasal airway lavage studies**

Ozone exposure causes not only bronchoalveolar inflammation; in 1988, Graham and co-workers [70] demonstrated nasal increase in neutrophil counts following 0.5 ppm ozone for 4 h on two consecutive days. Nasal neutrophils were not only increased following the first exposure, but also persisted immediately prior and 22 h following the second exposure. The design of the study allowed the authors to demonstrate that the cell recruitment remained following a second exposure, but the question of possible attenuation was not addressed. In a subsequent study, nasal and bronchoalveolar cell data were compared by Graham and Koren [71]. The exposure level was 0.4 ppm for NAL immediately following exposure and again prior to the BAL 18 h postexposure. Although proving qualitatively similar, with neutrophil increases in both locations, no quantitative correlation was found following O3 exposure. The authors presented the data on soluble mediators in the NAL fluid in separate papers the same year [66]. It was demonstrated that O3 caused a trypsin increase, indicating mast cell release, immediately postexposure. The albumin level was elevated only 18 h postexposure, indicating an elevated epithelial permeability in the nose. PGE2, C3a and U-PA were not elevated in the NAL, in contrast to the increase in BALF [66].

Bascom et al. [72] are so far alone in having reported on the use of NAL in allergic rhinitis subjects exposed to O3. Immediately following 0.5 ppm for 4 h, an influx predominantly of neutrophils, but also with striking increases in eosinophils, mononuclear cells and elevation of albumin was demonstrated. Thus, allergic rhinitis patients may have a partly different nasal response to O3 compared with healthy subjects [70]. Nasal allergen provocation was conducted within 30 min after exposure to O3 or air. The allergen challenge following O3 was not found to cause any significant addition in cells or mediator concentrations compared with that elicited by allergen and air, when the effect of the O3 exposure was subtracted. However, the study only provides information on the response to a certain exposure level, duration and timing of response and the issue of whether O3 may affect allergen response in the nose is by no means closed. We are likely to see an interesting continuation of investigations on this issue.

**Airway permeability**

99mTechnetium-diethylenetriamine penta-acetate (99mTc-DTPA) clearance is a method that has been widely used to investigate epithelial permeability. Since 99mTc-DTPA cannot penetrate through mucus, clearance analyses mainly provide information on alveolar permeability. The method has been used in investigating effects of O3 in animal studies, and so far only in one published study in humans. Keirn et al. [73] examined the effects of 400 ppb O3 for 2 h in eight healthy subjects. 99mTc-DTPA clearance showed a pronounced increase in alveolar permeability 75 min after exposure. This is in good agreement with the findings of Koren and co-workers [66] of early increases in BAL albumin and protein, and also with animal study data [74]. As a consequence, O3 exposure at this level may potentially facilitate the penetration of allergens, viruses and micro-organisms into the epithelium of the airways. An area of interest to explore are time-response data that are needed to assess the consequences of the reported findings. Furthermore, information on the effects at low O3 levels and whether adaptation develops after repeated exposures are issues that need to be elucidated.

**Particle clearance**

The effect of ozone on particle clearance has so far received little attention. Foster et al. [75] exposed their subjects to 0.2 or 0.4 ppm O3 for 2 h. The higher concentration produced a marked acceleration of particle clearance both from central and peripheral airways, together with a significant decrease in FVC. The lower concentration increased the particle clearance, but did not affect FVC. This contrasts slightly with the findings of Gerrity et al. [76], who recently reported mucociliary clearance of iron particles to be unaffected when measured 2–5 h after exposure to 0.4 ppm ozone.

Animal studies, on the other hand, have demonstrated retarded mucociliary clearance [77]. Thus, at the
participated. The subjects sensitive to developing bronchomild asthma and 28 healthy nonatopic control subjects. Nineteen subjects with allergic rhinitis, 41 subjects with O₃, whereas none of the healthy or rhinoconjunctivitis response to allergen inhalation after a prior exposure to ment, 9 out of 10 of the asthmatics showed an enhanced response. As a consequence, there are some issues that may have interfered with the conclusions made by the authors.

Most recently JóRRES et al. [82] presented a follow-up study to the investigation by MOLFINO et al. [81]. Nineteen subjects with allergic rhinitis, 41 subjects with mild asthma and 28 healthy nonatopic control subjects participated. The subjects sensitive to developing bronchostriction to 0.25 ppm O₃, "responders", were relatively equally distributed between the groups, without evident relationship to disease status. In the final experiment, 9 out of 10 of the asthmatics showed an enhanced response to allergen inhalation after a prior exposure to O₃, whereas none of the healthy or rhinoconjunctivitis subjects responded to allergen. This study, in accordance with the paper of MOLFINO et al. [81], implies that O₃ appears to enhance responsiveness to allergens. Further studies on this issue are needed, with different exposure conditions.

Effects on peripheral blood parameters

The use of flow cytometry to explore changes in peripheral blood lymphocyte subsets has, surprisingly, recei-

Allergen provocation

The response to allergens is well-known to be of importance in asthma [78]. There are indications from animal and in vitro studies that air pollutants may increase the response to allergens [79, 80]. Data from studies in humans have long been lacking. This issue was recently explored in a pivotal study by MOLFINO et al. [81]. In this much discussed paper, the authors demonstrated that exposure as low as 0.12 ppm O₃ for 1 h during rest increased the airway reactivity after allergen provocation. All seven allergic asthmatics underwent four different challenges with weekly intervals: 1) air and allergen diluent (placebo); 2) O₃ followed by inhalation of allergen diluent (placebo); 3) air followed by allergen; 4) O₃ followed by allergen. Methacholine tests were conducted the day before and after each challenge. The provocative dose of allergen causing a 15% decrease in FEV₁ (PC₁₅FEV₁), was reduced when the allergen challenge was preceded by O₃. Therefore, this clearly supports the hypothesis that air pollutants may alter the response to allergens in humans. However, it should be noted that the effects occurred after a very low O₃ concentration, which has only occasionally been reported to evoke any effect in resting humans [45]. The authors also used a lower significance level of hyperresponsiveness, PC₁₅, and not PC₂₀, which is commonly used. Furthermore, they also chose to put the combined exposure with O₃ followed by allergen last, in order to learn the magnitude of the response to the individual components in each subject and avoid the risk of a severe response. As a consequence, there are some issues that may have interfered with the conclusions made by the authors.

Nitric oxides

Chemical properties

The nitric oxides (NOₓ) include nitric oxide (NO) and nitrogen dioxide (NO₂). These gases are mainly produced during high temperature combustion and formed by reactions between nitrogen and oxygen [85]. NO is partly oxidized and converted to NO₂. In the atmosphere, the nitric oxides interact with other air pollutants, such as O₃ and hydrocarbons, during photochemical processes. During contact with water vapour and particulates in the atmosphere, or fluid on mucosal surfaces, nitric oxides form nitrous acid (HNO₂) and nitric acid (HNO₃). NO₂ is considered by far the most toxic of the nitrogen compounds, except for the formation of methaemoglobin. Consequently, this presentation is largely focused on the biological effects of this gas in humans [86, 87].

Solubility and deposition

NO₂ is a poorly water soluble gas, in contrast to the insoluble O₃ and highly water soluble SO₂. NO₂ is, therefore, deposited far more peripherally in the air-spaces compared with SO₂, but does not reach the alveoli in any significant quantities, except at extremely high exposure concentrations. This is an important consideration with regard to inhalation effects. The fate and distribution of inhaled NO₂ is relatively unknown. A study in
humans reported that approximately 80–90% of NO₂ was found to be absorbed in the airspaces of healthy volunteers during normal breathing [88].

With the use of dosimetry models for estimating the deposition of NO₂ in human and animal lungs, it has been indicated that there would be a relatively even distribution in the conducting airways but with a major deposition in the terminal bronchioles. Very little is believed to reach the alveolar spaces [89–92]. Even though the deposition of NO₂ is considerably less dependent upon the breathing mode than SO₂, ventilation factors are of some importance. Nasal breathing enables more NO₂ to be absorbed in the upper airway compared to oral breathing. Furthermore, exercise may cause more NO₂ to be delivered into the alveolar region [91–93].

Toxicological mechanisms

The formation of nitric and nitrous acids in aqueous solution on the moist surfaces of the airspaces is probably of importance for the toxicity of NO₂ [94]. The main mechanisms of pulmonary toxicity of the potent oxidant NO₂ have, however, been suggested to involve lipid peroxidation in cell membranes [95, 96], and various actions of free radicals on structural and functional molecules [97–99]. Particularly strong free radicals are formed when NO₂ oxidize lecithin, in cell membranes or surfactant, and by interaction with haeme [100]. The NO binding to the iron in haeme protein complexes may, therefore, be used as a biomarker for exposure with NO₂, as previously reported [101].

Sources and exposure

Nitrogen dioxide is discharged during burning of fossil fuels in motor vehicles, and for heating and power generation, and is a common air pollutant of community air in urban areas. The NO₂ concentrations in urban air are systematically characterized by two daily peaks, occurring during the morning and the afternoon hours when traffic patterns are greatest. Annual average concentrations in urban areas are usually less than 0.03 ppm, and thus below the 24 h average limit in most countries of 0.2 ppm (0.4 mg·m⁻³). During peaks, hourly averages may exceed 0.2 ppm, especially during periods of hot weather and stagnant air [102]. Peak concentrations of 0.6 ppm have been recorded during extreme situations.

NO₂ is also present in the indoor environment, and in many industries, particularly in chemical plants and workplaces where combustion processes or gas welding are in use [103]. The exposure limit for NO₂ for an 8 h workshift in most European countries is 2 ppm, and the peak exposure limit for workplaces is 5 ppm. Concentrations up to these levels occur and are sometimes exceeded. Extremely high concentrations may occur in silos, due to fermentation, especially if ventilation is inadequate, causing silo fillers disease [104]. Large populations are exposed to NO₂ from tobacco smoke, fireplaces, kerosene space heaters and gas stoves. In homes with gas stoves, 24 h averages may reach 0.5 ppm, and even peak concentrations of 1–2 ppm have been reported [105–107].

Lung function studies

A considerable number of studies have investigated the lung function response to NO₂ in healthy subjects, asthmatics and, to a lesser extent, patients with chronic obstructive pulmonary disease (COPD). The results have been quite variable over a wide range of concentrations, which makes the understanding of NO₂ effects in the lungs incomplete.

In humans, there has been only one study on NO looking at a workroom or extreme ambient situations [108]. A 2 h exposure to 1 ppm NO caused a significant increase in only one of a large number of investigated lung function parameters, which has not been estimated to be of practical importance [109]. Recently, the role of the NO produced in the vascular endothelium has become an intensive research area. Currently, studies are ongoing with therapeutic addition of NO in the breathing air of ventilators of patients with adult respiratory distress syndrome (ARDS).

Healthy subjects. In healthy subjects, several studies have reported that exposure to 1.5–5 ppm NO₂ significantly increases airway resistance [110, 111]. However, there have been occasional studies, like that from LINN et al. [112], who were unable to demonstrate any effects on lung function, despite an exposure dose as high as 4 ppm for 75 min with intermittent exercise. Concerning the effects of NO₂ concentrations below 1 ppm on lung mechanics, there is more controversy. Several studies have failed to demonstrate any significant effects at this low dose [113–118]. BYLIN et al. [116], in a well-designed study, were able to detect elevated airway resistance in healthy subjects exposed to a concentration as low as 0.24 ppm without exercise. However, a higher concentration of 0.51 ppm, examined in the study, did not cause any effect. It has been suggested that there may be a biphasic response to NO₂, with mechanisms eliciting bronchoconstriction at lower and higher concentrations, but bronchorelaxing mechanisms dominating at concentrations around 0.5 ppm. So far, this is only speculative, and a large scale study investigating a span of concentrations in the same individuals is needed.

BEIL and ULMER [110] demonstrated that healthy subjects developed an increase in airway reactivity following 7.5 ppm, but not 5 ppm, NO₂ for 2 h. Subsequent low dose studies with 0.1 ppm [119] and 0.48 ppm [116] were unable to demonstrate any effect. Recently, MOHSEND [120] observed an increased reactivity to methacholine in healthy subjects exposed to 2.0 ppm NO₂ for 1 h. This was further investigated by FRAMPTON et al. [121], who found 1.5 ppm for 3 h to increase airway reactivity, whereas three 15 min peaks of 2.0 ppm during a 3 h exposure to a basal concentration of 0.05 ppm produced no effect.
Asthmatics. There are indications that asthmatics are more susceptible to increased airway reactivity to NO₂ than healthy subjects. In a much discussed study, Orehuk et al. [122] used a body plethysmograph to demonstrate increased bronchial reactivity to carbachol in asthmatics after exposure to 0.1 ppm. The results were not reproduced in following studies with 0.1 and 0.25 ppm [119, 123]. Kleijn et al. [113], on the other hand, reported increased bronchial responsiveness after 0.2 ppm, as did Bylin and co-workers [116, 124] after 0.29 ppm and 0.51 ppm.

Bauer et al. [125] studied the effects of 0.3 ppm NO₂ on the airway response of asthmatics. Instead of methacholine or histamine challenge, they used exercise and cold air provocation, two common bronchoconstrictor stimuli in asthmatics. The authors demonstrated that the NO₂ exposure caused hyperresponsiveness to these provocations.

Chronic bronchitis and COPD. COPD patients were investigated by Von Nieding and Wagner [126] in 1975, and 1.6 ppm NO₂ was demonstrated to cause an increase in airway resistance. When compared to similarly aged healthy elderly people, Morrow et al. [127] did not find COPD patients to be more susceptible to develop lung function decrements by exposure to 0.3 ppm NO₂ for 4 h. Conflicting results have been noted by Lin and coworkers [128] in COPD patients from the Los Angeles area, who were not found to experience any lung function changes after 0.5–2.0 ppm for 1 h, or after 4 h exposure to 0.3 ppm with intermittent exercise [129].

Von Nieding et al. [130] investigated whether atropine s.c., meclastin i.v. or orciprenal inhalation would protect against increase in airway resistance by mouthpiece inhalation of 5–8 ppm NO₂. Studying a mixed population of healthy subjects and patients with chronic bronchitis, the authors concluded that the lung function impairment could be blocked by an unselective histamine antagonist, meclastine, leading to the assumption that mast cell degranulation and histamine release were involved in the bronchoconstriction caused by NO₂.

Transfer factor. Analysis of changes in the transfer factor for gas exchange in the lungs of humans has so far only been reported in a study from the early 1970s by Von Nieding et al. [130]. The group measured diffusion capacity for carbon monoxide (CO) using the single-breath method, and demonstrated a significant reduction, close to the detection limit, after exposure to 5 ppm for 15 min. Pulmonary perfusion changes were investigated by the same group, but were not seen at this concentration level.

Bronchoalveolar lavage studies

NO₂-induced pulmonary inflammation has long been studied in animal models, using histological specimens and BALF. In animals, the inflammation mainly involves high numbers of neutrophils, but also macrophages [131–133], and in some investigations also lymphocytes and mast cells [134, 135], as previously reviewed in the Journal [3]. Surprisingly, subsequent studies in humans failed to confirm increased numbers of neutrophils in BALF [3, 136–140]. Only in one study, with a very high total exposure dose of 2 ppm for 6 h, was a small but significant increase in BALF neutrophils observed [141]. In all these studies, the recovered BAL was pooled. No information regarding cell response in the proximal airways could, therefore, be gained. As a consequence, two groups have recently modified their BAL techniques, and have analysed a small volume proximal lavage separately from the following larger volume, which mainly sampled the peripheral airways. Hellday et al. [142] demonstrated a small, but significant, increase in neutrophils in the proximal airways of healthy subjects 24 h after exposure to 3.5 ppm NO₂ during relatively light work. Similar data were obtained by Becker et al. [143] at the US EPA, with a mild neutrophil increase in the proximal lavage portion 16 h after a 4 h exposure to 2 ppm NO₂ [143]. None of these studies demonstrated any significant elevation of neutrophil numbers in the peri-branchial portion. Hellday et al. [142] reported a more tendency towards increase. According to the deposition theories, the terminal bronchioli should be receiving the largest exposure dose in the airways, followed by a big dilution of the gas concentration once it enters the larger volumes in the alveoli. If NO₂ causes any recruitment of neutrophils from the bronchioli of healthy humans, it appears to be relatively small and becomes diluted when the BALF samples larger numbers of cells from the alveolar airspaces.

The cellular inflammation detected after higher concentrations over shorter time was investigated in a dose-response study by Sandström et al. [140]. Twenty four hours after 2.25–5.5 ppm for 20 min, small dose-dependent increases in mast cells and lymphocytes were found, without shift in CD4+/CD8+ ratio. Lysozyme positive macrophages, demonstrated with immunoperoxidase staining, were slightly elevated after 4 ppm. In a study following the same protocol, investigating the time course of cellular changes, mast cell and lymphocyte increases appeared early, 4–24 h after exposure, whereas a small elevation in the numbers of lysozyme positive macrophages occurred late, at 24–72 h [3]. At NO₂ concentrations below 2 ppm, no changes in cell numbers have been observed [136–139]. It is presently unclear what the recruitment of lymphocytes and small numbers of mast cells represents. The mere presence of the cells in BALF could be an answer to a recruitment stimulus, but not necessarily a sign of a more intense inflammatory response in the lungs. Studies on functional aspects of BALF cells and tissue will shed light on this question.

Jorres et al. [144] were recently the first to report a study investigating the effects of NO₂ in BAL and bronchial mucosal biopsies, not only in healthy controls but also in mild asthmatics. This was a logical step to extend the bronchoscopy studies on NO₂ to one of the groups that could be expected to react differently to normal subjects. Following a 3 h exposure to 1 ppm, no significant changes in cell numbers in BAL or biopsies could
be recognized, but in the asthmatics some effects could be demonstrated in prostanoid mediators. It is to be expected that in the near future there will be a series of studies in asthmatics using varying protocols and gases, in patients with different severity and medication. It is of considerable interest to evaluate how this growing population in our societies respond to air pollution.

Another group of interest is smokers. In a study involving both young smokers and healthy nonsmokers, as reference, Hellefday et al. [142] detected some differences between the two groups at the reference BAL as well as 24 h after a 20 min exposure to 3.5 ppm NO$_2$. Before exposure, the smokers had less T-cells and more alveolar macrophages (AMs) compared to nonsmokers. After exposure, the smokers reacted with a further increase of AMs and an elevation of neutrophils in the peripheral BAL portion. This may represent an enhancement of their already existing smoke-induced inflammation. The smokers did not experience the acute response with lymphocyte increase as did the nonsmokers, which could be due to some degree of protection by elevated antioxidant levels induced by smoking.

There are a number of indications from epidemiological and in vitro studies that air pollutants may increase susceptibility to airborne infections [145–147]. Frampton and co-workers [136] addressed this question in an experimental study in humans. They found an impaired inactivation of influenza virus by AMs from BALF together with increased interleukin-1 (IL-1) production in a subgroup of subjects following exposure to 0.6 ppm NO$_2$ for 3 h. In another important study, Gøngs et al. [148] inoculated attenuated Influenza A virus intranasally to healthy volunteers. Unfortunately, despite the large scale design of the study, the population was not large enough to detect statistical differences. It suggested, but did not prove, an increased susceptibility to respiratory virus infections following exposure to 1–2 ppm NO$_2$. The approach was novel and interesting, and is sure to be followed by further investigations.

Another approach to studying potentially adverse effects on the immune system has been used by Sandström and co-workers [149, 150]. Flow cytometry with monoclonal antibodies (MoAbs) for lymphocyte markers was used on BALF obtained before and following six repeated 20 min exposures, every other day, to the workroom concentrations, 1.5 and 4 ppm NO$_2$. In both investigations small but significant reductions were found in CD8$^+$/CD4$^+$ cells in BALF, causing modest elevations in the ratio of CD4$^+$/CD8$^+$ T-cells. Minor reductions in CD16$^+$/CD56$^+$ cells (NK cells) were also found. After 4 ppm NO$_2$, reductions were also seen in alveolar macrophage concentrations and CD19$^+$ cells (B-cells). The changes in cell numbers were relatively small, and verification of functional effects in the immune system are needed to clarify whether the findings indeed represent immune suppression. It is, however, interesting to note that Richters and co-workers [151, 152] in animal studies have reported NO$_2$ inhalation to cause adverse effects on the lymphocyte part of the immune system, not only within the lung, but also in the spleen, one of the major lymphoid organs. In their experience, the CD8$^+$ T-cells were most susceptible to NO$_2$ exposure. At present little seems to be known regarding NK cells in the lungs of animals exposed to NO$_2$; contrary to O$_3$ exposure, which has been shown to cause immunosuppression in terms of reduced NK cell activity [153].

The effect of repeated exposure to NO$_2$ has also been investigated in a study with lower concentration of NO$_2$ [154]. Rubenstein et al. [154] exposed subjects to 0.60 ppm NO$_2$ for 2 h, repeatedly, on 4 days during a 6 day period. The exact time intervals between exposures were not given. No effects on BAL cell numbers were seen, except for CD16$^+$/NK cells, which were slightly increased 2 h after the last exposure.

**Inflammatory markers.** During the course of the single exposure studies that have dominated the field of NO$_2$-BAL studies so far, the quest for useable markers of inflammation has long been meagre. In other pulmonary research areas, such as asthma and interstitial lung diseases, characterization of such markers has been successful far earlier. The levels of such substances as albumin, angiotensin converting enzyme (ACE), $\beta_2$-microglobulin, leucocyte elastase and lactate dehydrogenase, total protein [3, 137, 140] and LT$\beta$, PGE$_2$, thromboxane A$_2$ (TXA$_2$), tumour necrosis factor-α (TNF$\alpha$) (Sandström and co-workers, Henderson and co-workers, unpublished data) have all been assayed and found to be unaffected by exposure. Recently, Becker et al. [143] found the activity of plasminogen activator and the concentration of $\alpha_1$-protease inhibitor ($\alpha_1$-PI) ($\alpha_1$-antitrypsin) to be increased in the bronchial portion of BALF 16 h following 2 ppm for 4 h.

NO$_2$ can act as a potent oxidant as well as a free radical, and may affect structures in proteins and lipids. In studies with animal and in vitro models, there has been a considerable interest in how NO$_2$ may affect the protease-antiprotease balance. $\alpha_1$-PI is a protein of major importance for inactivating proteases in the body. Its primary function has been suggested to be control of neutrophil elastase [155]. Deficiency, low levels or inactivation of this protein by tobacco smoke or other oxidants, has been associated with development of emphysema [156, 157]. Moshein and Gee [138] were the first to study the functional activity of $\alpha_1$-protease inhibitor in pooled lavage fluid. The activity was found to be decreased after a 3–4 ppm NO$_2$ exposure for 3 h, as compared with air exposure. Using another protocol, with the highest examined concentration being 1.5 ppm for 3 h, Johnson et al. [158] were unable to confirm any trend towards reduced activity in pooled BALF. This may have been due to different exposure doses, processing and, in particular, the variability in activity relative to the immunological concentrations, as suggested by Johnson et al. [158]. Furthermore, in vitro exposure of $\alpha_1$-PI to very high concentrations of NO$_2$ did not affect its elastase inhibitory capacity [138]. This led to the hypothesis that NO$_2$ exerts its inhibitory action on $\alpha_1$-PI through peroxidation of cell surface and surfactant lipids, which are highly reactive towards the protease inhibitor, which indeed found support in a subsequent study [139]. Not only were lipid peroxidation products increased in BALF.
following 4 ppm for 3 h, the elastase inhibitory capacity was decreased. Noteworthy is the finding that supplements of vitamin C and E to one of two parallel exposed groups of subjects resulted in significant diminishment of these effects. The recent findings of Becker et al. [143] of an elevated concentration of α1-PI in the proximal (bronchial) portion of BALF adds interesting aspects to the issue of functional activity, which needs further work for full elucidation. Neutrophil numbers rise in the proximal airways following NO2 exposure [142, 143], and α1-PI production could, at least partly, compensate for the effects of increased neutrophil activity.

Alpha2-macroglobulin is a protein that acts as a local protease inhibitor in the lungs, but also has the ability to modify inflammatory courses [159, 160]. Increased lavage levels of α1-macroglobulin after exposure to 0.6 ppm NO2 have been reported by Frampton and coworkers [137]. In this early study, the BALF was not separated into different portions, so that comparisons with the elevation of α1-PI are not possible [143]. Until further studies are undertaken, the consequences of the response cannot be fully interpreted.

**Airway permeability**

By using the TE-DTPA clearance method, Rasmussen et al. [161] addressed the question of whether NO2 exposure would alter alveolar permeability in humans. A delayed reduction was observed at 11 h, but not 1 or 18 h after the end of a 5 h exposure to 2.3 ppm NO2 (4.4 mg.m⁻³). The reason for the NO2-induced reduction in alveolar permeability is not known, but a swelling of epithelial cells was among the possibilities suggested by the authors. The findings are in contrast to some animal studies, that have shown enhanced permeability of the epithelium in the trachea and alveoli after exposure with NO2 [74, 162, 163] and O3 [164]. The data are also in contrast to the increased alveolar permeability seen after ozone exposure in man [73]. Ozone causes increased levels of albumin in BALF, whereas NO2 does not, at the hitherto examined concentrations.

With regard to the suggested deposition of NO2, it would be surprising if a pronounced increase in permeability in the epithelium of the alveolar spaces would develop. The issue of whether NO2 may cause a greater permeability through the ciliated epithelium in the airways is perhaps of more interest. The methods of evaluation used in the two human studies mentioned have not sufficiently separated the permeability properties in the alveoli versus the ciliated airways. Animal studies have, indeed, found effects in the proximal airways. If this is also the case in humans, it would be of importance for the penetration of allergens, viruses and other micro-organisms in the airway epithelium. In the light of the studies by Molfino et al. [81] and Jørres et al. [82], showing that asthmatics experience a more pronounced airway responsiveness to allergen provocation if preceded by O3, this is an issue that needs further exploration both with regard to NO2 and O3.

**Antioxidants**

Mohsenin [139] highlighted an important issue regarding the antioxidant status of subjects who have undergone NO2 exposure. He found that supplements of vitamin C and E to one of two parallel exposed groups of subjects significantly diminished the effects on lung function. An important consequence of this protective effect is that spontaneous intake of vitamins of subjects in other studies may, potentially, have influenced the results in an unexpected fashion. These vitamins and other dietary antioxidants are confounding factors that could account for some of the differences in the outcomes of studies. Other reasons for diverging results between these studies are, of course, the differences in exposure protocols and methods of analysis, and the potential influence of clinical and subclinical viral infections. An important question is, whether the dietary antioxidant issue concerns only NO2. It could well be that other oxidants, such as O3, are also affected.

Antioxidants have been little evaluated after NO2 exposure. Rasmussen et al. [161] found 2.3 ppm NO2 for 5 h to significantly decrease glutathione peroxidase (GSH-Px) activity in serum, but not glutathione (GSH) or GSH-Px in whole blood. It was suggested that these findings showed that GSH-Px in serum is more accessible to the endothelial cells in the lungs and is taken up in response to lipid peroxidation of NO2.

**Biomarkers for exposure with nitric oxides**

Biological markers (biomarkers), by which inhaled biological exposure doses of gaseous pollutants can be measured, could be potentially beneficial when it is of importance to determine the biological dose, depending on activity and ventilation. Recently, two markers have been developed, one for measuring exposure to NO, [101], and one for SO2 in humans [165]. For developing a biomarker for NO2 and NO exposure, the property of NO to bind to the iron on haeme proteins was used. When NO2 is dissolved in ELF in the airways it is hydrolysed to form HNO2 and HNO3, which is in balance with NO in chemical reactions. A study by Maples et al. [10] investigated the NO/haeme protein complex as a biomarker, by measuring it with electron spin resonance (ESR) in tissue microsomes, rat lung, and BAL cell pellets from rats and humans. The ESR signal intensity to NO2 dose relationships were linear in all evaluation set-ups. Information on the usefulness of this biomarker in nasal lavage in humans is soon to be expected.

**Sulphur dioxide**

**Chemical properties**

SO2 is slowly oxidized in air to SO3. This process may be accelerated due to other pollutants and photochemical processes in the atmosphere. In contact with water vapour or water bound to particulate pollutants,
sulphurous acid (H$_2$SO$_3$) is formed. It is rapidly dissociated to hydrogen ions, HSO$_3^-$ and SO$_3^{2-}$, giving acidic solutions. The sulphurous anions are oxidized to SO$_4^{2-}$. H$_2$SO$_4$ may react with ammonia and eventually be neutralized by formation of a salt (NH$_4$)$_2$SO$_4$. An intermediate product, ammonium bisulphate (NH$_4$HSO$_4$), is a strong acid. The ammonium for neutralization may be available in the air in urban and agricultural areas, but not in forests and lakeland districts [166–168].

**Solubility and deposition**

Sulphur dioxide is a highly water soluble gas, in contrast to the common air pollutants NO$_2$ and O$_3$. Therefore, up to 98% may be absorbed in the nasopharynx during nasal breathing [169, 170]. Consequently, according to the current concept, only a minor portion will reach the proximal pulmonary airways and, due to the high water solubility, the rest will be rapidly absorbed in the mucus and epithelial lining fluid in the bronchi. Little or none of the SO$_2$ is believed to reach the lower airways. During mouth breathing, especially during hard work with high minute ventilation, the deposition is different. High concentrations may reach the trachea and proximal bronchi. Due to the solubility, the rest is probably rapidly absorbed before entering the most peripheral airspaces [170]. Only at extremely high SO$_2$ concentrations will alveolar deposition occur [167, 171].

**Toxicological mechanisms**

Several mechanisms have been suggested to mediate the noxious effects on cells by SO$_2$. It is still not completely identified which mechanisms dominate when SO$_2$ in gas phase affects cells laying superficially in the air spaces. SO$_2$ is probably absorbed by water vapour in the airways, and mucus and epithelial fluid in the bronchial walls, forming sulphuric acids and bisulphates. Whether the acids produce their main effects on the cells in the airways due to their acidity, ions or reaction products is still not completely resolved. At least with regard to the development of airway resistance, it does not seem that acidity itself, in aerosols with pH as low as 2, produces pronounced effects [172]. Furthermore, inhalation of H$_2$SO$_4$ aerosol, with unspecified pH, caused no cell influx measured with BAL [173]. However, this does not exclude the possibility that inflammatory reactions take place in the airways in response to acids. The mechanism of the SO$_2$–induced bronchoconstriction has been studied, but is not yet completely clarified. Strong evidence indicates that parasympathetic reflexes are important [174, 175]. Anticholinergics reduce the bronchoconstriction, but disodium cromoglycate also shares this effect. It has been suggested that neurokinins, such as substance P, and possibly mast cells, may be involved in mediating the effect [176].

**Sources and exposure.** Sulphur dioxide constitutes a considerable part of the gaseous portion of ambient air pollution. SO$_2$ is a common air pollutant, produced during combustion of sulphur rich fossil fuels in, e.g. oil refineries, motor vehicles, and for heating and power generation. It is also produced in paper pulp industries, blast furnaces, smelters, and during production of sulphur based chemicals, such as sulphuric acids. The highest workshift concentrations of SO$_2$ are encountered in indoor environments in, e.g. paper pulp industries, smelters and petroleum refineries [177, 178]. The SO$_2$ levels that people are exposed to in ambient air are low compared with the exposure in some indoor work situations, but may affect sensitive populations.

The short-term exposure limit (STEL) for workplaces is in Sweden, the United States (Occupational Safety and Health Act (OSHA)) and several other countries is 5 ppm (13 mg·m$^{-3}$). It has been reported from several sources that this limit has frequently been exceeded in brief peak exposures in workrooms in some industries [167, 177–179]. Exposure levels of 7–11 ppm SO$_2$ (20–30 mg·m$^{-3}$) have been reported to be common in some sulphite pulp mills [180], and peaks of 20–40 ppm have been reported from industrial plants [167, 171]. SO$_2$ concentrations in excess of 5 ppm have been reported to occur in all of the previously mentioned industries, but also in wineries and food-processing plants [176]. Kerosene heaters in homes may produce up to 1–2 ppm (2.5–5 mg·m$^{-3}$), depending on space and ventilation. Ambient air may contain slightly less SO$_2$, up to 0.3–0.4 ppm in peaks in very polluted urban areas [176]. With the exception of peaks, SO$_2$ concentrations in ambient air stays below 0.04 ppm (100 µg·m$^{-3}$).

**Lung function studies**

Environmental chamber exposures with controlled concentrations of SO$_2$ have been performed in numerous studies, with different designs regarding exposure concentration, workload and breathing pattern, both in healthy subjects and patients suffering from obstructive lung diseases.

The lung effects of SO$_2$ in concentrations ranging around the STEL for workplaces have been particularly well evaluated in chamber exposure studies. The response is highly dependent upon the exposure protocol, especially the breathing pattern. With oral breathing or inhalation via a mouthpiece, very high concentrations may enter deeper into the airways compared to nasal breathing, when the dominating part is absorbed in the upper airways before entering the lungs.

Most healthy nonhyperactive subjects seem to develop increased airflow resistance above 5 ppm SO$_2$, which is a concentration not encountered outside some polluted industrial workrooms [181–183]. Occasionally, sensitive subjects, reported to be nonasthmatics, have been found to react slightly after 1 ppm SO$_2$ [181]. Asthmatics have been much studied, and respond to lower concentrations than healthy subjects, with airway constriction and asthma symptoms at 0.25–0.5 ppm SO$_2$ (0.7–1.3 mg·m$^{-3}$) [184–187]. These levels seldom occur in ambient air over larger areas, except during occasions...
of pronounced air pollution. The concentrations can, however, occur during peaks for shorter time periods downwind of certain point sources [176]. Asthmatics may respond with a very rapid airway obstruction after brief exposures when exercising. Two minutes exposure to 1 ppm was reported to be sufficient to give significant response [188]. The response may increase over a longer exposure, 10–30 min [189]. It is not surprising that exercise so clearly enhances the bronchoconstrictive effects of SO₂. Firstly, the clearance of the main part of the SO₂ through the nose and nasopharynx is avoided, since exercise causes the subject to mouth breathe, with increased ventilation volumes. This causes vastly increased amounts of SO₂ to reach the airways. Secondly, the increased ventilation rate per se is a bronchoconstrictor by drying out the airways, which affects the ELF and ion balance over cell membranes. The combination of SO₂ and dry cold air enhances the bronchoconstrictive response [190, 191]. As a consequence of these circumstances, some asthmatics may be at risk of experiencing symptoms when exercising outdoors.

A method to predict the response to SO₂ in asthmatics has been an issue of evident interest. This was extensively explored by Magnussen et al. [192], in a large scale study with 46 asthmatics exposed to air and 0.5 ppm SO₂. After 10 min tidal volume breathing, the subjects performed isocapnic hyperventilation. Histamine challenge was used to measure hyperresponsiveness. A weak, though significant, correlation (r=0.48) was found between histamine PC₁₀₀ for airway resistance and SO₂, but no correlation with hyperventilation. It therefore appears that SO₂ and histamine cause bronchoconstriction, in part, through different mechanisms.

The symptoms associated with SO₂ exposure are usually mild, even at concentrations up to 10 mg·m⁻³ [193]. Only occasionally, more severe bronchoconstriction and dyspnoea develops. The symptoms are dependent on the solubility of the gas in moist mucosal membranes, and include nose irritation and an unpleasant smell of sulphur, as well as throat irritation. Eye and airway symptoms are less pronounced in healthy subjects. Tam et al. [194] investigated the nasal responsiveness in patients with allergic rhinitis and nonallergic rhinitis and nasal congestion, but were unable to detect any pronounced sensitivity to SO₂ in these groups up to a concentration of 10 mg·m⁻³ [194].

Bronchoalveolar lavage studies

Bronchoalveolar lavage has only been used in a few studies to investigate whether signs of acute inflammation would occur after single exposure to SO₂ at workroom concentrations [195–197]. After 20 min exposure to 10, 13, 20 and 30 mg SO₂·m⁻³, a dose-dependent increase in lymphocytes, alveolar macrophages, lysozyme positive macrophages and mast cells has been demonstrated. A time-kinetic study revealed that SO₂ had elicited an increase in inflammatory cells as soon as 4 h after exposure, which increased to peak values by 8–24 h, and had ceased by 72 h. The changes in cell numbers in BALF after SO₂ exposure were most pronounced in the alveolar macrophage numbers. The alveolar macrophage is by far the most numerous cell in BALF, around 90% of all cells under normal conditions. SO₂ exposure caused the total numbers to be doubled. Lymphocyte increases were moderate, without shift in CD4/CD8 T-cell ratios, and the changes in lysozyme positive macrophages and mast cells were miniscule. No changes in BAL concentrations of albumin, fibronecrtin, hyaluronic or β₂-microglobulin were detected in either study.

Biomarkers for SO₂ exposure

Until recently, there has been no biological marker (biomarker) available that corresponds with the inhaled dose of SO₂ in humans and would be beneficial for epidemiological studies, when the biological dose, depending on activity and other circumstances, needs to be measured. Bechthold et al. [165] developed such a marker by using cyanolytical extraction of sulphite from nasal lavages. The assay determines the s-sulphonate concentrations produced when inhaling SO₂. Single exposure in humans and rodents caused clearly detectable concentrations. Multiple exposures with SO₂ on separate days in humans did not cause any accumulation of the biomarker in the nose. Dose-response characteristics and clearance rates are expected in subsequent investigations.

Acid aerosols

There are epidemiological data that imply negative health effects in humans due to acidic aerosols [198–200]. Strong acids, such as HCl and HNO₃, do not commonly affect lung function at the ambient concentrations investigated in experimental studies [201]. Only a study by Koenig et al. [202] detected a bronchoconstrictive response to 50 ppb HNO₃. There is far more support for negative effects by sulphuric acids.

In experimental studies, normal subjects have been reported to experience throat irritation and increased airway reactivity to carbachol inhalation challenge at a H₂SO₄ concentration of 450 μg·m⁻³ [203, 204]. Lung function reduction has generally not been seen below 1,000 μg·m⁻³ in healthy subjects. Asthmatics may react considerably more profoundly to sulphuric acid aerosols, with lung function decrease after concentration as low as 51–100 μg·m⁻³ [202, 205, 206]; although a few contradictory results have been reported [207]. Mucociliary clearance is affected in a dual response pattern, depending on exposure concentration. High concentrations of 1,000 μg·m⁻³ and more impair clearance, whilst low concentrations stimulate the clearance rate [208, 209]. Frampton and co-workers [173] have presented the only study so far to evaluate the effects of sulphuric acid aerosol on BAL cells and function. Unlike findings following SO₂ exposure, no influx of inflammatory cells
was detected 18 h after 1,000 µg·m⁻³. Inactivation of influenza virus and superoxide anion production was not altered, in contrast to findings from this group after NO₂ exposure [136]. Interestingly, antibody-mediated cytotoxicity of AMs was found to increase after exposure.

**Combined exposures**

Combined exposures with air pollutants is the standard situation in outdoor air, and frequently also in indoor workrooms with air pollution. When human exposure studies are considered, it is apparent that studies with combined exposures are rare. This is probably an area where, for these obvious reasons, there will be an increased activity within the next few years. For regulatory reasons, it must be determined whether combinations of certain air pollutants at specific concentrations may cause effects that are different compared with the individual gases alone.

**Oxygen and nitrogen dioxide**

KOENIG et al. [210] investigated 12 healthy and 12 asthmatic subjects, exposed to ozone, 0.12 ppm O₃ and 0.30 ppm NO₂, alone or in combination during 60 min of intermittent moderate exercise according to a randomized scheme. The combination of the two gases did not produce any more pronounced effect than the individual gases alone. Other studies have come up with equivalent results [211, 212].

**Oxygen and sulphur dioxide**

In only one study have the interactive effect of a combination of ozone and SO₂ been found to cause an increased lung function decline compared with ozone alone [213]. In two follow-up studies, BEDI and co-workers [214, 215] were unable to confirm these findings. KOENIG and co-workers [216] recently evaluated the response to prior exposure with 0.12 ppm O₃ before exposing asthmatics to a concentration as low as 0.1 ppm SO₂. This combination elicited a significant bronchoconstrictive response, whereas using the same protocol, air followed by SO₂ exposure, or two sequential exposures with O₃, did not. An important circumstance, pointed out by FOLINSBEE [217] is that SO₂ and ozone only occasionally, e.g. in Southern California, co-exist at close to equivalent concentrations. Nevertheless, this study stimulates interest in exploring the effects of other O₃ and SO₂ concentration combinations.

**Ozone and nitric acid**

The effects of HNO₃ alone, and together with O₃ have been explored in one study. Nitric acid is even more water soluble than H₂SO₄, and is, therefore, believed to be cleared mainly in the upper airways. Healthy subjects were exposed to 500 mg·m⁻³ HNO₃ alone, and together with 0.2 ppm O₃, for 4 h. Åris et al. [62] were unable to find any changes in BALF constituents from isolated left mainstem bronchial lavage or mucosal biopsies sampled 18 h after exposure by HNO₃ alone. HNO₃ and O₃ caused similar effects to O₃ alone, as reported by KOREN and co-workers [66]. Neither, did the combination cause any potentiation of lung function effects compared with O₃ alone.

**Nitrogen dioxide and sulphur dioxide**

NO₂ has not been found to cause any potentiation of SO₂ effects on lung function, even at considerably high concentrations of both gases [219, 220]. So far, it seems that under most circumstances combinations of pollutants produce effects that are additive and not synergistic. However, there are numerous exposure situations that remain uninvestigated. For instance, it has not yet been determined whether repeated exposures may cause other effects, and if different intervals between exposures with different pollutants are of importance. The effects of a prior exposure with one pollutant may influence the effects of exposure to a subsequent pollutant, as shown by KOENIG et al. [221]. Commonly, automobile generated NO and NO₂ may be high during the morning traffic, and citizens may be re-exposed to NO₂ and NO during the late afternoon traffic, but then with the addition of ozone. Furthermore, suspended particulates may potentially alter the effects of the pollutants directly or indirectly by modifying immune reactions, e.g. via effects on alveolar macrophages.

Another circumstance that has not been sufficiently considered is determination of potential differences in O₃ sensitive subjects, "responders", and "nonresponders", with regard to lung function effects following exposures with combinations of ozone and other pollutants. It could well be that investigating a mixture of "responders" and "nonresponders" may dilute and conceal significant responses among the "responders".

There is clearly a need for specially designed studies to explore the effects of combinations with O₃ and other pollutants before the issue can be closed.

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