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# Symposium 13

# Environmental pathology

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## Environmental pathology of the nervous system

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### The negative public attitude toward environmenta agents

Harmful (adverse) effects on the nervous system can be produced by agents present in food, drinking water, beverages, air and the materials used to provide clothing, housing, entertainment, transportation, disinfection, cleaning, etc.. In contrast to medication, which is deliberately consumed due to its recognized health-improving and life-saving functions despite possible deleterious side effects, the exposure to environmental agents, especially to "unnatural" ones is inadvertent and unwanted. Consumers are not ready to accept any side effects of environmental agents. In general, such environmental agents can be considered chemicals, although with the recent advent of gene technology, differentiating between "chemical" and "nonchemical" agents is increasingly difficult. One could say that the potential effects of genetically engineered living organisms on humans are of microbiological concern, but toxicology cannot shun its responsibility for potentially harmful effects of substances produced by genetic engineering.

### The tasks and goals of neurotoxicology

To minimize the exposure to potentially harmful agents and to avoid such effects, the following actions must be taken: i) identification of neurotoxic potential (hazard) of particular agents, and ii) assessment and management of the obvious risk.

Originally, the overt effects of rather massive doses of today's notorious neurotoxins were a matter of concern. These agents produced severe neurotoxicity resulting in crippling, unbearable pain, blindness, deafness, or dementia. Since such agents have been recognized and appropriate measures for avoiding their effects implemented, interest is focused on subtle effects of chronic, low-level exposure, including potential carcinogenic effects and effects on developing and young organisms, especially the effects on neural functions essential for the human brain, such as learning and memory. The development of sensitive, quantitative and specific tests of cognitive function such as learning and memory has been identified as the highest research priority in neurotoxicology (1). Owing to the quite ubiquitous presence of the nervous system in the organism and to its integrative and steering function with regard to other organ systems, neurotoxicology is deeply involved in other areas of interest, such as endocrine disruption and immunotoxicity.

### Extrapolation to humans from experimental animal safety studies

Unfortunately, in the past neurotoxicity was mostly detected in cases of human disaster. Effects of neurotoxins such as methylmercury, organotins, triorthocresyl phosphate, gamma diketone solvents, or hexachiorophene were all initially manifest in humans and only subsequently confirmed in corresponding animal models. In response to this unsatisfactory situation, refined animal models for testing of novel chemicals were elaborated and implemented. The rat was selected as the standard species for testing neurotoxicity, because it is relatively easy to breed, keep and work with, and there is a vast database of pharmaceutical research on rats. However, it has not been demonstrated that the rat exhibits a generally high sensitivity to neurotoxicity. Actually, and unfortunately for dogs, the experience accumulated in our department indicates that the dog may be generally more susceptible to neurotoxicity than the rat.

The animal models presently available comprise highly advanced functional, biochemical and pathological methodologies. For example, the hen model for testing organophosphorus agents for their propensity to induce "delayed neuropathy" (OPIDN) has been developed to utmost perfection by appropriate functional testing, biochemical assay of inhibition of "neuropathy target esterase" (NTE) and examination of the most sensitive brain areas (the termination of spinocerebellar tract) using neuropathology (2, 3). In this model, the degree of NTE inhibition is a quantitative indicator of the biochemical effect of a tested agent, whereas the pathology evaluation reliably shows whether a neuropathy really has occurred. Studies conducted using this methodology show that neuropathic effects characteristic of OPIDN of both type I and type II can be detected with sufficient probability (4). Recent progress in pathological methods and molecular biology provides virtually unlimited technical possibilities. The difficulty lies in the appropriate choice of relevant and feasible methods of investigation. For instance, in the field of developmental neurotoxicity, it has been proposed that the brains of offspring should be submitted to morphometric examination in order to assess the size and cellular composition of particular areas of the brain. Such demands appear exaggerated since this kind of morphometric examination is tedious, and when used routinely would represent a dull exercise of duty. Other approaches, such as functional tests, biochemistry and semiquantitative pathology may be used as primary indicators of possible effects, which, if present, can be additionally characterized by morphometry.

Animal models, needless to say, have limitations due to the morphological, chemical, metabolic and functional differences among species. With respect to the extrapolation to man, there are purely human functions, such as language, which are untestable in animals. What is more, certain reactions of animal organisms have doubtful significance for the extrapolation to humans. An example lies in the alleged neurocarcinogenicity of the artificial sweetener aspartame. Olney *et ald* (5) analyzed National Cancer Institute data

on human central nervous system tumors from 1975 to 1992 and found that there was an increase in brain tumor incidence, which occurred in two phases, the first of which was attributable to the improved diagnostics, but the second more recent one, characterized by a shift toward greater malignancy, was related to an unknown factor. It was suggested that aspartame was a "promising candidate" compared to other environmental factors. The evidence potentially implicating aspartame includes an increased incidence of malignant astrocytic tumors in treated rats. However, one must be aware that there is no unanimity among the experimental pathologists as to the identity of rat astrocytic tumors. The genotype of rat lesions is unknown and their phenotype is hardly comparable to what is understood as astrocytoma in humans (6). Moreover, the observed incidence is dependent on the method of brain collecting and trimming for the examination: laboratories which carefully collect the rostral brain areas, including the olfactory bulbs, and examine multiple brain sections observe more such lesions than those that perform only a cursory examination. Solleveld and Zurcher (7) compared the incidence of astrocytomas in groups of untreated control rats from five different carcinogenicity studies and demonstrated a variation between 0% and 5.9%. There is an obvious need for better tools to assess the carcinogenic potential of chemicals than the expensive and time-consuming rodent experiments that yield equivocal results. As long as rodent carcinogenicity studies are mandatory, it appears desirable to perform them in a way that allows meaningful evaluation, using five or six dose groups in addition to two different control groups. With six dose groups exhibiting a dose-related trend of tumor incidence higher than both control groups, less equivocal evidence of positive carcinogenic effect could be obtained, but the problem of extrapolation among the species would not be solved. Incidentally, the example of aspartame shows that widely spread environmental agents will always be the focus of suspicious attention – recently a warning was distributed on the Internet about aspartame toxicity allegedly causing diseases mimicking multiple sclerosis and systemic lupus.

## Interpretation of experimental results
## The border between physiology and pathology

It is less difficult to obtain experimental data than to evaluate the data in terms of desirable or undesirable effects. Stimulation of neural receptors can result in their down-regulation, as well as possible changes of postreceptor messenger systems, and development of "pharmacological" tolerance. The tolerant individuals are then less susceptible to the effects of agents stimulating the receptors. Such a situation can occur, for example, with cholinesterase inhibitors. The dilemma for a pathologist then is to decide whether this kind of physiological adaptation can be classified as a pathological condition. Cholinesterase inhibition can improve memory but change sleep pattern (4). When exposed to cholinesterase inhibitors, individuals with deteriorated memory will welcome the improvement, while those with no memory problems may complain about the changes in sleep pattern. Organophosphorus pesticides, especially insecticides, are an example of widely spread environmental cholinesterase inhibitors. They are agents with potent biological activity. Their acute neurotoxic effects are well known, can be treated with medication and the risk of their occurrence is manageable. Recently, these agents were alleged to induce so-called "chronic syndrome" resulting from long-term, low-level, apparently asymptomatic exposure. The clinical features of this alleged syndrome are vaguely defined and include complaints about reduced attention, memory disturbance, reduced velocity of thinking, reduced velocity of psychomotor reactions, impaired dexterity, language disturbance, anxiety, irritability, depression, sleep disturbances, general fatigue and altered sexual habits. Taken together, these complaints very much resemble the changes commonly known to occur with advanced age. They are difficult to recognize as a nosological entity in the individuals who had been exposed for decades to low levels of cholinesterase inhibitors and aged naturally during this period. The controversial issue of chronic syndrome can hardly be solved by pathology, since no pathognomonic lesions were identified. The biochemical data indicate that changes related to cholinesterase inhibition are reversible and therefore physiological rather than pathological.

## The impact of recent legislation on the regulation of enyironmental agents

The simultaneous presence of multiple environmental agents gives rise to public concern about the potential toxicity of the combination of agents. An example can be found in recent United States legislation. The Food Quality Protection Act (FQPA), a new regulation setting stringent standards for pesticide residues in foods, became law on August 3,1996. The US Tolerance Reassessment Advisory Committee (TRAC) has identified nine science policy issues associated with its implementation. Among these, two items are of particular interest: aggregating exposure from all nonoccupational sources (exposure to the same agent from all such sources are put together to estimate total exposure), and cumulative risk assessment, especially for organophosphorus pesticides (exposures to various chemicals with common mechanism of toxicity put together). With respect to these items, it is important to consider differences among the chemicals that are apparently identical or appear to have a common mechanism of toxicity.

The following examples demonstrate the pitfalls in classifying agents and their mechanisms of action as "identical".

### *Mercury*

Neurotoxicity of mercury is widely known as Minimata disease, although the original denomination was Hunter-Russel syndrome. This condition is characterized by damage of neurons in the cerebral and cerebellar cortex and the dorsal root ganglia. It is induced by an organic form of mercury, methylmercury. Although inorganic mercury can be deposited in the nervous system, especially after exposure to mercury vapor, and induce neurologic signs, it does not produce any specific pathological lesions (8). It is believed that in order to produce Minimata-like lesions, inorganic mercury would have to be converted into its organic methylated form.

### *Organotins*

There are two neurotoxic trialkyltin agents: trimethyltin produces damage to the cerebral neurons, while triethyltin produces totally different lesions in the edema of white brain matter. Aldridge *etal.* used molecules containing either two methyl and one ethyl group (dimethylethyltin) or vice versa (methyldiethyltin) and demonstrated that such agents produce both kinds of neurotoxicity, whereby the predominating lesion is associated with two identical alkyl groups. However, it has not been demonstrated that addition of trimethyltin could modify the effects of triethyltin, or vice versa.

### *Hexacarbon solvents*

Certain agents with a aliphatic hexacarbon structure, which are mainly used as solvents, are neuropathic. Originally, this neuropathy was denominated "hexacarbon neuropathy". It was then demonstrated that only gamma diketones are neurotoxic, while diketones with other than gamma spacing are not. Therefore, it appears incorrect to consider all hexacarbons to be identical. Moreover, carbon disultide, chemically a quite different molecule, produces the same type ot neuropathy as gamma diketones (10).

### *Suppression and promotion of OPIDN*

Selected chemicals can protect against OPIDN when administered before the neuropathic agents. In contrast, when administered after the neuropathic agents these chemicals aggravate OPIDN (4). The protective effect may be associated with the interaction on the same target (NTE), but the promoting effects probably result from the interaction of promoters with a molecular target other than NTE. This example shows that different mechanisms can be involved in the pathogenesis of one type of lesion, and the order of events determines the level of toxicity, which cannot be assessed by simple cumulating exposure levels.

## New horizons in neurotoxicology

The requirement for aggregating and cumulating neurotoxic risks opens interesting, yet unanswered questions. Will simultaneous exposure to inorganic and organic mercury, to ethyl and methyl tin, to gamma diketones and other hexacarbons, or to multiple organophosphorus agents with other agents enhance, reduce, or otherwise modify the neurotoxicity? These questions of combination neurotoxicity cannot be solved without adequate experimental work. Experience shows that neurotoxins can be classitied by their cellular targets (neuronal damage – neuronopathy, axonal damage – axonopathy, myelin damage – myelinopathy, glia cell damage – gliopathy) rather than by their chemical structure and that similar lesions can be produced by chemically different agents. The type and pattern of the lesion is also determined by the type of exposure, as different lesions can be produced by identical agents tollowing short-term exposure to high levels or long-term exposure to low levels (11). The elucidation ot mechanisms instrumental in the production ot toxic neuropathies is the essential task of future activities. Their better understanding will improve the public attitude towards environmental agents, and allow full advantage to be taken of their benefits while avoiding undesired effects.

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# Test strategies for the identification of endocrine active chemicals. An industry point of view


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In recent years, possible adverse effects of endocrine active chemicals (EAC) on the environment and human health have been widely discussed in the public and in the scientific community. There is a general consensus that more scientific knowledge is necessary and that EACs should be identified for a refined risk assessment. In this regard, the following three-step approach seems to be the most appropriate:

i) Priority setting or initial assessment to identify from the large universe of chemicals those to be subjected to initial screening.
ii) Screening using a battery of *in vitro* and/or *in vivo* tests to identify chemicals for further detailed testing.
ii) Testing in definitive animal models for risk assessment, including not only adult animals but also *in utero* exposure.

Such a strategy has been proposed to the United States Environmental Protection Agency (EPA) by the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC), a multistakeholder advisory group, with participants from authorities, universities, environmental organizations and industry, and has recently been published by the US EPA as its Endocrine Disrupter Screening Program for public comment (EPA, 1998; Fed. Rag., 1998). A similar principle was developed by the Organization for Economic Cooperation and Development (OECD) Working Group on Endocrine Disrupter Testing and Assessment (EDTA).

The screening and definitive test procedures should address possible effects on wildlife and human health. The following will focus on the latter, human health. For the different levels, the tests found below have been proposed or are under discussion.

For priority setting, the US EPA proposes high throughput systems with transfected reporter gene cell lines for estrogens, androgens and thyroid hormones.

For *in vitro* screening, receptor-binding/transcriptional activation assays have been proposed both by the US EPA and the OECD for the *in vitro* part. In addition, the EPA suggests *in vitro* investigations on steroid metabolism.

As *in vivo* screening tests, the uterotropic (for (anti-)estrogenicity) and the Hershberger assay (for (anti-)androgenicity) have both been mentioned by the US EPA and the OECD. Further proposals of the EPA are for a 20-day pubertal female assay or a 14-day intact adult male assay, which also include testing for thyroid effects.

Definitive *in vivo* mammalian testing is focused on the two-generation test or another long-term procedure covering the whole reproductive cycle, including intrauterine development. In addition, the OECD is proposing classical subacute or subchronic tests for identification and possibly characterization of EACs. It may become necessary to enhance the existing test guidelines by additional hormone-specific parameters. This was proposed in the *Detailed Review Paper: Appraisal of Test Methods for Sex-Hormone Disrupting Chemicals,* prepared by UK authorities for the OECD (QEOD, 1997).

When looking at these proposals in detail, it becomes obvious that the suggested test methods have not been sufficiently validated. Thus, as a prerequisite for laying down definite test guidelines for regulatory purposes, a systemic validation effort is necessary. This regards both the relevance as well as the reliability.

The relevance of a test method is defined by its end point in relation to the different mechanisms by which chemicals may interact with the endocrine system, as follows: direct (cyto)toxicity on endocrine active or responsive tissues; interference with biosynthesis, metabolism or excretion/clearance of hormones; and interaction with hormone receptors, either as agonists or antagonists

Obviously, the relevance of each proposed test method may be different, as in the examples given below.

*In vitro* receptor-binding/transcriptional activation assays only address direct hormone receptor interactions. (Cyto)toxic effects or alterations in hormone biosynthesis for metabolism are not taken into account.

Two *in vitro* assays for hormone biosynthesis are under discussion in the EPA scheme, the "steroidogenesis assay with minced testes" and the placental aromatase assay". Both these assays only cover a small segment of the steroid hormone biosynthesis pathways and they do not give any indication at all of the metabolic deactivation of steroids. On the other hand, tests in the intact animal should, in principle, show any effect on the endocrine system regardless of the occurring mechanism, but offen the mode of action may remain unclear.

As regards the reliability of the test methods to be established, a worldwide validation process has now been initiated by the OECD for the uterotropic and the Hershberger assay as well as for the subacute 28-day test (OECD test guideline 407) enhanced by additional hormone-specific parameters. Laboratories used to routine testing for regulatory purposes, such as those from industry, must be predominantly involved in such a validation process. They must be capable of simultaneously handling a large number of animals and they must have a broad expertise in the clinical examination of animals, histopathology, clinical chemistry and hematology.

In the overall validation process of test methods for regulatory purposes of EACs, the following points are of utmost importance for the chemical industry:

i) The overall test strategy and each single test procedure should be accepted worldwide.
ii) Test methods must be specifically designed to a clearly defined purpose; there must be a differentiation between screening for effects, hazard identification, risk assessment purposes and finally mechanistic research tools.
ii) The test methods must have a sound scientific basis, they must be robust and yield reproducible results.
iv) The test methods should be "slim"; unnecessary parameters should be avoided, even if they interesting from a scientific point of view.
v) Costs and practicability of the tests should be an important, but of course not the decisive consideration, especially for screening tests and those to be used for routine investigations.
vi) The test guidelines should be broadly validated in an international effort to become accepted by all regulatory authorities worldwide.
vii) Validation of each test method must cover sensitivity, specificity and reproducibility.
viii) For the validation process, appropriate reference chemicals must be identifled and the same test materials should be used worldwide.

In summary, taking account of the uncertainties with regard to the possible impact of EACs on the environment or human health, it is necessary to establish screening and testing procedures for hazard identification and risk assessment purposes. Such tests should ideally cover all possible modes of action of EACs. Numerous regulatory authorities and organizations worldwide take part in this process, *e.g.,* the US EPA and the OECD. A critical review of the proposed test strategies or single test methods reveals they all lack sufficient validation. Before any test guideline can be finalized for regulatory purposes, an in-depth investigation of its relevance and reliability is necessary. Due to their specific expertise in handling large numbers of animals and measuring a wide variety of parameters simultaneously, industry laboratories must take an active part in this worldwide validation process.

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# Pulmonary toxicity and risk assessment of pesticides contained in house dust and smoke

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Pesticides in general and pyrethroids in particular have received considerable attention as they are widely used for numerous applications, ranging from food protection to general pest control in the indoor environment. The pyrethroids have been proven useful in the domestic environment as room and surface sprays, in passive and active evaporator systems, in incense products such as mosquito coils or to make textiles insect resistant. The specific physicochemical properties of most pyrethroids, *i.e.,* low vapor pressure and high lipophilicity, minimize the extent of passive inhalation exposure but favor their adsorption in house dust. Pyrethroids act directly on the axon through interference with the sodium channel

gating mechanism that underlies the generation and conduction of each nerve impulse (1, 2). One notable form of toxicity associated with some pyrethroids, *e.g.,* the type II pyrethroids containing an a-cyano group, has been facial cutaneous paraesthesia (transient sensations) and irritation-related respiratory symptoms mainly observed in highly exposed workers spraying pyrethroids or in occupational settings. The time course of these sensations is usually immediate or within a few minutes after contact (3). Such effects are considered to be the most prominent health symptoms known to accompany direct contact with this class of pesticide. Based on this rationale, it seems appropriate to utilize animal models to address this physiological end point and to analyze whether pyrethroids act as agents known to elicit upper respiratory tract sensory irritation (4) and whether this mode of action is changed when they are associated with particles.

Interest in indoor air quality is steadily increasing. The factors which affect indoor air quality and the health effects reportedly associated with it are the subject of intense debate. One of the issues which generates the most interest and emotion is related to the exposure to pesticides used indoors and their associated health effects. Indoor exposure to pesticides is complex and varies from short-term high-level exposure, as is likely to occur during the use of spray cans, to long-term low-level exposure to effluents of slow-release devices which are commonly used overnight, for example, mosquito coils or vaporiser systems (5, 6). Particularly for the latter group of indoor insecticides, the actual exposure pattern of humans is difficult to assess because the concentrations of airborne particulates containing pesticides vary with time, room ventilation, and proximity to the source. Since not all of their components are removed or translocated from the indoor environment at the same rate, the concentrations of volatile and nonvolatile components also vary in relation to each other over time. In addition, confounding factors attributing to the overall particle load, such as environmental tobacco smoke, have to be considered. These aspects demonstrate that well standardized sampling strategies are required for analytical determinations and differentiation of actual or potential sources of human exposure.

Due to the complexity of the pattern of indoor exposure to pesticides, sampling strategies differ considerably, ranging from determination of pesticides in sedimented house dust to measurements in airborne dust or body fluids. Because of the very low vapor pressure of most pyrethroids, their concentration in indoor air was found to be very low and passive and long-term exposure of adult humans is commonly thought to occur via resuspended contaminated house dust. This assumption has promoted the development of a number of sampling strategies for the determination and assessment of the external and internal dose of such pesticides. Approaches for the determination of the external dose ranged from taking dust from contaminated surfaces by wipe sampling, by analysis of house dust from vacuum cleaner bags or by taking samples from indoor air. Each medium may favor a specific route of exposure, *i.e.,* oral uptake by toddlers or small children through swallowing contaminated dust, licking hands or toys, direct dermal uptake via contaminated surfaces or inhalation uptake via airborne dust, smoke or aerosol.

The potential health risk arising from such complex exposure scenarios can only be evaluated by inhalation toxicity studies of adequate duration with the active ingredient, individual carrier substances likely to become airborne or even of the entire mixture. In this instance, hazard identification and risk assessment of smoke released from mosquito coils appears to be most complex because of the combustion of organic material from which the mosquito coil is made. Thus, a great number of ingredients are subject to evaporation and/or combustion, *e.g.,* sawdust, coconut shell powder, starch, binders, fungicides, insecticides, synergists and other additives that may, under certain circumstances, cause and/or exacerbate specific portal-of-entry effects which cannot be studied and assessed by noninhalation routes of exposure. Thus, the toxicological assessment of mosquito coil smoke atmospheres appears to be most challenging since the shuttle function of smoke particulates may facilitate the penetration of specific agents into the lower respiratory tract that would normally have been deposited in the upper respiratory tract. Thus, for mosquito coil smoke, the location of the major deposition of specific constituents contained in the smoke mixture may be contingent upon their behavior in the smoke mixture. Irritant combustion gases and an accumulation of particles are likely to affect the detoxification pathways of the respiratory tract which differ from location to location, *i.e.,* nasal cavities, larynx, trachea, bronchial airways, and alveolar region. This, in turn, may interfere with the pathomechanism(s) of the active ingredient and agents likely to accumulate in highly specialized cell populations present only in the alveolar region.

The objective of the studies presented is to analyze the suitability of various exposure regimens to evaluate which bioassay provides the relevant data for risk assessment for which household insecticide. Particular emphasis is made to test the most complex entity, namely mosquito coil smoke atmospheres, to characterize whether the acute sensory irritation inhalation rodent bioassay, a subacute or a subchronic inhalation study or even more in-depth approaches are required to allow evaluation and assessment of their toxic potency. Due to the complex nature of exposure atmospheres generated by mosquito coils, it is scientifically challenging to characterize the pathomechanism of most concern, since irritant combustion gases, volatile and semivolatile organic substances, particulates (soot), condensation aerosols and active substances recondensed onto particulates may act independently, synergistically or mixture specifically. Despite the presence of mild respiratory tract irritation, mosquito coil smoke did not demonstrate adverse effects caused by pyrethroid(s) or smoke particles, The histopathological effects elicited with mosquito coil smoke were confined to irritant effects in the upper respiratory tract (nasal cavities) which appeared to be causally related to wood combustion products, such as formaldehyde or acrolein, and are quite comparable to those obtained with environmental tobacco smoke. The data generated with mosquito coil smoke appear to suggest that the use of mosquito coils is not accompanied with any undue risk, although it has been shown that combustion effluents originating from the coil matrix may elicit upper and lower respiratory tract irritation.

Passive exposure to household insecticides may also raise concerns as nonvolatile active ingredients may be adsorbed by house dust. Many analytical surveys addressing indoor contamination have demonstrated appreciable amounts of pesticides bound to house dust and concerns have been raised as to whether this contamination is of any toxicological relevance. Accordingly, for the assessment of pyrethroid contamination of house dust, elaborate inhalation studies were conducted using "sprayed" or "dusted" carpets. The objective of these studies was to address the question as to whether pesticide contamination in sedimented house dust can serve as an indicator to predict potential inhalation exposure. Experimental evidence as well as human biomonitoring studies sug-

gest that, following treatment of carpet, only a very small fraction of the applied pyrethroid can indeed be regarded as potentially bioavailable by inhalation (max. 0.2% of the active ingredient applied to the carpet). Therefore, assessment of health hazards in the indoor environment based solely on "vacuum cleaner" sampling (as used by analytical chemists to search for indoor contaminants) rather than examination of the actual airborne concentration, including other relevant airborne materials, is prone to tremendous errors and misjudgments.

These examples demonstrate that new regulatory requirements, *e.g.,* the EU biocide directive, and public perception and concern may trigger complex and demanding toxicological examinations and standardization of such complex studies with regard to the generation of test atmospheres, mode and duration of exposure, and selection of adequate toxicological endpoints, as a basic prerequisite for state-of-the-art hazard identification and risk assessment.

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# Adverse effects of environmental exposures may occur not only in the individuals directly exposed, but also in their progeny


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Individual differences in the risk of cancer are ultimately determined by the interaction between the environment, that is the complex of factors that are not part of the genetic patrimony at the time of conception, and the genotype. Beginning prenatally, humans are exposed throughout life to a variety of environmental agents, among them many carcinogens and mutagens. Adverse effects of such exposure may occur not only in the individuals directly exposed, but also in their progeny.

Prenatal events may contribute to increase the cancer burden following either: i) exposure to a noxious agent during pregnancy with consequent direct exposure of fetal cells *in utero* (transplacental carcinogenesis), or ii) exposure of one or both parents to a carcinogen/mutagen before conception, resulting in a possible alteration of the germ cells (transgenerational carcinogenesis).

There is experimental evidence of a transplacental carcinogenic effect for a large number and variety of chemical carcinogens. Evidence in humans is instead limited to exposure during pregnancy to diethyisfilbestrol (DES) and X-rays. Studies in rodents, however, cover the entire lifespan of the progeny and the increase in cancer frequency is generally observed in adult or late life. Observations in humans are almost exclusively confined to the occurrence of Cancer in childhood.

Even if many experimental studies remain open to criticism, particularly due to the relatively small number of animals used and the consequent lack of statistical power of the data, there is convincing evidence for a transgenerational effect in laboratory animals of a few chemical Carcinogens and of internal and external exposure to radiation. The epidemiological data in humans are mostly derived from the investigation of paternal occupational exposures to chemicals, chemical mixtures or radiation and, although suggestive of a transgenerational effect, they remain Controversial. Observations in humans, again, are limited to the occurrence of childhood cancer.

The hypothesis has been proposed that certain environmental exposures of parents before conception may cause alterations of the germ cells which affect the susceptibility of the progeny to cancer. Most likely, different mechanisms underlie the high incidence of tumors appearing at an early age in certain familial syndromes, and the surfacing of an increased predisposition to cancer revealed by a relatively modest increase in tumor incidence late in life. A better understanding of pre- and postnatal gene-environment interactions in determining cancer risks would have important implications for public health, as it could considerably improve the efficacy of primary prevention.

# Experimental adenocarcinoma of the esophagus: Implications in the sequence Barrett's esophagus-adenocarcinoma


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No suitable models of esophageal carcinogenesis were available until the discovery by Duckrey *et al.* that N-methyl-N-nitrosoaniline given orally to rats resulted in a high incidence of squamous cell carcinomas of the esophagus. Since then, most of the experimentally induced squamous cell carcinomas of the esophagus have been due to the exposure of rats to several nitrosamines by various routes of administration.

In recent years, the incidence rates for adenocarcinoma of the esophagus have risen rapidly in Western countries, especially among white males. Most of these tumors arise from areas of columnar-lined esophagus (Barrett's esophagus), a condition clearly associated with a chronic gastroesophageal reflux of acid and duodenal-content secretions. These epidemiological observations prompted us

to study the possible role of duodenal-content reflux esophagitis in a model of esophageal carcinogenesis with 2,6-dimethylnitrosomorpholine (2,6-DMNM) in which squamous cell carcinoma was the histological type usually induced.

## Materials and methods

The induction of adenocarcinomas of the esophagus was accomplished in Sprague-Dawley rats under the combined influence of chronic esophagitis plus the carcinogenic effect of 2,6-DMNM. Chronic reflux esophagitis was produced by means of an esophagojejunostomy. This procedure, which diverts the biliary and pancreatic juice into the esophagus, significantly increased the number of animals with esophageal carcinomas (co-carcinogenic effect) after the chronic subcutaneous administration of 2,6-DMNM.

## Results

Most strikingly, this model resulted for the first time in the induction of a significant number of carcinomas with glandular differentiation. It is of interest that reflux esophagitis of long duration (20-30 weeks), without administration of carcinogen, induced the development of foci of glandular metaplasia in the esophagus of rats. This finding suggests that glandular metaplasia may represent a morphological substrate from which the adenocarcinomas originate, because only squamous cell carcinomas were observed when 2,6-DMNM was given to rats that did not have esophagojejunostomy.

## Discussion

In a subsequent study which aimed to determine which fraction of the duodenal-content reflux, pancreatic or biliary, contributed to the development of esophageal adenocarcinomas, it was found that adenocarcinomas developed only in those 2,6-DMNM-treated rats exposed to reflux of pancreatic secretions, either alone or in combination with bile. Adenocarcinomas were not observed in the group of carcinogen-treated rats exposed to bile reflux alone. Recent observations have demonstrated that duodenal-content reflux of longer duration (40-50 weeks) *per* se in the absence of exogenous carcinogens may induce the development of esophageal carcinomas, especially adenosquamous carcinomas. It was also common to observe the appearance of multiple foci of glandular metaplasia in the squamous epithelium. All these findings support the following hypothesis for the development of carcinomas arising in rat esophagus: pancreatic reflux injures the squamous epithelium, and unknown factors contained in this secretion promote a double differentiation capability in the proliferating stem cells of the basal layer of the squamous epithelium. In some cases foci of glandular metaplasia may also arise. Exogenous or endogenous carcinogens present in the biliopancreatic secretion, acting upon these stem cells, then induce the development of adenocarcinomas or adenosquamous carcinomas.

All these findings support the role of duodenal-content secretions on the process of mucus differentiation in the squamous epithelium. These observations may help to understand some aspects of the pathogenesis of Barrett's esophagus. In addition, the effect of biliopancreatic secretions on the sequence reflux esophagitis-mucus differentiation-adenocarcinoma/adenosquamous carcinoma in the rat model support the role of duodenal-content secretions in the process of malignant transformation in Barrett's esophagus as has recently been suggested.

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# Biological investigations of environmental and occupational compounds using an alternative *in vitro* concept


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Assessment of cytotoxicity of inhalable substances such as gaseous or particulate compounds and complex mixtures have traditionally involved animal experiments. Difficulties in the calculation of human risk from animal data and the high number of relevant substances raise the question of effective alternative test systems to analyze the biological effects of airborne matter. *In vitro* systems offer the unique possibility to analyze the cellular reactions dependent on substance, concentration and time and to compare the data of several substances in the same system. The use of human cells, in particular, reduces difficulties in interpreting and extrapolating animal data to the human situation.

New cultivation and exposure techniques in the field of *in vitro* toxicology also enhance the efficiency of such cellular studies, as demonstrated by two experimental setups which allow direct exposure of cells from the respiratory tract at the air/liquid interface. The basic feature in both cases is the cultivation of the cells on porous transwell membranes, which are permeable for the culture medium.

The first system, called "CULTEX", is based on an intermittent medium supply of the cells. We have developed this alternative cultivation system, which provides a flexible and reproducible experimental protocol, to cultivate and expose cells to airborne material at the air/liquid interface, The method as well as the culture chamber are already patented. The medium will be pumped into four special modular culture units, each housing three transwells, through the transwell membrane to support the cells. At certain time intervals, the medium is be removed completely and the cells can be maintained and exposed at the air/liquid interface until the next medium supply without loss of viability for up to 72 h. Both the wells and the individual modules are connected via a network of glass tubes and hoses. Starting from a central medium supply, the medium is directed by a peristaltic pump via distribution nozzles to the relevant

modules and the transwells inside. The fill level in the transwells is regulated by an infrared sensor connected to an outer tube of the whole inlet pipe for controlling the medium level in the culture containers. The sensor acts as a photoelectric beam system. Depending on the light refraction, there is a change in the current through the phototransistor in the photoelectric beam. Each module also has a second drainage nozzle, which allows separate removal of the medium via the drainage tube, thus ensuring continual medium analysis, e.g., for cell secretory products, such as metabolites, during an experiment.

The constant temperature of the module is ensured by a regulated flow of temperature-controllable water through the modules of the whole cultivation chamber.

For exposure of the cells, special equipment has been constructed consisting of an aerosol generation system, and a second part for the dilution and distribution of the smoke. The design of the *in vitro* exposure unit allows simultaneous treatment of the cells with three aerosol concentrations, using one module as control unit for exposure to clean air. The technical details and physical characteristics of the system are presented in pilot studies with sidestream smoke.

The second system called "MIPEX" (mobile *in vitro* exposure system) offers the possibility to treat cultivated cells under indoor or outdoor conditions using a transportable exposure unit for inhalable particulate and/or gaseous compounds. The transwells with the adherently growing cells are placed in a compendium plate directly located under a corresponding plate with nozzles. The compendium plate can be rotated by motor, thus guaranteeing a homogenous impaction of the cells with particulate material. This experimental setup is located in a plastic chamber, which can be controlled with regard to temperature and atmospheric humidity. The nozzles are connected to three flues, which, depending on the adjusted air flow, allows the impaction of particle fractions of different sizes. It is therefore possible to expose the cells to particles which, for example, are normally deposited in the thoracic or alveolar regions. The cells can be treated for a maximum of 20 min. Afterwards they can be analyzed for cellular reactions or replaced in medium for further experiments. In a series of studies, the functional stability and reproducibility of this mobile *in vitro* exposure system was shown with human cells.

The application of new cultivation and exposure techniques will, in future, offer new testing strategies for comparable toxicological evaluation of soluble and inert substances, as well as complex mixtures and specially designed aerosols.