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Viewpoint

An integrated *in vivo* and *in vitro* strategy to improve food safety evaluation

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Food safety evaluation and risk assessment is largely based on animal studies and is thereby limited by an overreliance on default assumptions that are used to address uncertainties resulting from the lack of human-relevant information. The recent development of new cellular, molecular and biochemical tools provides the opportunity to improve the scientific basis for risk assessments. In this Viewpoint article, we wish to question current practices in food safety evaluation, and propose a new approach that involves the strategic integration of *in vivo* and *in vitro* data from animals and humans; such data could form the basis for a more appropriate assessment of risk to humans, and consequently lead to the more effective use of experimental animals.

The standard approach used for the evaluation of the safety of food materials is principally based on toxicological data obtained through animal experimentation[1,2]. Although conventional testing strategies have generally been considered to be satisfactory for the identification of potentially hazardous food components, they are increasingly being subject to criticism for both ethical and scientific reasons. Over the past decade, public concern has been mounting about the use of live animals in experimental studies. This has led to the emergence of the 'Three Rs' principle, the philosophy of which is to *Reduce* animal use, to *Replace* animals, and to *Refine* methods so as to minimize pain[3].

It is widely accepted that the assessment of risk to humans solely from animal data is imprecise[4–6]. It is limited by an overreliance on general default assumptions to address uncertainties that result from the lack of human-relevant information. How can we attempt to overcome these limitations? In this Viewpoint article, we would like to outline a new strategy aimed at improving food safety evaluation. The key concept is the application of sensitive and diagnostic early markers of toxicity in an integrated and complementary *in vivo* and

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in vitro approach that uses both animal and human test systems[7]. We believe that the implementation of such a strategy will not only provide a more precise evaluation of risk to humans but also ensure a more efficient and hence more ethical use of animals in regulatory studies.

Food safety testing and risk assessment: conventional approaches

Hazard identification and characterization

The safety assessment of a food component involves the identification and characterization of its hazards. This involves the generation of experimental data that identify the most relevant indicator of toxicity and determine its relationship to the level of exposure to the test material (the dose–response relationship)[1,2]. However, the extent of toxicological testing and type of study required for the safety assessment can vary substantially depending on the nature of the food component under consideration and its potential application in food[1,2]. Thus, a first step in the safety evaluation process is the definition of the level of concern for the test material, which involves a prediction of the likelihood of its toxicity. This is determined on the basis of two main criteria[1,2]: first, the similarity of the molecular structure of the test component to known toxicants or conversely its resemblance to traditional components with a safe history of human use; and second, an estimate of its level of consumption. Finally, additional concerns arise when the component is intended for consumption by particular at-risk groups such as infants, which may require specific types of studies to be carried out[1].

In the case of those food components that are associated with a significant level of concern, characterized by high expected toxicity and high levels of exposure, an extensive toxicological database is usually required; this consists of:

- Genotoxicity data. Bacterial and mammalian *in vitro* test systems, and, if necessary, whole animals, are used to assess the potential of the test compound to damage the genetic material. This type of toxicity is considered an initial indicator of carcinogenic potential.
- *In vivo* mammalian bioassays. Internationally recognized, standardized guidelines for *in vivo* toxicological studies have been established to assess the potential of a test material to produce a variety of toxic effects. For example, acute, sub-acute (e.g. 28 days of treatment in rodents) and sub-chronic (e.g. 90 days in rodents) feeding studies are performed to identify the target organ(s) and potential differences between sexes or species, as well as to define the doses that are relevant for further studies. These studies may include investigation of the absorption, distribution and elimination of the test component. Chronic studies, such as 2-year feeding studies in rodents, are designed to assess the consequences of a life-span exposure to the compound of interest. Assessment of carcinogenicity may be performed as part of the chronic study or as a separate experiment. Potential adverse effects are identified by analysing a large number of parameters such as animal body weight, organ weights, blood-cell counts and morphology, and blood chemistry, including levels of sugars, lipids and proteins as well as of several enzymes that are diagnostic for tissue toxicity. Histopathological examinations of the tissues are performed to detect potential lesions such as necrosis or tumours. Teratogenicity and multigeneration studies are performed to determine the effects on developing organisms. Teratogenicity protocols assess whether structural abnormalities in developing foetuses are associated with the administration of the test component to pregnant females, whereas multigeneration studies provide information on the potential effects on reproductive function.

Risk assessment

A principal objective of toxicological studies is the identification of the major toxic effects of the component being tested. In the case of compounds whose toxicity is believed to be mediated by a threshold mechanism (i.e. below a certain dose there is no effect), the highest level of intake that does not result in any adverse effects is defined as the 'no observed adverse effect level' (NOAEL). A safe level for humans such as the acceptable daily intake (ADI) may sometimes be established from this safe level for the test animal[1]. The ADI corresponds to the amount of the compound that can be ingested daily over a lifetime without appreciable health risk. It is derived from the NOAEL by applying uncertainty factors to account for the potential differences in sensitivity between the animal test species and humans, and also for the potential variability in susceptibility to the test substance within the human population. The size of such uncertainty factors depends on several aspects including the extent and quality of the data available (e.g. human versus animal) and the nature of the toxicity (e.g. severity, irreversibility)[8]. In most cases, inter-species and intra-human differences are not well characterized and a default factor of 10 is applied for each uncertainty, resulting in a total factor of 100 (Refs 1 and 8). This factor assumes that humans are 10-fold more sensitive than the animal test species and that heterogeneity within the human population may result in some individuals being 10-fold more susceptible than the average.

In the case of compounds that act through a non-threshold mechanism, such as genotoxic carcinogens, the extrapolation from lifetime high-dose exposure in experimental animals to low-dose cancer risk for humans is generally performed using theoretical mathematical linear models (e.g. linearized multistage models), and inter-species extrapolation is carried out using a conversion factor based on body weight[9].

Limitations of the present risk assessment procedure

High-dose–low-dose extrapolation

Conventional safety testing strategies necessitate the use of large doses of the test material such that the laboratory animals are exposed to much higher levels than would be the case for humans[1,2]. This practice is followed because

larger doses are expected to facilitate the identification of target organs and also to reduce the need to use very large numbers of animals to detect small effects. Extrapolation of the observed dose–response relationship to lower doses is a difficult and usually imprecise task requiring critical consideration of several relevant data in order to reduce errors. For example, a large dose may overload the host's detoxification mechanisms, whereas adverse effects may not occur with a smaller dose[4,9].

The nature of the test compound may also limit its administration at high doses in experimental studies[10]. In particular, some novel food macrocomponents may represent a substantial proportion of the diet; thus, it is usually impossible to administer high doses because this would result in nutritional imbalances. Consequently, the effects observed may have little bearing on the inherent toxicity of the test material.

Inter-species extrapolation

In most cases dose–response data are obtained from animals. Accurately extrapolating from these data to predict the expected human response is perhaps the most difficult task in the risk assessment process. Usually, few data are available on the potential differences between the animal test species and humans. Furthermore, the human population is much more heterogeneous than the laboratory animals used in toxicology studies, and very often nothing is known about the degree of human variability in toxicant susceptibility.

In order to deal with the uncertainties resulting from the lack of relevant information, conventional risk assessment strategies employ conservative default options[4,6,8,9]. Some examples of default assumptions that are considered in cancer risk assessment are listed below:

- Humans are at least as susceptible as the most sensitive animal species (strain and sex) identified.
- Positive animal bioassay results for cancer induction are sufficient proof of cancer hazards in humans.
- Genotoxic chemicals act through a non-threshold mechanism at low doses (linear dose–response) such that intake of even one molecule is associated with a probability of cancer induction.

In general, the default assumptions result in an overestimation rather than an underestimation of the actual human risk.

The various options selected in the risk assessment procedure have profound consequences on the estimation of the risk to humans and on the subsequent regulatory decisions invoked to protect human health. Because practical actions to reduce the exposure of humans to certain toxicants may require significant technical and financial efforts and resources, strong justifications based on sound scientific information are clearly needed. There is an urgent need for new tools in food safety evaluation to increase the sensitivity and diagnostic capabilities of the toxicity tests and to improve the predictive ability of the risk assessment procedure.

How can safety testing and risk assessment strategies be improved?

It is now widely recognized that in order to improve the assessment of risk to humans there is a need to integrate scientific knowledge that will reduce the uncertainties and permit conservative assumptions to be superseded by more accurate models[4,6,8,9]. Three approaches appear to be the most promising:

- improvement of the sensitivity and diagnostic capabilities of the testing procedures;
- identification of the mechanism of toxicity;
- generation of human-relevant data.

We believe that the application of sensitive and diagnostic early markers of toxicity in an integrated and complementary *in vivo* and *in vitro* approach using both animal and human test systems is essential to address these issues[7].

Early markers of toxicity

We have defined two general types of early markers of toxicity (Fig. 1). The first type of early marker (the time-related marker) allows the detection of toxicities at much earlier time points than they would normally be detected in conventional studies. Examples of this type of marker are the events that occur during the early stages of the development of cancers. A conventional cancer bioassay in rats takes more than 2 years to perform; however, in the case of some tissues, it is possible to detect preneoplastic lesions within 2–3 months, which can provide an indication of the potential carcinogenicity of the test compound[11,12]. This type of early marker may be very useful for obtaining an initial indication from short-term studies of the potential long-term toxicity of the test compound. Such an approach will result in a more effective use of experimental animals because, in addition to the traditional information, extra relevant toxicological data will be produced from a single *in vivo* study. Furthermore, the information provided may lead to a more efficient design of long-term tests and in some cases may even obviate the need for such studies.

The second type of early marker (the dose-related marker) refers to a parameter that responds to a lower amount of the test material than the amount required to produce overt toxicity (i.e. toxicity that can be easily detected by conventional histopathology examinations). Living organisms possess an efficient battery of defence mechanisms, which enables them to survive exposure to various types of environmental changes[13]. The cellular response to chemical stress is thought to be a dose-dependent event. In the case of most chemicals, a low level of exposure can be dealt with by the cell without producing any adverse effects. At higher levels, when the constitutive cellular defence capacity starts to become overwhelmed, inducible systems are mobilized and appropriate proteins are rapidly produced with the aim of protecting cells against potential damage. Overt toxicity occurs at higher doses when the inducible defence mechanisms are also overcome. When an organism is

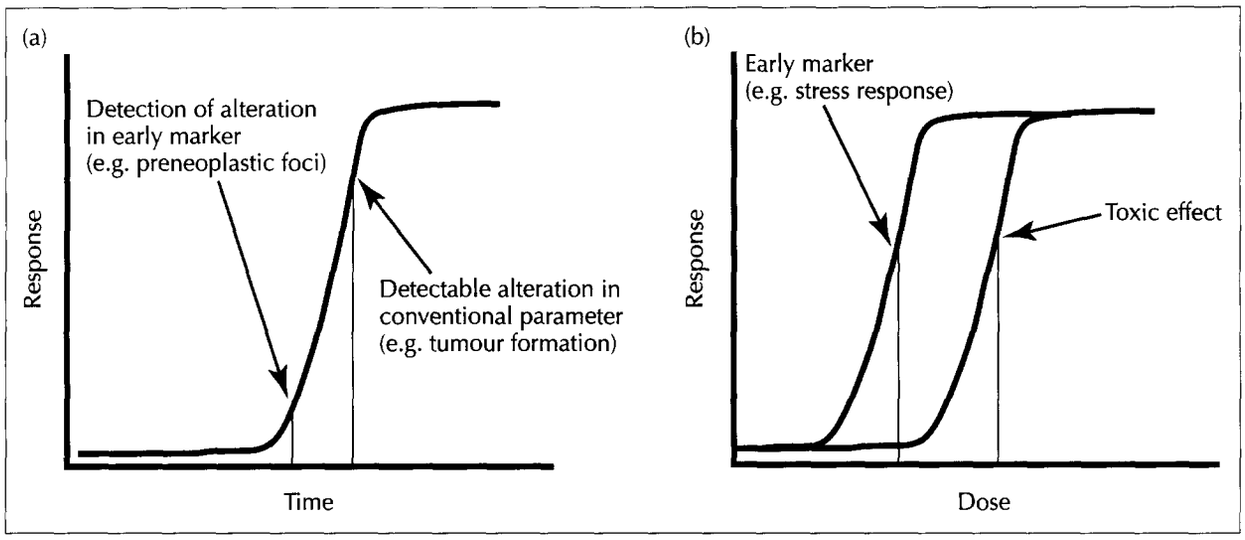

Fig. 1

We have defined two types of early markers of toxicity: (a), time-related markers, which are detectable effects that occur before the onset of alterations in conventional parameters; (b), dose-related markers, which are parameters that respond to a lower amount of the test component than the amount that is required to produce overt toxicity. In (b), the two curves represent two different responses: the response of an early marker, and the response of a conventional parameter that is associated with overt toxicity.

exposed to a toxic chemical, a common response is the activation of an array of xenobiotic metabolizing enzymes that detoxify and eliminate the chemical[14]. Other proteins, which also form part of the general cellular response to environmental stress, are involved at different levels of cell protection and repair. For example, some of the so-called heat-shock or stress proteins are rapidly induced as a response to a wide variety of stressors including xenobiotics[15]. Their precise functions have not yet been fully elucidated but some of them, such as HSP70, are involved in repairing denatured proteins and protecting cellular proteins from environmentally induced damage[16].

Although the parameters described above can be considered as general markers of stress that occur in most or all cell types, some responses may be specific to a particular tissue. For example, astrogliosis, which is characterized by hypertrophy of the astrocytes in the brain, has been observed to be a frequent reaction of the central nervous system to injuries such as toxic insult[17]. The monitoring of this reaction by measuring the astroglial marker glial fibrillary acidic protein is being increasingly applied in evaluations of neurological toxicity, and has been shown to be more sensitive than parameters studied in conventional neuropathological examinations[17].

The use of this second type of early marker of toxicity (the dose-related marker) may benefit food safety evaluations in several ways. The minimum dose of a chemical that is necessary to induce a cellular stress response is highly dependent on the inherent nature of the chemical and its toxic potency, and so may provide valuable information about its toxic potential. Moreover, because the molecular mechanisms involved in the regulation of the expression of some of these gene products are well characterized, the finding that they are inducible by the component of interest may provide an initial indication of its mechanism of toxic action. Because the stress response is an early event that is triggered by doses of chemicals that do not induce overt toxicity, its evaluation should result in an increase in the sensitivity of the toxicological assessment by allowing the detection of effects at lower doses of the test material. This approach should permit the use of doses that correspond more closely to the actual level of exposure in humans and it will thereby facilitate the high-dose–low-dose extrapolation. Furthermore, the application of markers that enhance the sensitivity of the toxicity test assay, in association with diets composed of interchangeable macroconstituents[10] (which allow an optimization of the amount of test material that can be administered) and the use of suitable extracts, will contribute to an important improvement in the ability to assess the safety of novel dietary macrocomponents.

Perhaps one of the most promising uses of these markers is their application as endpoints in comparative *in vitro* and *in vivo* studies. Often the findings from *in vitro* cell culture systems are of limited value owing to the difficulty of their extrapolation to the *in vivo* situation. The use of early markers as diagnostic endpoints of toxicity in both systems should facilitate extrapolation because direct effects on the same parameter can be compared. This application of early markers is one of the key concepts of the *in vivo* and *in vitro* parallelogram approach, as outlined below (Fig. 2).

Parallelogram approach

The first step of the parallelogram approach is the *in vivo* animal study aimed at identifying not only the key toxic effects and target tissues, but also appropriate early markers that are predictive of the toxicity. The next step is aimed at demonstrating the ability of *in vitro* systems to model the toxicity observed *in vivo*[7]. This is

performed by investigating the effects of the test compound on the selected early markers in appropriate cell cultures from the specific target tissues, obtained from the same animal species as that employed in the *in vivo* study. The early markers may also be used as *in vitro* endpoints in studies aimed at elucidating the toxic mechanisms of the compound under investigation. In the following step, the equivalent human *in vitro* system is employed to investigate the species specificity of the toxic effects and to check the relevance to humans of the putative mechanisms identified in the animal systems. This step permits an initial assessment of risk to humans, which is much improved compared with conventional approaches[7].

In some instances, it may be useful to perform human studies. The information obtained about the potential human response to the test compound may justify *in vivo* studies being performed on human volunteers. If a sensitive and diagnostic marker that was identified in the original study can be assessed by non-invasive procedures, it may be used to confirm the validity of the risk assessment.

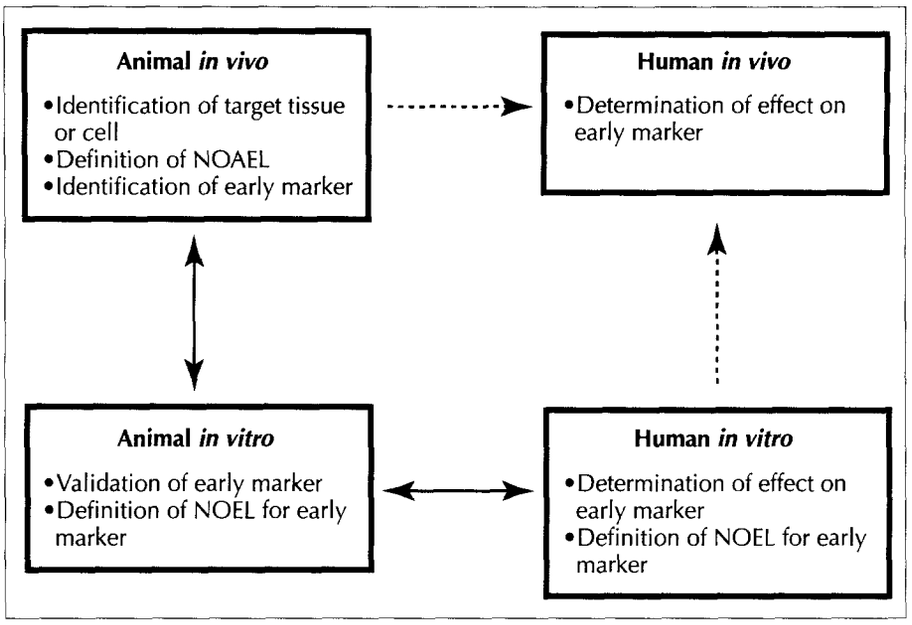

Fig. 2

The parallelogram approach involves the use of *in vivo* and *in vitro* data as part of an integrated strategy for hazard characterization and risk assessment. The *in vitro* systems are employed to obtain information about mechanisms of toxicity as well as to investigate the relevance of animal responses to humans. NOAEL, no observed adverse effect level; NOEL, no observed effect level. Solid lines represent data comparisons, and dotted lines represent extrapolations (where no appropriate *in vivo* human data are available).

This combined *in vivo* and *in vitro* approach may be integrated in physiologically based pharmacokinetics (PBPK) models. The application of these types of models is thought to be a critical approach to improve human risk assessment procedures, in particular to address issues such as inter-species and high-dose–low-dose extrapolations[18]. PBPK models use toxicokinetic data (i.e. absorption, metabolism and elimination) and some physicochemical properties of the test compound, in conjunction with pertinent physiological parameters and mathematical modelling to describe the dynamics of chemicals and their metabolites in the various organs[18]. The *in vivo* and *in vitro* parallelogram approach, using both animal and human systems, can be used to generate important toxicokinetic information that is required for the construction of PBPK models, such as the elucidation of the metabolic fate of the test chemical in different species.

In some cases, toxicological studies in a second animal species are required[2]. The comparison of the effects of the test component in *in vitro* systems from different species may provide an indication of the species that is likely to model the human situation most closely. Thus, the parallelogram approach can be used in association with data from *in vivo* pharmacokinetic studies or PBPK models for the selection of the animal model that is most appropriate for extrapolation to humans for subsequent toxicological investigations. Box 1 outlines a practical example of how the parallelogram approach can be used to provide useful integrated information in food safety evaluation.

Conclusions and outlook

There is a need to reconsider the way in which food safety evaluations and risk assessments are performed. In this article, we have proposed a new approach for food safety evaluation. Its basis is the application of sensitive and diagnostic early markers of toxicity in an integrated and complementary *in vivo* and *in vitro* approach that uses both animal and human test systems. The principal advantage of this strategy is that it should permit the generation of key scientific information that can be integrated into the risk assessment process in place of the default options currently applied. Efforts are now needed to validate, improve and apply this flexible approach[19]. We believe that if this approach is used in association with experimental designs based on sound preliminary statistical analysis[20], it will allow a more accurate and reliable assessment of risk to humans through a more effective use of laboratory animals in conjunction with focused animal and human *in vitro* studies. Furthermore, by aiding the exclusion of harmful substances at early stages in the testing procedure, the strategy may help to reduce the likelihood of expensive long-term animal studies being performed that ultimately lead to failed products.

A key issue that must be addressed is the acceptance of such an approach by regulators. Various national and international bodies, including the US Environmental Protection Agency and the Organisation for Economic Co-operation and Development, have expressed their openness to the inclusion of data from non-conventional

Box 1. An integrated approach to the safety evaluation of aflatoxin B_1

Aflatoxin B_1 (AFB_1), a mycotoxin produced by certain moulds of the genus *Aspergillus*, is a contaminant of particular foods such as peanuts and cereals. It has been shown to be a carcinogen in all animal species so far examined, including primates. The dose–response curves observed *in vivo* demonstrate marked species differences in response, limiting the value of the extrapolation of data from animal studies to humans.

Mechanistic *in vitro* investigations have demonstrated that the genotoxic response is related to the biotransformation process and therefore that the species differences in observed susceptibility reflect the species–species balance between activation of AFB_1 by cytochrome P450 and detoxification pathways.

Early markers of toxicity, in this particular case DNA adducts, applied in the parallelogram approach can be used for an improved extrapolation from animal studies to humans[7]:

Animal *in vivo*

- Toxicity: cancer in all animal species examined
- Target: liver
- Early marker: urinary N7 guanine adducts
- Species response: rat > mouse

Animal *in vitro*

- Model: subcellular fractions, hepatocytes
- Early marker: DNA adducts
- Species response: rat > mouse

Human *in vitro*

- Model: subcellular fractions, hepatocytes
- Early marker: DNA adducts
- Species response: rat > human > mouse

Human *in vivo*

- Extrapolation: the sensitivity of humans to AFB_1 is predicted to be intermediate between that of the rat and the mouse

Human *in vivo* data have been obtained to substantiate the validity of the extrapolation. A positive correlation between urinary N7 guanine adducts and cancer incidence has been observed. Humans demonstrate a response that is intermediate between that of the rat and the mouse for these adducts.

methods in addition to those from conventional studies. However, it is important that such data are both objective and scientifically valid, and that their limitations are acknowledged.

A further application of the strategy is the identification of food components that may confer beneficial effects on human health. For example, anticancer components identified by *in vivo* studies in animals could be investigated at the *in vitro* level to examine the relevance of the chemoprotective effects to humans. Thus, the parallelogram approach could be used to select components carefully before going on to perform costly human intervention studies.

In summary, we feel that the integration of more science into food toxicology through the use of modern cellular and molecular techniques in a defined and objective strategy will result in improved food safety evaluation.

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