In Vitro Tests for Respiratory Toxicity
The Report and Recommendations of ECVAM Workshop 18\textsuperscript{1,2}

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Preface

This is the report of the eighteenth of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM’s main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the

\textsuperscript{1}ECVAM — European Centre for the Validation of Alternative Methods. \textsuperscript{2}This document represents the agreed report of the participants as individual scientists.
current status of various types of in vitro tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on In Vitro Tests for Respiratory Toxicity was held in Angera, Italy, on 14–18 November 1994, under the chairmanship of Claude Lambré (Department of Toxicology-Ecotoxicology, INERIS, France). The objective of the workshop was to review the development and use of alternative methods for respiratory toxicology investigations with respect to their applicability for hazard assessment, and to formulate some recommendations about the best ways forward.

Introduction

Respiratory toxicity is a major health concern. With a population of $5 \times 10^9$ people worldwide, the total area of human pulmonary epithelium which is exposed to air represents about $2 \times 10^6$ km$^2$, that is, 1/200 of the total surface of the earth. Since the number of chemicals and products being developed continues to increase, a better understanding of the mechanisms of chemical-induced respiratory toxicity, and the development of efficient tests for predicting their effects on the respiratory system, are needed. For hazard assessment purposes, information derived from in vitro toxicology studies, combined with knowledge of structure-activity relationships (SAR) and biokinetics, should be employed more often than at present.

The respiratory tract has many different functions. In addition to olfactory, gaseous exchange and blood oxygenation functions, it plays a protective role against xenobiotics and invading microorganisms. In this respect, the respiratory tract is not only a passive and/or active physical barrier equipped with mucociliary clearance, but also plays a significant role in both non-specific and specific immune surveillance (2–4). The ability of the respiratory tract to carry out these various roles is dependent upon the presence of an intricate vascular, lymphatic and nervous network, and of a wide variety of cell types with many different functions. Since thin epithelial cell membranes are needed for gaseous exchange, the secretion of mucous, and the fluid lining the epithelia, provide a certain amount of protection following exposure, via inhalation, to xenobiotics. In addition, it must not be forgotten that the cells and tissues of the respiratory system can be exposed to xenobiotics via the systemic circulation.

Occupational and environmental exposure to chemicals, as either solids, liquids or gases, or as mixtures (for example, smokes, fumes and aerosols), can lead to pulmonary disorders (5–7). In this respect, cigarette smoking, in addition to its own direct effects on the respiratory tract, has frequently been demonstrated to have synergistic effects when combined with exposure to other chemicals.

Thus, inhalation toxicology is a complex subject. The workshop focused on discussion of the advantages and limitations of using various cell types and endpoints for identifying the potential toxic effects of airborne compounds. In this context, the recognised usefulness of the isolated perfused lung (8, 9) was not covered. The underlying basis for the discussions was the recognition that, at present, the application of in vitro methods is limited to investigations of acute respiratory toxicity.

Major Lung-specific Pathophysiological Effects

The major disorders of the respiratory tract which have been identified as being relevant to exposure to xenobiotics, and which are therefore necessary to take into consideration with respect to in vitro studies, are listed in Table I.

Pathophysiological Mechanisms Involved in Respiratory Disorders

When considering the means by which a given compound can produce toxic effects on respiratory cells, several initial factors have to be taken into account. Before reaching the cellular structures of the respiratory epithelium, the physicochemical properties of the xenobiotic may be such that it reacts with the intraluminal humoral material (that is, the various secretions of the respiratory tract). In this respect, the most relevant physicochemical properties are the granulometric and
Table I: Major disorders of the respiratory tract following exposure to xenobiotics

<table>
<thead>
<tr>
<th>Category</th>
<th>Details</th>
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<tbody>
<tr>
<td>Irritation</td>
<td></td>
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<tr>
<td>Sneezing, coughing, olfactory dysfunctions</td>
<td></td>
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<tr>
<td>Acute injury and repair of epithelium</td>
<td></td>
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<tr>
<td>— alteration in barrier function</td>
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<tr>
<td>— oedema</td>
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<tr>
<td>Altered clearance</td>
<td></td>
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<td>— decreased mucociliary escalator efficiency</td>
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<td></td>
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<tr>
<td>Gas exchange</td>
<td></td>
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<tr>
<td>— destruction of the alveolar septa: emphysema</td>
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<tr>
<td>— decreased elasticity: fibrosis</td>
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<tr>
<td>— decreased volume</td>
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<tr>
<td>— quantitative and qualitative alterations</td>
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<tr>
<td>— mucus: protein and sugar structure and</td>
<td></td>
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<tr>
<td>synthesis</td>
<td></td>
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<tr>
<td>— surfactant: composition, excretion and</td>
<td></td>
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<tr>
<td>resorption</td>
<td></td>
</tr>
<tr>
<td>— alveolitis (various cell types)</td>
<td></td>
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<tr>
<td>— hyper-reactivity/bronchoconstriction: asthma</td>
<td></td>
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<tr>
<td>— metaplasia</td>
<td></td>
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<tr>
<td>Neoplastic diseases (decreased immune functions):</td>
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<tr>
<td>— proliferation</td>
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<tr>
<td>— differentiation status</td>
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Adsorbing capacities of the compound, its solubility in the lining fluid, and its ability to interact with the body's various protection systems (for example, antioxidant compounds, such as uric acid, react with ozone [10]). These initial reactions may alter the toxic potential of the xenobiotic (11, 12).

Interaction of the xenobiotic with its primary target cells can damage the cells, resulting in modified cellular morphology and biochemical ultrastructures, altered metabolic activities (production of mediators, etc.), cytotoxicity, and/or genotoxicity. The xenobiotic can also undergo biotransformation, by either the target cells or by other cells in the vicinity which may, for example, have been attracted by mediators released by the primary target cells in response to exposure to the xenobiotic (13-17). The enzymatic (Phase I and Phase II) systems involved in biotransformation are necessary for the solubilisation and excretion of compounds and, in this respect, have a protective function. However, they can also produce reactive metabolites, leading to toxicity. In the respiratory tract, biotransformation reactions are often confined to specific cell types. It should also be remembered that there are considerable differences in biotransformation capacities between species and even between strains.

It is particularly important to characterise the in vitro system being used with respect to the presence of appropriate metabolising activity. Freshly isolated tissue slices and certain types of isolated cells maintained in suitable culture media, which are likely to possess biotransformation activities comparable to the situation in vivo, are better models than are long-term cultures or cell lines, which can lack the enzyme systems required for biotransformation (18, 19).
Target Cells from the Conducting Airways and Relevant Endpoints

Since the epithelium of the respiratory tract is a remarkably diverse and complicated layer, consisting of more than ten main types of cells, it was decided that the discussions at the workshop should concentrate on "respiratory epithelial cells". Thus, cartilage, neuro-epithelial, vascular and endothelial cells, fibroblasts, basal (undifferentiated) cells, mast cells and the bronchus-associated lymphatic tissue, although of importance in lung disorders induced by various airborne pollutants, have not been considered. Investigations are also being carried out with muscle cells; these cells are important in bronchial hyper-reactivity, a common manifestation of the toxic effects of airborne pollutants, and so the development of test systems employing muscle cells should be encouraged (20). Also, following uncontrolled cell proliferation as a result of the secretion of growth factors, fibroblasts play a prominent role in the development of lung fibrosis, another common pulmonary disease associated with chemical exposure. In addition, mesothelial pleural cells represent an important cell type in the context of lung diseases induced by fibres. Time constraints prevented proper discussion of these latter cell types at the workshop.

Nose

Cells from the nose can be harvested by surgical biopsy, curettage, and lavage. Several adverse effects of chemicals on olfactory cells from the rat have been reported. Ex vivo experiments, with cells from the nasal turbinate which possess biotransformation potential, were able to reproduce the effects observed in vivo.

Nasal polyps are a very useful source of epithelial cells, particularly from humans (21). Various endpoints, including mucin production, effects on enzymes, mucociliary clearance, and ciliogenesis (22), can be studied in these cells. Nasal lavage, which is easy to perform in humans, provides a useful source of cells. However, great care must be taken to standardise the technique, and confounding factors, such as the nasal cycle (23), should be taken into account. Nasal resistance is a questionable measurement in vivo and cannot be evaluated in vitro.

Trachea and bronchi

Epithelial cells

Various studies have been undertaken with epithelial cells derived from the trachea and bronchi (24, 25). Differences in both the species of origin (human, rat, rabbit, guinea-pig, hamster) and the endpoint measured, mean that the results obtained are not always comparable. Furthermore, the wide heterogeneity in the culture conditions employed results in different growth characteristics and even phenotypes. As an example, in addition to the species of origin of the cells, the proportion of ciliated cells depends mainly upon: a) the composition of the culture medium, including the presence of growth and differentiation factors, such as retinoids (26); b) the nature of the starting material, for example, explant, or mechanically or enzymatically isolated cells; and c) the presence of a supporting growth matrix in the culture flask (27). Secretory cell differentiation seems to be less dependent on the presence of a collagen substratum than does ciliated cell differentiation (27).

The epithelial nature of the cultured cells should be determined by either assessing their morphological characteristics (contact inhibition, tight junctions, presence of microvilli) or by immunostaining with, for example, anti-keratin antibodies (28). Fibroblast contamination can be prevented by various means, including: a) panning after isolation; b) culture under low-temperature (33°C) conditions; c) use of serum-free culture media; or d) use of special culture flasks.

This model has been very useful for studying ciliogenesis and ciliary repair, and effects on ciliary beating (29). Other endpoints which have been investigated are: a) cell proliferation (30); b) genotoxicity (31); c) carcinogenesis (32); d) cell transformation (33); e) mucin production; f) the biochemical composition of the cell membrane (34); g) the expression of receptors, including class I and II major histocompatibility complex (MHC) antigens (35), cell adhesion molecules (36), and adrenoreceptors (37); h) the release of mediators, such as cytokines (38) and arachidonic acid (39, 40); and i) the production of reactive oxygen metabolites. Cellular electrophysiological properties and ion transport have also been widely studied (41, 42).
The immunological roles of epithelial cells (4) warrant further investigation, since epithelial cells represent a major contact area for the organism with atmospheric pollutants. These roles include involvement in the host’s immune response to inhaled antigens, via the processing and presentation, in association with the MHC class II molecules, of these antigens. In addition, recognition by, and reaction with, components of the host’s immune system may occur, either as part of the regulation of cellular metabolism by immunocompetent molecules (43) or after xenobiotic-induced alterations of the cell membrane (44). This may play a significant role in the development of autoimmunity.

Differentiation parameters of epithelial cells are often poorly expressed, particularly for cells of human origin and for cells cultured on plastic. The possibility of using tissue slices and suspension cultures (18, 22), in which the structural properties and functions associated with differentiated cells (for example, the presence of cilia, and mucin production) are maintained, seems to be promising and should be encouraged. In addition, biphasic culture systems are suitable for mimicking naturally occurring exposures, and they should be developed and evaluated further (45–47). The recent production of immortalised human bronchial epithelial cells, by the introduction of exogenous DNA encoding, for example, SV40 T-antigen (48, 49), is an important development.

Submucosal glands
These cells warrant special attention with respect to studying respiratory toxicity, because hypertrophy of the tracheobronchial submucosal glands is a frequent observation in asthma, chronic bronchitis and cystic fibrosis. Data from physiological, histological and anatomical studies provide extensive evidence for the neuronal innervation of submucosal gland secretion, including adrenergic, cholinergic and peptidergic inputs. However, submucosal glands are not responsive to pharmacologically active compounds, and there is no evidence that they have neuronal inputs. In vivo, changes in the ratio of serous to mucous (goblet) cells have been reported in submucosal glands, as well as in the epithelium. Monolayer culture techniques for human tracheobronchial submucosal glands have been developed.

Bronchioli
The bronchioli are defined as those conducting airways which lack any surrounding cartilage. There are interspecies differences with respect to the distribution of Clara cells, the localisation of which may or may not be restricted to the bronchioli. Both in vivo and in vitro, the bronchioli are very sensitive to numerous xenobiotics and pollutants and, because of their role in forming hyperplastic lesions and the possibility that they may act as progenitors of adenocarcinomas, they have stimulated considerable interest.

Their involvement in biotransformation has been clearly established in rodents but, though very likely, still remains to be definitively demonstrated in humans. The bronchiolar cells possess a variety of enzymes, including cytochrome P450 mono-oxygenases, acid and alkaline phosphatases, non-specific esterases, hydroxylases, transferases, peroxidases and catalase. Clara cells show the highest rates of cytochrome P450-mediated metabolism of any pulmonary cell type; consequently, one of their main functions is the metabolism of airborne xenobiotics. Thus, they are an important target cell for any toxic, mutagenic or carcinogenic compounds which require bioactivation via the cytochrome P450 system. They are the only stem cells, and differentiate into further Clara cells, or into ciliated cells, in response to injury.

Clara cells have been isolated from various rodent species (50, 51). The purification of human Clara cells is still difficult. Protease digestion, cell elutriation and differential attachment to pre-conditioned collagen matrices have been demonstrated to yield cultures which are > 85% Clara cells (52). Alterations observed in enzymatic patterns and metabolic activities in vitro have been reported to be related to observations in vivo, and the secretion of chloride and hydrogen carbonate ions has been shown to be regulated by β-adrenergic agonists in vitro (52).

Alveoli
Squamous Type I pneumocytes cover 95% of the alveolar surface and are responsible for gaseous exchange. They have pinocytic activity and, in this respect, may play a role in antigen transport. In vivo, they are very sensitive to many compounds (either inhaled or parenterally administered), including vol-
canic ashes, butylated hydroxytoluene,
cyclophosphamide, nitrous oxide, and ozone.
In response to injury in vivo, Type I cells
detach from the basement membrane.
Thereafter, there is an increase in the
mitotic activities of Type II cells, followed, in
the absence of persistent damage, by differen-
tiation of these into new Type I cells (53).
Similarly, isolated Type II cells can differen-
tiate into Type I cells in vitro. It is possible to
select, in vitro, a population of replicating
Type II cells. Lung slices (54) and the organ
culture of lung transplants (55) have also
been used to study alveolar Type II cell dif-
ferentiation and proliferation.

Cuboidal Type II cells (originally called
granular pneumocytes) are metabolically
very active and contain most of the biotrans-
formation enzymes which are found in the
alveolar region. Purification of these cells for
culture purposes has been achieved by using
separation techniques based on size, differ-
ential adherence or cell sorting (56, 57).
In primary culture, following adherence to plas-
tic, the cells lose their ability to secrete surfac-
tant with time; this is associated with the
disappearance of intracellular lamellar bodies,
usually by day three in culture (58).
Maintenance of the cuboidal shape, which is
necessary for surfactant secretion, can be
achieved by culturing the cells on extracellu-
lar matrices (59).

The synthesis, secretion and re-uptake of
surfactant are good functional indicators of
Type II cell activity. The surfactant system
is essential for normal lung function. Surfactant
is a complex material which consists mainly of
phospholipids, but also contains carbohy-
drates and specific proteins. Its role in main-
taining the physical (by lowering the surface
tension) and biological (by acting as a bacteri-
cidal agent) integrity of the lung make this
system an important target with respect to
xenobiotic-induced toxicity (60, 61). Isolated
Type II cells in culture produce the various
components of lung surfactant; the rate of its
secretion has been shown to be stimulated by
β-adrenergic agonists in vitro (62). Transep-
thelial (ion) transport, the formation of secre-
tory products (growth factors, fibronectin,
complement components, etc.), and the
expression of intracellular adhesion molecules
(ICAMs [63]) have also been studied in vitro.

Fetal cells (from humans, rabbits and rats)
have been used to isolate pre-Type II cells
(64). The development of procedures for the
isolation of Type II pneumocytes now make
the use of the tumour cell line A 549 as a
model for studying surfactant production
less justified.

**Macrophages and monocytes**

Consideration must be given to both alveolar
and interstitial macrophages and recruited
circulating monocytes. These cells may be
present at various stages of activation (resi-
dent, elicited, primed, or fully activated) and,
therefore, will express different functional
properties (enzyme activities, membrane
markers, secretory products, etc.). The use-
fulness of cell lines (for example U 937, THP
1, HL 60 and RAW 264.7) is limited because of
the dedifferentiation which occurs follow-
ing cell transformation.

For alveolar macrophages, ex vivo experi-
ments with cells harvested by bronchoalveolar
lavage seem to be the most relevant. These
cells are commonly used for studying the toxic
effects of inhaled gases, dusts and particles.
The ability to culture these cells under gaseous
conditions (in biphasic cultures), which closely
mimic the naturally occurring exposure, is of
great importance. Since the secretory products
of these cells are considered to be involved in
many of the biological/morphological alter-
ations of the respiratory target cells which sub-
quently lead to pulmonary disorders, the
co-culture of monocytic cells together with, for
example, epithelial cells may provide a valu-
able test system.

Due to their many and varied physiological
and metabolic functions, an almost unlimited
number of endpoints can be studied,
including determination of cytotoxicity,
phagocytic index, motility, mediator release
(enzymes, cytokines, antioxidants, oxidation
products, hormones, lipids, nitric oxide, etc.),
and the expression/activity of membrane
receptors. A priority is therefore the identifi-
cation and validation of a limited number of
relevant endpoints and endpoint measure-
ments. It is suggested that a future ECVAM
workshop should be held on the topic of
macrophages and monocytes.

**A Practical Approach to In Vitro
Testing for Respiratory Toxicity**

In terms of adopting a practical strategy for
respiratory toxicity testing in vitro, the fol-
lowing stages could be envisaged:
1. Check the existing data which are available on the test material itself, or on related substances (for example, consultation of literature and data banks; in-house data).

2. Acquire knowledge on the physicochemical properties of the test material, in particular on: a) physical state (gas, liquid or solid); b) water solubility and partition coefficient (hydrophilicity, hydrophobicity); c) suitable solvents; and d) particulate size and shape (if appropriate).

3. Use computer modelling techniques (if available) to try to predict the likely toxic effects and target sites; for example, the prediction of irritation, cytotoxicity, receptor interactions, specific cellular uptake, and biotransformation.

4. Undertake first-phase in vitro tests to identify likely target cells; for example, the use of tracheal rings, lung slices and alveolar macrophages. Cell morphology should be determined and crude assessments of the cellular energy status could be undertaken. In this respect, it may be possible to undertake (semi) quantitative ranking studies of toxic potency.

5. Undertake second-phase in vitro tests. These should be selected, on the basis of results obtained in the first phase of in vitro testing, from the following: a) nasal olfactory cells; b) airway epithelial ciliated or non-ciliated cells; c) Clara cells; d) Type II cells; e) alveolar macrophages; f) vascular endothelial cells; g) fibroblasts; and h) mesothelial cells.

With respect to particulate size and shape (point 2), only respirable dusts with a mean aerodynamic diameter (MAD) of less than 3mm are able to efficiently reach the alveolar spaces in humans. However, although the MAD may enable the site of deposition of the particulate and, therefore, its likely targets, to be predicted, it must be remembered that fibrous and elongated particles (such as asbestos) may be intercepted at bifurcations in the airways, and thus their intrapulmonary deposition patterns are complex. This may result in them being toxic to cells other than the expected targets.

In the case of particulates, acellular tests can be carried out to assess: a) the presence of heavy metals at the particle surface, even in trace amounts; these are removable by low-molecular weight chelators; b) the adsorption of proteins; c) the potential of the test material to cause membrane lysis; d) the adsorption of other xenobiotics (for example, polycyclic aromatic hydrocarbons) which may have synergistic effects with the substance being investigated; and e) the release of free-radicals from aqueous suspensions at either pH 7.4 (that is, the pH of most body fluids) or at pH 4.5 (the pH found within the phagosomes of alveolar macrophages).

Several endpoints should be selected, to be used with the test systems indicated in points 4 and 5. It should be borne in mind that current in vitro systems are really limited to studying acute toxic effects. The endpoints to be used should be selected on the basis of the known or “expected” toxic effects of the material being investigated, and should always include cell viability measurements undertaken in at least two different cell types.

**Conclusions and Recommendations**

1. A tiered approach for respiratory toxicity testing in vitro has been proposed, which is based on the way in which mechanistic investigations on test materials are conducted at present.

2. Toxicological appraisal should commence with a systematic evaluation of existing data, assessment of the physicochemical characteristics of the test material, and the description of any structure-activity relationships. Mixtures and composites should be treated as a single entity, at least for initial toxicological screening purposes. If a positive result is obtained, additional in vitro methods can then be employed to investigate the effects of the individual components, utilising a tiered testing approach. Care must be taken not to exclude apparently toxic but potentially useful compounds on the basis of results from in vitro screens.

3. Standard reference samples, ideally accompanied by in vivo (human) and in vitro data, are needed for validation purposes. It is recommended that ECVAM should establish and maintain a data bank of these standard reference samples. The samples should include: a)
gases, for example, HCl (soluble, toxic), SO₂Cl₂ (very soluble, highly reactive, toxic), SO₃CO₂ (insoluble, toxic), and N₂O (insoluble, not particularly toxic); b) liquids (both aqueous and non-aqueous); and c) solids (both soluble and insoluble), including insoluble particulates, such as SiO₂ (toxic), Sikron F600 (toxic), TiO₂ ultrafine (toxic), and TiO₂ respirable (not particularly toxic).

4. The most appropriate cell system must be employed. Test systems with human cells would be very useful, provided that a consistent supply of suitable cells could be made available. Human fetal tissue may be useful for certain studies. Permanent cell lines need to be properly characterised to establish their usefulness as models of selected functions of the particular target cell of interest. The use of cells isolated from laboratory animals, such as alveolar macrophages, Type II cells and Clara cells, is unavoidable at present.

5. The use of primary cell cultures is encouraged, in order to mimic as closely as possible the in vivo situation and to avoid the problems which arise when trying to extrapolate data obtained with dedifferentiated continuous cell lines to the in vivo situation. The development of immortalised human epithelial cell lines, via the introduction of exogenous DNA, may eventually overcome the problems of cells dedifferentiating in culture.

6. In vitro test systems maintaining appropriate biotransformation activities are essential. In this respect, the use of organotypic cultures (lung slices and tracheal rings) is recommended.

7. Assays for cell viability and cell functionality need to be identified, optimised and validated. The use of at least two different cytotoxicity assays, selected from those which measure cell membrane integrity, various enzymatic activities (intracytoplasmic or enzyme leakage), the energy status of the cell (for example, ATP content) or cell proliferation, is recommended. Assays for cell function must be developed and validated for each cell type.

8. Relevant endpoints and markers of cell injury need to be identified and validated. Standardised cytological, bio-

chemical, immunological and molecular biological approaches should be applied to assess cellular viability, morphology, and metabolism, and other cellular functions. Effects on intercellular communication and genetic alterations can also be investigated.

9. The cell culture conditions for each cell type need to be optimised. The effects of culture media and extracellular matrices on the cellular phenotype expressed should be carefully assessed.

10. Biphasic test systems could be used to determine the influence of the depth of the layer of culture medium, and its composition, upon cell viability and susceptibility to chemicals.

11. Suspension cultures of cell clusters, such as those obtained from the nasal or upper respiratory tract epithelium, warrant further evaluation. When maintained in clusters, the epithelial cells appear to retain a number of the functions which they carry out in vivo.

12. The further standardisation and validation of procedures for exposing cell cultures to gases, liquids and particles is strongly encouraged. Reproducible and standardised in vitro delivery systems, which mimic inhalation exposure in vivo, are urgently needed. Biphasic (gas/liquid) systems are important in this respect.

13. The influence of the composition of the fluid which lines the respiratory tract with respect to modelling in vivo exposures in vitro should be determined, especially with regard to its possible impact on in vitro/in vivo correlations.

In conclusion, alternative methods could play a significant role in the study of direct acute respiratory toxicity. More knowledge is needed of the mechanisms of toxicity (for example, definition of relevant mediators, target structures, and cellular interactions) before the key stages in chronic respiratory conditions can be studied in vitro. It is suggested that ECVAM should organise other workshops, with more-narrowly defined objectives, on specific pulmonary cell types (for example, on macrophages), and on specific mechanisms of respiratory toxicity (for example, on hypersensitivity).
References


