

# 1

## General toxicology

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Toxicology is an essential part of the development process of new drugs in the pharmaceutical industry because ultimately the balance between safety and efficacy has to be established. The resulting information on drug-induced toxicity, including different types of side-effects and interactions, is of great concern for consumers of drugs as well as for pharmacists, healthcare practitioners, agencies regulating medicinal products and others that have responsibilities in different aspects related to the safe use of drugs. A lot of toxicological information can now be found easily on the Internet; unfortunately, this information is not always accurate. Moreover, knowledge about fundamental principles in toxicology is needed in order to evaluate even the most reliable information.

The fact that most toxicological data derive from studies on experimental animals reinforces the importance of knowledge of the premises for toxicity testing, as well as the way in which toxicological data are used in safety assessment. When evaluating the ‘toxicological profile’ of a chemical, information is gathered about its rate and pattern of absorption, distribution, metabolism and excretion (‘ADME’), as well as its immediate and delayed adverse health effects, target organs of toxicity, clinical manifestations of intoxication, mechanism(s) of action and dose–response relationships.

This chapter focuses on the basic principles in toxicology necessary for understanding how toxicity data are used in safety assessment of drugs for human use. Since quantitative aspects of dose–response relationships and pharmacokinetics of toxicants (‘toxicokinetics’) are on the whole very similar to those in pharmacology, those aspects are treated only briefly, outlining relevant differences where appropriate.

### **The concept of toxicity is not easily defined**

Toxicology (the science of ‘poisons’) deals with chemically induced adverse effects on living organisms. These chemicals (‘toxicants’) include

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both synthetic agents ('xenobiotics' or 'foreign compounds') and naturally occurring substances such as the poisons produced by bacteria, animals and plants (often referred to as 'toxins' or 'venoms'). Toxicology is a multidisciplinary science applying methods and traditions from several other disciplines (biochemistry, cell biology, pathology, pharmacology, physiology and analytical chemistry). The mainstream of toxicology focuses on describing and evaluating toxicity from the human health perspective, and safety assessment of drugs aims to predict human health hazard and risks.

Toxicity is often defined as the intrinsic ability of an agent to harm living organisms. This definition is not unequivocal because it will ultimately depend on how 'harm' is defined. Toxicity can also be defined as an adverse health effect associated with a change, reduction or loss of a vital function. This includes an impaired capacity to compensate for additional stress induced by other (e.g. environmental) factors. For example, many survivors of the Bhopal disaster in 1984 (in which severe lung toxicity occurred owing to the accidental release of methyl isocyanate) had a reduced lung function that made them more sensitive to otherwise mild infections. Although the accident occurred over 20 years ago, people may still die because of that tragic incident.

Clearly, drugs can induce a broad spectrum of undesired health effects, some of which are clearly deleterious, others that are not. In safety evaluation of drugs, toxicologists generally focus on direct adverse effects upon an exposure to therapeutic doses. But harmful effects, for example malformations or even death, may also be the result of an indirect effect such as drug-induced deficiency of an essential element (such as vitamin A or selenium). In addition, toxic agents (including drugs) may interact, which can result in both increased and decreased responses.

Changes in morphology, physiology, development, growth and lifespan leading to impairment of functional capacities are typical examples of 'toxic', 'deleterious', 'detrimental', 'harmful', 'injurious', 'damaging', 'unwanted', 'adverse' or 'side' effects. But should a subtle change in the blood pressure or a small change in a subset of lymphocytes be regarded as adverse effects? They could equally well be considered as 'just' biological indicators of exposure if they are (rapidly) reversible.

In conclusion, whereas severe adverse health effects are easily defined, there is also a 'grey zone' of effects of doubtful significance in terms of human health.

## **Each drug has a unique toxicological profile**

Drugs (which are often classified in terms of their therapeutic use) include many different types of agents, producing different types of adverse effects by various mechanisms of action. Therapeutic agents belonging to a given class of compounds often have some adverse effects in common ('class effects'). For instance, nonsteroidal anti-inflammatory drugs have gastrointestinal side-effects in common and may also be nephrotoxic, but as a rule each individual compound should be expected to have its own unique 'toxicological profile'. Since chemical and physical properties of the compound (water solubility, hydrogen bonding, reactivity, size, degree of ionisation, etc.) play an important role in the expression of this profile, knowledge of these properties is a prerequisite when testing and evaluating the toxicity of a chemical.

The biological effects of a drug are usually a function of the chemical structure of the parent compound or its metabolites, and the chemical and physical properties will to a large extent determine whether an agent will induce either local or systemic adverse effects. Whereas most drugs express their effects after they have been absorbed and distributed in the body (systemic effects), some chemicals (e.g. strong acids and bases, or highly reactive compounds such as epoxides) act primarily at the first site of contact. Typical examples are the severe burns to eyes and the skin following splashing of a strong alkaline agent in the face, the ulcers in the epithelia of the digestive system following ingestion of a corrosive agent, and the inflammatory reactions in the respiratory tract following the inhalation of an irritant agent.

In conclusion, each compound should be expected to have its own characteristic toxicological profile (including class effects); the route of exposure and physicochemical properties of the compound to a large extent determine the site of toxicity.

## **Is it only the 'dose' that makes the poison?**

One of the most fundamental concepts in toxicology is that it is the dose that makes the poison. This means that most chemicals will become toxic at some dose. Whereas some compounds are lethal if ingested in minute quantities (e.g. botulinum toxin), others will induce their adverse effects only if ingested in relatively large quantities (e.g. saccharin). In most cases, a chemical cannot induce any adverse effects unless it reaches a critical site at a sufficiently high concentration for a sufficiently

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long period of time. From this it follows that even an extremely toxic substance will be harmless as long as it is kept in a closed container, and that a relatively non-toxic chemical can be rather hazardous if handled carelessly.

Most drugs are taken orally and typical measures for the dose are then mg/kg or  $\mu\text{mol/kg}$  body weight (or per  $\text{cm}^2$  body surface area for interspecies comparisons). The same measures are used for intravenous injections and any other type of bolus dose. For inhalation experiments using sprays, it is not only the dose but also the concentration that is of importance, and the same applies for drugs administered on the skin. If a small amount of a drug is given at an extremely high concentration, the drug may have a strong effect locally (causing, for example, severe erythema), but if the same amount is administered in a much more diluted form it may not cause any local reactions at all.

Often it is more informative to talk about *dosage* than dose. The dosage can be defined as the amount of toxicant taken by, or given to, the organism over time (for example, in a repeat-dose toxicity study) and the typical measure is then mg/kg body weight per day. An even better measure of the actual exposure is the internal (systemic) dose, because this way of expressing the exposure is more directly related to the potential adverse health effects than is the dose or dosage (at least for toxicants with systemic effects; see below). The internal dose (usually the concentration of the toxicant in the blood) is therefore regularly monitored in toxicity studies of drugs. Choosing the appropriate dosage is very important when designing a toxicity study (especially a long-term study). Critical health effects may be overlooked if the dosage is too low. If the dosage is too high, this may lead to early deaths, which complicates the analysis of the study, especially when the interpretation of the outcome is dependent on a reasonable survival of the animals such as in 2-year carcinogenicity studies in rodents.

Clearly, the concentration of the toxicant at the site of action is related to the dosage. The final 'target dose' (i.e. the amount of toxicant present at the critical site for the necessary period of time) is governed by several factors such as the exposure situation and the fate of the toxicant in the body once it has been absorbed. There can be big differences in susceptibility between individuals (and species) exposed to a particular toxicant. Intra- and interindividual variations in susceptibility depend on several factors such as genetic constitution, age and sex, health condition and nutritional status, previous and ongoing exposures to other toxicants, and climate conditions. All these factors should be considered when using data obtained under one set of conditions to

predict what the outcome would become under another set of conditions.

In conclusion, although the concept of 'dose' looks quite simple, it is not easy to define unequivocally. It can relate to the 'external dose' (the amount actually ingested, inhaled or applied on the skin), the 'systemic (or internal) dose' (usually the concentration in blood), the 'tissue (or organ) dose' (the amount or concentration of the toxicant in various tissues after absorption, distribution and metabolism), or the 'target dose' (the amount of the ultimate toxicant actually present at the critical site for a sufficient period of time). Tissue and target doses are often very difficult to measure (especially in humans), so the systemic dose is usually the most precise measure of exposure in relation to the risk for adverse health effects.

### **Drugs can induce both immediate and delayed toxicity**

In the 'good old days', a compound was often considered harmless if it was without immediate adverse health effects when administered in a large single dose. Nowadays it is recognised that some toxicants accumulate in the body and that the 'tissue doses' will eventually become critically high if the exposure to such agents continues for a sufficiently long time, even at rather low doses. It has also been recognised that a short-term low-dose exposure to some types of toxicants (e.g. potent genotoxic agents) may be sufficient to induce delayed adverse effects (malignant tumours).

The terms 'acute' and 'chronic' are used to describe the duration and frequency of exposure in toxicity tests, but these terms can also be used to characterise the nature of the observed adverse health effects. Consequently, although a single dose exposure in most cases is associated with acute effects (i.e. immediately occurring adverse effects manifested within a few minutes up to a couple of days after the exposure), it can also induce delayed adverse effects manifested only after quite some time. One obvious example of a delayed effect following from an acute exposure is the lung cancer following from an acute inhalation of plutonium-239 (in the form of a  $\text{Pu}^{4+}$  salt). A less obvious, but still striking, example is the delayed neurotoxicity that was observed in humans who were exposed to the organophosphorus ester tri-*orthocresyl*phosphate (TOCP). In the latter case, severe axonopathy was observed both in the central and peripheral nervous systems several days after an accidental acute oral exposure without any signs of cholinergic

poisoning, the immediate acute effect typically induced by many other organophosphorus esters. Long-term chronic exposures are usually associated with chronic effects.

Depending on the duration and frequency of exposure, experimental studies on the toxicity of chemicals are usually referred to as either short-term or long-term toxicity studies (chronic studies). The route of administration of a drug in such tests should always include the intended human route of administration. The maximum duration of exposure in an acute study is usually limited to 24 hours. The compound may be administered orally (in most cases as a single dose), by inhalation (e.g. for 6 hours) or cutaneously (usually for 24 hours on a shaven area of the skin). The maximum duration of exposure in a short-term repeated dose study (formerly referred to as a 'subacute' study) is limited to one month. In a subchronic toxicity study, a period up to 10% of the normal lifespan of the animal is used (usually 90 days for rodents).

The duration of exposure in a long-term toxicity study should be at least 12 months, but is usually 18–24 months for mice and rats. In the long-term toxicity studies, the test compound can be given via the diet or administered in the drinking water (continuous exposure), by gavage or capsule (usually one oral dose/day, 5 days/week), on the skin (usually one daily application, 5 days/week), or in the inhaled air (e.g. 8 hours/day, 5 days/week). In some studies, the animals are exposed for several generations (e.g. in two-generation reproduction toxicity studies).

### **Bioavailability and toxicokinetics: two important factors for systemic toxicity**

Bioavailability represents the extent to which the chemical reaches the systemic circulation from the site of administration. Maximum bioavailability (and therefore the most intense and rapidly occurring toxic response) results after an intravenous injection when the bioavailability is by definition 100%. The route of entry along which a compound enters the body is a decisive factor determining the bioavailability. Chemical and physical properties determine its (rate of) absorption and first-pass metabolism. After oral administration, the bioavailability can be close to 100% for completely absorbed agents without first-pass metabolism, but usually it will be less.

Another important factor is the rate at which the toxicant is released from its environmental matrix (from nanoparticles, inhaled particles, slow-release preparations, creams, etc.). Since most toxicants

are absorbed by simple diffusion, small, lipid-soluble and non-ionised molecules will in general be more readily absorbed (i.e. have better bioavailability) than bulky, less lipid-soluble, ionised molecules.

Studies on the rates and patterns of absorption, distribution, metabolism, and excretion (ADME) of toxicants are known as toxicokinetic studies. They are essential for assessing the systemic exposure to a drug and its metabolites, because what is in the blood will reach the tissues. When studying the toxicokinetics of a chemical in experimental animals, the compound can be administered either as it is, or labelled with a radioactive isotope (usually tritium ( $^3\text{H}$ ) or carbon-14 ( $^{14}\text{C}$ )). The concentration of the toxicant (and/or its metabolites) is then usually determined after various intervals in different body fluids, organs and/or excreta, using gas or liquid chromatographic methods and/or mass spectrometry. Toxicokinetic studies should be performed using both high and low doses, single and repeated exposures, different routes of exposures, both sexes, different ages, pregnant and non-pregnant animals, and different species. Knowledge of the 'fate' of a toxicant in the body under different exposure conditions facilitates the selection of appropriate testing conditions when designing the subsequent toxicity studies.

The *kinetic* parameters determined in toxicokinetic studies are used in mathematical models to predict the time course of concentration of the toxicant (and/or its metabolites) in various 'compartments' of the organism. By using 'compartmental' or 'physiologically based' models it is, for example, possible to calculate various absorption and elimination rate constants, hepatic, renal and total body clearances, biological half-lives, apparent volumes of distribution, and steady-state concentrations of the toxicant in various organs. This is comparable to the pharmacokinetic approach and methodology.

Knowledge of the internal (systemic) exposure is essential when evaluating and comparing the toxicity of a given compound between different species (including humans). Toxicokinetic studies are therefore crucial when extrapolating animal toxicity data to assess human health risks. They will provide information to assess or predict, for example, possible interactions with various receptors and/or enzyme systems under different exposure conditions for different species. Consequently, estimates of margins of safety (see below) for adverse effects observed in animal studies are more reliable if they are based on toxicokinetic data on systemic exposures of a given drug rather than on the administered doses, when comparing the systemic toxicity between experimental animals and humans.

In conclusion, the intended route of administration in patients must always be included when establishing the toxicological profile of a particular drug. In general (but not always) the bioavailability for a given dose of the drug decreases in the following order: intravenous injection > inhalation > oral administration > dermal application.

### **Absorption**

There are several barriers a toxicant may have to pass before it is taken up into the blood and can induce its systemic toxicity: the skin, the lungs and the alimentary canal offer biological barriers after dermal, inhalatory or oral administration, respectively. Obviously these barriers are by-passed after an intravenous or intramuscular injection.

Some compounds enter the body by specialised transport systems (e.g. carriers for uptake of nutrients, electrolytes and other essential elements) but most toxicants appear to be absorbed by simple diffusion through the epithelial cell layers in the gut, lung or skin. Small, lipid-soluble and non-ionised molecules are therefore more readily absorbed than bulky, less lipid-soluble, ionised molecules. Very poorly lipid-soluble, highly water-soluble compounds are badly absorbed from the gut, and this may also be true for extremely lipid-soluble compounds because of their poor water solubility, both in the gut lumen and in blood. If the physicochemical properties of a compound are such that it is hardly absorbed from the gut, it will most likely not be able to induce any systemic toxicity. An example of such a drug is orlistat, a lipase inhibitor that acts on gut lipases to achieve weight reduction. Since its therapeutic action should occur only locally inside the gut lumen, it is an advantage that it is not absorbed into the systemic circulation.

Some substances given orally will hardly reach the general circulation because they are metabolised by enzymes in the intestinal mucosa or the liver. If an ingested compound is absorbed in the gastrointestinal tract, it will first pass to the liver through the portal vein, where it may be taken care of by various enzymes (the so-called first-pass effect). If the same substance enters the body via the lungs or through the skin, it will be taken up by the general circulation and may induce systemic toxicity if it is accumulated in sufficiently high concentrations.

There are other, internal, barriers that a toxicant may have to pass before it can induce its toxicity. The most important is probably the 'blood-brain barrier', but there are also other barriers such as the blood-testis barrier. These barriers are formed by sometimes highly specialised cell layers that – unless there are active transport mechanisms

available – prevent or impair the penetration of compounds with low lipid solubility.

Carriers in the cellular membranes, especially the P-glycoprotein pump, play a vital role in the maintenance of various barriers in the body that protect sensitive organs from the potential toxicity of different compounds. The blood–brain barrier is a permeability barrier that limits the influx of circulating substances to the brain. It is based on a number of anatomical and physiological characteristics (including pumps, tight junctions between the endothelial cells and a lack of fenestrations) that make this barrier impermeable to many toxicants (except small and lipophilic ones). The essential nutrients and components needed for brain function are usually provided by carrier-mediated uptake. Whether a toxicant can pass the blood–brain barrier will to a large extent decide whether this agent will induce CNS toxicity. The permeability of the blood–brain barrier in the embryo or fetus is not very tight, contributing to a more vulnerable CNS in the fetus/neonate than in the adult.

Formerly it was generally believed that the placenta protects the embryo and fetus from toxicants, but this ‘barrier’ will in fact only delay the passage of most drugs. Most toxicants will readily pass across the placenta, usually by passive diffusion but in some cases also by active transport; therefore, after some time, the mother and the fetus will have the same internal exposure. The blood–testis (or Sertoli cell) barrier is also believed to offer some protection of male germ cells (during their meiotic and post-meiotic stages), and female germ cells are possibly protected by the so-called zona pellucida surrounding the oocytes. However, the true effectiveness of these barriers is still uncertain.

## **Distribution**

Although some locally induced adverse health effects may indirectly lead to systemic effects (e.g. the kidney damage following severe acid burns on the skin), systemic toxicity cannot be induced unless the toxicant (and/or its toxic metabolites) is present in sufficiently high concentrations in the target organs. Studies of the distribution of a toxicant deal with the process(es) by which an absorbed toxicant (and/or its metabolites) circulates and distributes in the body. Three different types of distributions are of interest: within the body, within an organ, and within a cell.

If a compound is labelled with a radioactive isotope, it is possible to study its distribution using whole-body autoradiography and/or

microautoradiography. The concentration of an unlabelled test substance (and/or its metabolites) can also be measured using various analytical methods. If a particular organ accumulates the drug or its metabolites, this may raise concern. Some examples of high and selective accumulation of drugs elegantly demonstrated by whole-body autoradiography are the accumulation of chlorpromazine and chloroquine in the uvea (pigmented layers behind the retina of the eye), that of thiouracil in the thyroid gland of both the adult animal and the fetus, and that of gentamicin in the proximal tubular kidney cells.

After absorption has taken place and the compound has entered the blood, it is usually distributed rather rapidly throughout the body. The rate and pattern of distribution depend on several factors, including the solubility of the drug in the blood, the regional blood flow, the affinity of the toxicant to various serum proteins and tissue constituents, and carrier-mediated uptake by certain cells (e.g. the proximal tubular cells in the kidney). Whereas some toxicants accumulate in their target organs (e.g. chloroquine in the uvea), others will concentrate in tissues not primarily affected by toxicity. Highly lipid-soluble drugs, for example, accumulate in fat depots, resulting in a large volume of distribution and a long half-life of elimination but no local damage.

### **Biotransformation**

Since the biotransformation of xenobiotics plays a major role in their detoxification as well as their bioactivation to toxic metabolites (i.e. toxification), the rate and pattern of biotransformation is one of the most critical factors determining whether a given chemical will be able to induce toxicity. A number of factors influence the biotransformation of a toxicant, such as genetic constitution, age, sex, species, strain, nutritional status, underlying diseases and concomitant exposures to other xenobiotics with enzyme-inducing and/or enzyme-inhibiting activities.

During their evolution, mammals have developed rather specialised systems to deal with the plethora of foreign substances that enter the body every day. Biotransformation converts the xenobiotics to more water-soluble products so that they can be more readily eliminated from the body via the urine and/or faeces. Biotransformation is often assumed to be detoxification, leading to an increased elimination of less-toxic metabolites. However, sometimes this process can also lead to bioactivation. For a more detailed survey of the role of drug metabolism, see Chapter 2.

## Excretion

Elimination of a toxicant from the body is usually studied by measuring the amount of the toxicant and/or its metabolites in the excreta (typically urine, faeces and/or expired air). These measurements are usually performed until approximately 95% of the administered dose has been recovered. The kidney is the most important organ for the elimination of xenobiotics and their metabolites. The processes of elimination via the kidneys are rather complex, but there are at least three different pathways that are of interest: glomerular filtration, tubular excretion by passive diffusion, and active tubular secretion (mainly for organic acids and bases and some protein-bound toxicants). Compounds that are small enough to be filtered with the plasma in the glomeruli can be reabsorbed in the tubuli if they are sufficiently lipid-soluble (see Chapter 8).

Non-absorbed ingested materials as well as compounds excreted in bile from the liver or by intestinal cells in the gut wall are excreted in the faeces. Biliary excretion is an important route of elimination for some toxicants. When drugs are metabolised in the liver, the metabolites can either enter the bloodstream (and be excreted by the kidney) or be excreted into the bile (usually by carrier-mediated transport). Compounds with a fairly high molecular weight (above 300–500 in rats, guinea pigs and rabbits, and above 500–700 in humans) are primarily excreted in bile; those with lower molecular weights are excreted in the urine. Compounds excreted in the bile can also be reabsorbed from the small intestine (enterohepatic recirculation). Pulmonary excretion can be an important route of elimination for volatile compounds. The rate of elimination via the respiratory system depends on several factors, the most important ones being the volatility of the compound, its solubility in the blood, the blood flow to the lungs, and the rate of respiration.

In order to recover 95% of the administered dose, it may sometimes also be necessary to measure the amounts in breast milk, sweat, saliva, tears and hair. Excretion in breast milk is obviously important for mothers breast-feeding their baby because the baby may be exposed to a drug taken by the mother and/or to its metabolites. In the case of persistent environmental chemicals, this is an important route of unwanted exposure.

A toxicant will accumulate in the body if the rate of absorption exceeds the rates of biotransformation and/or elimination. The biological half-life ( $t_{1/2}$ ) of a compound can be defined as the time needed to reduce the absorbed amount in the body by 50%. More commonly it represents the elimination half-life of the concentration of toxicant in

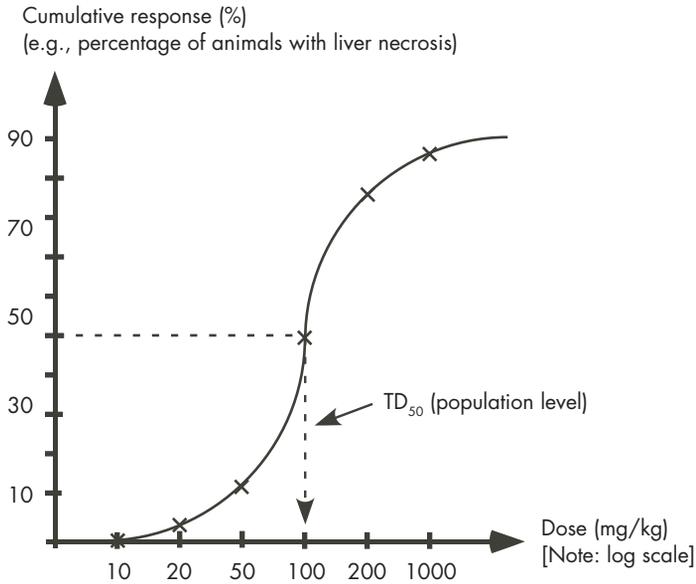
plasma (or in a specific organ). As in pharmacokinetics, a time equal to several half-lives may be required to characterise the complete elimination of a compound. Obviously, long half-lives may lead to prolonged toxic effects. The biological half-life varies considerably between various toxicants, from hours (e.g. for phenol), to years (e.g. for some dioxins) or even decades (e.g. for cadmium).

### **Dose–response and thresholds: fundamental concepts in toxicology**

As in pharmacology, there is a quantitative relationship between the ‘dose’ (the magnitude of exposure) and the ‘toxic response’ (the magnitude of the induced adverse effect). It is often useful to make a distinction between a ‘dose–effect’ relationship, i.e. the graded response after exposure to varying doses of a toxicant, and a ‘dose–response’ relationship, i.e. the incidence at which a ‘quantal’ response occurs in a population exposed to varying doses of the toxicant (Figures 1.1 and 1.2). Death and clinically manifest tumours are obviously quantal responses (‘all-or none’ effects), but all types of adverse effects can be converted into quantal responses if a cut-off procedure is used when distinguishing between a defined adverse effect level and a no-effect level (Figure 1.2). Consequently, whereas the dose–effect relationship expresses the extent of an effect in an individual or a group (e.g. the extent of liver toxicity expressed as the activity of transaminases in the blood, or the bilirubin level), the dose–response relationship expresses the incidence of a specific effect at a population level.

When an adverse effect is reproducibly and dose-dependently observed *in vitro* or *in vivo* (in animals or humans), there is a *causal* relationship with the (internal) exposure. This relationship can be quantitatively characterised in toxicokinetic and toxicodynamic studies. This implies that the adverse effect is induced by the toxicant and not by some unrelated factor. Unless the toxicant acts by affecting the microenvironment in the cells or tissues (see below), it is also usually implied that there is some kind of ‘critical receptor’ with which the toxicant interacts in order to induce its toxicity, that the effect or response magnitude is related to the concentration of the toxicant at this ‘receptor site’, and that this receptor concentration is related to the administered dose.

One example of toxicity following from a critical change in the microenvironment of cells or tissues, rather than a direct interaction between the toxicant and a specific endogenous target molecule, relates to the adverse effects induced by ethylene glycol through its metabolite

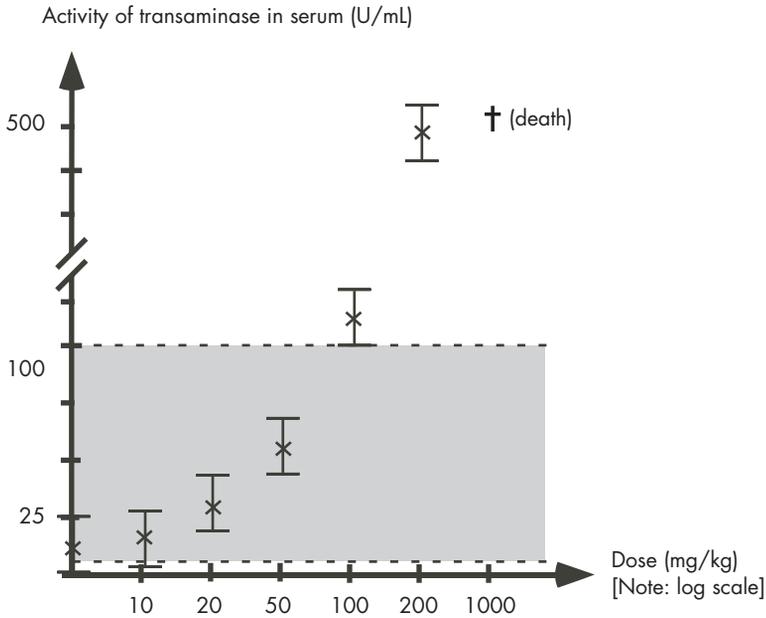


**Figure 1.1** Cumulative dose–response relationship. When animals are treated with increasing doses of a drug that causes, for instance, liver necrosis, the percentage of the animals that show an effect at each dose can be determined and plotted against the log of the dose. Experimental points are indicated in this sigmoid plot. The  $TD_{50}$ , the dose at which 50% of the treated animals show the predefined effect, is indicated. The predefined effect in this case may be that more than 10% of the cells on histological slides of liver samples taken 12 hours after treatment are necrotic. The threshold is around 10 mg/kg.

oxalic acid. This compound can induce hypocalcaemia due to calcium chelation; it forms water-insoluble calcium oxalate crystals, which are deposited in the kidney tubuli and in small blood vessels of the brain (i.e. in organs known to be damaged by ethylene glycol). Other examples include compounds that alter the hydrogen ion concentration in the aqueous biophase of the cells (e.g. acids shifting the pH) and agents that, in a non-specific way, alter the lipid phase of cellular membranes (e.g. detergents and organic solvents).

### Dose–response curves can be constructed in many different ways

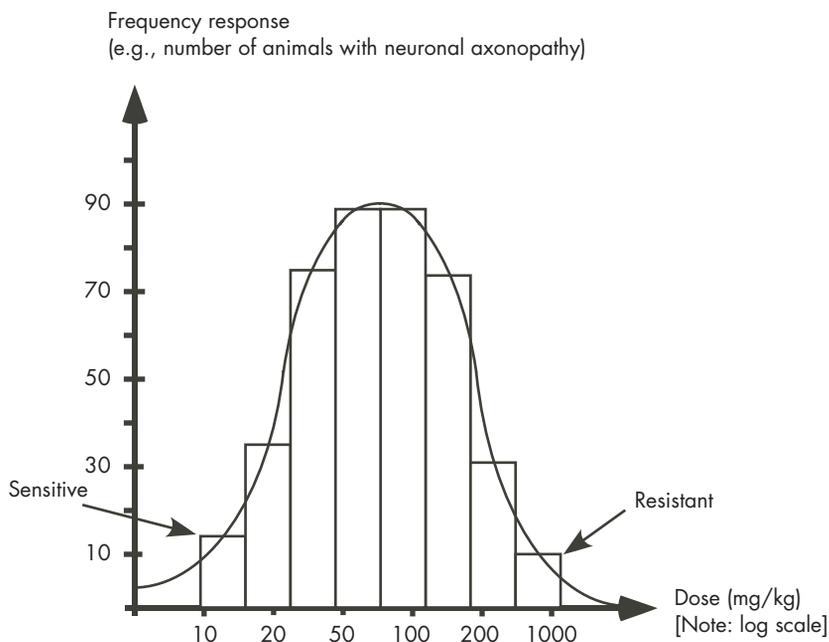
Figures 1.1–1.7 show different types of relationships between ‘dose’ and ‘response’ or ‘effect’. The magnitude of exposure (the independent



**Figure 1.2** Dose–effect relationship. Groups of animals were treated with increasing doses of a hepatotoxic drug and 24 hours after treatment the activity of transaminases was measured in serum. The mean activity per group is given (with standard deviations). The grey zone indicates the range of activities in historical controls (normal range) and activities above that are considered positive (in this case they indicate liver toxicity). To convert the graded response to a quantal response, animals with an activity above the grey level can be counted as positive in the choice: yes/no toxicity.

variable) is usually plotted semi-logarithmically on the  $x$ -axis, and the magnitude of response or effect (the dependent variable) on the  $y$ -axis. A major reason for using a log scale on the  $x$ -axis is that this produces a symmetrical curve and allows a broader range of doses on the graph. A dose–response curve (with a ‘quantal response’) shows which portion of the population will respond to a given dose of a toxicant. Many different units can be used on the  $y$ -axis, including cumulative percentages (as in Figure 1.1), frequencies (as in Figure 1.3), or so-called probit units (as in Figure 1.4). The units used on the  $y$ -axis in a dose–effect curve where the response (effect) varies continuously with the dose are usually arithmetic and can be actual (e.g. U/ml as in Figure 1.2) or derived (e.g. percentage of maximum response).

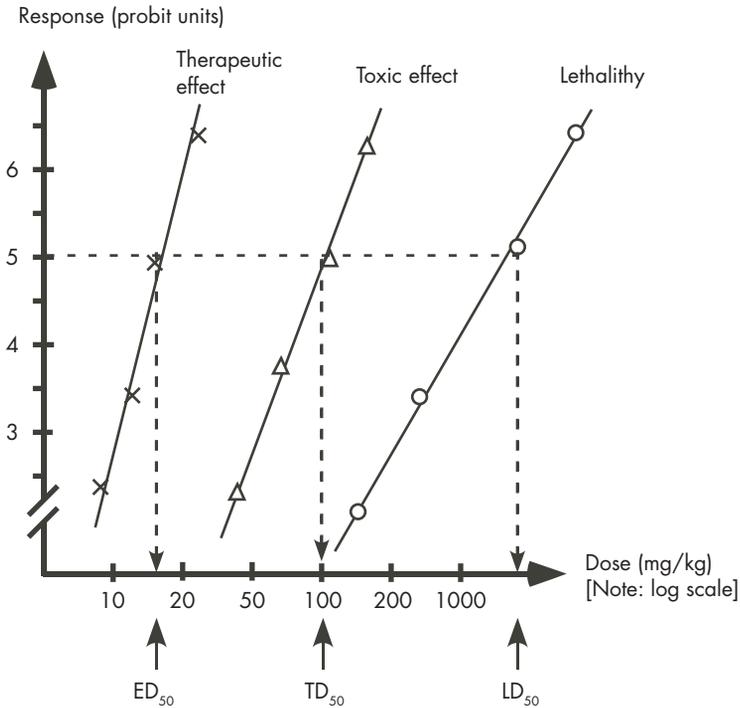
To establish a dose–response curve, the minimum dose required to



**Figure 1.3** Dose-response relationship: noncumulative. In individual animals the occurrence of a toxic effect (e.g. neuronal axonopathy) was 'titrated', so that for each individual animal the dose could be established at which a predefined pain effect occurred. The animals were subsequently grouped together in dose groups (the bars in the figure). The curve shows the sensitive individuals at the left and the resistant individuals at the right-hand side. When the bars are added up (accumulated), this results in the curve of Figure 1.1. The log normal distribution approximates a Gaussian distribution (i.e. it is normally distributed). Then a frequency response can be expressed in multiples of standard deviation (normal equivalent deviates). In such a case, the dose interval at which 50% of the population will respond  $\pm 2$  standard deviations will, for example, include 95% of the exposed population. The normal equivalent deviates are often transformed into probit (probability) units by adding a constant value of 5 to the standardised normal distributions (to avoid negative numbers) (see also Figure 1.4).

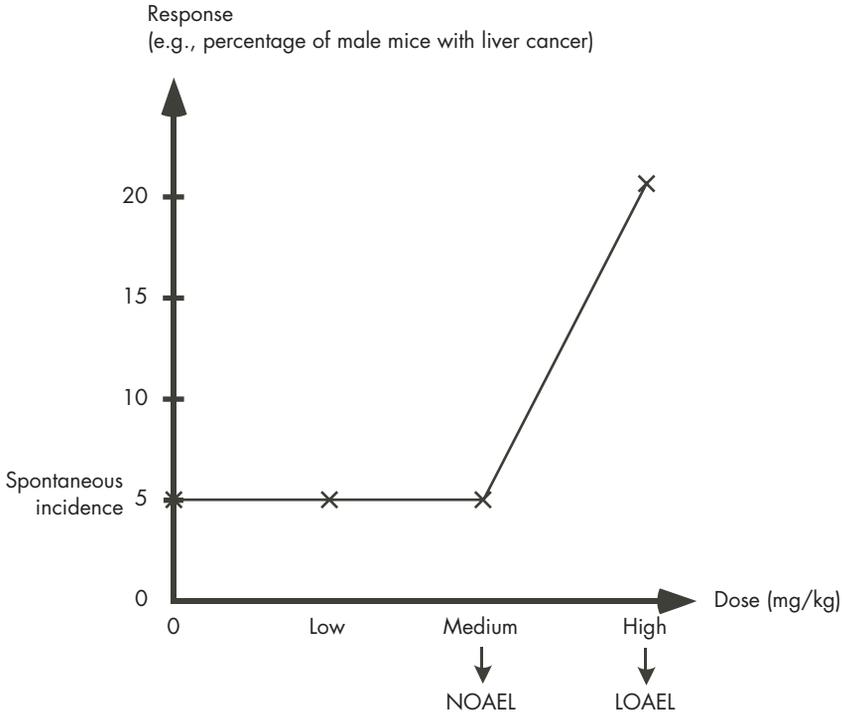
### Gaussian distribution (normally distributed data)

Per cent responding	Normal equivalent deviate (SD)	Probit unit
99.9	+3	8
97.7	+2	7
84.0	+1	6
50.0	0	5
16.0	-1	4
2.3	-2	3
0.1	-3	2



**Figure 1.4** Probit plot of various effects. Dose–response data can be plotted using a probit scale to extrapolate data: probability units obtained from standardised normal distributions are plotted against the logarithm of the dose of a substance when quantal (or graded) responses have been measured (in this case a therapeutic effect, a toxic effect and death, respectively). Log-dose data provide linear plots, which make it easy to extrapolate the data. ED<sub>50</sub> = the dose that produces the desired effect in 50% of the population; TD<sub>50</sub> = the dose that produces the defined toxicity in 50% of the population, and LD<sub>50</sub> = the dose that produces death in 50% of the population.

produce a predefined effect (e.g. a liver toxicity defined as 4 times the mean control level of transaminase activity in blood) in each individual of a group should be determined. If the population is very homogeneous (inbred rats without differences in lifestyle), this minimum dose will vary very little between the individual rats; but in a human population genetic and lifestyle differences will result in widely different sensitivities. To obtain this curve, one could titrate each individual of the entire population with increasing doses up to the dose that shows the predefined effect in each individual. The required dose for each individual is noted and the individuals are combined into dose groups to make a plot as in



**Figure 1.5** Dose-response for a carcinogenic effect. A typical dose-response curve obtained in a repeat dose toxicity study for carcinogenicity employing a control group (usually given vehicle only) and three groups of animals exposed to different doses of the test compound. NOAEL represent the 'no observed adverse effect level', and LOAEL the 'lowest observed adverse effect level'.

Figure 1.3. A more practical way is to randomly divide the test population into groups and give each group only one of a series of increasing doses. The response (percentage of responding animals) in each group is then recorded and plotted against the dose as in Figure 1.1.

The only requirements when establishing a dose-response curve in an experimental study (Figure 1.5) are that the toxicant can be administered accurately, and that the means of expressing the toxicity are precise. As indicated above, dose-effect relationships show the correlation between the extent of an effect (e.g. a decrease in respiratory volume) and the dose, either for an individual or for the group. In the latter case the mean of the individual responses is given, but this can of course only be done when the group is relatively homogenous (i.e. when the individual curves are distributed according to the Gaussian curve).

This is usually the case in experimental studies using inbred animals, but not necessarily in studies employing a group of human subjects. If such a group is very heterogeneous and is composed of two (or more) subgroups with pronounced genetic or lifestyle differences (for example in their metabolism) such means are rather useless.

A very sensitive subgroup may be defined as a 'special group at risk'. The individuals in such a group will, for various reasons, show an excessive toxic response to a certain exposure as compared to the majority of individuals in the general population. For instance, when asthmatic persons are exposed to an airway irritant or histamine at a low dose level that only marginally affects a healthy individual, they can experience a very severe, potentially life-threatening response. Clearly, a mean dose-effect curve for both groups together would not represent either group: each group would show a separate Gaussian distribution of sensitivities (Figure 1.3) peaking at different doses.

### **Thresholds or not?**

Dose-effect relationships are closely associated with a critical issue in toxicology – that of whether or not thresholds exist for various types of adverse effects. Below the threshold (a dose, dosage, concentration, exposure level, etc.) there should be no adverse health effects, but toxicity may occur above this 'minimally effective dose' (threshold). Obviously, the threshold level for a chemical may be different for various individuals and it is also difficult to establish precisely. This is a highly relevant issue when discussing 'safe' exposure levels. The therapeutic effect of a drug always requires a certain minimum dose, which may vary between individual patients. Similarly, the dose at which side-effects will occur can be quite different between different patients. A very sensitive patient may experience unacceptable adverse health effects at a therapeutically required dose of a drug that is safe for the majority of patients, implying that the safe use of this drug is impossible for this particular patient. This mainly applies to side-effects/adverse health effects that operate by a mechanism other than that of the therapeutic effect. Side-effects that are due to the same receptor as the therapeutic effect ('pharmacological side-effects') will be expected to follow the sensitivity for the therapeutic effect.

Most acute and chronic adverse effects are associated with thresholds. The threshold for a particular adverse effect will be determined by characteristics of each individual. In an inbred species (of experimental animals), each individual will show little variation in threshold, but

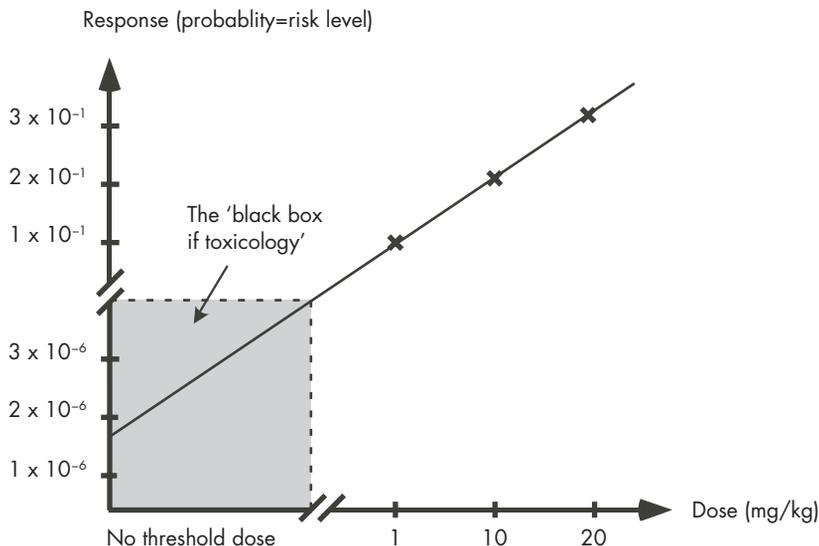
between species or even between different strains of one species there may be pronounced differences. As indicated in Figure 1.5, dose–response curves can be used to determine a ‘no observed adverse effect level’ (NOAEL) and a ‘lowest observed adverse effect level’ (LOAEL). The threshold is somewhere in the interval between NOAEL and LOAEL. It should be pointed out that the NOAELs and LOAELs are not absolute effect levels; they are highly dependent on the testing conditions, such as the number of animals in each group, the dose intervals used in the study (which are decided by the study director), the methods for registration of responses, the number of surviving animals, the number of animals and organs subjected to histopathological examinations, and so on.

For some type of adverse health effects (notably neoplasms and genetic diseases induced by mutagens interacting directly with the genetic material, and possibly also sensitisation), it cannot be excluded that there is no true threshold (Figure 1.6). At least theoretically, a single molecule may be sufficient to induce the critical response. ‘The black box’ of toxicology in Figure 1.6 refers to the true nature of the dose–response curve at very low doses (close to zero) which cannot realistically be studied experimentally (because of the extremely low response).

### The ‘black box’ of toxicology

Quantitative risk assessments concerning exposures to extremely low environmental concentrations of genotoxic carcinogens have to deal with this ‘black box’, because regulatory actions to reduce such an exposure may be initiated at risk levels as low as  $1 \times 10^{-6}$  to  $1 \times 10^{-5}$ . An extra cancer risk of  $1 \times 10^{-6}$  is equivalent to one extra case of cancer among one million exposed individuals (over a lifetime exposure) or, expressed on the individual level, an extra cancer risk of 0.0001%. This obviously cannot be determined experimentally. This is seldom an issue for the safety evaluation of drugs because most exposures that may lead to adverse health effects occur at therapeutic doses, far above the ‘close to the zero dose’ region (see below).

The continuing discussion of whether thresholds exist for genotoxic agents involves questions about, for example, the efficiency of detoxification at very low exposure levels and the importance of various DNA-repair pathways (e.g. the balance between the error-free, high-fidelity excision repair pathways and more error-prone pathways). In fact there is a constant and unavoidable exposure to ‘endogenous

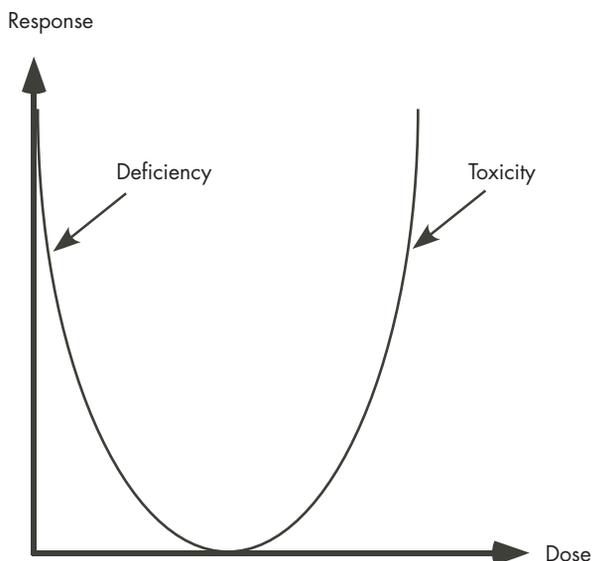


**Figure 1.6** Extrapolation from high to extremely low exposure for carcinogens. To calculate the risk for the general population of exposure to a carcinogenic compound, the data obtained at high exposure in experiments in, say, rats (when, for example, 20 of the 50 treated animals develop tumours compared to 3 per 50 in the controls) have to be extrapolated to the much lower exposure at which an incidence of perhaps one additional case of chemically induced cancer per 1 000 000 (risk level:  $10^{-6}$ ) exposed people is accepted (a 'political' and/or policy decision). These are calculated by linear extrapolation to zero exposure (the spontaneous incidence), which in principle is a worst-case approach because no threshold is assumed.

mutagens' and virtually all cells are subjected to spontaneous mutations and 'naturally' occurring DNA damage.

This issue is further complicated if there is an effect of 'hormesis' for genotoxic agents. The 'hormesis' theory states that a chemical that is known to be toxic at higher doses may be associated with a beneficial effect at low doses. For example, there are some data suggesting that a low level of exposure to a genotoxin stimulates the activity of the high-fidelity DNA repair, and it has also been shown that low levels of dioxin consistently decrease the incidence of certain tumours in animal experiments.

As shown in Figure 1.7, essential elements such as nutrients and vitamins (which also are toxic at high doses) have a rather unusual dose-response curve when it comes to their adverse health effects. A deficiency results in malfunction, while an excess results in toxicity.



**Figure 1.7** The toxicity response of an essential element. Typically a U-shaped dose–response curve (theoretical) is expected for an essential element such as selenium or oxygen: when the supply is too low a deficiency leads to disease, while an excess leads to toxicity.

### **Toxicity testing is necessary for hazard identification and safety assessment**

In toxicology, a distinction is made between hazard and risk. Whereas *hazard* is an ‘inherent’ potential of a chemical to be toxic at some site (e.g. it has the potential to cause malformations in rabbits or cancer in the forestomach of rats), *risk* includes the determination of the chance of the expression of an identified hazard given the exposure characteristics of the chemical. The concept of risk therefore includes two components: probability and outcome (in toxicology, an adverse health effect). Consequently, whereas risk is the probability that a compound will produce harm under specific conditions, safety (the reciprocal of risk) is the probability that harm will not occur under the specified conditions. The concept of risk is typically used for non-drugs; that of safety for drugs. Completely different, but very important, issues when discussing the risk/safety of chemicals are the processes of risk communication, risk perception and risk acceptance. For example, whether a risk is voluntary or not, is known or unknown, is of natural origin or human-made, etc., plays a decisive role in the degree of acceptance of a risk.

For example, a patient will most likely accept rather serious side-effects if the disease to be treated is directly life-threatening or debilitating. It is interesting to note that agents like botulinum toxin (BTX; an exotoxin with extremely high acute toxicity), arsenic trioxide ( $\text{As}_2\text{O}_3$ , ‘the king of poisons’) and thalidomide (a well-known human teratogen; see Chapter 4) are used today as drugs to treat various diseases, such as cervical dystonia (BTX), acute promyeloid leukaemia ( $\text{As}_2\text{O}_3$ ) and hypertrophic cutaneous lupus erythematosus (thalidomide).

A major purpose of toxicity testing is hazard identification, that is to identify the ‘toxicological profile’ of a drug (or any chemical) and characterise its ‘inherent’ potential to act as a toxicant. By adding data on toxicokinetics in animal studies, as well as pharmacokinetic data for the patient when treated with the intended dose, it is also possible to calculate a margin of safety for a drug (see below). The spectrum of adverse health effects (‘toxicological end-points’) that can be induced by a chemical includes both reversible and irreversible effects, local and systemic toxicity, immediate and delayed effects, and organ-specific and general adverse effects.

In combination with toxicokinetic studies, toxicity testing provides information about the shape of the dose–effect curves for the various types of toxic effects identified, including approximate lethal doses in acute toxicity studies,  $\text{TD}_{50}$  values as well as NOAELs and LOAELs (see Figures 1.4 and 1.5). The  $\text{LD}_{50}$ , that is the dose that kills 50% of the treated animals, is no longer used for drugs for reasons of saving the animals from unnecessary suffering; in any case, such values are not relevant for clinical side-effects of drugs in patients. The  $\text{TD}_{50}$  is the dose that induces an adverse effect in 50% of the exposed individuals.

The most valuable information about the toxicity of chemicals derives from observations made in exposed humans, but deliberate toxicity testing on human subjects is, of course, out of the question. Of necessity, toxicity data are therefore generated in studies on experimental animals. The results obtained in these studies are then extrapolated to the human exposure situation. The whole concept of toxicity testing is based on the presumption that experimental animals can be used to identify potential health hazards for humans. For drugs, the situation is usually much simpler: here human data become available during clinical trials. It is essential that all drug metabolites formed in humans also are present in the toxicity studies on animals, since every metabolite is expected to have its own unique toxicological profile.

As shown in Table 1.1, toxicity tests can be separated into two major categories: those designed to identify general toxicity (typically,

**Table 1.1** Toxicological end-points measured in an extensive battery of toxicity tests

<i>Adverse health effect</i>	<i>A representative sample of tests for which there are internationally accepted testing guidelines</i>
Acute toxicity and lethality	<ul style="list-style-type: none"> <li>• Acute oral toxicity study in rats</li> <li>• Acute inhalation toxicity study in mice</li> <li>• Acute dermal toxicity study in guinea pigs</li> </ul>
Short-term repeat dose and subchronic toxicity	<ul style="list-style-type: none"> <li>• 14-day oral toxicity study in mice</li> <li>• 21-day dermal toxicity study in rats</li> <li>• 90-day diet study in rats</li> <li>• 90-day inhalation toxicity study in guinea pigs</li> </ul>
Chronic toxicity	<ul style="list-style-type: none"> <li>• 12-month oral toxicity study in dogs</li> <li>• 24-month inhalation toxicity study in rats</li> </ul>
Genotoxicity and mutagenicity	<ul style="list-style-type: none"> <li>• <i>Salmonella typhimurium</i> reverse mutation assay</li> <li>• Mouse lymphoma TK-locus assay</li> <li>• Micronucleus test</li> </ul>
Carcinogenicity	<ul style="list-style-type: none"> <li>• 18-month dietary cancer bioassay in mice</li> <li>• 24-month inhalation cancer bioassay in rats</li> </ul>
Reproductive toxicity	<ul style="list-style-type: none"> <li>• Two-generation reproduction study in rats</li> </ul>
Embryotoxicity and teratogenicity	<ul style="list-style-type: none"> <li>• Teratogenicity study in rabbits</li> <li>• Teratogenicity study in rats</li> </ul>
Allergic sensitisation	<ul style="list-style-type: none"> <li>• Skin sensitisation study in guinea pigs</li> </ul>
Local effects on the skin	<ul style="list-style-type: none"> <li>• Acute dermal irritation and corrosion study in rabbits</li> </ul>
Neurotoxicity	(No specific testing guidelines available for neurotoxicity or behavioural toxicity; indirect evidence in general toxicity studies)
Immunotoxicity	(No specific testing guidelines available; thymus toxicity can be monitored in general toxicity studies)

toxic effects in various organs assessed by, for example, histopathology or clinical chemistry parameters), and those designed to identify specific types of adverse health effects (e.g. genotoxicity, cancer, reproductive toxicity and teratogenic effects). The general pharmacology studies on drugs also include what often is called ‘safety pharmacology’, focusing on effects of the drug on vital physiological functions such as those of the heart, the lungs and the central nervous system. In some cases,

special studies may be required when a certain toxic effect of a drug in animals raises concern and the drug company wants to prove that this effect is not relevant for the proposed human use. Nowadays drugs should also be evaluated with regard to their environmental safety (using different types of ecotoxicological studies). This need arises from concern about, for example, ethinylestradiol (a component of oral contraceptive pills) in waste water, and its oestrogenic effects in the environment.

For most toxicity tests there are internationally accepted guidelines describing how each individual test should be performed so as to obtain a well-defined framework for both the testing and the evaluation of toxicity; for instance, the OECD (Organisation for Economic Co-operation and Development) Guidelines for Testing of Chemicals. Much information about toxicity testing can be found on OECD's home page ([www.oecd.org](http://www.oecd.org)). The various guidelines specify the prerequisites, procedures (preferred species, group sizes, duration of treatment, etc.), testing conditions (dosages, routes of administration, clinical observations, haematology, pathology, etc.), statistical procedures, and how the test report should be formulated. The guidelines have also been developed in order to minimise suffering of experimental animals. Protection of the animals from unnecessary stress due to pain and discomfort is important not only from a humane point of view but also because stress may interfere with the 'true' toxic responses induced by the chemical under test.

It should be emphasised that all toxicity tests are designed to reveal potential toxicity and not to prove the harmlessness of chemicals. The tests are therefore usually designed to be as sensitive as possible. For ethical, practical and economic reasons, toxicity testing is usually performed using a restricted number of animals. Relatively high doses are used (for statistical reasons) in order to compensate for the limited number of animals. 'High' doses do not mean lethal doses. For example, the highest dose in a chronic cancer bioassay is often referred to as the 'maximum tolerated dose' (MTD). This dose should be high enough to induce some signs of toxicity (e.g. a slightly reduced body weight gain), but it should not substantially alter the normal lifespan of the animals as a result of effects other than tumour development.

The possible shortcomings of toxicity testing on animals and cultured cells must always be considered when evaluating the results of these tests. Data from *in vivo* studies in animals allow calculations of margins of safety but may not necessarily be predictive of the ultimately relevant adverse effects in the patients. Primates (especially great apes)

are more comparable to humans than rats, for example, but the use of primates is limited because of societal concern. Animal toxicity studies could, for example, predict that a drug will induce kidney toxicity in patients, but it might very well turn out later in clinical studies that this drug actually induces certain unacceptable CNS effects, and that it is the latter effects rather than the kidney toxicity that limit its human use.

Consequently, clinical side-effects observed in Phase II and Phase III studies will ultimately determine human toxicity profiles under therapeutic conditions. Unfortunately, rare side-effects may still be missed. Even in relatively large Phase III trials employing, say, 3000 patients, rare side-effects with an incidence of less than 1 in 1000 (risk level =  $1 \times 10^{-3}$ ) will almost certainly be missed unless they are extremely unusual (in the general population). This is why post-marketing surveillance and periodic safety updates ('PSUR') are extremely important after introducing a new drug to the market.

Several factors determine which (if any) side-effects will be observed in the general patient population at the therapeutic dosage. Among these are ethnicity, age, body weight, health condition, nutritional status, lifestyle (alcohol, smoking habits and drug abuse), co-medication, duration and frequency of exposure and/or climate conditions. Since ethnic differences can be quite pronounced in some cases, clinical studies in different ethnic groups are required. Many of the considerations mentioned above are also important when evaluating animal toxicity data, because they can influence the outcome in a toxicity study (along with species, strain, sex and route of exposure). Despite all the possible limitations of animal toxicity studies and the undeniable fact that different animal species may respond differently to various toxicants, there is a general consensus that animal studies are required and useful (but not sufficient) to guarantee the safe use of drugs.

*In vitro* assays can be used to study mechanisms and identify (potential) hazards, but they do not give information about the magnitude of risk.

## **Margins of safety**

A safe drug has a large margin of safety (MOS). At the group level (but not necessarily on the individual level) this means that unwanted side-effects of such a drug require a much higher dose than is required for the therapeutic effect. The MOS can be calculated in different ways depending on the type of chemical and the available data. If the MOS is based on toxicokinetic data for the internal exposure in experimental

animals and on pharmacokinetic data for the internal exposure in humans, one will get a rather reliable estimate of the MOS. For drugs, these data (blood levels, etc.) are usually readily available from both the toxicokinetic and toxicity studies in animals and the clinical trials.

In the past, a MOS for drugs was usually calculated as the ratio of the dose that is just within the lethal range ( $LD_1$ ) to the dose required to result in a therapeutic response in 99% of the exposed patients ( $ED_{99}$ ). Clearly,  $LD_1$  (the dose that kills 1% of exposed animals) and  $ED_{99}$  were statistically derived (e.g. by extrapolating from probit-transformed data) and not measured directly (an impossible task). The ratio  $LD_1/ED_{99}$  has at least one advantage over the therapeutic index ( $TI = LD_{50}/ED_{50}$ ), another statistically derived approximate estimate of the relative safety of drugs. Since TI is based on median values (see Figure 1.4), it only compares the mid-points of the dose–response curves and does not consider at all the slopes of the dose–response curves for the therapeutic and toxic effects. If there is an overlap in these two curves, a sensitive patient might experience adverse effects without the therapeutic effect, even if the average patient is effectively treated (without adverse effects).

Numerical values of  $LD_1/ED_{99}$  and  $LD_{50}/ED_{50}$  are no longer calculated; instead, margins of safety for drugs are based on toxicokinetic parameters that reflect the internal exposure much better. Consequently, MOS values for drugs are usually based on a toxic effect in animals (e.g. liver necrosis) occurring at a certain blood concentration and the blood concentrations reached in the patient on a therapeutic dosage schedule. If the difference is big enough, MOS is judged acceptable, but, obviously, the size of an acceptable (or unacceptable) MOS is to a large extent dependent on the severity of the disease to be treated.

For residues of pesticides in food, environmental pollutants in the air, and other types of non-drugs (for which there are no beneficial effective therapeutic doses), margins of safety (sometimes referred to as margins of exposure; MOE) can be calculated by comparing the difference between NOAEL (typically obtained in a toxicity study on animals) and the estimated exposure level in the human population at risk.

## **Studies on general toxicity**

### *Acute toxicity*

The main purpose of acute toxicity testing of drugs is to gather basic toxicological information before the more extensive toxicity testing and

Phase I clinical trials in healthy human volunteers (see Chapter 13). After exposure of a restricted number of animals (orally, intravenously or by inhalation), these are examined at least once a day for 14 days (including clinical chemistry in blood and urine). Animals showing severe signs of intoxication are killed prematurely to spare unnecessary suffering, and all animals are subjected to an autopsy at the end of the study.

The acute toxicity studies not only provide important information on immediate health hazards and clinical signs of intoxication; they can also identify possible target organs and conceivable modes of action. The results obtained in acute toxicity studies are used when establishing the dosage regimens in repeated dose toxicity studies. For chemicals in general, the results can also be used to classify them in terms of various types of toxicity ratings (see Table 1.2 and 1.3). Depending on the acute toxicity data, including its estimated lethality, a chemical can be classified as being 'very toxic', 'toxic', 'harmful' or 'moderately harmful' using a classification system based on LD<sub>50</sub> or LC<sub>50</sub> values in

**Table 1.2** Examples of single risk phrases for compounds that should be classified as very toxic (symbol T+) if after a single, brief exposure they can cause temporary or irreversible injuries or lead to death. (Taken from the Swedish National Chemicals Inspectorate)

<i>Code</i>	<i>Phrase</i>	<i>Criterion</i>
R28	Very toxic if swallowed	<ul style="list-style-type: none"> <li>• LD<sub>50</sub> oral, rat ≤ 25 mg/kg</li> <li>• Less than 100% survival at 5 mg/kg (oral rat) according to the discriminating dose (the fixed dose procedure)</li> </ul>
R27	Very toxic in contact with skin	<ul style="list-style-type: none"> <li>• LD<sub>50</sub> dermal, rat or rabbit ≤ 50 mg/kg</li> </ul>
R26	Very toxic by inhalation	<ul style="list-style-type: none"> <li>• LC<sub>50</sub> inhalation, rat, aerosols and particulates ≤ 0.25 mg/L, 4 h</li> <li>• LC<sub>50</sub> inhalation, rat, gases and vapours ≤ 0.5 mg/L, 4 h</li> </ul>
R39	Danger of very serious irreversible effects	<ul style="list-style-type: none"> <li>• Strong evidence that irreversible injuries can occur, generally within the above-mentioned dose ranges, after a single, brief exposure via a relevant route of administration.</li> </ul>

Note: Carcinogenicity, mutagenicity and reproductive toxicity excluded

**Table 1.3** The connection between classification in categories of danger and labelling: some examples. (Taken from the Swedish National Chemicals Inspectorate)

<i>Category of danger</i>	<i>Symbol letter</i>	<i>Symbol</i>
Very toxic (R26, R27, R28 and R39)	T+	Skull and crossbones
Toxic (R23, R24, R25, R39, R48)	T	Skull and crossbones
Corrosive (R34, R35)	C	Corrosion symbol
Harmful (R20, R21, R22, R65, R40, R48)	X <sub>n</sub>	St Andrew's cross

experimental animals (see Table 1.4), or as 'super toxic' (<5 mg/kg), 'extremely toxic' (5–50 mg/kg), 'very toxic' (50–500 mg/kg), 'moderately toxic' (0.5–5 g/kg), 'slightly toxic' (5–15 g/kg) or 'practically non-toxic' (>15 g/kg) based on its estimated oral lethal dose for humans (adults). Many recommendations regarding protective measures and possible need for medical attention when handling chemicals are based on such acute toxicity classification systems.

#### *Short-term repeated dose and subchronic toxicity*

Short-term repeated dose and subchronic toxicity studies provide information on immediate and delayed adverse effects, possible bioaccumulation, reversibility of damage, and development of tolerance. The clinical and histopathological examinations are quite extensive and it is therefore often possible to establish NOAELs and LOAELs that can be

**Table 1.4** Classification categories based on LD<sub>50</sub> or LC<sub>50</sub> values. (Taken from the Swedish National Chemicals Inspectorate)

<i>Classification category</i>	<i>Acute toxicity</i>	<i>LD<sub>50</sub> (oral) (mg/kg, rat)</i>	<i>LD<sub>50</sub> (dermal) (mg/kg, rat)</i>	<i>LC<sub>50</sub> (inhalation) (mg/kg, rat)</i>
Very toxic	Very high	≤25	≤50	≤0.5 <sup>a</sup> (≤0.25) <sup>b</sup>
Toxic	High	25–100	50–400	0.5–2 <sup>a</sup> (0.25–1) <sup>b</sup>
Harmful	Medium high	200–2000	400–2000	2–20 <sup>a</sup> (1–5) <sup>b</sup>
Moderately harmful	Moderate	>2000 <sup>c</sup>	—	—

<sup>a</sup> Applies to gases and vapours.

<sup>b</sup> Applies to aerosols and particulates.

<sup>c</sup> Applies to consumer products.

used when establishing the dosage regimen in other toxicity studies. For short-term human drug use (days or weeks), this information can be used to assess the safety/efficacy balance. Moreover, for short-term occupational exposures this type of information can also be used when establishing a threshold limit value and other types of safety criteria.

### *Chronic toxicity*

The main purpose of chronic toxicity testing is to identify adverse health effects that require long periods to develop, and to establish dose–response relationships for these effects. The chronic toxicity studies can provide important information on various types of biochemical, haematological, morphological, and physiological effects. Using rodents, each dose group (including a concurrent control group) should include at least 20 animals of each sex. When non-rodents are used (less commonly), a minimum of four animals (usually dogs or primates) of each sex is recommended per dose. The test compound should be administered on a daily basis in at least three different doses for at least 12 months. For drugs, toxicokinetic data should be collected at regular time intervals in so called ‘satellite groups’ that are treated for that specific purpose only.

A careful check should be made daily, and all clinical signs of toxicity (changes in body weights, food consumption, behaviour, etc.) should be recorded. Measurements of blood clinical chemistry, haematological examinations and urinalysis should be performed on a regular basis, and all animals should be subjected to a complete gross examination at necropsy. The histopathology should include at least a complete microscopic examination of most of the organs and tissues from the animals in the highest dose group and the control group, including all animals that died or were killed during the study period. The NOAELs and/or LOAELs obtained in a chronic toxicity study are typically used to establish a margin of safety for drugs or to establish various threshold limit values for environmental and/or occupational human exposures.

## **Studies on various types of specific adverse health effects**

### **Genotoxicity**

Genotoxicity (genetic toxicity) represents a diversity of genetic endpoints including primary DNA damage (DNA adducts, cross-linking,

intercalation, DNA strand breaks, etc.), gene mutations (changes in the nucleotide sequence at one or a few coding segments within a gene), structural chromosomal aberrations (e.g. translocations and inversions following chromosome or chromatid breakages), and numerical chromosomal aberrations (e.g. aneuploidy and other genomic mutations). There are numerous test systems available to detect various types of genetic end-points, using a broad spectrum of 'indicator' organisms (from plants, bacteria, yeast, insects, and cultured mammalian cells, to intact experimental animals). So far there are internationally accepted testing guidelines for approximately 15–20 of these test systems. The main purpose of genotoxicity testing is to establish whether a given compound has the 'inherent' ability of being mutagenic (usually with the aim of identifying potential carcinogens). For a more detailed survey of genetic toxicology, see Chapter 5.

### **Carcinogenicity**

Tumour development is a multistage process involving both permanent genetic alterations (i.e. mutations) and other ('epigenetic') events. The neoplasms ('tumours', 'cancers', 'malignancies') are a family of diseases characterised by aberrant control of cell proliferation and cell differentiation, and most malignant diseases are multifactorial in origin. The main purpose of a chronic cancer bioassay is to study the potential development of tumours in experimental animals exposed for a major portion of their lifespan. Typical indications of carcinogenicity are development of types of neoplasms not observed in controls; increased incidence of types of neoplasms also observed in controls; occurrence of neoplasms earlier than in the controls; and/or increased multiplicity of neoplasms in animals exposed to the test compound. Chronic cancer bioassays are usually performed on two different species (typically mice and rats), using at least 50 males and 50 females per dosage for each species (including an unexposed group of control animals). For a more detailed survey of chemical carcinogenesis, see Chapter 6.

### **Reproductive toxicity**

Toxic responses of the reproductive system can be the result of disruption of spermatogenesis or oogenesis (gametogenesis), or of adverse effects on libido, fertilisation, implantation, embryogenesis, organogenesis, fetal growth, or postnatal development. In a broad sense, reproductive toxicity studies include single- and multi-generation studies on

fertility and general reproductive performance (segment I studies), studies on embryotoxicity and teratogenicity (segment II studies; see below), and peri- and postnatal studies on effects occurring during late gestation and lactation (segment III studies).

The main purpose of a typical one- or two-generation reproductive toxicity study is to provide information on chemically induced adverse effects on the male and female reproductive performance. By studying parturition, duration of gestation, number and sex of pups, stillbirths, live births, microscopic alterations in the gonads of the adult animals, or gross anomalies in the offspring, for example, information can be obtained on adverse effects on gonadal function, oestrous cycle, mating behaviour, conception, parturition, lactation and weaning. These studies should also be able to provide some information on developmental toxicity, including neonatal morbidity and behaviour. In a typical segment I study, both sexes (usually rats) are exposed to graduated doses of the test compound (in order to cover important stages of both male and female gametogenesis). After mating, the females are continuously exposed during gestation and the nursing period as well. In a two-generation study, the test compound is also given to the offspring (the F<sub>1</sub> generation), starting at weaning and continuing until the second generation (the F<sub>2</sub> generation) is weaned. Some mechanisms behind reproductive effects are discussed in Chapter 4.

### **Embryotoxicity, teratogenicity and fetotoxicity**

Chemicals may affect the developing embryo or fetus without inducing any overt signs of maternal toxicity. For example, depending on the stage of embryonic or fetal development, a toxicant may induce early embryonic death, fetal death, malformations, retarded maturation or low birth weight, as well as various metabolic and physiological dysfunctions and cognitive deficiencies in the offspring. In the broadest sense, embryotoxicity can be defined as all types of adverse effects exhibited by the embryo (i.e. toxicity occurring from the formation of the blastula until the completion of organogenesis). Fetotoxicity (i.e. the adverse effects exhibited by the fetus) is induced after the completion of organogenesis. Typical examples of such effects are increased lethality at birth, low birth weight, various types of physiological and psychological dysfunction, and cognitive disturbances manifested after birth.

To be able to distinguish between the various types of adverse health effects that may follow from exposure to a chemical during gestation, 'embryotoxicity' has also come to mean the ability of a chemical

to impair embryonic growth or induce embryonic death. Teratogenicity is typified by permanent structural or functional abnormalities (including external malformations, skeletal abnormalities and/or visceral anomalies), but may also include behavioural changes (behavioural teratology) if a wider definition is used.

The main purpose of teratogenicity testing is to provide information on the potential hazards to the unborn following exposure during pregnancy. Embryotoxicity and teratogenicity without apparent maternal toxicity are particularly alarming. The test compound is given in different doses to pregnant animals (usually rats or rabbits), for a period including organogenesis. As in most other toxicity studies, the highest dose administered should elicit some maternal toxicity, and the lowest dose should be without apparent signs of toxicity. The pregnant animals are killed shortly before the expected time of delivery, and the offspring are examined for various embryotoxic and teratogenic effects. For a more detailed description of mechanisms behind teratogenic effects, see Chapter 4.

### **Neurotoxicity and behavioural toxicity**

Neurotoxicity can be defined as chemically induced adverse effects on any aspect of the central and peripheral nervous system, including various supportive structures. From this it follows that 'neurotoxicity' is associated with various types of pathological changes in the nervous system that are expressed as changes in morphology, physiology, biochemistry and/or neurochemistry, as well as various types of functional and neurobehavioural changes. Obviously, neurotoxicity is not a single end-point that can be evaluated in a single test system. Pathological changes in various regions of the brain, and/or clinical signs of intoxication deriving from CNS toxicity (e.g. piloerection, tremor or coma) can be monitored in acute and repeated dose toxicity studies. Chemically induced behavioural changes (sometimes very subtle) are more difficult to monitor. This usually requires a completely different type of testing procedure, with which a 'traditionally' trained toxicologist is not always familiar.

Test systems are available to detect various types of (subtle) CNS effects (e.g. changes in reflexive or schedule-controlled behaviours, or reduced performance in various learning and memory tasks). The concept of behavioural toxicology is based on the notion that a behaviour is the final functional expression of the whole nervous system (indirectly including the endocrine system and other organs as well) and that

behavioural changes therefore can be used as sensitive indicators of chemically induced neurotoxicity, both in adult animals and in animals exposed *in utero* or shortly after birth ('neurobehavioural teratology').

Behavioural toxicity tests are based on changes either in spontaneous behaviour of the animals (e.g. their natural social or exploratory behaviour) or in stimulus-oriented behaviour. The latter tests are either directed towards an operant-conditioned behaviour (the animals are trained to perform a task in order to avoid a punishment, or to obtain a reward) or towards classical conditioning (the animals are taught to associate a conditioning stimulus with a reflex action). Typical responses recorded in the various types of behavioural toxicity tests are 'passive avoidance', 'auditory startle', 'residential maze' and 'walking patterns'. It is not always easy to interpret the results from behavioural neurotoxicity tests in terms of (relevance to) human behaviour. Apart from the obvious problems associated with the functional reserve and adaptation of the nervous system, there is also an inherently large variability in behaviour. Since neurobehavioural testing usually involves multiple measurements in several different test systems, there is an obvious risk of getting some statistically significant result purely by chance.

### **Immunotoxicity including sensitisation**

Toxic effects mediated by the immune system are sometimes referred to as immunotoxicity. However, from a toxicological point of view, immunotoxicity is in most cases defined as chemically induced adverse effects on the immune system. The immune system is a highly complex and cooperative system of cells, tissues and organs, protecting the organism from infections and neoplastic alterations. Immunotoxic agents can interact with the immune system in many ways. They can, for example, interfere with the function, production or lifespan of the B- and T-lymphocytes, or interact with various antigens, antibodies and immunoglobulins. Basically, immunotoxic agents function either as 'immunosuppressive' agents or as 'immunostimulants'. Consequently, like several other toxicological 'end-points', immunotoxicity is not a single end-point that can be monitored with a single test system.

Skin sensitisation, i.e. allergic contact dermatitis, is an immunologically mediated reaction requiring an initial contact with an agent that induces sensitisation. It can be difficult to distinguish between an allergic contact dermatitis caused by skin sensitisation and an 'ordinary' contact dermatitis following skin irritation, because the symptoms (typically involving erythema and oedema, sometimes also including vesicles)

are quite similar. However, when sensitisation has occurred, responses are often more severe and are also elicited by rather low, non-irritating doses without apparent thresholds. Being very sensitive, the guinea pig is the preferred species when testing for skin sensitisation.

Several alternative tests are available: for example, Freund's complete adjuvant test, the guinea pig maximisation test and the open epicutaneous test. Most of these tests follow the same general outline. To make the animals hypersensitive, they are first exposed to a rather high dose of the test compound. After an 'induction period' without exposure, the animals are then exposed a second time to a low, non-irritating dose. After this challenge dose, the animals are examined with regard to possible development of allergic contact dermatitis. Sensitisation can also occur after other routes of exposure (notably inhalation), but internationally accepted testing guidelines have so far been developed only for allergic contact dermatitis.

Several drugs (e.g. penicillins and halothane) cause immune reactions ('allergy') because they can function as haptens. Since this type of allergic reaction is very difficult to predict in animal models, drug-related side-effects of this type are usually not detected until after the clinical trials or after the drug has been on the market for some time. Hypersensitivity responses to drugs are actually among the major types of unpredictable drug reactions and the effects can be drastic, including anaphylactic shock in the case of penicillins and hepatitis in the case of halothane. For a more detailed survey of immunotoxicology, see Chapter 10.

### **Skin and eye irritation**

The main purpose of testing for local toxicity on skin and eyes is to establish whether a chemical induces irritation (reversible changes) or corrosion (irreversible tissue damage) when applied as a single dose on the skin or to the anterior surface of the eye. Obviously, there is no point in testing a strongly acidic ( $\text{pH} \leq 2$ ) or alkaline ( $\text{pH} \geq 11.5$ ) agent for local toxicity on the skin and eyes. Equally, if an agent has been shown to be corrosive to the skin, it seems rather pointless to proceed with an acute eye irritation study. Testing for local effects on the skin is usually performed on albino rabbits (each animal serving as its own control). The degree of skin reaction is read and scored at various time points (up to 14 days after the application). Depending on the degree of erythema and oedema on the skin, the test chemical is classified as a non-irritant, irritant or corrosive agent. Currently, eye irritation tests

are often done *in vitro* rather than in the traditional Draize test on rabbits' eyes *in vivo*.

### **Toxicodynamics: there are many different mechanisms of toxicity**

In parallel with the concept of pharmacodynamics, one may summarise the adverse health effects of a drug and the mechanisms behind them as 'toxicodynamics'. Most toxicants induce their adverse effects by interacting with normal cellular processes, and many toxic responses are the ultimate result of cell death leading to loss of important organ functions. Other responses follow from interactions with various biochemical and physiological processes not affecting the survival of the cells. Common mechanisms of toxic action include receptor–ligand interactions, interference with cellular membrane functions, disturbed calcium homeostasis, disrupted cellular energy production, and reversible or irreversible binding to various proteins, nucleic acids and other 'biomolecules'. Toxicity can be the result of one specific physiological change in a single target organ, or can follow from multiple interactions at different sites in several organs and tissues.

Many toxicants induce their adverse effects by binding to a specific site on a biologically active molecule. This molecule can be a protein (e.g. a 'high-affinity' receptor, a bioactivating or detoxifying enzyme, a DNA-repair enzyme, a channel protein or a transport protein), a nucleic acid (DNA or RNA), a lipid, or another macromolecule with important biological functions. A 'receptor' is usually defined as a high-affinity binding site interacting with an endogenous ligand. Typical examples of such receptors are those interacting with various neurotransmitters in the CNS, and the intracellular receptors interacting with, for example, calcium or various steroid hormones. However, in a broad sense a receptor can be defined as any binding site available for a particular ligand, and in that sense the definition of a 'receptor' is broader in toxicology than in pharmacology.

When a toxicant binds to a high-affinity receptor for an endogenous ligand, it can either 'activate' the biological responses mediated by the receptor (acting as an 'agonist'), or block its function (acting as an 'antagonist'). The agonist can act directly by binding to the receptor or indirectly by increasing the concentration of the endogenous ligand at the receptor (e.g. by inhibiting its degradation, as with acetylcholinesterase inhibitors).

There are numerous examples of toxicants acting by binding to

various macromolecules. For example, the anoxia resulting from the high-affinity binding between carbon monoxide and haemoglobin is an example of an adverse effect that is due to binding to a protein, in this case non-covalent binding. 'Metabolic poisons' interfere with the biological activity of various enzymes. Some toxicants do this by binding to the enzymes and thereby changing their structure. Other types of metabolic poisons interfere with the metabolic pathways by competitive inhibition. Toxicants can also interfere with cellular energy production, for instance by inhibiting oxidative phosphorylation in the mitochondria. Such agents (e.g. several anti-AIDS drugs) are usually called 'mitochondrial poisons'. Other toxicants act as 'cellular poisons' by interfering with various membrane-bound functions and transport processes. Among those are many potent neurotoxins acting as ion channel blockers by binding to various channel proteins.

Several drugs form reactive intermediates during their biotransformation. These electrophilic intermediates can bind directly to various cellular macromolecules, but they can also induce 'oxidative stress' in the cells. This will eventually lead to the formation of various reactive oxygen species, including highly reactive hydroxyl radicals interacting with, for example, DNA (causing DNA damage) and unsaturated fatty acids in the cell membrane (causing lipid peroxidation). Oxidative stress has been implicated as an important factor in many biological processes, including ageing, inflammatory reactions and tumour development. Lipid peroxidation has been implicated as a mechanism of action for many hepatotoxic agents inducing centrilobular liver necrosis. For a more detailed survey of mechanisms behind cytotoxicity, see Chapter 3.

### **The evaluation of toxicity data is not always straightforward**

Most toxicological data are derived from animal experiments. Toxicity studies identify the nature of health damage that may be associated with a given compound, and the range of doses over which the damage is produced. When such data are used for safety assessment (of drugs intended for medical use) or risk assessment (of contaminants in occupational and environmental settings), the methods used in the toxicological evaluation should always be critically assessed. Was the toxicant administered accurately? Were the means of expressing the toxicity precise? Did the study include an adequate number of animals and dosages? Did the study include a control group? Were the results statistically significant? Were the responses biologically significant?

The results from one type of study should always be interpreted in conjunction with the results obtained in other toxicity studies and one should also be aware of the limitations when extrapolating animal data to a human exposure situation. For chemicals regulated in the occupational and environmental area, so-called uncertainty (or safety) factors are often used when extrapolating animal data to humans. Typically a NOAEL obtained in a toxicity study in animals is divided by a total uncertainty factor of 100 ( $10 \times 10$ ). One factor of 10 is to compensate for the possible interspecies variability (animals to humans) in toxicokinetics and toxicodynamics, the other factor of 10 is to compensate for the intraspecies variability (human to human). However, the uncertainty factors used may vary depending on the situation. For example, a factor of 3 may be used for a more homogenous worker population (instead of 10 for the general population), and additional safety factors may be added if the toxicity data are based on LOAELs only, or if the adverse health effect is considered to be particularly serious (e.g. a malignant disease).

For drugs intended for medical use in humans, an exceptionally wide range of toxicity data is available because of the requirements for registration: both animal and human data in relation to the intended therapeutic use and route of administration. The latter requirement means that new data have to be generated when a new route of administration of is considered for a drug that is already on the market. Moreover, if a drug has a registered indication for a short-term use (e.g. maximum three weeks for short-term treatment of pain) and the drug company wants an indication for chronic use (for example, to treat arthritic pain), this will require carcinogenicity data that were not needed for the short-term treatment.

The evaluation of toxicological data is often rather straightforward for immediate adverse health effects following from a well-characterised chemical exposure. Nevertheless, we still often do not know what to do during severe drug overdose because the mechanism behind the acute toxicity is unknown (unless it is a pharmacological side-effect). For continuous low-dose exposures (less of a problem for drugs, but a well-known issue in occupational and environmental medicine), the toxicological evaluation can become complicated (for example, involving questions of whether there is a threshold or not).

A complicating factor when evaluating toxicity data is the development of tolerance – a decreased responsiveness towards the toxicity of a chemical resulting from a previous exposure. Tolerance is a process of adaptation to the effects of a compound, which becomes obvious when,

for example, it is necessary to increase the dosage in order to obtain a given therapeutic response. Tolerance may be due to downregulation of receptors or to selection of resistant cells (not uncommon for cytotoxic cytostatics). Tolerance to morphine and ethanol are two well-known examples of adaptation. Similarly, initial irritation in the nose may disappear after an exposure period of a few minutes.

Another complicating factor is that chemicals, including drugs, can interact with each other. This issue is not usually addressed in conventional toxicity testing of chemicals, which focuses on one compound at a time. The interaction between chemicals can result in an additive effect (the combined effect equals the sum of the effect of each individual agent given alone); a synergistic effect (the combined effect is much greater than the sum of the effect of each agent given alone); a potentiating effect (one of the agents is without toxic effects of its own to the particular organ system, but when given together with a toxic agent, it will multiply the toxic effect); or an antagonistic effect (the combined effect is lower than the sum of the effects of each agent given alone).

During drug development, the company must address the possibility of interaction problems in relation to the drug's intended use, including both pharmacokinetic and pharmacodynamic interactions, as well as toxicokinetic and toxicodynamic interactions. For example, will the drug in question inhibit certain cytochrome P450 enzymes that are needed for the elimination of other drugs? Or is it possible that co-medication with other drugs might inhibit the metabolism of the new drug? Drugs that are frequently used by the intended patient population have to be tested with regard to their pharmacodynamic interactions.

## Conclusion

When a company submits the dossier of a certain indication for the therapeutic use of a new drug, it has to provide exhaustive evidence for the safety of that drug in comparison with its efficacy and intended use, taking into consideration the seriousness of the disease to be treated. Depending on the duration, the route of administration and the dose, the registration requires a certain set of data. Only when the registration authorities are convinced that the balance between safety and efficacy is positive will the drug be registered for that indication. For other, non-drug chemicals, much less is usually known about their toxicological profiles (especially as regards toxicokinetics and pharmacodynamics in humans). In such cases, experimental data from *in vivo* studies on animals and/or *in vitro* assays on isolated cells have to be

substituted for human data (making the conclusions about potential risks less certain). Only for bulk chemicals that have been in use for many years may enough epidemiological data be available to allow conclusions about their human toxicity.

The fact that most toxicity data are still generated in experimental studies (on animals and/or cells) makes it imperative that any professional working with different aspects related to the safe use of chemicals, including drugs intended for medical use, should understand fundamental toxicological principles and have a basic knowledge of the premises for toxicity testing.

### **Further reading**

- Klaasen CD, ed. (2001). *Casarett & Doull's Toxicology. The Basic Science of Poisons*, 6th edn. New York: McGraw-Hill.
- Hayes AW, ed. (2001). *Principles and Methods of Toxicology*, 4th edn. Philadelphia: Taylor and Francis.