# COURSE GUIDE

**EMT 407**
**PRINCIPLES OF TOXICOLOGY**

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## MODULE 1



## UNIT 1 HISTORY OF TOXICOLOGY

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### 1.0 INTRODUCTION

Toxicology has been defined as the study of the adverse effects of xenobiotics and thus is a borrowing science that has evolved from ancient poisoners. Modern toxicology goes beyond the study of the adverse effects of exogenous agents to the study of molecular biology, using toxicants as tools. Currently, many toxicologists are studying the mechanisms of endogenous compounds such as oxygen radicals and other reactive intermediates generated from xenobiotics and endobiotics. Historically, toxicology formed the basis of therapeutics and experimental medicine. Toxicology in this and last century (1900 to the present) continues to develop and expand by assimilating knowledge and techniques from most branches of biology, chemistry, mathematics, and physics. A recent addition to the field of toxicology (1975 to the present) is the application of this discipline to safety evaluation and risk assessment.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- define toxicology
- mention the major historical landmarks of toxicology
- understand the evolution of toxicology.

## 3.0 MAIN CONTENT

### 3.1 Antiquity

Toxicology dates back to the earliest humans, who used animal venom and plant extracts for hunting, warfare, and assassination. The knowledge of these poisons must have predated recorded history. It is safe to assume that prehistoric humans categorized some plants as harmful and others as safe. The same is probably true for the classification of snakes and other animals. The Ebers papyrus (circa 1500 BC) contains information pertaining to many recognized poisons, including hemlock (the state poison of the Greeks), aconite (a Chinese arrow poison), opium (used as both a poison and an antidote), andmetals such as lead, copper, and antimony. There is also an indication that plants containing substances similar to digitalis and belladonna alkaloids were known. Hippocrates (circa 400 BC) added a number of poisons and clinical toxicology principles pertaining to bioavailability in therapy and overdosage. In the literature of ancient Greece, there are several references to poisons and their use. Some interpretations of Homer have Odysseus obtaining poisons for his arrows (Homer, circa 600 BC). Theophrastus (370–286 BC), a student of Aristotle, included numerous references to poisonous plants in *De Historia Plantarum*. Dioscorides, a Greek physician in the court of the Roman emperor Nero, made the first attempt to classify poisons, which was accompanied by descriptions and drawings. His classification into plant, animal, and mineral poisons not only remained a standard for 16 centuries but is still a convenient classification (Gunther, 1934).

Dioscorides also dabbled in therapy, recognizing the use of emetics in poisoning and the use of caustic agents and cupping glasses in snakebite. Poisoning with plant and animal toxins was quite common. Perhaps the best-known recipient of poison used as a state method of execution was Socrates (470– 399 BC), whose cup of hemlock extract was apparently estimated to be the proper dose. Expeditious suicide on a voluntary basis also made use of toxicologic knowledge. Demosthenes (385–322 BC), who took poison hidden in his pen, was one of many examples. The mode of suicide calling for one to fall on his sword, although manly and noble, carried little appeal and less significance for the women of the day.

Cleopatra's (69–30 BC) knowledge of natural primitive toxicology permitted her to use the more genteel method of falling on her asp. The Romans too made considerable use of poisons in politics.

## 3.2 Middle Ages

Before the Renaissance, the writings of Maimonides (Moses ben Maimon, AD 1135–1204) included a treatise on the treatment of poisonings from insects, snakes, and mad dogs (Poisons and Their Antidotes, 1198). Maimonides, like Hippocrates before him, wrote on the subject of bioavailability, noting that milk, butter, and cream could delay intestinal absorption. Malmonides also refuted many of the popular remedies of the day and stated his doubts about others. It is rumored that alchemists of this period (circa AD 1200), in search of the universal antidote, learned to distill fermented products and made a 60% ethanol beverage that had many interesting powers. In the early Renaissance, the Italians, with characteristic pragmatism, brought the art of poisoning to its zenith. The poisoner became an integral part of the political scene. The records of the city councils of Florence, particularly those of the infamous Council of Ten of Venice, contain ample testimony about the political use of poisons. Victims were named, prices set, and contracts recorded; when the deed was accomplished, payment was made. An infamous figure of the time was a lady named Toffana who peddled specially prepared arsenic-containing cosmetics (*Agua Toffana*). Accompanying the product were appropriate instructions for its use. Toffana was succeeded by an imitator with organizational genius, Hieronyma Spara, who provided a new fillip by directing her activities toward specific marital and monetary objectives. A local club was formed of young, wealthy, married women, which soon became a club of eligible young wealthy widows, reminiscent of the matronly conspiracy of Rome centuries earlier. Incidentally, arseniccontaining cosmetics were reported to be responsible for deaths well into the twentieth century (Kallett and Schlink, 1933). Among the prominent families engaged in poisoning, the Borgias were the most notorious.

However, many deaths that were attributed to poisoning are now recognized as having resulted from infectious diseases such as malaria. It appears true, however, that Alexander VI, his son Cesare, and Lucrezia Borgia were quite active. The deft application of poisons to men of stature in the Catholic Church swelled the holdings of the papacy, which was their prime heir. In this period Catherine de Medici exported her skills from Italy to France, where the prime targets of women were their husbands. However, unlike poisoners of an earlier period, the circle represented by Catherine and epitomized by the notorious Marchioness de Brinvillers depended on developing direct evidence to arrive at the most effective compounds for their purposes. Under the guise of

delivering provender to the sick and the poor, Catherine tested toxic concoctions, carefully noting the rapidity of the toxic response (onset of action), the effectiveness of the compound (potency), the degree ofresponse of the parts of the body (specificity, site of action), and the complaints of the victim (clinical signs and symptoms). The culmination of the practice in France is represented by the commercialization of the service by Catherine Deshayes, who earned the title "La Voisine." Her business was dissolved by her execution. Her trial was one of the most famous of those held by the Chambre Ardente, a special judicial commission established by Louis XIV to try such cases without regard to age, sex, or national origin. La Voisine was convicted of many poisonings, with over 2000 infants among her victims.

## 3.3 Age of Enlightenment

A significant figure in the history of science and medicine in the late Middle Ages was the renaissance man Philippus Aureolus Theophrastus Bombastus von Hohenheim-Paracelsus (1493–1541). Between the time of Aristotle and the age of Paracelsus, there was little substantial change in the biomedical sciences. In the sixteenth century, the revolt against the authority of the Catholic Church was accompanied by a parallel attack on the godlike authority exercised by the followers of Hippocrates and Galen. Paracelsus personally and professionally embodied the qualities that forced numerous changes in this period. He and his age were pivotal, standing between the philosophy and magic of classical antiquity and the philosophy and science willed to us by figures of the seventeenth and eighteenth centuries. Clearly, one can identify in Paracelsus's approach, point of view, and breadth of interest numerous similarities to the discipline that is now called toxicology. Paracelsus, a physician-alchemist and the son of a physician, formulated many revolutionary views that remain an integral part of the structure of toxicology, pharmacology, and therapeutics today (Pagel, 1958). He promoted a focus on the "toxicon," the primary toxic agent, as a chemical entity, as opposed to the Grecian concept of the mixture or blend. A view initiated by Paracelsus that became a lasting contribution held as corollaries that (1) experimentation is essential in the examination of responses to chemicals, (2) one should make a distinction between the therapeutic and toxic properties of chemicals, (3) these properties are sometimes but not always indistinguishable except by dose, and (4) one can ascertain a degree of specificity of chemicals and their therapeutic or toxic effects. These principles led Paracelsus to introduce mercury as the drug of choice for the treatment of syphilis, a practice that survived 300 years but led to his famous trial. This viewpoint presaged the "magic bullet" (arsphenamine) of Paul Ehrlich and the introduction of the therapeutic index. Further, in a very real sense, this was the first sound articulation of the dose–response relation, a bulwark of toxicology (Pachter, 1961). The tradition of the

poisoners spread throughout Europe, and their deeds played a major role in the distribution of political power throughout the Middle Ages. Pharmacology as it is known today had its beginnings during the Middle Ages and early Renaissance. Concurrently, the study of the toxicity and the dose–response relationship of therapeutic agents was commencing. The occupational hazards associated with metalworking were recognized during the fifteenth century. Early publications by Ellenbog (circa 1480) warned of the toxicity of the mercury and lead exposures involved in goldsmithing. Agricola published a short treatise on mining diseases in 1556. However, the major work on the subject, *On the Miners' Sickness and Other Diseases of Miners* (1567), was published by Paracelsus. This treatise addressed the etiology of miners' disease, along with treatment and prevention strategies.

The 1800s witnessed the development of forensic toxicology as a scientific discipline. In 1814, Mathieiv J. B. Orfila (1787–1853), the "father of toxicology," published Traite des Poisons, ´ the first systematic approach to the study of the chemical and physiological nature of poisons (Gettler, 1977). Orfila's role as an expert witness in many famous murder trails, particularly his application of the Marsh test for arsenic in the trial of the poisoner Marie Lafarge, aroused both popular and scholarly interest in the new science. As dean of the medical faculty at the University of Paris, Orfila trained numerous students in forensic toxicology.

## 3.4 Modern Toxicology

Toxicology has evolved rapidly during the 1900s. The exponential growth of the discipline can be traced to the World War II era with its marked increase in the production of drugs, pesticides, munitions, synthetic fibers, and industrial chemicals. The history of many sciences represents an orderly transition based on theory, hypothesis testing, and synthesis of new ideas. Toxicology, as a gathering and an applied science, has, by contrast, developed in fits and starts. Toxicology calls on almost all the basic sciences to test its hypotheses. This fact, coupled with the health and occupational regulations that have driven toxicology research since 1900, has made this discipline exceptional in the history of science. The differentiation of toxicology as an art and a science, though arbitrary, permits the presentation of historical highlights along two major lines.

## 4.0 CONCLUSION

The major historical landmarks of toxicology have been rxamined in the unit. Further, the prominent scientists or toxicologists that contributed to the eolution of toxicology were discussed.

## 5.0 SUMMARY

In this unit, we have learnt the basic definition, historical landamrks and evolution of toxicology.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. What is Toxicology?
2. Evaluate the contributions of prominent scientists to the evolution of toxicology.

## 7.0 REFERENCES/FURTHER READING

Gettler, A.D. (1977). Poisoning and toxicology, forensic aspects: Part 1: Historical aspects. Inform 9:3–7.

Gunther, R.T. (1934). The Greek Herbal of Dioscorides. New York: Oxford University Press.

Kallet, A. and Schlink, F.J. (1933). 100,000,000 Guinea Pigs: Dangers in Everyday Foods, Drugs and Cosmetics. Vanguard, New York.

Pagel, W. (1958). Paracelsus: An Introduction to Philosophical Medicine in the Era of the Renaissance. Karger, New York.

# UNIT 2 BIOCHEMISTRY CELLULAR AND MOLECULAR TOXICOLOGY

## CONTENTS



## 1.0 INTRODUCTION

Most people have had the experience of looking through a microscope at a single cell. It may have been an amoeba, alive and oozing about like a blob of jelly on the microscope slide, or a cell of bacteria, stained with a dye to make it show up more plainly. Or it may have been a beautiful cell of algae with its bright green chlorophyll. Even the simplest of these cells is capable of carrying out a thousand or more chemical reactions. These life processes fall under the heading of biochemistry, the branch of chemistry that deals with the chemical properties, composition, and biologically mediated processes of complex substances in living systems. Biochemical phenomena that occur in living organisms are extremely sophisticated. In the human body, complex metabolic processes break down a variety of food materials to simpler chemicals, yielding energy and the raw materials to build body constituents, such as muscle, blood, and brain tissue. Impressive as this may be, consider a humble microscopic cell of photosynthetic cyanobacteria only about a micrometer in size, which requires only a few simple inorganic chemicals and sunlight for its existence. This cell uses sunlight energy to convert carbon from $CO_2$, hydrogen and oxygen from $H_2O$, nitrogen from $NO^{3-}$, sulfur from $SO_4^{2-}$, and phosphorus from inorganic phosphate into all the proteins,

nucleic acids, carbohydrates, and other materials that it requires to exist and reproduce. Such a simple cell accomplishes what could not be done by human endeavors even in a vast chemical factory costing billions of dollars. Ultimately, most environmental pollutants and hazardous substances are of concern because of their effects on living organisms. The study of the adverse effects of substances on life processes requires some basic knowledge of biochemistry. Biochemical processes not only are profoundly influenced by chemical species in the environment, but they largely determine the nature of these species, their degradation, and even their syntheses, particularly in the aquatic and soil environments. The study of such phenomena forms the basis of environmental biochemistry.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- understand the phenomena of biochemical, molecular and cellular toxicology
- understand the use of cell culture techniques in toxicology
- understand the use of molecular technniques in toxicology
- understand the use of immunochemical techniques in toxicology.

## 3.0 MAIN CONTENT

### 3.1 Cell Culture Techniques

While scientists have had the ability to culture many unicellular organisms for some time, recent advances in the culture of cells from multicellular organisms have played a pivotal role in recent advances in toxicology. Cells can be isolated and either maintained in a viable state for enough time to conduct informative experiments or, in some cases, propagated in culture. The advantages of cultured cells are that they can provide living systems for the investigation of toxicity that are simplified relative to the intact organism and they can be used as replacements for whole animal toxicity testing if the toxic end point can be validated. Human cells play an important role in the extrapolation of toxic effects, discovered in experimental animals, to humans. Cultured cells, from humans or other mammals, are utilized in many of the molecular methods. There are, however, limitations in the use of cellular methods. It has not been possible to culture many cell types, and of those that have been cultured, the loss of differentiated cell function is a common problem. Extrapolation of findings to the intact animal is often problematical and the use of undefined media constituents such as serum, often essential for cell viability, may have unwanted or undefined effects on cell function and toxicant bioavailability. Studies have been carried out on cells

isolated from tissues and maintained in suspension culture or on cells that have formed monolayers.

### 3.1.1 Suspension Cell Culture

Circulating blood cells or cells easily obtained by lavage such as peritoneal and alveolar macrophages can normally survive in suspension culture when provided with a suitable nutrient medium. Cells from organized solid organs or tissues must be separated from the tissue and, if possible, separated into cell types, before being suspended in such a medium. Cell association within organs depends on protein complex formation, which in turn is $Ca^{2+}$ dependent. Consequently, dissociation media generally contain a proteolytic enzyme and the $Ca^{2+}$ chelator EDTA. There are a number of methods available to separate cell types from the mixture of dispersed cells, the commonest being centrifugation without a density gradient, wherein cells are separated by size, or centrifugation through a density gradient wherein cells are separated on the basis of their buoyant density. Cells in suspension may be maintained for a limited period of time in defined media or for longer periods in nutrient, but less well-defined, media. In either case these cultures are often used for studies of xenobiotic metabolism.

### 3.1.2 Monolayer Cell Culture

Proliferation of most cells in culture requires attachment to a substrate and occurs until limited by cell-to-cell contact, resulting in the formation of a cellular monolayer. The substrate provided for attachment is usually polystyrene modified to carry a charge. The medium for continued maintenance and growth contains salts and glucose, usually with a bicarbonate buffer. Because of the bicarbonate buffering system these cultures are maintained in a 5–10% $CO_2$ atmosphere in a temperature and humidity controlled incubator. Many cells require serum for optimal growth, inducing considerable variability into the experimental system. Since the factors provided by serum are numerous and complex, defined serum substitutes are not always successful. The factors provided by serum include proteins such as growth factors, insulin and transferrin (to provide available iron), small organic molecules such as ethanolamine, and pyruvate and inorganic ions, such as selenium.

### 3.1.3 Indicators of Toxicity in Cultured Cells

Routine observation of cultured cells is usually carried out by phase contrast microscopy, utilizing the inverted phase contrast microscope. More recently, more detailed observations have become possible utilizing fluorescent tags and inverted fluorescent microscopes. Fluorescent tags currently in use permit the assessment of oxidant status and mitochondrial

function as well as the intracellular concentration of sulfhydryl groups, $Ca^{2+}$, $H^{+}$, $Na^{+}$, and $K^{+}$. Toxicity to cultured cells may be the result either of inadequacies in the culture or the toxicity effects of the chemical being investigated. Short-term toxicity is usually evaluated by examination of end points that indicate effects on cellular organelles such as leakage of cell constituents into the medium, uptake of dyes into the cell and the formation of surface "blebs."

Longer term assessments of cell toxicity are highly dependent on the relevant toxic end point. They may include measurement of growth competence, apoptosis, and/or necrosis, incorporation of radioactive precursors into essential cellular constituents such as RNA, DNA, and protein and specialized cellular functions.

## 3.2 Molecular Techniques

Recombinant DNA techniques, including molecular cloning, have provided recent dramatic advances in many areas of both fundamental and applied biology, toxicology not excepted. Responses to toxicants may involve changes in gene expression and the new microarray techniques enable the simultaneous examination of the level of expression of many genes. The completion of the Human Genome Project will permit toxic effects in humans to be investigated and will facilitate extrapolation from experimental animals. The human genome will also provide the essential genetic background information for studies of polymorphisms in xenobiotic-metabolizing and other enzymes. Such polymorphisms have already been shown to be very important in individual sensitivity to clinical drugs and in the definition of populations and/or individuals at increased risk from particular toxicants. Chemically induced mutations, particularly in oncogenes and tumor-suppressor genes are important in chemical carcinogenesis. The ability to develop "knockout" animals lacking a particular gene and transgenic animals with an additional transgene is also proving important in toxicological studies.

### 3.2.1 Molecular Cloning

The basic principle of molecular cloning is the insertion of a DNA segment into a suitable vector. The vector is an autonomously replicating DNA molecule and the inserted DNA segment may be as large as a gene or a small as a few nucleotides. The vector containing the DNA is inserted into a cell such as yeast, where it can be replicated many times, and either the DNA or the expressed protein subsequently isolated (Fig. 1).

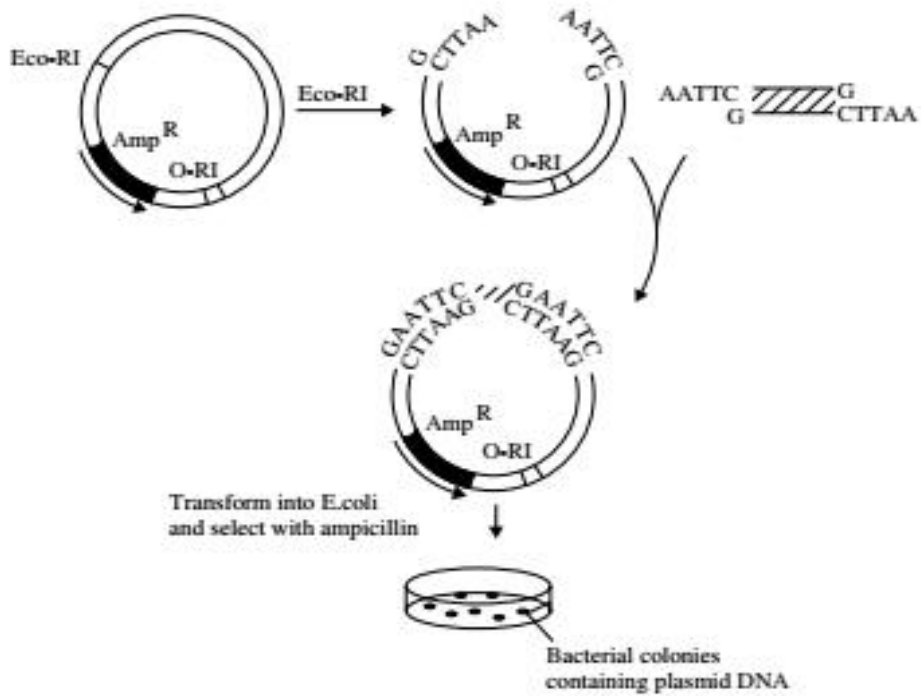


**Fig. 1:** Molecular cloning using a plasmid vector.

### 3.2.2 cDNA and Genomic Libraries

cDNA or genomic libraries are collections of DNA fragments incorporated into a recombinant vector and transformed into an appropriate host cell. In the case of cDNA libraries, the cDNAs complementary to all of the mRNAs in the tissue or cell sample are synthesized in a procedure using reverse transcriptase, before incorporation into the vector. With genomic DNA libraries the genomic DNA is digested, before cloning into the vector, with a restriction enzyme to produce an overlapping set of DNA fragments of some 12 to 20 kb.

### 3.2.3 Northern and Southern Blot Analyses

Northern analysis is usually used to identify and quantitate specific mRNAs in a sample. Southern analysis is used to determine whether or not a gene of interest is present as well as its copy number. Other uses for Southern analysis include identifying restriction fragment length polymorphisms and changes in heterozygosity. In both Southern and Northern analyses restriction-digested DNA fragments, mRNA, and polyA mRNA are separated by size when electrophoresed on agarose gel. The separated molecules are transferred, by electroblotting or capillary blotting, on to a nylon or nitrocellulose membrane. The immobilized RNA or DNA is reacted with a radiolabeled, chemiluminescent, or fluorescent probe that is complementary to the DNA/RNA of interest, unbound probe is washed off, and the membrane exposed, in the case of

radioactive probes, to radioautographic film to visualize the sample of interest.

### 3.2.4 Polymerase Chain Reaction (PCR)

PCR is a powerful technique that can, starting with amounts of DNA as small as those found in single cells, amplify the DNA until large amounts are available for many different kinds of research. Twenty to 40 cycles of can provide up to 105 times the original DNA sample. It is necessary to know as much of the sequence of the DNA of interest as possible in order to construct appropriate primers. These primers are complementary to the sequence at each end of the DNA sequence to be amplified. The DNA is incubated in a thermal cycler with thermostable DNA polymerase, all four dNTP, and the primers. The incubation temperature is raised to separate the DNA strands, lowered to permit annealing of the primers to the complementary regions of the DNA and then raised to permit the polymerase to synthesize DNA. This cycle is then repeated up to 40 times. The PCR technique has been used for many types of toxicological investigation including; uncovering polymorphisms in xenobiotic-metabolizing enzymes, isolating genes from cDNA and genomic libraries and for mutational analysis, to name only a few.

### 3.2.5 Evaluation of Gene Expression, Regulation, and Function

They include Northern analysis to determine levels of a particular mRNA, nuclear run on to determine whether an increase in mRNA is due to an increase in the rate of transcription, and promoter deletion analysis to identify specific elements in the promoter region responsible for the control of expression. Of much current interest is the use of microarrays that permit the study of the expression of hundreds to thousands of genes at the same time. Reverse transcriptase–polymerase chain reaction and RNase protection assay techniques are used to amplify and quantitate mRNAs, while the electrophoretic mobility shift assay is used to measure binding of a transcription factor to its specific DNA consensus sequence. Gene function in cultured cells can be investigated by expression of the gene product in a suitable expression system or, in vivo, by the creation of transgenic mice, either knockout mice in which the gene in question has been functionally deleted or mice into which a transgene has been introduced.

## 3.3 Immunochemical Techniques

Most of the recently developed methods for the detection, characterization, and quantitation of proteins are immunoassays based on the fact that proteins are antigens, compounds that can be recognized by an antibody. It is also true that by combining small molecules (haptens)

with a larger carrier molecule such as a protein, these methods can be extended to small molecules of interest since antibodies can be produced that recognize epitopes (specific sites on the antigen recognized by the antibody) that include the hapten. The antibodies used may be polyclonal or monoclonal, each with characteristics fitting them for use in particular immunochemical methods. Injection of a mammal with a foreign protein (immunogen) gives rise to an immune reaction that includes the generation of antibodies from B lymphocytes. Each B lymphocyte gives rise to only a single antibody type that recognizes a single epitope on the antigen. However, since these antibodies are derived from many different B lymphocytes the mixture of antibodies can recognize and bind to many different epitopes on the antigen. This mixture of antibodies can be isolated from the serum of the treated animal and is known, collectively, as polyclonal antibodies. However, if individual B lymphocytes from a treated animal can be isolated and cultured, because they are of a single clonal origin, they will produce a specific monoclonal antibody that recognizes only a single epitope on the antigen (Fig. 2). Because of the multiple sites for binding polyclonal antibodies are highly reactive. They are also relatively easy to produce. Monoclonal antibodies, although more difficult to produce, are, on the other hand, more specific. The advantages and disadvantages of each must be considered to determine which is the antibody of choice for a particular application. The most important immunochemical methods include the following:

Immunolocalization is a technique for identifying the presence of a protein within the cell, its relative abundance and its subcellular localization. After suitable preparation of the cells, they are treated with an antibody (the primary antibody) that binds to the protein of interest. An antibody that binds to the primary antibody (the secondary antibody) is then allowed to bind and form an antigen—primary antibody—secondary antibody complex. The detection system generally consists of the formation of a colored insoluble product of an enzymatic reaction, the enzyme, such as alkaline phosphatase or horseradish peroxidase, being covalently linked to the secondary antibody.

Immunoaffinity purification involves the use of antibodies, bound to an insoluble matrix, for chromatography. The advantage of this method is that it is highly specific, often permitting purification in a single step. Immunoprecipitation is a variant of immunoaffinity purification and is a means to remove a protein from a complex mixture in a highly specific manner.

Western blotting is a widely used technique in which antibodies are used to detect proteins following electrophoresis, generally SDS polyacrylamide gel electrophoresis that permits the separation of proteins

on the basis of their molecular weights (Fig. 3). Western blotting can be used to determine the presence and relative amount of a particular protein in a biological sample as well as its molecular weight.
Radioimmunoassay (RIA) is a very sensitive method used to measure minute quantities of an antigen. Since this method is most often used to measure drugs, toxicants, and other xenobiotics, the antigen used to produce the antibody is the small molecule (hapten) linked covalently to a protein. Among the techniques used in the actual measurement, the antigen capture method, in which the competition between radiolabeled antigen and the unlabeled antigen in the sample, is the most common. Depending on the design of the method *enzyme-linked immunoabsorbant assays* (ELISA) can be used to measure either antigens or antibodies in mixtures by using enzymatic-mediated detection of the corresponding immobilized immune complex. Even though this method has proved to be most useful for the rapid estimation of antibodies or antigens in complex biological mixtures, it has also been used for the quantitation of small molecules in a manner analogous to radioimmunoassays.

Inhibitory antibodies are frequently used in studies of xenobiotic metabolism, usually to estimate the contribution of particular enzymes in multienzyme mixtures. An important example is the use of antibodies to estimate the contribution of individual cytochrome P450 isoforms to the overall metabolism of a xenobiotic in microsomal preparations.

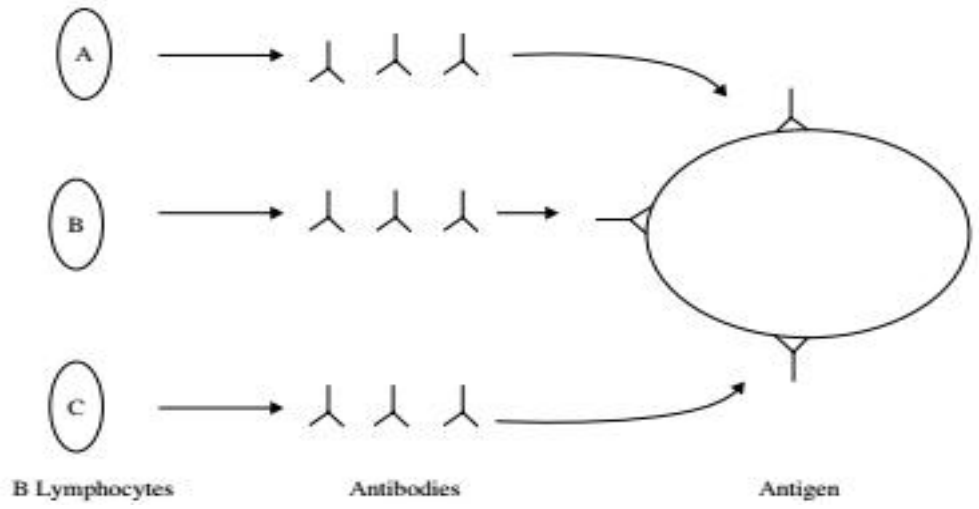


**Fig. 2:** The generation of antibodies of several clonal origins (polyclonal antibodies) with antibodies from each clonal origin (monoclonal antibodies A, B and C) recognizing a distinct epitope on the antigen.

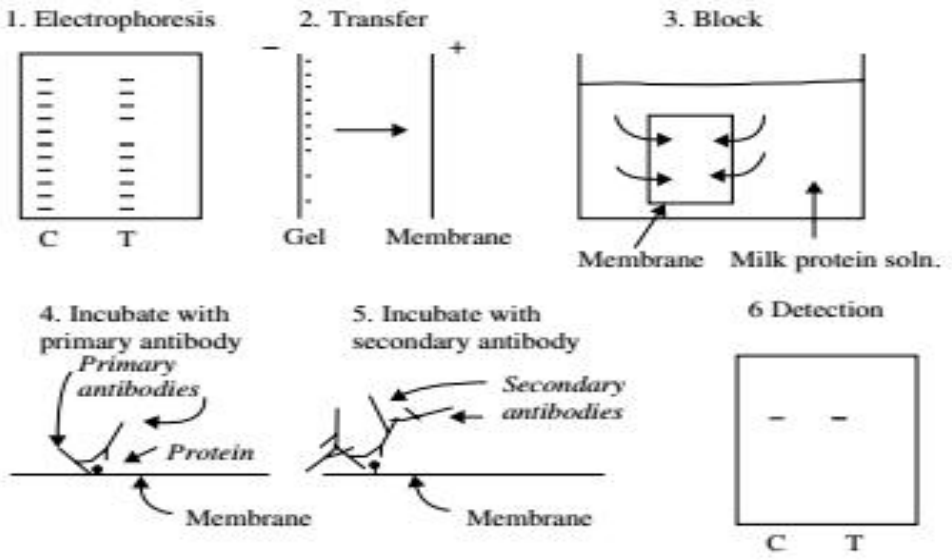


**Fig. 3:** Diagrammatic representation of the use of immunoblotting to assess relative levels of a P450 protein following treatment of rats with a PCB. C = hepatic microsomal proteins from a control, untreated rat; T = hepatic microsomal proteins from a rat treated with PCBs.

## 4.0 CONCLUSION

The various techiques involved in biochemical, cellular and molecular toxicology have been discussed in this unit. They are cell culture, molecular and immunochemical techiques.

## 5.0 SUMMARY

Ths unit has introduced the relevance of biochemistry in toxicology. In this unit, we have learnt the techniques used to assess the effects of toxicants at the biochemical, cellular and molecular levels.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. Briefly describe the importance of biochemistry to toxicology
2. Discuss succinctly the major techniques involved in biochemical, molecular and cellular toxicology.

## 7.0 REFERENCE/FURTHER READING

Hodgson, E., and R. C. Smart, eds. Introduction to Biochemical Toxicology, 3rd ed. New York: Wiley, 2001.

# UNIT 3 BIOTOXINS

## CONTENTS



## 1.0 INTRODUCTION

A toxin is a poisonous substance produced within living cells or organisms; synthetic toxicants created by artificial processes are thus excluded. The term was first used by organic chemist Ludwig Brieger (1849–1919), derived from the word toxic. Toxins can be small molecules, peptides, or proteins that are capable of causing disease on contact with or absorption by body tissues interacting with biological macromolecules such as enzymes or cellular receptors. Toxins vary greatly in their toxicity, ranging from usually minor (such as a bee sting) to almost immediately deadly (such as botulinum toxin). Toxins are often distinguished from other chemical agents by their method of production. The word toxin does not specify method of delivery. It simply means it is a biologically produced poison. Toxins are poisonous products of organisms; unlike biological agents, they are inanimate and not capable of reproducing themselves. A rather informal terminology of individual toxins relates them to the anatomical location where their effects are most notable: hemotoxin, causes destruction of red blood cells (hemolysis); phototoxin, causes dangerous photosensitivity. On a broader scale, toxins may be classified as either exotoxins, being excreted by an organism, or endotoxins, that are released mainly when bacteria are lysed.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- define biotoxin
- mention the categories of biotoxin
- describe the functions of biotoxins in animals.

## 3.0 MAIN CONTENT

### 3.1 Biotoxins

The term "biotoxin" is sometimes used to explicitly confirm the biological origin. Biotoxins can be further classified, for example, as fungal biotoxins, microbial toxins, plant biotoxins, or animal biotoxins. Toxins produced by microorganisms are important virulence determinants responsible for microbial pathogenicity and or evasion of the host immune response.

Biotoxins vary greatly in purpose and mechanism, and can be highly complex (the venom of the cone snail contains dozens of small proteins, each targeting a specific nerve channel or receptor), or relatively small protein.

Biotoxins in nature have two primary functions: (1). Predation, such as in the spider, snake, scorpion, jellyfish, and wasp. (2). Defense as in the bee, ant, termite, honey bee, wasp, and poison dart frog.

Some of the more well known types of biotoxins include cyanotoxins, produced by cyanobacteria; dinotoxins, produced by dinoflagellates; necrotoxins (they cause necrosis in the cells they encounter and destroy all types of tissue).

Necrotoxins spread through the bloodstream. In humans, skin and muscle tissues are most sensitive to necrotoxins. Organisms that possess necrotoxins include the brown recluse or "fiddle back" spider, rattlesnakes, and vipers.

Neurotoxins primarily affect the nervous systems of animals. The group neurotoxins generally consist of ion channel toxins that disrupt ion channel conductance. Organisms that possess neurotoxins include the black widow spider, most scorpions, the box jellyfish, elapid snakes, the cone snail, the blue-ringed octopus, frogs, and various different types of algae, cyanobacteria and dinoflagellates

Myotoxins are small, basic peptides found in snake and lizard venoms. They cause muscle tissue damage by a non enzymatic receptor based mechanism. Organisms that possess myotoxins include rattlesnakes and eastern bearded dragon.

Cytotoxins are toxic at the level of individual cells, either in a non-specific fashion or only in certain types of living cells: Ricin, from castor beans; Apitoxin, from honey bees; T-2 mycotoxin, from certain toxic mushrooms

### 3.2 Environmental Toxins

The term "environmental toxin" can sometimes explicitly include synthetic contaminants such as industrial pollutants and other artificially made toxic substances. As this contradicts most formal definitions of the term "toxin", it is important to confirm what the researcher means when encountering the term outside of microbiological contexts. Environmental toxins from food chains that may be dangerous to human health include Paralytic shellfish poisoning (PSP), Amnesic shellfish poisoning (ASP), Diarrheal shellfish poisoning (DSP), and Neurotoxic shellfish poisoning (NSP).

## 4.0 CONCLUSION

The categories and functions of biotoxins in living organisms have been decribed in this unit.

## 5.0 SUMMARY

In this unit, we have learnt the categories and functions of biotoxins in animals.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. What are biotoxins?
2. Mention five categories of biotoxins.
3. Describe the functions of biotoxins in two named animals.
4. Technically, toxins may not be the same as toxicants. Discuss.
5. All venoms are toxins but not all toxins are venoms. Discuss.

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# UNIT 4 CARCINOGENESIS

## CONTENTS



## 1.0 INTRODUCTION

Carcinogenesis is the process through which cancer develops. Chemical carcinogenesis is the study of the mechanisms through which chemical carcinogens induce cancer and also involves the development and utilization of experimental systems aimed at determining whether a substance is a potential human carcinogen. An important aspect of toxicology is the identification of potential human carcinogens. To begin to appreciate the complexity of this subject, it is important to first have some understanding of cancer and its etiologies.

Cancer is not a single disease but a large group of diseases, all of which can be characterized by the uncontrolled growth of an abnormal cell to produce a population of cells that have acquired the ability to multiply and invade surrounding and distant tissues. It is this invasive characteristic that imparts its lethality on the host. Epidemiology studies have revealed that the incidence of most cancers increase exponentially with age. Epidemiologists have interpreted this exponential increase in cancer incidence to denote that three to seven critical mutations or “hits” within a single cell are required for cancer development. Molecular analyses of human tumors have confirmed the accumulation of mutations in critical genes in the development of cancer. These mutations can be the result of imperfect DNA replication/repair, oxidative DNA damage, and DNA damage caused by environmental carcinogens. Most cancers are monoclonal in origin (derived from a single cell) and do not arise from a single critical mutation but from the accumulation of sequential critical

mutations in relevant target genes within a single cell. Initially a somatic mutation occurs in a critical gene, and this provides a growth advantage to the cell and results in the expansion of the mutant clone. Each additional critical mutation provides a further selective growth advantage resulting in clonal expansion of cells with mutations in multiple critical genes. It often requires decades for a cell clone to accumulate multiple critical mutations and for the progeny of this cell to clonally expand to produce a clinically detectable cancer. Thus the time required for accumulation of mutations in critical genes within a cell is likely related to the observation that cancer incidence increases exponentially with age.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- understand the concept of carcinogenesis
- mention the major causes of human cancer
- mention established human carcinogens.

## 3.0 MAIN CONTENT

### 3.1 Human Cancer

Although cancer is known to occur in many groups of animals, the primary interest and the focus of most research is in human cancer. Nevertheless, much of the mechanistic research and the hazard assessment is carried out in experimental animals. A consideration of the general aspects of human carcinogenesis follows.

#### 3.1.1 Causes, Incidence, and Mortality Rates of Human Cancer

Breast, lung, and colon and rectum cancers are the major cancers in females while prostate, lung, and colon and rectum are the major cancer sites in males. A comparison of cancer deaths versus incidence for a given site reveals that prognosis for lung cancer cases is poor while that for breast or prostate cancer cases is much better. The increase in the mortality rate associated with lung cancer in both females and males is striking and is due to cigarette smoking. It is estimated that 87% of lung cancers are due to smoking.

Lung cancer death rates in males and females began to increase in the mid-1930s and mid-1960s, respectively. These time differences are due to the fact that cigarette smoking among females did not become popular until the 1940s while smoking among males was popular in the early 1900s. Taking into account these differences along with a 20 to 25-year lag period for the cancer to develop explains the differences in the

temporal increase in lung cancer death rates in males and female. Another disturbing statistic is that lung cancer, a theoretically preventable cancer, has recently surpassed breast cancer as the cancer responsible for the greatest number of cancer deaths in women. In addition to lung cancer, smoking also plays a significant role in cancer of the mouth, esophagus, pancreas, pharynx, larynx, bladder, kidney, and uterine cervix. Overall, the age-adjusted national total cancer death rate is increasing. In 1930 the number of cancer deaths per 100,000 people was 143. In 1940, 1950, 1970, 1984, and 1992 the rate had increased to 152, 158, 163, 170, and 172, respectively. According to the American Cancer Society, when lung cancer deaths due to smoking are excluded, the total age-adjusted cancer mortality rate had actually decreased by 16% between 1950 and 1993. However, it is important to realize that death and incidence rates for some types of cancers are increasing while the rates for others are decreasing or remaining constant.

Major insights into the etiologies of cancer have been attained through epidemiological studies that relate the roles of hereditary, environmental, and cultural influences on cancer incidence as well as through laboratory studies using rodent/cellular systems. Cancer susceptibility is determined by complex interactions between age, environment, and an individual's genetic makeup. It is estimated from epidemiological studies that 35–80% of all cancers are associated with the environment in which we live and work. The geographic migration of immigrant populations and differences in cancer incidence among communities has provided a great deal of information regarding the role of the environment and specific cancer incidences. For example, Japanese immigrants and the sons of Japanese immigrants living in California begin to assume a cancer death rate similar to the California white population. These results implicate a role of the environment in the etiology of cancer. It should be noted that the term environment is not restricted to exposure to human-made chemicals in the environment but applies to all aspects of our lifestyle including smoking, diet, cultural and sexual behavior, occupation, natural and medical radiation, and exposure to substances in air, water, and soil. Only a small percentage of total cancer occurs in individuals with a hereditary mutation/hereditary cancer syndrome. However, an individual's genetic background is the "stage" in which the cancer develops and susceptibility genes have been identified in humans. For example, genetic polymorphisms in enzymes responsible for the activation of chemical carcinogens may represent a risk factor as is the case for polymorphisms in the *N*-acetyl-transferase gene and the risk of bladder cancer. These types of genetic risk factors are of low penetrance (low to moderate increased risk); however, increased risk is usually associated with environmental exposure If one considers all of the categories that pertain to human-made chemicals, it is estimated that their contribution to human cancer incidence is approximately 10%.

### 3.1.2 Known Human Carcinogens

Two of the earliest observations that exposure of humans to certain chemicals or substances is related to an increased incidence of cancer were made independently by two English physicians, John Hill in 1771 and Sir Percival Pott in 1776. Hill observed an increased incidence of nasal cancer among snuff users, while Pott observed that chimney sweeps had an increased incidence of scrotal cancer. Pott attributed this to topical exposure to soot and coal tar. It was not until nearly a century and a half later in 1915 when two Japanese scientists, K. Yamagiwa and K. J. Itchikawa, substantiated Pott's observation by demonstrating that multiple topical applications of coal tar to rabbit skin produced skin carcinomas. This experiment is important for two major reasons: (1) it was the first demonstration that a chemical or substance could produce cancer in animals, and (2) it confirmed Pott's initial observation and established a relationship between human epidemiology studies and animal carcinogenicity. Because of these important findings, Yamagiwa and Itchikawa are considered the fathers of experimental chemical carcinogenesis. In the 1930s Kennaway and coworkers isolated a single active carcinogenic chemical from coal tar and identified it as benzo[a]pyrene, a polycyclic aromatic hydrocarbon that results from the incomplete combustion of organic molecules. Benzo[a]pyrene has also been identified as one of the carcinogens in cigarette smoke. The p53 tumor suppressor gene can be mutationally inactivated by numerous carcinogens, including the carcinogenic metabolite of benzo[a]pyrene. Epidemiological studies have provided sufficient evidence that exposure to a variety of chemicals, agents, or processes are associated with human cancer. For example, the following causal associations have emerged between exposure and the development of specific cancers: vinyl chloride and hepatic cancer, amine dyes and bladder cancer, benzene and leukemia, diethylstilbestrol and clear cell carcinoma of the vagina, and cigarette smoking and lung cancer. Naturally occurring chemicals or agents such as asbestos, aflatoxin B1, betel nut, nickel, and certain arsenic compounds are also associated with an increased incidence of certain human cancers. Both epidemiological studies and rodent carcinogenicity studies are important in the identification and classification of potential human carcinogens. The strongest evidence for establishing whether exposure to a given chemical is carcinogenic in humans comes from epidemiological studies. However, these studies are complicated by the fact that it often takes 20 to 30 years after carcinogen exposure for a clinically detectable cancer to develop. This delay is problematic and can result in inaccurate historical exposure information and additional complexity due to the interference of a large number of confounding variables. This lag period can also prevent the timely identification of a putative carcinogen and result in unnecessary exposure. Therefore, methods to identify potential human carcinogens have been developed.

The long-term rodent bioassay also known as the two-year rodent carcinogenesis bioassay is currently used in an attempt to identify potential human carcinogens. It is clear that almost all human carcinogens identified to date are rodent carcinogens; however, it is not known if all rodent carcinogens are human carcinogens. Indeed, identification of possible human carcinogens based on rodent carcinogenicity can be extremely complicated.

## 3.2 Classes of Agents Associated with Carcinogenesis

Chemical agents that influence cancer development can be divided into two major categories based on whether or not they are mutagenic in in vitro mutagenicity assay. DNA-damaging agents (genotoxic) are mutagenic in in vitro mutagenicity assays and are considered to produce permanent alterations in the genetic material of the host in vivo, and epigenetic agents (nongenotoxic) are not mutagenic in in vitro assays. These agents are not believed to alter the primary sequence of DNA but are considered to alter the expression or repression of certain genes and/or to produce perturbations in signal transduction pathways that influence cellular events related to proliferation, differentiation, or apoptosis. Many epigenetic/nongenotoxic agents contribute to the clonal expansion of cells containing an altered genotype (DNA alterations) to form tumors, however in the absence of such DNA alterations these epigenetic agents have no effect on tumor formation.

## 3.3 DNA-Damaging Agents

DNA-damaging agents can be divided into four major categories. (1) Directacting carcinogens are intrinsically reactive compounds that do not require metabolic activation by cellular enzymes to covalently interact with DNA. Examples include *N*-methyl-*N*-nitrosourea and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; the alkyl alkanesulfonates such as methyl methanesulfonate; the lactones such as beta propiolactone and the nitrogen and sulfur mustards. (2) Indirect-acting carcinogens require metabolic activation by cellular enzymes to form the ultimate carcinogenic species that covalently binds to DNA. Examples include dimethylnitrosamine, benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, aflatoxin B1 and 2-acetylaminofluorene. (3) Radiation and oxidative DNA damage can occur directly or indirectly. Ionizing radiation produces DNA damage through direct ionization of DNA to produce DNA strand breaks or indirectly via the ionization of water to reactive oxygen species that damage DNA bases. Ultraviolet radiation (UVR) from the sun is responsible for approximately 1 million new cases of human basal and squamous cell skin cancer each year. Reactive oxygen species can also be produced by various chemicals and cellular process including respiration and lipid peroxidation. (4) Inorganic agents such as arsenic, chromium

and nickel are considered DNA-damaging agents although in many cases the definitive mechanism is unknown. DNA-damaging agents can produce three general types of genetic alterations: (1) gene mutations, which include point mutations involving single base pair substitutions that can result in amino acid substitutions in the encoded protein, and frame shift mutations involving the loss or gain of one or two base pairs, resulting in an altered reading frame and gross alterations in the encoded protein; (2) chromosome aberrations, including gross chromosomal rearrangement such as deletions, duplications, inversions, and translocations; and (3) aneuploidy and polyploidy, which involve the gain or loss of one or more chromosomes.

## 3.4 Epigenetic Agents

Epigenetic agents that influence carcinogenesis are not thought to alter the primary sequence of DNA, but rather they are considered to alter the expression or repression of certain genes and/or produce perturbations in signal transduction pathways that influence cellular events related to proliferation, differentiation, or apoptosis. Many epigenetic agents favor the proliferation of cells with an altered genotype (cells containing a mutated oncogene(s) and/or tumor suppressor gene(s)) and allow the clonal expansion of these altered or "initiated" cells. Epigenetic agents can be divided into four major categories: (1) hormones such as conjugated estrogens and diethylstilbestrol; (2) immunosuppressive xenobiotics such as azathioprine and cyclosporin A; (3) solidstate agents, which include plastic implants and asbestos; and (4) tumor promoters in rodent models, which include 12-*O*-tetradecanoylphorbol-13-acetate, peroxisome proliferators, TCDD and phenobarbital. In humans, diet (including caloric, fat, and protein intake), excess alcohol, and late age of pregnancy are considered to function through a promotion mechanism. While smoking and UVR have initiating activity, both are also considered to have tumor-promoting activity. By definition, tumor promoters are not classified as carcinogens since they are considered inactive in the absence of initiated cells. However, an altered genotype or an initiated cell can arise from spontaneous mutations resulting from imperfect DNA replication/repair oxidative DNA damage, or can result from environmental carcinogens. Theoretically, in the presence of a tumor promoter these mutant cells would clonally expand to form a tumor. Therefore, the nomenclature becomes somewhat a matter of semantics as to whether the tumor promoter should or should not be classified as a carcinogen. Certain hormones and immunosuppressive agents are classified as human carcinogens, although it is generally considered that these agents are not carcinogenic in the absence of initiated cells. Rather, like tumor promoters, they may only allow for the clonal expansion of cells with an altered genotype. Some nongenotoxic/epigenetic agents have been shown to induce DNA damage in vivo, for example, some

"nongenotoxic/epigenetic agents" can induce oxidative DNA damage in vivo through the direct or indirect production of reactive oxygen species. For example, certain estrogens may possess this ability and such a characteristic may contribute to their carcinogenicity. Thus, as we gain a better understanding of chemical carcinogenesis, we find that there is functional and mechanistic overlap and interaction between these two major categories of chemical carcinogens.

## 3.5 General Aspects of Chemical Carcinogenesis

A great deal of evidence has accumulated in support of the somatic mutation theory of carcinogenesis, which simply states that mutations within somatic cells are necessary for neoplasia. As stated earlier, cancer development (carcinogenesis) involves the accumulation of mutations in multiple critical genes. These mutations can be the result of imperfect DNA replication/repair, oxidative DNA damage, and/or DNA damage caused by environmental carcinogens. Many chemical carcinogens can alter DNA through covalent interaction (DNA adducts) or direct and/or indirect oxidative DNA damage. Some chemical carcinogens are intrinsically reactive and can directly covalently bind to DNA, while others require metabolic via cytochromes P450 to produce reactive electrophilic intermediates capable of covalently binding to DNA. In the 1950s Elizabeth and James Miller observed that a diverse array of chemicals could produce cancer in rodents. In an attempt to explain this, they hypothesized that many carcinogens are metabolically activated to electrophilic metabolites that are capable of interacting with nucleophilic sites in DNA. The Millers termed this the electrophilic theory of chemical carcinogenesis. From this concept of metabolic activation, the important terms parent, proximate, and ultimate carcinogen were developed. A parent carcinogen is a compound that must be metabolized in order to have carcinogenic activity; a proximate carcinogen is an intermediate metabolite requiring further metabolism and resulting in the ultimate carcinogen, which is the actual metabolite that covalently binds to the DNA. The cell has many defense systems to detoxify the carcinogenic species, including cellular antioxidants and nucleophiles as well as a whole host of phase I and phase II enzymes. In addition, reactive carcinogenic species may bind to noncritical sites in the cell, resulting in detoxification, or they can undergo spontaneous decomposition. If the carcinogenic species binds to DNA, the adducted DNA can be repaired and produce a normal cell. If there is error in the repair of the DNA or the DNA adduct is not repaired before the cell replicates, an error in the newly synthesized DNA could occur, and if so, a mutation would occur in the DNA of the daughter cell. If this change has occurred in a critical gene, for example, in a protooncogene or tumor suppressor gene, it would represent an important mutagenic event(s) in carcinogenesis. The mutationally altered cell or "initiated cell" has an altered genotype and

may remain dormant (not undergo clonal expansion) for the lifetime of the animal. However, additional mutations or "hits" in critical genes followed by clonal expansion could lead to tumor development. In addition to this mechanism, chemical carcinogenesis in experimental models can be divided into at least three stages: termed initiation, promotion, and progression; this model is thus often referred to as the initiation/promotion model of chemical carcinogenesis. The "initiated cell" may remain dormant (not undergo clonal expansion) for the lifetime of the animal. However, if the animal is repeatedly exposed to a tumor promoter, it will provide a selective growth advantage to the "initiated cell," which will clonally expand and eventually produce a benign tumor. This process is termed tumor promotion and is an epigenetic process favoring the growth of cells with an altered genotype. The development of a malignant tumor from a benign tumor encompasses a third step, termed progression and involves additional genetic changes.

## 4.0 CONCLUSION

The concept of carcinogenesis has been outlined in this unit. This unit has also shown the major causes of human cancer and known human carcinogens.

## 5.0 SUMMARY

In this unit, we have learnt the concept of carcinogenesis, the major causes of human cancer and established human carcinogens.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. Define Carcinogenesis
2. List two major causes of human cancer and justify why you you chose them.
3. State three human carcinogens.

## 7.0 REFERENCES/FURTHER READING

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# UNIT 5 TERATOGENESIS

## CONTENTS



## 1.0 INTRODUCTION

Developmental toxicity is any morphological or functional alteration caused by chemical or physical insult that interferes with normal growth, homeostasis, development, differentiation, and/or behavior. Teratology is a specialized area of embryology. It is the study of the etiology of abnormal development (the study of birth defects). Teratogens therefore are xenobiotics and other factors that cause malformations in the

developing conceptus. Examples of teratogens may include: pharmaceutical compounds, substances of abuse, hormones found in contraceptive agents, cigarette components, and heavy metals. Also included in this category are viral agents, altered metabolic states induced by stress, and nutrient deficiencies (e.g., folic acid deficiency).

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- mention the principles of teratology
- describe the embryology of mammals
- mention historical teratogens
- describe the application of teratology in Toxicology.

## 3.0 Main Content

### 3.1 Principles of Teratology

The six principles of teratology are as follows:

1. Susceptibility to teratogenesis depends on the embryo's genotype that interacts with adverse environmental factors (G × E interaction).
2. The developmental stage of exposure to the conceptus determines the outcome.
3. Teratogenic agents have specific mechanisms through which they exert their pathogenic effects.
4. The nature of the teratogenic compound or factor determines its access to the developing conceptus/tissue.
5. The four major categories of manifestations of altered development are death, malformation, growth retardation, and functional deficits.
6. The manifestations of the altered development increase with increasing dose (i.e., no effect to lethality).

When describing teratogens, one may think of three basic characteristics of teratogens:

1. A given teratogen may be organ specific.
2. It may be species specific.
3. It can be dose specific.

### 3.2 Mammalian Embryology Overview

Fig. 1 diagrams the events leading to the development of the three-layered embryo, the gastrula. The formation of the zygote marks the beginning of early embryonic development. The embryo proceeds from morula to the

blastocyst while still within the zona pellucida. The aforementioned morula will give rise to the structure that attaches the early embryo to the uterus and feeds the embryo (trophoblast). Mammalian development is characterized by the formation of the blastocele-bearing embryo, the blastula (Fig. 2). The blastula contains the mass of cells that will give rise to the actual embryo (conceptus). These cells, termed the inner cell mass (ICM), differentiate into ectoderm and endoderm prior to implantation. The ectoderm will eventually give rise to the epidermis and associated structures, the brain, and nervous system. The endoderm will give rise to glandular tissue such as the liver and pancreas and the linings of the gastrointestinal and respiratory tracts. The inner cell mass gives rise to the epiblast (develops into ectoderm) and the hypoblast (develops into endoderm). Cells of the epiblast migrate toward the midline of the early embryo. The primitive streak is active proliferation of the cells with a loss of the basement membrane separating the epiblast and endoderm. The epiblast cells migrate and intermingle with the endoderm cells. The anterior end of the streak is defined by the Hensen's node. This node (also termed the primitive node) is associated with the organization of the developing embryo. The cellular migration (involution) leads to the creation of the third germ layer (the mesoderm). Somites are derived from the mesoderm. Fig. 3 demonstrates the early stage embryo with visible somites and the succeeding embryonic to fetal stages. Somites are blocklike masses of mesoderm alongside the neural tube. They will form the vertebral column and segmental musculature. They will also develop into the excretory system, gonads, and the outer covering of internal organs. Also formed from mesoderm are mesenchymal cells. These are loose migratory cells forming the dermis (inner skin layer), bones and cartilage, and circulatory system.

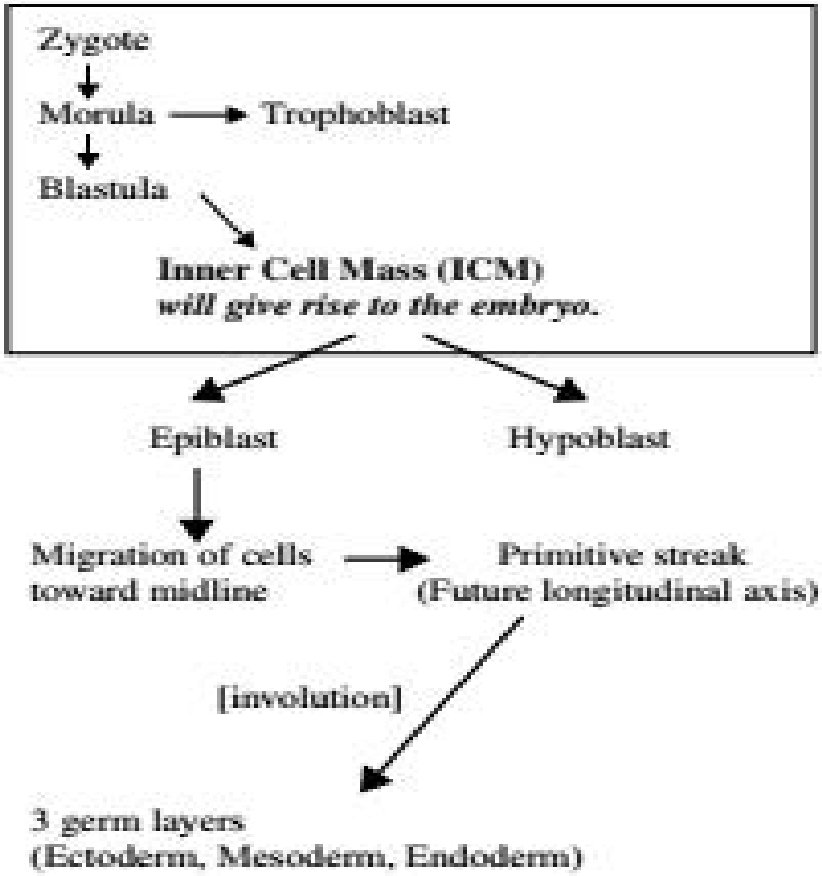



Fig. 1: Development of the zygote to a three germ cell layered embryo

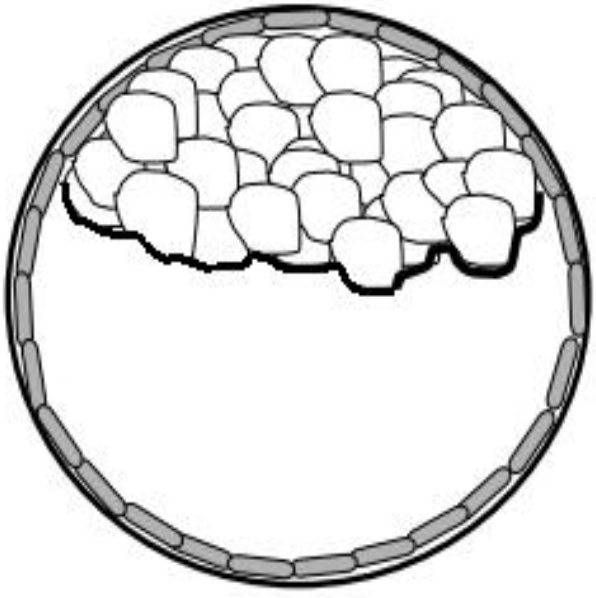
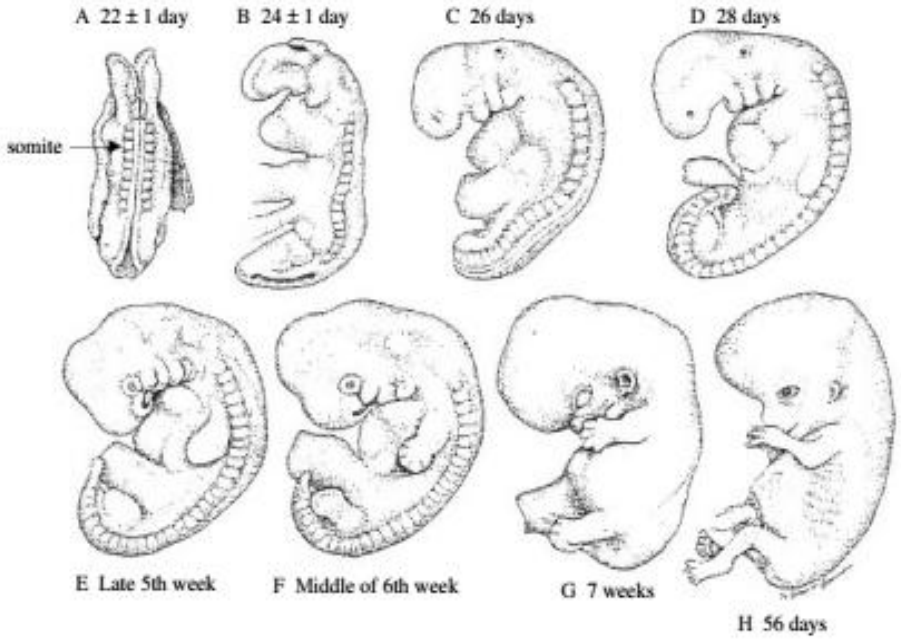


Fig. 2: Blastula containing the inner cell mass that gives rise to the embryo proper.




Somite: block-like mass of mesoderm alongside neural tube: forms vertebral column and segmental musculature

**Fig. 3:** Early stage human embryo with visible somites (represents a 22-day-old human embryo or an 8-day-old mouse embryo).

## 3.3 Historical Teratogens

### 3.3.1 Thalidomide

Thalidomide is a sedative-hypnotic drug used in Europe from 1957 to 1961. It was marketed for morning sickness, nausea, and insomnia. It went into general use and was widely prescribed in Europe, Australia, Asia, Africa, and the Americas. Women who had taken the drug from gestation days (GD) 35 to 50 gave birth to offspring suffering from a spectrum of different malformations, mainly amelia (absence of limbs) or phocomelia (severe shortening of limbs). Other malformations included: absence of the auricles with deafness, defects of the muscles of the eye and face, and malformations of the heart, bowel, uterus, and the gallbladder. The compound was withdrawn from the market in 1961 after about 10,000 cases had occurred.

### 3.3.2 Accutane (Isotetrinoin)

Accutane is a member of a family of drugs called retinoids, which are related to vitamin A. It is approved to treat serious forms of acne. These painful and disfiguring forms of acne do not respond to other acne treatments. Accutane is very effective, but its use is associated with a number of risks including birth defects. Exposure of pregnant women can

lead to birth defects such as facial malformations, heart defects, and mental retardation.

### 3.3.3 Diethylstilbestrol (DES)

DES is a synthetic estrogen that inhibits ovulation by affecting release of pituitary gonadotropins. Some of its uses include treatment for hypogonadism, primary ovarian failure, and in some cases of prostate cancer. From 1940 to 1970, DES was used to help maintain pregnancy. In utero exposure to DES has been associated with abnormal development of the uterus. It has also been associated with certain types of tumors. Women who were exposed in utero often developed vaginal neoplasia, vaginal adenosis, and cervical erosion. Effects were not seen in offspring until they reached puberty. Clear cell carcinoma of the vagina is a type of adenocarcinoma found in young women who are exposed to diethylstilbestrol in utero. The reproductive organ of males can alsobe affected subsequent to in utero exposure. The outcomes include hypotrophic testes, poor semen volume and quality.

### 3.3.4 Alcohol

#### 3.3.4.1 Fetal Alcohol Syndrome

Fetal alcohol syndrome (FAS) is a pattern of mental and physical defects that develops in some offspring when exposed to alcohol in utero. The first trimester is the most susceptible period. Some babies with alcohol-related birth defects, such as lower birth weight and body size and neurological impairments, do not have all of the classic FAS symptoms. These outcomes are often referred to as fetal alcohol effects (FAE). Currently there is not total agreement among medical scientists concerning the precise differences between FAE and FAS. In addition to growth retardation, the most common outcomes of fetal alcohol syndrome include psychomotor dysfunction and craniofacial anomalies. The observed growth deficiencies are associated with an inability of the baby to catch up due to a slower than normal rate of development. Other infrequent outcomes include skeletal malformations such as deformed ribs and sternum, scoliosis, malformed digits, and microcephaly. Distinctive facial anomalies have been associated with a diagnosis of fetal alcohol syndrome: small eye openings, epicanthal folds, failure of eyes to move in the same direction, short upturned nose, flat or absent groove between nose and upper lip, and thin upper lip. Visceral deformities may also be present: heart defects, genital malformations, kidney, and urinary defects. A common concurrent manifestation of FAS include central nervous system defects. These include irregular arrangement of neurons and connective tissue. Mental retardation may also be present and

associated with learning disabilities as well as difficulties in controlling body coordination.

### 3.3.5 ‘‘Non Chemical’’ Teratogens

Teratogens are not only xenobiotics. There may be other factors having the ability to cause malformations in the developing conceptus. Restraint stress in mice (12-hour restraint during early period of organogenesis) elicits axial skeletal defects (primarily supernumerary ribs). The Rubella virus (first reported in 1941, Austria) is associated with a number of fetal outcomes depending on the stage of development that the exposure occurs. Exposure during the first and second month of pregnancy was associated with heart and eye defects. Exposure during the third month was associated with hearing defects (and mental retardation in some cases).

## 3.4 Application of Teratology in Toxicology

### 3.4.1 Guidelines for Reproduction Studies for Safety Evaluation of Drugs for Human Use

#### 3.4.1.1 Multigenerational Study

This approach involves the continuous exposure of a rodent species (usually mice). The parental animals are exposed shortly after weaning (30–40 days of age). At reproductive maturity, the animals are mated. The first generation is produced (F1). From these an F2 is produced and then subsequently an F3 generation. The effects of the test is monitored through each generation. The measured parameters include fertility, litter size, and neonatal viability. This is a time-consuming effort that usually takes about two years to complete.

#### 3.4.1.2 Single-Generation Studies

Single-generation studies are short-term studies conducted in three segments:

##### 3.4.1.2.1 Segment I: Evaluation of Fertility and Reproductive Performance

Male rodents are treated for 70 days (to expose for one spermatogenic cycle), and nonpregnant females for 14 days (to exposure for several estrous cycles). Treatment is continued in the females during mating, pregnancy, and lactation. Fifty percent of the females are killed and the fetuses are examined for presence of malformations. The other 50% are

allowed to give birth. After weaning, these offspring are killed and necropsied.

#### 3.4.1.2.2 Segment II: Assessment of Developmental Toxicity

This involves the treatment of pregnant females only during the period covering implantation through organogenesis (typically from gestational days 6 to 15 in mice with 18-day gestational periods). One day prior to birth, the animals are killed and fetuses examined for viability, body weight, and presence of malformation.

#### 3.4.1.2.3 Segment II: Postnatal Evaluation

Pregnant animals are treated from the last trimester of pregnancy until weaning. Evaluated are parturition process, late fetal development, neonatal survival, and growth as well as presence of any malformations.

### 3.4.2 Technical Requirements for Registration of Pharmaceuticals for Human Use

As previously mentioned, new streamlined testing protocols with international acceptance have been developed. Below is a description of these guidelines as it relates to similarity to a particular segment-type study**.**

#### 3.4.2.1 Fertility Assessment

This study duration is typically shorter than segment I studies. Males are exposed for four weeks before mating and females two weeks before mating. Male reproductive organs are carefully evaluated: organ weights, histological analysis and sperm count, and mobility evaluation. For the females, fertility, litter size, and viability of conceptus are evaluated.

#### 3.4.2.2 Postnatal Evaluation and Pregnancy State Susceptibility

This study protocol is similar to the segment III study. Maternal toxicity is evaluated by comparing the degree of toxicity of the nonpregnant female to that of the pregnant female. Postnatal viability and growth are also evaluated. Offspring are also evaluated to assess functional development (i.e., presence of behavioral and reproductive deficits).

#### 3.4.2.3 Assessment of Developmental Toxicity

This is almost identical to the segment II study protocol. Pregnant animals are exposed from implantation through organogenesis. The parameters measured in the segment II study are similar. However, the study is

usually conducted using at least two species. More specifically, at least one rodent and one nonrodent species.

### 3.5 Alternative Test Methods

A number of alternative test methods have been developed to reduce the number of whole animals used in studies and/or to obtain more rapid information concerning the potential of a compound to be a reproductive/developmental toxicant. Validation of many of the methods has been problematic, since they do not address the contribution of maternal factors or multiorgan contributions to outcomes. Some of these alternative methods include the use of cell or embryo culture. For example, the micromass culture involves the use of limb bud cells from rat embryos grown in micromass culture for five days. The processes of differentiation and cell proliferation are assessed. In the Chernoff/Kavlock Assay, pregnant rodents are exposed during organogenesis and allowed to deliver. Postnatal growth, viability, and gross morphology of litters are recorded (detailed skeletal evaluations are not performed). Other alternative tests involve the use of nontraditional test species such as Xenopus embryos and Hydra. Xenopus embryos are exposed for 96 hours and then evaluated for morphological defects, viability, and growth. The cells of Hydra aggregate to form artificial embryos. The dose response in these "embryos" is compared to that of the adult Hydra.

## 4.0 CONCLUSION

Understanding the mechanisms of the induction of birth defects is pertinent to determine how to prevent these effects. Further, increasing the accuracy of experimental animal extrapolation will aid in the interpretation of experimental data in order to more accurately determine the risk of a given compound to elicit birth defects in humans.

## 5.0 SUMMARY

In this unit we have learnt the six principles of teratology. This unit has shown that historical teratogens include thalidomide, accutane, diethylstilbestrol and alcohol. We have also learnt the application of teratology in toxicology.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. State the principles of teratology.
2. Alcohol is a historical teratogen. Discuss.
3. Describe the application of teratology in toxicology.

## 7.0 REFERENCES/FURTHER READING

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**MODULE 2**



## UNIT 1 MUTAGENESIS

**CONTENTS**



### 1.0 INTRODUCTION

Mutagens are chemical and physical agents that are capable of producing a mutation. Mutagens include agents such as radiation, chemotherapeutic agents, and many carcinogens. A mutation is a permanent alteration in the genetic information (DNA) of the cell. DNA-damaging agents/mutagens can produce (1) point mutations involving single base pair substitutions that can result in amino acid substitutions in the encoded protein and frame-shift mutations involving the loss or gain of one or two base pairs, resulting in an altered reading frame and gross alterations in the encoded protein, (2) chromosome aberrations including gross chromosomal rearrangement such as deletions, duplications, inversions, and translocations, and (3) aneuploidy and polyploidy, which involve the gain or loss of one or more chromosomes. Point mutations are classified as missense or nonsense mutations.

A missense mutation produces an altered protein in which an incorrect amino acid has been substituted for the correct amino acid. A nonsense mutation is an alteration that produces a stop codon and results in a truncated protein. A point mutation can also be characterized based on the mutageninduced substitution of one base for another within the DNA. When a point mutation produces a substitution of a purine for another purine (i.e., guanine for adenine) or a pyrimidine for another pyrimidine

(i.e., thymine for cytosine), the mutation is referred as a transition. If a purine is substituted for a pyrimidine, and vice versa (i.e., thymine for adenine or guanine for cytosine), the mutation is referred to as a transversion.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- understand the usefulness of mutagenicity assays in identification of carcinogens
- understand the limitations of mutagenicity assays in identification of carcinogens.

## 3.0 MAIN CONTENT

### 3.1 Usefulness and Limitations of Mutagenicity Assays for the Identification of Carcinogens

The two-year rodent carcinogenesis bioassay is considered the "gold standard" and is utilized to determine whether a test compound has carcinogenic potential. Identification and classification of potential human carcinogens through the two-year rodent carcinogenesis bioassay is complicated by species differences, use of high doses (MTD, maximum tolerated dose), the short life span of the rodents, sample size, and the need to extrapolate from high to low doses for human risk assessment. In addition, the two-year rodent bioassay to is costly to conduct (>2 million dollars) and takes two to four years before complete results can be obtained. Since many carcinogens are mutagens, short-term test systems to evaluate the mutagenicity or genetic toxicity of compounds were developed with the idea that these tests could be used to quickly and inexpensively detect/identify chemical carcinogens. Short-term genotoxicity/mutagenicity assays were developed in a variety of organisms including bacteria, yeast, Drosophila, and human and rodent cells. These mutagenic assays or short-term genotoxicity tests directly or indirectly measure point mutations, frame-shift mutations, chromosomal damage, DNA damage and repair, and cell transformation. In the 1970s it was reported that mutagenicity could predict rodent carcinogenicity 90% of the time. However, after extensive evaluation, it is now considered that mutagenicity can predict rodent carcinogenicity approximately 60% of the time.

For certain classes of carcinogens such as the polycyclic aromatic hydrocarbons, short-term mutagenicity tests are generally highly accurate at predicting rodent carcinogenicity. For other classes of carcinogens such as the halogenated hydrocarbons, short-term genotoxicity tests often fail

to detect these rodent carcinogens. Many of these halogenated hydrocarbons probably function through an epigenetic mechanism/tumor promoting mechanism. In an important study published in 1987 by Tennant et al. (Science 236, pp. 933–941) 73 chemicals previously tested in the rodent two-year carcinogenesis bioassay were examined in four widely used short-term tests for genetic toxicity. The short-term assays measured mutagenesis in the Salmonella assay (Ames Assay) and mouse lymphoma assay, and chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells. The concordance (% agreement between short-term genotoxicity test and rodent bioassay results) of each assay with the rodent bioassay data was approximately 60%. Within the limits of the study there was no evidence of complementarity among the four tests, and no battery of tests constructed from these assays improved substantially on the overall performance of the Salmonella assay.

When interpreting the results of short-term test for genetic toxicity assays, it is important to consider (1) the structure and physical properties of the test compound, (2) the 60% concordance between the short-term test for genetic toxicity and rodent carcinogenicity, (3) epigenetic versus genetic mechanisms of carcinogenesis, and (4) the existence noncarcinogenic mutagens. It is also important to keep in mind that there is accumulating evidence that some compounds that are negative in short-term tests for mutagenicity can induce oxidative DNA damage in vivo through the direct or indirect production of reactive oxygen species. These compounds are in vivo mutagens but are negative in the short-term test of genetic toxicity.

## 4.0 CONCLUSION

The importance of mutagenicity assays in identifying carcinogenic compounds and their limitations have been discussed in this unit.

## 5.0 SUMMARY

This unit has shown that the two-year rodent carcinogenesis bioassay has been established as the standard for determining the carcinogenic poetential of a compound. However, there are limitations to the use mutagenic assays in the identification of carcinogenic compounds as discussed in this unit.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. Define Mutagenesis
2. Mutagenicity assays can be used to determine the carcinogenicity of a compound. Discuss the statement by highlighting the limitations to the use of assays.

## 7.0 REFERENCE/FURTHER READING

Smart, R. C. and Akunda, J. K. (2001). Carcinogenesis. *An Introduction to Biochemical Toxicology*, Wiley, New York.

# UNIT 2 BIOTRANSFORMATION OF TOXICANTS

## CONTENTS



## 1.0 INTRODUCTION

The enzymes that catalyze xenobiotic biotransformation are often called drug metabolizing enzymes. The acronym ADME stands for absorption, distribution, metabolism, and elimination. This acronym is used widely in the pharmaceutical industry to describe the four main processes governing drug disposition. One of the most important determinants of xenobiotic persistence in the body and subsequent toxicity to the organism is the extent to which they can be metabolized and excreted. Several families of metabolic enzymes, often with wide arrays of substrate specificity, are involved in xenobiotic metabolism. Some of the more important families of enzymes involved in xenobiotic metabolism include the cytochrome P450 monooxygenases (CYPs), flavincontaining monooxygenases (FMOs), alcohol and aldehyde dehydrogenases, amine oxidases, cyclooxygenases, reductases, hydrolases, and a variety of conjugating enzymes such as glucuronidases, sulfotransferases, methyltransferases, glutathione transferases, and acetyl transferases. Most xenobiotic metabolism occurs in the liver, an organ devoted to the synthesis of many important biologically functional proteins and thus with the capacity to

mediate chemical transformations of xenobiotics. Most xenobiotics that enter the body are lipophilic, a property that enables them to bind to lipid membranes and be transported by lipoproteins in the blood. After entrance into the liver, as well as in other organs, xenobiotics may undergo one or two phases of metabolism. In phase I a polar reactive group is introduced into the molecule rendering it a suitable substrate for phase II enzymes. Enzymes typically involved in phase I metabolism include the CYPs, FMOs, and hydrolases. Following the addition of a polar group, conjugating enzymes typically add much bulkier substituents, such as sugars, sulfates, or amino acids that result in a substantially increased water solubility of the xenobiotic, making it easily excreted. Although this process is generally a detoxication sequence, reactive intermediates may be formed that are much more toxic than the parent compound. It is, however, usually a sequence that increases water solubility and hence decreases the biological half-life (t0.5) of the xenobiotic in vivo. Phase I monooxygenations are more likely to form reactive intermediates than phase II metabolism because the products are usually potent electrophiles capable of reacting with nucleophilic substituents on macromolecules, unless detoxified by some subsequent reaction.

## 2.0 OBJECTIVE

By the end of this unit, you should be able to:

- describe the reactions involved in the biotransformation of toxicants.

## 3.0 MAIN CONTENT

### 3.1 Reductive Metabolism

A more satisfying classification system proposed by David Josephy, Fred Guengerich and John Miners sorts the chemical alterations sustained by foreign substances within the body into four groups instead of two as per the Williams model (Fig. 1). Fidelity to the actual chemical modification occurring during the enzymatic transformation is the primary concern in the Josephy system. The first class involves reductive metabolism, a type of transformation that involves the addition of extra hydrogen atoms to a molecule (or the removal of oxygen atoms) (Fig. 1a). A famous example is Prontosil which undergoes reductive metabolism to form sulfanilamide (Fig. 1a), the drug that when mixed with diethylene glycol triggered the 1930s nephrotoxicity epidemic in the USA. Reductive metabolism is not especially common in the big scheme of things, but it can inadvertently convert some important chemicals into toxic, DNA-damaging species. One class of suspected cancer causing chemicals, the nitrotoluenes,

undergo just this type of reductive metabolism during their conversion to harmful metabolites.

## 3.2 Oxidative Metabolism

A second and critically important – class of metabolic transformations are the oxidative reactions. These molecular transformations are familiar to organic chemists and are analogous to the alterations one produces in a test tube by treating organic compounds with an oxidising agent such as potassium permanganate or osmium tetroxide. In the body, such oxidative reactions are enzyme catalysed and typically proceed by adding oxygen to a foreign compound, as when a hydroxyl group is inserted in benzene to form phenol (Fig. 1b). Alternatively, oxidation can proceed by removing hydrogen atoms from a molecule, such as when ethanol from alcoholic beverages is converted to acetaldehyde by alcohol dehydrogenase (Fig. 1b). The most important catalysts of xenobiotic oxidations in the body are the cytochrome P450 (commonly abbreviated CYP or simply P450) enzymes, arguably the most highly researched family of proteins known to biology.

## 3.3 Conjugative Metabolism

Next to oxidative reactions, in terms of the sheer numbers of xenobiotics that undergo these reactions, the conjugative reactions are of high importance to toxicology (Fig. 1c). A defining characteristic is the formation of chemical bonds between foreign chemicals and hydrophilic substances already present in the liver, thereby forming a diverse class of metabolites known as conjugates. The first reaction of this kind was discovered in 1824 by the German researcher Friedrich Wohler, who administered benzoic acid to dogs and recovered hippuric acid from their urine (conjugate with glycine). Many foreign chemicals are directly metabolised by conjugative pathways, while others require oxidative metabolism before entering these pathways. Note that most of the conjugative pathways are not exclusively involved in the metabolism of foreign substances, since they also metabolise many endobiotics. In many cases, inherited genetic defects in these genes have been associated with clinical syndromes of varying degrees of severity, most of which manifest as alterations in the metabolism of key endogenous substrates.

### 3.3.1 Glucuronidation

The most commonly utilised pathway of conjugative transformation in humans involves glucuronidation reactions, facilitated by a family of enzymes known as glucuronosyltransferases. As is normally the case in biochemistry, their name reveals their primary function – glucuronosyltransferases transfer a glucuronic acid group from a

'cofactor' in the liver (UDPglucuronic acid, where UDP = uridine diphosphate) onto a nucleophilic foreign chemical, forming a glucuronide conjugate. These reactions are analogous to the nucleophilic substitution reactions known to chemists. These enzyme catalysts are commonly abbreviated as UGTs, or UDPglucuronosyltransferases. Within liver cells, UGT proteins are commonly – but not exclusively – located within the endoplasmic reticulum, a lipidrich subcellular compartment which is also home to the CYP enzymes. Since the endoplasmic reticulum acts as a miniature conveyer belt to deliver lipophilic xenobiotics to the catalytic chamber of CYP proteins, this location ensures UGT enzymes are well supplied with substrates. In addition to competing with CYP for the 'first bite' at some xenobiotics, UGT proteins metabolise many products of CYPcatalysed reactions. A substrate for glucuronidation is usually lipophilic while possessing a nucleophilic oxygen, sulfur or nitrogen atom(s). Such substrates are termed aglycones. Since glucuronic acid is highly polar, tacking this sugar onto a lipophilic xenobiotic ensures the resulting conjugate is more water soluble than the parent molecule. Normally, such conjugates lack significant biological activity, thus glucuronidation usually results in pharmacological or toxicological deactivation of the parent molecule. An example of such an outcome is paracetamol (acetaminophen), since approximately 50 % of a normal adult dose (1 g) is converted to an inactive glucuronic acid conjugate (Fig. 1c). In addition to their high levels in liver, UGP proteins are highly expressed in nasal tissue where they help deactivate inhaled airborne odorant molecules. UGT proteins are also expressed in the lungs and kidneys and other tissues. The human genome encodes multiple UGT isoforms which differ in terms of their sequence, substrate specificity, tissue distribution and expression patterns throughout the human lifespan. The term isoform rather than isoenzyme is used to denote individual members of most biotransformation enzyme families: while isoenzyme denotes multiple variants of an enzyme that carries out the same basic chemical transformation (e.g. lactate dehydrogenase isoenzymes all convert lactate to pyruvate, although they differ in their kinetic properties, tissue expression, etc.). By contrast, isoforms are related enzymes which carry out broadly similar chemical transformations yet differ significantly in their amino acid sequences and specific substrate preferences. Some 20 human UGT isoforms have been cloned and expressed in model systems, allowing study of their substrate preferences and enzymological properties. Some of these are expressed exclusively within liver – the hepatic UGTs – while others exhibit broader 'extrahepatic' expression patterns (Table 1). The highly conserved genetic locus of UGT1A family members is unusual compared to other xenobiotic metabolism genes since multiple family members are spliced from a common precursor mRNA transcript. mRNA editing of this long transcript yields mRNA species for each UGT1A isoform which contain four common exons from the constant region as well as a unique exon 5 from the variable region which

confers the distinctive substrate preferences on each isoform. This efficient method of storing genetic information is unfortunately prone to inactivation by frameshift mutations that cause deleterious changes to the reading frame of multiple isoforms. UGT1A promoter mutations of this kind accompany Crigler–Najjar syndrome, a fatal congenital condition which results in severely impaired clearance of the neurotoxic endobiotic bilirubin. While glucuronide conjugates normally lack biological activity, some important exceptions exist, most famous of which is morphine, the powerful painkiller extracted from the opium poppy. Much of the analgesic effectiveness of morphine is actually due to its metabolite morphine6glucuronide. Yet another conjugated metabolite, morphine glucuronide, probably produces such unwanted effects in morphine recipients as CNS excitation and mental confusion.

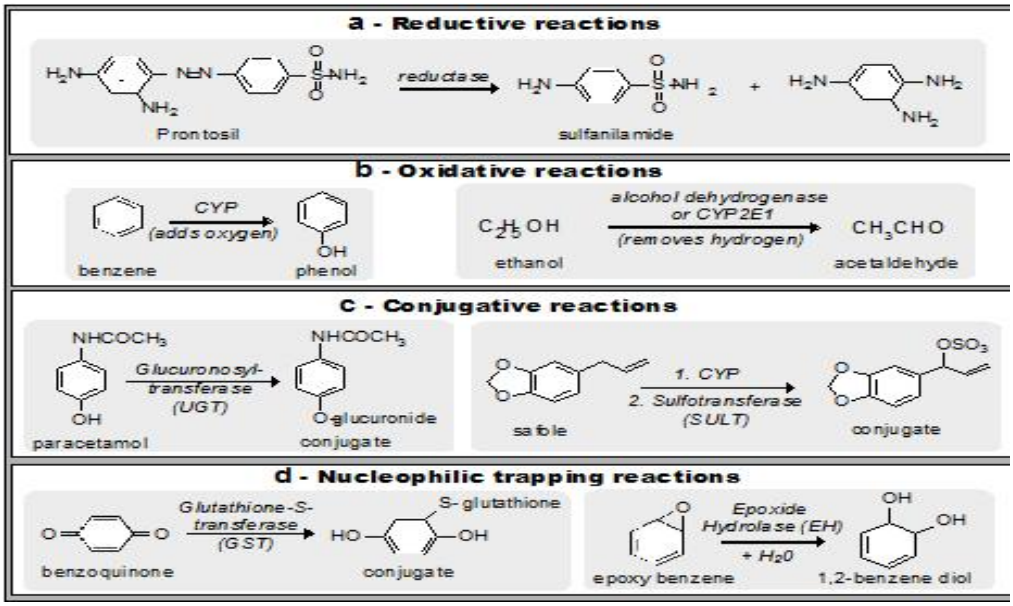


**Fig. 1:** A modern classification system developed by Josephy and colleagues recognises four broad types of metabolic fates for foreign chemicals. For the examples shown (e.g. paracetamol, benzene, safrole), the reactions depicted are only a subset of the total number of metabolites formed from the respective compounds in the body

**Table 1:** Some representative UGT enzymes within the human genome and their preferred substrates

| Isoform | Tissue | Substrates (examples) |
|---|---|---|
| UGT1A1 | Liver | Xenobiotics + endobiotics (e.g. bilirubin, oestrogens) |
| UGT1A4 | Liver | Mainly xenobiotics (e.g. aromatic amines) |
| UGT1A6 | Liver | Mainly xenobiotics (e.g. small phenolics) |
| UGT1A7 | GItract | Mostly endobiotics (e.g. steroids, bilirubin) |
| UGT2B4 | Liver + GI-tract | Xenobiotics + endobiotics |
| UGT2B17 | Liver + GI-tract | Xenobiotics + endobiotics |

### 3.3.2 Sulfonation

Sulfonation is an important class of conjugative reactions (Fig. 1c) which involve the transfer of a sulfonategroup ($-SO_3$) from a cofactor (PAPS–3′phosphoadenosine5′phosphosulfate) onto a nucleophilic xenobiotic. These reactions are often called sulfation pathways, due to the formation of a sulfate group during sulfonation of Ocontaining substrates (e.g. phenols). Yet since sulfonation also occurs on many nitrogen-containing substrates to form N-sulfonates, sulfation is an inadequate name for these reactions. These reactions are catalysed by the sulfotransferases (SULT), a diverse family of enzymes which are expressed strongly in the GI-tract, liver, kidneys, platelets and brain. Unlike many other biotransformation genes, SULT genes are strongly expressed in foetal tissues. Over 50 SULT genes are present within the human genome, which are further classified into 10 main families. SULT enzymes reside almost exclusively within the cytosolic fraction of cells, and the products of these reactions are generally very water soluble. In addition to metabolising thousands of xenobiotics, SULTs also convert important endobiotics such as dopamine and bile acids into water-soluble species. Compared to glucuronidation pathways which possess an insatiable appetite and can metabolise repeated doses of xenobiotics without experiencing saturation, sulfonation pathways have a low capacity due to limited reserves of PAPS (hepatic levels of UDP-glucuronic acid are typically much higher). Since their substrate preferences frequently overlap, UGT and SULT proteins often compete for the same xenobiotic substrate, with SULT-derived metabolites usually predominating at low xenobiotic concentrations. Sulfonation of a xenobiotic usually abolishes its biological activity, but some noteworthy exceptions exist. Safrole, a naturally occurring flavouring in nutmeg and cinnamon, causes cancer in laboratory animals

via a mechanism that involves oxidative metabolism followed by sulfonation to anoxious, DNA-damaging metabolite (Fig. 1c). Whether such reactions are relevant to human cancer is unclear. A more benign example of sulfonation generating a biologically active metabolite involves minoxidil, a heart drug which was introduced in the 1970s as a remedy for high blood pressure. Surprisingly, minoxidil caused unexpected hair growth in some patients, an effect that was due to a sulfonated metabolite.

### 3.3.3 Other Conjugative Pathways

Although glucuronidation and sulfonation are the most common routes of conjugative xenobiotic metabolism, other important pathways exist, including acetylation, methylation and conjugation with amino acids such as glycine or glutamine. While they are important to the toxicity of some select chemicals, these reactions lack the universal importance to xenobiotic metabolism as glucuronidation and sulfonation pathways.

### 3.3.4 Nucleophilic-Trapping Reactions

The final class of biotransformations under Josephy's naming system is the nucleophilic-trapping reactions (Fig. 1d). These are of great importance in toxicology since they protect the liver against chemically reactive, toxic metabolites that inadvertently form during the oxidative metabolism of some chemicals. Damaging metabolites of this kind are often unstable, electron-deficient species which attain chemical equilibrium by reacting with electron-dense centres in other molecules. Unfortunately for cells, certain amino acids in proteins, together with the nitrogen bases in DNA, contain electron-rich sites that are reactive with electrophiles. Interactions of this kind generate new covalent bonds between the metabolite and the macromolecule and the formation of a protein or DNA adduct.

### 3.3.5 Glutathione-S-Transferase

Tissues are not entirely at the mercy of electrophilic metabolites since the presence of small, electrondense scavenger molecules within cells can intercept these species before they damage proteins or DNA. The most important nucleophilic-trapping reactions involve glutathione, a small peptide comprising just three amino acids (glutamate, cysteine and glycine). Although small peptides are quickly degraded by peptidases within most cells, glutathione resists this fate since glutamate is attached to cysteine through an unusual γcarboxyl linkage (c.f. the αlinkage in conventional peptide bonds) ensuring glutathione resists proteolysis. As a result the intracellular concentrations of glutathione are high in many tissues, in the range of 5–10 mM. By contrast, glutathione levels within

extracellular fluids are typically within the low micromolar range or below (i.e. 1/1,000th or less). The possession of a strongly nucleophilic thiol group ensures glutathione reacts rapidly with many electrophiles as well as free radicals (Fig. 1d). As the thiol group readily forms disulfide bonds (–S–S–), the formation of glutathione disulfide (GSSG) accompanies cellular exposure to many prooxidants, electrophiles and reactive oxygen species. Under normal physiological conditions, glutathione reductase (GR) maintains glutathione predominantly within the reduced form (i.e. GSH, ~98 % of total cellular glutathione). The remaining ~ 2 % is present as mixed protein disulfides due to reactions with cysteine thiol groups in proteins (i.e.to form GSS-protein) as well as the disulfide form of glutathione (GSSG). In addition to direct oxidation of GSH, GSSG also forms during the enzymatic detoxication of inorganic and organic peroxides by glutathione peroxidise. During oxidative stress conditions in which GSSG formation is increased, GR helps restore the cellular thiol redox balance (i.e. GSH:GSSG) to normal values. The nucleophilic properties of the thiol group are most relevant to the cytoprotection GSH provides against electrophiles during xenobiotic biotransformation. While they usually proceed at a measurable rate in the absence of enzyme, electrophile trapping by GSH is accelerated by glutathione-S-transferases (GST), a ubiquitous enzyme family which protects cells against endogenous electrophiles and reactive intermediates formed during xenobiotic metabolism. GSTs are smaller than most xenobiotic-metabolising enzymes, exhibiting a typical monomeric mass of ~ 25 kDa, although they usually exist as cytosolic homodimers. These highly expressed proteins can comprise over 5 % of total protein in some cells, including the liver. The human genome contains at least 17 genes for cytosolic GSTs which are assigned to eight major classes, namely, A, K, M, P, S, T, Z and O. Each GST class typically contains multiple isoforms. In addition to their roles in xenobiotic detoxication, GST proteins play broad roles in the regulation of apoptosis, oxidative stress, cell proliferation, inflammatory responses, metabolic processes and the fine-tuning of many cell signalling pathways. Although most GSTs are mainly present in cytosol, some isoforms sustain posttranslational modifications that alter their subcellular distribution, as in the case of GSTA44 which undergoes phosphorylation followed by redistribution to mitochondria. Cytosolic GST isoforms that are also found in the nucleus, ER or plasma membrane are termed ‘echoproteins’. Some isoforms such as GSTA11 and GSTM11 are widely expressed in liver, lung, kidney, GItract and testis where they provide broad protection against electrophilic xenobiotics and reactive metabolites. Other isoforms such as GSTA44 mainly trap endogenous electrophiles and play key roles in diabetes, cardiovascular disease and neurological disorders such as Alzheimer’s and Parkinson’s neurodegeneration. Other isoforms such as GST P11 and T11 are upregulated in tumour cells and mediate multidrug resistance by accelerating the detoxication of cytotoxic chemotherapy

drugs. A range of polymorphisms have been identified for some GST isoforms including GST T1 and GST M1, ensuring considerable attention has been devoted to determining whether individuals with deficient GST activities are vulnerable to chemical toxicities or tumour responses. In the example of a glutathione-dependent reaction shown in Fig. 1d, the chem ically reactive benzene metabolite benzoquinone is trapped by glutathione to form a conjugate that is exported from the liver. Benzoquinone is just one of several toxic metabolites formed following the initial CYPcatalysed oxygenation of benzene to form phenol: it likely forms via subsequent oxidation of phenol by peroxidases within bone marrow. Although readily detoxicated by GSTs, glutathione-dependent protective pathways can be overwhelmed during chronic benzene exposure such as occurred among gas station attendants who manually ‘pumped’ gasoline in a bygone era. Benzene is added to automobile fuel to promote efficient combustion due to its ‘antiknock’ properties, yet workers who inhaled benzene fumes over an extended timeframe were sadly vulnerable to leukaemia, a serious form of haematopoietic cancer caused by DNA-damaging benzene metabolites. Although electrophile-trapping reactions initially generate glutathioneSconjugates, due to further metabolic processing, these species are often undetectable within the urine of animals following exposure to bioactivationprone xenobiotics. Subsequent to GST-catalysed trapping reactions, membrane transporters typically export glutathioneSconjugates out of hepatocytes into extracellular fluids (Fig. 2). The plasma membrane of many epithelial cells and especially those within renal tissues express various enzymes that further process glutathione conjugates in an effort to recycle amino acid components of the tripeptide (Fig. 2). In the first step, membrane-associated γglutamyltranspeptidase cleaves the glutamate residue to form a cysteinylglycine conjugate. The resulting product is then processed by dipeptidases such as aminopeptidaseM to release glycine, forming an Scysteine conjugate. Within kidney tissues, Sconjugates are converted to NacetylcysteineSconjugates by Nacetyl transferases (NAT). These metabolites are otherwise known as mercapturates. These metabolites are telltale signs of the formation of noxious electrophilic metabolites within the liver and can be detected in urine collected from animals and humans following exposure to many environmental pollutants, industrial chemicals and dietary constituents. Mercapturate detection during studies of the in vivo metabolism of candidate drugs during the drug discovery process is often a red flag precluding further preclinical development of the molecule(s).

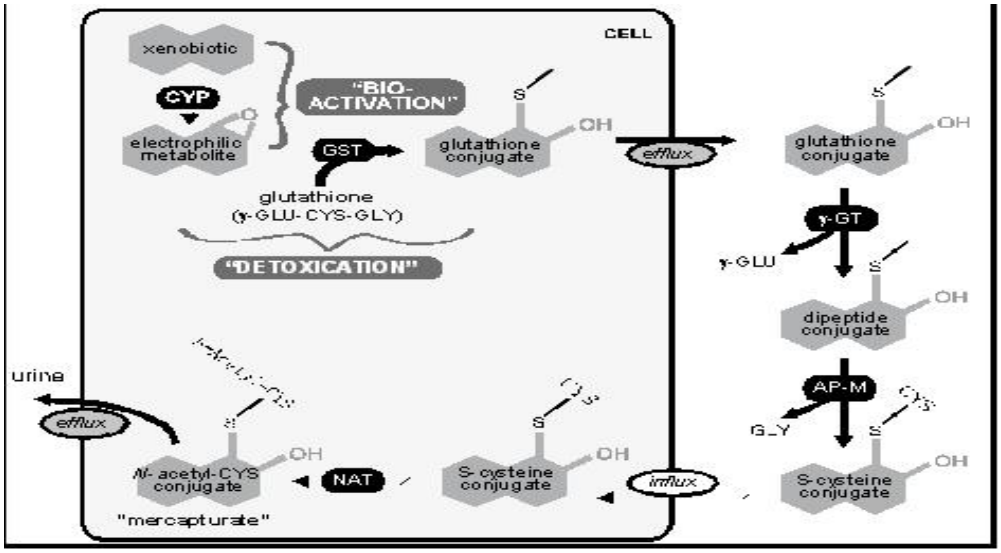


Fig. 2: Detoxication by glutathioneStransferases is a common fate for electrophilic metabolites (e.g. epoxides) which form during CYPmediated metabolism. Following export by membrane transporters, glutathioneSconjugates undergo proteolytic processing and *N*acetylation to form mercapturic acid metabolites that appear in urine (*N*acetylcysteine conjugates)

## 3.4 The Cytochrome P450 Superfamily

Most oxidative xenobiotic metabolism within the liver is catalysed by cytochrome P450 (CYP). Nicknamed ‘nature’s blowtorch’, this remarkable enzyme family converts a huge range of organic molecules into oxidised metabolites. The P450 system is very important to pharmacology since it catalyses some threequarters of all human drug biotransformations. The human genome contains 57 CYP genes, although this includes several inactive pseudogenes, species which are exclusively involved in endobiotic metabolism, and ‘orphans’ with as yet undiscovered substrates. The human liver makes a dozen or so CYP isoforms which accomplish xenobiotic metabolism, yet the workload is not shared equally among members of this subgroup since just five CYP isoforms likely account for 90 % of human drug metabolism – CYP1A2, 2C9, 2C19, 2D6 and 3A4. Yet even within this select group, the workload is unevenly distributed: CYP3A4 likely metabolises around one-half of medicinal agents in current use (Fig. 3). Yet toxicologists would insist on adding another elite CYP family member to form a Gang of Six: CYP2E1. While 2E1 plays a minor role in human drug metabolism, its contribution to the biotransformation of nontherapeutic xenobiotics is substantial.

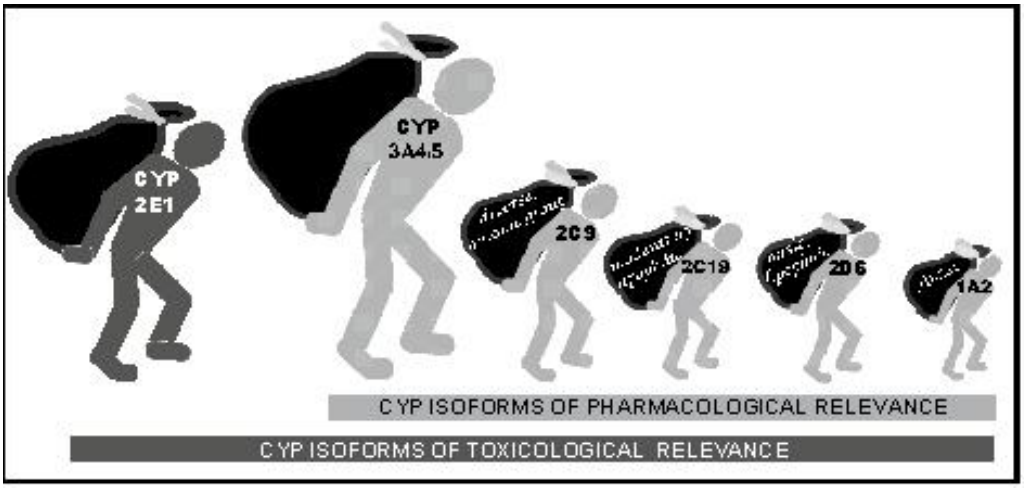


**Fig. 3:** Half a dozen CYP450 isoforms do most of the 'heavy lifting' during oxidative xenobiotic metabolism in humans, although the workload is shared unequally. CYP3A4/5 is most important during the metabolism of pharmaceuticals, while 2E1 metabolises many synthetic chemicals of relevance to toxicology.

### 3.4.1 Molecular Aspects of CYP450 Action

CYP isoforms are midsized proteins comprising approximately 500 amino acids and with a typical mass of 48–56 kDa. Members of this family are most strongly expressed in liver, but strong expression of CYP3A proteins also occurs in the gut wall. Other CYP-expressing tissues include the kidneys, lung, skin, testes and brain. The catalytic chamber represents the heart of the CYP structure since it contains the crucial heme group which is anchored to the protein near its carboxyterminus. The hydrophobic amino terminus tethers the structure within the lipid membranes of the endoplasmic reticulum. The iron atom within the heme is where the crucial redox chemistry occurs during the oxygenation of substrate molecules – after binding the substrate and molecular oxygen, the heme undergoes a rapid series of sequential redox reactions which are driven by the supply of reducing equivalents obtained from the cofactor NADPH by NADPH/cytochrome P450 reductase. Like a power plant providing electricity to an adjacent town, the reductase is an essential ancillary protein that is located in close proximity to the CYP complex, with each reductase likely supplying 'reducing equivalents' to up to 30 individual CYP proteins. The growing availability of crystal structures of CYP isoforms has provided valuable insights into the mechanistic basis for the oxidation of xenobiotics (Fig. 4). For a given xenobiotic to undergo oxidation by a CYP, it needs to be accommodated within the hydrophobic catalytic chamber, which it accesses via an access channel. The substrate must fit into the chamber in a manner allowing interactions between the molecule and critical amino acid residues which surround the active site. Several chemical forces contribute to these

interactions, including hydrogen-bonding interactions with key amino acid residues and Van der Waals interactions which are highly significant for xenobiotics containing aromatic rings. Ideally, the molecule must bind such that its most oxidation prone site is oriented towards the heme group, thereby allowing the high-valence iron complex that forms transiently during the CYP catalytic cycle – the perferryl complex (FeV = O) – to rapidly insert an oxygen atom, forming a metabolite which then diffuses out of the CYP complex. While our knowledge of 3dimensional CYP crystal structures has improved substantially, forecasting the types of oxidations sustained by substrates using docking software – techniques that have worked well in studies of more conventional enzymes has proved difficult. This is partly because the active sites of CYPs are surprisingly malleable, able to stretch and bloat to accommodate diverse molecules. Despite these ongoing challenges, structural biology has clarified the importance of the size of the active site in dictating the oxidative capabilities of individual CYP isoforms: as a rule, the larger the catalytic chamber, the greater the number of xenobiotics an individual CYP can metabolise. CYP3A4 – the isoform with the most voracious appetite for xenobiotics – has the largest active site of 1,440 cubic angstroms, compared to just 585 cubic angstroms in the fussier CYP2E1. These differ ences help explain why 2E1 prefers to metabolise small organic substrates. Most of the remaining CYPs occupy the middle ground between these extremes. The huge active site of 3A4 has further consequences for xenobiotic biotransformation – not only can it contain more than one substrate molecule simultaneously, it can also accommodate many substrates in different orientations, presenting different parts of the molecules to the all-important heme group. This capability explains why multiple oxidised metabolites can form from the same xenobiotic (e.g. oxygen atoms are inserted at multiple positions in an aromatic ring). The five CYP isoforms which metabolise human drugs are very important to the pharmaceutical industry which spends large sums each year investigating these pathways. Long before a new drug candidate is administered to humans, it will be screened for its susceptibility to oxidation by the major human CYPs. Many promising drug candidates are discarded because they are too quickly metabolised by CYPs: if hepatic metabolism is too extensive, a drug's effectiveness is diminished since a high proportion of an orally administered dose is destroyed in the liver before it accesses a remote tissue to elicit its therapeutic effect. Second, a rapidly metabo lised drug might need to be taken 3 or 4 times a day to ensure a consistent therapeutic response. The need for frequent dosing could lead to patient noncompliance but also might cause the drug to fail in the marketplace where it competes against rival drugs which are metabolised more slowly and need be taken just once or twice a day. While CYP3A enzymes can effectively metabolise many pharmaceuticals, CYP2E1 seems oddly intended to metabolise the sort of industrial substances that interest modern toxicologists, such as

benzene, acetone, styrene or vinyl chloride. Given the prevalence of alcohol consumption across human cultures, the most important CYP2E1 substrate is ethanol, which CYP2E1 readily converts to acetaldehyde (Fig. 1b).

### 3.4.2 Inhibition of CYP Pathways

Knowing which individual CYP isoform metabolises a drug of interest can help predict drug interactions within the body: if two co-administered drugs compete for the same hepatic CYP isoform, they may disrupt each other's removal from the body, leading to life-threatening outcomes. Many clinically significant 'DDIs' (drug–drug interactions) are directly attributable to interactions at the level of hepatic CYP isoforms. In the normal situation in which individuals are exposed to a single CYP substrate, molecules have unhindered access to the active site of a CYP (Fig. 5a). CYP inhibitory scenarios typically involve two main mecha nisms, the most common of which involves two xenobiotics competing for the same active site of a CYP isoform (Fig. 5). The molecule with the strongest affinity acts as a competitive inhibitor, blocking access by the other molecule (substrate) and preventing the oxidation chemistry from occurring (Fig. 5b). Competitive inhibitors and substrates often share structural similarities that facilitate competi tion for the same CYP isoform. Noncompetitive inhibitors, by contrast, are less likely to possess structural similarities and involve the inhibitor binding to an allo steric or modulatory site on the CYP molecule that is structurally and spatially dis tinct from the active site accessed by the substrate (Fig. 5c). While physical competition does not occur, these binding events change the structure of the CYP and disrupt the geometry of the active site, thereby diminishing its affinity for sub strates (Fig. 5c). The catalytic efficiency with respect to product formation is thereby decreased.

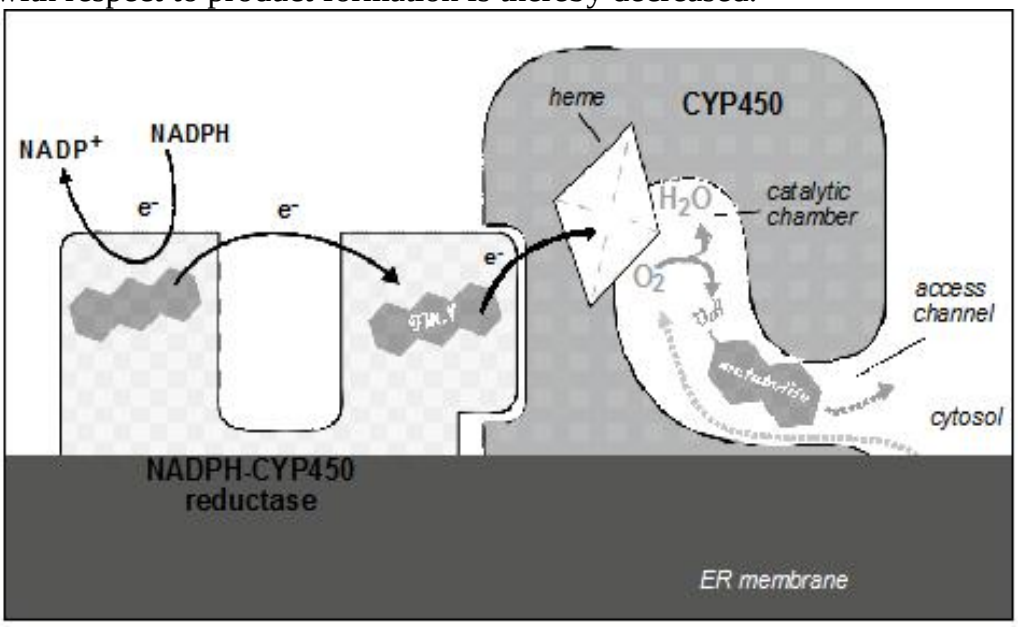

**Fig 4:** Cooperation by CYP450 and NADPH/CYP450 reductase during xenobiotic oxidation. As a monooxygenase, CYP typically incorporates one oxygen atom (O) from $O_2$ into the substrate to form a hydroxylated metabolite. The other O atom is converted to $H_2O$ using reducing equivalents donated by the flavoprotein NADH/CYP oxidoreductase. Substrate oxidation is enabled via redox changes in the central iron (Fe) atom in the heme group

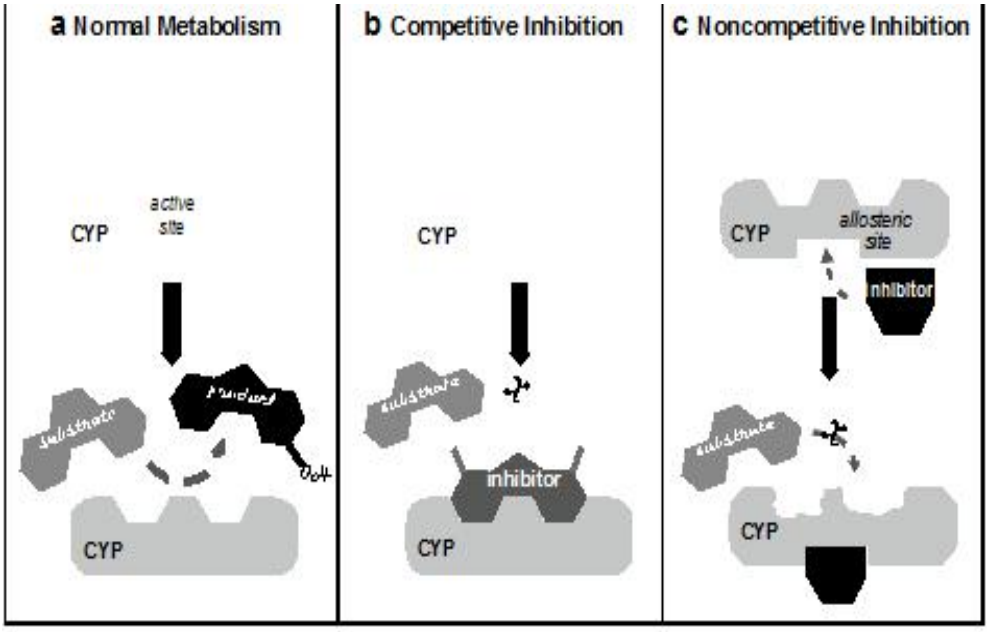


**Fig. 5:** In the normal situation, exposure to a single substrate allows unhindered access to the CYP catalyst (a). Toxicological interactions between foreign substances often involve competition between structurally related molecules for the same CYP isoform, with one toxicant acting as a competitive inhibitor which blocks access by a competing substrate (b). Less commonly, a toxicant may bind to a spatially distinct allosteric site, producing a change in the geometry of the active site that diminishes substrate affinity (c)

### 3.4.3 Induction of CYP Pathways

The capacity of the liver and other tissues to metabolise xenobiotics varies under the influence of diet, age, smoking and drug exposure. At the molecular level, pro longed xenobiotic exposure can boost levels of hepatic CYP enzymes due to a phenomenon termed induction. Polymorphisms in CYP Pathways Genetic diversity in xenobiotic biotransformation pathways is a major cause of inter-individual differences in the toxicokinetic properties of drugs and other xenobiotics. Genotypic variants within genes for xenobiotic metabolising enzymes that differ in a particular amino acid or possess other sequence differences can potentially alter an individual's susceptibility to toxic agents. The

branch of toxicology that investigates these phenomena is toxicogenetics or toxicogenomics. These sub disciplines devote considerable effort to clarifying the toxicological significance of gene polymorphisms, those genetic variants within xenobiotic biotransformation genes that are present within more than 1 % of the population. Single-nucleotide substitutions in the genomic DNA sequence (also known as SNPs) are the most common form of polymorphisms. Other sequence variations include insertion of deletion of runs of deoxynucleotides, gene copy variations (e.g. duplications) and gene conversions (e.g. via chromosomal recombination). The phenotypic impact of polymorphisms varies according to the gene and position of the mutated residue within the gene product: some polymorphisms exert minimal effects upon enzyme function, while others lead to completely nonfunctional protein products. In between these two poles are many polymorphic variants that can alter the metabolic fate of specific xenobiotics, sometimes in a highly significant manner. Polymorphisms have been identified for genes involved in virtually all aspects of toxicokinetics, ranging from xenobiotic transporters to enzyme catalysts involved in oxidative metabolism, glucuronidation, sulfonation, acetylation or glutathione conjugation pathways. Polymorphisms have also been reported for xenosensor pro teins that regulate the transcription of various biotransformation gene products. Polymorphisms within key conjugative pathways such as Nacetylation and glutathione conjugation have been investigated in hundreds of studies in efforts to associate particular alleles with particular cancer outcomes or other toxic responses to xenobiotics. In the case of CYP, documented polymorphisms reported in the literature are categorised by the Human Cytochrome P450 Allele Nomenclature Committee. The nomenclature followed when naming CYP polymorphisms usually indicates a polymorphic variant with an asterisk followed by a number and perhaps a letter (e.g. CYP2C19∗3A). By definition, the normal wildtype allele is designated with a ‘1’. Historically, much attention has been directed to CYP2D6 polymorphisms, due to the early discovery of patient subgroups that display exaggerated responses to the cardiovascular drugs debrisoquine and sparteine. The inability to metabolise debrisoquine was linked to a 2D6 polymorphism that was found to vary in its prevalence in different ethnic groups (e.g. 5–10 % of Caucasians are ‘poor metabolisers (PM)’, while the incidence in Asian populations is ~ 1 %). Using such techniques as restriction fragment length polymorphism, PCR and gene sequencing, over 110 polymorphisms were subsequently identified in the CYP2D6 gene. Genetic variants that exist at the same chromosomal locus are termed alleles. Although the number of 2D6 alleles is unusually large, allele numbers are typically high for most xenobiotic biotransformation genes compared to other genetic loci. Discovery of the wide range of CYP2D6 alleles allowed segregation of humans into distinct phenotypic groups, with at least four groups currently recognised, including poor

(PM), intermediate (IM), normal (NM) and ultrametabolisers (UM). Individuals within the 'PM' phenotype often possess a G → A transition mutation at an intron/exon boundary that disrupts splicing of RNA transcripts, impairing the production of functional CYP2D6 mRNA. These individuals usually fail to produce any 2D6 protein. CYP2D6 polymorphisms within the IM group can involve mutations within the protein coding sequence, leading to enzymes with altered activity towards xenobiotic substrates. The UM phenotype is often due to gene duplication, with 13 copies of the CYP2D6 gene observed in some members of a Swedish family caused by a base change that promotes gene duplication. A recent large study in the USA identified the UM genotype in around 1.5 % of the study population. Among the >110 known allelic CYP2D6 variants however, only a subset are prevalent or have major phenotypic consequences during pharmacological and toxicological phenomena. Promising to help clinicians during the selection of drugs for individual patients, the era of 'personalised medicine' began in 2004 when the US Food and Drug Administration approved the Roche AmpliChip CYP450 Test, the first microarray based diagnostic test for the detection of CYP mutations in human subjects. The test is able to detect 29 CYP2D6 polymorphisms and two CYP2C19 gene polymorphisms. Knowledge of 2D6 status may improve patient responses to anticancer drugs such as tamoxifen, while knowing a patient's 2C19 genotype can help minimise toxic responses to the blood thinner warfarin. While the promise of personalised medicine seemed high initially, clinical opinion is currently divided concerning the actual ben efits such diagnostic approaches bring to patient care. Such pharmacogenomic tools also hold promise during investigations of the factors that predispose individuals to toxicity caused by specific xenobiotics that are cleared by specific CYP pathways. However, the fact that CYP2D6 and 2C19 play relatively minor roles in toxicant detox ication or bioactivation may require the development of new arrays that assess poly morphisms in a wider range of CYP isoforms than the AmpliChip CYP450 Test allows.

## 3.5 Excretion

Altering the structure of lipophilic xenobiotics represents a shortterm solution to their tendency to accumulate in the body: eventually, both the metabolites and any remaining unmetabolised compound require permanent removal from the body. This process – the 'E' for excretion in ADME – is the critical final stage in the toxicokinetic fate of all chemical substances. Typically, most foreign compounds and their metabolites are removed via the kidney and/or the liver. However, for some chemicals, other minor routes including the lungs (for volatile substances such as alcohol), breast milk (important for basic drugs such as some antidepressants) or even sweat (relevant to some metallic substances such as nickel) can participate in toxicant elimination. In the main however,

excretion via urine and/or faeces represents the ultimate destination for most foreign chemicals that transit the human body.

### 3.5.1 Bile or Urine

The factors that determine whether a given xenobiotic is excreted via urine or bile have long fascinated researchers. In a classic 1975 study conducted by researchers at St Mary's Hospital in London, comparison of the excretory fates of 30 xenobiotics in rats revealed that the molecular weight of a compound determined whether it is excreted in urine or bile. The compounds essentially fell into three groups depending upon their molecular mass: the first group comprised molecules with a mass of 350 g/mol or less which were predominantly eliminated via the urine. A second set of molecules exhibited a mass of between 450 and 850 g/mol and were excreted predominantly in bile. When the bile duct was ligated via surgical intervention, alternative routes could not compensate for the loss of this pathway; hence these compounds accumulated to toxic levels in blood and tissues. A third group of xenobiotics comprised mid-sized molecules possessing a mass range of between 350 and 450 g/mol that were eliminated extensively in both urine and bile. Later work revealed that whether a xenobiotic is an organic acid or base also influences its excretory fate. In general, small, hydrophilic molecules are primarily excreted by the kidneys, whereas large, amphipathic organic compounds are mainly excreted by the bile.

### 3.5.2 Renal Excretion

The elimination of small, water-soluble substances including conjugates formed during xenobiotic biotransformation is performed by the one million or so nephrons in each adult kidney. Nephrons are highly vulnerable to chemical toxicity since these crucial structures only form during the foetal period of prenatal development, ensuring there is little capacity for the replacement of injured nephrons during the later stages of life. These crucial structures are constantly at work, with the entire blood volume passing through the kidneys once every 4 or 5 min. The basic functional unit of the kidney, a nephron comprises a glomerulus that acts as a filter to retain cells and large proteins within circulating blood. The resulting glomerular filtrate then drains into the long tubular structure in which the complex process of urine formation occurs. Anatomically and functionally distinct regions of the tubule are discernible and include the loop of Henle and distal tubule. After completing their migration through the nephron, the concentrated body wastes are delivered to the collecting duct from where they ultimately flow to the bladder (Fig. 6). Three main processes control the efficiency with which foreign chemicals are excreted via the kidneys (Fig. 6). First, most xenobiotics, with the exception of very large protein toxins, undergo filtration at the

glomerulus, the part of the nephron that interfaces directly with substances as they enter the kidney from the circulation. Since only unbound molecules are filtered in this manner, the extent of glomerular filtration is limited by any binding to plasma proteins such as albumin. A second contributing mechanism to renal excretion occurs in the proximal tubules – the end of the tubule directly adjacent to the glomerulus and involves the energy-dependent transport of chemicals from blood directly into the renal filtrate. This process varies greatly from one chemical to another due to the complex expression of xenobiotic transporters within the proximal tubules. Some transporters are especially good at 'pumping' positively charged molecules out of the blood (i.e. organic cation transporters), while others only transport negatively charged molecules (i.e. organic anion transporters). Finally, lipophilic non-ionised chemicals that undergo filtration at the glomerulus may return to the bloodstream via the passive reabsorption that occurs throughout the kidney nephron but is especially obvious in distal tubules (Fig. 6). Passive reabsorption is due to the dramatic reduction in fluid volume that occurs as renal filtrate proceeds through the nephron: in a healthy kidney, just a few ml of urine results from every 100 ml of blood that is filtered at the glomerulus, reflecting the effectiveness with which the kidneys salvage $H_2O$ and precious blood constituents. During this process, the progressive reduction in filtrate volume ensures that tubular concentrations of many toxicants increase to higher levels than those in circulating blood. If the chemical is sufficiently lipophilic and carries no ionic charge, it will likely diffuse down its concentration gradient back into the general circulation. For such chemicals, conversion to hydrophilic metabolites during a subsequent pass through the liver likely facilitates their eventual elimination.

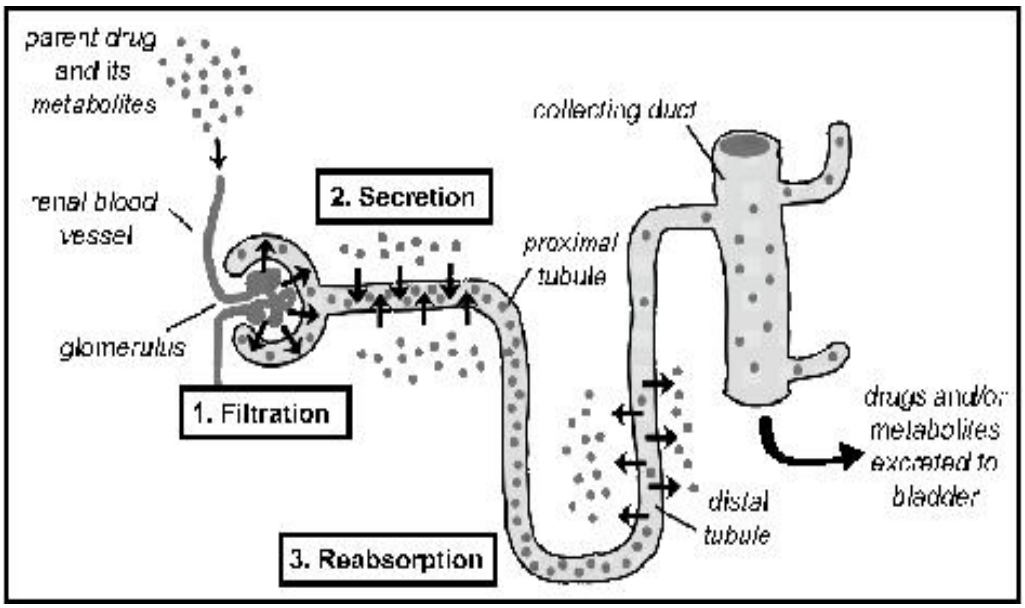


**Fig. 6:** Three processes occurring at kidney nephrons control the efficiency with which foreign chemicals and their

metabolites are excreted into urine: *glomerular filtration*, *active secretion* and *passive reabsorption*. The diagram is simplified and does not show the blood vessels that supply the proximal and distal tubules

## 4.0 CONCLUSION

The enzyme systems involved in the biotransformation of toxicants have been explained in this unit.

## 5.0 SUMMARY

This has shown the various enzymes involved in the biotransformation processes of toxicants.

## 6.0 TUTOR-MARKED ASSIGNMENT

Categorize the enzyme reactions involved in the biotransformation of toxicants into phase I and II reactions.

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# UNIT 3 TOXIC RESPONSES OF THE BLOOD

## CONTENTS



## 1.0 INTRODUCTION

Hematotoxicology is the study of adverse effects of drugs, nontherapeutic chemicals and other agents in our environment on blood and blood-forming tissues (Bloom, 1997). This subspecialty draws on the discipline of hematology and the principles of toxicology. Scientific understanding of the former began with the contributions of Leeuwenhoek and others in the seventeenth century with the Vmicroscopic examination of blood (Wintrobe, 1985). Hematology was later recognized as an applied laboratory science but limited to quantification of formed elements of the blood and the study of their morphology, along with that of bone marrow, spleen, and lymphoid tissues. It is now a diverse medical specialty, which, perhaps more than any other discipline, has made tremendous contributions to molecular medicine (Kaushansky, 2000). The vital functions that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication, makes the hematopoietic system unique as a target organ. Accordingly, it ranks with liver and kidney as one of the most important considerations in the risk assessment of individual patient populations exposed to potential toxicants in the environment, workplace, and medicine cabinet. The delivery of oxygen to tissues throughout the body, maintenance of vascular integrity, and provision of the many affector and effector immune functions necessary for host defense, requires a prodigious proliferative and regenerative

capacity. The various blood cells (erythrocytes, granulocytes, and platelets) are each produced at a rate of approximately 1–3 million per second in a healthy adult and up to several times that rate in conditions where demand for these cells is high, as in hemolytic anemia or suppurative inflammation (Kaushansky, 2006). As with intestinal mucosa and gonads, this characteristic makes hematopoietic tissue a particularly sensitive target for cytoreductive or antimitotic agents, such as those used to treat cancer, infection, and immune-mediated disorders. This tissue is also susceptible to secondary effects of toxic agents that affect the supply of nutrients, such as iron; the clearance of toxins and metabolites, such as urea; or the production of vital growth factors, such as erythropoietin and granulocyte colony stimulating factor (G-CSF). The consequences of direct or indirect damage to blood cells and their precursors are predictable and potentially life-threatening. They include hypoxia, hemorrhage, and infection. These effectsmay be subclinical and slowly progressive or acute and fulminant, with dramatic clinical presentations. Hematotoxicity is usually assessed in the context of risk versus benefit. It may be used to define dosage in treatment modalities in which these effects are limiting, such as those employing certain anticancer, antiviral, and antithrombotic agents. Hematotoxicity is generally regarded as unacceptable, however, in treatments for less serious illnesses, such as mild hypertension or arthritis or following exposure to contaminated foods or environmental contaminants. Risk-versus-benefit decisions involving hematotoxicity may be controversial, especially when the incidence of these effects is very low. Whether the effect is linked to the pharmacologic action of the agent, as with cytoreductive or thrombolytic chemicals, or unrelated to its intended action, the right balance between risk and benefit is not always clear. Hematotoxicity may be regarded as primary, where one or more blood components are directly affected, or secondary, where the toxic effect is a consequence of other tissue injury or systemic disturbances. Primary toxicity is regarded as among the more common serious effects of xenobiotics, particularly drugs (Vandendries and Drews, 2006). Secondary toxicity is exceedingly common, due to the propensity of blood cells to reflect a wide range of local and systemic effects of toxicants on other tissues. These secondary effects on hematopoietic tissue are often more reactive or compensatory than toxic, and provide the toxicologist with an important and accessible tool for monitoring and characterizing toxic responses.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- understand the toxicology of red blood cells
- understand the toxicology of white blood cells
- understand the toxicology of platelets.

## 3.0 MAIN CONTENT

### 3.1 Toxicology of the Erythron

#### 3.1.1 The Erythrocyte

Erythrocytes (red blood cells or RBCs) make up 40–45% of the circulating blood volume and serve as the principal vehicle of transportation of oxygen from the lungs to the peripheral tissues. In addition, erythrocytes are involved in the transport of carbon dioxide from tissues to the lung, maintenance of a constant pH in blood and regulation of blood flow to tissues (Kim-Shapiro *et al.*, 2005). Erythrocytes help modulate the inflammatory response through clearance of immune complexes containing complement components and through interaction with nitric oxide, a potent vasodilator (Kim-Shapiro *et al.*, 2005). An area of developing interest is the role of erythrocytes as a carrier and/or reservoir for drugs and toxins (Schrijvers *et al.*, 1999). The effect of xenobiotics on erythrocytes has been extensively evaluated, both because of the ready access to the tissue and the frequency with which xenobiotics cause changes in this critical tissue. Xenobiotics may affect the production, function and/or survival of erythrocytes. These effects are most frequently manifest as a change in the circulating red cell mass, usually resulting in a decrease (anemia). Occasionally, agents that increase oxygen affinity lead to an increase in red cell mass (erythrocytosis), but this is distinctly less common. Shifts in plasma volume can alter the relative concentration of erythrocytes/hemoglobin and can be easily confused with true anemia or erythrocytosis.

There are two general mechanisms that lead to true anemia— either decreased production or increased erythrocyte destruction.

Both mechanisms may be operative in some disorders, or a combination may arise due to the imposition of a second disorder on a compensated underlying problem. For example, patients with compensated congenital hemolytic anemias are very susceptible to additional insults thatmay precipitate an acute drop in a previously stable red cell mass, such as parvovirus-infection-associated suppression of erythropoiesis.

Evaluation of a peripheral blood sample can provide evidence for the underlying mechanism of anemia (Prchal, 2006). The usual parameters of a complete blood count (CBC)—including the red blood cell (RBC) count, hemoglobin concentration (Hbg) and hematocrit (also referred to as packed cell volume, or PCV)—can establish the presence of anemia. Two additional parameters helpful in classifying an anemia are the mean corpuscular volume (MCV) and the reticulocyte count. Increased destruction is usually accompanied by an increase in reticulocytes (young erythrocytes containing residual RNA), which are easily enumerated using appropriate stains. The introduction of automated methods has improved the precision of reticulocyte counting and introduced new parameters which aid in characterization of red cell production (Brugnara, 2000). With these new methods, reticulocyte counting may also be useful in conditions associated with decreased production, particularly when assessing response to therapy. Other readily performed parameters helpful in the evaluation of the human erythron include: erythrocyte morphology (e.g., megaloblastic changes, erythrocyte fragmentation, sickled RBCs); serum concentration of haptoglobin, lactic dehydrogenase (LD), free hemoglobin, vitamin B12, folate, iron, and ferritin; direct and indirect red cell antiglobulin tests; and bone marrow morphology (Prchal, 2006).

### 3.1.2 Alterations in Red Cell Production

Erythrocyte production is a continuous process that is dependent on frequent cell division and a high rate of hemoglobin synthesis. Adult hemoglobin (hemoglobin A), the major constituent of the erythrocyte cytoplasm, is a tetramer composed of two α- and two β-globin chains, each with a heme residue located in a stereospecific pocket of the globin chain. Synthesis of hemoglobin is dependent on coordinated production of globin chains and heme moieties. Abnormalities that lead to decreased hemoglobin synthesis are relatively common (e.g., iron deficiency) and are often associated with a decrease in the MCV and hypochromasia (increased central pallor of RBCs on stained blood films due to the low hemoglobin concentration).

An imbalance between α- and β-chain production is the basis of congenital thalassemia syndromes and results in decreased hemoglobin production and microcytosis (Weatherall, 2006). Xenobiotics can affect globin-chain synthesis and alter the composition of hemoglobin within erythrocytes. This is perhaps best demonstrated by hydroxyurea, which has been found to increase the synthesis of γ-globin chains. The γ-globin chains are a normal constituent of hemoglobin during fetal development, replacing the β chains in the hemoglobin tetramer (hemoglobin F, $\alpha 2\gamma 2$). Hemoglobin F has a higher affinity for oxygen than hemoglobin A and can protect against crystallization (sickling) of deoxyhemoglobin S in sickle cell disease (Steinberg, 2006).

Synthesis of heme requires incorporation of iron into a porphyrin ring. Iron deficiency is usually the result of dietary deficiency or increased blood loss. Any drug that contributes to blood loss, such as nonsteroidal anti-inflammatory drugs, with their increased risk of gastrointestinal ulceration and bleeding, may potentiate the risk of developing iron deficiency anemia. Defects in the synthesis of the porphyrin ring of heme can lead to sideroblastic anemia, with its characteristic accumulation of iron in bone marrow erythroblasts. The accumulated iron precipitates within mitochondria in a complex with mitochondria ferritin, causing the characteristic staining pattern of ringed sideroblasts evident on iron stains such as Prussian blue. A number of xenobiotics can interfere with one or more of the steps in erythroblast heme synthesis and result in sideroblastic anemia.

Hematopoiesis requires active DNA synthesis and frequent mitoses. Folate and vitamin B12 are necessary to maintain synthesis of thymidine for incorporation into DNA. Deficiency of folate and/or vitamin B12 results in megaloblastic anemia, with its characteristic morphologic and biochemical changes, which commonly affect erythroid, myeloid, and megakaryocytic lineages. A number of xenobiotics may contribute to a deficiency of vitamin B12 and/or folate, leading to megaloblastic anemia. Many of the antiproliferative drugs used in the treatment of malignancy predictably inhibit hematopoiesis, including erythropoiesis. New chemicals, such as amifostine, are being developed that may help protect against the marrow toxicity of these agents. The development of recombinant forms of some of the growth factors that regulate hematopoiesis has helped shorten the duration of bone marrow suppression. As with other therapeutic proteins, there is a risk of antibody formation in response to administration of these proteins; if the antibody reacts with the endogenous growth factor it may cause profound cytopenia. Erythropoietin is commonly used to support red cell production in patients undergoing chemotherapy and with renal failure. Following a change in formulation, a series of cases of red cell aplasia associated with erythropoietin use was reported. The etiology was antibodies to the synthetic protein that cross-reacted with endogenous erythropoietin. A change in formulation in combination with the nature of the storage container and route of administration is thought to have promoted the formation of protein aggregates, a phenomenon known to be associated with an increased risk of antibody formation.

The incidence of red cell aplasia appears to have diminished following a change in packaging and administration of erythropoietin by intravenous injection. Drug-induced *aplastic anemia* may represent either a predictable or idiosyncratic reaction to a xenobiotic. This lifethreatening disorder is characterized by peripheral blood pancytopenia, reticulocytopenia, and bone marrow hypoplasia. Chemicals such as

benzene and radiation have a predictable effect on hematopoietic progenitors, and the resulting aplastic anemia corresponds to the magnitude of the exposure to these chemicals. In contrast, idiosyncratic aplastic anemia does not appear to be related to the dose of the chemical initiating the process. A long list of chemicals has been associated with the development of aplastic anemia, many of which have been reported in only a few patients. The mechanisms of aplasia in affected patients are still unknown. Immune mechanisms have long been thought to contribute to the development of the idiosyncratic form of drug-induced aplastic anemia. However, it has been difficult to obtain definitive evidence for humoral and cellular mechanisms of marrow suppression.

Pure red cell aplasia is a syndrome in which the decrease in marrow production is limited to the erythroid lineage. Pure red cell aplasia is an uncommon disorder that may be due to genetic defects, infection (parvovirus B19), immune-mediated injury, myelodysplasia, drugs or other toxicants. As pure red cell aplasia occurs sporadically and infrequently, the linkage between drug exposure and pathogenesis of the aplasia remains speculative for some chemicals. The drugs most clearly implicated and for which there are multiple case reports, include isoniazid, phenytoin, and azathioprine. The mechanism of drug-induced pure red cell aplasia is unknown, but some evidence suggests that it may be immune-mediated. Patients with drug-induced red cell aplasia should not be re-exposed to the purported offending chemical. Pure red aplasia may also occur as a consequence of an immune response to therapeutic erythropoietin.

## 3.2 Toxicology of the Leukon

### 3.2.1 Components of Blood Leukocytes

The leukon consists of leukocytes, or white blood cells. They include granulocytes (which may be subdivided into neutrophils, eosinophils, and basophils), monocytes, and lymphocytes. Granulocytes and monocytes are nucleated ameboid cells that are phagocytic. They play a central role in the inflammatory response and host defense. Unlike the RBC, which resides exclusively within blood, granulocytes and monocytes generally pass through the blood on their way to the extravascular tissues, where they reside in large numbers; although it is now understood that senescent neutrophils that remain in the circulation return to the bone marrow through the SDF-1α/CXCR4 chemokine axis. Granulocytes are defined by the characteristics of their cytoplasmic granules as they appear on a blood smear stained with a polychromatic (Romanovsky) stain. Neutrophils, the largest component of blood leukocytes, are highly specialized in the mediation of inflammation and the ingestion and destruction of pathogenic microorganisms. The turnover of the neutrophil

is enormous and increases dramatically in times of inflammation and infection, elevating the number of these cells released from the bone marrow. Eosinophils and basophils modulate inflammation through the release of various mediators and play an important role in other homeostatic functions. Eosinophils and basophils are far more difficult to study, with changes in these populations most frequently associated with reactions to other target organ or systemic toxicity. Examples include the eosinophilia observed with the toxic oil syndrome in northwestern Spain that resulted from exposure to rapeseed oil denaturedwith aniline; and the eosinophilia-myalgia syndrome associated with λ-tryptophan preparations contaminated with 1, 1-ethylidene-bis [tryptophan]. Peripheral eosinophilia is often but not reliably observed with hypersensitivity reactions to drugs, while tissue eosinophilia can be diagnostic, in the context of a suggestive clinical course, in conditions such as drug-induced cutaneous vasculitis and eosinophilic pneumonia. Thisvariability in systemic response can be genetically predisposed, as demonstrated in studies using transgenic mice on genetic restrictions in people afflicted by the aforementioned toxic oil syndrome. The time course of the reaction can also influence whether eosinophilia can be demonstrated in hypersensitivity disease.

### 3.2.2 Evaluation of Granulocytes

The most informative test to assess the neutrophil compartment is the blood neutrophil count. Accurate interpretation requires an understanding of neutrophil kinetics and the response of this tissue to physiologic and pathologic changes. In the blood, neutrophils are distributed between *circulating* and *marginated* pools, which are of equal size in humans and in constant equilibrium. A blood neutrophil count assesses only the circulating pool, which remains between 1800 and 7500 μL-1 in a healthy adult human. This constancy is remarkable, considering that as many as 1011 neutrophils are released from the marrow daily, and this circulating pool represents only 1% of the total body neutrophils, and that the circulating half life of these cells is only approximately 6 hours. How this extraordinary regulation is achieved is only partially understood. Recent studies using knock-out mice suggest that G-CSF is an essential regulator of both granulopoiesis and neutrophil release from the bone marrow (Semerad *et al.*, 2002). It is induced by the T-cell derived cytokine IL-17 which, in turn, is controlled by IL-23 that is provided by dendritic cells and macrophages. The latter is down regulated by the phagocytosis of apoptotic neutrophils in the tissues, which provides an important negative feedback loop. Pharmaceutical companies are currently developing recombinant proteins that function as agonists and inhibitors of these mediators, which have great potential as exciting new therapies. Many will also be shown to cause unacceptable immunotoxicity and

hematotoxicity, which portends exciting times for the academic and industrial hematopathologist and toxicologist.

Neutrophil kinetics and response to disease will vary substantially among animal species (Feldman, 2000). Thus, a thorough understanding of these features in any animal model used in investigative toxicology is required before informed interpretations can be made. In humans, clinically significant neutropenia occurs when the blood neutrophil count is less than 1000 $\mu L^{-1}$, but serious recurrent infections do not usually occur until counts fall below 500 $\mu L^{-1}$. Morphologic assessment of peripheral blood granulocytes can be helpful in characterizing neutropenia. In humans and most healthy animal species, mature (segmented) and a few immature (band) neutrophils can be identified on blood films stained with Wright or Giemsa stain. During inflammation, a "shift to the left" may occur, which refers to an increased number of immature (nonsegmented) granulocytes in the peripheral blood, which may include bands, metamyelocytes, and occasionally myelocytes. During such times, neutrophils may also show "toxic" granulation, D¨ ohle bodies, and cytoplasmic vacuoles. These morphologic changes may be prominent in sepsis or as a result of drug or chemical intoxication. In order to fully characterize such changes or understand the pathogenesis of the abnormality, bone marrow must be examined using marrow aspirates and biopsies. These provide information on rates of production, bone marrow reserves, abnormalities in cell distribution and occasionally specific clues as to etiology. Normal human marrow specimens contain approximately 50–1000 CFU-GM per 106 nucleated cells cultured. Marrow stem cell reserves can be assessed in vitro after administration of G-CSF, which stimulates increased production and release of neutrophil precursors. Glucocorticoids and epinephrine (Babior and Golde, 1995) may also be used for this purpose but are rarely used in a clinical setting. The recent understanding that the CXC-chemokine ligand CXCL12/CXCL4 mediates the retention of granulopoietic stem cells within their bone marrow niche, as well as mature neutrophils within the bone marrow pool, has led to the development of an inhibitor of this ligand which, when administered with G-CSF, can cause a transient release of neutrophils and CD34+ (stem) cells into the circulation. The latter can be collected and re-engrafted to form new functioning bone marrow. The ability to manipulate this system in this way will likely provide important research, diagnostic and therapeutic tools for the hematologist, oncologist, and toxicologist. The degree of proliferation in the granulocyte-monocyte compartment can also be assessed using older techniques that employ 3H-thymidine suicide assays or DNA binding dyes with fluorescence-activated cell sorting analyses.

### 3.2.3 Toxic Effects on Granulocytes

The toxicologist is concerned with the effect of xenobiotics on granulocytes as relates to proliferation (granulopoiesis) and kinetics, the extent to which a drug or chemical contaminant can impair the vital functions these cells perform, and how neutrophils mediate or exacerbate inflammatory disease or other target organ toxicity. However, it is difficult to separate effects on granulopoiesis and neutrophil kinetics from that of function. Both are complex and highly regulated through an array of growth factors, chemokines, cytokines and interactions with monocytes, dendritic cells and lymphocytes in a bidirectional, multicompartmental manner.

Many of the mediators and interactions that enable this feat have now become targets for the therapeutic dysregulation of these processes, which has led to the development of candidate drugs that may prove to be uniquely efficacious and/or toxic.

## 3.3 Leukemogenesis as a Toxic Response

### 3.3.1 Human Leukemias

Leukemias are proliferative disorders of hematopoietic tissue that are monoclonal in origin and thus originate from individual bone marrow cells. Historically they have been classified as myeloid or lymphoid, referring to the major lineages for erythrocytes, granulocytes, thrombocytes or lymphocytes, respectively. Because the degree of leukemic cell differentiation has also loosely correlated with the rate of disease progression, poorly differentiated phenotypes have been designated as “acute,” whereas welldifferentiated ones are referred to as “chronic” leukemias. The classification of human leukemias proposed by the French-AmericanBritish (FAB) Cooperative Group has become convention. It provides the diagnostic framework for classifying chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), and the myelodysplastic syndromes (MDS), along with various subtypes of these disorders. The WHO has incorporated into these parameters more recent discoveries regarding the genetics and phenotypic features of disorders like AML in order to define subtypes that are biologically homogenous and that have prognostic and therapeutic relevance. These early correlations imply that the biology and clinical features of these proliferative disorders relate to the stage of differentiation of the target cell, which is now being linked to individual gene alterations, as well as epigenetic factors such as cytokine stimulation.

There is considerable evidence supporting the notion that leukemogenesis is a multievent progression. These studies suggest that factors involved in the regulation of hematopoiesis also influence neoplastic transformation. Such factors include cellular growth factors (cytokines), protooncogenes and other growth-promoting genes, as well as additional genetic and epigenetic factors that govern survival, proliferation, and differentiation.

## 3.4 Toxicology of Platelets and Hemostasis

Hemostasis is a multicomponent system responsible for preventing the loss of blood from sites of vascular injury and maintaining circulating blood in a fluid state. Loss of blood is prevented by formation of stable hemostatic plugs mediated by the procoagulant arm of hemostasis. This procoagulant response is normally limited to sites of vascular injury by the multicomponent regulatory arm of hemostasis. The dynamically modulated balance between procoagulant and regulatory pathways permits a rapid, localized response to injury. The major constituents of the hemostatic system include circulating platelets, a variety of plasma proteins, and vascular endothelial cells. More recently the role of other cells in hemostasis, especially leukocytes, has become apparent. Alterations in these components or systemic activation of this system can lead to the clinical manifestations of deranged hemostasis, including excessive bleeding and thrombosis. The hemostatic system is a frequent target of therapeutic intervention as well as inadvertent expression of the toxic effect of a variety of xenobiotics.

### 3.4.1 Toxic Effects on Platelets

Platelets are essential for formation of a stable hemostatic plug in response to vascular injury. Platelets initially adhere to the damaged wall through binding of von Willebrand factor (vWF) with the platelet glycoprotein Ib/IX/V (GP Ib/IX/V) receptor complex. Ligand binding to GP Ib/IX/V or interaction of other platelet agonists (e.g., thrombin, collagen, ADP, thromboxane A2) with their specific receptors initiates biochemical response pathways that lead to shape change, platelet contraction, platelet secretion of granule contents, activation of the GPIIb/IIIareceptor, and externalization of phosphatidylserine. Activation of the GP IIb/IIIa receptor permits fibrinogen and other multivalent adhesive molecules to form cross-links between nearby platelets, resulting in platelet aggregation. Xenobiotics may interfere with the platelet response by causing thrombocytopenia or interfering with platelet function; some chemicals are capable of affecting both platelet number and function.

## 4.0 CONCLUSION

The understanding of the toxic responses of the blood enables toxicologists to use haematological parameters to assess the toxicities of substances

## 5.0 SUMMARY

In this unit we have learnt that haematological parameters can be to evaluate the toxicity of a susbstance.

## 6.0 TUTOR-MARKED ASSIGNMENT

Briefly explain how the haematologcal parameters of a named animal can be used to assess the toxicity of named chemical.

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# UNIT 4 TOXIC RESPONSES OF THE LIVER

## CONTENTS



## 1.0 INTRODUCTION

After absorption into the systemic circulation and distribution throughout the body, few toxicants equally damage each of the 60 trillion or so cells possessed by a grown human: instead, their noxious effects are usually manifest within select target organs. These organs are chosen for attention as they are more commonly involved in overt organ toxicity than most other tissues. While some toxicity is manifest in excretory organs because it disrupts a distinctive capability of the target tissue (e.g. cessation of bile flow in the liver, impairment of proximal tubular function in the kidney), other pathologies resemble toxicant-induced responses that can occur in any tissue (e.g. cancer, inflammation, fibrosis). If space permitted, a broader range of tissues (e.g. brain, lungs, heart) could be considered since many important toxic syndromes also involve these organs.

The liver's status as the largest organ in the body reflects its key roles in many physiological processes, ensuring its undisputed position as 'metabolic coordinator' of the entire body. Due to the organ's importance to many body functions, any tendency for a chemical to damage the liver is taken very seriously in modern toxicology and risk assessment.

Several factors predispose the liver to xenobiotic toxicity. Firstly, for chemicals entering the body via the oral route, anatomical proximity to the GI-tract ensures the liver is the 'first port of call' for ingested xenobiotics. Secondly, chemicals and nutrients are not the only substances that enter portal blood as it perfuses the intestines: it also accumulates products of the degradation of intestinal microorganisms

such as inflammogenic lipopolysaccharide components of the bacterial cell wall (i.e. endotoxin). Since endotoxin delivery may increase during xenobiotic intoxication, immunological responses to co-absorbed endotoxin can exacerbate the hepatotoxicity of ingested chemicals. Thirdly, in addition to entry via the portal circulation, chemicals can access the liver via arterial blood that mixes with venous blood in the hepatic sinusoids. For example, inhaled tobacco constituents that enter via the lungs are efficiently delivered to the liver via the arterial route. Fourthly, the vast metabolic capacities of the liver also paradoxically heighten its vulnerability to chemical toxicity: by functioning as a miniaturised chemical factory that performs many diverse chemical modifications on foreign molecules, CYPs and other hepatic enzymes can inadvertently generate noxious metabolites that induce 'bioactivation-dependent' hepatotoxicity.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- understand the toxic responses of the liver
- mention examples of hepatoxicants.

## 3.0 MAIN CONTENT

### 3.1 Major Toxic Responses of the Liver

Traditionally, chemicals that cause liver injury were labelled intrinsic hepatotoxicants if they induced predictable, dose-related liver injury in a majority of exposed individuals. Idiosyncratic hepatotoxicants on the other hand typically elicit hepatotoxicity in a small minority (approximately <1 in 100,000) of exposed individuals. The severity of the hepatic response to an idiosyncratic hepatotoxicant is often assumed to bear little relation to the administered dose, although recent studies suggest this is not necessarily always the case for drug-induced allergic hepatotoxicity. Hepatotoxic responses can often be distinguished on the grounds of whether they can be reproduced in rodent species: as a rule, intrinsic hepatotoxicants usually induce a comparable hepatotoxicity in lab animals, whereas idiosyncratic toxicity is often hard to reproduce in rodents. Historically, chemicals inducing intrinsic hepatotoxicity were often assumed to inflict hepatotoxicity via non-immunologic mechanisms, while idiosyncratic toxicity was typically attributed to immune-mediated responses such as antibody production and T-cell activation. While these broad categorisations retain some use during the classification of hepatotoxic chemicals, the assumption that the presence or absence of immune mechanisms is of defining importance is no longer valid. Immunologic mechanisms are now thought to contribute to the

pathogenesis of many hepatotoxic syndromes, including acute intrinsic hepatotoxic syndromes that were once thought to exclude immunological pathways. Studies of the effects of hepatotoxicants upon liver histology have revealed a surprisingly constrained cluster of toxic responses. Thus, toxic responses to hundreds of chemically diverse hepatotoxicants typically feature a handful of welldefined pathological responses.

### 3.1.1 Fatty Liver

An early sign of liver injury upon exposure to diverse hepatotoxicants is steatosis, a reversible condition in which small or large fatty vesicles (i.e. micro- or macrovesicular droplets) emerge within the cytoplasm of parenchymal cells throughout the liver lobule. These typically comprise triglyceride-filled droplets that are coated with phospholipids and specific lipid droplet-associated proteins, most notably members of the PAT protein family that assist triglyceride storage in adipocytes. Ongoing exposure to hepatotoxicants that induce droplet formation often triggers the emergence of more overt liver pathology. Diagnosing xenobiotic-associated steatosis is challenging for clinicians since fatty liver occurs in a wide range of common health disorders, including nonalcoholic fatty liver disease (NAFLD) which afflicts patients suffering from obesity and metabolic syndrome, as well as diverse steatotic conditions that occur in patients with micronutrient deficiencies or chronic viral infections. The mechanisms underlying fatty droplet accumulation in intoxicated liver are complex. Long-standing biochemical explanations attributed fat deposition to toxicant- induced shifts in the hepatocellular redox state that favour the accumulation of fatty acids rather than their oxidation, but this mechanism is hard to prove for all steatogenic compounds. Newer insights gained from microarray studies of steatotic livers have identified changes in the expression of numerous proteins involved in hepatic lipid metabolism, including dysregulation of transcription factors that control fatty acid synthesis (e.g. SREBP, sterol regulatory element binding protein) as well as fatty acid oxidation (e.g. PPAR, peroxisome proliferatoractivated receptor). Such microarray studies of steatogenesis suggest a transcriptional shift towards an ‘adipogenic’ state in which the liver boosts its capacity for fatty acid synthesis and simultaneously downregulates fatty acid oxidation and secretion of very-low-density lipoproteins (VLDLs).

### 3.1.2 Cell Death

Cell death within the liver typically proceeds via either necrosis or apoptosis. Apoptotic cell death involves small clusters of hepatocytes and proceeds via a tightly orchestrated sequence of molecular events that involve the controlled digestion of cellular components by cell death enzymes such as caspases. Alternatively, heavier intoxication with

hepatotoxicants can induce a more overt, uncontrolled form of cell death known as *hepatic necrosis*. An irreversible process involving the death of many hepatocytes, necrotic liver injury may be 'focal' (i.e. localised to specific zones as in the case of centrilobular or periportal necrosis) or 'fulminant' (rapid in onset and affecting a majority of liver cells). Although its early stages involve reversible cell swelling, events quickly progress to irreversible necrotic tissue destruction. Typical signs of cell death by necrosis include loss of membrane integrity, swelling of mitochondria and other intracellular organelles, ATP depletion and loss of calcium homeostasis secondary to calcium influx. The latter change activates calcium- dependent endonucleases, proteases and phospholipases that begin digesting key cell components, leading to cytoskeletal derangement and cell blebbing.

Cell lysis and striking changes in the organisation of nuclear DNA (e.g. pyknosis, karyorrhexis and karyolysis) are also conspicuous in necrotic liver. Hepatic necrosis occurs upon intoxication with many noxious substances including medicinal agents, natural substances and synthetic chemicals. In industrialised nations, the leading cause of hepatic necrosis in emergency department patients is often paracetamol intoxication. Although the liver has a remarkable regenerative capacity that ensures rapid regrowth after acute intoxication with paracetamol and other hepatotoxicants, some types of hepatic necrosis trigger the formation of persistent scar tissue. Since liver necrosis releases hepatocellular constituents into the bloodstream, measuring the levels of common liver enzymes such as alanine transaminase (ALT) or aspartate transaminase (AST) within blood samples is commonly used during the evaluation of xenobiotic-exposed patients. The greater the liver injury sustained by a patient, the higher the ALT and AST levels in their blood, with up to 1,000-fold elevations seen in serious cases of hepatic injury. Some caution is needed when interpreting clinical 'liver transaminase' data since these enzyme markers are not entirely liver specific; their expression in skeletal and cardiac muscle ensures damage to these tissues can also elevate blood transaminase levels. Moreover, modest elevations in plasma transaminases are not necessarily predictive of a progressive hepatotoxic response: for patients receiving some drugs such as the Alzheimer's medication tacrine, doctors may tolerate a modest 'asymptomatic' elevation in ALT levels. Plasma markers can sometimes provide subtle insights into the nature of the hepatic insult: elevations in alkaline phosphatase and γ-glutamyltranspeptidase (GGT), for example, can indicate impaired biliary excretion rather than hepatocellular necrosis. Measuring an array of enzyme markers can thus clarify the nature of the hepatic damage occurring in poisoned patients. Recent years have witnessed testing of new diagnostic approaches to detect chemically induced liver toxicity. Changes in the abundance of circulating microRNAs – short pieces of RNA that help regulate the expression of

gene networks – represent one attractive possibility. Animal experiments suggest that hepatotoxic doses of model toxicants such as acetaminophen or carbon tetrachloride up- or downregulate particular clusters of microRNA molecules in both blood and urine samples. While these approaches seem promising, whether their sensitivity or specificity in intoxicated human subjects is better than traditional enzymological approaches largely awaits future clarification.

### 3.1.3 Impaired Bile Flow

As the largest gland in the body, the bile-secreting capacity of the liver rids the circulation of diverse endogenous waste products as well as foreign xenobiotics and their metabolites. Bile also contains cholesterol-derived detergent-like substances that help emulsify ingested fats. Some hepatotoxic chemicals disrupt these core hepatic functions by eliciting cholestasis, namely, a partial or complete arrest of bile flow. This has immediate physiological consequences since the bile is a major elimination route for bilirubin and biliverdin, toxic chromogenic pigments which form during the degradation of heme-rich red blood cells. During normal liver function, bilirubin and biliverdin are actively exported into the tubular canalicular network that eventually drains into the bile duct. Since some hepatotoxic chemicals inhibit the membrane transporters that export these pigments across canalicular membranes, the impaired hepatic clearance of biliverdin and bilirubin causes their deposition in blood and body tissues, with their accumulation in dermal layers conferring a yellow tinge to the patient's skin. Jaundice – a hyperbilirubinaemic state – is also common in newborn babies due to poor conjugative metabolism within neonatal liver. Jaundice also accompanies some drug-related hepatotoxicity syndromes (e.g. it is particularly common among heavy users of anabolic steroids). Unresolved jaundice can precipitate a medical emergency since the accumulation of unconjugated bilirubin in body tissues and particularly the brain can elicit cellular injury.

### 3.1.4 Liver Fibrosis

Continuing exposure to hepatotoxicants frequently promotes excessive deposition of extracellular matrix proteins such as collagen, leading to the serious condition known as liver fibrosis. Although the histology accompanying fibrotic responses to structurally diverse hepatotoxicants is often similar, the mechanisms involved are frequently dissimilar and complex. Activated hepatic stellate cells (HSCs) are the main effectors of fibrosis, although myofibroblasts play a substantial supporting role, especially in the deposition of collagen fibres throughout the canalicular tracts of the liver lobule. Fibroblasts originating in extrahepatic tissues including bone marrow may also assist fibrogenesis.

The molecular factors that trigger fibrosis have received much attention. Although a complex cocktail of cytokines and other mediators can arouse HSCs from their normal quiescent state to become profibrogenic factories, platelet-derived growth factor (PGDF) released from activated Kupffer cells is likely most important. Other mediators assist by suppressing the normal apoptotic death of HSCs, thereby prolonging their duration of fibrogenic activity. The complexity of the biology underlying fibrosis unfortunately means few effective therapies are available for this condition, beyond cessation of xenobiotic exposure. In the case of alcoholic fibrosis, continued drinking achieves transition to the cirrhotic phase of alcoholic liver disease, a terminal condition in which uncontrolled fibre deposition and widespread hepatocellular death leaves the liver a shrunken mass of dysfunctional tissue.

### 3.1.5 Liver Cancer

Primary liver cancer is relatively uncommon in Western nations but more prevalent in African and eastern Asian populations. Variations in contamination of the food supply with fungal carcinogens likely contribute to these geographical differences. Liver cancer arises from tumour cell clusters that are typically *monoclonal* in origin, meaning they originate when cells acquire genetic changes that confer growth advantages upon their descendants. Within the liver, tumours can arise in cells of the bile duct or hepatic blood vessels, although they most commonly originate in hepatocytes. The prognosis following the detection of liver cancer is usually dire since it is typically a highly metastatic, aggressive form of cancer. The disease is often highly advanced by the time of diagnosis. Liver tumours can accompany chronic exposure to alcohol and a wide range of occupational hazards including the toxic metal arsenic and the industrial reagent vinyl chloride.

## 3.2 Major Hepatotoxicant Classes

Many structurally diverse chemicals have been associated with hepatotoxicity in humans. These include chemicals that are used as medicinal agents, reagents that are employed during particular occupational practices in the workplace and hepatotoxicants that arise from natural sources such as plants and fungi. A growing contribution to the global burden of human liver injury reflects the rising popularity of herbal remedies and dietary supplements. Due to the diversity of substances that induce liver injury, geographical differences are seen in the relative importance of different causative agents: antibiotics, anticonvulsants and psychotropic drugs are leading causes of hepatotoxicity in Western societies, whereas in Asia, ‘herbs’ and ‘health foods or dietary supplements’ represent a leading cause.

## 4.0 CONCLUSION

In this unit, the toxicological responses of the liver have been examined. The liver is an important organ for toxicological assessment.

## 5.0 SUMMARY

In this unit, we have learnt the parameters that are used to assess hepatotoxic damages caused by toxicants.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. Describe the parameters that can be used to assess hepatotoxic damage induced by a named chemical
2. Any tendency for a chemical to damage the liver is taken seriously in toxicology. Discuss.

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# UNIT 5 TOXIC RESPONSES OF KIDNEY

## CONTENTS



## 1.0 INTRODUCTION

Chemically induced nephrotoxicity is closely related to hepatotoxicity in that these two excretory organs often incur simultaneous damage by the same toxicant. For example, upon human exposure to cadmium – a heavy metal constituent of batteries, paints and plastics – the balance between nephrotoxicity and hepatotoxicity varies according to the magnitude and duration of exposure: the liver typically sustains damage by large, acute doses of cadmium, while the kidneys are vulnerable during extended exposure to low doses. Since the kidney lacks the regenerative capacity of liver, nephrotoxic episodes that diminish the number of functional nephrons often condemn victims to either long-term renal dialysis or renal transplants. Several physiological considerations predispose the kidneys to chemical toxicity. The strong expression of xenobiotic transporters within the luminal membranes of the renal nephron renders the kidneys highly vulnerable to nephrotoxicants since it means local toxicant concentrations can significantly exceed their levels in circulating blood. The large surface area of the luminal membranes and strong expression of xenobiotic metabolising enzymes in proximal tubules is a further

exacerbating factor. The vulnerability of the renal vasculature to vasoactive compounds also predisposes the kidneys to injury, since blood flow changes can further maximise local xenobiotic concentrations within renal tissue. These collective factors ensure human exposure to nephrotoxic substances is of high clinical relevance. Indeed, xenobiotic intoxications contribute to around one-half of acute and chronic renal failures, while between 10 % and 15 % of intensive care unit admissions involve acute renal failure. Since mortality rates for acute renal failure have barely budged in the past 50 years, the need for basic and clinical research in this area remains high. As with the liver, normal kidney function underpins the wellbeing of many physiological systems, with important roles in the maintenance of electrolyte homeostasis via regulation of the volume and ionic composition of total body fluid (e.g. levels of water, sodium, potassium or hydrogen ions). The kidneys also produce crucial hormones that regulate blood pressure (e.g. rennin), red blood cell production (erythropoietin) and blood calcium levels (calcitrol). The renal capacity to excrete foreign chemicals as well as metabolic waste products (e.g. urea, ammonia and uric acid) is also crucial to bodily health.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- describe the consequences of toxicity to the kidney
- mention substancesthat induce toxicity to the kidney
- explain the parameters used to assess toxicity to the kidney.

## 3.0 MAIN CONTENT

### 3.1 Functional Consequences of Nephrotoxicity

As a rule, functional changes accompanying toxicant-induced nephrotoxicity is acute in nature, developing rapidly following initial exposure to a noxious drug or chemical, or progressive, developing slowly and insidiously during chronic exposure to nephrotoxicants. For still other toxic substances, renal cancer is a major long-term toxic outcome. Xenobiotic-induced renal injury often makes its presence known via changes in the quantity or quality of urine: symptoms can involve overproduction of dilute urine (polyuria), excretion of low quantities of urine (anuria) or the passage of bloodstained urine (haematuria). Alterations in the composition of urine that are indicative of renal damage include the appearance of glucose (glucosuria) or blood proteins in urine (proteinuria). Levels of specific proteins such as albumin (albuminuria) are useful indicators of nephrotoxicity in the clinical setting. Unfortunately, renal injury is often far advanced before such

changes become obvious to patients, a situation that drives a search for early markers of drug- or toxicant- induced nephrotoxicity. Specific urinary markers that pinpoint kidney damage to particular renal zones are of particular interest since relying upon gross urinary changes to detect nephrotoxicity is often unreliable (e.g. both glomerular and tubular toxicity can alter urine volumes).

In recent decades, researchers within the pharmaceutical industry and academic laboratories have studied specific proteins within urine as markers of injury to specific renal structures. Since these approaches initially used traditional biochemicalmapproaches with limited scope, their clinical uptake in diagnostic settings was typically low. More recently, the use of broad-based metabolomic and proteomic methods for monitoring large numbers of molecules in urine has identified many promising nephrotoxicity biomarkers. While most of these markers are still undergoing validation in animal-based studies and human trials, they may have a significant clinical impact in coming years.

## 3.2 Major Human Nephrotoxicants

Development of a unified mechanistic understanding of chemically induced nephrotoxicity has been hampered by the dissimilarities between nephrotoxicants in terms of their chemical structures and physicochemical properties. Thus, while the clinical signs accompanying kidney damage are often similar, few chemical or structural similarities exist between toxicants as diverse as the heavy metal mercury, the mycotoxin fumonisin B1, the immunosuppressant cyclosporine A or the aminoglycoside gentamicin.

## 3.3 Susceptibility of the Kidney to Toxic Injury

### 3.3.1 Incidence and Severity of Toxic Nephropathy

A wide variety of drugs, environmental chemicals, and metals can cause nephrotoxicity. It has been estimated that ischemia/reperfusion and nephrotoxicants are responsible for 35% of AKI. Nephrotoxicity is a recognized clinical liability of certain classes of drugs; in particular, antibiotics, angiotensinconverting enzyme (ACE) inhibitors and angiotensinreceptor blockers, analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs), radiocontrast media, and anti-cancer agents. Approximately 70% of the patients presenting with drug-induced ARF were nonoliguric; the pathologic findings revealed acute tubular necrosis in 60%. Approximately 50% recovered completely. A myriad of risk factors appear to contribute to the incidence/severity of ARF, including genetic/hereditary factors, volume depletion, septic shock, hypotension, multiple chemical insults, age, diabetes, and preexisting

renal disease. The consequences of ARF can be profound, as permanentrenal damage may result and dialysis or renal transplantation may be required. Chronic renal failure leading to end-stage renal failure has been associated with long-term abuse of analgesics. The incidence of analgesic nephropathy has been reported to be as high as 20–25% in certain countries (e.g., Switzerland). Other agents, such as lithium, cyclosporine, NSAIDs, lead, and cadmium, may produce chronic tubulointerstitial nephropathy with progressive loss of renal function.

### 3.3.2 Reasons for the Susceptibility of the Kidney to Toxicity

The unusual susceptibility of the mammalian kidney to the toxic effects of noxious chemicals can be attributed in part to the unique physiologic and anatomic features of this organ. Although the kidneys constitute only 0.5% of total body mass, they receive about 20–25% of the resting cardiac output. Consequently, any drug or chemical in the systemic circulation will be delivered to these organs in relatively high amounts. The processes involved in forming concentrated urine also serve to concentrate potential toxicants in the tubular fluid. As water and electrolytes are reabsorbed from the glomerular filtrate, chemicals in the tubular fluid may be concentrated, thereby driving passive diffusion of toxicants into tubular cells. Therefore, a nontoxic concentration of a chemical in the plasma may reach toxic concentrations in the kidney. Progressive concentration of toxicants along the nephron may result in intraluminal precipitation of relatively insoluble compounds, causing ARF secondary to tubular obstruction. Finally, renal transport, accumulation, and metabolism of xenobiotics contribute significantly to the susceptibility of the kidney (and specific nephron segments) to toxic injury. In addition to intrarenal factors, the incidence and/or severity of chemically induced nephrotoxicity may be related to the sensitivity of the kidney to circulating vasoactive substances. Under these conditions, vasoconstrictors such as angiotensin II or vasopressin are increased. Normally, the actions of high circulating levels of vasoconstrictor hormones are counterbalanced by the actions of increased vasodilatory prostaglandins; thus, RBF and GFR are maintained. However, when prostaglandin synthesis is suppressed by NSAIDs, RBF declines markedly and ARF ensues, due to the unopposed actions of vasoconstrictors. Another example of predisposing risk factors relates to the clinical use of ACE inhibitors. ACE inhibitors have been reported to produce ARF in patients with severe hypertension, due either to bilateral renal artery stenosis or to renal artery stenosis in a solitary kidney. Under these conditions, glomerular filtration pressure is dependent on angiotensin II-induced efferent arteriolar constriction. ACE inhibitors will block this vasoconstriction, resulting in a precipitous decline in filtration pressure and ARF.

## 3.4 Assessment of Renal Function

Evaluation of the effects of a chemical on the kidney can be accomplished using a variety of both in vivo and in vitro methods. Initially, nephrotoxicity can be assessed by evaluating serum and urine chemistries following treatment with the chemical in question. The standard battery of noninvasive tests includes measurement of urine volume and osmolality, pH, and urinary composition (e.g., electrolytes, glucose, and protein). Although specificity is often lacking in such an assessment, urinalysis provides a relatively easy and noninvasive assessment of overall renal functional integrity and can provide some insight into the nature of the nephrotoxic insult. For example, chemically induced increases in urine volume accompanied by decreases in osmolality may suggest an impaired concentrating ability, possibly via a defect in ADH synthesis, release, and/or action. To determine whether the impaired concentrating ability is due to an altered tubular response to ADH, concentrating ability can be determined before and after an exogenous ADH challenge. Glucosuria may reflect chemically induced defects in proximal tubular reabsorption of sugars; however, because glucosuria also may be secondary to hyperglycemia, measurement of serum glucose concentrations also must be evaluated. Urinary excretion of high-molecular-weight proteins, such as albumin, is suggestive of glomerular damage, whereas excretion of low-molecular-weight proteins, such as β2-microglobulin, suggests proximal tubular injury. Urinary excretion of enzymes localized in the brush border (e.g., alkaline phosphatase, γ -glutamyl transpeptidase) may reflect brush-border damage, whereas urinary excretion of other enzymes (e.g., lactate dehydrogenase) may reflect more generalized cell damage. Enzymuria is often a transient phenomenon, as chemically induced damage may result in an early loss of most of the enzyme available. Thus, the absence of enzymuria does not necessarily reflect an absence of damage. The simultaneous analysis of cellular metabolites in sera and urine using nuclear magnetic analysis (metabonomics) has matured over the past few years and may provide an additional technology to identify and monitor nephrotoxicity (Lindon et al., 2006). For example, rats treated with the nephrotoxicant HgCl2 exhibited increased levels of threonine, isobutyric acid, glutamate, and lysine in renal cortical tissue (Wang et al., 2006) and increased levels of isoleucine and lysine and decreased levels of fumarate in the urine, and that these changes are associated with renal dysfunction (Holmes et al., 2006). However, this technology will require further development and validation using different species and renal insults in the presence and absence of underlying diseases prior to greater use. GFR can be measured directly by determining creatinine or inulin clearance. Creatinine is an endogenous compound released from skeletal muscle at a constant rate under most circumstances. Further, it is completely filtered with limited

secretion. Inulin is an exogenous compound that is completely filtered with no reabsorption or secretion.

## 3.5 Biochemical Mechanisms/Mediators of Renal Cell Injury

### 3.5.1 Cell Death

In many cases, renal cell injury may culminate in cell death. In general, cell death is thought to occur through either oncosis or apoptosis. The morphologic and biochemical characteristics of oncosis ("necrotic cell death") and apoptosis arevery different. For example, apoptosis is a tightly controlled, organized process that usually affects scattered individual cells. The organelles retain integrity while cell volume decreases. Ultimately, the cell breaks into small fragments that are phagocytosed by adjacent cells or macrophages without producing an inflammatory response. In contrast, oncosis often affects many contiguous cells; the organelles swell, cell volume increases, and the cell ruptures with the release of cellular contents, followed by inflammation. With many toxicants, lower but injurious concentrations produce cell death through apoptosis. As the concentration of the toxicant increases, oncosis plays a predominant role. However, because apoptosis is an ATP-dependent process, for those toxicants that target the mitochondrion, oncosis may be the predominant pathway with only limited apoptosis occurring. In general, nephrotoxicants produce cell death through apoptosis and oncosis, and it is likely that both forms of cell death contribute to AKI.

### 3.5.2 Mediators of Toxicity

A chemical can initiate cell injury by a variety of mechanisms. In some cases the chemical may initiate toxicity due to its intrinsic reactivity with cellular macromolecules. For example, amphotericin B reacts with plasma membrane sterols, increasing membrane permeability; fumonisin B1 inhibits sphinganine (sphingosine) *N*-acyltransferase; and $Hg^{2+}$ binds to sulfhydryl groups on cellular proteins. In contrast, some chemicals are not toxic until they are biotransformed to a reactive intermediate. Biologically reactive intermediates, also known as alkylating agents, are electron-deficient compounds (electrophiles) that bind to cellular nucleophiles (electron-rich compounds) such as proteins and lipids. For example, acetaminophen and chloroform are metabolized in the mouse kidney by cytochrome P450 to the reactive intermediates, *N*-acetyl-*p*-benzoquinoneimine and phosgene, respectively. The covalent binding of the reactive intermediate to critical cellular macromolecules is thought to interfere with the normal biological activity of the macromolecule and thereby initiate cellular injury. In other instances, extrarenal biotransformation may be required prior to the delivery of the penultimate

nephrotoxic species to the proximal tubule, where it is metabolized further to a reactive intermediate. Finally, chemicals may initiate injury indirectly by inducing oxidative stress via increased production of ROS, such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. ROS can react with a variety of cellular constituents to induce toxicity. For example, ROS are capable of inducing lipid peroxidation, which may result in altered membrane fluidity, enzyme activity, and membrane permeability and transport characteristics; inactivating cellular enzymes by directly oxidizing critical protein sulfhydryl or amino groups; depolymerizing polysaccharides; and inducing DNA strand breaks and chromosome breakage. Each of these events could lead to cell injury and/or death. Oxidative stress has been proposed to contribute, at least in part, to the nephrotoxicity associated with ischemia/reperfusion injury, gentamicin, cyclosporine, cisplatin, and haloalkene cysteine conjugates (Ueda *et al.*, 2001). While nitric oxide is an important second messenger in a number of physiologic pathways, recent studies suggest that in the presence of oxidative stress, nitric oxide can be converted into reactive nitrogen species that contribute to cellular injury and death. For example, in the presence of superoxide anion, nitric oxide can be transformed into peroxynitrite (ONOO–), a strong oxidant and nitrating species. Proteins, lipids, and DNA are all targets of peroxynitrite. The primary evidence for a role of peroxynitrite in renal ischemia/reperfusion injury is the formation of nitrotyrosine-protein adducts and the attenuation of renal dysfunction through the inhibition of the inducible form of nitric oxide synthase (Ueda *et al.*, 2001).

### 3.5.3 Cellular/Subcellular and Molecular Targets

A number of cellular targets have been identified to play a role in cell death. It is generally thought that an intracellular interaction (e.g., an alkylating agent or ROS with a macromolecule) initiates a sequence of events that leads to cell death. In the case of oncosis, a “point of no return” is reached in which the cell will die regardless of any intervention. The idea of a single sequence of events is probably simplistic for most toxicants, given the extensive number of targets available for alkylating species and ROS. Rather multiple pathways, with both distinct and common sequences of events, may lead to cell death.

### 3.5.4 Cell Volume and Ion Homeostasis

Cell volume and ion homeostasis are tightly regulated and are critical for the reabsorptive properties of the tubular epithelial cells. Toxicants generally disrupt cell volume and ion homeostasis by interacting with the plasmamembrane and increasing ion permeability or by inhibiting energy production. The loss of ATP, for example, results in the inhibition of membrane transporters that maintain the internal ion balance and drive

transmembrane ion movement. Following ATP depletion, Na+, K+-ATPase activity decreases, resulting in K+ efflux, Na+ and Cl– influx, cell swelling, and ultimately cell membrane rupture. Miller and Schnellmann (1995) have proposed that ATP depletion in rabbit renal proximal tubule segments initially results in K+ efflux and Na+ influx followed by a lag period before Cl– influx occurs. Cl– influx occurs during the late stages of cell injury produced by a diverse group of toxicants and appears to be due to the volume-sensitive, outwardly rectifying (VSOR) Cl– channel (Okada *et al.*, 2004). Cl– influx may be a trigger for cell swelling, because decreasing Cl– influx decreased cell swelling and cell death, and inhibition of cell swelling decreased cell lysis but not Cl– influx. Meng and Reeves (2000) have reported similar findings using hydrogen peroxide as the toxicant and LLCPK1 cells. In contrast, the cell shrinkage that occurs during apoptosis is mediated by K+ and Cl- efflux through respective channels and inhibition of these channels is cytoprotective (Okada *et al.*, 2004).

### 3.5.5 Cytoskeleton and Cell Polarity

Toxicants may cause early changes in membrane integrity such as loss of the brush border, blebbing of the plasma membrane, or alterations inmembrane polarity. These changes canresultfrom toxicantinduced alterations in cytoskeleton components and cytoskeletalmembrane interactions, or theymay be associated with perturbations in energy metabolism or calcium and phospholipid homeostasis. Marked changes in the polarity of tubular epithelium occur following an ischemic insult. Under controlled conditions, the tubular epithelial cell is polarized with respect to certain transporters and enzymes. During in vivo ischemia and in vitro ATP depletion there is a dissociation of Na+, K+-ATPase from the actin cytoskeleton and redistribution from the basolateral membrane to the apical domain in renal proximal tubule cells. The redistribution of this enzyme has been postulated to explain decreased $Na^+$ and water reabsorption during ischemic injury.

### 3.5.6 Mitochondria

Many cellular processes depend on mitochondrial ATP and thus become compromised simultaneously with inhibition of respiration. Conversely, mitochondrial dysfunction may be a consequence of some other cellular process altered by the toxicant. Numerous nephrotoxicants causemitochondrial dysfunction. For example, following an in vivo exposure, $HgCl_2$ altered isolated renal cortical mitochondrial function and mitochondrial morphology prior to the appearance of tubular necrosis. Furthermore, $HgCl_2$ produced similar changes in various respiratory parameters when added to isolated rat renal cortical mitochondria. Different toxicants also produce different types of mitochondrial

dysfunction. For example, pentachlorobutadienyl-l-cysteine initially uncouples oxidative phosphorylation in renal proximal tubular cells by dissipating the proton gradient, whereas TFEC does not uncouple oxidative phosphorylation but rather inhibits state 3 respiration by inhibiting sites I and II of the electron transport chain. Whether toxicants target mitochondria directly or indirectly, it is clear that mitochondria play a critical role in determining whether cells die by apoptosis or oncosis. The mitochondrial permeability transition (MPT) is characterized by the opening of a highconductance pore that allows solutes of <1500 molecular weight to pass. It is thought that the MPT occurs during cell injury and ultimately progresses to apoptosis if sufficient ATP is available or oncosis if ATP is depleted. Further, the release of apoptotic proteins such as apoptosis inducing factor (AIF), cytochrome c, Smac/Diablo, Omi and Endonuclease G following MPT play a key role in activating downstream caspases and executing apoptosis.

### 3.5.7 $Ca^{2+}$ Homeostasis

$Ca^{2+}$ is a second messenger and plays a critical role in a variety of cellular functions. The distribution of $Ca^{2+}$ within renal cells is complex and involves binding to anionic sites on macromolecules and compartmentation within subcellular organelles. The cytosolic free $Ca^{2+}$ concentration of this pool is approximately 100 nM and is maintained at this level against a large extracellular/intracellular gradient (10,000:1) by a series of pumps and channels located on the plasma membrane and endoplasmic reticulum (ER). Because the proximal tubular cells reabsorb approximately 50–60% of the filtered load of $Ca^{2+}$, they must maintain low cytosolic $Ca^{2+}$ concentrations during a large $Ca^{2+}$ flux. Sustained elevations or abnormally large increases in cytosolic free $Ca^{2+}$ can exert a number of detrimental effects on the cell. For example, an increase in cytosolic free $Ca^{2+}$ can activate a number of degradative $Ca^{2+}$-dependent enzymes, such as phospholipases and proteinases (e.g., calpains), and can produce aberrations in the structure and function of cytoskeletal elements. While the precise role of $Ca^{2+}$ in toxicant-induced injury remains unclear, release of ER $Ca^{2+}$ stores may be a key step in initiating the injury process and increasing cytosolic free $Ca^{2+}$ concentrations (Harriman *et al.*, 2002). For example, prior depletion of ER $Ca^{2+}$ stores protects renal proximal tubules from extracellular $Ca^{2+}$ influx and cell death produced by mitochondrial inhibition and hypoxia). Further, the release of ER $Ca^{2+}$ activates calpains which leads to further disruption of ion homeostasis, cleavage of cytoskeleton proteins, cell swelling, and, ultimately oncosis. Mitochondria are known to accumulate $Ca^{2+}$ in lethally injured cells through a low-affinity, high-capacity $Ca^{2+}$ transport system. While this system plays a minor role in normal cellular $Ca^{2+}$ regulation, under injurious conditions the uptake of $Ca^{2+}$ may facilitate ROS formation and damage.

## 4.0 CONCLUSION

In this unit, the toxic responses of the kidney have been examined. The kidney is vulnerable vulnerable to chronic toxicity.

## 5.0 SUMMARY

In this unit, we have learnt some of the substances that are toxic to the kidney and the paraemeters that can be used to examine nephrotoxicity.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. Mention 10 substances that can be toxic to the human kidney with refence to scientific articles.
2. Evaluation of the effects of a chemical on the kidney can be accomplished using a variety of both in vivo and in vitro methods. Discuss.

## 7.0 REFERENCES/FURTHER READING

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**MODULE 3**

Unit 1 Immune Systems
Unit 2 Respiratory Systems
Unit 3 Nervous System
Unit 4 Eye

## UNIT 1 IMMUNE SYSTEMS

**CONTENTS**



## 1.0 INTRODUCTION

For many years, it has been widely established that xenobiotics can have significant effects on the immune system. More recently, and during the establishment of the subdiscipline of immunotoxicology, a significant emphasis was placed on the development of a standardized battery of tests to evaluate immune competence. Among the unique features of the immune system is the ability of immune cells to be removed from the body and to function in vitro. This unique quality makes it possible to comprehensively evaluate the actions of xenobiotics on the immune system employing in vivo, ex vivo, and in vitro approaches to dissect the cellular, biochemical, and molecular mechanisms of action of xenobiotics. While standard toxicological end points such as organ weights, cellularity, and enumeration of cell subpopulations are important components in assessing when an agent is capable of altering the immune system, by far the most sensitive indicators of immunotoxicity are the tests that challenge the various immune cells to respond functionally to

exogenous stimuli. Employing such a battery of functional assays whereby different cell types can be evaluated not only for their effector functions, but also in certain cases for their ability to participate as accessory cells in an immune response, can provide important mechanistic information concerningwhich cell type(s) within the immune system are, in fact, targeted by a xenobiotic.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- understand the methods of using immunocompetence in toxicological studies
- understand the modulation of the immune system by xenobiotics.

## 3.0 MAIN CONTENT

### 3.1 Methods to Assess Immunocompetence

#### 3.1.1 General Assessment

Central to any series of studies evaluating immunocompetence is the inclusion of standard toxicological studies, because any immunologic finding should be interpreted in conjunction with effects observed on other target organs. Standard toxicological studies that are usually evaluated include body and selected organ weights, general observations of overall animal health, selected serum chemistries, hematologic parameters, and status of the bone marrow (ability to generate specific colony-forming units). In addition, histopathology of lymphoid organs, such as the spleen, thymus, and lymph nodes, may provide insight into potential immunotoxicants. Because of the unique nature of the immune system, there are several experimental approaches that may be taken to assess immunotoxicity and to evaluate the mechanisms of action of xenobiotics.

#### 3.1.2 Functional Assessment

##### 3.1.2.1 Innate Immunity

Innate immunity encompasses all those immunologic responses that do not require prior exposure to an antigen and that are nonspecific in nature. These responses include recognition of tumor cells by NK cells, phagocytosis of pathogens by macrophages, and the lytic activity of the components of the complement cascade. To evaluate phagocytic activity, macrophages are harvested from the peritoneal cavity (peritoneal exudate cells) and are allowed to adhere in tissue culture plates. The cells are then

incubated with chromated chicken red blood cells (51Cr-cRBCs). Following incubation, the supernatant, containing 51Cr-cRBCs that have not been bound by macrophages, is removed. The cRBCs which are bound to the macrophages, but which have not been phagocytized, are removed by a brief incubation with ammonium chloride. Finally, macrophages are lysed with NaOH and radioactivity in the lysate iscounted to determine the amount of phagocytosis that occurred. A set of control wells is needed to determine DNA content for each set of wells. Data are presented as a specific activity for adherence and phagocytosis (adhered or phagocytized counts per minute (cpm)/DNA content) because xenobiotics altering adherence will have a significant effect on the results. Another method to evaluate phagocytosis, but which does not require radioactivity, begins similarly to the 51Cr-cRBC assay. Peritoneal macrophages are allowed to adhere to each chamber of a tissue culture slide. After adherence, macrophages are washed and incubated with latex covaspheres. At the end of incubation, cells are fixed in methanol and stained in methylene chloride. Macrophages containing five covaspheres or more are counted as positive and data are expressed as a percentage of phagocytosis (the ratio of macrophages with ≥5 covaspheres to total macrophages counted).

The previous macrophage assays are conducted in vitro after chemical exposure either in vivo or in vitro. If an in vivo assay to assess the ability of tissue macrophages to phagocytose a foreign antigen is required, the functional activity of the reticuloendothelial system can be evaluated. Intravenously injected radiolabeled sRBCs (51Cr-sRBCs) are removed by the tissue macrophage from the circulation and sequestered for degradation in organs such as the liver, spleen, lymph nodes, lung, and thymus. Clearance of the 51CrsRBCs is monitored by sampling of the peripheral blood. When steady state has been attained, animals are killed and organs are removed and counted in a gamma counter to assess uptake of the 51Cr-sRBCs.

### 3.1.2.2 Acquired Immunity—Cell Mediated

While there are numerous assays used to assess CMI, three primary tests are used routinely in the National Toxicology Program (NTP) test battery. The test battery includes the CTL assay, DTH response, and the T-cell proliferative responses to antigens (anti-CD3 + IL-2), mitogens (phytohemagluttinin and concanavalin A), and allogeneic cell antigens [mixed lymphocyte responses (MLRs)]. The CTL assay measures the in vitro ability of splenic T cells to recognize allogeneic target cells by evaluating the ability of the CTLs to proliferate and then lyse the target cells. Splenocytes are incubated with P815 mastocytoma cells, which serve as target cells. These target cells are pretreated with mitomycin C so that they cannot proliferate themselves. During this sensitization phase,

the CTLs recognize the targets and undergo proliferation. At 5 days after sensitization, the CTLs are harvested and incubated in microtiter plates with radiolabeled (51Cr) P815 mastocytoma cells. During this elicitation phase, the CTLs that have acquired memory recognize the foreign MHC class I on the P815 cells and lyse the targets. At the end of the incubation, plates are centrifuged, the supernatant is removed, and radioactivity released into the supernatant is counted on a gamma counter. After correcting for spontaneous release, the percent cytotoxicity is calculated for each effector-to-target ratio and compared to that from control animals. The DTH response evaluates the ability of memory T cells to recognize foreign antigen, proliferate and migrate to the site of the antigen, and secrete cytokines and chemokines, which result in the influx of other inflammatory cells. The assay itself quantifies the influx of radiolabeled monocytes into the sensitization site. During xenobiotic exposure, mice are sensitized twice with keyhole limpet hemocyanin subcutaneously between the shoulders. On the last day of exposure, mononuclear cells are labeled in vivowith anIV injection of 125I-5-iododeoxyuridine. One day later, mice are challenged intradermally in one ear with keyhole limpet hemocyanin. Twenty-four hours after challenge, animals are killed, the ears are biopsied, and radiolabeled cells are counted in a gamma counter. Data are expressed as a stimulation index, which represents the cpm of 125I activity in the challenged ear divided by the cpm in the unchallenged ear.

T cells play a central role in CMI and the ability of T cells to undergo blastogenesis and proliferation is critical to this role. Several mechanisms exist to evaluate proliferative capacity. The MLR measures the ability of T cells to recognize foreign MHC class I on splenocytes from an MHC-incompatible mouse (allogeneic cells) and undergo proliferation. For example, splenocytes from B6C3F1 mice (responders) are incubated with splenocytes from mitomycin C-treated DBA/2 mice (stimulators). Proliferation is evaluated 4–5 days after stimulation by measuring uptake of 3H-thymidine into the DNA of the cultured responder cells. Cells are collected from each well using a cell harvester and counted in a scintillation counter. Data may be expressed as either the mean cpm for each treatment group or as a stimulation index where the index is calculated by dividing the cpm of wells containing responders and stimulators by the cpm of wells containing responders alone. Splenocytes are stimulated in microtiter plates with a monoclonal antibody to the CD3 complex of the TCR (anti-CD3) in the presence of IL-2, or with the T-cell mitogens concanavalin A or phytohemagluttinin. Proliferation is evaluated 2–3 days after stimulation by measuring uptake of 3H-thymidine into the DNA of the cultured T cells. Data are usually expressed as mean cpm for each treatment group. These studies are usually done in conjunction with B-cell proliferative responses.

### 3.1.2.3 Flow Cytometric Analysis

One of the most rapidly advancing areas and powerful tools in immunotoxicology has been the application of fluorescence-activated cell sorting, or more commonly referred to as flow cytometry. In the most general sense, flow cytometry is a method that employs light scatter, fluorescence, and absorbance measurements to analyze large numbers of cells (typically 5000–20,000/sample) on an individual basis. Most commonly, fluorochrome-conjugated monoclonal antibodies raised against a specific protein of interest are employed for detection. The strength of the approach is that a wide variety of measurements can be made on large numbers of cells, rapidly, and with a high level of precision. In addition, methods are now available that allow for the analysis of specific proteins in cell-free preparations such as cell lysates and culture supernatants. A broad selection of monoclonal antibodies is now available to cell surface markers, intracellular proteins, and secreted proteins. The most common application of flow cytometry in immunotoxicology is to enumerate specific leukocyte populations and subpopulations. For example, antibodies are available to the T-cell surface markers CD4, CD8, and CD3 (among others). Because flow cytometers can detect light emission of multiple wavelengths simultaneously, multiple-colored fluorochromes can be used concurrently facilitating an analysis of more than one protein simultaneously This approach can be used to provide insight into which specific T-cell subsets are targeted after exposure to a xenobiotic, and to identify putative effects on T-cell maturation. Similarly, antibodies are readily available to identify other leukocyte subpopulations including to surface immunoglobulin and to B220 (the CD45 phosphatase on B cells) for enumerating B cells, Mac1, and F4/80 for macrophages, CD16/CD56 for NK cells in humans and CD161 (NK-1.1) in mice. Surface marker analysis of heterogenous cell preparations can reveal significant alterations in lymphoid subpopulations, and in many instances this is indicative of alterations in immunologic integrity. Indeed, an indicator of AIDS is the changes observed in CD4+ T-cell numbers. Luster *et al.*, (1992) reported that, in conjunction with two or three functional tests, the enumeration of lymphocyte subsets can greatly enhance the detection of immunotoxic chemicals. However, it is important to emphasize that although surface marker analysis can identify changes in leukocyte populations, functional analysis of the immune system is more definitive for the detection of immunotoxicity because the ability of immunocompetent cells to mount an effector response is assessed directly. With technical advancements of flow cytometers (i.e., an increased number of wavelengths that can be simultaneously detected, enhanced sensitivity of detection and more rapid analysis) coupled with a steady growth of applications, reagents, and methods, flow cytometry has become an integral tool in elucidating the cellular and molecular mechanisms of action produced by immunotoxicants. Current

applications go well beyond strictly evaluating cell surface markers and include measurements of cell cycle, intracellular free calcium, cellular viability, induction of apoptosis, DNA strand breaks (TUNEL assay), intracellular proteins, membrane potential, intracellular pH, oxidative stress, and membrane lipophilicity. A major recent advancement in flow cytometry-based analyses has been the development of fluorescent microspheres that are individually identified by the instrument. By coating the surface of microspheres with various concentrations of two fluorescent dyes, sets of microspheres can be generated with each set possessing a unique spectral signature. Subsequently, various materials (i.e., proteins, antibodies, or nucleic acids) can be covalently conjugated to the surface of these microspheres in order to create unique detection systems. This technology is being widely applied for analyzing a broad variety of soluble cellular components including proteins in cell-free preparations by flow cytometry. For example, up to 15 different cytokines can be measured simultaneously in cell supernatants or biological fluids (e.g., bronchoalveolar lavage). A major advantage of this method over more traditional approaches, such as ELISA-based assays, is that only small amounts of biological samples and reagents are required as multiple proteins are assayed simultaneously. The same technology is also being applied for analyzing cell lysates for changes in protein phosphorylation in investigations of cell signaling. In addition, flow cytometry is routinely used to purify and isolate leukocyte subpopulation from heterogeneous cellular preparations. Thus, flow cytometry has become a powerful tool for characterizing the cellular and molecular mechanisms associated with immunotoxicants.

### 3.1.2.4 Measurements of Cytokines and Cytokine Profiling

Development, maturation, differentiation, and effector responses of the immune system are highly dependent on a multitude of small secreted proteins termed cytokines. In most cases, these immunologic processes are controlled by the production of multiple cytokines, some of which are released simultaneously, whereas others are released in a very defined temporal sequence. Many of these cytokines are produced by T cells and are the mechanism by which a wide variety of functions by T cells are mediated. Due to the importance of cytokines in regulating the immune system, xenobiotics that alter the production and release of these mediators can significantly affect immune competence. Therefore, measurement of multiple cytokines, often referred to as cytokine profiling, has become routine in immunotoxicology and can provide significant insights into the mechanisms by which a xenobiotic produces its immunotoxicity. For example, cytokine profiling has been explored more recently as an approach for identifying chemical allergens, either contact sensitizers, which typically induce a Th1 profile of cytokines (IL-2, IFN-γ , and TNF-β), or respiratory sensitizers, which typically produce

a strong Th2 cytokine profile (IL-4, IL-5, IL-6, and IL-10). Cytokines are most commonly measured in cell culture supernatants or biological fluids (i.e., serum or bronchoalveolar lavage) by ELISA. Quantification of test samples is accomplished by comparison to a standard curve employing recombinant cytokine standards. There is also a relatively new ELISA-type assay, the ELISPOT, which measures the number of cells producing the cytokine of interest. Cytokines in media or biological fluids can also be accurately assayed and quantified by flow cytometery with the main advantage being that many cytokines can be assayed simultaneously from one sample. Because cytokines are regulated transcriptionally and then actively synthesized by cells at the time they are secreted, rather than existing as stored proteins, measurements of cytokine mRNA levels has become another common approach of assessing which cytokines are being expressed at a given time and the putative effects xenobiotics exert on their regulation and expression. Real-time polymerase chain reaction is most commonly used for quantifying mRNA levels of specific cytokines, as well as for other genes of interest in cells or tissues but other approaches including RNase protection and microarrays are also being more widely employed. Major advantages to quantifying cytokine expression at the mRNA rather than protein level include significantly greater sensitivity, the ability to quantify expression in solid tissues, and the capability to rapidly design appropriate reagents (PCR primers) specific for any cytokine. The major disadvantage is that changes at the mRNA level for a given cytokine may not necessarily correlate with changes in protein. One additional limitation to cytokine measurements by ELISA or via quantification of cytokine mRNA levels is that neither of these approaches provide information concerning the biological activity of the proteins being measured.

### 3.1.2.5 Host Resistance Assays

Host resistance assays represent a way of assessing how xenobiotic exposure affects the ability of the host to combat infection by a variety of pathogens. Although host resistance studies provide significant insight into the mechanisms by which an immunotoxicant is acting, these assays are not used as a first or only choice for evaluating immunocompetence. The results from host resistance assays are typically more variable than other immune function assays already discussed, and therefore require markedly greater numbers of animals in order to obtain statistical power. The increased number of animals required also raises ethical considerations as well as cost. In addition, as with other immune function tests, no single host resistance model can predict overall immunocompetence of the host, primarily because each model uses different mechanisms for elimination of various pathogens. Typically, three challenge levels of pathogen (approximating the LD20, LD50, and LD80) for each concentration of xenobiotic are used in order to be able to

detect both increases and decreases in resistance. End point analyses are lethality (for bacterial and viral pathogens), changes in tumor burden, and increased or decreased parasitemia. In host resistance studies, it is also important to consider the following: (1) strain, route of administration, and challenge size of the pathogen; (2) strain, age, and sex of the host; (3) physiological state of the host and the pathogen; and (4) time of challenge with the pathogen (prior to, during, or after xenobiotic exposure). All of these can have significant effects on the results from any individual study.

Assessment of Developmental Immunotoxicology Interest in developmental immunotoxicology is predicated on the recognition that the developing immune system represents a novel target for xenobiotic-induced toxicity that presents some special considerations when it comes to assessment. This unique susceptibility may be manifested as a qualitative difference, in the sense that a chemical could affect the developing immune system without affecting the adult immune system, or as a quantitative difference, in the sense that a chemical could affect the developing immune system at lower doses than the adult immune system, or as a temporal difference, in the sense that a chemical could produce either a more persistent effect in younger animals than adults, or trigger a delayed effect (i.e., the consequences of early exposure are not manifested until early adulthood). As a result, while effective assessment of developmental immunotoxicology should certainly draw upon the prior experience with adult-exposure immunotoxicity assessment, it is important to examine the database of known immune changes that reflect the potential for unique susceptibility, and are specific for the developing immune. One of the most comprehensive reports to date attempted to compare the immunotoxicity following developmental or adult exposure to the following compounds: diethylstilbestrol, diazepam, lead, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and tributyltin oxide (Luebke et al., 2006).

The selection of these five compounds was reported to be based on the availability of some human data. The authors concluded that for all five chemicals, the developing immune system was found to be at greater risk than the adult, either because lower doses produced immunotoxicity, adverse effects were persistent, or both. A better understanding of the developing immune system, and in particular, an understanding of critical developmental landmarks has prompted some to speculate about the existence of five critical “windows” of vulnerability (Dietert et al., 2000). The first window encompasses a period of hematopoietic stem cell formation from undifferentiated mesenchymal cells. Exposure of the embryo to toxic chemicals during this period could result in failures of stem cell formation, abnormalities in production of all hematopoietic lineages, and immune failure. The second window is characterized by migration of hematopoietic cells to the fetal liver and thymus,

differentiation of lineage-restricted stem cells, and expansion of progenitor cells for each leukocyte lineage. This developmental window is likely to be particularly sensitive to agents that interrupt cell migration, adhesion, and proliferation. The critical developmental events during the third window are the establishment of bone marrow as the primary hematopoietic site and the establishment of the bone marrow and the thymus as the primary lymphopoietic sites for Band T cells, respectively. The fourth window addresses the critical periods of immune system functional development, including the initial period of perinatal immunodeficiency, and the maturation of the immune system to adult levels of competence. The final window, addresses the subsequent period during which mature immune responses are manifest, and functional pools of protective memory cells are established. Most recently, considerable attention has been focused on the perinatal period (i.e., prior to and just after birth) because this window of development is known to be replete with dynamic immune changes, many of which do not occur in adults. Indeed, one reality associated with developmental immunotoxicology windows is that the developing immune system exists in an unbalanced state through the latter portion of gestation with certain functional CMI capacities deliberately impaired. In fact, Taylor et al. (2006) demonstrated that placentally induced immune skewing via the release of Fas-ligand-containing exosomes is one hallmark of a successful pregnancy brought to full term. Upon birth, restoring effective immune balance through the enhancement of Th1 capacity in the newborn is critical for protecting childhood health.

Prenatal maturation and functional skewing of the fetal immune system followed by the rapid reversal of the imbalance at birth has features that are not effectively modeled using adult exposure–assessment, and developmental immunotoxicity is best viewed as a continuum of alterations. Suppression of the developing immune system, manifested as increased susceptibility to infections and cancer, is not the only concern, and immunotoxic changes that increase the risk for allergic or autoimmune responses in later life should also be considered. Adding to the complexity is the demonstration that some developmental immunotoxicants seem capable of inducing targeted immune suppression while at the same time elevating the risk of allergy and/or autoimmunity. Because either significant immune suppression or disrupted immune regulation is a concern and needs to be detected, the most effective methodology for assessing developmental immunotoxicity must also be capable of assessing significant changes in immune balance. In spite of the increased interest in assessing the potential for developmental immunotoxicity, it must be emphasized that neither validated nor widely accepted methods currently exist for evaluating the effects of a chemical on the developing immune system. In constructing a developmental immunotoxicity testingframework, one of the first points to consider is

the selection of an animal model. Consistently, the rat has been identified as the preferred species for evaluations of developmental immunotoxicity, largely due to the fact that the rat has been utilized extensively in guideline developmental and reproductive toxicology testing.

A review compared the anatomical and functional differences in the immune systems of the mouse, rat, dog, primate, and human, and their use as models for developmental immunotoxicity testing (Holsapple et al., 2003). The review concluded that the developing immune systems of mice and humans have been best characterized to date. In addition, the review also concluded that immune ontogeny in the mouse and rat is likely similar. Another important consideration when selecting a species is that the development of the immune system in the rodent is delayed relative to the human, and how this differential maturation will impact data extrapolation for predicting human risk. For example, some developmental landmarks observed in utero in humans, occur after parturition in the rat. A second consideration when constructing a framework for assessing developmental immunotoxicity concerns gender-specific effects. Results from perinatal exposure to xenobiotics suggest that significant sex-based differences in immunotoxic sensitivity are common and are at least as prevalent, if not more frequent, compared with the incidence observed following adult exposure–assessment (Luebke et al., 2006). As such, for the evaluation of developmental immunotoxicity, testing of both sexes is critical.

A third major consideration in developmental immunotoxicology testing framework is a consideration of exposure. The best exposure protocol is one where exposure occurs across all non-adult developmental windows followed either by immediate assessment or assessment after a few weeks. Exposure to pregnant dams has been a hallmark feature of most developmental immunotoxicology protocols, and the maternal influences on exposure to the fetus/newborn pups would be dependent on transfer of the xenobiotic either across the placenta or via lactation. The gestational (e.g., transplacental) and lactational periods in the rat would result in exposure from conception to early postweaning in the pup, approximately 3 weeks of age. Direct exposure of pups via the diet would generally commence at about 3 weeks after birth. An unresolved issue is whether direct exposure of the pups, which is generally accepted as a routine procedure at around postnatal Day 7, should occur during the lactational period as well (Ladics et al., 2005); however, any decisions concerning this issue will require consideration of how humans would be exposed and the specific properties of the chemical being studied. For example, if exposure is only oral and there is no reason to believe that the lactational transfer differs significantly for a class of compounds between rat and human, then direct dosing of pups could be initiated postweaning. In addition, information on pharmacokinetic and dosimetry could be useful

in determining whether any direct dosing of pups during lactation is necessary. However, it is important to emphasize that this type of information is not routinely available for most xenobiotics. A fourth consideration in creating a developmental immunotoxicology testing framework concerns which specific end points to measure. Immune organs, like the thymus, spleen, and bone marrow, are not typically assessed in routine developmental and reproductive toxicology studies, and it has been the consensus of recent workshops that histopathological evaluation of these immune organs could be easily integrated into these protocols (Luster et al., 2003; Holsapple et al., 2005).

However, presently, there is uncertainty whether routine histopathology is sufficiently sensitive to detect all potential immunotoxic effects, especially when the unique characteristics of the developing immune system are considered. Indeed, there are examples where morphometric histopathologic findings do not predict functional impairments due to toxicity produced on the developing immune system. Ultimately, while the use of specific rather than general histopathology has been recommended, it has been also suggested that functional tests be employed in the assessment of developmental immunotoxicity. Unfortunately, few if any functional assays have been validated for detection of developmental immunotoxicity, even for routine assays like the Tcell-dependent antibody response, which has largely been confined to adult exposure protocols. Results published on several chemicals and drugs in recent years suggest that functional tests are a front-line priority for perinatal immunotoxicity detection and that a combination of at least two functional tests, such as a multi-isotype T-cell-dependent antibody response, and a cell-mediated immune response assay, such as the DTH assay and/or CTL or NK cytotoxicity assays, should be paired with histopathological analysis and phenotypic analysis of lymphocyte subsets using flow cytometry. One final point regarding the evaluation of developmental immunotoxicity needs to be considered. As developmental immunotoxicity protocols are inserted into exising toxicology testing regimes, such as developmental and reproductive toxicology protocols, it is likely to be necessary to incorporate immunization protocols. This approach has raised concerns among those evaluating other physiological systems (e.g., reproductive and neurological) in terms of potential immunization-induced changes. However, investigations addressing this potential by determining the impact of the incorporation of immunotoxicological functional assays on standard toxicological studies in rats have been largely negative.

## 4.0 CONCLUSION

The approaches to assess immuntoxicity have been examined in this unit.

## 5.0 SUMMARY

In the unit we have learnt processes involved in evaluating immunotoxicity.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. What is immunocompetence?
2. Describe the approaches that are taken to examine immunotoxicity.

## 7.0 REFERENCES/FURTHER READING

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# UNIT 2 RESPIRATORY SYSTEMS

## CONTENTS



## 1.0 INTRODUCTION

Lung injury caused by chemicals was first recognized as an occupational disease. In 1713, the Italian physician Bernardino Ramazzini provided

detailed and harrowing accounts of the sufferings of miners. Two of his quotations remain noteworthy. With regard to miners of metals, he stated, "the lungs and brains of that class of workers are badly affected, the lungs especially, since they take in with the air mineral spirits and are the first to be keenly aware of injury." Ramazzini was also aware of the important concept of exposure: "They (workers who shovel, melt, cast and refine mined material) are liable of the same diseases, though in less acute form, because they perform their tasks in open air (Ramazzini, 1964)." Thus, exposure to chemicals by inhalation can have two effects: on the lung tissues and on distant organs that are reached after chemicals enter the body by means of inhalation. Indeed, "inhalation toxicology" refers to the route of exposure, whereas "respiratory tract toxicology" refers to target organ toxicity, in this case abnormal changes in the respiratory tract produced by airborne (and occasionally blood-borne) agents. Examples of lung diseases prompted by occupational exposures include black lung in coal miners, silicosis and silicotuberculosis in sandblasters and tunnel miners, and asbestosis in shipyard workers and asbestos miners. Occupational exposures to asbestos or metals such as nickel, beryllium, and cadmium can also cause lung cancer. In the twentieth century, it has become obvious that disease caused by airborne agents may not be limited to certain trades. The ubiquitous presence of airborne chemicals is a matter of concern, since "air pollution" adversely affects human health and may be an important contributor to morbidity and mortality.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- describe the pathogenesis of lung damage induced by toxicants
- describe the methods of assessing the toxicity of chemical to the respiratory system.

## 3.0 MAIN CONTENT

### 3.1 General Principles in the Pathogenesis of Lung Damage Caused by Chemicals

#### 3.1.1 Toxic Inhalants, Gases, and Dosimetry

The sites of deposition of gases in the respiratory tract define the pattern of toxicity of those gases. Water solubility is the critical factor in determining how deeply a given gas penetrates into the lung. Highly soluble gases such as $SO_2$ do not penetrate farther than the nose unless doses are very high, and are therefore relatively nontoxic to animals, especially obligatory nose breathers, such as the rat. Relatively insoluble gases such as ozone and $NO_2$ penetrate deeply into the lung and reach the

smallest airways and the alveoli (centriacinar region), where they can elicit toxic responses. Mathematical models of gas entry and deposition in the lung that are based solely on the aqueous solubility of a gas predict sites of lung lesions fairly accurately. These models may be useful for extrapolating findings made in laboratory animals to humans. Very insoluble gases such as CO and $H_2S$ efficiently pass through the respiratory tract and are taken up by the pulmonary blood supply to be distributed throughout the body.

### 3.1.2 Particle Deposition

Particle size is usually the critical factor that determines the region of the respiratory tract in which a particle or an aerosol will be deposited. Deposition of particles on the surface of the lung and airways is brought about by a combination of lung anatomy and the patterns of airflow in the respiratory system.

### 3.1.3 Particle Size

The median diameter that is determined may reflect the number of particles, as in the count median diameter (CMD), or reflect mass, as in the mass median aerodynamic diameter (MMAD). If more particles actually reach the deep lung, the higher is the probability of a toxic effect. Particle surface area is of special importance when toxic materials are adsorbed on particles and thus are carried into the lung. Large particles (larger than 5 μm MMAD) are usually trapped in the upper respiratory tract (nasopharyngeal region and large conducting airways), whereas smaller particles (0.2–5 μm MMAD) can be transported to the smaller airways and the alveoli. Patterns of breathing can change the site of deposition of a particle of a given size. Inhaled ambient particles and aerosols are most frequently polydisperse in size. The size distribution of many aerosols approximates a log-normal distribution that may be described by the median or geometric mean and the geometric standard deviation. A plot of the frequency of occurrence of a given size against the log of the size produces a bell-shaped probability curve. Particle data frequently are analyzed by plotting the cumulative percentage of particles smaller than a stated size on log-probability paper. This results in a straight line that may be fitted by eye or mathematically. In actual practice, it is not unusual to have some deviation from a straight line at the largest or smallest particle sizes measured. The geometric mean is the 50% size as the mean bisects the curve. The geometric standard deviation (σ g) is calculated as σg = 84.1% size/50% size The σ g of the particle size distribution is a measure of the polydispersity of the aerosol. In the laboratory, values for σ g of 1.8–3.0 are encountered frequently. In the field, value for σ g may range up to 4.5. An aerosol with a σ g below 1.2 may be considered monodispersed. Particles that are nonspherical in

shape are frequently characterized in terms of equivalent spheres on the basis of equal mass, volume, or aerodynamic drag. The MMAD takes into account both the density of the particle and aerodynamic drag. It represents the diameter of a unit density sphere with the same terminal settling velocity as the particle, regardless of its size, shape, and density. Aerodynamic diameter is the proper measurement for particles that are deposited by impaction and sedimentation. For very small particles, which are deposited primarily by diffusion, the critical factor is particle size, not density. It must be kept in mind that the size of a particle may change before its deposition in the respiratory tract. Materials that are hygroscopic, such as sodium chloride, sulfuric acid, and glycerol, take on water and grow in size in the warm, saturated atmosphere of the upper and lower respiratory tract.

### 3.1.4 Nanotoxicology

There is intense current interest in the lung toxicity of nanoparticles, particles with diameters of <100 nm. Ultrafine particles of this size range are increasingly being used in manufactured products, and synthesis and release of particles of this size to the environment, and exposure of individuals in the workplace, is increasing exponentially. In addition, emissions control devices on internal combustion engines, diesel engines, and industrial furnaces and burners can efficiently trap larger particles; however, technology that decreases emissions of larger particles (which contain most of the mass of the particulate fraction) generally produces greater amounts of ultrafine particles (on a particle number basis). The toxicological concerns reflect threemajor issues: (1) the enormous surface area of these nanoparticles relative to their mass, especially with regard to the adsorption of co-pollutants and the presence of reactive metals on their surfaces, (2) commercially important forms of nanoparticles include nanotubes, which are high axial ratio rods that provoke concerns that they might be far more toxic than spheres of the same MMAD, and (3) the question of whether normal host defenses are effective against particles this small, which can be readily transported through and out of the lung to other tissues via pathways that are not normally accessible to larger particles. A major technical issue in interpretation of the emerging literature that tries to address this topic is whether intratracheally (IT) administered nanoparticles behave similarly to inhaled nanoparticles, as most of the studies thus far of “nanotoxicology” have used IT instillation as the mode of delivery.

### 3.1.5 Deposition Mechanisms

Deposition of particles occurs primarily by interception, impaction, sedimentation, and diffusion (Brownian movement). Interception occurs when the trajectory of a particle brings it near enough to a surface so that an edge of the particle contacts the airway surface. Interception is important for the deposition of fibers. Whereas fiber diameter determines the probability of deposition by impaction and sedimentation, interception is dependent on fiber length. Thus, a fiber with a diameter of 1 μm and a length of 200 μm will be deposited in the bronchial tree primarily by interception rather than impaction. As a result of inertia, particles suspended in air tend to continue to travel along their original path. In an airstream that is not straight, such as at an airway bifurcation, a particle may be impacted on the surface. At relatively symmetrical bifurcations, which typically occur in the human lung, the deposition rate is likely to be high for particles that move in the center of the airway. In the average adult, most particles larger than 10 μm in aerodynamic diameter are deposited in the nose or oral pharynx and cannot penetrate to tissues distal to the larynx. Very fine particles (0.01 μm and smaller) are also trapped relatively efficiently in the upper airways by diffusion. Particles that penetrate beyond the upper airways are available to be deposited in the bronchial region and the deep-lying airways. Therefore, the alveolar region has significant deposition efficiencies for particles smaller than 5 μm and larger than 0.01 μm. Sedimentation brings about deposition in the smaller bronchi, the bronchioles, and the alveolar spaces, where the airways are small and the velocity of airflow is low. As a particle moves through air, buoyancy and the resistance of air act on the particle in an upward direction while gravitational force acts on the particle in a downward direction. Eventually, the gravitational force equilibrates with the sum of the buoyancy and the air resistance, and the particle continues to settle with a constant velocity known as the terminal settling velocity. Sedimentation is not a significant route of particle deposition when the aerodynamic diameter is below 0.5 μm. Diffusion is an important factor in the deposition of submicrometer particles. A random motion is imparted to these particles by the impact of gas molecules. This Brownian motion increases with decreasing particle size, so diffusion is an important deposition mechanism in the nose and in other airways and alveoli for particles smaller than about 0.5 μm. An important factor in particle deposition is the pattern of breathing. During quiet breathing, in which the TV is only two to three times the volume of the anatomic dead space (i.e, the volume of the conducting airways where gas exchange does not occur), a large proportion of the inhaled particles may be exhaled. During exercise, when larger volumes are inhaled at higher velocities, impaction in the large airways and sedimentation and diffusion in the smaller airways and alveoli increase. Breath holding also increases deposition from sedimentation and diffusion. Factors that modify the

diameter of the conducting airways can alter particle deposition. In patients with chronic bronchitis, the mucous layer is greatly thickened and extended peripherally and may partially block the airways in some areas. Jets formed by air flowing through such partially occluded airways have the potential to increase the deposition of particles by impaction and diffusion in the small airways. Irritant materials that produce bronchoconstriction tend to increase the tracheobronchial deposition of particles. Cigarette smoking has been shown experimentally to produce such an effect.

### 3.1.6 Particle Clearance

The clearance of deposited particles is an important aspect of lung defense; Rapid removal lessens the time available to cause damage to the pulmonary tissues or permit local absorption. It is important to emphasize that clearance of particles from the respiratory tract is not synonymous with clearance from the body. The only mechanisms by which deposited particles can truly be removed from the respiratory system are coughing and blowing of the nose.

### 3.1.7 Nasal Clearance

Particles deposited in the nose are cleared depending on their site of deposition and solubility in mucus. Particles deposited in the anterior portion of the nose are removed by extrinsic actions such as wiping and blowing. The other regions of the nose are largely covered by a mucociliary epithelium that propels mucus toward the glottis, where it is swallowed. Insoluble particles generally are cleared from this region in healthy adults and swallowed within an hour of deposition. Particles that are soluble in mucus may dissolve and enter the epithelium and/or blood before they can be mechanically removed.

### 3.1.8 Tracheobronchial Clearance

The mucus layer covering the tracheobronchial tree is moved upward by the beating of the underlying cilia. This mucociliary escalator transports deposited particles and particle-laden macrophages upward to the oropharynx, where they are swallowed and pass through the GI tract. Mucociliary clearance is relatively rapid in healthy individuals and is completed within 24–48 hours for particles deposited in the lower airways. Infection and other injuries can greatly impair clearance.

### 3.1.9 Pulmonary Clearance

There are several ways by which particulate material is removed from the lower respiratory tract once it has been deposited: 1. Particles may be directly trapped on the lining layer of the conducting airways by impaction and cleared upward in the tracheobronchial tree via the mucociliary escalator. 2. Particles may be phagocytized by macrophages and cleared via the mucociliary escalator. 3. Particles may be phagocytized by alveolar macrophages and removed via the lymphatic drainage. 4. Materials may dissolve from the surfaces of particles and be removed via the bloodstream or lymphatics. 5. Small particles may directly penetrate epithelial membranes. 6. Minutes after particles are inhaled, they may be found in alveolar macrophages. Insoluble particles, especially long narrow fibers, may be sequestered in the lung for very long periods, often in macrophages located in the interstitium.

## 3.2 Acute Responses of the Lung to Injury

### 3.2.1 Mechanisms of Respiratory Tract Injury

Airborne agents can come in contact with cells lining the respiratory tract from the nostrils to the gas-exchanging region. The sites of interaction of toxicants in the respiratory tract have important implications for evaluation of the risk to humans posed by inhalants. For example, rats have much more nasal surface on a per body weight basis than do humans. Measurement of DNA-protein cross-links formed in nasal tissue by the highly reactive gas formaldehyde has demonstrated that rats, which readily develop nasal tumors, have many more DNA cross-links per unit of exposure (concentration of formaldehyde × duration of exposure) than do monkeys. Because the breathing pattern of humans resembles that of monkeys more than that of rats, it was concluded that extrapolation of tumor data from rats to humans on the basis of formaldehyde concentration may overestimate doses of formaldehyde to humans. Patterns of animal activity can affect dose to the lung; nocturnally active animals such as rats receive a greater dose per unit of exposure at night than during the day, whereas humans show the opposite diurnal relationships of exposure concentration to dose. Certain gases and vapors stimulate nerve endings in the nose, particularly those of the trigeminal nerve (Alarie et al., 1998). The result is holding of the breath or changes in breathing patterns, to avoid or reduce further exposure. If continued exposure cannot be avoided, many acidic or alkaline irritants produce cell necrosis and increased permeability of the alveolar walls. Other inhaled chemicals can be more insidious; inhalation of high concentrations of HCl, $NO_2$, $NH_3$, or phosgene may at first produce very little apparent damage in the respiratory tract. The epithelial barrier in the alveolar zone, after a latency period of several hours, begins to leak, flooding the alveoli

and producing a delayed pulmonary edema that is often fatal. A different pathogenetic mechanism is typical of highly reactive molecules such as ozone. It is unlikely that ozone as such can penetrate beyond the layer of fluid covering the cells of the lung. Instead, ozone lesions are propagated by a cascade of secondary reaction products, such as aldehydes and hydroxyperoxides produced by ozonolysis of fatty acids and other substrates in the lung lining fluid, and by reactive oxygen species arising from free radical reactions. Metabolism of foreign compounds can be involved in the pathogenesis of lung injury. The balance of activation and detoxification plays a key role in determining whether a given chemical ultimately will cause damage. The lung contains most of the enzymes involved in xenobiotic metabolism that have been identified in other tissues, such as the liver (Cascorbi, 2006). These enzymes are highly concentrated in specific cell populations of the respiratory tract and the content of particular cytochrome P-450 isozymes may be much higher in lung. Thus, the turnover of a particular substrate by a lung P-450 may be far more rapid than occurs in liver. Many isozymes of the cytochrome P-450 complex have been identified in and isolated from the lungs of rabbits, rats, hamsters, and humans. Cytochrome P-450 1A1 is present in low amounts in normal rat and rabbit lungs but is highly inducible by polycyclic aromatic hydrocarbons, flavones, and mixtures of polyhalogenated biphenyls. By inference, this P-450 isozyme may play a role in the pathogenesis of lung cancer. Cytochrome P-450 2B1, which is readily inducible in rat liver by phenobarbital, is not inducible in lung tissue. Other isozymes identified in human lung are cytochrome P-450 2F1, 4B1, and 3A4, as well as NADPH cytochrome P-450 reductase, epoxide hydrolase, and flavin-containing monoxygenases. Two important cytosolic enzymes involved in lung xenobiotic metabolism are glutathione-S-transferases and glutathione peroxidase.

### 3.2.2 Oxidative Burden

An undue oxidative burden that often is mediated by free radicals, such as those generated by ozone, NO2, tobacco smoke, and lung defense cells (Rahman, 2003), can directly and indirectly cause lung damage. Numerous studies have reported increases in the activity of free radical scavenging enzymes in the lungs of animals exposed to $O_3$, $NO_2$, and other toxicants, indirectly supporting this hypothesis. Theories of lung oxidant toxicity suggest the formation of reactive and unstablefreeradicals and active oxygen species. Subsequent chain reactions can lead to uncontrolled destructive oxidation. Recent work has emphasized the pivotal roles of superoxide, nitric oxide, peroxynitrate, hydroxyl radicals, hydrogen peroxide, and even possibly singlet oxygen in mediating tissue damage. Reduction of O2 to active O2metabolites normally occurs as a by-product of cellular metabolism during both microsomal and mitochondrial electron transfer reactions; considerable amounts of

superoxide anion are generated by NADPH cytochrome P-450 reductase reactions. Because these oxidant species are potentially cytotoxic, they may mediate or promote the actions of various pneumotoxicants. Such mechanisms have been proposed for paraquat- and nitrofurantoininduced lung injury. Among mammalian cells, neutrophils, monocytes, and macrophages are particularly adept at converting molecular O2to reactive $O_2$ metabolites; this is likely related to their phagocytotic and antimicrobial activities. As a by-product of this capability, toxic $O_2$ species are released into surrounding tissues. As most forms of toxic pulmonary edema are accompanied by phagocyte accumulation in the lung microcirculation (pulmonary leukostasis) and parenchyma, oxidative damage may represent a significant component of pneumotoxic lung injury. Chemotactic and phagocytic activation processes result in a substantial increase in the release of potent oxidants by stimulated phagocytes; these radicals cause oxidative damage to the surrounding tissues. A key role of hydrogen peroxide as the mediator of the extracellular cytotoxic mechanism of activated phagocytes has been well documented. In addition, hydrogen peroxide is a potent intracellular signaling molecule that readily crosses cell membranes, and can thereby amplify cell damage. Phagocytic production of active oxygen species causes inactivation of proteinase inhibitors and degranulation of mast cells. Platelets (and platelet microthrombi) also have the ability to generate activated $O_2$ species. The lung can respond with specific defense mechanisms that may be stimulated by constant exposure to airborne microorganisms, as well as by a variety of low- and high-molecular-weight antigenic materials. The immune system can mount either cellular or humorally mediated responses to these inhaled antigens. Direct immunologic effects occur when inhaled foreign material sensitizes the respiratory system to further exposure to the samematerial. Lymphocytes reside in the hilar or mediastinal lymph nodes, lymphoid aggregates, and lymphoepithelial nodules, as well as in aggregates or as single cells throughout the airways. Bronchoconstriction and chronic pulmonary disease can result from the inhalation of materials that appear to act wholly or partly through an allergic response. Frequently, chemical components of the sensitizing dusts or gases are responsible for the allergic response. Low-molecular-weight compounds can act as haptens that combine with native proteins to form a complex that is recognized as an antigen by the immune system. Further exposure to the sensitizing compound can result in an allergic reaction that is characterized by the release of various inflammatory mediators that produce an early and/or a late bronchoconstrictor response. Such a response is observed in sensitized workers exposed to toluene diisocyanate (TDI), a chemical widely used in the manufacture of polyurethane plastics (Matheson et al., 2001). Increased susceptibility of asthmatic individuals to air pollutants such as ozone and sulfur dioxide may be mediated by indirect immune effects.

### 3.2.3 Mediators of Lung Toxicity

Interleukin 1 (IL-1β), transforming growth factor (TGF-beta), and tumor necrosis factor (TNF-alpha) have all been implicated in the cascade of reactions that are thought to be responsible for the pathogenesis of pulmonary fibrosis (Zhang and Phan, 1999). Several members of the interleukin family, especially IL-1, IL-2, IL-5, IL-8, and IL-13, are thought to be essential components of the lung's response to epithelial cell injury. Various specific prostaglandins, especially PGE2, and leukotrienes have been implicated in intracellular signaling pathways in the lung. The roles of cell surface adhesion molecules and their interaction with cell matrix components and with control of inflammatory cell migration (particularly neutrophil influx to the lung) have been studied intensively. Analysis of normal lung homogenates suggests that the lung contains large amounts of endogenous cytokines and inflammatory mediators, far more than enough for these potent compounds to elicit effects. Thus, these agents must be compartmentalized in a healthy lung to control their potent bioactivity. How these processes are regulated normally, what exactly goes wrong with homeostasis in a damaged lung, the temporal and geographic relationship of different cytokines in the amplification of an initial injurious event, and detailed mechanisms of resolution of lung injury are not well understood and represent the current focus of much research on mechanisms of lung injury by toxic agents.

### 3.2.4 Airway Reactivity

Large airways are surrounded by bronchial smooth muscle, which helps maintain airway tone and diameter during expansion and contraction of the lung. Bronchial smooth muscle tone is normally regulated by the autonomic nervous system. Bronchoconstriction can be provoked by irritants such as cigarette smoke and air pollutants, and by cholinergic drugs such as acetylcholine. This phenomenon serves as the basis for a sensitive measure of whether a toxicant can cause bronchoconstriction in animals or humans primed by a prior dose of an acetylcholine-like agent (bronchoprovocation testing). These chemicals bind to cell surface receptors (cholinergic receptors) and trigger an increase in the intracellular concentration of cyclic guanosine monophosphate (cGMP), which in turn facilitates smooth muscle contraction. The actions of cGMP can be antagonized by cyclic adenosine monophospate (cAMP), which has bronchodilatory activity, and can be increased by agents that bind to beta-adrenergic receptors on the cell surface. Other important mediators of airway smooth muscle tone include histamine, various prostaglandins and leukotrienes, substance P, and nitric oxide. The bronchial smooth muscles of individuals with asthma contract with much less provocation than do those of normal subjects. Bronchoconstriction causes a decrease in airway diameter and a corresponding increase in resistance to airflow.

Characteristic associated symptoms include wheezing, coughing, a sensation of chest tightness, and dyspnea. Exercise potentiates these problems. Because the major component of airway resistance usually is contributed by large bronchi, inhaled chemicals that cause reflex bronchoconstriction are generally irritant gases with moderate solubility. Asthmatic individuals may represent a population that is particularly susceptible to the adverse health effects of ambient air pollution, especially ozone, other respiratory irritant gases, and respirable particles.

### 3.2.5 Pulmonary Edema

Toxic pulmonary edema represents an acute, exudative phase of lung injury that generally produces a thickening of the alveolar-capillary barrier. Edema fluid alters ventilation-perfusion relationships and limits diffusive transfer of $O_2$ and $CO_2$ even in otherwise structurally normal alveoli. Toxic pulmonary edema may not only induce acute compromise of lung structure and function but may also cause abnormalities that remain after resolution of the edematous process. After exposure to some toxic chemicals in which the alveolar-capillary surface is denuded (such as alloxan), recovery is unlikely, whereas in situations of more modest injury (such as histamine administration), full recovery is readily achievable. Between these two extremes there are forms of severe lung injury accompanied by amplified inflammatory damage and exaggerated restorative-reparative processes (e.g., after paraquat ingestion). In these severe forms, the extensive interstitial and intraalveolar inflammatory exudate resolves via fibrogenesis, an outcome that may be beneficial or damaging to the lung. Accumulation and turnover of inflammatory cells and related immune responses in an edematous lung probably play a role in eliciting both mitogenic activity and fibrogenic responses. Pulmonary edema is customarily quantified in experimental animals by measurement of lung water content. Very commonly, the wet (undesiccated) weight of the whole lung or that of a single lung lobe is determined. This value is often normalized to the weight of the animal from which the lung was taken. Alternatively, some investigators determine lung water content by weighing whole lungs or lung slices before and after complete drying in an oven or desiccator. Commonly used methods for expressing such data include (1) percentage water content [100 ×(wet weight -dry weight)/(wet weight)], (2) percentage dry weight [100 × (dry weight)/(wet weight)], and (3) water content [(mL of water)/(dry weight)].

## 3.3 Chronic Responses of the Lung to Injury

### 3.3.1 Emphysema

Emphysema is commonly defined as “an abnormal enlargement of the airspaces distal to the terminal bronchiole accompanied by destruction of

the walls without obvious fibrosis" (Snider et al., 1985). Destruction of the gas-exchanging surface area results in a distended, hyperinflated lung that no longer effectively exchanges oxygen and carbon dioxide as a result of both losses of tissue and air trapping. The major cause of human emphysema is, by far, cigarette smoke inhalation, although other toxicants also can elicit this response. A feature of toxicant-induced emphysema is severe or recurrent inflammation. A unifying hypothesis that explains the pathogenesis of emphysema has emerged from studies by several investigators. Early clinical research on screening blood protein phenotypes identified a rare mutation giving rise to a hereditary deficiency of the serum globulin alpha1-antitrypsin. Homozygotes for this mutation had no circulating levels of this protein, which can prevent the proteolytic activity of serine proteases such as trypsin, and tended to get emphysema at a very young age. Alpha1-antitrypsin (now called alpha1-antiprotease) is one of the body'smain defenses against uncontrolled proteolytic digestion by this class of enzymes, which includes elastase. Studies in smokers led to the hypothesis that neutrophil (and perhaps alveolar macrophage) elastases can break down lung elastin and thus cause emphysema; these elastases usually are kept in check by alpha1-antiprotease that diffuses into the lung from the blood. As an individual ages, an accumulation of random elastolytic events can cause the emphysematous changes in the lungs that are normally associated with aging. Toxicants that cause inflammatory cell influx and thus increase the burden of neutrophil elastase can accelerate this process. In accord with this hypothesis are a large number of experimental studies in animals instilled intratracheally with pancreatic or neutrophil elastase or with other proteolytic enzymes that can digest elastin. A pathological condition then develops that has some of the characteristics of emphysema, including destruction of alveolar walls and airspace enlargement in the lung parenchyma. Mice with defects in genes that code for elastin and collagen modifying enzymes develop emphysema (O'Byrne and Postma, 1999). These observations suggest that problems with elastin synthesismay play an important role in the pathogenesis of emphysema, and that in its simplest form the elastase-antiprotease model alone cannot fully explain the detailed biochemical mechanisms that underlie the etiology of emphysema.

### 3.3.2 Fibrosis

The pathological hallmark of pulmonary fibrosis is increased focal staining of collagen fibers in the alveolar interstitium. Fibrotic lungs from humans with acute or chronic pulmonary fibrosis contain increased amounts of collagen as evaluated biochemically. In lungs damaged by toxicants, the response resembles adult or infant respiratory distress syndrome more closely than it resembles chronic interstitial fibrosis.

Excess lung collagen is usually observed not only in the alveolar interstitium but also throughout the centriacinar region, including the alveolar ducts and respiratory bronchioles. The relationship between increased collagen deposition around small airways and lung mechanics is not understood either theoretically or empirically. Types I and III collagen are major lung interstitial components, representing about 90% or more of the total lung collagen, that are found in the normal lungs of all mammals in an approximate ratio of 2:1. There is an increase in type I collagen relative to type III collagen in patients with idiopathic pulmonary fibrosis and in patients dying of acute respiratory distress syndrome. It is not known whether shifts in collagen types, compared with absolute increases in collagen content, account for the increased stiffness of fibrotic lungs. Type III collagen is more compliant than is type I; thus, increasing type I relative to type III collagen may result in a stiffer lung. Changes in collagen cross-linking in fibrotic lungs also may contribute to the increased stiffness. Increased collagen type I:type III ratios and altered collagen cross-linking have been observed in collagen in several animal models of acute pulmonary fibrosis.

### 3.3.3 Asthma

Asthma is becoming increasingly prevalent in the United States and Europe especially in crowded urban areas. The clinical literature describes “an epidemic” of childhood asthma, with prevalence rates reportedly as high as 40% in children living in the inner city. This alarming increase in reported asthma in children has stimulated public health concerns, many of which have focused on air pollution (especially ultrafine particulate air pollution) as a possible cause of the observed increase in asthma. Asthma is characterized clinically by attacks of shortness of breath, which may be mild or severe. The clinical hallmark of asthma is increased airway reactivity: the smooth muscle around the large airways contracts in response to exposure to irritants. There are well-established links between occupational and environmental exposure to antigens or to chemicals that can act as haptens and the pathogenesis of asthma. There may be common mechanisms, which are shared between asthma and pulmonary fibrosis, especially with regard to the role of recurrent or chronic inflammation in disease pathogenesis (Quan et al., 2006).

### 3.3.4 Lung Cancer

At the begining of the twentieth century, lung cancer was an extremely rare disease. It is now the leading cause of death from cancer among both men and women. Retrospective and, more conclusively, prospective epidemiological studies unequivocally show an association between tobacco smoking and lung cancer. It has been estimated that

approximately 80–90% of lung cancers (and several other cancers, such as cancer of the bladder, esophagus, oral cavity, and pancreas) are caused by cigarette smoking. Average smokers have a 10-fold, and heavy smokers a 20-fold, increased risk of developing lung cancer compared with nonsmokers. Quitting the habit will reduce the risk (IARC 2004). Exposure to many chemicals encountered in industrial settings also pose a lung cancer risk. Inhalation of asbestos fibers and metallic dusts or fumes, such as arsenic, beryllium, cadmium, chromium, and nickel, encountered in smelting and manufacturing operations has been associated with cancer of therespiratory tract (IARC 1993).

## 3.4 Evaluation of Toxic Lung Damage

### 3.4.1 Studies Being Done in Humans

While the lung is susceptible to multiple toxic injuries, it is also amenable to a number of tests that allow evaluation of proper functioning (Frampton et al., 2006). Commonly used tests include measurement of VC, TLC, functional RV, tidal volume, airway resistance, and maximum flow. Additional tests evaluate the distribution of ventilation, lung and chest wall compliance, diffusion capacity, and the oxygen and carbon dioxide content of the arterial and venous blood. Many pulmonary function tests require active collaboration by the subject examined, for example, the so-called FEV1 (forced expiratory volume) during the first second of an active exhalation. This is an easy test to administer to humans, does not require sophisticated equipment or a hospital setting, and is completely noninvasive. The subject is asked first to inhale deeply and then to exhale the air as quickly as possible. The test is often used in epidemiological studies or controlled clinical studies designed to assess the potential adverse effects of air pollutants. A reduction in FEV1 is usually indicative of impaired ventilation such as that found in restrictive (increased lung stiffness) or obstructive (obstructed airflow) lung disease. To accomplish proper oxygenation of venous blood and elimination of $CO_2$, the gases have to diffuse across the air-blood barrier. Gas exchange may be hindered by the accumulation of fluids or cellular elements in the alveoli (edema, pneumonic infiltrates), thickening of the alveolar wall (fibrosis), insufficient ventilation of the alveolar region (emphysema), or insufficient presence of oxygen transport elements (reduced alveolar blood volume or reduced amount of hemoglobin in the blood). Gas exchange can be evaluated by measuring the arterial partial pressure of both oxygen and $CO_2$. In general, blood gas analysis is a comparatively insensitive assay for disturbed ventilation because of the organisms' buffering and reserve capacities, but may be a useful tool in clinical medicine. Measurement of diffusion capacity with CO, a gas that binds with 250 times higher affinity to hemoglobin than does oxygen, is more sensitive. The test is easy to perform in humans and laboratory animals

and is widely used in clinical studies. Proper lung function in humans can be evaluated with several additional techniques. Computed tomography provides detailed roentgenographic information of airways and lung parenchyma. Increased concentrations of nitric oxide are often found in exhaled air when inflammatory processes have led to induction of iNOS. Fiberoptic bronchoscopy has become one of the most valuable tools for the detection of toxic lung injury. The procedure allows direct visual inspection of the major lobar and segmental airways; the depth of penetration is limited by the external diameter of the bronchoscope, usually 5 mm. Bronchoscopy also allows the introduction and retrieval of saline solutions into the lung and subsequent analysis for cellular and molecular constituents (broncholveolar lavage). Excision of small tissue samples (biopsies) during bronchoscopy is an additional diagnostic tool, most helpful in the evaluation and staging of precancerous and cancerous lesions.

### 3.4.2 Studies Being Done in Animals

The toxicology of inhaled materials has been and continues to be extensively studied in experimental animals. In such studies, selection of animals with a respiratory system similar to that of humans is particularly desirable. The respiratory system of monkeys most closely resembles that of humans. However, the availability and cost of these animals and the necessity for special facilities for housing monkeys and performing long-term exposures, along with ethical considerations, including the confinement of primates in small exposure chambers for prolonged periods, severely limit the use of primates. Rats and mice are widely used, although fundamental differences in respiratory anatomy (for example, lack of respiratory bronchioles) and function (rats and mice are obligate nose breathers) can complicate the extrapolation of effects to humans. Experimental studies with guinea pigs and rabbits provided the first conclusive evidence that sulfuric acid and $SO_2$ may damage human lungs (Amdur, 1989). The following techniques are used to study the effects of inhaled toxicants in animals.

#### 3.4.2.1 In Vitro Approaches

In vitro systems with materials originally obtained either from human tissues or from experimental animals are particularly suited for the study of mechanisms that cause lung injury. The following systems are widely used (Allen, 2006).

### 3.4.3 Isolated Perfused Lung

The isolated perfused lung method is applicable to lungs from many laboratory animal species (rabbit, rat, mouse, guinea pig). The lung, in

situ or excised, is perfusedwith blood or a blood substitute through the pulmonary arterial bed. At the same time, the lung is actively (through rhythmic inflation-deflation cycles with positive pressure) or passively (by creating negative pressure with an artificial thorax in which the lung is suspended) ventilated. Toxic agents can be introduced into the perfusate or the inspired air. Repeated sampling of the perfusate allows one to determine the rate of metabolism of drugs and the metabolic activity of the lung.

### 3.4.4 Microdissection

Many inhalants act in circumscribed regions of the respiratory tract, such as the terminal bronchioles, a region especially rich in the highly metabolically competent Clara cells. Microdissection of the airways consists of the stripping of the surrounding parenchyma away from the small bronchi and terminal bronchioli. This facilitates study of the metabolically active Clara cells found in the airways. Microdissected airways can be maintained in culture for up to 1 week, can be used to study site specific gene expression, morphologic changes in response to toxicants or during repair or can be used for biochemical reactions including enzyme activity measures and determination of native antioxidant concentrations (such as glutathione).

### 3.4.5 Organotypic Cell Culture Systems

Tissue culture systems have been developed in which epithelial cells maintain their polarity, differentiation, and normal function similar to what is observed in vivo. Epithelial cell surfaces are exposed to air (or a gas phase containing an airborne toxic agent), while the basal portion is bathed by a tissue culture medium. Maintenance of the epithelial cells at the air–liquid interface is important to maintain polarity and differentiation. Epithelial cells may be seeded on top of a suitable supporting material (e.g., collagen or nitrocellulose membranes) with mesenchymal cells seeded on the other side to observe epithelial cell–fibroblast interactions.

### 3.4.6 Isolated Lung Cell Populations

Many specific lung cell types have been isolated and maintained as primary cultures in vitro. Alveolar macrophages are easily obtained from human and animal lungs by lavage. Their function can be examined in vitro with or without exposure to appropriate toxic stimuli. Type II alveolar epithelial cells are isolated after digestion of the lung. Direct isolation of type I epithelial cells has also been successful. Systems for the isolation and culture of Clara cells and neuroepithelial cells are available. Lung fibroblasts are easily grown and have been studied in

coculture with epithelial cells. Multiple primary cell cultures and cell lines have been established from lung tumors found in experimental animals and humans. Isolated cell techniques suffer from possible enzymatic digestion of critical cellular components and the loss of the normal integration of the many cell types within the tissue layers which may be important for maintenance of normal function. Caution should be exercised in the final interpretation of experiments utilizing this approach.

## 4.0 CONLUSION

The pathogenesis of lung damage caused by toxicants has been discussed in this unit.

## 5.0 SUMMARY

In this unit, we have learnt that the pathogenicitiy lungs and the respiratory tract can be used to assess the toxicants.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. Define inhalation toxicology and respiratory tract toxicology.
2. Describe how lung damage induced by chemicals can be assessed in animals.

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# UNIT 3 NERVOUS SYSTEM

## CONTENTS



## 1.0 INTRODUCTION

Neurotoxicants and toxins have been extensively studied, both because of their toxic effects on humans and because of their utility in the study of the nervous system (NS). Many insights into the organization and function of the NS are based on observations derived from the action of neurotoxicants. The binding of exogenous compounds to membranes has been the basis for the definition of specific receptors within the brain; an understanding of the roles of different cell types in the function of the NS has stemmed from the selectivity of certain toxicants in injuring specific cell types while sparing others; and important differences in basic metabolic requirements of different subpopulations of neurons have been inferred from the effects of toxicants. It is estimated that millions of people worldwide are exposed to known neurotoxicants each year, a fact underscored by repeated outbreaks of neurologic disease (Federal Register, 1994). An even larger potential problem stems from the

incomplete information on many compounds that may have neurotoxic effects. Unknown is the extent to which neurologic disability may be related to chronic low-level exposures, nor do we understand the overall impact of environmental contaminants on brain function. In order to study neurotoxicologic consequences of chemical exposures, one must understand the structure, function, and development of the NS. These features can be quite complex, with differential anatomy, physiology, and cell types specific for location and function. Several general aspects modulate the NS response to chemicals, including (1) the privileged status of the NS with the maintenance of a biochemical barrier between the brain and the blood, (2) the importance of the high energy requirements of the brain, (3) the spatial extensions of the NS as long cellular processes and the requirements of cells with such a complex geometry, (4) the maintenance of an environment rich in lipids, (5) the transmission of information across extracellular space at the synapse, (6) the distances over which electrical impulses must be transmitted, coordinated and integrated, and (7) development and regenerative patterns of the nervous system. Each of these features of the NS carries with it specialized metabolic/physiological requirements and unique vulnerabilities to toxic compounds.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- understand the environmental factors to neurogenerative diseases
- understand the mechanisms of neurotoxicity
- mention chemicals that are neurotoxic and their modes of action.

## 3.0 MAIN CONTENT

### 3.1 Environmental Factors Relevant to Neurodegenerative Diseases

Individuals exposed to insufficient 1,2,3,6-tetrahydro-1-methyl-4-phenylpyridine (MPTP) to result in immediate Parkinsonism have developed early signs of the disease years later (Calne et al., 1985). This observation presents the possibility that the onset of a neurotoxic problem may follow toxic exposure for many years (Landrigan et al., 2005). It does not seem likely that an early sublethal injury to dopaminergic neurons later becomes lethal. Rather, smaller exposures to MPTP may cause a decrement in the population of dopaminergic neurons within the substantia nigra. Such a loss would most likely be silent, because the symptoms of Parkinson’s disease (PD) do not develop until approximately 80–90% of the substantia nigra neurons are lost. These individuals with a diminished number of neurons may be more vulnerable

to further loss of opaminergic neurons. The neurologic picture of PD develops at an earlier age than in unexposed individuals, as a further loss of catecholaminergic neurons occurs during the process of aging. The relationship between MPTP intoxication and Parkinsonism has stimulated investigations into the role that environmental and occupational exposures may play in the pathogenesis of Parkinson's disease. While several families with early-onset Parkinson's disease demonstrate autosomal dominant inheritance, with identification of candidate genes, twin studies indicate that environmental exposures play a more significant role than genetics in the vast majority of Parkinson's disease patients, particularly those with late-onset disease. Epidemiologic studies implicate exposure to herbicides, pesticides, and metals as risk factors for Parkinson's disease (Gorell et al., 1998; Liou et al., 1997). Dithiocarbamates also play an important role. Cigarette smoking may have a protective effect against both Alzheimer's disease and Parkinson's disease, but alternative explanations have been offered. An epidemic of dialysis-related dementia with some pathologic resemblance to Alzheimer's disease appears to have been related to aluminum in the dialysate, and its removal has prevented further instances of dialysis dementia. However, there is no substantial evidence to date that aluminum is in any way related to sporadic Alzheimer's disease in the general population (Letzel et al., 2000). The expanding field of the excitotoxic amino acids embodies many of the same attributes that characterize the entire discipline of neurotoxicology. Neurotoxicology is generally viewed as the study of compounds that are deleterious to the NS, and the effects of glutamate and kainate may be viewed as examples of this type of deleterious toxicity. Exposure to these excitotoxic amino acids leads to neuronal injury and—when of sufficient degree—may kill neurons. Many of these neurotoxic compounds have become tools for neurobiologists who seek to explore the anatomy and function of the NS. Kainate, through its selective action on neuronal cell bodies, has provided a greater understanding of the functions of cells within a specific region of the brain, while previous lesioning techniques addressed only regional functions. Finally, the questions surrounding domoic acid poisoning and the Guamanian neurodegenerative complex serve to remind the student of neurotoxicology and that the causes of many neurologic diseases remain unknown. This void in understanding and the epidemiologic evidence that some neurodegenerative diseases may have environmental contributors provide a heightened desire to appreciate more fully the effects of elements of our environment on the NS.

## 3.2 Functional Manifestations of Neurotoxicity

While knowledge of a toxicant's complete biochemical or molecular mechanism(s) is the ultimate goal of neurotoxicology, a full understanding of the toxicity also requires knowledge of the functional

outcomes of those changes. Being the final output or manifestation of the NS, function includes motor, sensory, autonomic, and cognitive capabilities. The strength of functional assessments has been exploited by many investigators and regulatory agencies, and they are now routinely used in the assessment of the neurological effects of chemicals. Tilson (1993) has proposed two distinct tiers of functional testing of neurotoxicants: a first tier in which observational batteries or motor activity tests may be used to identify the presence of a neurotoxic substance, and a second tier that involves more complete description of the effects. An overall assessment of behavior may be described using a series, or battery, of tests. These tests typically evaluate a variety of neurological functions, and are often used to screen for potential neurotoxicity in regulatory and safety pharmacology testing. Specific methods include functional observational batteries (FOBs), Irwin screens, tests of motor activity, and expanded clinical observations. These tests have the advantage over biochemical and pathologic measures in that they permit evaluation of a single animal over longitudinal studies to determine the onset, progression, duration, and reversibility of a neurotoxic injury. Comparisons of defined protocols of FOBs with limited numbers of compounds (Moser et al., 1997a,b) suggest that these methods can identify neurotoxic compounds reliably. Some functional tests are more specific than observations and motor activity, and may be used to more fully characterize neurotoxic effects. Many of these functions have a clinical or behavioral correlate in humans, thus improving extrapolation of the outcomes. Measures of sensory function tap specific neuronal pathways that govern stimuli-dependent reflexes. For example, the acoustic startle response is a sensory-evoked motor reflex with a defined neuronal pathway. Treatment effects could indicate sensory, motor, or muscle fiber alterations with little or no central involvement. Autonomic function includes evaluations of cardiovascular status and cholinergic/adrenergic balance. Acetylcholinesterase-inhibiting pesticides produce marked effects on multiple aspects of cholinergic homeostasis. Deficits in cognitive function, especially in the context of developmental toxicity, represent an endpoint of great public concern and rhetoric. Behavioral toxicologists have incorporated methodologies from behavioral pharmacology and psychology to develop a range of tests of learning and memory for laboratory animals. These procedures include spatial navigation of mazes, associations with shock, conditioned responses, and appetite-motivated operant responses. In most cases, deficits in human cognitive function may be detected in laboratory animals as well, although the affected cognitive domain may vary. For example, in humans, exposure to lead in early childhood is known to lower IQ and alter behavioral control. Studies in rats have reported deficits in spatial learning, sustained attention, activity levels, and other behaviors (Morgan et al., 2001; Nihei et al., 2000). Detailed assessments such as these provide valuable insights into the damage caused by

neurotoxicants. Ultimately, neurotoxicants identified by behavioral methods are evaluated at a cellular and molecular level to provide an understanding of the events in the NS that cause the neurological dysfunction.

## 3.3 Mechanisms of Neurotoxicity

Efforts to understand the mechanism of action of individual neurotoxic compounds have begun with the identification of the cellular target. In the nervous system, this has most often been one of four targets: the neuron, the axon, the myelinating cell, or the neurotransmitter system. As a result, neurotoxic compounds may be identified which cause neuronopathies, axonopathies, myelinopathies, or neurotransmitter-associated toxicity. This is the classification system that is utilized here to organize the discussion of neurotoxic compounds and their mechanisms of action.

### 3.3.1 Neuronopathies

Certain toxicants are specific for neurons, or sometimes a particular group of neurons, resulting in their injury or, when intoxication is severe enough, their death. The loss of a neuron is irreversible and includes degeneration of all of its cytoplasmic extensions, dendrites and axons, and of the myelin ensheathing the axon. Although the neuron is similar to other cell types in many respects, some features of the neuron are unique, placing it at risk for the action of cellular toxicants. Some of the unique features of the neuron include a high metabolic rate, a long cellular process that is supported by the cell body, and an excitable membrane that is rapidly depolarized and repolarized. Because many neurotoxic compounds act at the site of the cell body, when massive loss of axons and myelin are discovered in the PNS or CNS, the first question is whether the neuronal cell bodies themselves have been destroyed. Although a large number of compounds are known to result in toxic neuronopathies, all these toxicants share certain features. Each toxic condition is the result of a cellular toxicant that has a predilection for neurons, most likely due to one of the neuron’s peculiar vulnerabilities. The initial injury to neurons is followed by apoptosis or necrosis, leading to permanent loss of the neuron. These chemicals tend to be diffuse in their action, although they may show some selectivity in the degree of injury of different neuronal subpopulations or at times an exquisite selectivity for such a subpopulation. The expression of these cellular events is often a diffuse encephalopathy, with global dysfunctions; however, the symptomatology reflects the injury to the brain, so neurotoxicants that are selective in their action and affect only a subpopulation of neurons may lead to interruption of only a particular functionality. Doxorubicin Doxorubicin (Adriamycin), a quinone-containing anthracycline antibiotic, is one of the most effective antimitotics in cancer chemotherapy. Unfortunately,

clinical application of doxorubicin is greatly limited by its acute and chronic cardiotoxicity. In addition to its cardiac toxicity that limits the quantity of doxorubicin that can be given to cancer patients, doxorubicin also injures neurons in the PNS, specifically those of the dorsal root ganglia and autonomic ganglia. This selective vulnerability of peripheral ganglion cells is particularly dramatic in experimental animals. Doxorubicin's antineoplastic properties derive from its ability to intercalate into grooves of DNA, interfering with transcription. Other important mechanisms of action of doxorubicin include its interaction with topoisomerase II, which forms a DNAcleavable complex generation of reactive oxygen species (ROS) by enzymatic electron reduction of doxorubicin by variety of oxidases, reductases, and dehydrogenases. The neurotoxicity of doxorubicin is quite limited in its extent, despite the fact that all neurons are dependent on the ability to transcribe DNA. The particular vulnerability of sensory and autonomic neurons appears to reflect the lack of protection of these neurons by a blood-tissue barrier within ganglia. If the blood–brain barrier is temporarily opened by the use of mannitol, the toxicity of doxorubicin is expressed in a much more diffuse manner, with injury of neurons in the cortex and subcortical nuclei of the brain.

### 3.3.2 Methyl Mercury

The neuronal toxicity of organomercurial compounds, such as methyl mercury (MeHg), was tragically revealed in large numbers of poisonings in Japan and Iraq. The residents of Minamata Bay in Japan, whose diet was largely composed of fish from the bay, were exposed to massive amounts of methyl mercury when mercury-laden industrial effluent was rerouted into the bay. Methyl mercury injured even more people in Iraq, with more than 400 deaths and 6000 people hospitalized. In this epidemic, as well as in several smaller ones, the effects occurred after the consumption of grain that had been dusted with methyl mercury as an inexpensive pesticide. Typically, environmental exposure to mercury occurs via the food chain due to accumulation of MeHg in fish. Latest statistics in the United States indicate that 46 states have fish consumption advisories covering 40% of the nation's rivers, lakes, and streams. In addition, mercury is a common pollutant in hazardous waste sites in the nation. It is estimated that 3–4 million children live within one mile of at least one of the 1300+ active hazardous waste sites in the United States. The clinical picture of MeHg poisoning varies both with the severity of exposure and the age of the individual at the time of exposure. In adults, the most dramatic sites of injury are the neurons of the visual cortex and the small internal granular cell neurons of the cerebellar cortex, whose massive degeneration results in blindness and marked ataxia. In children, developmental disabilities, retardation, and cognitive deficits occur. Such age-related differences are seen also in other mammals, although the

specific areas damaged may differ. It has been suggested that these differences are caused by an immature blood–brain barrier causing a more generalized distribution of mercury in the developing brain. Neurons that are most sensitive to the toxic effects of methyl mercury are those that reside in the dorsal root ganglia, perhaps again reflecting the vulnerability of neurons not shielded by blood-tissue barriers. The mechanism of MeHg toxicity has been the subject of intense investigation. However, it remains unknown whether the ultimate toxicant is methyl mercury or the liberated mercuric ion. Whereas $Hg^{2+}$ is known to bind strongly to sulfhydryl groups, it is not clear that MeHg results in cell death through sulfhydryl binding. A variety of aberrations in cellular function have been noted, including impaired glycolysis, nucleic acid biosynthesis, aerobic respiration, protein synthesis, and neurotransmitter release. In addition, there is evidence for enhanced oxidative injury (Shanker et al., 2002) and altered calcium homeostasis (Marty and Atchison, 1997). Exposure to MeHg leads to widespread neuronal injury and subsequently to a diffuse encephalopathy. However, there is relative selectivity of the toxicant for some groups of neurons over others. The distribution of neuronal injury does not appear to be related to the tissue distribution of either MeHg or ionic mercury but rather to particular vulnerabilities of these neurons. Susceptibility of different brain regions or cell types to MeHg may also be dependent on factors such as the intracellular reduced glutathione (GSH) concentration and the ability to increase glycolytic flux in the face of mitochondrial damage.

### 3.3.3 Trimethyltin

Organotins are used industrially as plasticizers, antifungal agents, or pesticides. Intoxication with trimethyltin has been associated with a potentially irreversible limbic-cerebellar syndrome in humans and similar behavioral changes in primates. Trimethyltin gains access to the nervous system where, by an undefined mechanism, it leads to diffuse neuronal injury. Trimethyltin triggers selective apoptosis in specific subregions of the mammalian CNS and specific subsets of immune system cells. The hippocampus is particularly vulnerable to this process. Following acute intoxication, the cells of the fascia dentata degenerate; with chronic intoxication, the cells of the corpus ammonis are lost. Ganglion cells and hair cells of the cochlea are similarly sensitive. Several hypotheses are suggested for the mechanism of trimethyltin neurotoxicity, including energy deprivation and excitotoxic damage. Evidence to date suggests that organotins, such as trimethyltin, interact with the CXC region of stannin, and that trimethyltin treatment significantly alters its expression. Stannin is located on human chromosome 16p13, and has a syntenic relationship to the murine chromosomal homolog.

### 3.3.4 Axonopathies

The neurotoxic disorders termed axonopathies are those in which the primary site of toxicity is the axon itself. The axon degenerates, and with it the myelin surrounding that axon; however, the neuron cell body remains intact. John Cavanagh coined the term dying-back neuropathy as a synonym for axonopathy (Cavanagh, 1964). The concept of "dying back" postulated that the focus of toxicity was the neuronal cell body itself and that the distal axon degenerated progressively from the synapse, back toward the cell body with increasing injury. It now appears that, in the best-studied axonopathies, a different pathogenetic sequence occurs; the toxicant results in a "chemical transection" of the axon at some point along its length, and the axon distal to the transection, biologically separated from its cell body, degenerates. Because longer axons have more targets for toxic damage than shorter axons, one would predict that longer axons would be more affected in toxic axonopathies. Indeed, such is the case. The involvement of long axons of the CNS, such as ascending sensory axons in the posterior columns or descending motor axons, along with long sensory and motor axons of the PNS, prompted Spencer and Schaumburg (1976) to suggest that the toxic axonopathies in which the distal axon was most vulnerable be called central peripheral distal axonopathies, which, though cumbersome, accurately depicts the pathologic loci. Axonopathies can be considered to result from a chemical transection of the axon. The number of axonal toxicants is large and increasing in number; however, they may be viewed as a group, all of which result in the pathologic loss of distal axons with the survival of the cell body. Because the axonopathies pathologically resemble the actual physical transection of the axon, axonal transport appears to be a likely target in many of the toxic axonopathies. Furthermore, as these axons degenerate, the result is most often the clinical condition of peripheral neuropathy, in which sensations and motor strength are first impaired in the most distal extent of the axonal processes, the feet and hands. With time and continued injury, the deficit progresses to involve more proximal areas of the body and the long axons of the spinal cord. The potential for regeneration is great when the insult is limited to peripheral nerves and may be complete in axonopathies in which the initiating event can be determined and removed.

### 3.3.5 Gamma-Diketones

It was first noted in the late 1960s that humans with chronic high exposures to n-hexane, a simple alkane, in a work setting develop a progressive sensorimotor distal axonopathy (Yamamura, 1969). Intentional inhalation of materials containing n-hexane is also common, and produces the same neurotoxic effects. An identical axonopathy was also produced by methyl n-butyl ketone (2-hexanone), leading to the

discovery of the mechanism by which these two compounds are similarly metabolized. The carbon chain undergoes ω-1 oxidation, resulting in 2,5-hexanedione, a γ- diketone. This metabolite is ultimately the toxic species produced from n-hexane and 2-hexanone. Other γ-diketones or precursors also produced the same axonopathy, whereas α- or β-diketones are not toxic to the nervous system. γ-Diketones, including 2,5-hexanedione, react with amino groups on all proteins, forming pyrrole adducts. This is an important step in development of axonopathy, as evidenced by the inability of 3,3-dimethyl-2,5-hexanedione, a γ-diketone which is unable to form pyrrole adducts, to cause neurotoxicity. After forming, these pyrroles are oxidized and cross-linking occurs between neurofilament subunits. The inability of 3-acetyl- 2,5-hexanedione to cross-link prevents toxicity, suggesting that pyrrole oxidation and cross-linking is a necessary step in the development of axonopathy. Neurofilaments accumulate in the distal axon, usually just proximal to a node of Ranvier, and form massive axonal swellings leading to retraction of myelin from the nodes. In addition to swelling, axonal atrophy is also a pathological feature of γ-diketone neurotoxicity. This axonal atrophy was previously thought to occur secondary to swelling; however, more recent studies have suggested that it may be the more relevant pathophysiologic feature. In one study, rats dosed at a lower rate (100–250 mg/kg/d) developed axonal swelling and atrophy, and rats given a higher dose rate (400 mg/kg/d) failed to consistently develop swellings, while atrophy and behavioral alterations were nearly universal. These data suggest that axonal atrophy is the pathologic change that leads to nerve dysfunction and behavioral changes. The mechanism responsible for axonal atrophy is still unknown; however, a depletion of tubulin subunits has been reported, and is a likely contributor to the overall neuropathologic picture. The dimethyl analog of HD, 3,4-dimethyl-2,5-hexanedione (DMHD), produces a similar neuropathy. However, due to the methyl groups, DMHD forms the cyclic adduct much more rapidly than HD, forming pyrrole adducts that oxidize and lead to crosslinking faster than HD. DMHD is more potent than HD, and produces proximal axonal swellings similar to those seen in β,β-iminodipropionitrile, and is thought to be the product of faster rates of adduct formation. Carbon Disulfide The most significant exposures of humans to CS2 have occurred in the vulcan rubber and viscose rayon industries. Manic psychoses were observed in the former setting and were correlatedwith very high levels of exposure. In recent decades, interest in the human health effects has been focused on the NS and the cardiovascular system, where injury has been documented in workers exposed to much higher levels than those that are allowed today. What is clearly established is the capacity of CS2 to cause a distal axonopathy that is identical pathologically to that caused by n-hexane. There is growing evidence that covalent cross-linking of neurofilaments also underlies CS2 neuropathy through a series of reactions that parallel the sequence of events in n-hexane neuropathy. While n-hexane requires metabolism to

2,5-hexanedione, CS2 is itself the ultimate toxicant, reacting with protein amino groups to form dithiocarbamate adducts. The dithiocarbamate adducts of lysyl amino groups undergo decomposition to isothiocyanate adducts, electrophiles that then react with protein nucleophiles to yield covalent cross-linking. The reaction of the isothiocyanate adducts with cysteinyl sulfhydryls to form N,S-dialkyldithiocarbamate ester cross-links is reversible, while the reaction with protein amino functions forms thiourea cross-links irreversibly. Over time, the thiourea cross-links predominate and are most likely the most biologically significant. As with n-hexane neuropathy, it has been postulated that the stability and long transport distance of neurofilaments determine that the neurofilament subunit proteins are the toxicologically relevant targets in chronic CS2 intoxication. Nonetheless, proteins throughout the organism are derivatized and cross-linked as well. Crosslinking has been identified in erythrocyte-associated proteins including spectrin and globin as well as in the putative neurotoxic target neurofilament subunit proteins. Analysis of cross-linking in erythrocyte proteins has verified that cross-linking occurs through thiourea bridges that accumulate with continuing exposure. Neurofilament crosslinking involves all three subunits and also demonstrates a cumulative dose response and temporal relationship consistent with a contributing event in the development of the axonal neurofilamentous swellings. The correlation of protein cross-linking in erythrocyte proteins and axonal proteins together with the ability to detect covalent modifications on peripheral proteins at subneurotoxic levels and at preneurotoxic time points suggests that modifications on peripheral proteins can be used as biomarkers of effect for CS2 exposure. These biomarkers together with morphologic changes have been used to establish CS2 as the ultimate neurotoxic species in the peripheral neuropathy produced by oral administration of N, Ndiethyldithiocarbamate. The clinical effects of exposure to CS2 in the chronic setting are very similar to those of hexane exposure, with the development of sensory and motor symptoms occurring initially in a stockingand-glove distribution. In addition to this chronic axonopathy, CS2 can also lead to aberrations in mood and signs of diffuse encephalopathic disease. Some of these are transient at first and subsequently become more long lasting, a feature that is common in vascular insufficiency in the nervous system. This fact, in combination with the knowledge that CS2 may accelerate the process of atherosclerosis, suggests that some of the effects of CS2 on the CNS are vascular in origin.

### 3.3.6 β,β-Iminodipropionitrile (IDPN)

β,β-iminodipropionitrile (IDPN) is a synthetic, bifunctional nitrile that causes a “waltzing syndrome” in rats and other mammals, although

human exposure has never been documented. Features of this "waltzing syndrome" include excitement, circling, head twitching, and over-alertness, and are observed after a single large intraperitoneal injection to rats (1.5–2.0 g/kg). While the cause of this behavior has not been conclusively determined, it has been suggested that degeneration of vestibular sensory hair cells is responsible. Pathologic changes also follow administration of IDPN, most notably in large caliber axons, the primary target of neurotoxicity. The accumulation of neurofilaments in the proximal axon occurs, leading to swelling without degeneration in most animals. Quails deficient in neurofilaments demonstrate no swellings when administered IDPN, suggesting that the toxicity is due to a selective effect on neurofilaments. These neurofilament swellings are similar to those observed in carbon disulfide or γ-diketones toxicity. Repeated exposure to IDPN leads to demyelination and onion bulb formation, and eventually can produce distal axonal atrophy due to a reduction in anterograde neurofilament transport to the distal axon. This impairment of axonal transport results from the disruption of the association between microtubules and neurofilaments by IDPN, causing neurofilament accumulation. This leads to complete disturbance of the cytoskeleton of the axon. Although unclear, the mechanism responsible for this interference is hypothesized to result from the direct alteration of neurofilament proteins by IDPN, possibly by changing their chemical properties and causing aggregation. Acrylamide Acrylamide is a vinyl monomer used widely in water purification, paper manufacturing, mining, and waterproofing. It is also used extensively in biochemical laboratories, and is present in many foods prepared at high temperatures. Although it can be dangerous if not handled carefully, most toxic events in humans have been observed as peripheral neuropathies in factory workers exposed to high doses.

### 3.3.7 Organophosphorus (OP) Compounds

OP compounds are used not only as insecticides and chemical warfare agents, but also as chemical intermediates, flame retardants, fuel additives, hydraulic fluids, lubricants, pharmaceuticals, and plasticizers. The OP insecticides and nerve agents are designed to inhibit acetylcholinesterase (AChE), thereby causing accumulation of acetylcholine in cholinergic synapses resulting in cholinergic toxicity and death. However, apart from the insecticides, nerve agents, and some of the pharmaceuticals, OP compounds produced for other applications often have little or no anti-AChE activity. Some OP compounds, such as tri-o-cresyl phosphate (TOCP), are neuropathic and can cause a severe sensorimotor central peripheral distal axonopathy called OP compound-induced delayed neurotoxicity (OPIDN) without inducing cholinergic poisoning. This condition is also referred to as a delayed neuropathy or delayed polyneuropathy (OPIDP). However, neuropathy usually connotes

peripheral nerve disease, whereas OPIDN also involves degeneration of ascending and descending spinal cord tracts. An OPIDN epidemic of massive proportions occurred during Prohibition in the United States, when Jamaica Ginger extract (Ginger Jake), a popular source of alcohol, was adulterated with TOCP. Another outbreak occurred in Morocco where olive oil was contaminated with TOCP. Human cases have also occurred after exposure to certain formerly used OP insecticides, such as EPN (O-ethyl-O-4-nitrophenyl phenylphosphonothionate) and leptophos [O-(4-bromo-2,5-dichlorophenyl)-O-methyl phenylphosphonothionate]. Many OP compounds are hydrophobic and readily enter the NS. If the parent compound and/or metabolites have suitable reactivity, they can phosphorylate neural target proteins, such as various serine hydrolases. When the principal target is acetylcholinesterase (AChE), cholinergic toxicity can ensue, either because of suprathreshold levels of inhibition or inhibition plus aging. A substantial level of AChE inhibition on its own is sufficient to produce cholinergic toxicity and death. When aging of inhibited AChE also occurs (net loss of a ligand from the phosphorus of the OP-enzyme conjugate, leaving a negatively charged phosphoryl moiety attached to the active site), the qualitative nature of the toxicity does not change. Instead, the inhibited AChE becomes intractable to reactivation, rendering therapy with oximes, such as 2-pralidoxime methiodide (2-PAM) ineffective. When the principal target is neuropathy target esterase (neurotoxic esterase, NTE), OPIDN can result only if both suprathreshold (>70%) inhibition occurs and the inhibited enzyme undergoes aging. Thus, in the case of NTE and OPIDN, inhibition alone is insufficient to precipitate toxicity. It appears that the biochemical lesion is not simply a blockade of the active site. Instead, axonopathy is triggered by specific chemical modification of the NTE protein. Neuropathic (aging) inhibitors of NTE include compounds from the phosphate, phosphonate, and phosphoramidate classes of OP compounds. Certain NTE inhibitors, including members of the phosphinate, carbamate, and sulfonylfluoride classes, do not age and do not cause OPIDN. However, pretreatment with a nonaging NTE inhibitor prevents OPIDN from occurring after a challenge dose of a neuropathic (aging) NTE inhibitor. It has been proposed that these nonaging compounds protect against OPIDN by blocking the active site of NTE, and preventing inhibition and aging by a subsequent dose of a neuropathic (aging) inhibitor. In contrast, when protective NTE inhibitors are administered following exposure to a near-threshold subclinical dose of a neuropathic OP compound, OPIDN is fully expressed. Because the initial treatment involves a compound that can produce OPIDN on its own and the disease is likely to be incipient rather than absent, this effect should be called potentiation; however, some authors refer to the phenomenon as promotion. Although the potentiating agents inhibit NTE, this enzyme is not thought to be the target of potentiation. The level of NTE inhibition produced by the potentiator is not related to the level of potentiation observed, and these potentiators

appear to exacerbate axonopathies from other causes as well, such as trauma and 2,5-hexanedione exposure. These results have been interpreted to indicate that potentiation enhances progression of the axonopathic process, inhibits repair, or both. Axonal degeneration does not commence immediately after acute exposure to a neuropathic OP compound, but is delayed for at least 8 days between the acute high-dose exposure and clinical signs of axonopathy. Some effective regeneration of axons occurs in the PNS, for example, excitatory inputs to skeletal muscle from lower motor neurons in the spinal cord. In contrast, axonal degeneration is progressive and persistent in long tracts of the spinal cord, for example, inhibitory pathways from upper motor neurons in the motor cortex to lower motor neurons in the spinal cord anterior horn. Accordingly, the clinical picture of OPIDN changes from flaccid to spastic paralysis during a course of months to years. Fortunately, studies of the initiation steps of OPIDN and structure-activity relationships of neuropathic OP compounds have led to highly accurate prediction of the neuropathic potential of these chemicals. Consequently, human cases of OPIDN are now rare and usually arise from intentional ingestion of massive doses of OP insecticides in suicide attempts. Nevertheless, the fact remains that OPIDN is a debilitating and incurable condition. Moreover, the mechanism linking aged NTE to axonopathy is unknown.

### 3.3.8 Pyridinethione

This compound is a chelating agent that is usually encountered as the zinc complex. Two molecules of pyridinethione are complexed with zinc to form bis[1-hydroxy-2(1H)-pyridinethionato] zinc, commonly known as zinc pyridinethione or zinc pyrithione (ZPT). ZPT is a biocide that has antibacterial and antifungal properties. It is the active ingredient in shampoos and other preparations for the treatment of seborrheic dermatitis and dandruff. ZPT is also used as an antifouling agent for ship paints, drywall, and tarps, and as an antibacterial agent for incorporation into cleaning sponges. Thus, the intended uses of ZPT can lead to human exposures through direct dermal contact and potential exposure to biota through leaching into marine and freshwater environments. Because the compound is directly applied to the human scalp in antidandruff shampoos, the finding that ZPT produced limb weakness and peripheral neuropathy in rodents after oral administration raised concern about potential neurotoxicity in humans. Rats, rabbits, and guinea pigs all develop a distal axonopathy when exposed to ZPT in the diet. Fortunately, however, dermal absorption of ZPT is minimal, and there have been no reports of neurological findings in humans attributable to occupational or consumer ZPT exposures. Although the zinc ion appears to be an important component of the therapeutic action of ZPT, only the pyridinethione moiety is absorbed following ingestion, with the majority of zinc eliminated in the feces. In addition, oral sodium pyridinethione is

also neurotoxic, indicating that the pyridinethione moiety is responsible for the neurotoxicity. Pyridinethione chelates zinc, copper, and other metal ions and, once oxidized to the disulfide, may lead to the formation of protein-pyridinethione mixed disulfides. However, which of these properties, if any, is responsible for the molecular mechanism of its neurotoxicity remains unknown. Although these molecular issues remain to be resolved, pyridinethione appears to interfere with the fast axonal transport systems. While the fast anterograde system is less affected, pyridinethione impairs the turnaround of rapidly transported vesicles and slows their retrograde transport This aberration of the fast axonal transport systems is the most likely physiologic basis of the accumulation of tubulovesicular structures in the distal axon. As these materials accumulate in one region of the axon, they distend the axonal diameter, resulting in axonal swellings filled with membranous profiles. As in many other distal axonopathies, the axon degenerates in its more distal regions beyond the accumulated structures. The earliest signs are diminished grip strength and electrophysiologic changes of the axon terminal, with normal conduction along the proximal axon in the early stages of exposure.

### 3.3.9 Microtubule-Associated Neurotoxicity

A number of plant alkaloids alter the assembly and depolymerization of microtubules in nerve axons, causing neurotoxicity. The oldest known of these are colchicine and the vinca alkaloids, which bind to tubulin and cause depolymerization of microtubules. Colchicine is an alkaloid pharmaceutical used in the treatment of gout, familial Mediterranean fever, and other disorders. A common side effect of treatment in patients with abnormal renal function is a peripheral axonal neuropathy. While this neuropathy is generally mild, it is often accompanied by a disabling myopathy that can lead to the inability to walk. A number of vinca alkaloids, including vincristine and vinblastine, both chemotherapeutic agents, produce a peripheral axonopathy very similar to that induced by colchicine. Vincristine is commonly used to treat leukemias and lymphomas, and also has greater potential for adverse toxic effects than vinblastine. The agent binds to tubulin subunits and prevents the polymerization into microtubules. Nearly all evidence of vincristine-induced neuropathy has been observed in humans. Most treated patients develop neurotoxicity to some extent, beginning with parasthesias of the fingers. General weakness and clumsiness is common, but this improves quickly with removal of treatment. Parasthesias may persist, however, and some distal sensory loss may be permanent. More recently paclitaxel (Taxol), another plant alkaloid, has become a popular chemotherapeutic agent used to treat a variety of neoplasms. However, side effects include a predominantly sensory neuropathy, beginning in the hands and feet. Like colchicine and the vinca alkaloids, paclitaxel binds to tubulin; however, instead of leading to depolymerization, it promotes the

formation of microtubules. Once formed, these microtubules remain stabilized by paclitaxel even in conditions that normally lead to dissociation of tubulin subunits, including cold temperatures or the presence of calcium. When paclitaxel is injected directly into the sciatic nerve of rats, microtubules aggregate along the axon, causing axonal degeneration, demyelination, and impairment of regeneration. The pathologies of the axon induced by these drugs are different. While colchicine leads to atrophy of the axon and a decrease in the number of microtubules, paclitaxel causes the aggregation to form a matrix that may inhibit fast axonal transport, which has been demonstrated with both colchicine and paclitaxel. A change in the number of microtubules has been observed in some reports and absent from others. While the mechanisms may differ slightly, both exposures result in a peripheral neuropathy which must be taken into account in medical treatments.

### 3.3.10 Neurotransmission-Associated Neurotoxicity

A wide variety of naturally occurring toxins, as well as synthetic chemicals, alters specific mechanisms of intercellular communication. Whereas neurotransmitter-associated actions may be well understood for some chemicals, the specificity of the mechanisms should not be assumed. For example, organophosphorus (OP) and carbamate pesticides produce their insecticidal actions by inhibiting acetylcholinesterase, the catalytic enzyme that ends the postsynaptic action of acetylcholine. The resultant cholinergic overstimulation produces signs of acute toxicity ranging from flu-like symptoms to gastrointestinal distress, ataxia, twitching, convulsions, coma, and death. These effects are not as well correlated with acetylcholinesterase inhibition as might be expected for all such pesticides, leading to suggestions of additional mechanisms of actions that have since been verified in animal and in vitro studies. These include direct actions on pre- and postsynaptic cholinergic receptors and altered reuptake of choline; such actions serve to modulate the downstream impact of cholinergic overstimulation. Thus, multiple neurotransmitter targets may be more common than was once expected.

### 3.3.11 Nicotine

Widely available in tobacco products and in certain pesticides, nicotine has diverse pharmacologic actions and may be the source of considerable toxicity. These toxic effects range from acute poisoning tomore chronic effects. Nicotine exerts its effects by binding to a subset of cholinergicreceptors, the nicotinicreceptors. These receptors are located in ganglia, at the neuromuscular junction, and also within the CNS, where the psychoactive and addictive properties most likely reside. Smoking and “pharmacologic” doses of nicotine accelerate heart rate, elevate blood pressure, and constrict blood vessels within the skin. Because the majority

of these effects may be prevented by the administration of α- and β-adrenergic blockade, these consequences may be viewed as the result of stimulation of the ganglionic sympathetic nervous system. At the same time, nicotine leads to a sensation of "relaxation" and is associated with alterations of electroencephalographic (EEG) recordings in humans. These effects are probably related to the binding of nicotine with nicotinic receptors within the CNS, and the EEG changes may be blocked with mecamylamine, a nicotinic antagonist. Acute overdose of nicotine has occurred in children who accidentally ingest tobacco products, in tobacco workers exposed to wet tobacco leaves, and in workers exposed to nicotine-containing pesticides. In each of these settings, the rapid rise in circulating levels of nicotine leads to excessive stimulation of nicotinic receptors, a process that is followed rapidly by ganglionic paralysis. Initial nausea, rapid heart rate, and perspiration are followed shortly by marked slowing of heart rate with a fall in blood pressure. Somnolence and confusion may occur, followed by coma; if death results, it is often the result of paralysis of the muscles of respiration. Such acute poisoning with nicotine fortunately is uncommon. Exposure to lower levels for longer duration, in contrast, is very common, and the health effects of this exposure are of considerable epidemiologic concern. In humans, however, it has been difficult to separate the effects of nicotine from those of other components of cigarette smoke. The complications of smoking include cardiovascular disease, cancers (especiallymalignancies of the lung and upper airway), chronic pulmonary disease, and attention deficit disorders in children of women who smoke during pregnancy. Nicotine may be a factor in some of these problems. For example, an increased propensity for platelets to aggregate is seen in smokers, and this platelet abnormality correlates with the level of nicotine. Nicotine also places an increased burden on the heart through its acceleration of heart rate and blood pressure, suggesting that nicotine may play a role in the onset of myocardial ischemia. In addition, nicotine also inhibits apoptosis and may play a direct role in tumor promotion and tobacco-related cancers.

### 3.3.12 Developmentally Neurotoxic Chemicals

Replication, migration, differentiation, myelination, and synapse formation are the basic processes that underlie development of the NS. There are a variety of insults known to disrupt NS development, the outcomes of which may be very different depending on the time of exposure, including exposures to certain metals, solvents, antimetabolites, persistent organic pollutants, pesticides, pharmaceuticals, and ionizing radiation. Multiple mechanisms of action may be present, producing a wide array of effects in the offspring. The impact on the developing NS may be very different, and often cannot be predicted, from effects observed in adults. Ethanol exposure during pregnancy can result in abnormalities in the fetus, including abnormal

neuronal migration and facial development, and diffuse abnormalities in the development of neuronal processes, especially the dendritic spines. While the exposure may be of little consequence to the mother, it can be devastating to the fetus. There is an effect on NMDA glutamate receptors and excessive activation of GABA receptors, with induction of apoptosis throughout the brain. The clinical result of fetal alcohol exposure is often mental retardation, with malformations of the brain and delayed myelination of white matter. Although there remains a great deal of uncertainty concerning the molecular basis of this developmental aberration, it occurs in a variety of experimental animals, and it appears that acetaldehyde, a product of ethanol catabolism, can produce migration defects in developing animals similar to those that occur in the fetal alcohol syndrome. Some developmental neurotoxicants have been revealed by human studies or tragic poisoning occurrences. The methyl mercury contamination of fish in Minamata Bay, Japan, led to the birth of many children with developmental disabilities, including cerebral palsy, mental retardation, and seizures. Since then, it was shown that children exposed to methyl mercury in utero show widespread neuronal loss, disruption of cellular migration, profound mental retardation, and paralysis. Studies on primates exposed in utero also have demonstrated abnormal social development (Burbacher et al., 1990). The earlier the exposure, the more generalized the damage that is observed. As with methyl mercury, ethanol and lead are known to produce frank neuropathology in highly exposed populations. However, in recent years the concept has emerged that extremely low levels of exposure to these substances in “asymptomatic” children may have an effect on their behavioral and cognitive development. The association between lead exposure and brain dysfunction has received experimental support in animal models and has prompted screening for lead in children. There is no proven safe lower limit for lead, and recent studies have attributed lower IQ scores to blood lead levels less than 5–10 μg/dl (Canfield et al., 2003). Similarly, the debate regarding “safe” level of drinking during pregnancy is ongoing, with recent reports of no threshold for subtle cognitive effects (Sampson et al., 2000). There is considerable evidence that chronic exposure to nicotine has effects on the developing fetus. Along with decreased birth weights, attention deficit disorders are more common in children whose mothers smoke cigarettes during pregnancy, and nicotine has been shown to lead to analogous neurobehavioral abnormalities in animals exposed prenatally to nicotine. Nicotinic receptors are expressed early in the development of the NS, beginning in the developing brainstem and later expressed in the diencephalon. The role of these nicotinic receptors during development is unclear; however, it appears that prenatal exposure to nicotine alters the development of nicotinic receptors in the CNS changes that may be related to subsequent attention and cognitive disorders in animals and children. Cocaine use during pregnancy is a major concern, especially in urban areas, where use

can lead to a variety of acute and chronic adverse events in offspring. Cocaine is able to cross the placental barrier and the fetal blood–brain barrier, and also causes reduced blood flow in the uterus. In severe events at large doses taken by the mother, the fetus may develop hypoxia, leading to a higher rate of birth defects. Maternal cocaine use is associated with low birth weight and behavioral defects, including a decreased awareness of the surroundings and altered response to stress and pain sensitivity. These persistent pollutants produce endocrine disruptions, cognitive deficits, and changes in activity levels in exposed offspring; however, the specific outcomes depend on the congener or mixture tested as well as the timing of exposure. Changes in estrogen or thyroid hormone, neurotransmitter function, and second messenger systems have been proposed as cellular bases for PCB toxicity. Polybrominated diphenyl ethers (PBDEs) have shown similarities in altering thyroid hormone metabolism and cholinergic function, and it has thus been proposed that this chemical class would also be developmentally neurotoxic. Thyroid hormone is critical to NS development, and the developing brain may be vulnerable to environmental thyrotoxicants of all sorts. Finally, reversible changes in neurotransmission, such as those produced by nicotine or cholinesterase inhibitors, may alter specific growth processes and produce long-lasting deficits.

## 3.4 Chemicals that Induce Depression of Nervous System Function

Generalized depression of central nervous system function is produced by a variety of volatile solvents. These solvents include several chemical classes—aliphatic and aromatic hydrocarbons, halogenated hydrocarbons, ketones, esters, alcohols, and ethers—that are small, lipophilic molecules. They are widely found in industry, medicine, and commercial products. Human exposures range from chronic low-level concentrations encountered in environmental or occupational setting to high-level concentrations intentionally generated through solvent abuse. There are several theories as to the mechanism of this generalized depression, but none is fully explanatory. Solvent potency correlates well with the olive oil: water or octanol:water partition coefficients, leading to the once-popular Meyer-Overton hypothesis that CNS depressants exert their actions through nonspecific disruption of the lipid portions of cell membranes (e.g., Janoff et al., 1981). Anesthesia could occur as a consequence of membrane expansion or perturbations of mitochondrial calcium transport. More recent research has implicated interactions with ligand-gated ion channels as well as voltage-gated calcium channels. Specific receptors regulating these channels include gamma-aminobutyric acid type A (GABAA), N-methyl-D-aspartate (NMDA), and glycine receptors. These actions relate the effects of solvents to those of pharmaceutical agents such as barbiturates and benzodiazepines.

## 4.0 CONCLUSION

Chemicals that are neurotoxic and their modes of action have been examined in this unit as well as the environmental factors that are relevant to neurodegenerative diseases.

## 5.0 SUMMARY

In this unit, we have learnt the mechanisms of action of some neurotoxicants.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. What is neutoxicity?
2. List three chemicals that are neutoxic and briefly explain their mechanisms of action in a named animal.

## 7.0 REFERENCES/FURTHER READING

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## UNIT 4 EYE

## CONTENTS



## 1.0 INTRODUCTION

Environmental and occupational exposure to toxic chemicals, gases, and vapors as well as side effects resulting from systemic and ocular therapeutic drugs frequently result in structural and functional alterations in the eye and central visual system. Almost half of all neurotoxic chemicals affect some aspect of sensory function. The most frequently sensory system alterations occur in the visual system (Fox, 1998). There are approximately 2800 substances that are reportedly toxic to the eye (Grant, 1986). In many cases, alterations in visual function are the initial symptoms following chemical exposure. Even more relevant is the fact that these alterations often occur in the absence of any clinical signs of toxicity. This suggests that sensory systems, and in particular the retina and central visual system, may be especially vulnerable to toxic insult. In fact, alterations in the structure and/or function of the eye or central visual system are among the criteria utilized for setting permissible occupational or environmental exposure levels for many different chemicals in the United States. In addition, numerous new drugs used for the treatment of ocular diseases or ocular complications of systemic diseases recently entered the marketplace. Moreover, subtle alterations in visual processing of information (e.g., visual perceptual, visual motor) can have profound immediate, long-term, and in some cases delayed effects on the mental, social, and physical health and performance of an individual. Finally,

ocular and visual system impairments can lead to increased occupational injuries, loss of productive work time, costs for providing medical and social services, lost productivity, and a distinct decrease in the overall quality of life.

## 2.0 OBJECTIVES

By the end of this unit, you should be able to:

- understand how the eye is exposed to toxicants
- explain the mehanisms of action of toxicants in the target sites of the eye.

## 3.0 MAIN CONTENT

### 3.1 Exposure to the Eye and Visual System

#### 3.1.1 Ocular Pharmacodynamics and Pharmacokinetics

Toxic chemicals and systemic drugs can affect all parts of the eye. Several factors determine whether a chemical can reach a particular ocular site of action, including the physiochemical properties of the chemical, concentration and duration of exposure, route of exposure, and the movement of the chemical into and across the different ocular compartments and barriers. The cornea and external adnexa of the eye, including the conjunctiva (the delicate membranes covering the inner surface of the eyelids and the exposed surface of the sclera) and eyelids are often exposed directly to chemicals (i.e., acids, bases, solvents), gases and particles, and drugs. The first site of action is the tear film—a threelayered structure with both hydrophobic and hydrophilic properties. The outermost tear film layer is a thin (0.1 μm) hydrophobic layer that is secreted by the meibomian (sebaceous) glands. This superficial lipid layer protects the underlying thicker (7 μm) aqueous layer that is produced by the lacrimal glands. The third layer, which has both hydrophobic and hydrophilic properties, is the very thin (0.02 to 0.05 μm) mucoid layer. It is secreted by the goblet cells of the conjunctiva and acts as an interface between the hydrophilic layer of the tears and the hydrophobic layer of the corneal epithelial cells. Thus, the aqueous layer is the largest portion of the tear film, and therefore water-soluble chemical compounds more readily mix with the tears and gain access to the cornea. However, a large proportion of the compounds that are splashed into the eyes is washed away by the tears and thus not absorbed. The cornea, an avascular tissue, is considered the external barrier to the internal ocular structures. Once a chemical interacts with the tear film and subsequently contacts the cornea and conjunctiva, the majority of what is absorbed locally enters the anterior segment by passing across the cornea. In contrast, a greater

systemic absorption and higher blood concentration occurs through contact with the vascularized conjunctiva. The human cornea, which is approximately 500 μm thick, has several distinct layers, or barriers, through which a chemical must pass in order to reach the anterior chamber. The first is the corneal epithelium. It is a stratified squamous, nonkeratinized, and multicellular hydrophobic layer. These cells have a relatively low ionic conductance through apical cell membranes, and due to the tight junctions (desmosomes), they have a high resistance paracellular pathway. The primary barrier to chemical penetration of the cornea is the set of tight junctions at the superficial layer of the corneal epithelial cells. Thus, the permeability of the corneal epithelium as a whole is low and only lipid soluble chemicals readily pass through this layer. Bowmann's membrane separates the epithelium from the stroma. The corneal stroma makes up 90% of the corneal thickness and is composed of water, collagen, and glycosaminoglycans. It contains approximately 200 lamellae, each about 1.5 to 2.0 μm thick. Due to the composition and structure of the stroma, hydrophilic chemicals easily dissolve in this thick layer, which can also act as a reservoir for these chemicals. The inner edge of the corneal stroma is bounded by a thin, limiting basement membrane, called Descemet's membrane, which is secreted by the corneal endothelium. The innermost layer of the cornea, the corneal endothelium, is composed of a single layer of large diameter hexagonal cells connected by terminal bars and surrounded by lipid membranes. The endothelial cells have a relatively low ionic conductance through apical cell surface and a high-resistance paracellular pathway. Although, the permeability of the corneal endothelial cells to ionized chemicals is relatively low, it is still 100 to 200 times more permeable than the corneal epithelium. There are two separate vascular systems in the eye: (1) the uveal blood vessels, which include the vascular beds of the iris, ciliary body, and choroid, and (2) the retinal vessels. In humans, the ocular vessels are derived from the ophthalmic artery, which is a branch of the internal carotid artery. The ophthalmic artery branches into (1) the central retinal artery, which enters the eye and then further branches into four major vessels serving each of the retinal quadrants; (2) two posterior ciliary arteries; and (3) several anterior arteries. In the anterior segment of the eye, there is a blood–aqueous barrier that has relatively tight junctions between the endothelial cells of the iris capillaries and nonpigmented cells of the ciliary epithelium. The major function of the ciliary epithelium is the production of aqueous humor from the plasma filtrate present in the stroma of the ciliary processes. In humans and several widely used experimental animals (e.g., monkeys, pigs, dogs, rats, mice), the retina has a dual circulatory supply: choroidal and retinal. The retinal blood vessels are distributed within the inner or proximal portion of the retina, which consists of the outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). The endothelial cells of capillaries of the

retinal vessels have tight junctions similar to those that form the blood–brain barrier in the cerebral capillaries. These capillaries form the blood-retinal barrier and under normal physiologic conditions, they are largely impermeable to chemicals such as glucose and amino acids. However, at the level of the optic disk, the blood-retinal barrier lacks these tight-junction types of capillaries and thus hydrophilic molecules can enter the optic nerve head by diffusion from the extravascular space and cause selective damage at this site of action. The outer or distal retina, which consists of the retinal pigment epithelium (RPE), rod, and cone photoreceptor outer segments (ROS, COS) and inner segments (RIS, CIS), and the photoreceptor outer nuclear layer (ONL), are avascular. These areas of the retina are supplied by the choriocapillaris: a dense, one-layered network of fenestrated vessels formed by the short posterior ciliary arteries and located next to the RPE. Consistent with their known structure, these capillaries have loose endothelial junctions and abundant fenestrae; they are highly permeable to large proteins. Thus, the extravascular space contains a high concentration of albumin and γ - globulin (Sears, 1992). Following systemic exposure to drugs and chemicals by the oral, inhalation, dermal, or parenteral route, these compounds are distributed to all parts of the eye by the blood in the uveal blood vessels and retinal vessels. Most of these drugs and chemicals can rapidly equilibrate with the extravascular space of the choroid where they are separated from the retina and vitreous body by the RPE and endothelial cells of the retinal capillaries, respectively. Hydrophilic molecules with molecular weights less than 200 to 300 Da can cross the ciliary epithelium and iris capillaries and enter the aqueous humor. Thus, the corneal endothelium—the cells responsible for maintaining normal hydration and transparency of the corneal stroma—could be exposed to chemical compounds by the aqueous humor and limbal capillaries. Similarly, the anterior surface of the lens can also be exposed as a result of its contact with the aqueous humor. The most likely retinal target sites following systemic drug and chemical exposure appear to be the RPE and photoreceptors in the distal retina because the endothelial cells of the choriocapillaris are permeable to proteins smaller than 50 to 70 kDa. However, the cells of the RPE are joined on their basolateral surface by tight junctions' zonula occludens that limit the passive penetration of large molecules into the neural retina. The presence of intraocular melanin plays a special role in ocular toxicology. First, it is found in several different locations in the eye: pigmented cells of the iris, ciliary body, RPE, and uveal tract. Second, it has a high binding affinity for polycyclic aromatic hydrocarbons, electrophiles, calcium, and toxic heavy metals such as aluminum, iron, lead, and mercury. Although this initially may play a protective role, it also results in the excessive accumulation, long-term storage, and slow release of numerous drugs and chemicals from melanin. For example, atropine binds more avidly to pigmented irides and thus its duration of action is prolonged. In addition, the accumulation of

chloroquine in the RPE produces an 80-fold higher concentration of chloroquine in the retina relative to liver. Similarly, lead accumulates in the human retina such that its concentration is 5 to 750 times that in other ocular tissues.

### 3.1.2 Evaluation of Ocular Irritancy and Toxicity

Standard procedures for evaluating ocular irritation have been based on a method originally by Draize et al. (1944) over a half a century ago. Over this time, the Draize test with some additions and revisions formed the basis of safety evaluations in data submitted to several government regulatory bodies including the European Economic Community and several federal agencies within the United States. Traditionally, albino rabbits were the subjects evaluated in the Draize test, although the Environmental Protection Agency (EPA) protocol allows different test species to be used if sufficient justification is provided. The standard procedure involves instillation of 0.1 mL of a liquid or 100 mg of a solid into the conjunctival sac of one eye and then gently holding the eye closed for 1 second. The untreated eye serves as a control. Both eyes are evaluated at 1, 24, 48, and 72 hours, respectively, after treatment. If there is evidence of damage in the treated eye at 72 hours, the examination time may be extended. The cornea, iris, and conjunctiva are evaluated and scored according to a weighted scale. The cornea is scored for both the degree of opacity and area of involvement, with each measure having a potential range from 0 (none) to 4 (most severe). The iris receives a single score (0 to 2) for irritation, including degree of swelling, congestion, and degree of reaction to light. The conjunctiva is scored for the redness (0 to 3), chemosis (swelling: 0 to 4), and discharge (0 to 3). The individual scores are then multiplied by a weighting factor: 5 for the cornea, 2 for the iris, and 5 for the conjunctiva. The results are summed for a maximum total score of 110. In this scale, the cornea accounts for 80 (73%) of the total possible points, in accordance with the severity associated with corneal injury. The Draize test, although a standard for decades, has been criticized on several grounds, including high interlaboratory variability, the subjective nature of the scoring, poor predictive value for human irritants, and most significantly, for causing undue pain and distress to the tested animals. These criticisms have spawned a concerted effort to develop alternative methods or strategies to evaluate compounds for their potential to cause ocular irritation. These alternatives include modifications of the traditional Draize test to reduce the number of test animals required, reduce the volume of the compound administered, and increase objectivity of scoring. In addition, several alternative test procedures have been proposed, including the use of skin irritancy tests as substitutes for ocular irritancy and the use of in vitro assays. The Draize results are used as a standard because information available from human ocular exposures almost invariably comes from accidental exposure

episodes in which the dose levels, durations and conditions of exposure are unknown. However, the Draize test cannot be considered a "gold standard" due to the variability of results within and between studies, subjectivity of scoring outcomes, and inter-species differences in the ability to predict human ocular toxic potency from testing rabbit eyes.

### 3.1.3 Ophthalmologic Evaluations

There are many ophthalmologic procedures for evaluating the health of the eye. These should be conducted by a trained ophthalmologist or optometrist experienced in evaluating the species of interest. Procedures available range from fairly routine clinical screening evaluations to sophisticated techniques for very targeted purposes. A clinical evaluation of the eye addresses the adnexa and both the anterior and posterior structures in the eye. Examination of the adnexa includes evaluating the eyelids, lacrimal apparatus, and palpebral (covering the eyelid) and bulbar (covering the eye) conjunctiva. The anterior structures or anterior segment include the cornea, iris, lens, and anterior chamber. The posterior structures, referred to as the ocular fundus, include the retina, retinal vasculature, choroid, optic nerve, and sclera. The adnexa and surface of the cornea can be examined initially with the naked eye and a hand-held light. Closer examination requires a slit-lamp biomicroscope, using a mydriatic drug (causes pupil dilation) if the lens is to be observed. The width of the reflection of a thin beam of light projected from the slit lamp is an indication of the thickness of the cornea and may be used to evaluate corneal edema. Lesions of the cornea can be better visualized with the use of fluorescein dye, which is retained where there is an ulceration of the corneal epithelium. Examination of the fundus requires use of a mydriatic drug. Fundoscopic examination is conducted using a direct or an indirect ophthalmoscope. An ophthalmologic examination of the eye may also involve, prior to introducing mydriatics, an examination of the pupillary light reflex. The direct pupillary reflex involves shining a bright light into the eye and observing the reflexive pupil constriction in the same eye. The consensual pupillary reflex is observed in the eye not stimulated. Both the direct and consensual pupillary light reflexes are dependent on function of a reflex arc involving cells in the retina, which travel through the optic nerve, optic chiasm, and optic tract to project to neurons in the pretectal area. Pretectal neurons travel to both ipsilateral (for the direct reflex) and contralateral (for the consensual reflex) parasympathetic neurons of the midbrain accessory oculomotor (Edinger–Westphal) nucleus. Preganglion neurons from the Edinger–Westphal nucleus project through the oculomotor nerve to the ciliary ganglion. Postganglionic neurons from the ciliary ganglion then innervate the smooth muscle fibers of the iridal pupillary sphincter. The absence of a pupillary reflex is indicative of damage somewhere in the reflex pathway, and differential impairment of the direct or consensual reflexes can indicate the location of the lesion.

The presence of a pupillary light reflex, however, is not synonymous with normal visual function. Pupillary reflexes can be maintained even with substantial retinal damage. In addition, lesions in visual areas outside of the reflex pathway, such as in the visual cortex, may also leave the reflex function intact.

## 3.2 Target Sites and Mechanisms of Action: Cornea

The cornea provides the anterior covering of the eye and as such must provide three essential functions. First, it must provide a clear refractive surface. The air-to-fluid/tissue interface at the cornea is the principal refractive surface of the eye, providing approximately 48 diopters of refraction. The curvature of the cornea must be correct for the visual image to be focused at the retina. Second, the cornea provides tensile strength to maintain the appropriate shape of the globe. Third, the cornea protects the eye from external factors, including potentially toxic chemicals. The cornea is transparent to wavelengths of light ranging between 310 nm (UV) to 2500 nm (IR) in wavelengths. Exposure to UV light below this range can damage the cornea. It is most sensitive to wavelengths of approximately 270 nm. Excessive UV exposure leads to photokeratitis and corneal pathology, the classic example being welder's arc burns. The cornea can be damaged by topical or systemic exposure to drugs and chemicals. One summary analysis, of approximately 600 agricultural and industrial chemicals (raw materials, intermediates, formulation components, and sales products), evaluated using the Draize procedure, reported that over half of the materials tested caused no (18–31%) or minimal (42–51%) irritation. Depending on the chemical category, 9–17% of compounds were graded as slightly irritant, whereas 1–6% were graded as strong or extreme irritants (Kobel and Gfeller, 1985). Direct chemical exposure to the eye requires emergency medical attention. Acid and alkali chemicals that come into contact with the cornea can be extremely destructive. Products at pH extremes ≤2.5 or ≥11.5 are considered as extreme ocular irritants. They can cause severe ocular damage and permanent loss of vision. Damage that extends to the corneal endothelium is associated with poor repair and recovery. The most important therapy is immediate and adequate irrigation with large amounts of water or saline, whichever is most readily available. The extent of damage to the eye and the ability to achieve a full recovery are dependent upon the nature of the chemical, the concentration and duration of exposure, and the speed and magnitude of the initial irrigation. Acids Strong acids with a pH ≤2.5 can be highly injurious. Among the most significant acidic chemicals in terms of the tendency to cause clinical ocular damage are hydrofluoric acid, sulfurous acid, sulfuric acid, chromic acid, hydrochloric acid and nitric acid and acetic acid (McCulley, 1998). Injuries may be mild if contact is with weak acids or with dilute solutions of strong acids. Compounds with a pH between 2.5 and 7,

produce pain or stinging; but with only brief contact, they will cause no lasting damage. Following mild burns, the corneal epithelium may become turbid as the corneal stroma swells. Mild burns are typicallyfollowed by rapid regeneration of the corneal epithelium and full recovery. In more severe burns, the epithelium of the cornea and conjunctiva become opaque and necrotic and may disintegrate over the course of a few days. In severe burns, there may be no sensation of pain because the corneal nerve endings are destroyed. Acid chemical burns of the cornea occur through hydrogen ion–induced denaturing and coagulation of proteins. As epithelial cell proteins coagulate, glycosaminoglycans precipitate and stromal collagen fibers shrink. These events cause the cornea to become cloudy. The protein coagulation and shrinkage of the collagen is protective in that it forms a barrier and reduces further penetration of the acid. The collagen shrinkage, however, contracts the eye and can lead to a dangerous acute increase in intraocular pressure. The pH of the acid is not the only determinant of the severity of injury; however, as equimolar solutions of several chemicals adjusted to the same pH of 2 produce a wide range of outcomes. Both the hydrogen ion and anionic portions of the acid molecules contribute to protein coagulation and precipitation. The tissue proteins also tend to act as buffers.

## 3.3 Target Sites and Mechanisms of Action: Lens

The lens of the eye plays a critical role in focusing the visual image on the retina. While the cornea is the primary refractive surface for bending incoming light rays, the lens is capable of being reshaped to adjust the focal point to adapt for the distance of visual objects. The lens is a biconvex transparent body, encased in an elastic capsule, and located between the pupil and the vitreous humor. The mature lens has a dense inner nuclear region surrounded by the lens cortex. The high transparency of the lens to visible wavelengths of light is a function of its chemical composition, approximately twothirds water and one-third protein, and the special organizational structure of the lenticular proteins. The water-soluble crystallins are a set of proteins particular to the lens that, through their close intermolecular structure, give the lens both transparency and the proper refractive index. The lens fibers are laid down during development, as the epithelial cells grow and elongate along meridian pathways between the anterior and posterior poles of the lens. As the epithelial cells continue to grow, the nuclei recede and, in the central portions of the lens, disappear, such that the inner lens substance is composed of nonnucleated cells that form long proteinaceous fibers. The lens fibers are arranged within the lens in an onion-like fashion of concentric rings that have a prismatic arrangement in cross section. The regular geometric organization of the lens fibers is essential for the refractive index and transparency of the lens. At birth, the lens has no

blood supply and no innervation. Nutrients are provided from the aqueous and vitreous fluids, and are transported into the lens substance through a system of intercellular gap-type junctions. The lens is a metabolically active tissue that maintains careful electrolyte and ionic balance. The lens continues to grow throughout life, with new cells added to the epithelial margin of the lens as the older cells condense into a central nuclear region. The dramatic growth of the lens is illustrated by increasing its weight, from approximately 150 mg at 20 years of age to approximately 250 mg at 80 years of age. Cataracts are decreases in the optical transparency of the lens that ultimately can lead to functional visual disturbances. They are the leading cause of blindness worldwide, affecting an estimated 30 to 45 million people. In the United States, approximately 400,000 people develop cataracts each year. This accounts for about 35% of existing visual impairments. Cataracts can occur at any age; they can also be congenital. However, they are much more frequent with advancing age. Senile cataracts develop most frequently in the cortical or nuclear regions of the lens and less frequently in the posterior subcapsular region. Senile cataracts in the cortical region of the lens are associated with disruptions of water and electrolyte homeostasis, whereas nuclear cataracts are characterized by an increase in the water-insoluble fraction of lens proteins. Both genetic and environmental factors contribute to age-related and environmentally mediated cataracts and that these involve several different mechanisms of action. Risk factorsfor the development of cataracts includes aging, diabetes, low antioxidant levels, and exposure to a variety of environmental factors. Environmental factors include exposure to UV radiation and visible light, trauma, smoking, and exposure to a large variety of topical and systemic drugs and chemicals. Several different mechanisms of action have been hypothesized to account for the development of cataracts. These include the disruption of lens energy metabolism, hydration and/or electrolyte balance, the occurrence of oxidative stress due to the generation of free radicals and reactive oxygen species, and the occurrence of oxidative stress due a decrease in antioxidant defense mechanisms such as glutathione, superoxide dismutase, catalase, ascorbic acid, or vitamin E. The generation of reactive oxygen species leads to oxidation of lens membrane proteins and lipids. A critical pathway in the development of highmolecular-weight aggregates involves the oxidation of protein thiol groups, particularly inmethionine or cysteine amino acids, that leads to the formation of polypeptide links through disulfide bonds, and in turn, high-molecular-weight protein aggregates. These large aggregations of proteins can attain a size sufficient to scatter light, thus reducing lens transparency. Oxidation of membrane lipids and proteins may also impair membrane transport and permeability.

## 3.4 Target Sites and Mechanisms of Action: Retina

The adult mammalian retina is a highly differentiated tissue containing nine distinct layers plus the RPE, ten major types of neurons, and three cells with glial functions. The nine layers of the neural retina, which originate from the cells of the inner layer of the embryonic optic cup, are the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), rod and cone photoreceptor inner segment layer (RIS; CIS), and the rod and cone photoreceptor outer segment layer (ROS; COS). The RPE, which originates from the cells of the outer layer of the embryonic optic cup, is a single layer of cuboidal epithelial cells that lies on Bruch's membrane adjacent to the vascular choroid. Between the RPE and photoreceptor outer segments lies the subretinal space, which is similar to the brain ventricles. The ten major types of neurons are the rod (R) and cone (C) photoreceptors, (depolarizing) ON-rod and ON-cone bipolar cells (BC), (hyperpolarizing) OFF-cone bipolar cells, horizontal cells (HC), numerous subtypes of amacrine cells (AC), an interplexiform cell (IPC), and ON-RGCs and OFF-RGCs. The three cells with glial functions are the M¨ uller cells (MC), fibrous astrocytes, and microglia. The somas of the MCs are in the INL. The end feet of the MCs in the proximal or inner retina along with a basal lamina form the internal limiting membrane (ILM) of the retina, which is similar to the pial surface of the brain. In the distal retina, the MC end feet join with the photoreceptors and zonula adherens to form the external limiting membrane (ELM), which is located between the ONL and RIS/CIS. The interested reader is referred to the excellent references in the Introduction as well as to numerous outstanding websites devoted exclusively to the retina for basic information on the anatomic, biochemical, cell and molecular biological, and physiologic aspects of retinal structure and function. The mammalian retina is highly vulnerable to toxicant-induced structural and/or functional damage due to (1) the presence of a highly fenestrated choriocapillaris that supplies the distal or outer retina as well as a portion of the inner retina; (2) the very high rate of oxidative mitochondrial metabolism, especially that in the photoreceptors; (3) high daily turnover of rod and cone outer segments; (4) high susceptibility of the rod and cones to degeneration due to inherited retinal dystrophies as well as associated syndromes and metabolic disorders; (5) presence of specialized ribbon synapses and synaptic contact sites; (6) presence of numerous neurotransmitter and neuromodulatory systems, including extensive glutamatergic, GABAergic and glycinergic systems; (7) presence of numerous and highly specialized gap junctions used in the information signaling process; (8) presence of melanin in the choroid and RPE and also in the iris; (9) a very high choroidal blood flow rate, as high as ten times that of the gray matter of the brain; and (10) the additive or synergistic toxic action of certain chemicals with light. The retina is also

an excellent model system for studying the effects of chemicals on the developing and mature CNS. Its structure– function relations are well established. The histogenic steps of development of the neurons and glial components are well characterized. The development of the CNS and most retinal cells occurs early during gestation in humans and continues for an additional 7 to 14 days postnatally in the rat. Therefore, toxicological effects in the rodent retina have relevance for chemical exposure during the early gestation period in humans as well as during early postnatal development. The retina contains a wide diversity of synaptic transmitters and second messengers whose developmental patterns are well described. Moreover, the rodent retina is easily accessible, it has most of the same anatomical and functional features found in the developing and mature human retina, and the rat rod pathway is similar to that in other mammals. Finally, rat rods have similar dimensions, photochemistry, and photocurrents as human and monkey rods. These general and specific features underscore the relevance and applicability of using the rodent retina to investigate the effects of chemicals on this target site as well as a model to investigate the neurotoxic effects of chemicals during development. Each of the retinal layers can undergo specific as well as general toxic effects. These alterations and deficits include, but are not limited to visual field deficits, scotopic vision deficits such as night blindness and increases in the threshold for dark adaptation, cone-mediated (photopic) deficits such as decreased color perception, decreased visual acuity, macular and general retina edema, retinal hemorrhages and vasoconstriction, and pigmentary changes.

## 3.5 Target Sites and Mechanisms of Action: Optic Nerve and Tract

The optic nerve consists primarily of RGC axons carrying visual information from the retina to several distinct anatomic destinations in the CNS. Both myelinated and nonmyelinated axons are present and grouped into bundles of axons that maintain a topographic distribution with respect to the site of origin in the retina. At the optic chiasm, the fibers split, so that, in humans and other primates, those fibers originating from the temporal retina continue in the optic tract toward the ipsilateral side of the brain, while those fibers originating in the nasal half of the retina, cross the midline and project to the contralateral side of the brain. In species with sideward-facing eyes such as the rat, a larger proportion of the optic nerve fibers (up to 90%) cross the midline. Fibers from the optic nerve terminate in the dorsal LGN, the superior colliculus, and pretectal areas. Information passing through the LGN to visual cortex gives rise to conscious visual perception. Information traveling to the superior colliculus is used to generate eye movements. Pathways leading to the pretectal areas subserve the pupil response. The LGN of primates contains

six histologic layers that are alternately innervated by cells from the contralateral and ipsilateral eyes. The cells projecting to and from the ventral two layers of the LGN have large cell bodies, and consequently, this pathway is referred to as the magnocellular system. Retinal ganglion cells projecting to the magnocellular layers of the LGN are referred to as either M-type or Pα cells. Magnocellular neurons are sensitive to fast moving stimuli and to low levels of luminance contrast, but are insensitive to differences in color. The cells from the magnocellular pathway are involved in motion perception. On the dorsal side of the LGN, the cells are smaller and form the parvocellular pathway. Retinal ganglion cells projecting to the parvocellular layers of the LGN are referred to as P-type or Pβ cells. Parvocellular neurons are sensitive to color and to fine detailed patterns, have slower conduction velocities, and are involved in perception of color and form. Disorders of the optic nerve may be termed optic neuritis, optic neuropathy, or optic nerve atrophy, referring to inflammation, damage, or degeneration, respectively, of the optic nerve. Retrobulbar optic neuritis refers to inflammation of the portion of the optic nerve posterior to the globe. Among the symptoms of optic nerve disease are reduced visual acuity, contrast sensitivity, and color vision. Toxic effects observed in the optic nerve may originate from damage to the optic nerve fibers themselves or to the RGC somas that provide axons to the optic nerve. A number of nutritional disorders can adversely affect the optic nerve. Deficiency of thiamine, vitamin B12, or zinc results in degenerative changes in optic nerve fibers. Nutritional and toxic factors can interact to produce optic nerve damage. A condition referred to as alcohol–tobacco amblyopia or simply as toxic amblyopia is observed in habitually heavy users of these substances and is associated nutritional deficiency. Dietary supplementation with vitamin B12 is therapeutically helpful, even when patients continue to consume large amounts of alcohol and tobacco.

## 3.6 Target Sites and Mechanisms of Action: The Central Visual System

Many areas of the cerebral cortex are involved in the perception of visual information. The primary visual cortex called V1, Brodmann's area 17, or striate cortex receives the primary projections of visual information from the LGN and also from the superior colliculus. Neurons from the LGN project to visual cortex maintaining a topographic representation of the receptive field origin in the retina. The receptive fields in the left and right sides of area 17 reflect the contralateral visual world and representations of the upper and lower regions of the visual field are separated below and above, respectively, the calcarine fissure. Cells in the posterior aspects of the calcarine fissure have receptive fields located in the central part of the retina. Cortical cells progressively deeper in the calcarine fissure have retinal receptive fields that are located more and

more peripherally in the retina. The central part of the fovea has tightly packed photoreceptors for resolution of fine detailed images, and the cortical representation of the central fovea is proportionately larger than the peripheral retina in order to accommodate a proportionately larger need for neural image processing. The magnocelluar and parvocellular pathways project differently to the histologically defined layers of primary striate visual cortex and then to extrastriate visual areas. The receptive fields of neurons in visual cortex are more complex than the circular center-surround arrangement found in the retina and LGN. Cortical cells respond better to lines of a particular orientation than to simple spots. The receptive fields of cortical cells are thought to represent computational summaries of a number of simpler input signals. As the visual information proceeds from area V1 to extrastriate visual cortical areas, the representation of the visual world reflected in the receptive fields of individual neurons becomes progressively more complex.

## 4.0 CONCLUSION

The mechanisms of action of some toxicants in the target sites of the eye have been examined in this unit.

## 5.0 SUMMARY

In this unit, we have learnt that how the eye can be exposed to toxicants and the mechansms of action of toxicants in the target sites of the eye.

## 6.0 TUTOR-MARKED ASSIGNMENT

1. Explain how the human eye can be exposed a named chemical.
2. Explain the mehanisms of action of toxicants in the target sites of the eye.

## 7.0 REFERENCES/FURTHER READING

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