PART V

ORGAN TOXICITY

CHAPTER 13

Hepatotoxicity

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13.1 INTRODUCTION

Hepatotoxicity is a consequence of exposure to natural toxins and many man-made chemicals including industrial compounds, pesticides, and pharmaceutical drugs. Mechanisms of hepatotoxicity are well understood for several chemicals such as halogenated solvent CCl_4 and analgesic acetaminophen. Drug induced liver injury (DILI) remains one the major reasons for new drugs to fail to meet regulatory approval. The progressive injury to the liver due to repeated exposure to toxic doses of ethanol remains a leading human health concern. The liver has many critical functions in the body, and the unique structures and functions of the liver are important reasons for the liver's susceptibility to chemical toxicity.

13.1.1 Liver Structure

The liver consists of a variety of cell types, but the basic architecture of the hepatic parenchyma consists of rows of functionally diverse hepatocytes separated by spaces called sinusoids (see Chapter 9, Figure 9.2). Blood flows into the sinusoidal spaces via the hepatic portal vein blood from the gastrointestinal (GI) tract, which is the main blood supply, and oxygenated blood also enters from the hepatic artery. Blood subdivides and drains into the sinusoids then exits via the terminal hepatic venule (THV) or central vein. The blood that perfuses the liver exits by these hepatic veins, which merge into the inferior vena cava and return blood to the heart. The hepatocytes located near the THV are referred to as centrilobular, while those near the portal vein are periportal hepatocytes, and these hepatocytes differ in size and functions.

Although hepatocytes comprise the majority of liver cells, other nonparenchymal cells are present in sizable numbers at specific locations (Figure 13.1). Bile duct epithelial cells are located in portal triads and endothelial cells line the sinusoids. Kupffer cells are macrophages, which engulf and destroy materials such as solid particles, bacteria, and dead blood cells, and are attached to the intralumenal side of the sinusoidal wall, while hepatic stellate cells (HSCs) (also known as fat-storing

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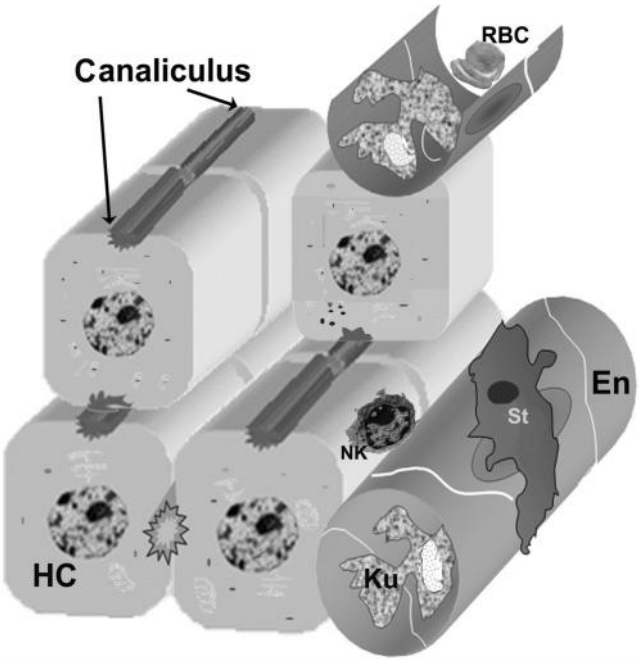

Figure 13.1 Diagram illustrating different types of liver cells and their spatial relationship. HC, hepatocytes; Ku, Kupffer cells; En, vascular endothelial cells; St, Stellate (Ito) cells; NK, lymphocytes.

or Ito cells) are in the perisinusoidal space of Disse, a region between the sinusoidal endothelium and hepatocytes. In chemically injured liver, the periportal region can become populated with a morphologically distinct cell, the "oval" cell, which is thought to be a stem cell capable of differentiating into either hepatocytes or bile duct epithelia.

Other materials, such as bile acids and many xenobiotics, move from the hepatocytes into the bile from their sites of synthesis at the hepatocyte canalicular membrane, which merge into larger ducts that follow the portal vein branches. The ducts merge into the hepatic duct from which bile drains into the upper part of the small intestine, the duodenum. The gall bladder, in all species but rat, serves to hold bile until it is emptied into the intestine.

13.1.2 Liver Function

The liver has many important physiological functions that impact the body, but the liver's three main functions include storage, metabolism, and biosynthesis, and the heterogeneity of hepatocytes in the conduct of these functions occurs largely differentiated by position along the sinusoid. Glucose is converted to glycogen and stored as needed for energy, and is converted back to glucose as the need arises by periportal hepatocytes due to their enrichment in gluconeogenic enzymes. Fat-soluble vitamins and other nutrients are also stored in the liver. Fatty acids are metabolized and converted to lipids, which are then conjugated with proteins

synthesized in the liver and released into the bloodstream as lipoproteins. The liver also synthesizes numerous functional proteins, such as enzymes and plasma proteins including blood-coagulating factors. In addition, the liver, which contains numerous xenobiotic metabolizing enzymes, is the main site of xenobiotic metabolism, which predominates in the centrilobular hepatocytes. Liver metabolism of xenobiotics absorbed from the gut can greatly reduce the xenobiotic blood levels reaching systemic circulation and is known as the first-pass effect.

13.2 SUSCEPTIBILITY OF THE LIVER

The liver, the largest organ in the body, is often the target organ for chemically induced injuries. Several important factors are known to contribute to the liver's susceptibility. First, most xenobiotics enter the body through the GI tract and, after absorption, are transported by the hepatic portal vein to the liver. Thus, the liver is the first organ perfused by chemicals that are absorbed in the gut and is exposed to the highest concentrations of xenobiotics. A second factor is the high concentration in the liver of xenobiotic metabolizing enzymes, primarily the cytochrome P450-dependent monooxygenase system. Although most biotransformations of xenobiotics act as detoxification reactions, many oxidative reactions produce reactive metabolites (Chapters 7 and 8) that can induce lesions within the liver. Often, areas of damage are in the centrilobular region, as hepatocytes in this localization have the highest concentration of cytochrome P450s (CYPs), and therefore, the greatest amount of reactive metabolites are produced in this region. Third, the process of bile formation and movement of bile to the GI tract can concentrate xenobiotics that are transported with the bile. Xenobiotics and most of the bile released into the intestines are reabsorbed and transported back to the liver by the hepatic portal circulation, which can increase the concentration of xenobiotics in hepatocytes.

13.3 TYPES OF LIVER INJURY

The classification of hepatotoxicity is primarily based on the pattern of incidence and the histopathological morphology. *Intrinsic* hepatotoxicants demonstrate broad incidence, dose-dependent relationship, and usually similar toxicities are seen in humans and animal models. *Idiosyncratic* hepatotoxicants demonstrate limited toxicity seen in susceptible individuals and results from hypersensitivity or unusual metabolic conversions that may occur due to polymorphisms in drug metabolizing genes. The types of injury to the liver depend on the type of toxic agent, the severity of intoxication, and whether the type of exposure is acute or chronic. The main types of liver damage are discussed briefly in this section. The hallmarks of hepatotoxicity are impaired hepatocyte function and viability that are observed histopathologically as steatosis (fatty liver), cholestasis, fibrosis, and necrosis, or apoptosis. Whereas some types of damage—for example, cholestasis—are liver specific, others such as necrosis and carcinogenesis are a more general phenomena. Damaged liver cells release liver-specific enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase into the blood.

The enzymes ALT and AST are used as biomarkers of injured hepatocytes, while alkaline phosphatase indicates bile duct epithelial damage. These enzymes are commonly monitored clinically and in animal studies to detect hepatotoxicity.

13.3.1 Fatty Liver

Fatty liver or steatosis refers to the abnormal accumulation of lipid in hepatocytes, primarily as triglycerides, due to an imbalance between the uptake of extrahepatic triglycercides and the hepatic secretion of triglyceride-containing lipoproteins and fatty acid catabolism. Although many toxicants may cause lipid accumulation in the liver (Table 13.1), the mechanisms may be different. Basically, lipid accumulation is related to disturbances in either the synthesis or the secretion of lipoproteins. Excess lipid can result from an oversupply of free fatty acids from adipose tissues or, more commonly, from impaired release of triglycerides from the liver into the plasma. Triglycerides are secreted from the liver as lipoproteins, such as very low density lipoprotein (VLDL). As might be expected, there are a number of points

TABLE 13.1 Examples of Hepatotoxic Agents and Associated Liver Injury

Necrosis and Fatty Liver		
Carbon tetrachloride	Dimethylnitrosamine	Phosphorous
Chloroform	Cyclohexamide	Beryllium
Trichloroethylene	Tetracycline	Allyl alcohol
Tetrachloroethylene	Acetaminophen	Galactosamine
Bromobenzene	Mitomycin	Azaserine
Thioacetamide	Puromycin	Aflatoxin
Ethionine	Tannic acid	Pyrrolizidine alkaloids
Troglitazone	Zidovudine (AZT)	
Cholestasis (Drug Induced)		
Chlorpromazine	Imipramine	Carbarsone
Promazine	Diazepam	Chlorthiazide
Thioridazine	Methandrolone	Methimazole
Mepazine	Mestranol	Sulfanilamide
Amitriptyline	Estradiol	Phenindione
Phenytoin		
Hepatitis (Drug Induced)		
Iproniazid	Methoxyflurane	Halothane
Isoniazid	Papaverine	Zoxazolamine
Imipramine	Phenyl butazone	Indomethacin
6-Mercaptopurine	Colchicine	Methyldopa
Carcinogenesis (Experimental Animals)		
Aflatoxin B1	Dimethylbenzanthracene	Acetylaminofluorene
Pyrrolizidine alkaloids	Dialkyl nitrosamines	Urethane
Cycasin	Polychlorinated biphenyls	
Safrole	Vinyl chloride	

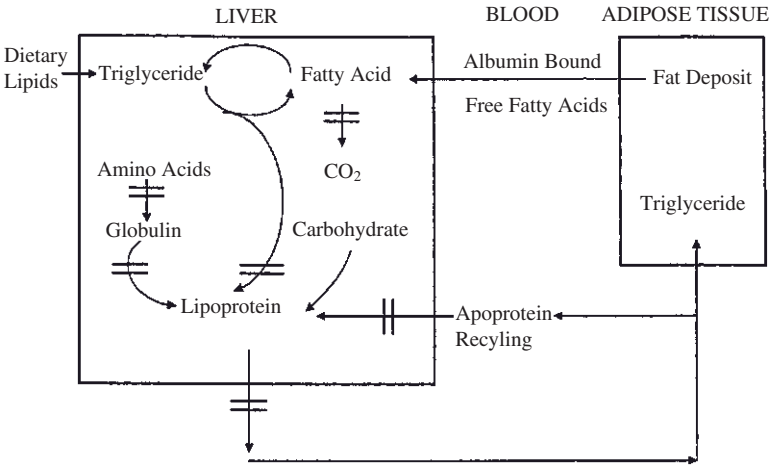

Figure 13.2 Triglyceride cycle in the pathogenesis of fatty liver. "=" are metabolic blocks. From Wallace, A. D. and S. A. Meyer. *Molecular and Biochemical Toxicology*, 4th ed. Wiley, 2008.

at which this process can be disrupted. Some of the more important ones are as follows (Figure 13.2):

- Interference with synthesis of proteins
- Impaired conjugation of triglyceride with lipoprotein
- Interference with transfer of VLDL across cell membranes
- Decreased synthesis of phospholipids
- Impaired β-oxidation of lipids by mitochondria
- Inadequate energy (adenosine triphosphate [ATP] for lipid and protein synthesis)

The role that fatty liver plays in liver injury is not clearly understood, and fatty liver in itself does not necessarily mean liver dysfunction. The onset of lipid accumulation in the liver is accompanied by changes in blood biochemistry, as indicated by changes in ALT and AST, and for this reason, blood chemistry analysis can be a useful diagnostic tool.

13.3.2 Cholestasis

Cholestasis is the suppression or stoppage of bile flow, and may have either intrahepatic or extrahepatic causes. Inflammation or blockage of the bile ducts results in retention of bile salts as well as bilirubin accumulation, an event that leads to jaundice. Other mechanisms causing cholestasis include changes in membranes permeability of either hepatocytes or biliary canaliculi. The formation of bile depends on ATP-dependent transport of bile into the canalicular lumen. Chemicals that have effects on membrane permeability and disrupt cellular Na^+ and K^+ gradients can cause cholestatis by their impact on the ATP-dependent movement of bile.

Cholestasis is usually drug induced (Table 13.1) and is difficult to produce in experimental animals. Again, changes in blood chemistry can be a useful diagnostic tool.

13.3.3 Fibrosis and Cirrhosis

Chemicals that are hepatotoxicants cause damage to hepatocytes that results in hepatic fibrosis as part of the wound-healing response. Fibrosis is characterized by the deposition of collagen, proteoglycans, and glycoproteins, and chronic fibrosis results in formation of an extracellular matrix (ECM) that can be observed histopathologically. After a toxicant exposure, hepatic stellate cells (HSC) proliferate and differentiate into fibroblast-like cells that secrete the components of the ECM. Extensive fibrosis can disrupt the liver architecture and blood flow resulting in irreversible liver damage. Reversibility of fibrosis is possible upon HSC becoming quiescent or undergoing apoptosis, breakdown of ECM, and hepatocyte regeneration.

Cirrhosis is a result of hepatotoxicant exposure that is characterized by fibrosis to the extent that deposition of collagen is found throughout the liver and results in the formation of scar tissue. In most cases, cirrhosis results from chronic chemical injury, which results in the accumulation of ECM that causes severe restriction in blood flow and also inhibits the liver's normal metabolic and detoxication processes. This situation can in turn cause further damage and eventually lead to liver failure. In humans, chronic use of ethanol is the single most important cause of cirrhosis, although there is some dispute as to whether the effect is due to ethanol alone or is also related to the nutritional deficiencies that usually accompany alcoholism.

13.3.4 Necrosis

Necrosis refers to an irreversible loss of cell viability that occurs due to loss of normal cellular function. Necrosis, usually an acute injury, may be localized and affect only a few hepatocytes (focal necrosis), or it may involve an entire lobe (massive necrosis). Cell death is "unordered" and occurs along with rupture of the plasma membrane, and is preceded by a number of morphologic changes such as cellular swelling, dilation of the endoplasmic reticulum, accumulation of triglycerides, swelling of mitochondria with disruption of cristae, and dissolution of organelles and a shrunken nucleus. In areas of necrosis, increased eosinophilic staining of the cytoplasm and an immune response is seen as neutrophils infiltrate the damaged area. Biochemical events that may lead to these changes include binding of reactive metabolites to proteins and unsaturated lipids (inducing lipid peroxidation and subsequent membrane destruction, disturbance of cellular Ca^{+2} homeostasis, inference with metabolic pathways, shifts in Na^{+} and K^{+} balance, and inhibition of protein synthesis. Changes in blood chemistry resemble those seen with fatty liver, except they are quantitatively larger. Because of the regenerating capability of the liver, necrotic lesions are not necessarily critical. Massive areas of necrosis, however, can lead to severe liver damage and failure.

13.3.5 Apoptosis

Apoptosis is a controlled form of cell death that serves as a regulation point for biologic processes and can be thought of as the counterpoint of cell division by

mitosis. This "ordered" mechanism of cell death, unlike necrosis, is particularly active during development and senescence. Although apoptosis is a normal physiological process, it can also be induced by a number of exogenous factors such as xenobiotic chemicals, oxidative stress, anoxia, and radiation. (A stimulus that induces a cell to undergo apoptosis is known as an apogen.) If, however, apoptosis is suppressed in some cell types, it can lead to accumulation of these cells. For example, in some instances, clonal expansion of malignant cells and subsequent tumor growth results primarily from inhibition of apoptosis.

Apoptosis can be distinguished from necrosis by morphologic criteria, using either light or electron microscopy. A hallmark of apoptosis is the absence of inflammatory infiltrate. Toxicants, however, do not always act in a clear-cut fashion, and some toxicants can induce both apoptosis and necrosis either concurrently or sequentially.

13.3.6 Hepatitis

Hepatitis is an inflammation of the liver and is usually viral in origin; however, certain chemicals, usually drugs, can induce a hepatitis that closely resembles that produced by viral infections (Table 13.1). It is characterized by the increase in immune cells and this type of liver injury is sometimes associated with idiosyncratic hepatotoxicants, such as diclofenac. This type of idiosyncratic response is not usually demonstrable in laboratory animals and is often manifest only in susceptible individuals. Fortunately, the incidence of this type of disease is very low.

13.3.7 Carcinogenesis

The most common type of primary liver cancer is hepatocellular carcinoma; other types include cholangiocarcinoma, biliary cystadenocarcinoma, and undifferentiated liver cell carcinoma. Although a wide variety of chemicals are known to induce liver cancer in laboratory animals (Table 13.1), the incidence of primary liver cancer in humans in the United States is very low.

Some naturally occurring liver carcinogens are aflatoxin, cycasin, and safrole. A number of synthetic chemicals have been shown to cause liver cancer in animals, including the dialkylnitrosamines, dimethylbenzanthracene, aromatic amines such as 2-naphthylamine and acetylaminofluorene, and vinyl chloride. The structure and activation of these compounds can be found in Chapters 6 and 7. In humans, the most noted case of occupation-related liver cancer is the development of angiosarcoma, a rare malignancy of blood vessels, among workers exposed to high levels of vinyl chloride in manufacturing plants. For a discussion of chemical carcinogenesis, see Chapter 11.

13.4 MECHANISMS OF HEPATOTOXICITY

Chemically induced cell injury can be thought of as involving a series of events occurring in the affected animal and often in the target organ itself:

- The chemical agent is activated to form the initiating toxic agent.
- The initiating toxic agent is either detoxified or causes molecular changes in the cell.

- The cell recovers or there are irreversible changes.
- Irreversible changes may culminate in cell death.

Cell injury can be initiated by a number of mechanisms, such as inhibition of enzymes, depletion of cofactors or metabolites, depletion of energy (ATP) stores, interaction with receptors, elevated intracellular free calcium, formation of a reactive metabolite, and alteration of cell membranes. In recent years, attention has focused on the role of biotransformation of chemicals to highly reactive metabolites that initiate cellular toxicity. Many compounds, including clinically useful drugs, can cause cellular damage through metabolic activation of the chemical to highly reactive compounds such as free radicals, carbenes, and nitrenes causing oxidative stress (Chapters 6 and 7).

These reactive metabolites can bind covalently to cellular macromolecules such as nucleic acids, proteins, cofactors, lipids, and polysaccharides, thereby changing their biologic properties. The liver is particularly vulnerable to toxicity produced by reactive metabolites because it is the major site of xenobiotic metabolism. Most activation reactions are catalyzed by CYP enzymes, and agents that induce these enzymes, such as phenobarbital and 3-methylcholanthrene, often increase toxicity. Conversely, inhibitors of CYPs, such as SKF-525A and piperonyl butoxide, frequently decrease toxicity.

Formation of reactive metabolites can result in oxidative stress, which has been defined as an imbalance between the pro-oxidant/antioxidant steady state in the cell, with the excess of pro-oxidants being available to interact with cellular macromolecules to cause damage to the cell, often resulting in cell death. To date, a number of liver diseases, including alcoholic liver disease, metal storage diseases, and cholestatic liver disease, have been shown to have an oxidative stress component.

Reactive oxygen and reactive nitrogen radicals can be formed in a number of ways (Figure 13.3), the former primarily as a by-product of mitochondrial electron transport. Superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl can all arise

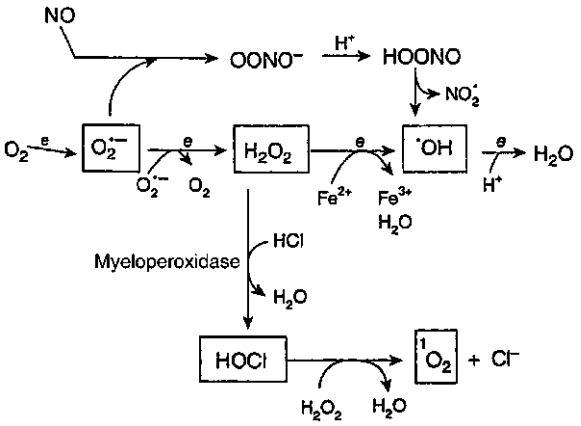

Figure 13.3 Origin of reactive oxygen and nitrogen species and sites of blocking their oxidant challenges by antioxidant defenses. From Reed, D. J. *Molecular and Biochemical Toxicology*, 4th ed. Wiley, 2008.

from this source. Other sources include monooxygenases and peroxisomes. If not detoxified, reactive oxygen species can interact with biological macromolecules such as DNA and protein or with lipids. Once lipid peroxidation of unsaturated fatty acids in phospholipids is initiated, it is propagated in such a way as to have a major damaging effect on cellular membranes. The formation, detoxication by superoxide dismutase and by glutathione-dependent mechanisms, and interaction at sites of toxic action are illustrated in Figure 13.3.

Mechanisms such as conjugation of the reactive chemical with glutathione are protective mechanisms that exist within the cell for the rapid removal and inactivation of many potentially toxic compounds. Because of these interactions, cellular toxicity is a function of the balance between the rate of formation of reactive metabolites and the rate of their removal. Examples of these interactions are presented in the following discussions of specific hepatotoxicants.

13.5 EXAMPLES OF HEPATOTOXICANTS

13.5.1 Carbon Tetrachloride

Carbon tetrachloride has probably been studied more extensively, both biochemically and pathologically, than any other hepatotoxicant. It is a classic example of a chemical activated by CYPs to form a highly reactive free radical (Figure 13.4). First, CCl_4 is converted to the trichloromethyl radical ($CCl_3\bullet$) and then to the trichloromethylperoxy radical ($CCl_3O_2\bullet$). Such radicals are highly reactive and generally have a small radius of action. For this reason, the necrosis induced by CCl_4 is most severe in the centrilobular liver cells that contain the highest concentration of the CYP isozyme responsible for CCl_4 activation.

Typically free radicals may participate in a number of events (Figure 13.5), such as covalent binding to lipids, proteins, or nucleotides as well as lipid peroxidation. It is now thought that $CCl_3\bullet$, which forms relatively stable adducts, is responsible for covalent binding to macromolecules, and the more reactive $CCl_3O_2\bullet$, which is formed when $CCl_3\bullet$ reacts with oxygen, is the prime initiator of lipid peroxidation. Lipid peroxidation (Figure 13.6) is the initiating reaction in a cascade of events, starting with the oxidation of unsaturated fatty acids to form lipid hydroperoxides, which then break down to yield a variety of end products, mainly aldehydes, which can go on to produce toxicity in distal tissues. For this reason, cellular damage

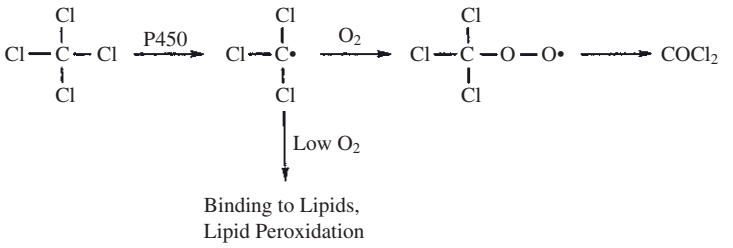

Figure 13.4 Metabolism of carbon tetrachloride and formation of reactive metabolites. From Hodgson, E. and Levi, P. E. *A Textbook of Modern Toxicology*, 3rd ed., Wiley, 2004.

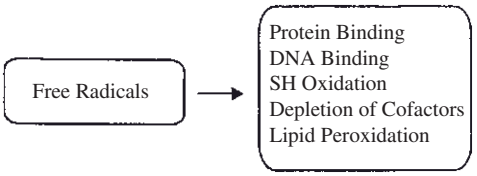

Figure 13.5 Summary of targets for free radicals. From Hodgson, E. and Levi, P. E. *A Textbook of Modern Toxicology*, 3rd ed., Wiley, 2004.

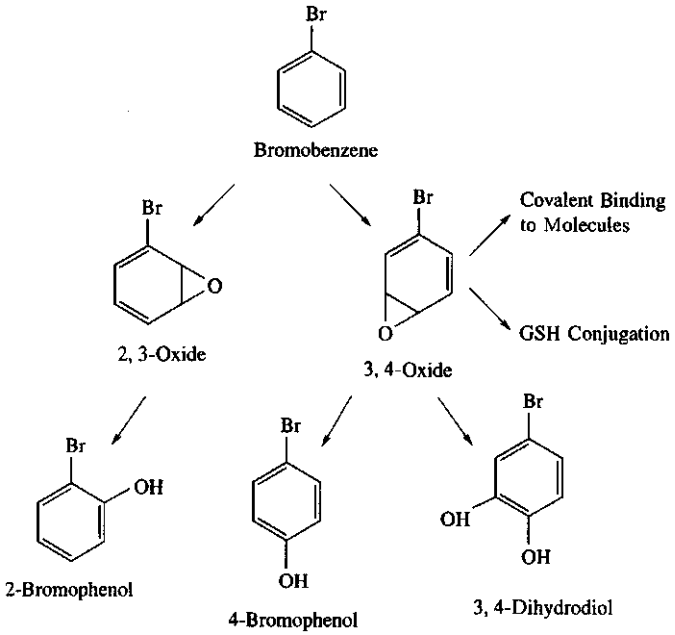

Figure 13.6 Metabolism of bromobenzene. From Hodgson, E. and Levi, P. E. *A Textbook of Modern Toxicology*, 3rd ed., Wiley, 2004.

results not only from the breakdown of membranes such as those of the endoplasmic reticulum, mitochondria, and lysosomes but also from the production of reactive aldehydes that can travel to other tissues. It is now thought that many types of tissue injury, including inflammation, may involve lipid peroxidation.

13.5.2 Ethanol

Alcohol-related liver diseases are complex, and ethanol has been shown to interact with a large number of molecular targets. Ethanol can interfere with hepatic lipid metabolism in a number of ways and is known to induce both inflammation and necrosis in the liver. Ethanol increases the formation of superoxide by Kupffer cells thus implicating oxidative stress in ethanol-induced liver disease. Similarly,

pro-oxidants (reactive oxygen species) are produced in the hepatocytes by partial reactions in the catalytic cycle of CYP2E1, an ethanol-induced CYP isoform. The formation of protein adducts in the microtubules by acetaldehyde, the metabolic product formed from ethanol by alcohol dehydrogenase, plays a role in the impairment of VLDL secretion associated with ethanol.

13.5.3 Bromobenzene

Bromobenzene is a toxic industrial solvent that is known to produce centrilobular hepatic necrosis through the formation of reactive epoxides. Figure 13.6 summarizes the major pathways of bromobenzene metabolism. Both bromobenzene 2,3-epoxide and bromobenzene 3,4-epoxide are produced by CYP oxidations. The 2,3-epoxide, however, is the less toxic of the two species, reacting readily with cellular water to form the nontoxic 2-bromophenol. The more stable 3,4-epoxide is the form most responsible for covalent binding to cellular proteins. A number of pathways exist for detoxication of the 3,4-epoxide: rearrangement to the 4-bromophenol, hydration to the 3,4-dihydrodiol catalyzed by epoxide hydrolase, or conjugation with glutathione. When more 3,4-epoxide is produced than can readily be detoxified, cell injury increases. Pretreatment of animals with inhibitors of CYPs is known to decrease tissue necrosis by slowing down the rate of formation of the reactive metabolite, whereas pretreatment of animals with certain CYP inducers can increase the toxicity of bromobenzene, as the CYP inducer phenobarbital increases hepatotoxicity by inducting a P450 isozyme that preferentially forms the 3,4-epoxide. However, pretreatment with another CYP inducer, 3-methylcholanthrene, decreases bromobenzene hepatotoxicity by inducing a form of CYP that produces primarily the less toxic 2,3-epoxide.

13.5.4 Acetaminophen

Acetaminophen is a widely used analgesic that is normally safe when taken at therapeutic doses. Overdoses, however, may cause an acute centrilobular hepatic necrosis that can be fatal. Although acetaminophen is eliminated primarily by formation of glucuronide and sulfate conjugates, a small proportion is metabolized by CYPs to a reactive electrophilic intermediate believed to be a quinoneimine (see Chapter 8). This reactive intermediate is usually inactivated by conjugation with reduced glutathione and excreted. Higher doses of acetaminophen will progressively deplete hepatic glutathione levels, however, resulting in extensive covalent binding of the reactive metabolite to liver macromolecules with subsequent hepatic necrosis. The early administration of sulfhydryl compounds such as cysteamine, methionine, and *N*-acetylcysteine is very effective in preventing liver damage, renal failure, and death that would otherwise follow an acetaminophen overdose. These agents are thought to act primarily by stimulating glutathione synthesis.

In laboratory animals, the formation of the acetaminophen-reactive metabolite, the extent of covalent binding, and the severity of hepatotoxicity can be influenced by altering the activity of various CYP isozymes. Induction of CYP isozymes with phenobarbital, 3-methylcholanthrene, or ethanol increases toxicity, whereas inhibition of CYPs with piperonyl butoxide, cobalt chloride, or metyrapone decreases toxicity. Consistent with these effects in animals, it appears that the severity of liver

damage after acetaminophen overdose is greater in chronic alcoholics and patients taking drugs that induce the levels of the CYP isozymes responsible for the activation of acetaminophen.

13.5.5 Troglitazone

Troglitazone (Rezulin®; Pfizer, Inc., New York, NY, USA) was a type II diabetes drug approved for use in 1997 and subsequently withdrawn from the market due to hepatotoxicity, which was seen in susceptible patients, but was not observed in preclinical animal studies. Troglitazone represented a new type of drug treatment for diabetes and acted as a peroxisome proliferator-activated receptor (PPAR) gamma agonist. In a small number of cases, complete liver failure was seen resulting in liver transplant or death. During therapy elevations of blood liver enzymes indicating hepatic injury were not seen until months after the initiation of treatment. The spectrum of liver injury in patients was broad with a heterogenous pattern of injury that included steatosis, cholestasis, fibrosis, cirrhosis, inflammation, and necrosis. Much effort has been made to elucidate if the mechanism(s) of toxicity involve genetic differences of metabolic enzymes in susceptible patients, formation of toxic metabolites, mitochondrial toxicity, oxidative stress, apoptosis, or a combination of these mechanisms. While it remains unclear the exact mechanisms responsible for troglitazone hepatotoxicity, evidence suggests a combination of unknown genetic and/or environmental factors lead to mitochondrial dysfunction. Fortunately, this type of idiosyncratic hepatotoxicity is rare, but much research still needs to be done to understand the mechanisms responsible.

13.6 METABOLIC ACTIVATION OF HEPATOTOXICANTS

Studies of liver toxicity caused by bromobenzene, acetaminophen, and other compounds have led to some important observations concerning tissue damage:

- Toxicity may be correlated with the formation of a minor but highly reactive intermediate.
- A threshold tissue concentration of the reactive metabolite must be attained before tissue injury occurs.
- Endogenous substances, such as glutathione, play an essential role in protecting the cell from injury by removing chemically reactive intermediates and by keeping the sulfhydryl groups of proteins in the reduced state.
- Pathways such as those catalyzed by glutathione transferase and epoxide hydrolases play an important role in protecting the cell.
- Agents that selectively induce or inhibit the xenobiotic metabolizing enzymes may alter the toxicity of xenobiotic chemicals.

These same principles are applicable to the toxicity caused by reactive metabolites in other organs, such as kidney and lung as will be illustrated in the following sections.

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SAMPLE QUESTIONS

1. Sinusoidal endothelial cells form loose connections such that the sinusoids are relatively leaky. When intra-sinusoidal Kupffer cells encounter certain agents, such as bacterial lipopolysaccharide (endotoxin), they become activated and secrete various small protein molecules, the cytokines. These cytokines can cause toxic responses in hepatocytes. Discuss how these spatially separated liver cells interact to mediate endotoxin-mediated hepatocellular toxicity.

2. How would you determine in an experimental animal study whether a hepatotoxicant required metabolic activation by cytochrome P450?

3. Hepatotoxicants can be classified into two different groups based on the pattern of injury. Name these two groups and describe them.

4. What are the hallmarks of hepatotoxicity and what tests can be done to detect hepatotoxicity?

CHAPTER 14

Nephrotoxicity

JOAN B. TARLOFF and ANDREW D. WALLACE

14.1 INTRODUCTION

Nephrotoxicity can be a potentially serious complication of drug therapy or chemical exposure. Although in most instances the mechanisms mediating nephrotoxicity are unclear, susceptibility of the kidney to toxic injury appears to be related, at least in part, to the complexities of renal anatomy and physiology.

14.1.1 Structural Organization of the Kidney

Upon gross examination, three major anatomical areas of the kidney are apparent: cortex, medulla, and papilla. The cortex is the outermost portion of the kidney and contains proximal and distal tubules, glomeruli, and peritubular capillaries. Cortical blood flow is high relative to cortical volume and oxygen consumption; the cortex receives about 90% of total renal blood flow. A blood-borne toxicant will be delivered preferentially to the renal cortex and therefore have a greater potential to influence cortical, rather than medullary or papillary, functions.

The renal medulla is the middle portion of the kidney and consists of the loops of Henle, vasa recta, and collecting ducts. Medullary blood flow (about 6% of total renal blood flow) is considerably lower than cortical flow. However, by virtue of its countercurrent arrangement between tubular and vascular components, the medulla may be exposed to high concentrations of toxicants within tubular and interstitial structures.

The papilla is the smallest anatomical portion of the kidney. Papillary tissue consists primarily of terminal portions of the collecting duct system and the vasa recta. Papillary blood flow is low relative to cortex and medulla; less than 1% of total renal blood flow reaches the papilla. However, tubular fluid is maximally concentrated, and the volume of luminal fluid is maximally reduced within the papilla. Potential toxicants trapped in tubular lumens may attain extremely high concentrations within the papilla during the process of urinary concentration. High intraluminal concentrations of potential toxicants may result in diffusion of these chemicals into papillary tubular epithelial and/or interstitial cells, leading to cellular injury.

The nephron is the functional unit of the kidney and consists of vascular and tubular elements. Both elements have multiple specific functions, which may be

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influenced by toxicants. The glomerulus is the portion of the nephron where ultrafiltrate of the plasma is formed and is governed by physical processes across capillaries. The renal tubule begins as a blind pouch surrounding the glomerulus, and consists of multiple segments which modify the composition of the ultrafiltrate. The segments of the renal tubule include the proximal tubule, loop of Henle, distal tubule, and collecting duct. The unique properties and functions of the cells that compose these segments can lead to susceptibility to toxicants.

14.1.2 Function of the Renal System

The kidneys participate in regulation of extracellular fluid volume, blood pressure, acid–base balance, and electrolyte balance. Blood-borne substances are exposed to kidney cells through the processes of filtration and reabsorption. A primary function of the kidneys is to eliminate waste products. During the process of reabsorption, potentially toxic chemicals may achieve higher concentrations than present in plasma, which may predispose the kidney to injury.

Renal tubules consist of multiple segments. These tubular elements selectively modify the composition of glomerular filtrate, enabling conservation of electrolytes and metabolic substrates while allowing elimination of waste products. For example, renal tubules reabsorb 98–99% of filtered electrolytes and water, and virtually 100% of filtered glucose and amino acids. Additionally, renal tubules participate in the reabsorption of bicarbonate and secretion of protons, thereby participating in acid–base balance.

Other functions of the kidney include synthesis of hormones. For example, 25-hydroxy-vitamin D_3 requires metabolism by the kidneys to the active 1,25-hydroxy-vitamin D_3. The kidney also secretes erythropoietin, which is involved in differentiation and development of red blood cells. Renin is an important enzyme released by the kidney in response to low blood pressure and catalyzes a step in the formation of angiotensin II, a powerful vasoconstrictor hormone.

Kidney toxicity is usually diagnosed by changes in excretory function, such as increases in urinary glucose, amino acid, or protein excretion, changes in urine volume, osmolarity, or pH. Changes in blood urea nitrogen (BUN) or serum creatinine concentrations are also indicators of altered renal function. Recently, several biomarkers have been approved by the Food and Drug Administration (FDA) as reliable indicators of kidney toxicity. A biomarker is a biochemical feature that can be used to diagnose a disease or monitor the effects of treatment. The biomarkers approved by the FDA are all proteins that appear in the urine when kidney damage has occurred and include proteins such as kidney injury molecule-1 (KIM-1), β_2-microglobulin, and albumin. Excretion of higher molecular weight proteins in the urine such as albumin is suggestive of injury to the glomerulus, while the presence of low molecular weight proteins, such as β_2-microglobulin is more suggestive of proximal tubule injury.

14.2 FACTORS CONTRIBUTING TO NEPHROTOXICITY

Several factors contribute to the unique susceptibility of the kidney to toxicants (Table 14.1). First, renal blood flow is high relative to organ weight. For an organ constituting less than 1% of body weight, the kidneys receive about 25% of the

TABLE 14.1 Factors Influencing Susceptibility of the Kidney to Toxicants

High renal blood flow
Concentration of chemicals in intraluminal fluid
Reabsorption and/or secretion of chemicals through tubular cells
Biotransformation of protoxicants to reactive intermediates

resting cardiac output. Thus, the kidneys will receive higher concentrations of toxicants (per gram of tissue) than poorly perfused tissue such as skeletal muscle, skin, and fat. Renal blood flow is unequally distributed, with cortex receiving a disproportionately high flow compared to medulla and papilla. Therefore, a blood-borne toxicant will be delivered preferentially to the renal cortex and thereby have a greater potential to influence cortical, rather than medullary or papillary, functions.

Second, the processes involved in forming concentrated urine also will serve to concentrate potential toxicants present in the glomerular filtrate. Reabsorptive processes along the nephron may raise the intraluminal concentration of a toxicant from 10 mM to 50 mM by the end of the proximal tubule, 66 mM at the hairpin turn of the loop of Henle, 200 mM at the end of the distal tubule, and as high as 2000 mM in the collecting duct. Progressive concentration of toxicants may result in intraluminal precipitation of poorly soluble compounds, causing acute renal failure secondary to obstruction. The potentially tremendous concentration gradient for passive diffusion between lumen and cell may drive even a relatively nondiffusible toxicant into tubular cells.

Third, active transport processes within the proximal tubule may further raise the intracellular concentration of an actively transported toxicant. During active secretion and/or reabsorption, substrates generally accumulate in proximal tubular cells in much higher concentrations than present in either luminal fluid or peritubular blood.

Fourth, certain segments of the nephron have a capacity for metabolic bioactivation. For example, the proximal and distal tubules contain isozymes of the cytochrome P450 monooxygenase system that may mediate intrarenal bioactivation of several protoxicants. Additionally, prostaglandin synthase activity in medullary and papillary interstitial cells may be involved in co-oxidation of protoxicants, resulting in selective papillary injury.

14.3 EXAMPLES OF NEPHROTOXICANTS

Many compounds have been implicated as nephrotoxicants (Table 14.2). Only rarely have specific receptors for specific nephrotoxicants been identified. Rather, in many cases it appears that toxicants exert multiple effects on intracellular systems. This is not to say, however, that there are not specific targets for certain nephrotoxicants in the kidney. For example, the proximal convoluted tubule seems to be more susceptible than other nephron segments to certain metals, such as chromium. The straight portion of the proximal tubule seems to be more susceptible to damage due to halogenated hydrocarbons (i.e., hexachlorobutadiene and dichlorovinyl-*L*-cysteine). Some agents, such as analgesic mixtures (usually aspirin, phenacetin, and caffeine) taken over long periods can produce a unique toxicity characterized

TABLE 14.2 Segments of the Nephron Affected by Selected Toxicants

Glomerulus
Immune complexes
Aminoglycoside antibiotics
Puromycin aminonucleoside
Adriamycin
Penicillamine
Proximal Tubule
Antibiotics
Cephalosporins
Aminoglycosides
Antineoplastic agents
Nitrosoureas
Cisplatin and analogs
Radiographic contrast agents
Halogenated hydrocarbons
Chlorotrifluoroethylene
Hexafluropropene
Hexachlorobutadiene
Trichloroethylene
Chloroform
Carbon tetrachloride
Maleic acid
Citrinin
Metals
Mercury
Uranyl nitrate
Cadmium
Chromium
Distal Tubule/Collecting Duct
Lithium
Tetracyclines
Amphotericin
Fluoride
Methoxyflurane
Papilla
Aspirin
Phenacetin
Acetaminophen
Nonsteroidal anti-inflammatory agents
2-bromoethylamine

by renal medullary and papillary necrosis. Histological evaluation following intoxication with analgesic mixtures reveals damage to the ascending limbs of the loop of Henle. Likewise, fluoride ion and outdated tetracyclines produce damage in this area.

14.3.1 Metals

Many heavy metals are potent toxicants. Exposure to relatively low amounts of metal can produce renal toxicity characterized by functional changes such as glucosuria, aminoaciduria, and polyuria. As the exposure level increases, renal necrosis, anuria, increased BUN, and overt renal failure may occur. Several mechanisms operate to protect the kidney from heavy metal toxicity. After low level exposure and often before detectable signs or symptoms of nephrotoxicity, metals may be found in renal lysosomes. Accumulation in lysosomes occurs following uptake of metal–protein complexes, digestion of metal-damaged organelles such as mitochondria, or interactions of metals with lipoproteins within lysosomes.

Cadmium Human exposure to cadmium is through food or industrial processes. Cadmium is excreted in urine complexed with metallothionein (MT), a low molecular weight protein synthesized in liver. MT contains free sulfhydryl groups that bind metals such as cadmium. Binding of cadmium to MT may protect some organs, such as testis and brain, from toxicity. However, the cadmium–MT complex may be taken up by kidney cells more readily than unbound cadmium. Thus, complexing of cadmium with MT may contribute to selective renal toxicity of cadmium. Cadmium–MT probably accumulates in lysosomes following uptake into kidney cells. Once in lysosomes, the cadmium may dissociate and persist in cells as free metal. The half-life of cadmium is extremely long in humans, 10–12 years, so that low level exposure to cadmium over time may result in renal accumulation and toxicity.

In Japan, Itai-itai Byo (literally, ouch-ouch disease) occurred among women who consumed rice grown in cadmium-contaminated areas. The disease is characterized by anemia, bone and joint pains, and kidney failure, and the severity of disease is correlated with the extent of cadmium contamination.

Mercury Mercury is found in the environment and many industrial settings, and exposure may occur from dietary sources such as contaminated water or food items such as large predator fish. Mercury can exist as elemental (Hg), mercury salts ($HgCl_2$), or organic mercury (R-Hg). In the body, elemental mercury is a cation (Hg^{2+}) that binds to sulfhydryl-containing molecules including glutathione, cysteine, homocysteine, and metallothionein. Within the kidney, inorganic and organic mercury accumulate rapidly.

The nephrotoxicity of mercury is characterized by increasing excretion levels of enzymes, such as alkaline phosphatase and γ-glutamyltransferase, amino acids, and albumin in the urine. Intracellular toxicity of mercury occurs due to its high affinity for thiol-containing proteins that can lead to oxidative stress involving mitochondrial dysfunction. Thiol-containing metal chelating agents such as *meso*-2,3-dimercaptosuccinic acid or 2 2,3-dimercapto-1-propanesulfonic acid are often utilized as antidotes for mercury poisoning and allow excretion of mercuric conjugates in the urine.

14.3.2 Antimicrobial Agents

Aminoglycosides Aminoglycoside antibiotics, such as gentamicin, amikacin, and netilmicin, are powerful drugs for the treatment of serious gram-negative

infections. However, about 10% of patients treated with aminoglycosides will develop moderate but significant signs and symptoms of renal toxicity. Aminoglycoside nephrotoxicity is characterized by proximal tubular necrosis, proteinuria, and a profound decline in glomerular filtration rate.

Aminoglycoside antibiotics are organic polycations and carry net positive charges. The primary route of elimination of aminoglycosides is by renal excretion. Gentamicin, a typical nephrotoxic aminoglycoside, is filtered at the glomerulus and appears to be reabsorbed via active transport processes at the proximal tubular brush border. Intracellular accumulation of gentamicin appears to occur following binding to plasma luminal membrane sites and incorporation of bound drug into apical vesicles such as lysosomes. Lysosomal alterations and the presence of myelin bodies and cytosegresomes are characteristic of aminoglycoside nephrotoxicity (Figure 14.1).

The sequence of biochemical events leading to gentamicin-induced proximal tubular dysfunction is unknown. Perhaps owing to its polycationic structure, gentamicin interferes with a number of intracellular proteins and macromolecules, producing a variety of biochemical effects. Several mechanisms have been proposed to account for gentamicin cytotoxicity, including (1) lysosomal damage, (2) altered phospholipid metabolism, (3) inhibition of critical intracellular enzymes, (4) inhibition of mitochondrial respiration, (5) lipid peroxidation, and (6) misreading of mRNA.

Cephalosporins Cephalosporins are broad-spectrum antibiotics similar in structure to penicillin. For several cephalosporins, therapy is limited by the development of nephrotoxicity.

Cephaloridine is zwitterionic and the principal route of elimination is by the kidneys. Cephaloridine clearance approximates inulin clearance, indicating absence

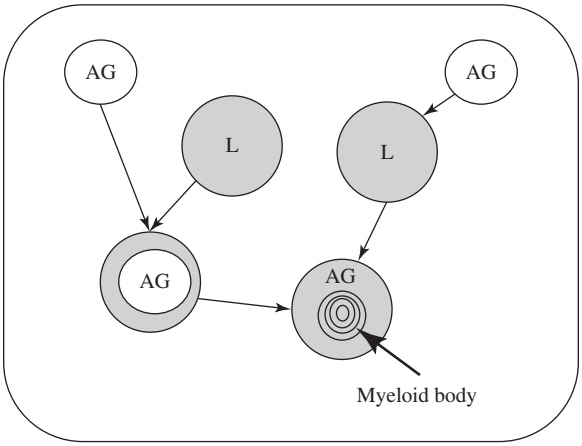


Figure 14.1 Interaction of aminoglycosides with lysosomes. Aminoglycosides (AG) enter the cell by pinocytosis and endocytosis, subsequently fusing with a primary lysosome (L). Aminoglycosides may interfere with normal lysosomal function, forming myeloid bodies (arrow).

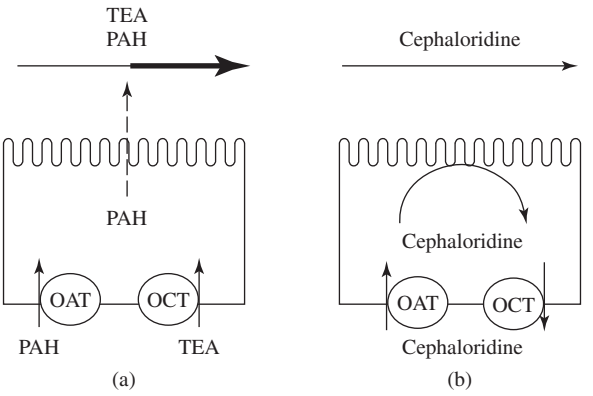


Figure 14.2 Schematic representation of proximal tubular transport and urinary excretion of *para*-aminohippurate (PAH), tetraethylammonium (TEA), and cephaloridine in the kidney. (a) PAH and TEA are excreted following both filtration and active secretion by the proximal tubule. PAH is transported across the basolateral membrane by organic anion transporter(s) (OAT) and TEA is secreted by organic cation transporter(s) (OCT). Intracellular concentrations of PAH and TEA may become great enough to drive passive diffusion from intracellular fluid to tubular fluid. Alternately, anion and cation exchangers may facilitate movement across the luminal membrane. (b) Cephaloridine is excreted primarily following filtration. Active cortical uptake of cephaloridine, inhibited by probenecid and PAH, indicates a secretory component for cephaloridine transport. However, diffusion of cephaloridine from proximal tubular cell to lumen is restricted, leading to high intracellular concentrations of cephaloridine. Some efflux of cephaloridine from proximal tubular cells appears to be mediated by organic cation transporter(s) since inhibitors of this transport system potentiate cephaloridine nephrotoxicity.

of net secretion for cephaloridine. However, inhibitors of organic anion transport, such as penicillin and probenecid, attenuate nephrotoxicity of cephaloridine while inhibitors of organic cation transport, such as cyanine 863 and mepiperphenidol, exacerbate toxicity. Taken together, these data suggest that, owing to its zwitterionic charge, cephaloridine is actively accumulated into proximal tubular cells via the organic anion transport system (inhibited by probenecid, PAH) and that a portion of cephaloridine efflux occurs via the organic cation transport system (inhibited by mepiperphenidol, cyanine). Once cephaloridine is transported into proximal tubular cells, it diffuses across the luminal membrane into tubular fluid only to a limited extent. Thus, active transport of cephaloridine into proximal tubular cells results in extremely high intracellular cephaloridine concentrations compared to other organs, which, in turn, contributes to selective nephrotoxicity (Figure 14.2).

Although the role of renal tubular transport in cephaloridine nephrotoxicity has been well defined, the exact molecular mechanisms mediating cephaloridine nephrotoxicity are less well understood. Several mechanisms have been postulated to mediate cephaloridine nephrotoxicity, including: (1) production of a highly reactive acylating metabolite(s) by cytochrome P450-dependent monooxygenases, (2) production of mitochondrial respiratory toxicity, and (3) production of lipid peroxidation.

Amphotericin B Amphotericin B is a polyene antifungal agent used in the treatment of systemic mycoses caused by opportunistic fungi. Clinical utility of amphotericin B is limited by its nephrotoxicity, characterized functionally by polyuria resistant to antidiuretic hormone administration, hyposthenuria, hypokalemia, and mild renal tubular acidosis.

Amphotericin B is highly lipophilic and interacts with membrane lipid sterols, such as cholesterol, to disrupt membrane permeability. Since amphotericin is freely filtered, it achieves high concentrations in distal tubular fluid and easily forms complexes with cholesterol and other lipids present in distal tubular luminal membranes. Amphotericin effectively transforms the "tight" distal tubular epithelium into an epithelium leaky to water, H^+ and K^+. Functional abnormalities observed with amphotericin B are attenuated when the antifungal agent is administered as an emulsion formulation whereby amphotericin is incorporated into lipid micelles. Antifungal activity of emulsion-formulated amphotericin B is equivalent to the standard non-emulsion formulation, whereas polyuria and hyposthenuria are significantly reduced by emulsion formulation.

14.3.3 Agents that Precipitate in Renal Tubules

The kidneys are responsible for producing small volumes of waste products and are involved in maintenance of water balance by antidiuretic hormone-dependent water reabsorption. However, this function may lead to relatively high concentrations of poorly soluble substances and in some cases, these poorly soluble substances may precipitate and obstruct urine outflow. Kidney stones represent a form of precipitate formation. The most common type of kidney stones contains calcium in combination with either oxalate or phosphate.

Ethylene Glycol Ethylene glycol is commonly found in antifreeze and hydraulic brake fluids. The cause of toxicity is not ethylene glycol but its metabolites, particularly oxalic acid. Ethylene glycol is metabolized initially to glycolic acid and ultimately to oxalic acid (Figure 14.3). Oxalic acid binds with calcium to form a poorly soluble product that precipitates and blocks urine flow. In addition, oxalic acid may be directly toxic to kidney cells.

Melamine Recalls of pet food in 2007 and infant formula in 2008 focused interest on the toxicity of melamine. Melamine is a nitrogen-containing compound used in

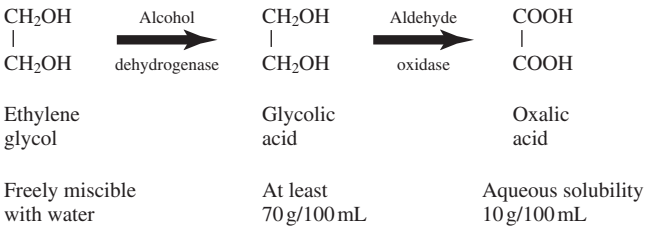
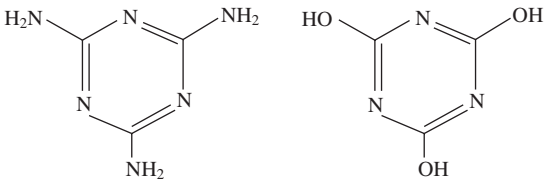


Figure 14.3 Metabolism of ethylene glycol, showing solubility of parent compound and metabolites.



Melamine Cyanuric acid

Figure 14.4 Chemical structures of melamine and cyanuric acid.

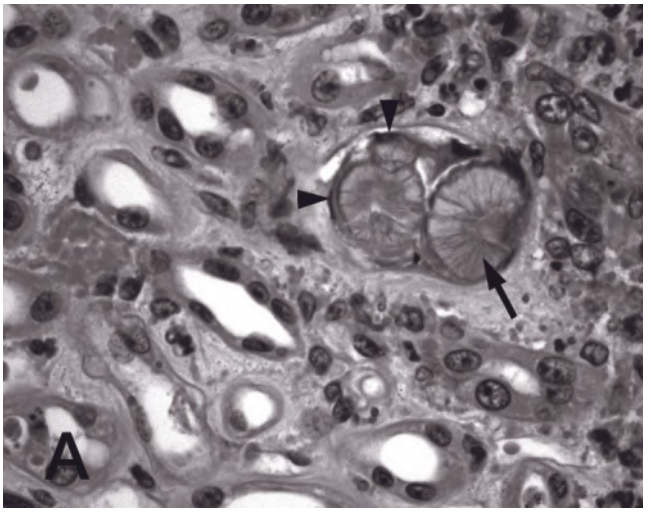


Figure 14.5 A hematoxylin and eosin stained paraffin embedded kidney tissue depicts the renal parenchyma from an adult cat, which was presented with acute renal failure during the recent outbreak of commercial pet food-associated melamine toxicosis. Characteristic melamine-containing crystals (arrow) occluding the lumen of a renal tubule and necrosis of tubular epithelial cells (arrowheads) are visible. Image courtesy of Drs. Ronald Baynes and Keith Linder (North Carolina State University).

commercial applications as a component of plastics. Because of its high nitrogen content, it is incorporated into animal feed as a nonprotein source of nitrogen. Cyanuric acid is also a nitrogen-containing compound used in the manufacture of bleaches. It can serve as a nonprotein nitrogen source in animal feed (Figure 14.4).

While both melamine and cyanuric acid have been reported as relatively safe, necropsies of animals fed contaminated pet food revealed yellowish-brown crystals present in kidney tissue (Figure 14.5). Subsequently, investigators found that a combination of melamine and cyanuric acid produced renal toxicity in rats and observed crystals containing both components in kidney tissue and urine from rats.

14.3.4 Halogenated Hydrocarbons

Chloroform Chloroform is a nephrotoxicant that most likely undergoes metabolic bioactivation within the kidney. Chloroform ($CHCl_3$), a common organic solvent

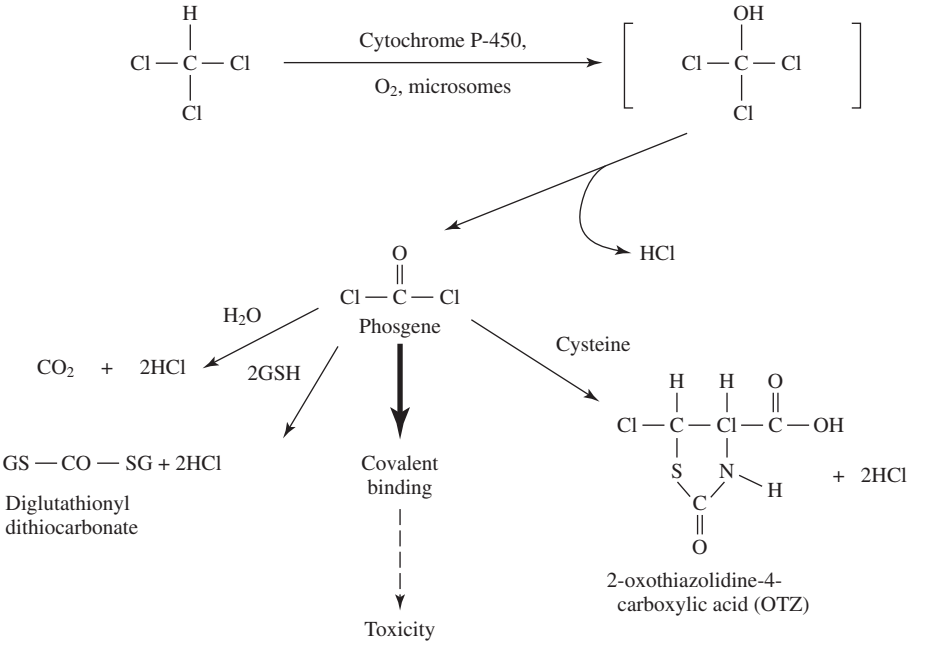


Figure 14.6 Proposed mechanism of chloroform biotransformation. Chloroform undergoes cytochrome P450-catalyzed conversion to trichloromethanol (CCl_3-OH), which spontaneously decomposes to form phosgene. Phosgene is highly reactive and may be detoxified by reacting with sulfhydryl-containing chemicals (cysteine, glutathione [GSH]). Alternately, phosgene can react with sulfhydryl groups on protein, leading to covalent binding and possibly to toxicity.

widely used in the chemical industry, produces hepatic and renal injury in humans and experimental animals. Tissue injury by chloroform is probably not due to chloroform per se, but is mediated by a chloroform metabolite. The initial step leading to chloroform-induced tissue injury is believed to be the biotransformation of chloroform to a reactive intermediate, phosgene ($COCl_2$). Phosgene is a highly reactive intermediate and may react with intracellular macromolecules to induce cell damage (Figure 14.6).

Hexachlorobutadiene Hexachlorobutadiene is an industrial solvent used in various applications. It is a widespread environmental contaminant and a relatively specific nephrotoxicant. The nephrotoxicity of hexachlorobutadiene is of interest because it is an example of formation of a more toxic compound due to glutathione conjugation. Glutathione is a major intracellular antioxidant and conjugation with glutathione is thought to represent a detoxification or protective pathway. However, hexachlorobutadiene–glutathione conjugates are further processed into species that can be accumulated by kidney cells. Once inside cells, the conjugate is metabolized by a specific renal enzyme, cysteine conjugate β-lyase, into a reactive intermediate.

14.3.5 Analgesics

Chronic consumption of large dosages of combination analgesics, typically phenacetin and/or caffeine-containing preparations, may be associated with renal papillary necrosis.

Renal function may be compromised modestly by a loss of concentrating ability or, in severe cases, anuria, sepsis, and rapid deterioration of renal function may occur. Morphologically, there is loss of renal papilla (containing terminal collecting ducts), medullary inflammation, and interstitial fibrosis, and loss of renomedullary interstitial cells. A variety of nonnarcotic analgesics have been implicated in the etiology of renal papillary necrosis, including acetaminophen, aspirin, acetanilid, and nonsteroidal anti-inflammatory agents such as ibuprofen, phenylbutazone, and indomethacin.

The mechanism of renal injury of these compounds is unclear. Chronic consumption over a period of many years is required to demonstrate loss of concentrating ability. Although these agents are dissimilar structurally and chemically, they share a common mechanism of action, acting as analgesics by inhibiting prostaglandin synthesis. In the kidney, prostaglandin H synthase activity is distributed asymmetrically, with highest activity in renal medulla and lowest activity in renal cortex. The renal papilla may be injured selectively by nonnarcotic analgesic agents due to the combination of high concentrations of potential toxicants present in tubular fluid and specialized enzymes capable of biotransforming protoxicants to active intermediates.

14.4 SUMMARY

Susceptibility of the kidney to chemically induced toxicity is related, at least in part, to several unique aspects of renal anatomy and physiology. By virtue of high renal blood flow, active transport processes for secretion and reabsorption, and progressive concentration of the glomerular filtrate following water removal during the formation of urine, renal tubular cells may be exposed to higher concentrations of potential toxicants than are cells in other organs. Additionally, intrarenal metabolism, via cytochrome P450 or prostaglandin H synthase, may contribute to the generation of toxic metabolites within the kidney.

The precise biochemical mechanisms leading to irreversible cell injury and nephrotoxicity are not well defined. Many diverse biochemical activities occur within the kidney, and interference with one or more of these functions may lead to irreversible cell injury. Rather than any one single mechanism mediating chemically induced nephrotoxicity, it is likely that a toxicant alters a number of critical intracellular functions, ultimately leading to cytotoxicity and cellular necrosis.

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SAMPLE QUESTIONS

1. Discuss how aspects of renal physiology, particularly water reabsorption, can contribute to selective kidney toxicity.

2. Discuss the role of renal transporters in exposure of kidney cells to potentially toxic concentrations of chemicals.

3. Compare and contrast the toxicity of chloroform and hexachlorobutadiene.

CHAPTER 15

Toxicology of the Nervous System

BONITA L. BLAKE

15.1 INTRODUCTION

Many substances alter the normal activity of the nervous system. Sometimes these effects are immediate and transient, like the stimulatory effect of a cup of coffee, or a headache from smelling fresh paint. Other effects can be much more insidious, like the movement disorders suffered by miners after years of breathing toxic manganese dust. Many agents are safe and even therapeutic at lower doses, but become neurotoxic at higher levels. Trace metals and pyridoxine (vitamin B6) fall into this category of dose-dependent neurotoxicants. Since these agents affirm the maxim, "the dose makes the poison," it becomes necessary to have a meaningful definition of nervous system poisoning, or neurotoxicity. *Neurotoxicity refers to the ability of an agent to adversely affect the structural or functional integrity of the nervous system.* It is often easier to identify changes in the structure or function of the nervous system than it is to say whether or not these events are adverse. For example, while some individuals need the stimulant effect of a morning cup of coffee, the same amount of coffee might provoke anxiety in others. Certainly, the function of the central nervous system (CNS) is altered (albeit temporarily) in both cases, but only those people who became jittery or nervous would characterize the effect as adverse.

In this chapter, a brief introduction to the nervous system and how it functions is described. A discussion of some of the mechanisms of structural and functional neurotoxicant effects follows. These descriptions are not exhaustive, but are meant to illustrate the concepts of toxicant interaction with the nervous system. Finally, some methods for testing toxicant effects in the nervous system are explored.

15.2 THE NERVOUS SYSTEM

Most multicellular animals possess a nervous system. In each case, the function of the nervous system is to receive signals about the external and internal environment,

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integrate this information, and then to coordinate a response that is appropriate to the environmental stimulus. All of the other organ systems of the body are subject to control by the nervous system, thus damage to this "master system" by toxicants can have far-reaching and even devastating effects. In addition to these basic, vital functions, the nervous systems of higher organisms are responsible for thinking and learning.

In vertebrates, there are two major components of the nervous system. The brain and spinal cord comprise the CNS, and the nervous tissue (ganglia and peripheral nerves) outside the brain and spinal cord comprise the peripheral nervous system (PNS). Although these two systems are thought of as separate anatomical divisions, they are contiguous and function interactively. The PNS can be further divided into the somatic nervous system (SNS) and the autonomic nervous system (ANS). The somatic division consists of neurons that carry sensory information from the skin, muscle, and joints to the CNS, and motor nerves that originate in the CNS and innervate skeletal muscle to cause contractive movement. The ANS is often thought of as an involuntary motor system for visceral organs, since it innervates and controls the function of smooth muscle, cardiac muscle, and endocrine and exocrine glands. The ANS consists of sympathetic and parasympathetic subdivisions that control functions that are needed in preparation for expending energy ("fight or flight," sympathetic) or conserving energy ("rest and digest," parasympathetic). For example, stimulation of sympathetic nerves increases heart rate, while stimulation of the vagus nerve, the primary parasympathetic innervation of the heart, slows the rate of cardiac contraction. Nearly all glands and organs are innervated by both sympathetic and parasympathetic nerves, and their influences generally oppose one another.

15.2.1 The Neuron

The basic unit of the nervous system is the neuron, a type of cell that is structurally and functionally specialized to receive, integrate, conduct, and transmit information. Although neurons are a far more diverse group than any other cell type in the body, some common features can be found. Neurons are polarized cells, meaning that they have different characteristics on one end of the cell compared to the other (Figure 15.1). A typical neuron has a receiving end and a transmitting end. The end of the neuron that receives information from other neurons, usually in the form of neurotransmitter stimulation, is highly branched and is known as the dendritic tree. The branches are sometimes studded with tiny projections, known as spines, which contain clusters of neurotransmitter receptors on the surface. In such areas of high receptor density, the neuron is in close contact with other neurons via specialized structures called synapses. Synapses are areas of close apposition where one neuron (called the presynaptic neuron) releases neurotransmitter into the gap between the two neurons. The receptors on the dendritic spine of the receiving neuron (called the postsynaptic neuron) are selective for certain types of neurotransmitters. Receptor stimulation by neurotransmitter is translated into intracellular and electrochemical signals, and these signals from multiple regions of the dendritic tree are integrated together intracellularly. Neurotransmitters and their receptors are discussed in more detail below. In the typical neuron, the arborizations of the dendritic tree converge on the soma, or cell body, where the nucleus and most of

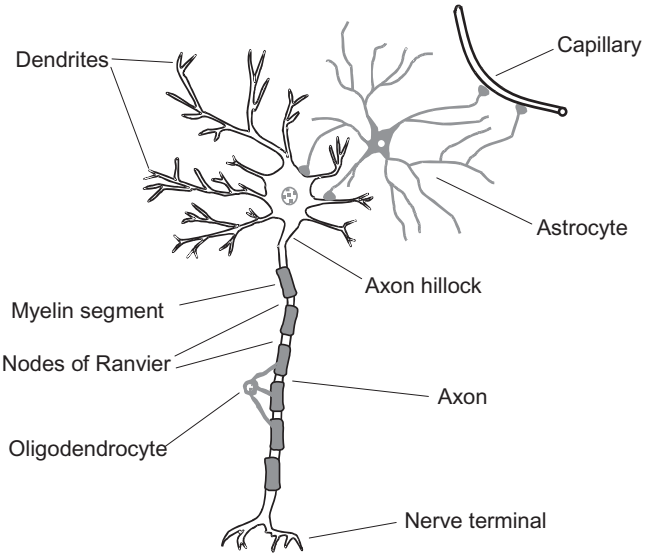

Figure 15.1 A typical neuron with myelinated axon. The neuron is shown with two types of glial cells, the myelinating oligodendrocyte and an astrocyte that is also interacting with a capillary.

RNA- and protein-synthesizing machinery exist. Integrated signals that reach the nucleus modulate the expression of a multitude of molecules within the neuron, many of which help to fashion the neuron's responsiveness to further neurotransmitter stimulation.

The area of the neuron designed to transmit information is the axon, and most neurons have only a single axon. The initial segment of the axon as it leaves the cell body is called the axon hillock. This area is particularly sensitive to the summation of signals from dendritic regions that arrive at the cell body. If enough signals arrive over a short period of time to reach a certain threshold, an action potential will be formed in the hillock. Here it is thus determined whether the neuron will transmit its information (or "fire"), causing the release of neurotransmitter at its terminal (see below).

In the resting state, the interior of the neuronal membrane is negatively charged compared to the exterior surface and with this difference in charge, the resting membrane is said to be "polarized." The charge difference, or potential, across the membrane in the resting state is approximately –70 mV, due primarily to an excess of sodium ions on the exterior which have been actively pumped out of the neuron by the energy-dependent Na^+/K^+ ATPase pump. Sodium, however, can be transferred back across the membrane through selective channels on the membrane surface. These channels are normally closed, but are sensitive to changes in the charge difference across the membrane, as well as to intracellular signaling pathways. Signals arriving from the dendritic regions of the neuron stimulate the opening of these channels and sodium moves inward down its own concentration gradient. The incoming sodium brings its positive charges with it, and this alters the resting state potential. The net charge difference across the membrane is thus reduced as

positive ions pour inward, and the membrane is said to be "depolarized." When the summation of these depolarization signals over a short time period reaches a certain threshold at the axon hillock (generally about +50 mV), the axon will generate an action potential. Once this occurs, all of the sodium channels in the nearby vicinity are stimulated to open, allowing a massive influx of sodium. Sodium channels stay open for only a short period of time, and once they close, they cannot reopen for a while, so the amount of time sodium can flow inward through a single channel is limited. However, as sodium channels a little further down the axon sense the voltage change across the membrane, they also open and thus, a feed-forward effect is created (Figure 15.2). The membrane is repolarized by the opening of potassium channels, which respond to the very same signals that stimulated the sodium channels but are slightly delayed in time. Therefore, as sodium channels begin to close after being stimulated, the potassium channels open, and potassium rushes out of

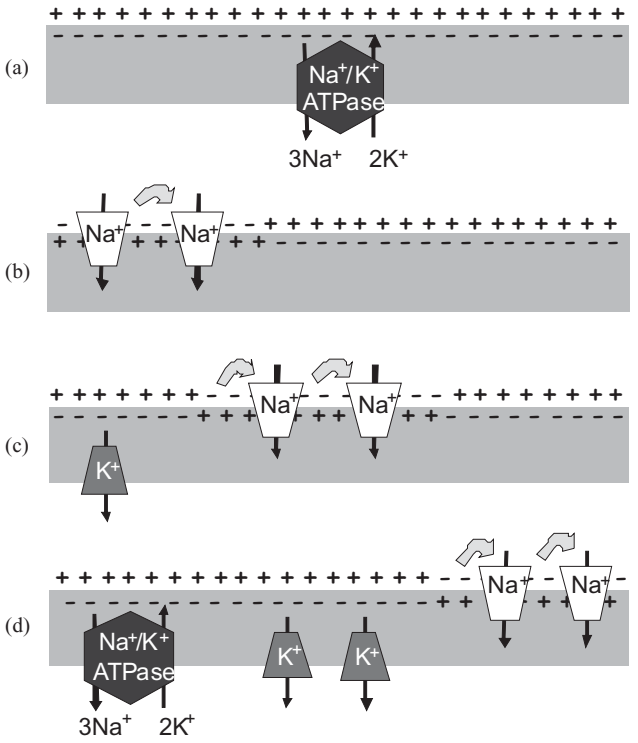

Figure 15.2 An action potential is a wave of electrical impulse that is propagated down an axon only in one direction. The ATP-dependent sodium/potassium pump (a) exchanges three sodium ions (transported to the outside of the cell) for two potassium ions (transported inward), maintaining a polarized membrane. When an action potential is initiated at the axon hillock, nearby voltage-gated sodium channels temporarily open and allow sodium to enter the neuron, depolarizing more of the membrane. This stimulates other channels to open, propagating the depolarization (b and c). Potassium channels open more slowly than sodium channels, and these allow potassium to exit, restoring the polarized state of the membrane (c and d). The ATPase pump reestablishes the sodium and potassium gradient needed to drive the next impulse (d).

the cell down its own concentration gradient. This produces a net outflow of positive charge, restoring the resting state condition of more positive charges on the outside and repolarizing the membrane. This process of depolarization/repolarization continues propagating itself down the length of the axon. Behind the action potential, the resting state sodium and potassium ion concentrations are restored by the ongoing activity of the Na^+/K^+ ATPase pump, pumping sodium back out and potassium back into the cell.

A segmented sheath of myelin (see Section 15.2.3) is found around the trunk of some axons. In myelinated axons, the ion channels that mediate action potential are clustered in regions between the segments of myelin. These regions are known as nodes of Ranvier. Myelin protects and insulates the axon, preventing any leakage of charge across the membrane and allowing the current to flow from one node to the next. The action potential is in effect regenerated at each node. This process of action potential jumping from node to node is called saltatory conduction (Figure 15.3) and results in much faster conduction velocity down the length of the axon.

Axons terminate at synapses with other neurons, at neuromuscular junctions, or in effector organs such as a gland or the heart. When the action potential reaches the terminal of the axon, the depolarizing impulse stimulates the release of neurotransmitter from the terminal into the cleft between the presynaptic membrane and its effector or receiving neuron (Figure 15.4). Neurotransmitter is packaged into vesicles docked at the presynaptic membrane. Upon stimulation by an incoming action potential, these vesicles fuse with the membrane to release their contents

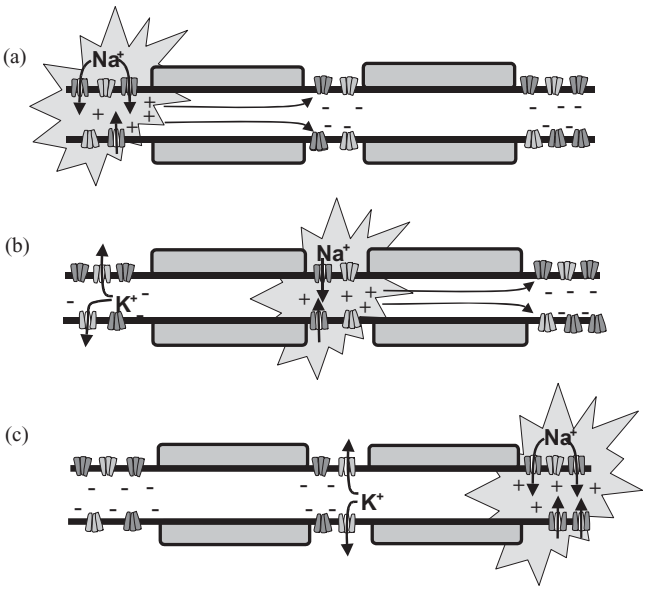

Figure 15.3 Saltatory conduction. (a) Myelin acts as an insulator to prevent current loss as the action potential travels down the axon. (b) Sodium and potassium channels are clustered at the Nodes of Ranvier, where there is no myelin. (c) Action potentials jump from one node to the next, reducing the overall membrane area involved in conduction, and speeding up electrical transmission.

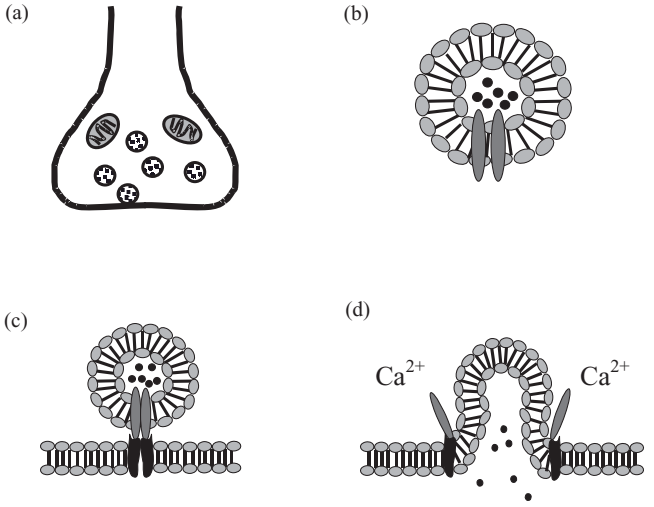

Figure 15.4 Neurotransmitter release. (a) Presynaptic nerve terminal is shown containing vesicles and other organelles. (b) Neurotransmitter-containing vesicles are made of lipid bilayers and contain membrane-associated proteins that participate in the release process. (c) These proteins form a complex with proteins on the presynaptic membrane to dock the vessels as they wait for the signal to release neurotransmitter. (d) The protein complexes alter their conformation when stimulated by calcium promoting fusion of the vesicle with the presynaptic membrane. Neurotransmitter within the vesicle is then free to diffuse into the synapse (d).

into the synaptic cleft. The actual primary signal to fuse is an influx of calcium, mediated by calcium channels on the presynaptic membrane that, like the sodium channels described above, are sensitive to changes in voltage across the membrane. Specific proteins on the vesicle membrane and on the presynaptic membrane form complexes with one another, and when stimulated by the localized increase in calcium ion concentration, mediate the fusion and pulling apart of the two membranes to release neurotransmitter. Electrical signals transferring information within the neuron are thus converted to chemical signals that transfer information between neurons in the form of neurotransmitters.

15.2.2 Neurotransmitters and Their Receptors

Neurotransmitters are recognized by receptors that lie on the postsynaptic membrane of receiving neurons, at neuromuscular junctions, or on end effector organs. Receptors are generally selective for the neurotransmitter that they bind, just like the lock-and-key mechanism of an enzyme/substrate interaction. Often, more than one selective receptor is associated with a specific neurotransmitter. An example of this is acetylcholine, which binds to two very different subclasses of selective receptors, the nicotinic and muscarinic acetylcholine receptors. The acetylcholine receptor found in neuromuscular junctions belongs to the nicotinic subclass, and these receptors are ion channels that are permeable to sodium. Stimulation of nicotinic receptors by acetylcholine results in the opening of the channel, and the influx of

sodium serves to rapidly depolarize the muscle membrane that receives acetylcholinergic innervation. Neurotransmitter receptors that are ion channels thus mediate very fast and short-lived neurotransmission. This is particularly evident when one compares its signaling to that of the other major type of neurotransmitter receptor, the G protein-coupled receptor. Unlike nicotinic receptors, muscarinic acetylcholine receptors are coupled intracellularly to G proteins, which then activate a variety of intracellular signaling pathways. G protein-coupled receptors thus produce a more slow and sustained response to neurotransmitter stimulation. G protein-coupled receptors can modulate ion channel neurotransmission by stimulating kinase and phosphatase pathways, altering the phosphorylation state, and thus the activity, of ion channels. G protein-coupled receptors also signal to the nucleus to maintain and mediate changes in RNA and protein expression, and promote cellular survival.

Neurotransmitters stimulate receptors on postsynaptic membranes, but the message mediated by the receptor may be either excitatory or inhibitory to the receiving neuron. For example, the neurotransmitter glutamate binds to selective ion channel receptors and G protein-coupled receptors, and both of these receptor types transmit a signal that enhances the excitability of the receiving neuron. On the other hand, the neurotransmitter GABA (for gamma-amino butyric acid), while also binding both ion channel GABA receptors and G protein-coupled GABA receptors, is known for its ability to decrease the excitability of the postsynaptic neuron. Its message is therefore inhibitory to the propagation of signaling within a group of neurons. The nervous system works on a balance of excitatory and inhibitory neurotransmission, primarily mediated in the brain by glutamate and GABA, respectively.

15.2.3 Glial Cells

While neurons constitute the definitive unit of the nervous system, their function is critically dependent on the presence of glial cells. In fact, glial cells make up about 90% of cells in the nervous system. Glial cells perform many functions, including nutritive and protective support, electrical insulation, modulation of synaptic function, and guidance of migration during development.

Astrocytes are the most numerous of all glial cells, and their roles in the nervous system are probably the most diverse. Of critical importance to toxicology, astrocytes make up the interface between the bloodstream and neurons. They help comprise part of the blood–brain barrier by extending processes that enwrap and interact with blood vessels, prohibiting some substances from reaching neurons while actively transporting glucose and other substances to neurons. Astrocytes also signal changes in neuronal activity to blood vessels, resulting in changes in regional blood flow. This allows more glucose and oxygen to be delivered to neurons when they are highly active, a mechanism that is the basis for the study of brain activity by functional magnetic resonance imaging or fMRI.

Astrocytes are also intimately associated with synapses, where they take up excess neurotransmitter and ions, and serve as a physical barrier to isolate synaptic connections between neighboring neurons. In this manner, private signals are transmitted between two communicating neurons while diffusion of neurotransmitter into the extrasynaptic space (where it could interact with other neurons) is limited. Astrocytes express many of the same neurotransmitter receptors that neurons do.

Upon stimulation, glutamate that has been taken up by astrocytes can be released to interact with neurons, thus these cells are active participants in synaptic signaling. Other molecules released by astrocytes include growth factors and neuromodulators (usually peptides or small molecules like ATP) that inhibit or enhance overall levels of neuronal activity.

Metabolic enzymes expressed within and on the surface of astrocytes regulate neuronal signaling by catabolizing excessive amounts of neurotransmitter. Monoamine oxidases, for example, catalyze the biotransformation of dopamine, norepinephrine, and serotonin into oxidation products that are substrates for further enzymatic reactions *en route* to excretion. Several drugs and neurotoxicants are also substrates of these enzymes.

Astrocytes are very sensitive to the homeostatic status of the tissue in which they reside. In response to a toxic insult or other injury, astrocytes are activated to multiply and undergo morphological changes. Activated astrocytes have greatly enlarged cytoplasmic processes, and produce increased amounts of a protein known as glial fibrillary acidic protein (GFAP). GFAP is often used as a quantitative histochemical marker for toxicant-mediated injury in the nervous system.

Another class of glial cell performs the important function of insulating axons with myelin. The myelinating cells in the CNS are oligodendrocytes, while Schwann cells myelinate axons in the PNS. These cells wrap layer upon layer of their plasma membrane around an axon with very little cytoplasm between layers; thus, myelin is composed chiefly of lipids. The white matter areas of the brain appear white because they are dense in myelinated axons. Myelin aids in speeding electrical transmission by insulating axons from leakage of current. The loss of myelin can disrupt neurotransmission between different areas of the brain, or between the brain and the body. Several neurotoxicants that target myelin or myelinating glial cells are discussed in the following sections.

A third class of glial cell is called microglia. Unlike neurons and other glial cells that are derived from neuroectoderm, microglial cells are derived from hematopoietic precursor cells that migrate to the nervous system during development. Microglial cells are the resident immune cells of the nervous system, monitoring neural tissue for signs of injury or infection. When they encounter signals of injury, such as changes in ionic balance or inflammatory factors, microglia can migrate toward the source of such signals. At the same time, they change their morphology in a process known as activation and begin secreting inflammatory factors that attract other microglia and stimulate astrocyte involvement. At the final stage of activation, microglial cells transform into macrophages capable of engulfing cellular debris. While many of the functions of microglia are beneficial, they can also release factors that are cytotoxic to neural tissue, such as damaging inflammatory cytokines and reactive oxygen species. Often, the most devastating consequences of toxicant action in the nervous system arise indirectly due to inflammatory responses that have spiraled out of control.

15.2.4 The Blood–Brain Barrier

The blood–brain barrier was conceptualized when it was noted that dyes injected into the bloodstream of animals stained nearly all tissues except the brain. This barrier and its PNS equivalent, the blood–nerve barrier, prevent all but a select few

molecules from entering the nervous system. The barrier itself is not a single unitary structure but is a combination of unique anatomical and biochemical features that prevent the translocation of blood-borne agents from brain capillaries into the surrounding tissue. As mentioned above, astrocytes help form the barrier, surrounding capillary endothelial cells with extensions of their cytoplasm known as endfeet. There are also pericytes, the function of which is not well-known, that associate with the capillaries and may participate in blood flow regulation and inflammation. Another component of the barrier is the relatively impermeable nature of the endothelial cells that line the interior of capillaries in the nervous system. For example, capillary endothelial cells in the brain are different from those in the periphery in at least three ways. First, brain capillaries form tight junctions of very high resistance between cells. In contrast, peripheral capillaries have low resistance tight junctions, and even openings, or fenestrations, which allow compounds to pass between cells. Second, compared to peripheral endothelial cells, brain endothelial cells are deficient in their ability to transport agents by pinocytosis, and only small lipophilic molecules are transported transcellularly by this mechanism. For larger molecules, carrier-mediated transport mechanisms are highly selective, and allow only one-way transport. Third, there is an enzymatic barrier that metabolizes nutrients and other compounds. Enzymes such as gamma-glutamyl transpeptidase, alkaline phosphatase, and aromatic acid decarboxylase are more prevalent in cerebral microvessels than in non-neuronal capillaries. Most of these enzymes are present at the lumenal side of the endothelium. Additionally, the P-glycoprotein (P-gp) multidrug efflux transporter is presently thought to exist at the interior surface of the capillary, although some scientists argue that P-gp is actually associated with astrocytes. Finally, the CNS endothelial cell displays a net negative charge at its luminal side and at the basement membrane. This provides an additional selective mechanism by impeding passage of anionic molecules across the membrane.

Most of the toxicants that enter the nervous system do so by exploiting mechanisms designed to allow entry of essential molecules, such as nutrients, ions, neurotransmitter precursors, and the like. Small, lipophilic molecules are able to cross the blood–brain barrier relatively easily. Some agents can be recognized by active transport systems and thereby traverse the blood–brain barrier along with endogenous ligands. For example, the neurotoxicant methylmercury forms a complex with cysteine and enters the brain through amino acid transporters due to its structural similarity to methionine. In some cases, the blood–brain barrier is itself subject to damage by neurotoxicants. Metals such as lead, cadmium, mercury, and manganese accumulate in endothelial cells and damage their membranes, leading to brain hemorrhage and edema.

15.2.5 The Energy-Dependent Nervous System

Nervous tissue has a high demand for energy, yet nerve cells can only synthesize ATP through glucose metabolism in the presence of oxygen. Critical ATP-dependent processes in the nervous system include regulation of ion gradients, release and uptake of neurotransmitters, anterograde and retrograde axonal transport, active transport of nutrients across the blood–brain barrier, P-gp function, phosphorylation reactions, assembly of mitochondria, and many others. The highest demand for energy (up to 70%) is created by the maintenance of resting potential in the

form of sodium and potassium concentration gradients across the nerve cell membrane. As discussed earlier, these gradients are maintained primarily by the activity of the Na^+/K^+ ATPase pump. The pump uses the energy of hydrolyzing each ATP molecule to transport three sodium ions out of the cell and two potassium ions into the cell. Maintenance of the resting potential is not the only benefit of this pump's activity, however. The gradients created by the pump are also important for maintaining osmotic balance, and for the activity of indirect pumps that make use of the sodium gradient to transport other molecules against their own concentration gradient. Neurotransmission is thus heavily dependent on the proper functioning of the Na^+/K^+ ATPase pump.

Another process dependent on energy metabolism is axonal transport. Axonal transport carries organelles, vesicles, viruses, and neurotrophins between the nerve nucleus and the terminal. This distance can be quite long when one considers that the length of the sciatic nerve, for example, can be up to one meter. Anterograde transport (from cell body to terminal) is accomplished by two mechanisms defined by their rate: fast axonal transport and slow axonal transport. Fast axonal transport proceeds at rates of approximately 400 mm/day, and is mediated by the ATP-dependent motor protein kinesin. Kinesin forms cross-bridges between vesicles or organelles and microtubules, and dual projections of these cross-bridges shift back-to-front in a coordinated, ATP-dependent manner, such that the entire molecule "walks" along the microtubule. Slow axonal transport is used to carry cytoskeletal elements such as tubulin and neurofilaments to the far ends of the axon, and it proceeds at approximately 0.2–5 mm/day. Traditionally, slow transport has been regarded as passively dependent on axoplasmic flow; however, recent evidence suggests that the cytoskeletal elements actually move rather quickly, but frequently stall in a stop-and-go fashion. Fast axonal transport also proceeds retrogradely, mediated by the ATP-dependent motor protein dynein. The rate of retrograde transport is about 200 mm/day. Neurons use retrograde transport for recycling membranes, vesicles, and their associated proteins. Neurotrophic factors, and some viruses and toxins (e.g., tetanus toxin) are also transported by this mechanism.

15.3 TOXICANT EFFECTS ON THE NERVOUS SYSTEM

Neurotoxicants affect the nervous system in a number of different ways. Some neurotoxicants damage the distal portions of axons without much effect on the remainder of the cell, while others produce outright cell death. Still others affect signaling processes in the nervous system without causing structural damage. Neurons may also be secondarily affected by neurotoxicants that target other cells in the nervous system, disrupting normal homeostatic function and causing structural or functional damage.

15.3.1 Structural Effects of Toxicants on Neurons

Demyelination The role of myelin in the nervous system is to aid in signal transduction. Myelin acts like an electrical insulator by preventing loss of ionic currents, and intact myelin is critical for the rapid saltatory nerve conduction as discussed above. Neurotoxicants that target the synthesis or integrity of PNS myelin may

cause numbness and tingling, muscle weakness, poor coordination, and paralysis. The nerve disorder associated with the loss of myelin from peripheral nerves is called myelinopathy. In the brain, white matter tracts that connect neurons within and between hemispheres may be destroyed, in a syndrome known as toxic leukoencephalopathy. Clinical manifestations of toxic leukoencephalopathy are extremely varied; some of these include headaches, to mild to severe cognitive dysfunction, paralysis, and death.

Neurotoxicants that produce primary demyelination are uncommon but may be divided into those that affect the integrity of the myelin sheath without or prior to damage to the myelinating cells, and those that directly injure myelin-producing cells. The former include agents like hexachlorophene, isoniazid, and the organotins. These compounds cause reversible edema between the layers of myelin by a mechanism that is yet unclear. The optic nerve is particularly susceptible to demyelination by hexachlorophene and organic solvents, whereas other cranial nerves, such as the trigeminal and vestibulocochlear, are vulnerable to styrene, xylene, and to tricholoroethylene, an agent used in dry-cleaning. The metalloid tellurium damages myelin by inhibiting an enzyme involved in the synthesis of cholesterol, a major component of myelin. In many cases, complete recovery from the effects of these agents is possible once the source of exposure is removed.

In contrast to agents that target the integrity of the myelin sheath, chronic exposure to cyanide and carbon monoxide is thought to directly injure myelin-producing Schwann cell bodies in the PNS and oligodendrocytes in the CNS. Inorganic lead also causes direct damage myelinating cells. Oligodendrocytes appear more sensitive to lead toxicity than astrocytes or neurons. One mechanism for the devastating developmental effects of lead exposure may be the preferential inhibition of oligodendrocyte precursor cell differentiation.

Axonopathy Axonopathy is a specialized form of neuronal damage, involving selective degeneration of the axon while leaving the cell body intact. In many cases, the most distal portions of the longest and largest diameter axons are most vulnerable to this type of toxicity, and these areas degenerate first. With continued exposure to the toxicant, however, the degeneration progresses proximally and may eventually affect the entire neuron. This distal-to-proximal degeneration is called "dying back neuropathy." As the axon degenerates, the myelin associated with it breaks down as well; yet Schwann cells may survive and guide regeneration of the axon in some cases. If exposure to the toxicant is discontinued before death of the entire proximal axon and cell body, axons in the PNS will often regenerate, but axonal regeneration does not occur within the CNS.

It has been speculated that the reason for the enhanced vulnerability of distal axons to toxic effects is because these regions are the most heavily dependent on intact axonal transport mechanisms. Since axonal transport is energy-dependent, toxicants that interfere with ATP production, such as the nicotinamide analog Vacor, may cause distal regions to degenerate initially. Agents that target tubulin, like the vinca alkaloids, also cause this type of injury, because the tubulin-derived microtubules are critically important for axonal transport.

In the 1850s Augustus Waller described the sequence of degenerative events that occurred following transection, or slicing in half, of a nerve fiber. These events have subsequently become known as Wallerian degeneration. The essential features of

this type of degeneration include swelling of the axon in the proximal segment at the, dissolution and phagocytosis by inflammatory cells of the axon segment distal to the transection, and dissolution of myelin, with preservation and proliferation of Schwann cells along the length of the former axon. Certain neurotoxicants are capable of chemically transecting an axon, producing Wallerian degeneration similar to that occurring after slicing the nerve in half. Hexane, for example, forms covalent adducts with neurofilament proteins resulting in secondary cross-linking of neurofilaments. This cross-linking is thought be the source of axonal swellings that contain high levels of neurofilament. These swellings essentially block transport to regions of the axon distal to the swelling, performing in effect a chemical transection. The distal regions then die due to lack of communication with the neuron cell body, undergoing Wallerian degeneration.

Axonopathy can manifest as defects in sensory or motor functions, or a combination of the two. For most neurotoxicants, sensory changes are noticed first, followed by progressive involvement of motor neurons. One historically important case that illustrates these effects is that of the epidemic poisoning resulting from the consumption of "Ginger Jake" during Prohibition. Tonics containing extracts of ginger were legally required to contain 5 g of ginger per milliliter of alcohol. To check for compliance with this requirement, the Department of Agriculture sampled the tonics by boiling off the ethanol and weighing the solid content. Bootleggers soon discovered that money could be saved by cutting back on the ginger and substituting it with adulterating agents like castor oil and molasses that would give the tonics the same amount of solid content. It was such an attempt at adulterating Ginger Jake that led to the addition of Lyndol, a triorthocresyl phosphate (TOCP)-containing oil used in lacquers and varnishes, to tonic that was consumed by hundreds of thousands of people. Days to weeks after consuming the product, people developed problems beginning with tingling and numbness in the hands and feet. In many, this progressed to leg cramps, weakness of the legs and arms, and loss of coordination and balance. Those with minor symptoms improved, but perhaps thousands of people were left permanently paralyzed by the incident. Today, TOCP is used to study the syndrome of delayed effects caused by some organophosphate compounds, commonly known as organophosphate-induced delayed neuropathy (OPIDN). The nature of OPIDN is still poorly understood. It appears not to be associated with organophosphate inhibition of acetylcholinesterase, but rather with another neuronal enzyme, the neuropathy target esterase (NTE). Recently, a physiological role for NTE in phospholipid homeostasis has been proposed.

Neuronopathy Neuronopathy refers to generalized damage to nerve cells, with the primary damage occurring at the nerve cell body. Many neurotoxicants produce their effects by promoting cell death in neurons. One area of intense research focus has been the toxic effects of excessive signaling by glutamate and other excitatory amino acids (EAAs), and the role that EAAs may play in neurodegenerative disorders. Glutamate activates ion channel receptors that open to allow influx of calcium and other ions into the neuron. This influx of ions, combined with other second messenger events that promote further intracellular release of calcium, contribute to calcium overload. Signaling cascades are then activated in response to the intracellular calcium, and these pathways eventually lead to oxidative stress and cell death. This type of injury, known as excitotoxicity, has been extensively

studied for its role in ischemic and seizure-induced brain damage. Domoic acid, a toxin produced by algae, binds to glutamate receptors and produces excitotoxic cell death. In 1987, several people died and dozens became ill with dizziness, seizures, and memory loss after consuming shellfish that were contaminated with domoic acid. The domoic acid had been produced at high levels following an algae bloom near Prince Edward Island, Canada, contaminating the shellfish. More recently, domoic acid produced by algae blooms has been blamed for episodes of abnormal behavior and deaths of pelicans, cormorants, and sea lions on the California coast.

15.3.2 Toxicant-Mediated Alterations in Synaptic Function

Nervous system function may be adversely affected by neurotoxicants without necessarily causing structural damage to tissue. In many cases, neurotoxicants interfere with signaling processes within the nervous system by activating or inhibiting receptors, or altering the amount of neurotransmitter available to activate receptors. This type of neurotoxicity is illustrated by the well-characterized actions of the organophosphates and carbamates on acetylcholine signaling.

Organophosphates inhibit acetylcholinesterase, the enzyme responsible for breaking down acetylcholine into acetic acid and choline. After acetylcholine has been released into the synapse or the neuromuscular junction, acetylcholinesterase terminates receptor-stimulating activity by binding acetylcholine in its active site. Separate sites within the binding pocket of acetylcholinesterase bind the quaternary nitrogen of the choline group, and the carbonyl of the ester group. A hydrolytic reaction results in the loss of choline, leaving an acylated serine residue, which is then rapidly hydrolyzed. The biologically active oxon forms of organophosphates also bind to the active site of acetylcholinesterase, covalently phosphorylating the serine residue in the catalytic site of the enzyme. The phosphorylation of acetylcholinesterase creates a relatively stable inactive enzyme that persists for hours to days before hydrolysis of the phosphate moiety occurs spontaneously, restoring acetylcholinesterase activity. The rate of spontaneous hydrolysis is increased with larger alkyl groups attached to the phosphate moiety. When one or more of these alkyl groups is lost, in a process known as “aging,” spontaneous reactivation of acetylcholinesterase by hydrolysis of the phosphate moiety is impossible, and the enzyme is permanently inactivated. Carbamates similarly inhibit acetylcholinesterase by carbamylating the enzyme active site. The stability of carbamylation is much less than phosphorylation, however, and spontaneous reactivation thus occurs faster than with organophosphates.

The effects of acetylcholinesterase inhibition can be seen throughout the nervous system. Acetylcholine and its receptors mediate neurotransmission in sympathetic and parasympathetic autonomic ganglia, in the effector organs where autonomic nerves terminate, in neuromuscular junctions, and in the brain and spinal cord. The signs of hypercholinergic activity are thus very diverse, and include effects mediated by both nicotinic and muscarinic types of acetylcholine receptor. Hyperstimulation of nicotinic receptors in neuromuscular junctions results in muscle weakness, in rapid, localized contractions called fasciculations, and in paralysis. Nicotinic receptors are also found in sympathetic and parasympathetic ganglia, and so stimulation of both divisions of the autonomic system is apparent as hypertension, increased heart rate, and dilation of the pupils. Muscarinic receptors in the PNS mediate

postganglionic parasympathetic effects on the smooth muscle present in the end organs such as the lung, gastrointestinal tract, eye, bladder, and secretory glands. Hyperstimulation of these receptors results in a pattern of toxicity known by the mnemonic SLUDGE (salivation, lacrimation, urination, defecation, g.i. upset, emesis). Bronchospasm and bradycardia are also muscarinic effects. In the CNS, confusion, anxiety, restlessness, ataxia, seizures, and coma are effects of both muscarinic and nicotinic receptor overstimulation. Death generally occurs from respiratory paralysis.

Anticholinergic toxicity by organophosphates and carbamates is directed at counteracting hyperstimulation and regenerating acetylcholinesterase enzymatic activity. Atropine is a muscarinic receptor antagonist (it blocks acetylcholine from binding to the muscarinic receptor), and is used to counteract the effects of cholinergic overactivity. Since muscarinic receptors are found primarily at parasympathetic sites, atropine blocks parasympathetic symptoms of organophosphate toxicity. Atropine has no effect at the nicotinic receptor, however, so the effects on skeletal muscle and some of the sympathetic responses to cholinergic hyperstimulation will remain after administration of atropine. Inhibition of acetylcholinesterase activity by organophosphates can be reversed by administration of oxime compounds (such as pralidoxime and 2-PAM). These compounds contain a quaternary nitrogen that binds to the choline binding site of acetylcholinesterase, positioning the oxime portion of the molecule near the esteratic site. Oximes are themselves reversible inhibitors of acetylcholinesterase, but their mechanism of organophosphate reversal is by attack of the covalent phosphoserine bond, releasing the phosphate group. Oximes are not effective on dealkylated or "aged" enzymes, so they must be administered soon after organophosphate intoxication in order to be effective. They are also ineffective against carbamate-mediated toxicity, and some researchers believe they actually worsen carbamate effects by stabilizing the carbamylation of the enzyme.

Whereas organophosphates enhance neurotransmitter activity by inhibiting the breakdown of acetylcholine, many biological toxins produce hyperstimulation of receptors by directly binding and activating them (agonism). Others reduce receptor stimulation by prohibiting the neurotransmitter from activating them (antagonism). A number of natural toxins such as snake and spider venoms, mushroom and plant alkaloids affect nervous system function by these mechanisms. As the binding of receptors by these agents is usually reversible, their effects are reversible as well, although some may still cause death by massively altering neuronal signaling.

On the other hand, the *Clostridium* bacterial toxins, botulinum (causing botulism) and tetanospasmin (causing tetanus), block neurotransmission by inhibiting release of neurotransmitter into synapses and at motor end plates in muscle. Both of these agents are structurally similar proteases, but the effects they cause are vastly different. Botulinum toxin enters presynaptic motor neurons in the PNS, where it cleaves proteins that are involved in the fusion of synaptic vesicles with membranes. This cleavage results in the inhibition of acetylcholine release from the presynaptic terminal, and thus muscles cannot be stimulated to contract. The clinical result of botulinum intoxication (usually by ingestion) is a flaccid paralysis. Since the release of acetylcholine onto muscles is blocked, the muscles are unable to contract and are thus flaccid or limp. Recovery occurs when the presynaptic neuron sprouts new nerve endings that contact the muscle and create new motor end plates. Tetanospasmin causes a completely different clinical picture, even though its

substrate specificity for the cleavage of proteins is very similar. Once taken up into the presynaptic nerve endings, tetanospasmin is transported retrogradely toward the neuron cell body and then to the dendritic regions of neurons. There it is released into synapses within the spinal cord. In the spinal cord, tetanospasmin prevents the release of the inhibitory neurotransmitter GABA onto the motor neurons. GABA normally acts as an inhibitory "brake" to keep motor neurons from becoming hyperactive, but when tetanospasmin blocks GABA release, the neurons fire readily in response to the many excitatory signals they receive. This firing of motor neurons results in overstimulation of the muscles with acetylcholine, resulting in spasms, stiffness, and whole-body paralysis. Thus, the clinical effect of tetanus toxin is a spastic paralysis that is quite the opposite of the effects of botulinum toxin. Similar to botulinum toxin, however, the interneurons themselves do not die, but they must form new synapses with the motor neurons to regain their ability to inhibit them. Fortunately, in all but the most severe cases, recovery is complete. The reformation of new synapses by neurons, even in the CNS, is an example of the remarkable plasticity of the nervous system. The continual formation and reformation of synaptic connections allows the organism to change and adapt to an inconstant environment.

15.4 NEUROTOXICITY TESTING

A large number of the chemicals used in industry today remain poorly characterized with respect to their toxic effects on the nervous system. In order to determine potential risks to human and environmental well-being, existing neurotoxicants must be identified, and the approximately 2000 new chemicals introduced each year must be screened for their potential neurotoxic effects. A tiered approach is recommended, with the first tier consisting of general screening tests to assess neurotoxic exposures. These include a functional observational battery (FOB, see below) to evaluate sensory, motor, and autonomic effects, as well as quantitative tests that identify changes in motor activity, and neuropathological or postmortem assessment. More selective testing and examining the effects of repeated exposures are used to characterize effects in the second tier. Specialized tests for behavioral effects, developmental neurotoxicity, or delayed organophosphate effects may be required. If necessary, a third tier of testing characterizes dose–response effects and identifies mechanisms of neurotoxicant-induced injury. Complete and comprehensive evaluation of potential neurotoxicant effects requires that data from different types of sources be considered; this can range from molecular interactions to whole animal and human exposure analysis. Some examples of techniques commonly used for testing neurotoxic effects are described below.

15.4.1 *In Vivo* Tests of Animal Exposure

The primary approach currently used to detect and characterize potential neurotoxicants involves the use of animal models, particularly rodents. Behavioral and neurophysiological tests, often similar to the ones used in humans, are typically administered. The sensitivity of these measures to neurotoxicant exposure is widely accepted. Although it is often not possible to test toxicant effects on some higher behavioral functions in animals (e.g., verbal ability, cognitive flexibility), there are

other neurobehavioral outcomes such as memory loss, motivational defects, somatosensory deficits, and motor dysfunction that can be successfully modeled in rodents. These behaviors are based on the ability of the nervous system to integrate multiple inputs and outputs, factors that are difficult to model adequately *in vitro*. Although the bulk of neurotoxicity data has been collected in rodents, birds and primates are also used to model human behavioral outcomes.

As mentioned above, a FOB is a useful screening tool for the effects of drugs and chemicals that are potentially neurotoxic. Designed to assess autonomic, neuromuscular, sensorimotor, and behavioral status of animals, FOBs are a noninvasive method of detecting overt changes in behavior and physiology of animals that have been exposed to neurotoxicants. In the typical exam, an observer documents cageside observations regarding the appearance and activity of the animal, such as whether the animal is sitting, running, lying on its side, having seizures, etc. Then, the animal is handled and examined for obvious signs such as lacrimation, salivation, or piloerection. The ease of handling the animal is noted at this time. The animal is then placed in an open field, such as the top of a laboratory cart, and observed for a set period of time, during which the observer records exploratory behaviors, excretion rate, mobility, and level of arousal. Following the open field measurements, the animal's response to various types of stimulation is tested. The latter tests are designed to assess hearing, sensitivity to touch and noise, righting reflex, coordination, and grip strength. Pupillary light responses, weight, and temperature are recorded. A more thorough test of locomotor activity can be administered along with the FOB, consisting of quantitative evaluations of the animal's movement in either an open field or a maze. A number of agents, such as toluene, triadimefon, and chlorinated hydrocarbons increase or decrease motor activity in a toxicant-specific manner, unrelated to their general effects on the health of the animal.

More in-depth behavioral tests are required if dose-related toxicant effects are noted in screening tests. These tests may also be required as part of more selective toxicological screening, such as for developmental neurotoxicity. Focused tests of neuromotor function and activity, sensory functions, memory, attention, and motivation help to identify sites of toxicant-mediated lesioning, aid in the classification of neurotoxicants, and may suggest mechanisms of action. Some of these tests, like the schedule-controlled operant behavior tests for cognitive function, require animal training and extensive operator interaction with the animals.

15.4.2 *In Vivo* Tests of Human Exposure

Historically, the first signs indicating neurotoxic potential by a chemical have often followed accidental human exposure in the workplace. Case reports of incidents involving individuals, or clusters of individuals, are useful for documentation, but generally provide a limited amount of information about the specific details of an exposure. Procedures included in most case reports include a patient's medical history and clinical neurological exam, sometimes supplemented with psychiatric or neurophysiological tests, and/or neuroimaging. Although the specific tests involved vary depending on the clinician, most basic clinical neurological exams rely heavily on evaluation of mental status (level of consciousness, orientation, mood, etc.) and sensorimotor function (gait, coordination, muscle tone, sensitivity to touch, reflexes).

Human epidemiological studies generally represent a deeper investigation into the causal relationship between an exposure and neurotoxicological effects. Some of the methods used to identify neurotoxic effects in epidemiological studies include behavioral assessments, neurophysiological evaluations, and neuroimaging techniques. Neurobehavioral assessments examine a variety of psychological and cognitive functions such as mood, attention, memory, perceptual and visuospatial ability, and psychomotor performance. In an effort to standardize neurotoxicological testing of human behavioral effects, particularly for studies involving worksite exposure, the World Health Organization (WHO) and the U.S. National Institute for Occupational Safety and Health (NIOSH) devised a the Neurobehavioral Core Test Battery (NCBT). The NCBT (Table 15.1) consists of seven tests that were shown previously to be sensitive indicators of neurotoxicant exposure. The battery is designed to be administered one-on-one by an examiner. Although this battery has a relatively narrow focus (primarily on the effects most commonly seen in CNS toxicity), it also provides suggestions for the selection of further testing depending on the exposure setting. The NCBT has been widely used because of its ease of administration, relatively low cost, and its large base of control data. A broader battery of cognitive and psychomotor tests that is often used is the Neurobehavioral Evaluation System (NES). The NES consists of a combination of automated

TABLE 15.1 The WHO Neurobehavioral Core Test Battery (NBCT)

Domain	Analysis	Test	Task
Psychomotor performance	Motor speed, motor steadiness	Pursuit aiming	Follow a pattern of small circles, placing a dot in each circle around a pattern; subject's score is number of taps in circle within one minute.
	Manual dexterity, hand–eye coordination	Santa Ana Dexterity test	Perform skillful movements with hands and arms.
Perceptual coding and perceptual motor speed		Wechsler digit Symbol test	Each number in a list is associated with a simple symbol. On a list of random digits with blank spaces below them, write the correct symbols in blank spaces as fast as possible.
Attention and short-term memory	Attention and response speed	Simple reaction time	Reactions of hands or feet from visual and auditory signals.
	Visual perception and memory	Benton visual retention test	Recall and reproduce figures.
	Auditory memory	Wechsler digit span test	Recall digits in series forward and backward immediately after hearing them.
Mood and affect		Profile of mood states	Questionnaire to evaluate anger, tension, confusion, depression, etc.

(computerized) and hand-administered tests. The sensitivity of the NES to effects caused by neurotoxicants in industrial settings has been validated internationally.

Neurobehavioral examinations are useful for identifying neurotoxicant-mediated deficits, but it is often difficult to localize the site of toxic action from such tests. For example, sensorimotor tests of reaction time, manual dexterity, hand–eye coordination, and finger tapping can indicate either neuromuscular or psychomotor damage. The results of these tests should thus be interpreted in the context of other experiments. For example, electrophysiological techniques can help to focus an investigation to the site of the lesion and characterize electrical dysfunction within the damaged nerves. Electrophysiological nerve conduction studies can distinguish between proximal and distal axonal lesions in peripheral nerves and can be performed noninvasively (i.e., with skin surface electrodes). Characteristic changes in the velocity, duration, amplitude, waveform, or refractory period of peripheral nerves may be detected, depending on the agent. Evoked potentials represent another useful electrophysiological end point. These procedures measure the function of an entire system, such as the visual, auditory, or motor systems. The specific pathway is stimulated by an evoking stimulus, such as a flash of light or electrical nerve stimulation. In response to the stimulation, evoked potentials are read as changes in ongoing electroencephalograms (EEGs) measuring electrical brain activity or electromyograms measuring electrical muscle activity. Evoked potentials can be very sensitive indicators of changes in neural activity when performed in a carefully controlled environment, and when interpreted in light of behavioral or other physiological findings.

An increasingly popular method of documenting brain pathology is the use of neuroimaging methods. Computerized axial tomography (CAT) and magnetic resonance imaging (MRI) can produce images of the brain that can show structural changes in the volume or density of a specific region or ventricle. Other techniques, such as positron emission tomography (PET) and single photon emission computerized tomography (SPECT), use radioactive tracer molecules to determine functional biochemical changes in processes like glucose utilization or receptor binding. The number of cases so far analyzed with neuroimaging techniques is still relatively small and thus, specific toxicant-mediated effects are not well characterized. Nevertheless, this growing field promises to contribute significantly to neurotoxicity studies in the future.

15.4.3 *In Vitro* Neurochemical and Histopathological End points

In vitro methods for studying neurotoxicant effects are a valuable part of whole animal and human testing, allowing the researcher to supplement findings, test hypotheses, and reduce the number of animals used for toxicity testing. Much of the neurochemical and histopathological data on neurotoxicant effects in humans and animals is gathered concomitantly with, or immediately after, performing behavioral tests. This may involve collection of bodily fluids or samples from living subjects for the purpose of analyzing acetylcholinesterase or NTE activity in blood, determining hormone or neurotransmitter concentration, or detecting the presence of toxicant or metabolite in the cerebrospinal fluid.

Postmortem tissues can provide a wealth of information about the location, timing, extent, and mechanism of neurotoxicant-induced damage. For example,

changes in the gross morphology and weight of brain or nerves may be seen at higher levels of toxicant exposure. Microscopically, fixed and stained tissues reveal characteristics regarding the type of damage to target cells, such as axonopathic or demyelinating lesions. Degenerative changes in cells may be indicative of the processes leading to injury, and may indicate whether cells are dying by necrosis or apoptosis. Typical stains such as Nissl stain (cresyl violet) and Golgi impregnation (with potassium dichromate and silver nitrate) are useful for cell morphology and counting. Other stains are selective for damaged cells, like the specialized silver degeneration staining techniques that are frequently used to identify neurotoxicant-mediated injury to neurons.

Tissue sections may also be processed for immunohistochemical staining. A frequently used immunochemical marker for neuropathologic insult is GFAP. GFAP is produced in large amounts by reactive astrocytes that proliferate in response to tissue injury. Stress proteins, apoptotic signals, and immediate early genes are also utilized as markers of neuronal activity and injury. Other protein markers can be used to quantitatively identify specific types of neurons, which may be reduced in numbers after selective neurotoxicant-induced cell death. For example, tyrosine hydroxylase (TH) is an enzyme involved in dopamine synthesis, and as such, is selectively expressed in dopamine-containing neurons. Loss of TH immunoreactivity is used to identify dopaminergic cell death.

In homogenized tissue preparations, mechanistic information can be obtained from analyzing tissue levels of neurotransmitter and metabolites, signaling proteins, and receptor binding affinities. Protein and lipid peroxidation and oxygen radical formation are commonly seen with toxicants that target mitochondrial function. Neurotoxicants may alter the levels or activation state of many proteins, including kinases, phosphatases, and proteases, quantifiable with activity or immunological techniques.

Cell culture protocols are a useful adjunct to neurotoxicity testing. Individual cell lines are particularly well suited for identifying selective cellular and molecular toxicity and for studying the mechanistic aspects of neurotoxicant injury. Clonal cell lines, as well as primary cultures of neurons or glial cells may be used, and the choice of cell type or particular clonal line depends on the particular end points under study. For example, if a researcher wished to study the effects of a given neurotoxicant on neurotransmitter release, he or she might choose the rat pheochromocytoma PC12 cell, which releases catecholamine neurotransmitter upon stimulation with a variety of agents. The relative inexpense and ease of manipulating exposure make cellular techniques an attractive alternative for many types of studies. Cultured cell studies cannot, however, reproduce systemic metabolic and kinetic effects, or mimic the complex neuronal circuitry that is present *in vivo*. Thus, while cell studies provide a vehicle for in-depth examination of the nature of toxicant–cellular interactions, extrapolation to *in vivo* conditions is often not possible.

15.5 SUMMARY

The nervous system is at once unique in structure and staggeringly complex, exquisitely sensitive, yet capable of amazing adaptability. Because of these attributes, the neurotoxic potential of many agents, to say little of their underlying mechanisms,

remains unknown. Particularly concerning are the possibilities that chronic low levels of chemical exposure are having an effect on the behavioral development of children, and contributing subtly to neurodegenerative diseases in the elderly. The huge task of testing natural and synthetic chemicals for neurotoxic effects has been facilitated in recent years with the development of behavioral testing batteries, advances in pathological and biochemical techniques, and a more focused attention of regulatory agencies on issues relating to neurotoxicology.

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SAMPLE QUESTIONS

1. Which of the following components of a neuron is specialized for receiving chemical signals from other neurons?

a. Nodes of Ranvier

b. Dendrites

c. Somas

d. Axons

2. Which cell type is considered the resident immune cells of the nervous system? Why?

3. Describe four components of the blood–brain barrier.

4. Atropine administration is unable to reverse all of the neurotoxic effects of organophosphates because it has no effect on …

a. Nicotinic receptors in the brain.

b. Nicotinic receptors in skeletal muscle.

c. Nicotinic receptors in the sympathetic nervous system.

d. Muscarinic receptors in the parasympathetic nervous system.

e. a, b and c.

5. Compare and contrast botulinum and tetanospasmin.

6. What is the purpose of a rodent functional observational battery?

CHAPTER 16

Reproductive System

HEATHER PATISAUL

16.1 INTRODUCTION

In her 1985 dystopian novel, *The Handmaid's Tale*, Margret Atwood described a human population rendered largely sterile by overwhelming nuclear, biological, and chemical pollution. Science fiction or foreshadowing? In 1992, Carlsen and colleagues conducted a comprehensive review of the literature on human semen quality. Their systematic analysis of 61 published papers, incorporating data collected from nearly 15,000 men, revealed a statistically significant decline in mean seminal volume and sperm concentration over the last 50 years (Carlsen et al., 1992). This finding was recently confirmed by a different group of investigators (Swan et al., 2000). In Demark, it is now estimated that more than 10% of men have sperm counts in the infertile range and up to 30% are in the subfertile range (Joensen et al., 2008). The rapidity of the decline suggests an environmental, rather than a genetic, etiology. It is now widely hypothesized that exposure to endocrine disrupting compounds, both naturally occurring as well as synthetic, are at least partially responsible for this decline and may also be contributing to a decline in female fecundity. Is this a plausible hypothesis? If so, did this decline in fecundity result from exposure in the womb, when the gonads were forming, puberty, when the reproductive system was maturing, or adulthood, when conception is desired? Can such a hypothesis be tested? Can decreased fecundity which results from environmental factors be improved or corrected? Could it affect subsequent generations? This chapter aims to illustrate both the major principles and complexity of reproductive toxicity, a field which spans conception through adulthood and includes the unborn.

16.1.1 Defining Reproductive Toxicity

What is reproductive toxicity? Reproductive toxicity is the occurrence of adverse effects on male or female sexual anatomy, function, maturation, or behavior resulting from exposure to exogenous chemical agents. This definition includes lactation, sexual maturation, the ability to produce viable, fertile offspring, sex-specific

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

behavior, and premature reproductive senescence. There is now speculation that gender preference and gender identity may also be influenced by toxic insult. Because the potential for successful reproduction begins in the womb, it can be difficult to distinguish reproductive toxicity from developmental toxicity. This chapter will first provide an overview of normal male and female reproductive physiology and discuss how the ontogeny and function of this system might be affected by toxicants, particularly endocrine disrupting compounds.

16.1.2 Defining Endocrine Disruption

Endocrine disruption is thought to be one of the primary ways in which the developing and adult reproductive system is altered by exposure to toxicants. The U.S. Environmental Protection Agency defines an endocrine disrupting compounds (EDC) as an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects. Hundreds of compounds have now been identified as possible endocrine disruptors. There are six basic mechanisms of endocrine disruption.

1. Alteration of the production and/or secretion of hormones.
2. Altered sensitivity of the target cell to the hormone. For example, this could result from interference with the ability to upregulate or downregulate a hormone receptor.
3. Agonism of the hormone receptor, effectively mimicking the effect of the hormone.
4. Antagonism of the hormone receptor, effectively blocking the effect of the hormone.
5. Alteration of hormone metabolism and/or clearance resulting in abnormally high or low levels of the hormone.
6. Displacement of a hormone from its binding proteins.

Think about these mechanisms as you read the rest of the chapter and how a compound, through any of these five mechanisms, could disrupt either the development or function of the reproductive system across the lifespan (Li et al., 2008).

16.2 THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

In mammals, reproductive maturation and function is coordinated by the hypothalamic-pituitary-gonadal (HPG) axis (Figure 16.1). The hypothalamus is a sexually dimorphic brain region that lies just below the thalamus. It forms the ventral part of the forebrain (diencephalon) and is responsible for coordinating the majority of neuroendocrine functions including hunger, thirst, circadian cycles, emotion, body temperature, stress, and reproduction. It is responsive to a number of external signals including day length, hormones, olfactory cues, and glucose levels among others. Mammalian reproduction is regulated by the temporal release of gonadotropin releasing hormone (GnRH) from the hypothalamus to the anterior portion of the pituitary gland (adenohypophysis). GnRH is released from the hypothalamus in

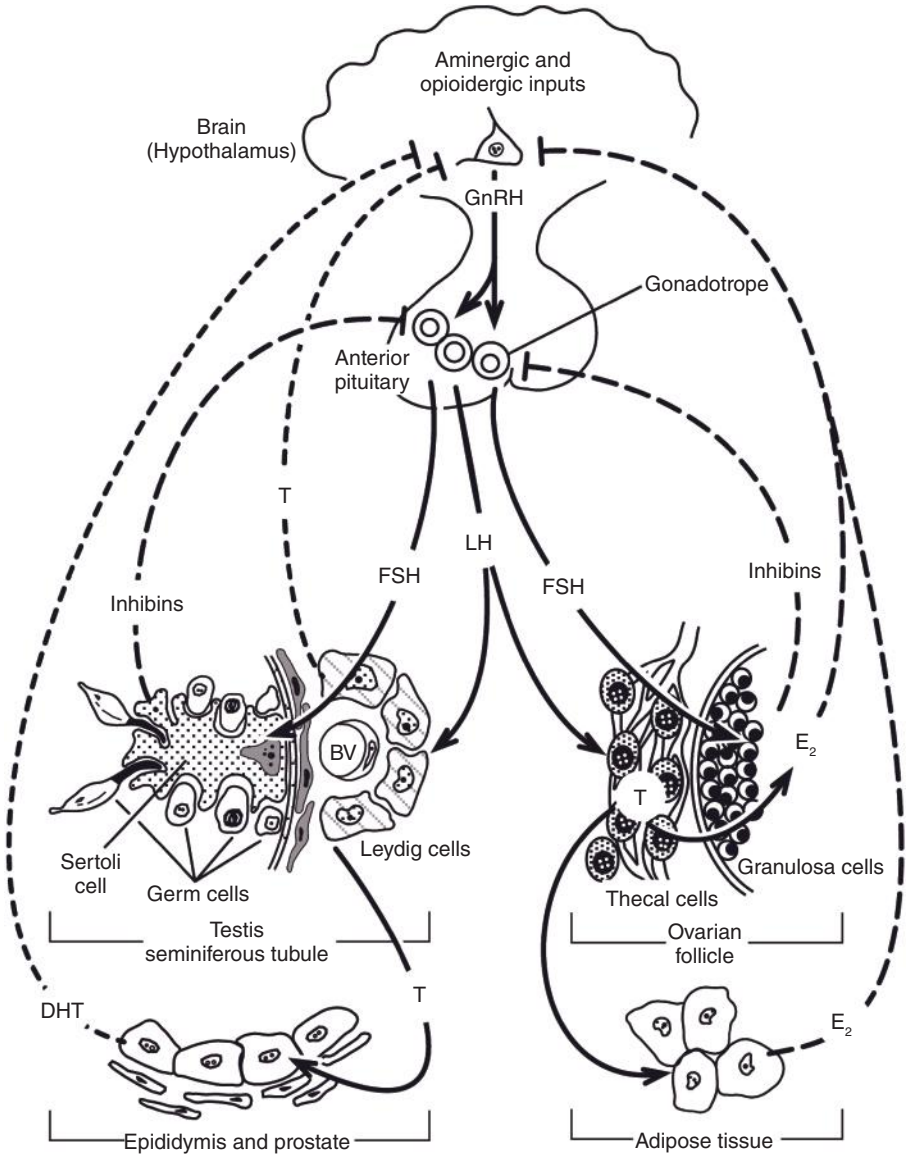


Figure 16.1 Steroid positive and negative feedback loops of the mammalian hypothalamic-pituitary-gonadal (HPG) axis. (E_2, estradiol; T, testosterone, DHT, dihydrotestosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone.) Reprinted with permission from Couse, J. F. Reproductive toxicology. In *Molecular and Biochemical Toxicology,* 4th ed., eds. R. C. Smart and E. Hodgson, p. 810. Hoboken, NJ: Wiley, 2008.

pulses, approximately once per hour in humans, and stimulates the synthesis and secretion of the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary into the bloodstream. FSH and LH then stimulate the production of steroid hormones, primarily estrogens in females and androgens (such as testosterone) in males, by the gonads. Elevated steroid hormone

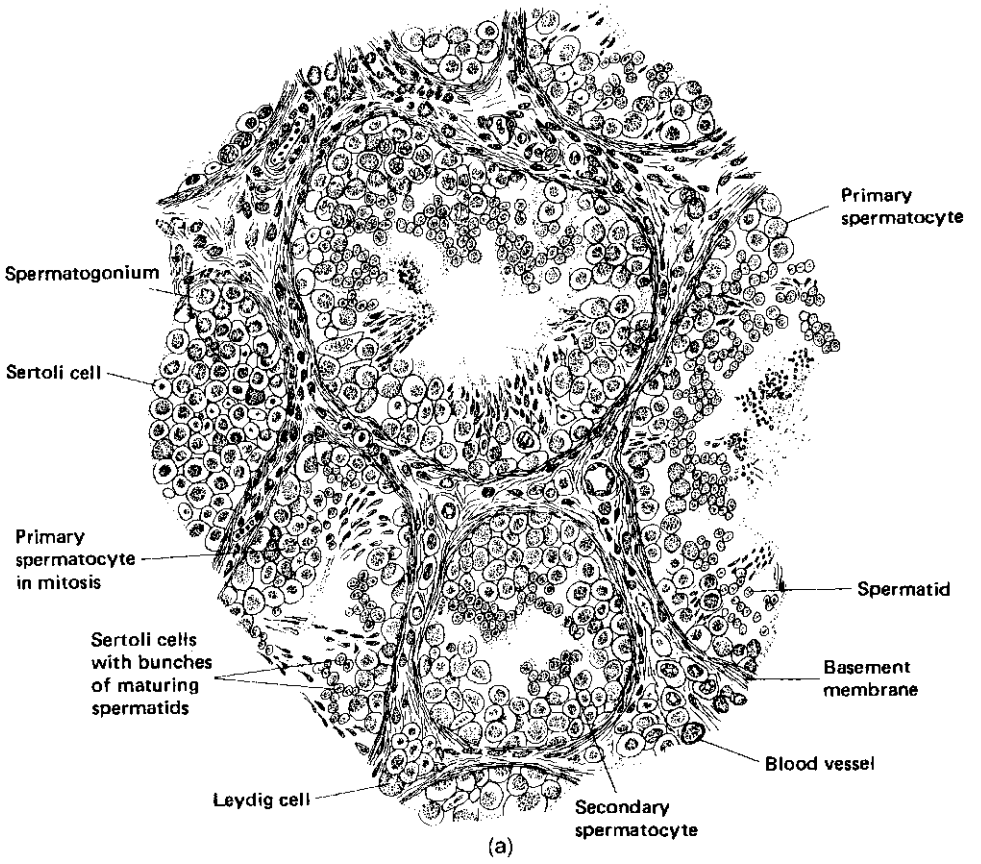
levels then feed back on the hypothalamus to suppress the production of GnRH in a process called steroid negative feedback. In females, ovulation is triggered by steroid positive feedback, the specifics of which will be discussed later in this chapter.

GnRH neurons also coordinate the timing of pubertal onset. Early in fetal development, GnRH neurons arise in the olfactory portion of the brain, migrate to their ultimate position in the hypothalamus, and begin secreting GnRH in low amplitude pulses. At puberty, the amplitude of GnRH secretion increases dramatically which, in turn, signals the HPG axis to undergo maturation. Pubertal transformation includes the development of secondary sex characteristics, the ability to produce and release gametes, and, in females, support a full-term pregnancy. Disruption of GnRH release can impair HPG axis function. Effects may be permanent if GnRH neurons, their axonal projections to the anterior pituitary gland, or synaptic inputs on GnRH neurons are damaged. In contrast, effects may be reversible if exposure occurs in adulthood because the neural circuitry surrounding GnRH neurons has already developed. For example, opiate drugs such as morphine and other narcotics, are tonic inhibitors of GnRH secretion and men who abuse narcotics often develop impotence as a result. Cessation of drug taking eliminates the suppression and normal, pulsatile release of GnRH resumes. Many industrial solvents, such as toluene and other aromatic hydrocarbons, and some pesticides can also suppress GnRH secretion. Polychlorinated biphenyl (PCB) mixtures (such as Aroclor 1221 and Aroclor 1254), organochlorine pesticides (such as methoxychlor and chlorpyrifos), and the plastic component bisphenol A have also been found to affect hypothalamic GnRH gene expression, cell survival, and neurite outgrowth (Gore, 2001). Exposure to the phytoestrogen genistein, which is found in soy and soy-based products, during early development can result in impaired steroid positive feedback in adult female rats and thus compromised reproductive capacity.

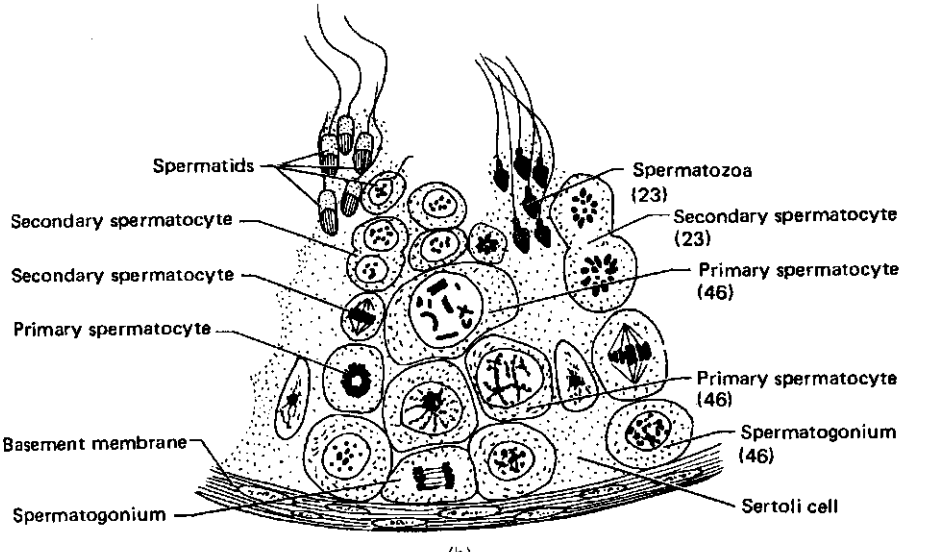
16.3 MALE REPRODUCTIVE PHYSIOLOGY

The presence of the SRY gene on the Y chromosome initiates a gene cascade that ultimately triggers the development of the testes from the mammalian fetal urogenital tract. In humans, the testes begin to develop around the eight week of pregnancy. Once formed, they begin secreting androgens and anti-Müllerian hormone. Androgens produce their effects by binding to androgen receptors (ARs) on target tissues throughout the reproductive tract and the brain. Androgens promote the maturation of the Wolffian ducts, which ultimately develop into the epididymis, vas deferens, and seminal vesicles (Figure 16.2). Anti-Müllerian hormone suppresses the development of the Müllerian ducts, which ultimately form

Figure 16.2 A cross-sectional diagram of the human testis depicting the overall structure of the seminiferous tubules and interstitial tissue. Leydig cells, along with connective tissue and blood vessels lie in the interstitial tissue. Sertoli cells and the germ cells lie within the seminiferous tubles. Maturation of the germ cells progresses from the basement membrane toward the lumen. Each stage is depicted with the requisite number of chromosomes indicated. Reprinted with permission from Jones, R. E. *Human Reproductive Biology*. San Diego, CA: Academic Press, 1991, p. 75.


Primary spermatocyte
Spermatogonium
Sertoli cell
Primary spermatocyte in mitosis
Spermatid
Sertoli cells with bunches of maturing spermatids
Basement membrane
Blood vessel
Leydig cell
Secondary spermatocyte

(a)


Spermatids
Spermatozoa (23)
Secondary spermatocyte
Secondary spermatocyte (23)
Secondary spermatocyte
Primary spermatocyte (46)
Primary spermatocyte
Primary spermatocyte (46)
Basement membrane
Spermatogonium (46)
Spermatogonium
Sertoli cell

(b)

the uterus and fallopian tubes in females. Differentiation of the prostate and external genitalia requires 5α-dihydrotestosterone (DHT), which is the most potent androgen produced by the testis and is a metabolite of testosterone. Failure to produce DHT, or sufficient levels of DHT, can lead to poorly developed or malformed external genitalia. The enzyme 5α-reductase catalyzes the conversion of testosterone to DHT; therefore, toxicants that interfere with the action of this enzyme, or the interaction of DHT with ARs during development can impair proper development of male genitalia and the prostate.

Androgen levels are relatively high during early fetal development, drop toward the end of pregnancy, and then rise again in infancy. The functional significance of these androgens in infancy is not known but has been shown to be critical for the masculinization of the brain and sex-specific behavior in other mammals. In late infancy, androgen levels fall precipitously and remain low through adolescence, then rise at puberty as GnRH pulse amplitude increases. Elevated androgen levels following pubertal onset ultimately initiate the development of secondary sex characteristics, the maturation of the external genitalia, and sperm production.

The testis contains three major cell types: Leydig cells, Sertoli cells, and germ cells. Leydig cells reside in the interstitial space of the testis, outside of the seminiferous tubules (Figure 16.2). The principal function of Leydig cells is androgen production. Androgen production is stimulated by LH which binds to its receptor within Leydig cells. Cholesterol, the basic building block of all steroid hormones, is delivered to the mitochondria by the steroidogenic acute regulatory (StAR) protein. Cholesterol is then cleaved to form pregnenolone and ultimately, after many steps, testosterone (Figure 16.3). Most of the enzymes needed for this conversion process belong to the cytochrome P450 family of enzymes. Interference with enzyme action within this pathway can result in insufficient androgen production. For example, some therapeutics for prostate cancer suppress the activity of the enzymes needed to convert androstenedione to testosterone or testosterone to dihydrotestosterone, therefore reducing androgen levels. Ketoconazole, an antifungal agent, is a potent inhibitor of the enzyme CYP17A1, which is needed for the production of androgens (and estrogens).

Sertoli cells are sometimes called the somatic nurse cells of the testis because their primary function is to support the germ cells as they differentiate into sperm. Sperm count is directly proportional to the number of Sertoli cells contained within the testis and this population is nonrenewable in the adult. Sertoli cells reside within the seminiferous tubule (Figure 16.2), are irregular in shape, and envelop the maturing germ cells. Tight junctions, called occluding junctional complexes, bind neighboring Sertoli cells together and ring the seminiferous tubule, forming the blood–testis barrier. This barrier is selectively permeable, much like the blood–brain barrier, and protects the developing germ cells from the immune system. In addition to providing a physical scaffold for the seminiferous tubules, Sertoli cells play a vital role in the development of the male reproductive tract and germ cell differentiation. During development, anti-Müllerian hormone is produced and secreted by the Sertoli cells. In adulthood, Sertoli cells synthesize a number of critical hormones and growth factors, most notably inhibin, which plays an important role in steroid negative feedback in the HPG axis (Figure 16.1). Sertoli cells express ARs, and are thus the primary targets for androgens in the testes. They also express the receptor for FSH, which is a critical regulator of Sertoli cell function.

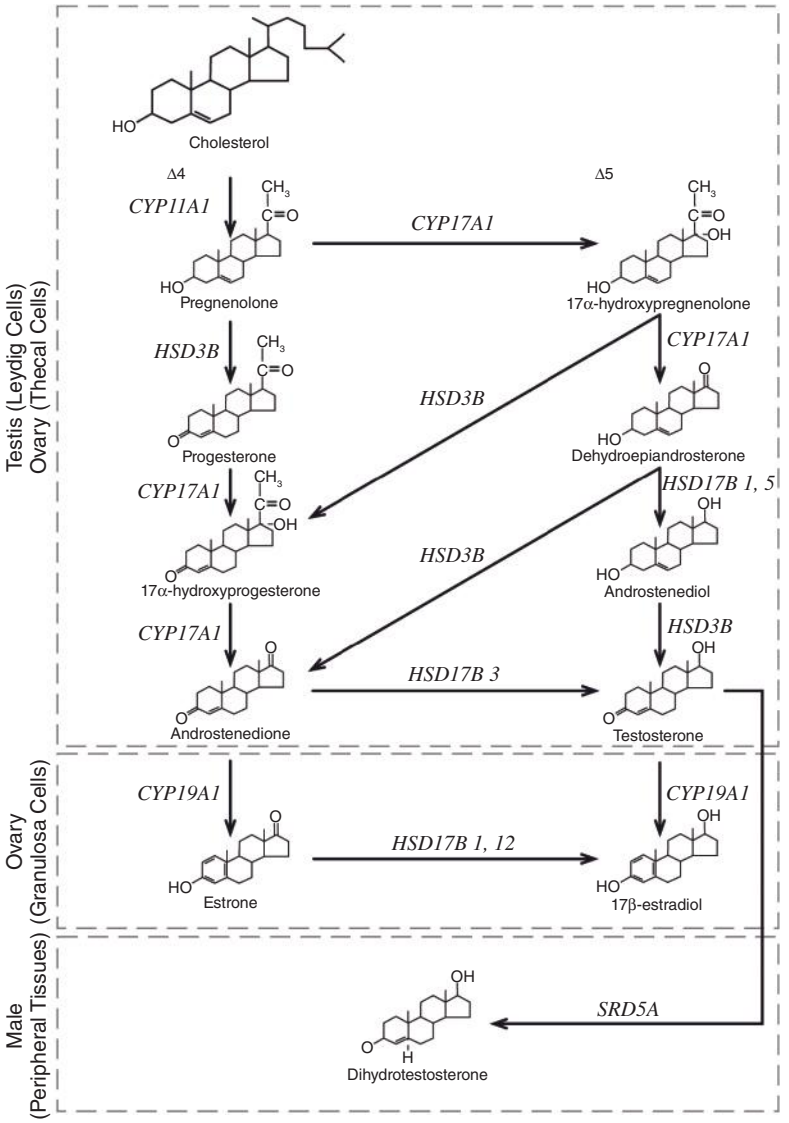


Figure 16.3 Biosynthetic pathway for sex steroid hormones indicating the enzyme required for each conversion.

Germ cells make up approximately 95% of the seminiferous epithelium and are especially sensitive to toxicological damage because, in the adult, they are undergoing a high rate of proliferation. Early in testicular development, primordial germ cells and Sertoli cell precursors aggregate and initiate the development of the seminiferous tubules. As the testis differentiates, the germ cells undergo mitotic division to form the primary spermatogonia. Division then slows considerably until pubertal onset. Spermatogonia then differentiate into sperm (spermatozoa) through a multistep process called spermatogenesis. In the mature testis, there are two

major types of spermatogonia, Type A and Type B (Figure 16.2) which lie along the basement membrane of the seminiferous tubules. Maturation of the germ cells then proceeds inward, toward the lumen, and concludes with the production of spermatozoa. Type A spermatogonia are the precursors of Type B spermatogonia which ultimately undergo meiosis and develop into primary spermatocytes. Type A spermatogonia undergo mitosis to produce Type B spermatogonia and replenish the supply of Type A spermatogonia. Type B spermatogonia then divide by mitosis to produce primary spermatocytes. These cells then undergo meiosis to produce haploid, secondary spermatocytes. The progression from Type A spermatogonia through secondary spermatocytes is called spermatocytogenesis. The haploid secondary spermatocytes then differentiate into round spermatids and then, ultimately, spermatozoan through a process called spermiogenesis. In human males, the entire process takes 70 days.

The immature spermatids then move into the epididymis where they mature, become more motile, and ultimately become viable. The prostate and seminal vesicles (secretory organs of the male reproductive tract) contribute to the maturation of the sperm and the chemical composition of the semen. Sperm are only viable after they mature in the epididymis.

16.4 DISRUPTION OF MALE REPRODUCTION BY TOXICANTS

Male reproductive physiology is vulnerable to disruption by toxicants during all stages of development and function, including gestation. Toxic insult to the testes can result in numerous effects including reduced sperm count and blood androgen levels that are either above or below the normal range. Damage to the epididymis can result in poor sperm motility or inadequate semen quality. Because sperm production is directly proportional to Sertoli cell number and once lost, cannot be replenished, failure to properly differentiate this critical cell population can irreversibly limit the capacity to produce viable sperm. Disruption to the testis or accessory sex glands can occur via a number of different mechanisms including DNA-damage, inhibition of cell division, interference with GnRH secretion, or alteration of testicular vasculature. The most widely studied and best understood mechanism of action is interference with ARs and/or androgen production.

16.4.1 Pesticides

One of the earliest animal studies designed to test the hypothesis that chemical agents could interfere with androgen action was conducted in 1950 using chickens. Injection of the pesticide dichlorodiphenyltrichloroethane (DDT) resulted in markedly undersized testes and inhibited the development of the comb and wattle. It was later determined that DDT and its metabolites function as anti-androgens and compete with endogenous androgens for access to the ARs. Because androgens, especially DHT, are essential for the development of male genitalia, it is widely speculated that gestational exposure to compounds like DDT with anti-androgen properties may be responsible for the documented increase in the frequency of developmental defects in male genitalia including hypospadias (misplaced urethral opening on the penis) and cryptorchidism (undescended testes) in boys.

Impaired fertility from pesticide exposure has also been documented in humans. For example, one of the earliest known examples of reduced male fertility in humans following pesticide exposure emerged in the 1970s in a group of 25 Californian men exposed occupationally to the pesticide 1,2-dibromo-3-chloropropane (DBCP). DBCP was a potent nematocide that went into commercial production in 1956 and was later banned in 1977. The exact mechanism by which DBCP impairs male reproduction has still not been elucidated, but it is hypothesized that one of its metabolites acts directly on germ cells, thereby impairing spermatogenesis. Of the 25 occupationally exposed men, most had reduced sperm counts and 9 had no viable sperm. Subsequent studies, which examined the long-term effects of exposure on these and other occupationally exposed men, found that sperm production ultimately improved in most cases, but not in individuals whose sperm counts were lowest. These studies illustrate two important concepts. First, effects on the male reproductive system from exposure to pesticides and other toxicants during adulthood are largely temporary and reversible because the system is already developed and, in most cases, can recover. Second, recovery is unlikely if primary spermatogonia or Sertoli cells are killed. In the case of DBCP, long-term exposure depleted the pool of healthy spermatogonia, ultimately resulting in irreversible infertility.

Other pesticides, herbicides, and fungicides are also known to affect male reproductive development and function. Parathion, an organophosphate, used on nine commercial crops in the United States, most notably corn, and vinclozolin, a fungicide commonly used on vineyards, are two examples. Parathion is a potent inhibitor of cholinesterase, an enzyme that facilitates the transmission of nerve impulses. Therefore, acute exposure to high doses can cause neurotoxicity and result in death. At lower doses, it is suspected of being an endocrine disruptor but the specific mechanism(s) by which it acts is not yet known. Vinclozolin is an androgen antagonist. Animal studies have shown that exposure to vinclozolin in the womb, when the male reproductive organs are forming, can result in the impaired development of the male reproductive organs. This process is called demasculinization.

16.4.2 Metals

Of the metals, the effects of lead on the male reproductive system are the best documented and understood. Lead exposure can reduce sperm counts and increase the frequency of malformed sperm, resulting in reduced fertility. Lead can affect the testis in several ways. For example, it can directly interfere with the HPG axis, resulting in hypogonadism or low testosterone levels. It can also disrupt the formation and function of the vascular system within the testis, ultimately causing damage to the seminiferous tubules. Lead exposure most frequently occurs in industrialized settings but is also common in older homes by inhalation or ingestion of lead paint residues. Children are most likely to be exposed through this route or through unacceptably high levels in drinking water. There is some concern that exposure to lead levels currently considered to be “safe” can affect brain development and impair male fertility but, to date, the data remain inconclusive.

Cadmium is also well-known to impair male reproduction but only at near fatal doses. It causes Sertoli cell death, testicular necrosis, and germinal cell damage through a direct effect on the vascular system within the testis. Exposures of this

magnitude likely only occur in industrial settings. Other metals suspected of having reproductive effects in men are mercury and boron.

16.4.3 Plastics

Considerable attention is now being placed on the potential for chemicals with endocrine disrupting properties to leach from plastics into food. Of these, the most widely studied group that pertains to male reproductive health is the phthalates. There are many different kinds of phthalates, and the two considered to have the greatest potential to impact male reproduction are dibutyl phthalate (DBP) and diethylhexyl phthalate (DEHP). DBP is used in many personal care products such as lotions, cosmetics, nail polish, and perfume. It is also found in the coatings of many time release pharmaceuticals. DEHP is primarily used as a plasticizer in the production of flexible products including vinyl, medical tubing, and other medical devices, baby toys, and flooring.

A series of studies conducted in rats in the late 1990s was the first to demonstrate that phthalates could interfere with the ability of testosterone to masculinize the male reproductive tract. Exposure *in utero*, when the genitals are being formed and the action of DHT is critical, resulted in a number of genital malformations in laboratory animals including hypospadias and hemorrhagic testes (Gray et al., 1999; Wolf et al., 2000). Interestingly, the phthalates do not produce their effects by antagonizing ARs, but rather by interfering with the production of testosterone in the fetal testis. Exposure to phthalates during pregnancy has now been associated with a greater risk of genital malformations in infant boys (Swan et al., 2005). Epidemiological evidence has also positively correlated higher urinary phthalate levels with lower sperm counts and an increased likelihood of sperm with damaged DNA in adult men. Phthalate exposure is also linked with lower circulating levels of estrogens and androgens, indicating that the HPG axis may be impaired. Although it is important to keep in mind that correlation does not prove causation, these newly emerging epidemiology studies are the best evidence to data that phthalates have the potential to affect male reproductive health.

Studies of phthalate exposure in animals and humans emphasize the significance of when exposure occurs. In late 2008, the United States banned the use of phthalates in toys. Although this will help remove a source of exposure for children, it fails to eliminate the potential for exposure when the developing male is most vulnerable—in the womb (Lottrup et al., 2006). Exposure during development is more likely to result in permanent effects because disrupted organization of the male reproductive system is largely irreparable. Therefore, it is important to always be mindful of the mechanism by which a compound is suspected of impacting the male reproductive system. Understanding the mechanism is critical for making regulatory decisions that could protect human health.

16.5 FEMALE REPRODUCTIVE PHYSIOLOGY

Absence of the SRY gene allows the bipotential gonad, contained within the fetal urogenital tract, to develop into an ovary. As in the male, both the Wolffian and Müllerian ducts are present during early fetal life. In the female, the Wolffian ducts

regress because the androgens needed to promote their differentiation are not present (Figure 16.2). Similarly, absence of anti-Müllerian hormone preserves this structure which ultimately differentiates into the oviducts, uterus, cervix, and upper vagina. The absence of androgens also results in the formation of the female genitalia. Because androgens are required for the masculinization of the genitalia, exposure to androgenic compounds can cause masculinization, also called virialization, of the female genitalia. Generally, this virialization is incomplete and can lead to the formation of ambiguous genitalia, including an enlarged clitoris, enlarged labia, or partial fusion of the labia.

The ovary contains three major cell types: germ cells, granulosa cells, and thecal cells. Early in gestation, primordial germ cells, termed gonocytes, migrate to the genital ridge and induce the formation of the ovary. As the ovary forms, the gonocytes then undergo rapid mitotic divisions producing oogonia. In humans, by the second trimester of gestation, the fetal ovary contains several million oogonia, the most the fetus will ever have in her lifetime. At this point in development, the mitotic divisions end and most oogonia enter the prophase of meiosis I, becoming primary oocytes. The primary oocytes remain in the diplotene stage prophase (meiosis I) until they are recruited for maturation during the ovulatory cycle in adulthood. Over time, the number of germ cells decline through a process called atresia, decreasing to about a million at birth, approximately 200,000 at puberty, and only a few thousand in early adulthood. It has been estimated that, of the approximately 7 million oogonia produced in human fetal life, only 400 will ever be ovulated. This phenomenon appears to be universal, since all vertebrate species examined to date are born with far fewer oocytes than their peak number generated during early gestation.

The primary oocytes lie within ovarian follicles, each of which comprises a single oocyte surrounded by follicular cells (Figure 16.4). Thecal cells form the outer layer of follicular cells. They are similar to the Leydig cells of the testes in that they constitutively express the receptor for LH and their primary role is to produce androgens. Granulosa cells lie inwardly adjacent to the thecal cells and line the interior of the ovarian follicle. The functional role of these cells is similar to the Sertoli cells. They are tightly linked together by gap junctions, express the receptor for FSH, and convert the androgens, produced by the thecal cells, into estrogens, most significantly estradiol. The enzyme need to complete this conversion is cytochrome P450 (or CYP19), otherwise know as aromatase. Aromatase inhibitors are now commonly used to treat hormone-dependent breast cancers.

Estrogens produce their effects by binding to estrogen receptors (ERs) of which there are two major subtypes in mammals, ERα and ERβ. ERβ was discovered relatively recently, and its functional role in the reproductive system is still being described. Thecal cells express ERα while granulosa cells express ERβ. Mice lacking ERα (ERα knockout mice) are infertile and have numerous ovarian malformations. In contrast, mice lacking ERβ have relatively normal ovulatory cycles and are capable of getting pregnant, although they appear to be subfertile compared to wild-type control mice.

16.5.1 The Ovulatory Cycle

The HPG axis signals the ovary to initiate follicular growth by the secretion of FSH. Several follicles are recruited, enlarge, and develop into primary follicles. Granulosa

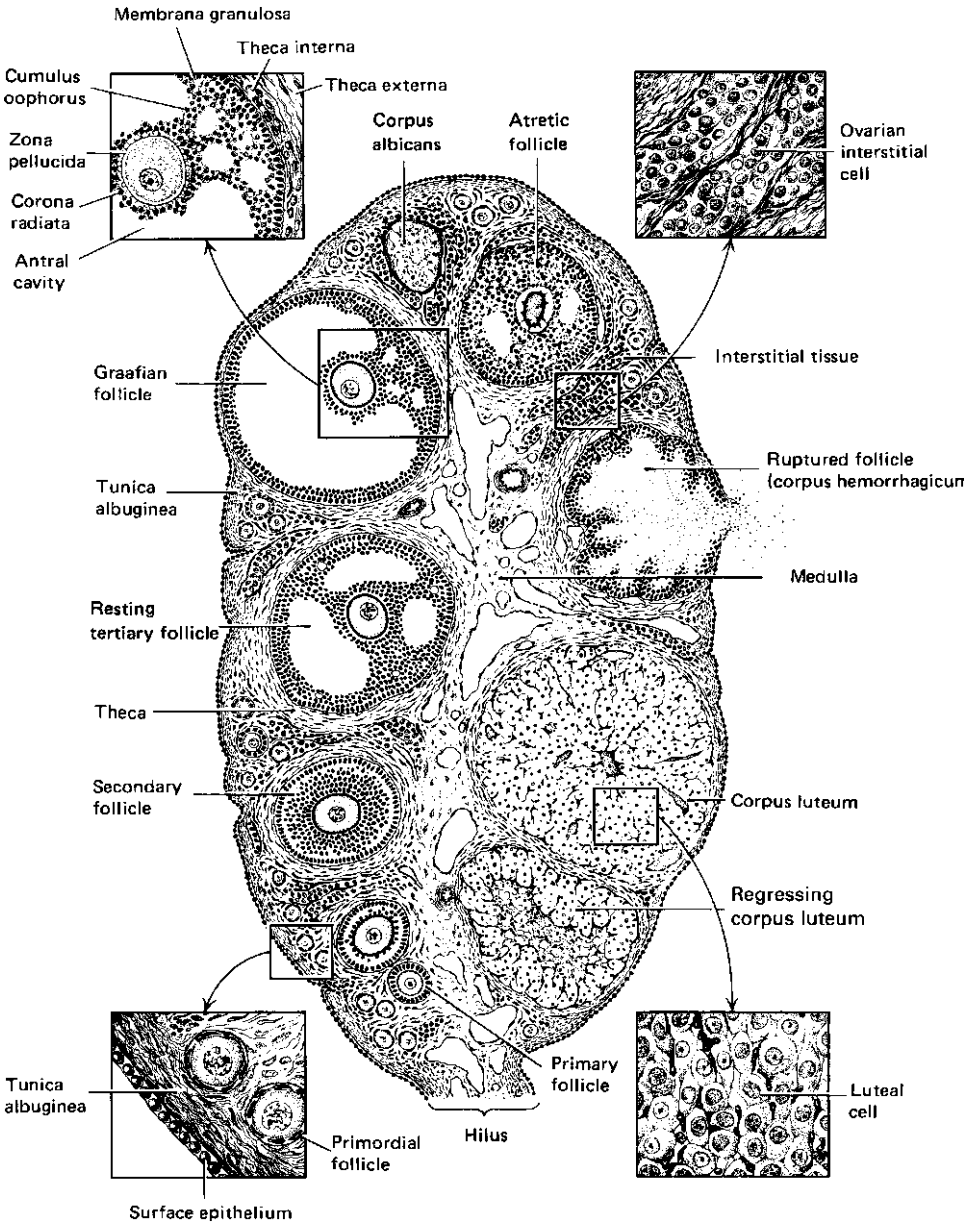


Figure 16.4 A cross-sectional diagram of the ovary illustrating its overall structure and component parts. The different phases of follicular development are also depicted along with the cellular components of the mature follicle. Reprinted with permission from Jones, R. E. *Human Reproductive Biology*. San Diego, CA: Academic Press, 1991, p. 42.

cells proliferate, increase their estrogen production, and support the growing oocyte. The thecal cells surrounding each developing primary follicle become heavily vascularized and help coordinate the flow of nutrients to the developing follicle. As the follicular phase progresses, for reasons that still remain largely unknown, most

of these primary follicles ultimately begin to degenerate, as does the oocyte within them. In humans, only one a month will ultimately reach maturity. A cavity, called the antrum, begins to form within the remaining primary follicles at which point these follicles are called secondary follicles. The granulosa cells also promote the development of a clear membrane, called the zona pellucida, around the oocyte. When the follicle is ready to be ovulated, it is called a mature or graafian follicle. Elevated estradiol levels, produced by the granulosa cells, stimulate GnRH neurons to release a surge of gonadotropins, which in turn generates the surge of pituitary LH needed to trigger ovulation (Figure 16.1). This process is called steroid positive feedback.

At ovulation, the secondary oocyte, surrounded by the zona pelludica, erupts from the graafian follicle and begins its migration down the oviduct. The remaining follicular cells enlarge and transform into a corpus luteum, a glandular structure that secretes progesterone along with some estrogens. If the oocyte is ultimately fertilized, the progesterone produced by the corpus luteum will sustain the first several days of conception and be stimulated by the fetus to remain through the early part of pregnancy until the placenta forms and takes over the production of progesterone. If fertilization does not take place, the corpus luteum will regress. In humans, this process takes approximately 10 days.

In humans, the menstrual cycle is approximately 28 days but in rodents, it is only about 4 days. Hormone profiles across the ovulatory cycle vary somewhat across different species, and these differences should be kept in mind when considering the effects of toxicant exposure on ovarian function. Hormone profiles also differ during development. For example, the role of estrogen in the development of the female reproductive tract is species specific. In rodents, estrogen is not required, and exposure to estrogens during development, including the neonatal period, can in fact masculinize the female reproductive system, particularly the brain. This may not be the case in humans, however, as androgens, rather than estrogens, appear to be more important for masculinizing the primate brain. Therefore, caution must be taken when extrapolating data from female rodents to women.

16.6 DISRUPTION OF FEMALE REPRODUCTION BY TOXICANTS

Data on female reproductive health trends are limited, but recent data suggest that conception rates are declining in the developing world and the prevalence of reproductive disorders such as advanced pubertal onset, premature ovarian failure, and polycystic ovary syndrome are becoming more common (Hamilton and Ventura, 2006). Delayed childbearing resulting from cultural changes arguably has the biggest impact on fertility trends in Western countries. Obesity, dietary habits, and chronic stress may also be important factors affecting female reproductive health. These characteristics do not adequately explain, however, why the sharpest increase in reported infertility is among women under the age of 25. It is now widely hypothesized that environmental exposure, either to the mother or her developing fetus, may also contribute.

Female reproductive physiology is vulnerable to disruption by toxicants during all stages of development and function, including gestation. Toxic insult to the ovaries can result in structural abnormalities or damage to the follicles. Compounds

that act as androgen agonists could masculinize aspects of the female reproductive tract or brain. For example, exposure to low levels of androgens during fetal life is thought to increase the risk of developing polycystic ovary syndrome. Disruption of uterine development can occur via a number of different mechanisms including endocrine disruption, inhibition of cell division, or improper maturation of the Müllerian ducts. Improper ductal differentiation could result in malformations within the fallopian tubes, potentially compromising the ability to sustain pregnancy. The most widely studied and best-understood mechanism of action is interference with estrogen receptors and/or estrogen production.

16.6.1 Cigarette Smoke

Smoking has long been known to induce premature menopause and reduce fertility. Smokers have fewer eggs, fewer quality eggs, an impaired capacity to recruit quality eggs when administered fertility drugs, and decreased rates of successful *in vitro* fertilization. Cigarette smoke contains several hundred known toxicants, all of which could, either individually or in combination, produce these effects. In animal models, nicotine has been shown to have disruptive effects on ovulation rates, oocyte maturation, and implantation success. Chromosomal abnormalities within oocytes are also more prevalent in smokers compared to the general population and may contribute to lower conception rates among smokers. Fertility in the offspring of smokers may also be compromised. Studies in mice have found that *in utero* exposure to polycyclic aromatic hydrocarbons, a group of more than 100 different chemicals found in cigarette smoke, results in significantly fewer ovarian follicles and therefore compromised reproductive capacity (Jurisicova et al., 2007).

16.6.2 Diethylstilbestrol (DES)

The most well understood example of how the administration of a synthetic estrogen can affect female reproduction is the tragic case of diethylstilbestrol (DES) used by pregnant women in the middle of the twentieth century. DES, a potent synthetic estrogen, was first manufactured in 1938 and prescribed to women for the prevention of miscarriage until 1971. It is estimated that 4–6 million women were prescribed DES in the United States alone. The consequences of *in utero* DES exposure were first identified in 1971 by a group of keen-eyed physicians who noticed that DES daughters were more likely to develop an extremely rare type of cervicovaginal cancer (Herbst et al., 1970, 1971). Since that initial discovery, other abnormalities in DES daughters have been described, including uterine malformations, increased risk of ectopic pregnancy, premature menopause, increased risk of developing uterine fibroids and endometriosis, increased risk of breast cancer, and decreased fertility. There is also newly emerging evidence that the children of the DES daughters (referred to as DES granddaughters) are also experiencing reproductive problems. For these women, their exposure occurred when they were germ cells in their mothers' developing ovary in the womb of their grandmother. It is one of the first instances in humans which demonstrate that persistent, generational effects can result from an *in utero* exposure to a potent estrogen (Newbold et al., 1998, 2006). Nearly all of these outcomes were replicated in or predicted by

laboratory animal studies. This unfortunate event in human medical history illustrates both the vulnerability of the developing fetus to estrogenic endocrine disruptors and the importance of animal models for predicting these vulnerabilities. The tragedy of DES introduced the concept of "fetal origins of adult disease," a principle that is still used in reproductive toxicology.

What remains to be determined is if chronic, low-dose exposures to far weaker estrogens can also impair female reproduction. DES has a higher binding affinity for ERα and ERβ than estradiol and is therefore a potent estrogen agonist. Other compounds, some of which will be discussed in detail below, have binding affinities 100–10,000 times lower and are thus considered "weak" estrogens. In some cases, however, blood levels of these compounds can be severalfold higher than endogenous estrogen levels. Therefore, understanding the potential impact of these compounds on reproductive health is paramount.

16.6.3 Pesticides

A variety of pesticides and herbicides have been found to interfere with hormone action, especially estrogen. The most famous of these is DDT (still sold under many names including Anofex, Dicophane, Dinocide, Neocid, and Neocidol among others). DDT is usually sold and used as a mixture of several, closely related compounds of which dichlorodiphenyldichloroethylene (DDE) is a component and the principle breakdown product. Created in 1874 by Othmar Zeidler, the insecticidal properties were not revealed until 1939 by Hermann Müller, for which he was awarded the 1948 Nobel Prize in Physiology and Medicine. This insecticide, which is structurally similar to DES, was widely used all over the globe until the early 1970s to eradicate lice and mosquitoes. It was, and continues to be, a critical weapon in the fight against malaria and other mosquito-borne illnesses which, collectively, cause the greatest number of preventable deaths in the world. More people die from malaria than HIV, heart disease, or cancer. For that reason, it is still used in many places where the risk of contracting malaria remains high although some mosquito populations have developed resistance to the insecticide. The effects of DDT on wildlife populations was popularized by the landmark 1962 book *Silent Spring* by biologist Rachel Carson. The book became an instant best seller and launched the environmental movement in the United States as well as a governmental investigation into its claims that persistent pesticides, including DDT, were poisoning wildlife and, potentially, humans. DDT and its metabolites are toxic to a wide range of animals and insects. It thins eggshells in birds, and is believed to be one of the primary culprits for the decline of the bald eagle and other large birds of prey.

DDT has multiple modes of action. It is moderately toxic to mammals with a rat oral lethal dose 50 (LD_{50}) of 113 mg/kg and is similar to the pyrethroids in that it is a nervous system stimulant which acts by opening neuronal sodium channels, causing spasms, seizure, and death. At far lower doses, it is also thought to be both an estrogen agonist and androgen antagonist. Whether or not DDT can impair reproductive development in women is still the subject of investigation. Exposure *in utero* or early in life is associated with an increased risk of breast cancer (Cohn et al., 2007). There is also emerging evidence from areas where DDT is still used, that DDT exposure is associated with preterm birth, early pregnancy loss, reduced semen quality,

disrupted menstruation, and problems with lactation (Rogan and Chen, 2005; Venners et al., 2005). Infants from these regions are frequently exposed to levels which exceed the acceptable daily intake established by the World Health Organization via breast milk. Therefore, the potential reproductive risk of DDT exposure is ongoing for many populations outside the United States.

Synthetic pyrethroids also appear to have endocrine disrupting properties in female vertebrates. Natural pyrethroids are derived from chrysanthemums, and their synthetic brethren are similar in structure but more lipophilic and environmentally persistent, making them potent weapons against ticks, mites, lice, and mosquitoes. Synthetic pyrethroids can act either as estrogen agonists or estrogen antagonists, depending on the level of exposure and the endogenous hormone levels of the exposed individual.

16.6.4 Plastics

A large number of chemicals are used in plastics manufacturing to make them more useful. For example, compounds may be added to reduce microbial growth, or to improve the stability, clarity, durability, or pliability of the product. One additive that has been the subject of intense scientific and political attention is bisphenol A (BPA). BPA was developed in the 1930s as a synthetic estrogen but was abandoned as a pharmaceutical with the invention of DES. It is now used in a variety of products, most notably polycarbonate plastics, to increase their strength and durability. It is also present in dental sealants, epoxy resins, and the linings of food cans and drink cartons, including soda cans. Annual worldwide production of BPA is estimated to exceed 6 billion pounds and in 2008, the U.S. Center for Disease Control (CDC) estimated that BPA was present in approximately 95% of the U.S. population. A study published in early 2009 found that exposure to BPA, along with the phthalates and other endocrine disruptors that leach from plastics, is particularly high among premature infants being cared for in neonatal intensive care units (Weuve et al., 2006). BPA does not persist in the environment like DDT or the PCBs, but daily human exposure is estimated to be much higher because it readily leaches from plastic containers, especially if heated. Laboratory rodents exposed to BPA *in utero* or just after birth develop a variety of reproductive abnormalities including accelerated puberty, abnormal ovarian follicles, premature loss of the ovulatory cycle, and alterations in mammary gland formation that suggest an elevated risk of developing breast cancer (Crain et al., 2008). In 2008, the U.S. Food and Drug Administration concluded that the reference dose of 50 μg/kg body weight was adequate, a conclusion that was widely criticized and revised in January, 2010. The FDA now states that it has "some concern" for neuroendocrine effects in infants and children. Public attention has resulted in considerable pressure upon manufacturers and as a result, many products advertised to be "BPA free" are now widely available. The history of BPA is an informative example of how scientific discovery, both by academic and industry scientists, policymakers, and public pressure interact to affect how a compound is used (Box 16.1).

16.6.5 Phytoestrogens

Not all endocrine disruptors are produced by humans. Phytoestrogens are a class of compounds produced by plants, primarily legumes. Legumes, such as soy, use these

BOX 16.1 THE HISTORY OF BISPHENOL-A (BPA): AN EXAMPLE OF HOW SCIENCE, THE MEDIA, AND PUBLIC SCRUTINY SHAPE PUBLIC POLICY

1891	BPA synthesized by Aleksandr Dianin, a chemist in Saint Petersburg, Russia.
1953	Polycarbonate plastic developed by Bayer and General Electric.
1957	BPA enters commercial production and is incorporated in epoxy resins.
1982	The National Toxicology Program (NTP) establishes a lowest observed adverse effect level (LOAEL) of 50 mg/kg body weight or 1000 parts per million.
1988	The Environmental Protection Agency (EPA) sets the safe dose (reference dose) at 50 μg/kg body weight. This standard remains today.
1996	The Food and Drug Administration (FDA) concludes that adults are exposed to approximately 11 μg per day and infants are exposed to 7 μg per day.
1997	First laboratory animal study showing adverse reproductive effects at doses equal or lower than those seen in humans is published by Fred vom Saal at the University of Missouri-Columbia.
1997	FDA finds BPA contamination in infant formula, demonstrating for the first time that BPA can leach into food.
1999	The Consumers Union, which publishes Consumer Reports, finds that BPA can leach from baby bottles, especially when heated.
1999	FDA asserts that BPA is safe and human doses are too low to cause adverse effects.
2003	An advisory panel for the NTP is convened to evaluate the safety of BPA. A company called Sciences International is hired to help organize the evaluation.
2003	Worldwide production of BPA exceeds 6 million pounds.
2006	A draft report from the NTP advisory panel is published and generally concludes that BPA is safe. Many scientists are critical of the report noting that some low-dose animal studies were excluded from the evaluation.
February–March 2007	The advocacy group Environmental Working Group discovers that the largest BPA manufacturers are clients of Sciences International, a revelation that launches a Congressional investigation into how the NTP advisory panel evaluation was conducted. A series of investigational reports by the *LA Times* heightens public and media awareness of the issue.

April 2007	The NTP panel continues working on the report, but the contract with Sciences International is canceled. Scrutiny of the panel by scientists, private citizens, the media, and advocacy groups intensifies.
August 2007	A separate, NIH-funded, panel of 38 scientists considered to be experts in BPA research (known as the "Chapel Hill Panel") are convened to conduct their own evaluation and publish a consensus statement. This panel produced five peer-reviewed articles published in the journal *Reproductive Toxicology*.
November 2007	The NTP advisory panel issues its final report and concludes that there is "some concern" about the neural and behavioral impacts of fetal exposure to BPA. This is considered a substantial change from the initial draft report.
February 2008	Congress requests that the FDA "clarify" its position on BPA.
April 2008	The NTP, weighing the conclusions of both the advisory panel and Chapel Hill Panel, concludes that BPA may pose a threat to humans. Canada announces its intention to ban the chemical from baby bottles and ultimately declares it a "hazardous substance." 10 States now have legislation pending to minimize use of or ban BPA. Major manufacturers including Playtex and Nalgene announce that they will stop using BPA and Wal-Mart announces that it will phase out BPA-containing bottles.
January 2010	the FDA reverses it's prior decision and declares that it has "some concern" for developmental toxicity in fetuses, infants and children.

compounds to recruit and store nitrogen-fixing bacteria. They generally have a higher binding affinity for both forms of the estrogen receptor than most man-made products (with the exception of DES) and, like most endocrine disruptors, readily cross the placenta. Consumption of a soy-rich diet is associated with a lower risk of heart disease and osteoporosis, but the reproductive health impacts of soy phytoestrogen exposure either *in utero* or after birth (through the use of soy infant formula) is generally unknown in humans. Evidence from rodents suggests that the phytoestrogen genistein can inhibit oocyte development resulting in multinucleated follicles and premature loss of the ovulatory cycle. In 2001, a team of epidemiologists examined the reproductive health of females that, as infants, had taken part in a controlled feeding study at the University of Iowa between 1965 and 1978 (Strom et al., 2001). The 2001 survey took place when the women were between 20 and 34 years of age. Women fed soy formula were more likely to have a longer period of menstrual bleeding each month, abnormally painful menstrual periods, or

to have visited a gynecologist for pain or discomfort during menstruation. It was not possible to examine any potential effects on pregnancy outcomes because at the time of the study, most of the women had not yet attempted a first pregnancy. These findings are generally consistent with studies done in animals. In rodents, the premature cessation of ovulatory cycles is commonly observed. It has yet to be determined if phytoestrogen consumption in infancy results in premature menopause in women.

16.6.6 Others

PCBs were banned in the 1970s because of their toxicity and environmental persistence, but they remain in the environment and human tissues. Once used as coolants and within insulation for electrical equipment, they are present at alarmingly high levels in places thought to be pristine and free from environmental contamination, such as the arctic. A group of Inuit women and their children in Arctic Quebec is currently under study because they are known to have significantly elevated blood levels of PCBs and other contaminants compared to the general population. Dioxins, produced from incineration processes, many flame retardants, phthalates, and some fungicides are also thought to interfere with female reproduction, especially when exposure occurs *in utero*.

16.7 SUMMARY

This chapter has provided an overview of reproductive toxicology. However, as a whole, reproductive toxicology overlaps with neurotoxicology, endocrine toxicology, and developmental toxicology, all of which are covered in depth in prior or subsequent chapters. The next chapter will explore the endocrine system in more detail and build upon the concepts presented in this chapter.

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SAMPLE QUESTIONS

1. What is meant by the terms "steroid positive" and "steroid negative" feedback?

2. List three different mechanisms by which endocrine disruptors can impact the reproductive system and give an example of each.

3. Leydig cells are most similar to which cell type in the ovary? Why?

4. You are asked to test the toxicity of a compound on male sperm production. In your experiments you find that the compound damages Sertoli cells. Will this likely affect sperm production?

5. True or false: all oocytes are made prior to birth.

6. What is Bisphenol-A and why is there so much public pressure to discontinue using it?

CHAPTER 17

Endocrine Toxicology

GERALD A. LEBLANC

17.1 INTRODUCTION

Among the various organ systems of the body, the endocrine system is somewhat unique. While most systems are associated with a specific physiological task (i.e., respiration, reproduction, excretion) the endocrine system functions to regulate many of the activities associated with these other systems. Accordingly, the endocrine system is integral to the maintenance of total normal bodily function (homeostasis), and disruption of normal endocrine function by exogenous chemicals can result in multiple, diverse, and dire consequences. Toxicity to the endocrine system is most commonly associated with altered development, growth, maturation, and reproduction (Table 17.1). However, endocrine toxicity also can present as gastrointestinal dysfunction, malaise, neurological disorders, etc. (Table 17.1). Accordingly, endocrine toxicity often can be misconstrued as toxicity to some other endocrine-regulated system of the body.

The endocrine system, as an authentic target of chemical toxicity, tragically entered the limelight as a consequence of the widespread use of the drug diethylstilbestrol (DES). DES, a nonsteroidal synthetic estrogen, was prescribed to pregnant women from the 1940s to the 1960s as a prophylactic against miscarriage (see Section 17.4.1). Following the discovery of the endocrine toxicity of this drug, many additional drugs and environmental chemicals have been shown to mimic the action of hormones or interfere with their normal function. These activities often have been clearly shown, in laboratory studies, to result in endocrine-related toxicity. In some instances, drug use or exposure to ambient environmental chemicals also have been shown to result in endocrine toxicity. Such examples will be presented at the end of this chapter.

17.2 ENDOCRINE SYSTEM

The endocrine system can be broadly described as *an assemblage of organs (glands) that produce chemical messengers (hormones) that regulate various bodily*

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TABLE 17.1 Processes Regulated by Some Hormones of the Endocrine System and, Accordingly, That Are Susceptible to Disruption by Endocrine Toxicants

Hormone Group	Example	Origin	Regulated Process
Androgens	Testosterone	Testes, adrenals	Sexual differentiation, fertility, secondary sex characteristics, sexual function, libido
Estrogens	17β-estradiol	Ovaries, testes	Sexual differentiation, fertility, secondary sex characteristics, bone density maintenance, blood coagulation
Glucocorticoids	Cortisol	Adrenals	Bone formation, wound healing growth, development
Thyroid hormones	Thyroxine	Thyroid gland	Fetal brain and bone development oxygen consumption, gut motility

functions. The bodily functions regulated by the endocrine system can be categorized as those involved in the maintenance of homeostasis and those involved in physiological progression. Functions regulated by the endocrine system resulting in homeostasis include maintenance of the reproductive system, energy production, and metabolism. Functions regulated by the endocrine system resulting in physiological progression include fetal development, growth, and maturation. Endocrine processes related to physiological progression historically have received the greatest attention in endocrine toxicology and will be emphasized in this chapter.

Both the maintenance of homeostasis and the regulation of physiological progression require that the endocrine system detect signals, either external or internal, and transduce these signals to the appropriate target sites within the body. These target sites then respond in the appropriate manner to maintain homeostasis or institute change related to development, maturation, etc. In many species, these initial signals are of external origin. For example, many species initiate reproductive maturation in response to changes in environmental temperature, day length, etc. Reproductively mature organisms often respond to external visual or olfactory stimuli produced by sexually receptive individual to initiate sexual behavior.

The signal to be transduced by the endocrine system initiates in the central nervous system. In mammals, the hypothalamus commonly initiates the endocrine signaling pathway by secreting peptide hormones. These neuroendocrine hormones can be rapidly synthesized, secreted, and degraded to allow near instantaneous, short-lived responses to the stimulatory signal. Accordingly, they can be present in the body in pulses and secretory rhythms that often contribute to their signaling function. For example, the hypothalamic peptide hormones "growth hormone releasing hormone" (GHRH) and somatostatin are secreted in an alternating pulsatile fashion. Both hormones target the pituitary gland, though GHRH stimulates and somatostatin inhibits growth hormone secretion by the pituitary. As a result, the secretory pattern of the secondary hormone messenger in this cascade,

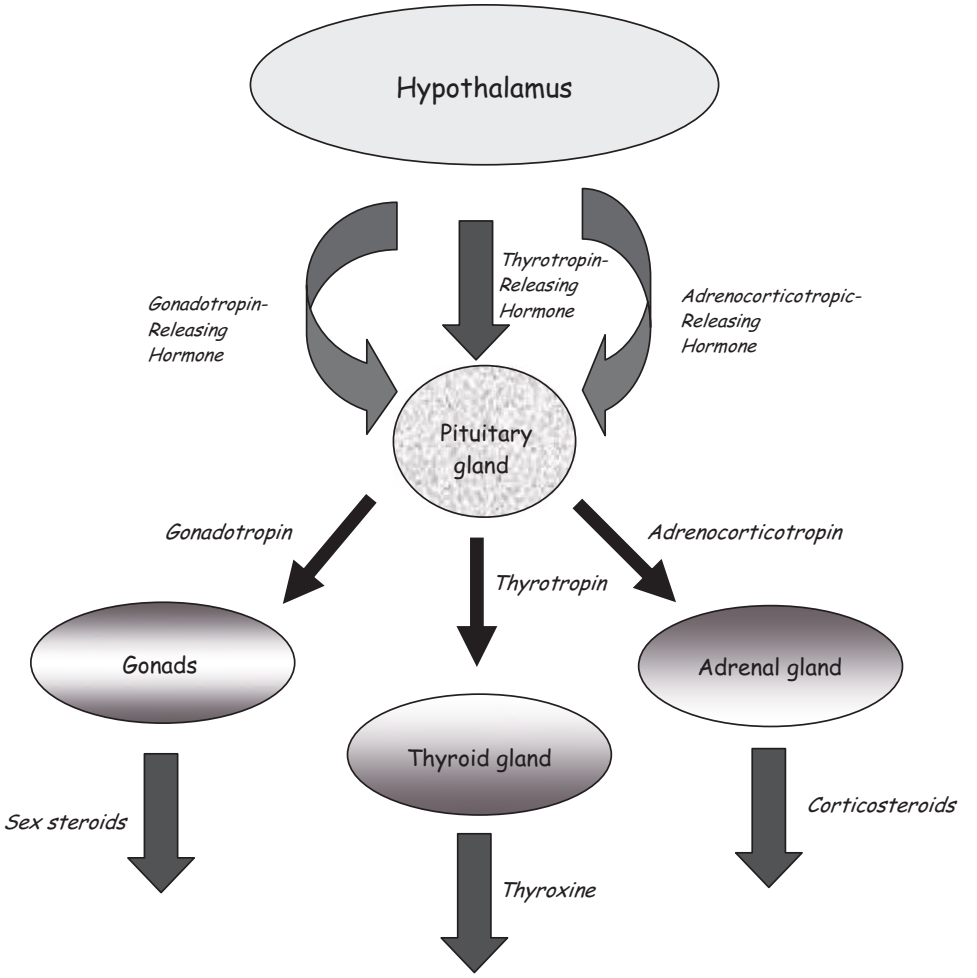


Figure 17.1 Some major neuroendocrine axes that transduce endocrine signals to target organs. Neuroendocrine signaling is initiated by the secretion of releasing hormones or, in some instances, inhibiting hormones that regulate secretion of the secondary hormone signal by the pituitary. Pituitary hormones then regulate secretion of the tertiary hormone, often a steroid hormone, by the appropriate endocrine gland. The tertiary hormones then stimulate gene transcription at target organs.

growth hormone, is highly controlled. Disruption of this rhythm in rodent models can alter hepatic enzyme expression and other dynamic processes. Disruption of the growth hormone secretory rhythm associated with sleep has been shown to interfere with normal growth in children. Hormone secretory rhythms have been associated with other physiological processes including sleep, sexual behavior, and ovulation.

Endocrine signaling pathways from the central nervous system to the target organ typically occur along axes (Figure 17.1). An axis is defined by the endocrine glands that produce signaling hormones along the cascade (e.g.,

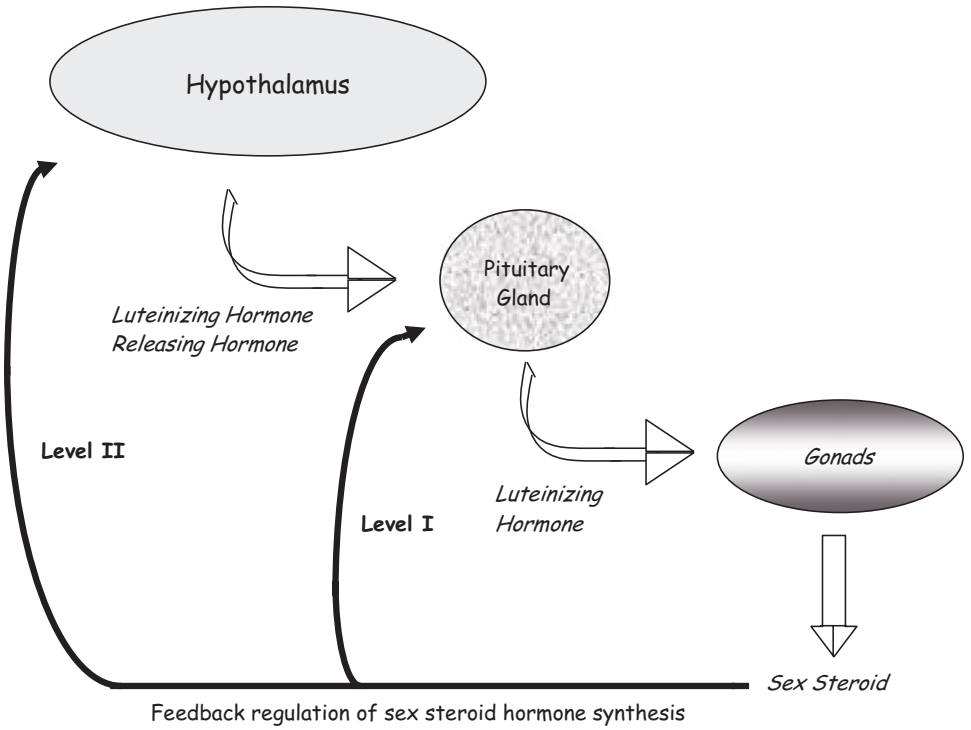


Figure 17.2 The hypothalamic-pituitary-gonadal axis. Endocrine signaling cascades provide multiple sites for regulation and to ensure optimum signaling.

hypothalamic-pituitary-gonadal axis), and sometimes, a terminal target organ of the signaling pathway (e.g., hypothalamic-pituitary-gonadal-hepatic axis).

Endocrine signaling cascades offer several advantages over a single hormone signaling strategy. Cascades provide several sites at which the signal can be regulated thus ensuring maintenance of the appropriate endocrine signal (Figure 17.2). For example, testosterone is secreted by the testis, but regulates its own secretion by acting upstream in the axis at the pituitary gland and hypothalamic gland. Signaling cascades also utilize multiple hormones with differing properties to contribute to the process. Peptide hormones are commonly the intermediate messengers along a signaling cascade while the terminal hormone is often of non-peptide origin (e.g., steroids). Peptide hormones offer advantages as intermediate messengers in that they can be rapidly synthesized and degraded (i.e., turned "on" and "off"). Peptide hormones also do not require cell entry to elicit activity, but rather bind to cell surface receptors. This facilitates a rapid physiological response to the hormone. Steroid and other non-peptide hormones are typically more stable; they are maintained in circulation at a relatively constant, physiologically appropriate level; they can be stored as precursor molecules or apolar conjugates; they can be mobilized as polar conjugates; and most often, they require cell entry to interact with its receptor and elicit a response. Accordingly, the non-peptide terminal

hormones offer the advantages of constant availability but lack the advantages of rapid modulation.

17.2.1 Nuclear Receptors

Toxicologically, the function of the terminal hormones of endocrine cascades (i.e., steroid, retinoid, thyroid hormones) appear to be most susceptible to disruption by chemicals. This is because many foreign molecules share sufficient characteristics with these hormone molecules to allow binding to the nuclear receptors of these hormones in either an agonistic or antagonistic fashion. The binding of the xenobiotic to the receptor results in aberrant receptor function with associated toxicological outcome. The nuclear receptors are so-called since these receptors initiate their classical physiological responses within the cell nucleus. Cell surface receptors to peptide hormones, on the other hand, can likely discriminate between peptide molecules and non-peptide xenobiotics thus minimizing the likelihood of interaction and associated disruption of function.

Nuclear receptors comprise a superfamily of transcription factors divided among seven distinct subfamilies. Humans express at least 48 different nuclear receptors, many of which are considered orphan receptors because no hormone is known to activate the receptor. Many of these orphan receptors may indeed be ligand-independent transcription factors. Others may have, as of yet, unrecognized ligands. Androgen, estrogen, progesterone, glucocorticoid, and mineralocorticoid receptors are all members of the nuclear receptor subfamily NR3. The retinoic acid, vitamin D, and others are members of the NR1 subfamily. The NR3 subfamily receptors typically homodimerize to form active transcription factors while the NR1 family members typically heterodimerize with RXR (an NR2 subfamily member) to form the active transcription factor (Figure 17.3a).

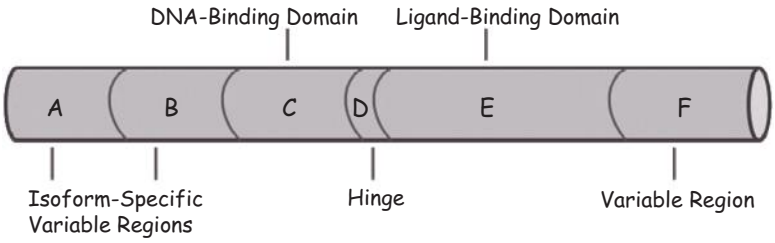
Nuclear receptors typically share a common five-domain structure (Figure 17.3b). The DNA-binding (domain C) and ligand-binding (domain E) are most highly conserved among receptors. The remaining domains are highly variable among receptors and, in the case of domain F, is absent from some receptors. Nuclear receptors modulate transcription of target genes by binding to hormone response elements in the promoter region of the target gene. Hormone response elements typically consist of two half-sites each consisting of a core sequence of AGGTCA or some derivative thereof (Figure 17.3c). Half-sites are separated by nucleotide spacers, and the number of spacers contributes to the receptor specificity of the response element. Each partner receptor in a dimeric complex binds to one of the half-sites to properly stabilize the complex on the DNA. Some orphan receptors bind DNA as a monomer. Response elements for these receptors consist of a single half-site with an adjacent A/T-rich region that further promotes stabilization of the complex. Binding of hormone-activated receptor on the response element initiates the recruitment of coactivators and other factors required to initiation gene transcription.

The NR3 subfamily members typically exist in the extranuclear matrix of the cell in association with various accessory proteins. These accessory proteins stabilize the receptor molecule and help maintain the molecule's integrity. Binding of hormone ligand to the receptor protein stimulates dissociation with the accessory

(a)

Receptor	Group	Major partnership	Example ligand
Thyroid hormone (THR)	NR1A	THR:RXR	Thyroxine
Retinoic acid (RAR)	NR1B	RAR:RXR	*all trans*-retinoic acid
Vitamine D (VDR)	NR1I	VDR:RXR	Vitamin D_3
Retinoid X (RXR)	NR2B	RXR:RXR	9-*cis*-retinoic acid
Estrogen (ER)	NR3A	ER:ER	17b-estradiol
Androgen (AR)	NR3C	AR:AR	Dihydrotestosterone
Progesterone (PR)	NR3C	PR:PR	Progesterone
Glucocorticoid (GR)	NR3C	GR:GR	Cortisol
Mineralcorticoid (MR)	NR3C	MR:MR	Aldosterone

(b)



(c)

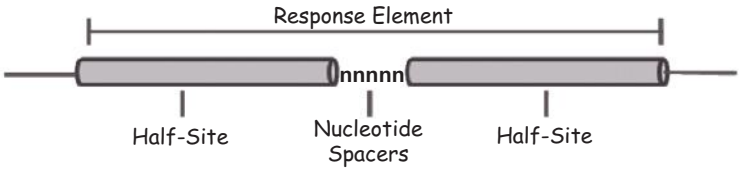


Figure 17.3 (a) Some hormone-activated nuclear receptors of vertebrates. (b) Basic common structure of nuclear receptors. (c) General characteristics of hormone response elements to which receptors bind.

proteins, homodimerization of two receptor molecules, and nuclear localization (Figure 17.4). The NR1 group members typically form heterodimeric combinations with the retinoid X receptor (RXR). RXR also is capable of homodimerization in association with its ligand.

17.2.2 Membrane-Bound Steroid Hormone Receptors

Some cellular responses occur too rapidly following steroid hormone exposure to involve the multistep process of nuclear receptor activation. For example, 17β-estradiol can rapidly stimulate adenylate cyclase and cause a near instantaneous increase in intracellular cyclic adenosine monophosphate (cAMP) in cultured prostate cells. These effects are mediated by the interaction of steroid hormones with cell surface proteins.

Due to their lipophilic nature, steroid hormones are mobilized in the circulatory system by transfer proteins. Sex hormone-binding globulin (SHBG) is one such

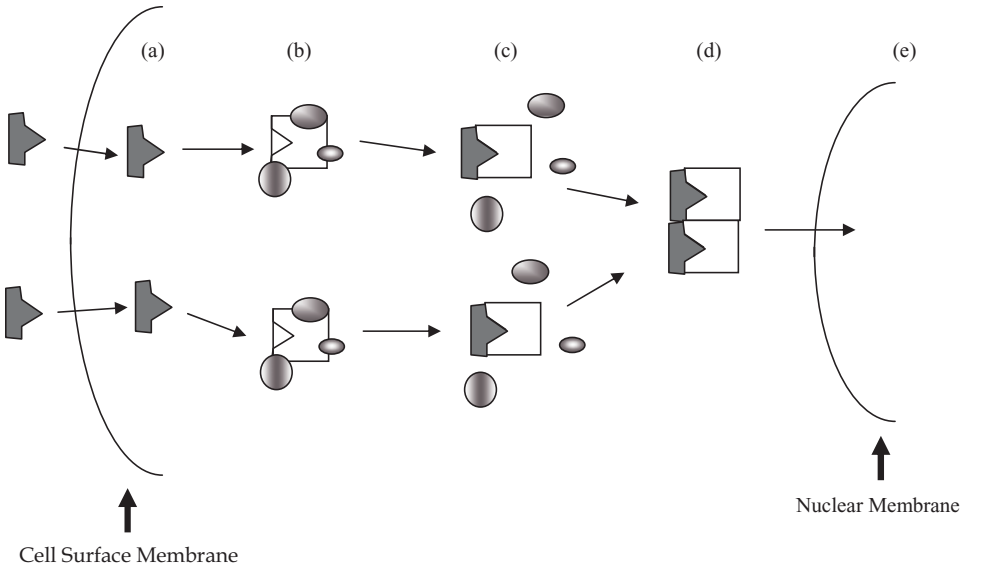


Figure 17.4 Intracellular steroid receptor activation by hormone ligands. (a) Steroid hormones diffuse across the cell membrane into the cell. (b) Steroid hormone receptors in the basal state bound to accessory proteins. (c) Steroid hormones bind to receptors and accessory proteins are dissociated from the receptors. (d) Hormone–receptor complexes dimerize. (e) Dimer complexes enter the nucleus and initiate transcription of responsive genes.

transfer protein that binds testosterone, 17β-estradiol, and other sex steroids. Receptors exist on the surface of some cells that are capable of binding unliganded SHBG (Figure 17.5). Unliganded SHBG, that is bound to the cell surface receptor, can subsequently bind steroid hormone. Binding of an appropriate hormone to the SHBG then stimulates a signal-transduction pathway within the cell. Some steroid hormones (17β-estradiol, 5α-androstan-3α,17β-diol) function as SHBG:SHBG-receptor agonists while others (testosterone, 5α-dihydrotestosterone) function as antagonists. Interestingly, 5α-androstan-3α,17β-diol had previously been considered an inactivation product of the potent androgen 5α-dihydrotestosterone (DHT). Studies in human prostate cells have shown that activation of this SHBG-dependent pathway stimulates DNA synthesis and cell growth. These observations, in combination with studies in dogs that have shown 5α-androstan-3α,17β-diol to stimulate benign prostatic hyperplasia, have led to suggestions that the SHBG-receptor pathway is involved in this disease condition. The susceptibility of these membrane-bound receptor pathways in endocrine toxicology has received little attention, although conceivably, toxicants could perturb these pathways by competing with endogenous hormone for binding to surface membrane receptors resulting in the loss of stimulatory activity (antagonists) or inappropriate stimulation of activity (agonists). Several xeno-estrogens have been shown to stimulate calcium influx in cultured cells through interaction with a surface membrane-bound estrogen receptor at exposure concentrations much lower than is required to activate the nuclear estrogen receptor.

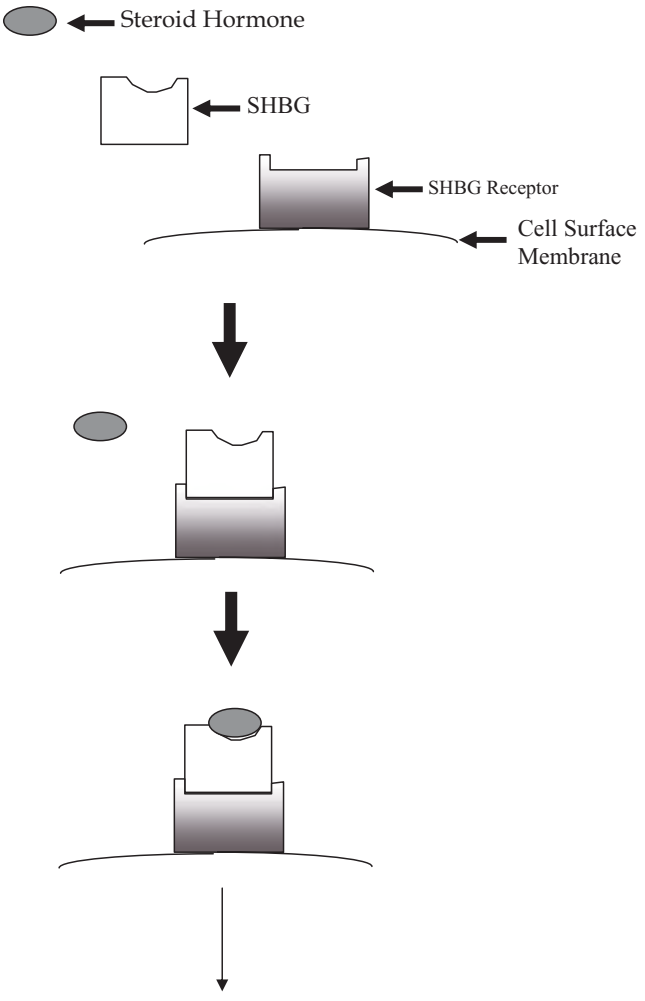


Figure 17.5 Endocrine signaling pathway involving steroid hormone, sex hormone-binding globulin (SHBG), and the SHBG receptor.

17.3 ENDOCRINE DISRUPTION

Xenobiotics have the ability to disrupt hormone activity through a variety of mechanisms though the predominant mechanisms appear to involve binding to the hormone receptor, either as an agonist or antagonist, or by modulating endogenous steroid hormone levels.

17.3.1 Hormone Receptor Agonists

A hormone receptor agonist is defined as *a compound that binds to and activates a hormone receptor*. Endogenous hormones function as agonist to their respective

receptors. Xenobiotics can act as receptor agonist and stimulate receptor-dependent physiological processes in the absence of the endogenous receptor ligand (hormone). Such inappropriate stimulation can result in the errant expression of hormone-dependent processes such as breast development in males (gynecomastia).

Estrogen Receptor Among the steroid hormone receptors, the estrogen receptor appears most susceptible to the agonistic action of xenobiotics. Estrogen receptor agonists are quite diverse in molecular structure (Figure 17.6). Several steric considerations, associated with the steroid structure, in conjunction with electrostatic (charge) properties of the outer surface of the molecule, seem to dictate whether a xenobiotic can fit into the binding pocket of the receptor and function as a receptor agonist. It is not clear why the estrogen receptor would be more susceptible to the agonistic action of xenobiotics as compared to other steroid hormone receptors. The estrogen receptor is often referred to as a promiscuous receptor because of this susceptibility to agonistic interactions with xenobiotics.

Some drugs are rather potent estrogens (e.g., DES); however, environmental chemicals with estrogenic activity are typically weak agonists with activity several

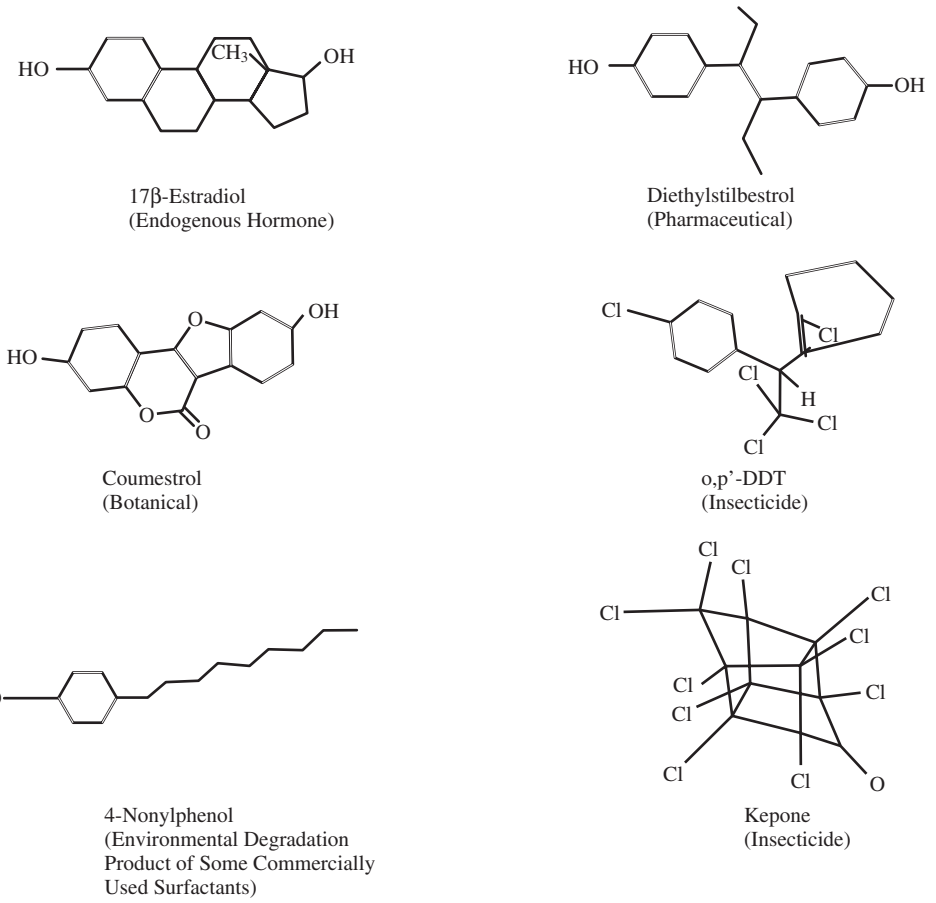


Figure 17.6 Diverse structures of estrogen receptor agonists.

TABLE 17.2 Potency of Some Xeno-Estrogens Relative to 17β-Estradiol

Chemical	Potency
17β-Estradiol	100
Diethylstilbestrol	74
4-Nonylphenol	0.005
4-Octylphenol	0.003
4-*tert*-Octylphenol	0.00036
o′,p′-DDT	0.00011
o′,p′-DDE	0.00004
2′,5′-Dichloro-4-biphenylol	0.62
2′,4′,6-Trichloro-4-biphenylol	1.0
2′,3′,4′,5′-Tetrachloro-4-biphenylol	0.82
Bisphenol A	0.005
Butylbenzylphthalate	0.0004

Note: Estrogenic potency of the compounds was measured using a recombinant yeast cell bioassay (Coldham et al. *Environ. Health Perspect.* **105** (7): 734–742, 1997).

orders of magnitude less than that of 17β-estradiol (Table 17.2). Because of this weak activity, xeno-estrogens are typically not associated with endocrine toxicity to adult females owing to the large amount of 17β-estradiol in these individuals. However, adult males, immature individuals, and embryos all have been shown to exhibit endocrine toxicity resulting from xeno-estrogen exposure. For example, *in utero* exposure of male or female rodents and humans to DES causes proliferation of epithelial cells associated with the reproductive system, resulting in abnormalities of this system. Gynecomastia is a common side effect of estrogenic drugs such as fosfestrol when administered to adult males. The physiological consequences of xeno-estrogenic activity is typically characteristic of feminization, that is, the acquisition of female characteristics.

Ecdysteroid Receptor Ecdysteroids are a class of steroid hormones that regulate a variety of processes related to development, growth, and reproduction in insects and other arthropods but are not utilized by vertebrates. Many compounds of plant origin, or derivations thereof, have been identified that are ecdysteroid receptor agonists (i.e., cucurbitacins, withasteroids). The ecdysteroid agonists are presumed to have evolved in plants as a means of protection against insect predation. Some environmental chemicals of anthropogenic origin also have been shown to exhibit ecdysteroid receptor agonistic activity (e.g., tebufenozide) and have been exploited as insecticides due to their ability to interfere with insect development and growth.

Retinoic Acid Receptor Most of the biological effects of retinoids are mediated through the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). Both all-*trans*-retinoic acid and 9-*cis*-retinoic acid serve as agonists of RAR while only 9-*cis*-retinoic acid functions as an agonist of RXR. The functional RAR exists as a heterodimer with RXR, while functional RXR exists as a homodimer. Methoprene

is a juvenile hormone III analog that mimics the activity of this insect hormone. Exposure of juvenile insects to methoprene results in various abnormalities associated with development and ultimately death. An environmental degradation product of methoprene, methoprenic acid, was found to serve as an RXR agonist and specifically activate genes responsive to RXR homodimers. In addition, exposure of frog larvae to methoprenic acid caused developmental deformities consistent with those that have been observed in recent years in wild populations and consistent with those caused by exposure to retinoic acid under laboratory conditions. The biocide tributyltin is a high-affinity agonist of the RXR. This activity has been causally associated with a condition, documented worldwide, where females of some marine snail species have acquired male sex characteristics. These observations indicate that activation of the RXR by xeno-agonist may contribute to the occurrence of various deformities documented in wildlife species.

17.3.2 Hormone Receptor Antagonists

While the estrogen receptor appears somewhat unique among vertebrate nuclear hormone receptors in its promiscuity toward receptor agonists, many nuclear hormone receptors have been shown to be susceptible to chemical antagonism. Receptor antagonists are defined as *chemicals that bind to a hormone receptor but do not activate the receptor*. Rather, these chemicals inhibit receptor activity by preventing the endogenous hormone from binding to and activating the receptor.

Estrogen Receptor Chemicals often bind to the estrogen receptor and function as mixed agonists/antagonists (discussed below). Drugs that bind to the estrogen receptor as an antagonist or mixed agonist/antagonist include tamoxifen, raloxifene, ICI 164,384, and toremifene. Environmental estrogen receptor antagonists include some phytochemicals (e.g., flavonoids) and industrial chemicals such as some polychlorinated biphenyls (PCBs). Consequences of estrogen receptor antagonism are typically considered defeminization (loss of female traits). In laboratory animal studies, estrogen receptor antagonists have been shown in females to disrupt estrous cycles, impair fertility, increase preimplantation loss, and cause embryo lethality.

Androgen Receptor Chemicals that bind to the androgen receptor in an antagonistic fashion include the pharmaceuticals spironolactone, cimetidine, cyproterone acetate, and hydroxyflutamide. Environmental chemicals that have been shown to act as androgen receptor antagonists include the metabolites of the agricultural fungicide vinclozolin, the DDT metabolite *p,p′*-DDE, some hydroxylated PCBs, and the organophosphate insecticide fenitrothion. The consequence of androgen receptor antagonism is typically considered demasculinization (loss of male traits). Demasculinizing effects of anti-androgens in laboratory animal studies have included reductions in the size of the ventral prostate and seminal vesicle weights along with deformities of the penis.

Glucocorticoid Receptor Some drugs (e.g., mifepristone) elicit antagonistic activity toward the glucocorticoid receptor. This property has been associated with adverse side effects of the drugs and also has been capitalized upon therapeutically

for the modulation of the glucocorticoid receptor. Anti-glucocorticoids typically are steroidal compounds that are capable of binding to the receptors but are relatively ineffective in activating the receptor. As such, these compounds are typically mixed agonists/antagonists (see below). Glucocorticoid receptor antagonists can adversely affect growth, development, and other glucocorticoid-regulated processes (Table 17.1). Little is known of the ability of environmental chemicals to function as glucocorticoid receptor antagonists.

Mixed Agonists/Antagonists Chemicals often can function as either a receptor agonist or antagonist depending upon the level of endogenous hormone. A weak agonist may bind to a receptor and stimulate some low-level receptor-mediated activity in the absence of the endogenous hormone. However, in the presence of the hormone, binding of the xenobiotic to the receptor may prevent binding of the endogenous hormone and, if the xenobiotic is a much weaker activator of receptor-mediated activity, then the net effect is loss of activity. Thus, in the presence of the endogenous hormone, the xenobiotic functions as a receptor antagonist. Whether a weak xeno-agonist functions as an agonist or antagonist typically depends upon (1) the concentration of the xeno-agonist, (2) the potency of the xeno-agonist, and (3) the concentration of the endogenous hormone. These compounds are classified as mixed agonists/antagonists.

17.3.3 Organizational Versus Activational Effects of Endocrine Toxicants

Effects of receptor agonists or antagonists on endocrine-related processes are often described as being either organizational or activational. An organizational effect of an endocrine toxicant is one that typically results from neonatal or prenatal exposure during which time hormones are directing various irreversible aspects of development. Accordingly, the disrupting effect of the toxicant also is irreversible. These organizational effects may be evident only later in life during maturation or reproduction. Neonatal exposure to DES resulting in proliferation of epithelial cells of the reproductive tract at reproductive maturity is an example of an organizational effect of an endocrine toxicant. Organizational effects of endocrine toxicants have been of great concern to toxicologists and are the most difficult type of toxicity to diagnose, owing to the temporal separation between exposure and effect.

An activational effect of an endocrine toxicant occurs in the same time frame as the exposure and is the consequence of the toxicant disrupting the immediate role of a hormone in some physiological process. Activational effects are typically reversible following cessation of exposure to the toxicant. For example, androgens contribute to maintenance of the prostate gland in the adult male. Exposure of adult males to an anti-androgen can result in a decrease in prostate size. Cessation of exposure to the anti-androgen then results in restoration of the prostate gland to its normal size.

17.3.4 Inhibitors of Hormone Synthesis

Endocrine toxicants can elicit antihormone activity by lowering levels of endogenous hormone in the body. With steroid hormones, chemicals typically elicit this

effect by inhibiting enzymes necessary for synthesis of the hormone. For example, the cytochrome P450 enzyme CYP19 is responsible for the aromatization of testosterone to form 17β-estradiol. CYP19 inhibitors such as fadrozol, anastrozole, and letrozole, can lower endogenous 17β-estradiol levels resulting in defeminization. Cytochrome P450 enzymes also are critical to various hydroxylation reactions that contribute to the synthesis of androgens and other steroid hormones, and inhibition of these enzymes can result in a variety of anti-steroid hormone effects. For example, the agricultural and medicinal fungicides propiconazole, ketoconazole, and fenarimol are capable of inhibiting P450 enzymes and reducing synthesis and circulating levels of testosterone and other steroid hormones. Toxicological consequences of the lowering of endogenous steroid hormone levels are typically comparable to those effects elicited by antagonists of the hormone's receptor.

17.3.5 Inducers of Hormone Clearance

In most species, steroid and thyroid hormones are inactivated and cleared from the body by the same biotransformation processes that are involved in chemical detoxification (see Chapter 6). Predominant among the hormone biotransformation processes in vertebrates are hydroxylation, glucuronic acid conjugation, and sulfate conjugation. The thyroid hormones T_3 and T_4 are inactivated and cleared following sulfate and glucuronic acid conjugation, respectively. The glucuronosyl transferase enzymes that are responsible for the elimination of T_4 are induced following exposure to phenobarbital-type inducers and Ah receptor ligands (see Chapter 8). Thus, exposure to chemicals such as some dioxins and PCBs can result in enhanced clearance of thyroid hormone resulting in low circulating thyroid hormone levels. The resulting hypothyroid state can result in a variety of pathological conditions (Table 17.3). In newborn infants, hypothyroidism is associated with cretinism. This organizational syndrome is characterized by mental retardation, short stature, and various neurological abnormalities. In children, hypothyroidism can cause delayed growth and mental development while advancing the onset of puberty in adolescents. Hypothyroidism in adults results in various activational abnormalities including impaired cardiovascular, pulmonary, intestinal, and renal function. Chronic fatigue, lethargy, and difficulty in concentration are also associated with hypothyroidism in adults.

TABLE 17.3 Clinical Manifestations of Hypothyroidism

Organ System	Manifestation
Skin	Puffy appearance, dry, course, yellow tinted skin Brittle nails, wound healing slowed, hair loss
Cardiovascular	Enlarged heart, changes in electrocardiographs
Respiratory	Maximal breathing capacity reduced, obstructive sleep apnea, fluid accumulation in the pleural cavity
Digestive system	Reduced appetite with modest weight gain
Muscle	Stiffness, aching
Nervous	Slowing of intellectual functions, lethargy, headaches

Increased clearance of steroid hormones due to induction of hepatic biotransformation enzymes following chemical exposure often has been cited as a possible mechanism by which toxicants could lower circulating testosterone or 17β-estradiol levels. While enhanced clearance of sex steroids has been demonstrated following chemical exposure and induction of hepatic biotransformation enzymes, elegant feedback control mechanisms tend to ensure that more hormone is produced and homeostasis is maintained (Figure 17.2). Enhanced clearance of sex steroids can contribute to endocrine disruption if the toxicity also results in impaired hormone synthesis (i.e., gonadal toxicity or interference with the feedback control of hormone synthesis). 2,3,7,8-Tetrachlorodibenzodioxin appears to lower circulating sex steroid levels via this dual effect.

17.3.6 Hormone Displacement from Binding Proteins

Steroid and thyroid hormones are distributed throughout the body while bound to serum-binding proteins such as sex hormone-binding globulin, corticosteroid-binding globulin, thyroxine-binding globulin (transthyretin), and albumin. Most steroid and thyroid hormones (>95%) are present in the blood reversibly bound to proteins. This bound hormone is not available for cell entry where it may interact with nuclear receptors or undergo inactivation/elimination reactions. Rather, the bound hormone serves as a reservoir from which hormone can be liberated (free hormone) for cell entry.

Some xenobiotics can compete with hormones for binding to the blood proteins. As a result, the circulating hormone reservoir can be depleted and free hormone becomes limited. A variety of phenolic compounds including hydroxylated metabolites of PCBs, chlorophenols, chlorophenoxy acids, and nitrophenols have been shown to interfere with thyroxine binding to thyroxine-binding globulin during *in vitro* experiments. In some instances, compounds that displace thyroxine from the binding protein also have been shown to decrease circulating thyroxine levels in exposed animal models or in humans. *In vitro* experiments also have revealed that testosterone and 17β-estradiol can be displaced from sex hormone-binding globulin by some chemicals such as 4-nonylphenol, 4-tert-octylphenol, bisphenol A, *O*-hydroxybiphenyl, and pyrethroid insecticides. However, it is not clear whether these chemicals would significantly displace sex steroids from the binding globulin at concentrations typically measured in human blood.

17.4 INCIDENTS OF ENDOCRINE TOXICITY

17.4.1 Organizational Toxicity

In utero exposure to estrogens or anti-androgens has been shown, in animal models, to elicit a variety of organizational effects associated with development of the reproductive system. The best-described example of the organizational effects of a drug administered to humans involves the synthetic estrogen DES. DES was prescribed to over two million pregnant women in the United States between the 1940s and 1960s to prevent miscarriage. Offspring exposed to DES during fetal development experienced a variety of problems upon attainment of sexual maturity.

DES daughters experience a significantly increased risk of clear cell adenocarcinoma of the vagina and cervix. DES daughters had increased risk of a variety of reproductive disorders including structural abnormalities of the reproductive tract, infertility, ectopic pregnancy, miscarriage, and preterm delivery.

Less is known of the risks faced by males exposed to DES during fetal development. Animal studies have revealed that male rodents exposed to DES have increased incidence of prostatic metaplasia. Epidemiological studies of DES sons have suggested increased risk of various testicular abnormalities including epididymal cysts, testicular varicoceles, and undescended testis. Hyperplasia and metaplasia of the prostatic ducts in DES sons also have been reported.

The effects elicited by fetal exposure to DES appear to be largely the consequence of the estrogenic activity of this drug. Estrogens orchestrate organizational events during fetal development that promote female reproductive tract development. Excess estrogen exposure resulting from DES treatment of either female or male fetuses resulted in permanent alterations, many of which became evident only upon attainment of reproductive maturity.

Organizational effects on reproductive development resulting from perinatal exposure to endocrine toxicants of environmental origin also have been reported to occur. In 1973, a fire retardant containing polybrominated biphenyls (PBBs) was mistakenly added to cattle feed in Michigan. An estimated 4000 people subsequently were exposed to the PBBs by consuming dairy products derived from these cattle. PBBs are long-lived chemicals that are stored in the fat of exposed individuals. PBBs have been reported to elicit endocrine toxicity-like symptoms in animal models consistent with hypothyroidism. For example, offspring from maternal rats provided PBBs during gestation and lactation showed signs of neurological deficit and growth retardation. Daughters of mothers that were exposed to PBBs during the Michigan incident exhibited an earlier initiation of menarche (menstruation) that correlated with the likely severity of *in utero* PBB exposure. The most highly exposed daughters began menstruating approximately 1 year ahead of females that were less severely exposed. Early initiation of menarche is consistent with precocious puberty associated with hypothyroidism.

The distance between the anus and the genitals is a sexually dimorphic trait that is hormonally regulated during fetal development. Males have a greater anogenital distance than do females. A study published in 2005 revealed that anogenital distance among male babies was inversely correlated to the level of phthalate ester metabolites in the urine of their mothers. Babies produced by mothers with the highest phthalate ester metabolite concentrations were seven times more likely to have a shortened anogenital distance. Phthalate esters are widely used in plastics to confer flexibility to the plastic. Studies in rats have shown that some phthalate esters interfere with fetal testosterone production and thus have anti-androgenic effects in the developing fetus. Results with human babies suggest that levels of phthalate esters associated with the human population may be sufficient to disrupt male reproductive development.

17.4.2 Activational Toxicity

Estrogenic Pharmaceuticals Administration of estrogenic pharmaceuticals to children or adults can result in a variety of abnormalities associated largely

with secondary sex characteristics that are reversible upon cessation of drug treatment.

Gynecomastia, the development of breast tissue in males, is often the consequence of perturbations in the normal androgen/estrogen ratio. Prolonged administration of drugs with estrogenic or anti-androgenic activity can cause gynecomastia. Gynecomastia had been reported in the medical literature to occur as a result of frequent intercourse when an estrogen-containing cream was used as a vaginal lubricant and among morticians who applied estrogen-containing skin creams to corpses without the use of gloves.

Similar to gynecomastia in adult males, activational toxicity from estrogenic drugs has been reported to cause pseudoprecocious puberty in children. Pseudoprecocious puberty is characterized by the development of some indicators of puberty (e.g., pubic or facial hair, morphological changes in sex organs, breast development) in preadolescent individuals. An outbreak of pseudoprecocious puberty was reported among a group of children ranging in age from 4 months to 2 years of age following application of a skin cream to treat dermatitis. Symptoms included pigmentation of the nipples, breast development, the presence of pubic hair, and vaginal discharge and bleeding among the females. Breast development also was reported in prepubertal boys following use of an estrogen-containing hair cream. These reports highlight the fact that dermal exposure can be adequate to attain a sufficient dose of endocrine-active compound to elicit adverse responses. In all of these cases, the symptoms of endocrine toxicity resolved following cessation of exposure to the causative agent.

Environmental Estrogens Thelarche is defined as the development of breast tissue in preadolescent females (typically <8 years of age). An epidemic level of thelarche occurred on the island of Puerto Rico during the 1980s and 1990s. The cause of the outbreak was never discerned; however, evidence strongly implicated exposure to endocrine-disrupting agents. Analyses of blood samples from thelarche and non-thelarche children for environmental chemicals with known estrogenic activity revealed that 68% of the thelarche children contained significantly high levels of several types of phthalate esters. Only a single non-thelarche child contained a significant amount of phthalate ester and only one type of phthalate ester was found in this individual. The association between phthalate ester exposure and the high incidence of thelarche in Puerto Rico does not establish causality but has generated concern that environmental agents were responsible for this condition.

Kepone (chlordecone) is an organochlorine insecticide (Figure 17.5) that was manufactured in Hopewell, Virginia from the mid 1960s to 1975. In 1975, the Center for Disease Control determined that employees of the manufacturing facility and other residents of Hopewell, totaling over 200 individuals, had been significantly contaminated with this insecticide. Exposed individuals reported a variety of symptoms. Foremost, among the symptoms of "Kepone sickness" were neurological disorders presenting as tremors, weight loss, and nervousness. However, subsequent evaluations revealed that males exposed to Kepone also experienced testicular dysfunction that was characteristic of estrogen exposure. Later laboratory studies demonstrated that Kepone was an estrogen receptor agonist, which could explain its adverse effects on the male reproductive system.

17.4.3 Hypothyroidism

Hypothyroidism describes the clinical state arising from a deficiency in thyroid hormone. Toxicity resulting in hypothyroidism is manifested at several organ systems as described in Table 17.3, and individual effects may be misdiagnosed as organ-specific toxicity. Hypothyroidism can result from various causes other than chemical toxicity including diseases of the hypothalamic-pituitary-thyroidal axis, iodine deficiency, and heritable defects in thyroid hormone production. Chemical agents that have historically been recognized for their ability to cause hypothyroidism include phenylbutazone, resorcinol, lithium, and para-aminosalicylic acid.

Disruptions in thyroid hormone levels can occur through chemical-induced increases in the metabolic inactivation and elimination of the hormone. Chemicals that are capable of increasing the metabolic clearance of thryroid hormone include the polycyclic halogenated hydrocarbons (i.e., dioxins, furans, PCBs, polybrominated biphenyls). A study reported in *The New England Journal of Medicine* suggested that environmental or occupational exposure to such chemicals can result in hypothyroidism in humans. The study consisted of a comparison of thyroid status in workers who were occupationally exposed to polybrominated biphenyls as compared to workers who were not exposed to any polyhalogenated hydrocarbons. Four of 35 exposed workers and none of 89 unexposed workers exhibited signs of hypothyroidism that included increased plasma levels of thyrotropin and decreased plasma levels of thyroxine. Thyrotropin is secreted by the pituitary gland and stimulates the thyroid gland to produce thyroxine (see Figure 17.1). The increase in thyrotropin and decrease in thyroxine is consistent with hypothyroidism caused by increased clearance of the thyroxine. As discussed earlier in this chapter, perinatal exposure to PBBs during the Michigan milk contamination also produced symptoms characteristic of hypothyroidism.

17.5 CONCLUSION

The endocrine system possesses many targets at which toxicants can elicit either reversible or permanent effects on an individual. Effects of chemicals on endocrine-regulated processes such as development, maturation, growth, and reproduction have been well documented in both laboratory and epidemiological studies. Less is known of the potential effects of endocrine toxicants on more generalized endocrine-regulated processes such as bone maintenance, general organ function, and metabolism. The U.S. Environmental Protection Agency (EPA) has been mandated by the U.S. Congress to develop and implement a program for the screening and testing of chemicals for endocrine-disrupting toxicity. The EPA has developed a program that focuses upon the effects of chemicals on androgen, estrogen, and thyroid hormone-regulated processes. This required testing will greatly expand our knowledge of the extent to which humans are exposed to chemicals that interfere with processes regulated by these hormones. However, it is important to recognize that chemicals have the potential ability to interfere with other hormone cascades including those involving mineralocorticoids, glucocorticoids, retinoids, and perhaps some peptide hormones. Research is needed to increase our understanding of the

susceptibility of endocrine signaling pathways involving these hormones to chemical toxicity and, ultimately, establishing chemical exposure limits that include these considerations.

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SAMPLE QUESTIONS

1. Endocrine signaling typically occurs through neuroendocrine cascades that involve both peptide and non-peptide (e.g., steroid) hormones. What is the value of having both peptide and steroid hormones in the same endocrine cascade?

2. Why are steroid hormone receptors often the most susceptible link in an endocrine cascade to disruption by toxicants?

3. What are two mechanisms through which a chemical might act as an anti-androgen?

4. Define “organizational” and “activational” actions of endocrine toxicants.

CHAPTER 18

Respiratory Toxicology

JAMES C. BONNER

18.1 INTRODUCTION

The respiratory system represents a unique target for the potential toxicity of toxicants due to the fact that the lungs are the primary portal of entry for inhaled gases and particles. In addition, the lungs receive the entire cardiac output, and therefore, toxicants that enter the bloodstream also have the potential to cause lung injury. Because of the essential function of the respiratory system in transporting atmospheric oxygen to the bloodstream, the alveolar region of the lung is the primary interface for exposure to a vast array of toxins. The mammalian lung has evolved exquisite cellular defense mechanisms to cope with the clearance of inhaled agents and finely tuned repair mechanisms to restore the delicate architecture of the lung after injury. Despite these protective mechanisms, toxic exposures to both natural and man-made toxicants result in lung diseases such as asthma, fibrosis, chronic obstructive pulmonary disease (COPD), and cancer.

18.2 ANATOMY AND FUNCTION OF THE RESPIRATORY TRACT

The general features of upper and lower regions of the respiratory tract are illustrated in Figure 18.1. The upper respiratory tract consists of the mouth, nose, and pharyngeal region. Air enters the respiratory systems of mammals through the nose or mouth in humans, while some species such as rodents are obligate nasal breathers. The primary functions of the upper respiratory tract are olfaction, temperature equilibration and humidification of inspired air, and uptake of inhaled particles and irritant gases. The lower respiratory tract begins distal to the pharyngeal region and consists of the tracheobronchial region and the pulmonary parenchyma or alveolar region. A primary function of the lower respiratory tract is harvesting oxygen from the atmosphere and the transfer of the metabolic gas CO_2 from the blood to the exhaled air. A second important function of the lower respiratory tract is defense against inhaled toxicants.

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

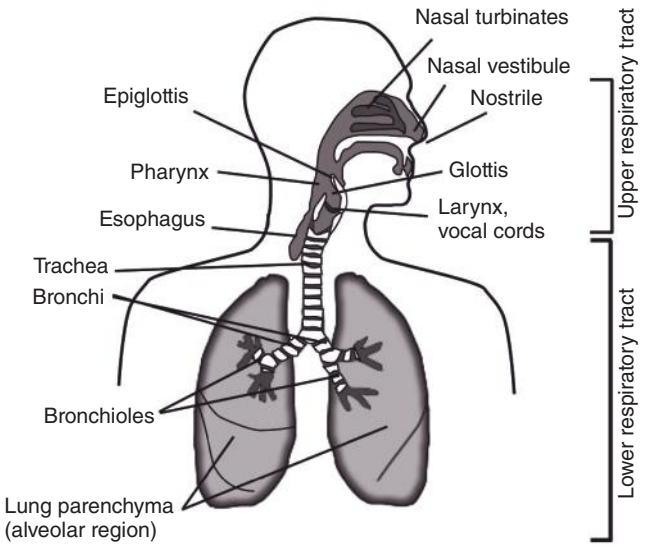

Figure 18.1 Anatomy of the human respiratory system. Adapted from *LifeART Illustration Series*. Hagerstown, MD: Lippincott Williams & Wilkins, 1994.

As the goal of the lung is to provide oxygen to the tissues and remove CO_2, the inflow and outflow of air between the atmosphere and the alveoli, termed *ventilation*, is a critical function. A simple method for studying pulmonary ventilation is to record the volume movement of air into and out of the lungs, a process called *spirometry*. Four different pulmonary lung volumes are defined as follows: (1) the *tidal volume* is the volume of air inspired or expired with each normal breath; (2) the *inspiratory reserve volume* is the maximum extra volume of air that can be inspired over and above the normal tidal volume; (3) the *expiratory reserve volume* is the maximum extra volume of air that can be expired by forceful expiration after the end of a normal tidal expiration; and (4) The *residual volume* is the volume of air remaining in the lungs after the most forceful expiration. Two or more volumes together are called *pulmonary capacities*, which are described as follows: (1) the *inspiratory capacity* equals the *tidal volume* plus the *inspiratory reserve volume*; (2) the *functional residual capacity* equals the *expiratory reserve volume* plus the *residual volume*; (3) the *vital capacity* equals the *inspiratory reserve volume* plus the *tidal volume* plus the *expiratory reserve volume*; and (4) the *total lung capacity* is the maximum volume to which the lungs can be expanded, equal to the *vital capacity* plus the *residual volume*.

Measures of the lung to expand and fill with air during inspiration and to deflate during exhalation are termed lung mechanics. These properties depend on the elasticity of the lung and the caliber of the airways. Lung mechanics are commonly reported as compliance and resistance. *Compliance* is the volume change per unit pressure change. *Resistance* is the pressure difference per change in airflow. Compliance and resistance are generally measured during steady tidal breathing by recording transpulmonary pressure, tidal volume, and airflow rate. Because the

direct measurement of transpulmonary pressure require invasive procedures such as an intrapleural catheter, indirect measures approximating compliance and resistance are typically applied to humans using *plethysmography*.

Toxicity to the respiratory tract can interfere with this gas-exchange function by (1) altering the tone of the airways resulting in decreased airflow; (2) damaging the delicate architecture of the alveolar/capillary barrier of the deep lung resulting in impaired gas exchange; or (3) causing tissue damage that leads to chronic structural changes and decreased lung volumes or lung mechanics. The total lung capacity and vital capacity are reduced in fibrotic lung disease in which the lung becomes smaller and stiffer. This type of change in the lung is called *restrictive lung disease* and is marked by smaller lung volumes and little change in airflow. In an emphysematous lung, on the other hand, the total lung capacity may increase as a result of the breakdown of alveolar walls and loss of elastin fibers that allow the lung to deflate on exhalation, but vital capacity is reduced due to airway collapse during exhalation. This type of change in the lung is called *obstructive lung disease* and is marked by reduced airflow.

18.2.1 Upper Respiratory Tract as a Site of Toxicity

The upper respiratory tract, particularly the nose, has a unique anatomy that performs normal physiologic functions as well as innate defense against inhaled toxins. The nose extends from the nostrils to the pharynx. The nasal cavity is divided longitudinally by a septum into two nasal compartments. In most mammalian species, each nasal cavity is divided into a dorsal, ventral, and middle (lateral) meatus by two turbinate bones, the nasoturbinate and maxilloturbinate. These turbinates project from the dorsolateral and ventrolateral wall of the cavity, respectively. In the posterior portion of the nose, the ethmoid recess contains the ethmoturbinate. The nasal cavity is lined by a vascular mucosa that consists of four distinct types of epithelia. In rodents, these epithelia are (1) the stratified squamous epithelium that line the nasal vestibule and the floor of the ventral meatus in the anterior portion of the nose; (2) the nonciliated, pseudostratified, transitional epithelium that lies between the squamous epithelium and the respiratory epithelium and lines the lateral meatus; (3) the ciliated respiratory epithelium that lines the remainder of the nasal cavity anterior and ventral to the olfactory epithelium; and (4) the olfactory epithelium (neuroepithelium) that lines the dorsal meatus and ethmoturbinates in the caudal portion of the nose.

The olfactory epithelium is composed of basal, neuronal (olfactory), and sustentacular (support) cells (Figure 18.2). The portion of each olfactory cell that responds to the olfactory chemical stimuli is the cilia. The odorant substance first diffuses into the mucus that covers the cilia and then binds to specific receptor proteins in the membrane of each cilium. Next, receptor activation by the odorant activates multiple molecules of the G-protein complex in the olfactory epithelial cell. This in turn activates adenylyl cyclase inside the olfactory cell membrane, which in turn causes formation of a greater multitude of cyclic adenosine monophosphate (cAMP) molecules. Finally, the cAMP molecules trigger the opening of yet an even greater multitude of sodium ion channels. This amplification mechanism accounts for the exquisite sensitivity of the olfactory neurons to extremely small amounts of odorant. The olfactory epithelium is an important target of certain inhaled toxicants. Certain

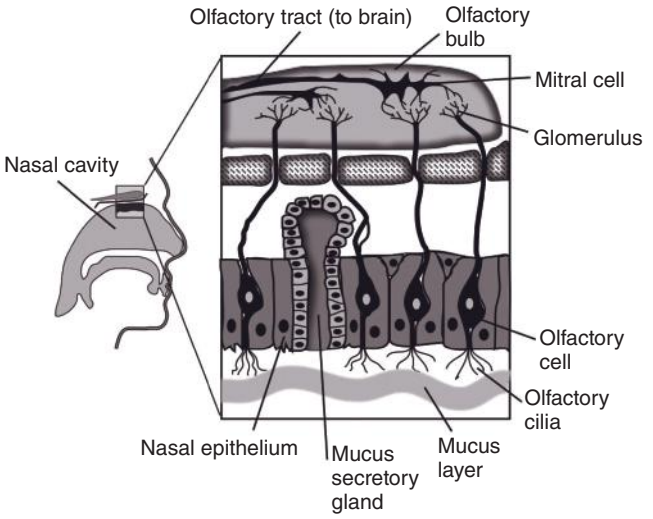

Figure 18.2 Anatomy of the olfactory apparatus in the nasal cavity of the upper respiratory tract. Adapted from *LifeART Illustration Series*. Hagerstown, MD: Lippincott Williams & Wilkins, 1994.

metals, solvents, proteins, and viruses are transported to the brain via transport from the olfactory epithelium to the olfactory tract and exert neurotoxicity.

As air passes through the nose, essential normal respiratory functions are performed by the nasal cavities. These functions are collectively referred to as the air conditioning function of the upper respiratory tract. First, the air is warmed and is almost completely humidified by the extensive mucosal surfaces of the nasal conchae and septum. Second, the nose plays a critical role in trapping the majority of inhaled particles before they reach the lower lung. Large particles (>5 μm in diameter) are stopped from entering the lower respiratory tract by *impaction* of particles on the surfaces of the nasal turbinates (also referred to as nasal conchae), which are folds of osseous tissue lined with ciliated and mucous-producing epithelial cells. Impaction means that particles in the inspired air passing through the nasal passageways collide with many obstructions, including the nasal turbinates and septum, and stick in the mucous lining of the nasal epithelium. The term "turbinates" was derived from the fact that these structures cause turbulence of the inspired air. As air encounters the turbinates, it changes direction of movement. However, particles that are suspended in the air have greater mass than the air itself and continue forward, impacting the surface of the obstruction. Impacted particles are transported by the beating cilia of the nasal epithelium in a unidirectional manner to the pharynx where they are swallowed.

18.2.2 Lower Respiratory Tract as a Site of Toxicity

The lower respiratory tract is composed of the conducting airways (trachea, bronchi, and bronchioles) and the lung parenchyma which consists primarily of gas exchange units (alveoli). The trachea, bronchi, and bronchioles conduct air to the pulmonary

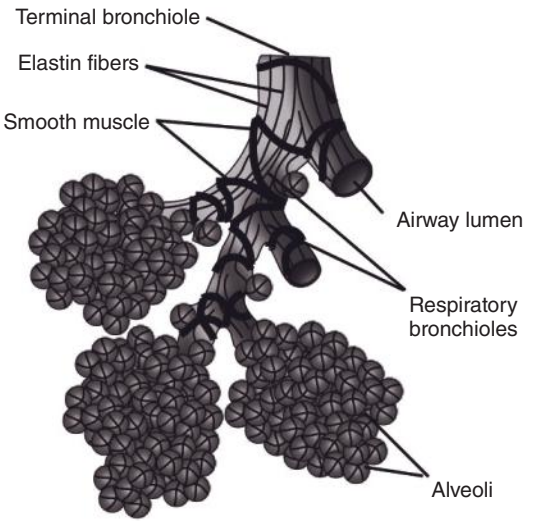

Figure 18.3 Anatomy of the distal airways and functional units (alveoli) in the lower respiratory tract. Adapted from *LifeART Illustration Series*. Hagerstown, MD: Lippincott Williams & Wilkins, 1994.

parenchyma. The trachea extends from the larynx distally where it divides to form the two main bronchi, which enter the right and left lungs. The bronchi bifurcate to form bronchioles and continue to progressively bifurcate in a tree-like fashion to form bronchioles of decreasing diameter. The most distal conducting segment of the tracheobronchial tree is called the terminal bronchiole, which bifurcates to form respiratory bronchioles that contain some alveolar ducts and terminate in clusters of alveolar sacs. This distal region of the lung where airways transition to alveoli is illustrated in Figure 18.3.

18.2.3 Airways of the Lower Respiratory Tract

The trachea and bronchi contain bands of cartilage in the airway wall that prevent collapse. In contrast, the walls of the intrapulmonary airways do not contain cartilage but are supported by flexible elastic fibers and bands of smooth muscle. These smaller airways are susceptible to bronchoconstriction in diseases such as asthma, where allergens provoke a neurogenic response that results in airway smooth muscle contraction. The airways of the lungs are lined with pseudostratified, columnar cells that are predominantly ciliated or mucus-producing serous cells (Figure 18.4). Together, these epithelial cell types contribute to the clearance of particles from the airways through a mechanism termed the "mucociliary escalator." Throughout the tracheobronchial region, mucociliary clearance is an important clearance and defense mechanism for moving inhaled particles up the airway tree where they are expelled from the trachea and swallowed. In humans, submucosal glands in the bronchi also contribute to mucus production, especially during chronic irritation such as cigarette smoking or during chronic respiratory viral infections. In the

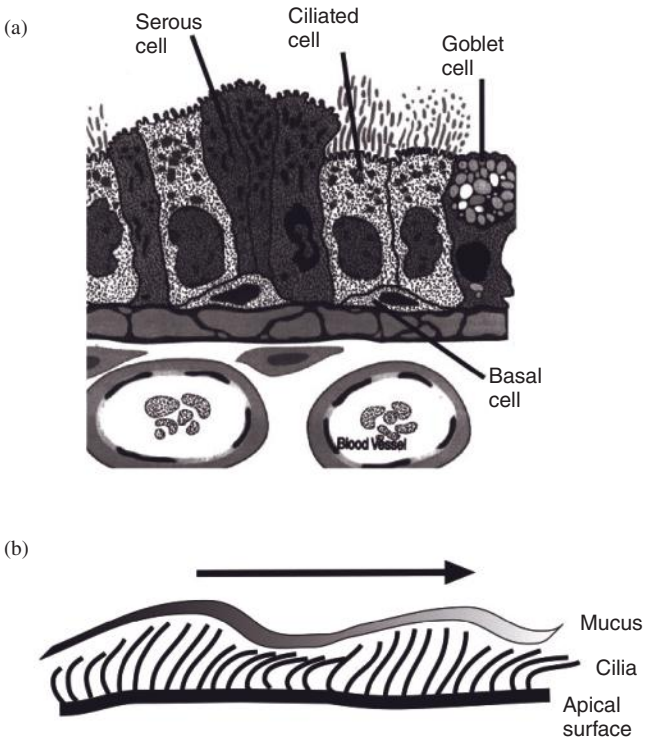

Figure 18.4 Structure and dynamics of the airway epithelium. (a) Illustration of tracheal and bronchial epithelial cell types. (b) The mucociliary escalator wherein epithelial cell cilia move in a low viscosity periciliary layer to propel mucus with their tips.

terminal bronchioles, the population of mucus and serous cells gradually transition to nonciliated cuboidal (Clara) cells. Clara cells have relatively high levels of cytochrome P450 enzymes and may be selectively damaged by toxicants that require metabolic activation by P450s. The epithelium of large and small airways contain triangular-shaped basal cells, which are thought to give rise to ciliated and mucous cells following toxicant injury. All airway epithelial cells are attached basally to a basement membrane or lamina composed of extracellular matrix.

The airway epithelium forms a continuous lining for the conducting airways. The varied composition of the epithelium allows it to perform a variety of functions. First, the epithelium, along with its apical mucus layer and its basal lamina, comprise an important barrier against inhaled toxicants and xenobiotics. The apical surfaces of the airway epithelial cells are connected by tight junctions and effectively provide a barrier that isolates the airway lumen. Second, the various airway epithelial cells produce a mixture of secretions composed of (a) an aqueous "sol" phase containing proteins, lipids, and ions and (b) a gel phase containing mucus. Third, ciliated cells comprise the largest proportion of exposed cells in the normal airway and as discussed above, they propel the mucus within the airway lumen proximally, thereby mediating clearance of inhaled particles and debris (Figure 18.4). Fourth, the airway epithelium exhibits repair following injury, thereby establishing normal airway architecture. Fifth, the airway epithelium can produce a variety of soluble mediators

(cytokines, growth factors, proteinase, and lipid mediators) that modulate the responses of other lung cells including airway smooth muscle cells, fibroblasts, immune cells and phagocytes.

18.2.4 Parenchyma of the Lower Respiratory Tract

The primary function of the lung parenchymal region is gas exchange. The major structures are the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. The respiratory bronchioles are lined with cuboidal ciliated and Clara cells, and have alveoli opening into their lumina. Therefore, the respiratory bronchioles function both as conducting passages and as a gas exchange region. A number of mammalian species, including humans, have respiratory bronchioles whereas other species, including rats, have no respiratory bronchioles. In the latter case, the terminal bronchioles end in alveolar ducts. Alveolar ducts are tubular structures whose walls are covered by alveoli. The alveoli open polyhedral chambers lined with thin type I epithelial cells interspersed with cuboidal type II cells. Type I cells comprise 8–11% of the structural cells found in the alveolar region and yet cover 90–95% of the alveolar surface. Their major function is to allow gases to equilibrate across the air–blood barrier and to prevent leakage of fluids across the alveolar wall into the lumen. The type I epithelium is particularly sensitive to damage from a variety of inhaled toxicants due to their large surface area. Moreover, their repair capacity is limited because they have few organelles associated with energy production and macromolecular synthesis.

Type II cells comprise 12–16% of the structural cells in the alveolar region but cover only about 7% of the alveolar surface. They are cuboidal cells with a microvillus surface and unique organelles called lamellar bodies that store surfactant. The major function of type II cells is to secrete surfactant to lower the surface tension in the alveoli, thereby reducing the filling of the alveolar compartment with fluid and alveolar collapse. Type II cells also serve as a progenitor cell for type I cells, which cannot replicate. Therefore, type II cells are critical to alveolar epithelial repair after injury. An interesting third pneumocyte, called the brush cell, is sparsely distributed and appears at alveolar duct bifurcations. Even though this cell type was identified decades ago, essentially nothing is known about its function.

The wall of the alveolus is composed of the alveolar epithelium, a thin layer of collagenous and elastic connective tissue interspersed with fibroblasts (termed the pulmonary interstitium) and a network of capillaries lined by endothelial cells. This distance between the alveolar space and the capillary lumen is known as the air–blood barrier. The air–blood barrier is a multilayered structure approximately 0.4 μm in thickness that consists of an alveolar type I cell, alveolar basement membrane, interstitial space, endothelial basement membrane, and a capillary endothelial cell (Figure 18.5). CO_2 and O_2 are exchanged between air and blood by diffusion across the air–blood barrier. A number of factors determine how rapidly a gas will pass through the respiratory membrane. First, the rate of gas diffusion through the membrane is inversely proportional to the thickness of the membrane. In situations where the thickness of the membrane increases, gas exchange between the air and blood is decreased. For example, edema fluid in the alveolar space results in gases requiring passage not only through the cellular membrane but also through a fluid layer. Another example is thickening of the lung interstitial space between the

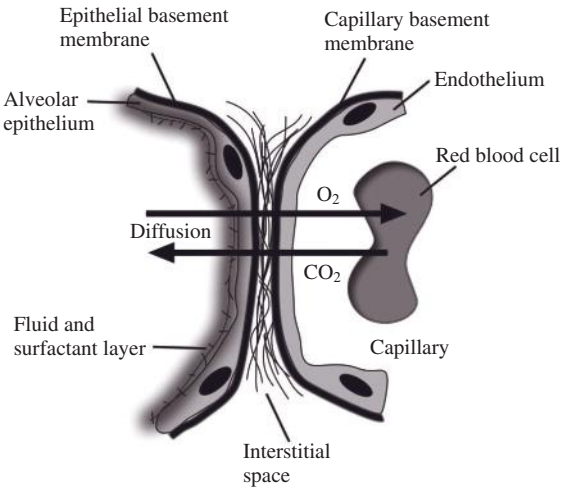

Figure 18.5 Ultrastructure of the alveolar respiratory membrane shown in cross section. Adapted from Guyton, A. C. and J. E. Hall. *Textbook of Medical Physiology*, 10th ed., ed. Philadelphia: Saunders, 2000.

alveolar membrane and the blood capillary membrane during pulmonary fibrogenesis. In general, any factor that increases the thickness of the interstitial space more than two to three times normal can significantly interfere with normal respiratory exchange of gases.

The pulmonary interstitium consists of extracellluar matrix (collagen and elastin) and resident interstitial cells. Although the amount of collagen and elastin in the pulmonary parenchyma is small, these structural proteins are key to normal pulmonary mechanics. Increases or decreases in these proteins lead to impairment, as in pulmonary fibrosis or emphysema. The major resident cell type are fibroblasts, although interstitial macrophages, lymphocytes, plasma cells, and mast cells are also present in the interstitium. Resident interstitial cells comprise about 35% of the structural cells in the alveolar region. However, during an inflammatory response, the relative abundance of these interstitial cells, as well as neutrophils, monocytes, and lymphocytes infiltrating from the blood greatly increase.

Capillary endothelial cells comprise 30–42% of cells in the alveolar region and comprise the walls of the extensive network of blood capillaries in the lung parenchyma. The endothelium forms a continuous, attenuated cell layer that transports respiratory gases, water, and solutes. However, it also forms a barrier to the leakage of excess water and macromolecules into the pulmonary interstitial space. Pulmonary endothelial cells, like type I cells, are vulnerable to injury from inhaled substances and substances in the systemic circulation. Injury to the endothelium results in fluid and protein leakage into the pulmonary interstitium and alveolar spaces, resulting in pulmonary edema.

The lung parenchyma is constantly surveyed by mobile phagocytes, which provide an essential defense against inhaled foreign materials. The most common of these is the alveolar macrophage, which patrol the surface of the alveolar spaces. Their major defense roles are phagocytosis, killing, and clearing of microorganisms, such as bacteria, as well as phagocytosis and clearance of a wide variety of inhaled

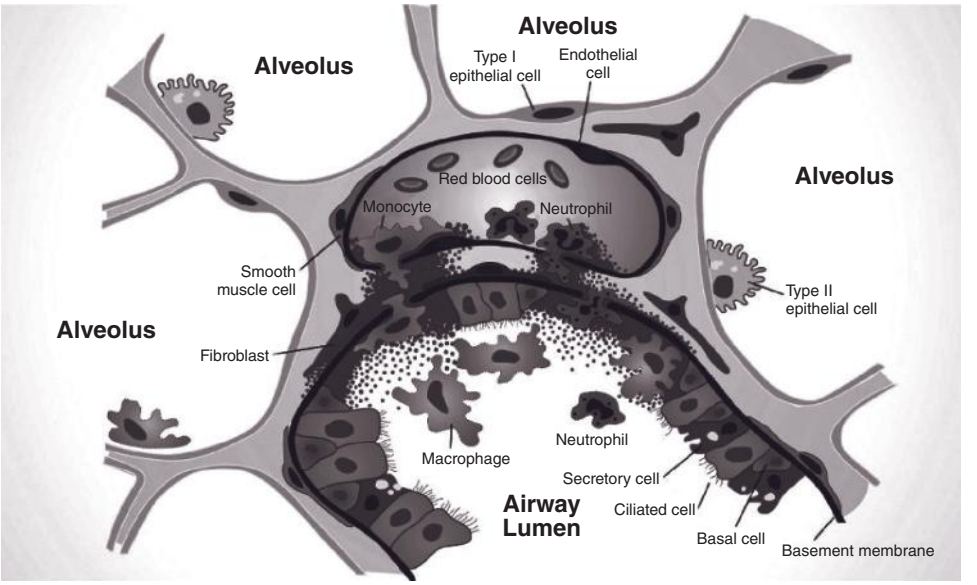

Figure 18.6 Macrophages migrate to a site of injury in the wall of a distal airway. Alveolar macrophages normally residing in the alveolar spaces migrate to chemoattractants released by injured epithelial cells. Other leukocytes such as monocytes and neutrophils also respond to chemotactic molecules and migrate from the blood across the pulmonary interstitium.

particulate matter. After engulfing a foreign particle, clearance may be accomplished via the *mucociliary escalator*. Particle-laden macrophages migrate from the alveolar spaces to the distal airways and are taken up the airway by the unidirectional beating of cilia on the airway epithelium, which moves the macrophages and their cargo up and out of the lungs where they are expelled into the pharynx and swallowed. In addition to the mucociliary escalator, macrophages may also clear engulfed foreign material by migrating into the pulmonary interstitium and into the lymphatics. Phagocytosis triggers the release of cytokines and chemokines, growth factors, proteolytic enzymes, and reactive oxygen species. These mediators recruit and activate other cells that participate either in the resolution of an inflammatory response or structural alterations in lung tissue that lead to a pathological disease outcome. The macrophage population in the lung is replenished by recruitment of bone marrow-derived monocytes via the bloodstream (Figure 18.6). Once in the lung, monocytes proliferate in response to specific growth factors and mature into alveolar macrophages under the direction of other specific cytokines and differentiation factors. Other subpopulations of macrophages that are less recognized are the pulmonary interstitial macrophage that resides beneath the epithelial lining and the intravascular macrophage that is present in humans and some other mammals, but not in rodents.

18.2.5 Circulatory, Lymphatic, and Nervous System of the Lung

The respiratory system communicates with other organ system primarily through direct connections with the circulatory, lymphatic, and nervous systems. The

circulatory system bridges the closely adjacent heart and lungs and the entire cardiac output of the heart enters the lung to be replenished with oxygen. Oxygen-poor blood from the right ventricle travels through the pulmonary arteries to supply capillary beds of the respiratory bronchioles, alveolar ducts, and alveoli where gas is exchanged. Oxygenated blood then returns to the heart via pulmonary venules in the lung parenchyma that merge into pulmonary veins that feed the left atrium. A second arterial system, the bronchial system, supplies oxygenated blood from the left ventricle of the heart through the aorta and the bronchial arteries to the large airways of the lung, the pleura, and large pulmonary vessels.

The *pulmonary lymphatic system* is a vascular network that serves to remove excess fluid from the connective tissue spaces of the lung parenchyma. The lymphatic system is also important in clearing particulate material from the lung to the lymph nodes. The lymphatic system in the lung is divided into superficial and deep portions, but these two portions are connected. The superficial portion is located in the connective tissue of the pleural lining of the lung. The deep portion is in the connective tissue surrounding the bronchovascular tree. The two portions connect in the interlobular septa. The lymphatic vessels are structurally similar to thin-walled veins. The presence of valves in the lymphatic vessels and the movement of the lung during respiration promote the flow of lymph from the periphery and pleura toward the hilus. Afferent lymphatics from the lung drain into the tracheobronchial lymph nodes. Lymph from tracheobronchial and hilar nodes drain into the thoracic, right, and left lymphatic ducts and from these ducts drain into the systemic venous system.

The respiratory tract contains both sensory (afferent) and motor (efferent) innervation. Both the parasympathetic and sympathetic portions of the autonomic *nervous system* provide the motor innervation. Preganglionic parasympathetic fibers descend in the vagus nerves to ganglia located around airways and blood vessels. The postganglionic fibers innervate the smooth muscle of the airways and blood vessels, bronchial glands, and epithelial mucous cells. In general, the same structures are also innervated by postganglionic fibers from the sympathetic ganglia. Vagal stimulation causes airway constriction, dilation of the pulmonary circulation, and increased glandular secretion. Conversely, sympathetic nerve stimulation causes bronchial relaxation, constriction of pulmonary blood vessels, and inhibition of glandular secretion. Sensory receptors that respond to irritants or mechanical stress are located throughout the respiratory tract. Stimulation of these receptors leads to reflex responses such as stimulation of nasal receptors to cause sneezing. Three principal vagal sensory reflexes and their corresponding receptors are known as (1) bronchopulmonary stretch receptors, (2) irritant receptors, and (3) C-fiber receptors. Stretch receptors are associated with smooth muscle of the trachea and bronchi, are stimulated by lung inflation, and normally function to terminate inspiration. Rapidly adapting irritant receptors are located in the epithelium of extrapulmonary and, to a lesser extent, intrapulmonary bronchi. They respond to a variety of stimuli, including inhalation of irritant gases and mechanical stimulation of the airways, to cause bronchoconstriction, cough, and increased mucus secretion. C-fiber receptors are located both in the parenchyma and along conducting airways. Bronchial C-fibers along conducting airways respond to stimuli near the bronchial arterial system and when stimulated cause airway constriction. Pulmonary C-fibers may contribute to the sensation of dyspnea that accompanies pulmonary edema,

pneumonia, and inhalation of noxious gases. Stimulation of both pulmonary and bronchial C-fibers causes a reflex increase in airway secretion.

18.3 TOXICANT-INDUCED LUNG INJURY, REMODELING, AND REPAIR

The respiratory tract is exposed to many environmental factors (particles, gases, infectious microbial agents) and has evolved sophisticated defense and repair systems. The response to injury by foreign agents that enter the respiratory tract involves *host recognition* of the toxic insult followed by *acute inflammation* and then *tissue remodeling* that can result in one of two general outcomes. First, an inflammatory response may resolve and lead to a tissue repair process where normal respiratory architecture and function are restored. Alternatively, an inflammatory response may not resolve but instead may progress to an abnormal tissue remodeling response leading to diseases such as fibrosis and emphysema. This general concept is illustrated in Figure 18.7.

A number of factors determine whether tissue in the lung parenchyma is successfully repaired after injury or whether an inflammatory response progresses to a pathologic outcome. As mentioned previously, the alveolar region is especially vulnerable to damage due to the delicate architecture of the type I epithelium and blood capillary endothelial membranes. An appropriate balance of catabolic and anabolic activity involving cytokines, growth factors, lipid mediators, and proteinases is required for tissue repair. Following injury, damaged cells are triggered to undergo apoptosis by specific cytokine signals (e.g., tumor necrosis factor (TNF)-α) released

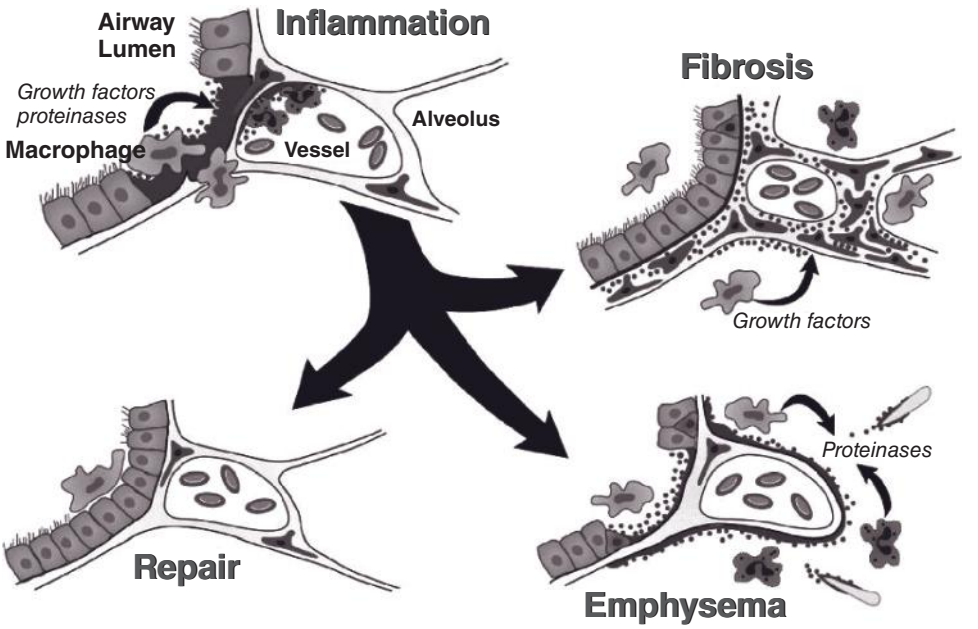

Figure 18.7 Tissue remodeling outcomes following injury and inflammation in the lower respiratory tract that result in repair or disease. (See text for details.)

from phagocytes or the damaged cells themselves release apoptotic factors in an autocrine manner. At the same time, extracellular debris is degraded by specific proteinases (e.g., elastases, collagenases) released by infiltrating neutrophils and mononuclear cells. Resident macrophages then mediate the clearance of the resulting cellular and extracellular debris. The rebuilding process involves a precise balance of growth and differentiation factors to stimulate repopulation of the epithelium and restore connective tissue, vascular tissue, and nerves. An over-exuberant production of growth factors (e.g., transforming growth factor beta (TGF-β), connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF)) may lead to a fibrotic response characterized by increased fibroblasts and collagen. Alternatively, an imbalance in proteinase/anti-proteinase systems may lead to a progressive degradation of structural proteins (e.g., elastin) in the alveolar wall and may cause emphysema.

18.3.1 Oxidative Stress and Lung Injury

There is considerable evidence that links oxidants to the development of a number of human lung diseases. Oxidants can be generated by endogenous mechanisms involving nicotinamide adenine dinucleotide phosphate (NADPH) oxidase systems in phagocytic cells, as well as xanthine oxidase. Lung oxidants can also be increased from exogenous sources, such as inhaled air pollution (gases or particles) and cigarette smoke. The intake of oxygen through the lungs is required for aerobic life, and yet conversion of oxygen to reactive oxygen species (ROS) can have profound detrimental effects on tissues of the respiratory tract. Oxygen is converted to the oxidizing agent superoxide anion ($O_2^{\bullet -}$) by cellular NADPH oxidase systems (principally in phagocytes) and by xanthine oxidase (Figure 18.8). Hydrogen peroxide (H_2O_2) is formed by the further oxidation of $O_2^{\bullet -}$ by superoxide dismutase (SOD):

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Three types of SODs occur: (1) extracellular (EC)-SOD, (2) manganese SOD found in the mitochondria, and (3) copper-zinc SOD found in the cytosol and nucleus. H_2O_2 can be converted to the highly toxic hydroxyl radical (•OH) via the iron-catalyzed Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

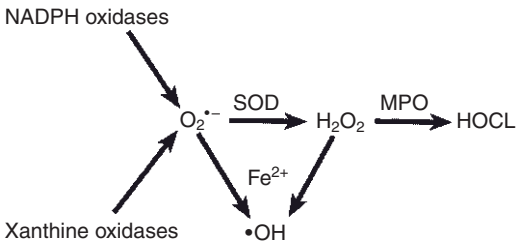

Figure 18.8 Conversion of superoxide anion ($O_2^{\bullet -}$) to hydroxyl radical (•OH) in the presence of iron, or to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), which is converted to hypochlorous acid by myeloperoxidase (MPO).

Moreover, H_2O_2 in the presence of $O_2^{\bullet-}$ and a divalent metal can also produce •OH via the iron-catalyzed Haber–Weiss reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$
$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$$
$$O_2^{\bullet-} + H_2O_2 \rightarrow \bullet OH + OH^-$$

At least three different sources of ROS contribute to oxidative stress in the respiratory system. First, macrophage-mediated phagocytosis involves the release of $O_2^{\bullet-}$, which is primarily generated by the NADPH oxidase system in these cells. This "respiratory burst" likely evolved as a microbial killing mechanism. However, the phagocytosis of a variety of particles and fibers also activate macrophages to undergo a respiratory burst. Some of these agents (e.g., asbestos fibers) are not easily cleared by macrophages and therefore stimulate chronic activation of phagocytes. Second, particles containing metal oxides generate an additional burden of reactive oxygen species via the Fenton and Haber–Weiss reactions shown above. Third, ozone and nitrogen dioxide gases in the environment are reactive species that further contribute to oxidative stress. Finally, cigarette smoke contains a multitude of oxidizing compounds, including nitrogen oxides, quinones, semiquinone radical, and hydroquinone moieties. The combined burden of ROS from these sources results in pulmonary cell damage by lipid peroxidation of the polyunsaturated fatty acids in cell membranes.

While the majority of toxic agents that generate ROS enter via inhalation, some chemicals cause injury via entry through the circulation. Ingestion of the herbicide paraquat causes pulmonary fibrosis by accumulating in the epithelium of the lung and causes oxidant formation as a result of redox cycling (Figure 18.9). The reduction of paraquat (PQ^{2+}) by NADPH is dependent on cytochrome P450 reductase present in the endoplasmic reticulum and mitochondria. The intravenous administration of the chemotherapeutic drug bleomycin also causes lung fibrosis via redox cycling, although it is strictly Fe-dependent. Because bleomycin binds avidly to DNA, much of the site-directed •OH formation leads to DNA damage. Finally, ionizing radiation directly produces •OH without the necessity for a transition metal.

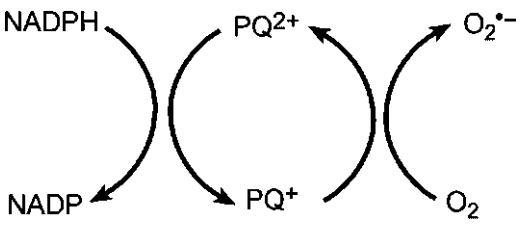

Figure 18.9 Formation of superoxide anion ($O_2^{\bullet-}$) by the herbicide paraquat (PQ^{2+}) via redox cycling.

18.3.2 Antioxidant Mechanisms in the Lung

There are a number of enzymatic and nonenzymatic antioxidant defense mechanisms that counterbalance the deleterious effects of oxidative stress in the lung. Several general concepts have emerged regarding the action of antioxidants. First, there is specificity in the scavenging ability of various antioxidants. Nearly all cells contain a number of enzymatic scavengers, including *superoxide dismutase* (SOD), *catalase*, and *glutathione redox systems* which degrade specific oxidants in specific ways. For example, SOD converts superoxide anion to H_2O_2, whereas catalase specifically degrades H_2O_2. Second, there is usually a selective localization of antioxidants within cells. Manganese SOD is localized in mitochondria, whereas copper-zinc SOD is primarily located in the cytoplasm. Third, antioxidant levels and activities are dynamic. Antioxidants can be inactivated by oxidants. For example, H_2O_2 can inactivate SOD and superoxide anion can inactivate catalase. Moreover, oxidative stress can induce the transcription of genes that encode oxidant-generating systems such as NADPH oxidase or alternatively induce antioxidant enzymes as a protective feedback mechanism. Catalase, SOD, and enzymes of the glutathione redox cycle are primary intracellular antioxidant defense mechanisms that eliminate oxygen radicals and hydroperoxides that may pose a threat to the cell by oxidizing cellular structures. Catalase is located primarily in peroxisomes which contain many of the enzymes that generate H_2O_2 in aerobic cells. Catalase reduces H_2O_2 to water and oxygen. In the lung, catalase is present mainly in type II cells, Clara cells, and macrophages.

The glutathione redox cycle is a central mechanism for reduction of intracellular fatty acid hydroperoxides and complements catalase as a reducing system for H_2O_2. Glutathione metabolism also degrades large molecule lipid peroxides formed by free radical action on polyunsaturated lipid membranes. The key enzyme in the glutathione redox cycle responsible for the reduction of hydroperoxides is glutathione peroxidase. Nonstressed cells contain a high intracellular ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). This high GSH/GSSG ratio ensures availability of GSH for reduction of hydroperoxides via the glutathione redox cycle. Regeneration of GSH requires nicotinamide-adenine dinucleotide phosphate (NADP)-reducing equivalents that are supplied through the glucose-6-phosphate dehydrogenase (G6PD) activity in the hexose monophosphate shunt. NADPH is an important antioxidant molecule as it is the cofactor for the regeneration of GSH from its oxidized form, GSSG. The NADPH, in turn, is regenerated by the enzyme, G6PD as part of the hexose monophosphate shunt pathway of energy metabolism.

18.3.3 Respiratory Tract Injury from Inhaled Particles and Fibers

A variety of naturally occurring and man-made particles and fibers pose a threat to the respiratory tract, including air pollution particulates, allergens such as pollen, soot from the industrial burning of oil, diesel exhaust particles, metal oxides, particles in cigarette smoke, and asbestos fibers. Many of these, including air pollution particulates and cigarette smoke, are complex mixtures of organic and inorganic chemical substances. Several different types of particles and fibers will be discussed below to highlight the heterogeneous nature of particle exposure in environmental or occupational settings.

The inhalation of urban air particles has been associated with increased morbidity and mortality principally due to the physiologic impact on the pulmonary and cardiovascular systems. Adverse respiratory effects in exposed human populations include increased asthmatic episodes and an increase in the prevalence of chronic bronchitis. Moreover, chronic exposure to air pollution particles could contribute to the increasing prevalence of COPD. A mixture of organic and inorganic agents contribute to the composition of air pollution particles, including transition metals released during the burning of petrochemicals, polycyclic aromatic hydrocarbons derived from diesel exhaust, and endotoxins from bacterial sources. Many of these constituents are known to stimulate a variety of intracellular signaling pathways that mediate cellular stress responses leading to the pathologic phenotypes that characterize airway remodeling.

Many of the pathophysiologic effects of inhaled particles are due to oxidative stress. The oxidative potential of air pollution particles is generally attributed to transition metals (e.g., zinc, copper, vanadium, iron) which can induce generation of ROS either via Fenton-like reactions, or by stimulating an oxidative burst in leukocytes that engulf particles via phagocytosis. Particles from a number of sources have been shown to induce oxidant generation that is associated with metal content of the sample.

18.3.4 Particle and Fiber Deposition and Clearance

While chemical composition is important in determining the toxicity of particles and fibers, it is equally or more important to determine where a particle or fiber will deposit in the respiratory tract and how long it will stay there. The mechanism of deposition is determined by the physical (size, shape, and density) and chemical (hygroscopicity and charge) characteristics of the inhaled particles. Particle deposition is also affected by biological factors inherent to the exposed individual such as breathing pattern (volume and rate), route of breathing (mouth vs. nose), and the anatomy of the airways.

Particle deposition in the respiratory tract occurs primarily by three mechanisms: *impaction*, *sedimentation*, and *diffusion*. Impaction and sedimentation depend on the particle's *aerodynamic diameter*. The aerodynamic diameter of a particle is the size parameter of greatest importance for deposition considerations. It is equal to the diameter of a unit-density sphere having the same terminal settling velocity as the particle in question. *Impaction* is the collision of a moving particle with a static structure. It occurs when an inhaled particle has too much momentum to change course with the directional change in airflow and as a result impacts against the airway surface. Most inhaled particles greater than 5 μm in aerodynamic diameter impact on the surfaces of the pharyngeal tracheobronchial regions of the respiratory tract and do not reach the distal lung. *Sedimentation* occurs by the gravitational settling of particles on a respiratory tract surface. Diffusion takes place when a particle reaches an airway surface by random *Brownian movement*. This is an important mechanism for particles with diameters in the nanometer range that reach the distal lung (terminal bronchioles and alveoli) where there is almost no airflow. The combined processes of diffusive and sedimentary deposition are important for particles in the range 0.1–1 μm. Impaction and sedimentation predominate above and diffusion predominates below this range (Figure 18.10). Air pollution particulate

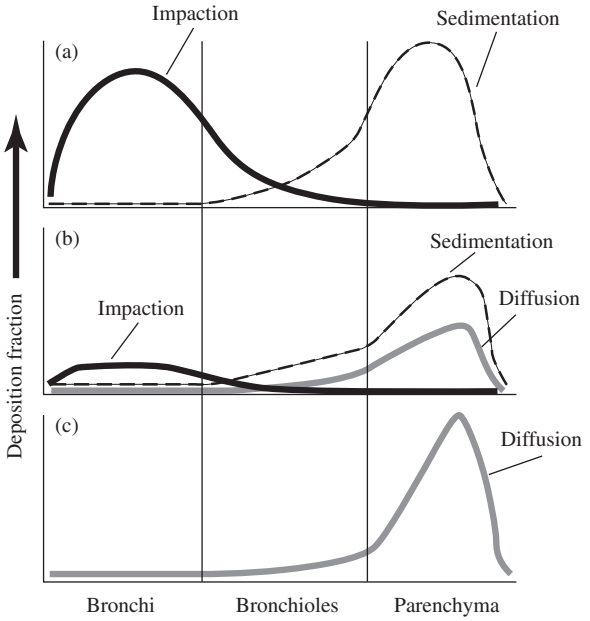

Figure 18.10 The deposition site of particles in the lung depends on particle size. Shown are predicted deposition patterns of (a) 5 μm particles, (b) 1 μm particles, and (c) 0.1 μm particles. Adapted from Bennett, W. D. and J. S Brown. Particulate dosimetry in the respiratory tract. In *Air Pollutants and the Respiratory Tract*, 2nd ed., eds. W. M Foster and D. L. Costa, pp. 21–73. New York: Taylor & Francis, 2005.

matter has been characterized into three categories: (1) ultrafine (<0.1 μm diameter), also referred to as nanosized particulates, (2) fine particles (ranging from 0.1 to 2.5 μm diameter), and course particles (>2.5 μm diameter). Epidemiological studies suggest that ultrafine and fine particles are better correlated with adverse health effects when compared to course particles.

Other mechanisms of deposition are *interception* and *electrostatic charge*. *Interception* is most important for fibers and occurs when an inhaled fiber contacts an airway wall or when long, thin fibers intercept the airway bifurcations. The likelihood of interception is enhanced with increasing fiber length. Such is the case for chrysotile asbestos fibers, which primarily deposit at alveolar duct bifurcations in the distal lung. While spherical particles with a diameter greater than 5 μm do not reach this region of the lung due to impaction in the upper respiratory tract, asbestos fibers with a length exceeding 20 μm can reach the alveolar region of the lung and persist for months or even years due to the inability of macrophages to effectively clear such long, thin structures. Therefore, for particles, decreasing diameter correlates with increasing toxicity because (1) smaller particles in the submicron or nanometer range reach the alveolar region (distal lung), and (2) an equivalent mass of smaller particles have a greater surface area per unit mass and therefore a greater potential to generate ROS and oxidative stress (Figure 18.11). For fibers, increasing

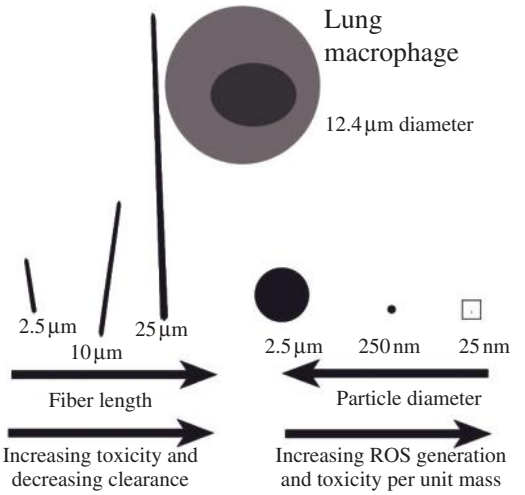

Figure 18.11 Size and shape characteristics of particles that determine deposition, clearance and toxicity in the respiratory tract. In general, smaller particles are more toxic whereas longer fibers are more toxic. A lung macrophage is shown as a size reference. (See text for details.)

length corresponds to greater toxicity given that the fiber is respirable (i.e., one that can be inhaled and be deposited in the alveolar region of the lung) because longer fibers are more difficult to clear from the lung and persist in the interstitial tissues to cause damage.

18.3.5 Respiratory Tract Injury from Gases and Vapors

The respiratory toxicity of gases and vapors is determined by several different physical and *chemical* properties. These factors include (1) chemical *dose*, (2) *water solubility* (hydrophilicity vs. lipophilicity), and (3) *chemical reactivity*.

The *dose* of the toxic substance to which an individual is exposed generally determines severity of injury. For example, the occupational exposure to gases (e.g., chlorine, ammonia, HCl) can be divided into three levels of dose exposure that directly correlate to the degree of airway injury and severity of symptoms. Low exposures to these gases cause sneezing, rhinitis, and sore throat. Repeated low exposures or mild exposure causes persistent cough and bronchitis. Accidental exposure to high concentrations of these gases cause laryngeal edema, acute respiratory distress syndrome, and possibly death. Moreover, high dose exposure to toxic gases and vapors can cause substantial airway epithelial cell damage in the respiratory tract, resulting in a reactive airways dysfunction syndrome (RADS) that is characterized by denuded epithelium and airway luminal fibroproliferative lesions.

The *solubility* of an inhaled substance influences the deposition pattern in the respiratory tract and the site of injury. Gases such as formaldehyde, hydrochloric acid, and sulfur dioxide are taken up by the mucosal surfaces of the upper

TABLE 18.1 Water Solubility, Irritant Classification, and Site of Injury for Selected Highly Reactive Gases

Gas/Vapor	Water Solubility	Irritant Class	Site of Injury
Chlorine	High	Sensory	Nasal, trachea
Formaldehyde	High	Sensory	Nasal, trachea
HCL	High	Sensory	Nasal, trachea
Ammonia	High	Sensory, bronchoconstrictor	Nasal, trachea
Phosgene	Low	Pulmonary	Terminal bronchioles
Ozone	Low	Pulmonary	Terminal bronchioles
Nitrogen dioxide	Low	Pulmonary	Terminal bronchioles
Sulfur dioxide	High	Sensory, bronchoconstrictor	Nasal, trachea

respiratory tract and will exert most of their toxicity in the nasal region. This is because a highly water-soluble chemical will tend to leave the air in the respiratory tract and enter the mucus lining. The relative amount of a chemical in two compartments (e.g., air and mucus lining) at equilibrium is called the *partition coefficient* for that chemical in those two compartments. Gases and vapors with low water solubility such as nitrogen dioxide and ozone tend to deposit farther down the respiratory tract and cause damage to terminal and respiratory bronchioles. Selected water-soluble and insoluble gases are listed in Table 18.1.

The *reactivity* of an inhaled chemical refers to a more unstable conformation (high-energy state) such as formaldehyde that can easily bond with other molecules. Also, chemical reactivity often means that the reactive substance has the ability to generate reactive oxygen or reactive nitrogen species as a consequence of its reaction with other molecules.

A number of gases also cause *irritant responses* in the respiratory tract. These can be classified as *sensory irritants*, *pulmonary irritants*, or *bronchoconstrictors*. Sensory irritants stimulate the trigeminal nerve endings in the upper respiratory tract, leading to a burning sensation in the nose. Examples of sensory irritants are ammonia, acrolein, formaldehyde, and sulfur dioxide. A *pulmonary irritant* is one that stimulates sensory receptors (C-fiber receptors) in the airways, thereby increasing respiratory rate and causing dyspnea (difficulty in breathing) or rapid, shallow breathing. Examples of pulmonary irritants are phosgene, nitrogen dioxide, ozone, and sulfuric acid mist. Bronchoconstrictors stimulate nerve endings in airways to cause contraction of airway smooth muscle, thereby narrowing the airway lumen and increasing resistance to airflow in conducting airways. Examples of bronchoconstrictors are sulfur dioxide and ammonia. In addition to gases and vapors, a number of particles and allergens stimulate bronchoconstriction in individuals with asthma.

18.4 OCCUPATIONAL AND ENVIRONMENTAL LUNG DISEASES

While the respiratory system is well-equipped to defend against exposure to a vast array of toxic substances, the intricate cellular and molecular mechanisms designed

to repair injured lung tissues often fail, resulting in a number of chronic lung diseases, including cancer, fibrosis, asthma, hypersensitivity pneumonitis (HP), and COPD, which is a combination of bronchitis and emphysema.

18.4.1 Pulmonary Fibrosis

Fibrotic interstitial lung diseases are restrictive disorders that involve the proliferation of myofibroblasts in the interstitium, including the alveolar walls and perivascular and peribronchial tissues. These include the chronic progressive disorders such as idiopathic pulmonary fibrosis (fibrotic disorders of unknown etiology) and a number of fibrotic diseases that occur as a result of environmental or occupational exposures, including asbestosis and silicosis. A chronic fibrotic disease caused by exposure to a specific airborne inorganic dust (e.g., asbestos) is often referred to as a *pneumoconiosis*. Fibrosis can also occur following acute lung injury, as in the adult respiratory distress syndrome, where destruction of the epithelium and endothelium destroys the permeability barrier and permits flooding of the airspaces with proteinaceous edema and the infiltration of myofibroblasts. Myofibroblasts may also infiltrate into small airways of the lung, as occurs during the progression of obliterative bronchiolitis following tissue rejection after lung transplantation. Finally, it is becoming increasingly clear that more subtle fibrotic reactions are associated with airway remodeling in diseases such as asthma, chronic bronchitis, and COPD.

18.4.2 Asthma

Asthma is an *obstructive* allergic airways disease that is hallmarked by *acute bronchospasm* of airways called an "asthma attack," but also features *chronic airway inflammation and remodeling* that occurs over a period of years. Both the acute bronchospasm and the chronic airway remodeling process contribute to the obstructive nature of this disease. Asthma is common. Over the past few decades, asthma prevalence has increased and affects up to 10% of the population in developed countries. The reason for the increase in asthma is not entirely clear, but children are primarily affected. Some cases of asthma in children resolve, while some individuals with asthma develop irreversible changes in lung function due to the chronic airway remodeling process. A variety of allergens cause asthma and act as sensitizing agents (Table 18.2). High-molecular weight allergens from plants, animals, bacteria, or mold trigger an IgE-mediated immune response. Most cases of asthma are caused by indoor allergens, particularly components of chitin exoskeleton from house dust mite or cockroaches. Low-molecular weight allergens such as metals, anhydrides, and penicillin generally act as "haptens" (incomplete allergen) and must combine with serum proteins to elicit an allergic response.

Asthma features a chronic airway remodeling response that is characterized by: (1) eosinophilic inflammation, (2) airway smooth muscle thickening, (3) mucus cell hyperplasia and mucus hypersecretion, and (4) subepithelial fibrosis. Allergic diseases, including asthma, are thought to result from a dysregulated immune response to commonly encountered antigens in genetically predisposed individuals. Immunological research into the mechanisms of allergy has identified cytokine production by T-helper 2 (Th2) effector lymphocytes as being critical for

TABLE 18.2 Agents that Cause or Exacerbate Asthma

Agent
Allergens causing asthma
Cockroach
House dust mite
Cat
Plant debris
Endotoxin
Molds
Exacerbation of asthma
Environmental tobacco smoke
Sulfur dioxide
Nitrogen dioxide
Ozone
Particulates

orchestrating allergic inflammation rich in eosinophils. Upon recognition of their cognate antigen, Th2 lymphocytes produce cytokines that regulate IgE synthesis, growth and activation of eosinophils and mast cells, and expression of endothelial cell adhesion molecules. The first step in the allergic immune response is the uptake and presentation of allergen by antigen-presenting cells called dendritic cells. Macrophages and B lymphocytes may also serve as antigen-presenting cells. Following recognition and uptake, dendritic cells migrate to the T-cell-rich area of draining lymph nodes, display an array of antigen-derived peptides on the surface of major histocompatibility complex (MHC) molecules, and acquire the cellular specialization to select and activate naïve antigen-specific T cells. Allergen targeting to the dendritic cells occurs via membrane-bound IgE. Dendritic cells interact with many cell types, including mast cells, epithelial cells, and fibroblasts. Mediators released by these cells can activate the dendritic cells so that it is induced to mature and attract memory Th2 cells through release of Th2-selective chemokines. Mature effector Th2 cells play a central role in asthma pathogenesis by releasing cytokines (e.g., IL-13) that stimulate eosinophil recruitment, smooth muscle cell cytokine and chemokine production, and goblet cell hyperplasia.

18.4.3 Hypersensitivity Pneumonitis (HP)

Hypersensitivity pneumonitis (HP) is an exaggerated adaptive immune response that occurs in susceptible individuals. Unlike asthma, which affects the airways of the lung, HP is a disease of the lower lung (terminal bronchioles and alveoli) caused by the inhalation of allergens that elicit lymphocytic inflammation. The pathogenesis of HP involves three major steps. First, there is an *acute phase* that features lymphocyte and macrophage accumulation and activation. CD8+ cytotoxic lymphocytes and monocytes accumulate widely in the alveolar spaces. After inhalation, soluble antigens bind IgG antibody, and immune complex initiate the complement cascade, and the resulting C5 activates macrophages. Activated resident macrophages in the lung then secrete chemokines that attract circulating

TABLE 18.3 Causative Agents of Hypersensitivity Pneumonitis

Agent	Source	Disease
Microbes		
Thermophilic actinomycetes	Moldy plant materials	Farmer's lung
Saccharopolyspora rectivirgula	Moldy hay	Farmer's lung
Thermoactinomycetes vulgaris	Compost	Mushroom worker's lung
Aspergillus species	Tobacco mold	Tobacco worker's lung
Penicillium chrysogenum	Moldy wood dust	Woodworker's lung
Bacteria and fungi	Metalworking fluids	Machine operator's lung
Animals		
Avian proteins	Bird excreta, feathers	Bird fancier's lung
Animal fur protein	Animal fur	Furrier's lung
Plants		
Soybean	Soybean hulls	Soybean worker's lung
Coffee	Coffee bean dust	Coffee worker's lung
Chemicals		
Isocyanates	Paints, plastics	Paint refinisher's lung
Anhydrides	Plastics	Chemical worker's lung
Pyrethrum	Insecticides	Insecticide lung
Metals		
Cobalt	—	Hard metal lung disease
Beryllium	—	Chronic beryllium disease

Source: Patel et al. *J. Allergy Clin. Immunol.* **108**:661–670, 2001.

T lymphocytes and monocytes. In contrast to IgE-mediated allergic responses (i.e., asthma), the IgG-mediated response in HP does not feature eosinophilic inflammation. Second, a *subacute phase* occurs when the monocytes mature into foamy macrophages and develop into *granulomas* widely dispersed throughout the lung. Third, a *chronic phase* begins as the inflammatory cells produce growth factors such as TGF-β that, over time, stimulate fibroblasts and myofibroblasts to proliferate and deposit collagen to form scar tissue. The end result is interstitial fibrosis. A variety of agents cause HP and many of these agents are encountered occupationally (Table 18.3). Most of the causative allergens have been recognized in a wide variety of occupations. Consequently, once the problem has been identified, the exposure can be controlled and the disease prevented. As a result of this reduction in occupational exposures, the disease is now less common than it was 20 years ago. It is also important to recognize that many existing exposures also occur at home. They are especially associated with pet birds, contaminated humidifiers, and heavy concentrations of indoor molds.

18.4.4 COPD

COPD is a progressive disease that encompasses both *chronic bronchitis* and *emphysema*. The major cause of COPD is cigarette smoking. Chronic bronchitis

is defined clinically as the presence of chronic productive cough for 3 months in each of 2 successive years in a person in whom other causes of cough have been excluded. Emphysema is defined anatomically by enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of alveolar walls. Reduced airflow in COPD is caused by increased resistance to airflow due to inflammation, fibrosis, goblet cell metaplasia, and smooth muscle cell hypertrophy in small airways. A major factor in reducing airway function is loss of elastic recoil due to inflammation and the progressive loss of elastin-dependent attachment of alveoli to bronchioles. The progressive destruction of elastin in the distal lung is the major cause for loss of alveolar walls in emphysema. The loss of elastin is thought to be due to an imbalance between proteinases released from inflammatory cells and anti-proteinases in the lung that normally serve to protect against excessive proteolytic degradation. This is referred to as the *proteinase-anti-proteinase hypothesis* for the development of emphysema. This hypothesis is supported by the following: (1) individuals with a genetic deficiency in α1-antitrypsin (the major inhibitor of neutrophil elastase) are predisposed to the emphysema whether they smoke or not, and (2) emphysema can be induced experimentally in animals by the intratracheal instillation of neutrophil elastase. Moreover, ROS released by cigarette smoke inactivate the inhibitor of elastase (α1-antitrypsin) by oxidizing amino acids within the active site of the enzyme. While elastase appears to be a major proteinase involved in emphysema, other proteinases may be contributory, including cathepsins, matrix metalloproteinases, and collagenases. In addition to α1-antitrypsin, other anti-proteinases include secretory leukoproteinase inhibitor (SLPI), tissue inhibitors of metalloproteinases (TIMPs), and α2-macroglobulin.

18.4.5 Lung Cancer

Lung cancer is the leading cause of death from cancer in the United States. The major risk factor for lung cancer is cigarette smoke. A higher incidence of lung cancer in men versus women is directly correlated with a higher rate of smoking in men versus women. However, an increase in smoking women has led to a doubling in lung cancer incidence in women over the past 20 years, whereas a decline in smoking among men has led to a slight decline in lung cancer incidence in men over the same period. In addition to cigarette smoke, occupational exposures to a variety of agents, including arsenic, asbestos, polycyclic aromatic hydrocarbons, chromium, and nickel are also associated with increased incidence of lung cancer. The major histopathologic types of human lung cancer are squamous cell carcinoma (29%), adenocarcinoma (32%), small cell carcinoma (18%), and large cell carcinoma (9%). Adenocarcinomas and large cell carcinomas tend to occur more in the peripheral lung, whereas squamous cell carcinomas and small cell carcinomas are more likely to occur in the central lung adjacent to large airways. Mesothelioma is an unusual type of lung cancer that develops along the pleural lining of the lung as a result of asbestos exposure. The major histopathologic types of lung cancer found in rats and mice include adenomas, adenocarcinomas, and squamous cell carcinomas. The mouse has proven to be a suitable model for the study of lung cancer progression and most agents that cause cancer in humans also cause cancer in mice (Table 18.4).

TABLE 18.4 Pulmonary Tumor Response of Laboratory Rodents to Inhalation of Known Human Pulmonary Carcinogens

Agent	Human	Rat	Mouse
Chemicals			
Arsenic	+[a]	ND	ND
Asbestos	+	+	±
Beryllium	+	+	±
Chromium	+	±[b]	ND
Coal tar	+	+	+
Mustard gas	+	ND[c]	±
Nickel	+	+	±
Soots	+	+	ND
Vinyl chloride	+	+	+
Environmental agents			
Tobacco smoke	+	+	±
Radon	+	+	—[d]

Source: From Hahn, F. F. Lung carcinogenesis. In *Carcinogenicity*, ed. K. T. Kitchum. New York: Marcel Dekker, 1998.

[a] +, positive; [b] ±, limited data; [c] ND, no data; [d] –, negative.

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SAMPLE QUESTIONS

1. The lung is equipped with unique cellular defenses that operate to remove foreign inhaled materials such as particles and fibers. What are two mechanisms that act coordinately to remove inhaled particles from the lungs?

2. Give two reasons why the lung is a major site of toxicity from environmental exposures.

3. What are the three major factors that determine the toxicity of gases and vapors?

4. An antioxidant enzyme that converts hydrogen peroxide to water and oxygen is: (a) superoxide dismutase, (b) catalase, (c) elastase, (d) all of the above.

5. Which mechanism is most important for the deposition of inhaled particles in the nanometer size range? (a) Impaction, (b) Sedimentation, (c) Diffusion, (d) all of the above.

6. Which of the following lung diseases is caused by repeated exposures to agents such as *thermophilic actinomycetes* or beryllium that result in proliferation of Th1 lymphocytes? (a) Asthma, (b) Hypersensitivity pneumonitis, (c) Cystic fibrosis, (d) Chronic obstructive pulmonary disease.

CHAPTER 19

Immune System*

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19.1 INTRODUCTION

A properly functioning immune system is essential to good health. It defends the body against infectious agents and, in some cases, tumor cells. Individuals with immune deficiencies resulting from genetic defects, diseases (e.g., AIDS, leukemia), or drug therapies are more susceptible to infections and certain types of cancer, the consequences of which can be life-threatening. On the other hand, the immune system may react to foreign substances that would otherwise be relatively innocuous, such as certain chemicals, pollens, or house dust. The resulting allergic reactions can produce an array of pathologies ranging from skin rashes and rhinitis to more life-threatening asthmatic and anaphylactic reactions. A crucial part of immune function is the ability to distinguish endogenous components ("self") from potentially harmful exogenous components ("nonself"). Failure to make this distinction results in autoimmune disease.

Immunotoxicology is the study of undesired effects resulting from the interactions of xenobiotics with the immune system (Figure 19.1). There is evidence that some xenobiotics can cause immune suppression. Xenobiotics can also interact with the immune system to either cause or exacerbate allergic disease. Finally, there is also evidence that xenobiotics have some involvement in autoimmune disease. This chapter provides a brief overview of the immune system, chemicals associated with immune suppression and immune pathologies, and approaches to testing for these effects.

*Disclaimer: This chapter has been reviewed by the National Health and Environmental Effects Research Laboratory and the U.S. Environmental Protection Agency, and has been approved for publication. Approval does not signify that the contents necessarily reflects the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

A Textbook of Modern Toxicology, *Fourth Edition*. Edited by Ernest Hodgson

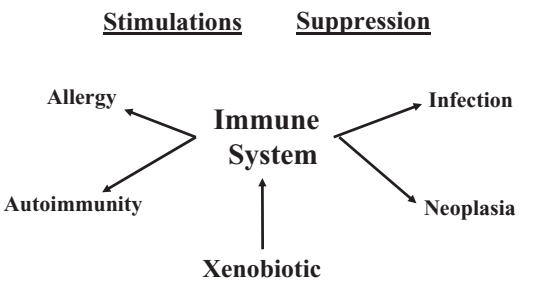

Figure 19.1 Potential consequences of immunotoxicity.

TABLE 19.1 Leukocytes

Granulocytes (Polymorphonuclear Leukocytes)
Neutrophils
Eosinophils
Basophils/mast cells[a]
Mononuclear Leukocytes
Lymphocytes
Monocytes/macrophages[a]
Natural killer cells

[a] Found in blood/more activated form found in tissues.

19.2 THE IMMUNE SYSTEM

Cells of the immune system include several types of leukocytes (white blood cells) (Table 19.1), which are derived from bone marrow. T lymphocytes, a subset of immune cells, undergo differentiation and maturation in the thymus. Leukocytes circulate throughout the body in blood and lymph and populate other lymphoid tissues including the spleen, lymph nodes (scattered throughout the body), tonsils, and adenoids, as well as aggregates of lymphoid tissue in the lung, gut, and skin referred to as bronchus-, gut- and skin-associated lymphoid tissue (BALT, GALT, and SALT). Also, immune cells can be recruited to almost any tissue in the body where there is injury or infection. Accumulation of leukocytes in tissues in response to injury is known as inflammation. Cytokines (e.g., interleukins, interferons, and chemokines), soluble mediators produced by immune cells, as well as cells outside the immune system, control the maturation, differentiation, and mobilization of immune cells. Immune responses are divided into innate responses directed non-specifically against foreign substances, and acquired responses directed against specific antigens. There is considerable interaction between these two types of immunity.

Innate immunity provides a rapid, although usually incomplete, antimicrobial defense and is characterized by inflammation. Granulocytes, natural killer cells, and macrophages are important mediators of innate immunity. Neutrophils (and to a

limited extent eosinophils) have the capacity to phagocytose (engulf) infectious agents or other types of particles and destroy or remove them from the tissue. Granulocytes release a variety of soluble mediators that can kill invading organisms, increase vascular permeability, and recruit more leukocytes to the tissue. Natural killer cells are large granular lymphocytes that nonspecifically kill tumor and virus infected cells. Macrophages are also phagocytic, can release chemotactic and cytotoxic cytokines, and, when activated, can kill tumor or virus-infected cells. Mediators released from all of these cells during the acute inflammatory response influence the development of acquired immune responses.

Although the innate immune system does not recognize specific antigens, proteins known as pattern recognition receptors occur on macrophages, neutrophils, and dendritic cells and recognize features common to many pathogens. When the receptors bind to these highly conserved microbial constituents, a cascade of events is triggered that culminates in phagocytosis, chemotaxis, and production of molecules that influence the initiation and nature of subsequent adaptive immune responses. Toll-like receptors (TLR) are the most well studied of the pattern recognition receptors. Different TLRs recognize pathogen-associated molecular patterns (PAMPs) that are present in many pathogens. For example, TLR4 recognizes bacterial lipopolysaccharide, a component of the outer membrane of gram-negative bacteria, and TLR3 recognizes double-stranded RNA, a component of some viruses. Physical tissue damage, including damage that may occur as a result of exposure to a toxicant, also causes inflammatory responses. The release of nuclear components from cells that have died as a result of tissue trauma also appears to be recognized by certain TLRs.

Acquired immunity *specifically* recognizes foreign substances (called antigens) and *selectively* eliminates them. On re-encountering the same antigen, there is an enhanced response providing protection against reinfection. Vaccination against infectious agents is based on this principle. T lymphocytes and B lymphocytes (T cells and B cells) are the major players in acquired immunity (Figure 19.2). In both cases, there are millions of different clones, groups of immune cells that have specific receptors for a particular antigen. When a cell encounters that specific antigen, clonal expansion occurs; that is, B and T cells with that particular specificity divide and differentiate and are thus activated to respond to the current crisis (e.g., infection). Memory cells also develop that represent an enlarged clone of long-lived cells that are committed to respond rapidly, by clonal expansion, upon reexposure to the same antigen.

B cells recognize native or denatured forms of proteins or carbohydrates in soluble, particulate, or cell-bound form. Activated B cells differentiate into plasma cells and produce antibodies—soluble proteins known as immunoglobulins (Ig)—that circulate freely and react specifically with the invoking antigen. There are several classes (called isotypes) of Ig molecules—IgM, IgG, IgA, IgE, and IgD. IgM is the predominant antibody in the primary immune response (following initial exposure to an antigen). IgG usually appears later following a primary infection but is the predominant antibody in the response to subsequent exposures. IgE acts as a mediator of allergy and parasitic immunity. IgA is found in secretions such as mucous, tears, saliva, and milk, as well as serum, and acts locally to block entrance of pathogens through mucous membranes. IgD is mainly membrane bound on B cells. Little is known about the function of this isotype. It does not appear to have a unique role that affects host immunity.

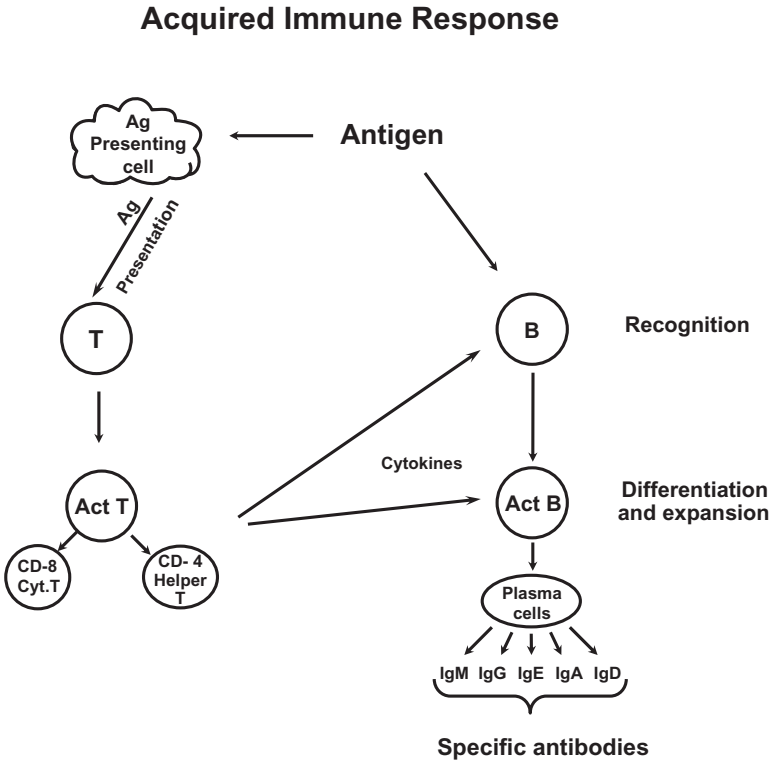

Figure 19.2 The acquired immune response: In response to a specific antigen, there is clonal expansion of B cells and subsequent production of antibodies (Ig) specific for that antigen. Antigen presenting cells process and present antigen to T cells. Again, there is clonal expansion of cells specific for that antigen.

A given B cell will form antibody against just one single antigen; however, during the lifetime of this cell, it can switch to make a different class of antibody. Isotype switching is mediated by T-helper (Th) cells. B cells recognize two types of antigen: T-independent antigens, which activate the cell without T cell help (predominantly an IgM response), and T-dependent antigens, which require T cell help in order to activate B cells. Most antigens belong to this latter category. Antibodies that specifically recognize microbial antigens can, in combination with plasma proteins known as complement, lyse bacterial cells or neutralize virus. Also, microbes complexed with antibody are more readily phagocytized.

T cells recognize antigen that is presented via an antigen-presenting cell (APC), usually a dendritic cell. APCs process and present short peptide fragments complexed with major histocompatibility complex (MHC) molecules on the surface of the APC. This processing and presentation is required for T cell activation. There are two major divisions of T cells that are distinguished by expression of different cell surface markers (CD4 and CD8) and recognize different MHC molecules. CD4 cells, also known as Th cells because they provide help for B cell activation, recognize antigen presented by MHC II. CD8 cells, also known as cytotoxic T cells because they lyse cells expressing specific viral or tumor antigens, recognize antigen

presented by MHC I. There are two major subpopulations of CD4 cells, Th1 and Th2 that are distinguished by the cytokines they produce and the functions they carry out. There are also a smaller number to CD4 cells collectively known as regulatory T cells that have suppressive effects that control or turn off the effector cells when the crisis has passed.

As indicated above, the thymus plays a key role in T cell differentiation. Pre-T cells migrate from the bone marrow to the thymus. As relatively immature cells, T cells express both CD4 and CD8 molecules. As maturation progresses in the thymus, these cells undergo both positive and negative selection. During positive selection, only cells that bind to MHC with a certain affinity survive. As a result of this process, T cells become MHC restricted; that is, they will only respond to antigen presented in association with MHC. Cells that survive positive selection are potentially able to respond to self proteins. However, before T cells leave the thymus, negative selection occurs during which self-reactive cells are removed or are functionally inactivated. During the course of positive and negative selection, CD4+ CD8+ cells downregulate the expression of one of these molecules such that mature T cells express only CD4 or CD8. Mature T cells leave the thymus and populate secondary lymphoid organs.

19.3 IMMUNE SUPPRESSION

Experimental studies in laboratory rodents have demonstrated that a diverse array of chemical exposures suppress immune function (Table 19.2). In addition, a limited

TABLE 19.2 Selected Examples of Immunosuppressive Agents

Drugs
Cyclosporin A, cyclophosphamide, glucocorticoids (Dexamethazone), azothioprine
Metals
Arsenic, lead, cadmium, methylmercury, organotins[a]
Pesticides
Chlorodane[a], DDT[a], dieldrin[a]
Industrial compounds
2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated and polybrominated biphenyls (PCBs and PBBs), benzene, polyaromatic hydrocarbons[a]
Addictive substances
Cocaine, ethanol, opiates, cannabinoids, nicotine
Air pollutants
Environmental tobacco smoke, ozone, nitrogen dioxide
Microbial toxins
Aflatoxin[b], ochratoxin A[b], trichothecenes T-2 toxin[b]
Radiation
Ionizing, UV
Other
Asbestos, diethylstilbestrol (DES), dimethylnitrosamine

[a] Effects in humans are unknown; for all other compound without superscripts, changes have been demonstrated in both rodents and humans.

[b] Effects in humans unknown, but veterinary clinicians have noted immunosuppression in livestock ingesting mycotoxins at levels below those that cause overt toxicity.

TABLE 19.3 Tier I Tests (Screen) for Immune Suppression[a]

Immunopathology	Hematology: complete blood count and differential
	Weights: body, spleen, thymus
	Histology: spleen, thymus, lymph node
Antibody-mediated immunity	IgM plaque-forming cell (PFC) response to T cell-immune dependent antigen, e.g., SRBC, (TDAR)
Cell-mediated immunity	Lymphoproliferative response: T cell mitogens (Concanavalin A and phytohemagglutinin)
	Allogeneic mixed leukocyte response (MLR)
Nonspecific immunity	Natural killer (NK) cell activity

[a] For details on specific assays, see Luster et al. *Fund. Appl. Toxicol.* **10**:2–19, 1988.

number of clinical and epidemiologic studies have reported suppression of immune function (including responses to vaccines) and/or increased frequency of infectious and/or neoplastic disease following exposure of humans to some of these agents. From the above description, it is clear there are a number of cellular and molecular targets for chemicals that act as immunosuppressants. Clearly, a chemical that disrupts cell proliferation would impact clonal expansion. Disruption of T cell maturation in the thymus is another potential mechanism for immune suppression. Chemicals may also interfere with receptor ligand binding at the cell surface and/or the cascade of signals that lead to transcription of genes responsible for generating and regulating the appropriate immune responses.

Because of the complexity of the immune system, tiered approaches to testing chemicals for immunosuppressive potential have been developed. Like other types of toxicity testing, the first level of the tier (Table 19.3) frequently relies solely on structural end points, including changes in the weight of thymus and other lymphoid organs, histopathology of these organs, or differential blood cell counts. This type of evaluation is convenient because it can be carried out along with an evaluation for other organ systems during routine toxicity testing using one set of animals. However, although these nonfunctional end points may be effective in identifying gross (high dose) immunotoxic effects, they are not very accurate in predicting changes in immune function or alterations in susceptibility to challenge with infectious agents or tumor cells at lower chemical doses. Hence, the first testing tier (Table 19.3) often includes functional end points designed to assess (1) antibody-mediated responses, (2) T cell-mediated responses, and (3) NK cell activity. The most commonly used immune function assay in laboratory animals assesses the ability of a mouse or rat to respond to challenge with an antigen, usually sheep red blood cells (SRBC) (Figure 19.3). The response is assessed by determining the number of antigen-specific antibody (IgM)-forming cells (AFC) in the spleen (Jerne assay) or by assessing antigen-specific antibodies in serum using an enzyme-linked immunosorbent assay (ELISA). Because the SRBC is a T-dependent antigen, these assays are often referred to as T-dependent antibody response (TDAR) assays. Both T and B cells, as well as antigen presenting cells, must be functional to have a successful immunization. Suppression of this response is highly predictive of suppression of other immune function tests and also correlates well with tests that assess resistance to challenge with an infectious agent or tumor cells. The disadvan-

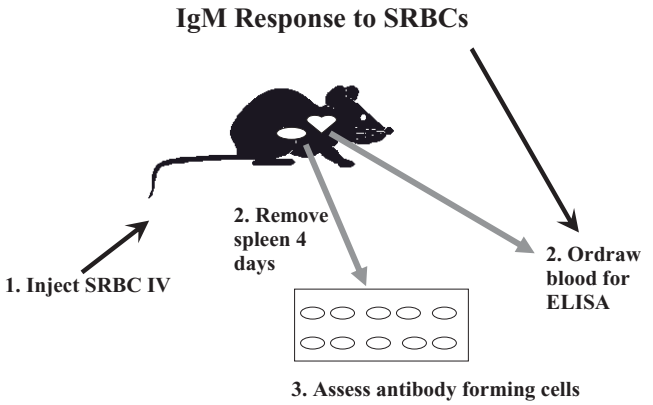

Figure 19.3 Assessing chemicals for immunosuppressive effects: The most common approach to accomplish this goal is to inject chemical and vehicle-treated mice or rats with antigen and assess the antibody response. Most often, the antigen injected is sheep red blood cells (SRBC); 4 days later, slides are made with a single cell suspension of spleen cells, sheep red blood cells, and complement immobilized in agar. Slides are incubated and spleen cells making antibody against SRBC lyse the surrounding RBCs generating plaques. Plaques are counted to determine the number of antibody-forming cells. Alternatively, serum can be obtained and an ELISA assay performed to detect SRBC specific antibody.

TABLE 19.4 Tier II More In-Depth Evaluation of Immunosuppressive Chemicals[a]

Immunopathology	Quantitation of B and T cell numbers using flow cytometry
Antibody-mediated immunity	IgG PFC to SRBC
	IgM PFC to T cell-independent antigen (e.g., TNP-LPS)
Cell-mediated immunity	Cytotoxic T lymphocyte (CTL) cytolysis
	Delayed hypersensitivity response (DHR)
Nonspecific immunity	Macrophage: phagocytosis, bactericidal/tumoricidal activity)
	Neutrophil: function (phagocytosis and bactericidal activity)
Host resistance models	Response to challenge with infectious agent or tumor cells

[a] For details on specific assays, see Luster et al. *Fund. Appl. Toxicol.* **10**:2–19, 1988.

tage to this test is that it usually requires a dedicated set of animals because of the antigen challenge. The most common approach has been to treat the animals for 14–28 days with the xenobiotic of interest, inject the antigen at the end of that exposure, and collect spleen or serum 4–5 days later. Unlike the tests for antibody-mediated immunity, tier 1 tests for cell-mediated immunity and natural killer cell activity can be done *ex vivo* and do not require a dedicated set of animals. However, these tests focus on one cell type and are not as predictive of overall immunocompetence as the TDAR.

When immunosuppressive effects are noted in tier 1, an in depth evaluation using more sophisticated tests may be carried out (tier 2, Table 19.4). This might include

enumeration of lymphocyte subsets (B cells, total T cells, and CD4+ and CD8+ T cells) using flow cytometry or assessment of the IgM response to a T-independent antigen in an effort to determine what portion of the immune response is the actual target. Unlike tier 1, tests of cell-mediated immunity in tier 2 require administration of an antigen and subsequent test for cytotoxic T cells (e.g., against an immunizing tumor cell) or a delayed type hypersensitivity response (similar to the response to a tuberculin test). In order to understand the mechanisms underlying immune suppression, a host of other tests can be carried out, including expression of an assortment of cytokines.

Tier 2 also include host resistance models, tests in which an animal is exposed to a xenobiotic and then challenged with an infectious agent or tumor cells. This is considered the ultimate test for an adverse effect on the immune system. However, it should be noted that the amount of immune suppression that can be tolerated is greatly dependent on the dose and virulence of the challenging agent, as well as the genetics of the host. Manipulation of these variables can affect greatly results obtained in host resistance tests.

The tiered approach assesses systemic effects on immune responses. For inhaled compounds and dermally applied compounds, it is also important to consider effects on local immune responses in the lung and skin, respectively. For example, the alveolar macrophage provides the first line of defense against inhaled microbes, and a number of air pollutants suppress this response leading to increased risk of respiratory infections that would not necessarily be detected using the classic tiered approach. Ultraviolet radiation and some chemicals suppress local responses in the skin and or cause the release of mediators from the skin that affect cell-mediated but not antibody-mediated responses.

As in animal studies, human clinical data obtained from routine hematology (differential cell counts) and clinical chemistry (serum immunoglobulin levels) may provide general information on the status of the immune system in humans. However, as with the animal studies, these may not be as sensitive nor as informative as assays that target specific components of the immune system and/or assess function. The assessment of certain lymphocyte surface antigens has been successfully used in the clinic to detect and monitor the progression or regression of leukemias, lymphomas, and HIV infections, all diseases associated with severe immunosuppression. However, there is considerable variability in the "normal" human population, such that the clinical significance of slight to moderate quantitative changes in the numbers of immune cell populations is difficult to interpret. There is consensus within the immunotoxicology community that tests that measure the response to an actual antigen challenge are likely to be more reliable predictors of immunotoxicity than flow cytometric assays for cell surface markers because the latter generally only assesses the state of the immune system at rest. For ethical reasons, it is not possible to immunize humans with SRBC. One approach is to assess responses to vaccines in chemically exposed populations. This approach has been used successfully to demonstrate a link between mild, stress-induced suppression of the antibody response to influenza vaccine and enhanced risk of infectious disease. There are also a limited number of studies where this approach has been used to demonstrate immune suppression in populations exposed to toxic chemicals. Most notably, a semiquantitative relationship between polychlorinated biphenyls (PCBs) exposure and suppressed responses to diphtheria and tetanus toxoid

was demonstrated in a population of children exposed *in utero* and postnatally. Increased susceptibility to infection has also been demonstrated in children exposed to PCBs.

There is some debate over how to interpret immunotoxicity data with respect to adversity. The most conservative interpretation is that any significant suppression of an immune response is adverse because a linear relationship between immune suppression and susceptibility is assumed. Supporting this notion is the fact that apparently immunocompetent individuals suffer from infections, suggesting that adverse effects can occur even when known immune suppression is zero. Others argue that there is clearly redundancy and reserve capacity in the immune response and that some suppression should be tolerable. It is impossible to establish a quantitative relationship between immune suppression and increased risk of infection because both the genetics of the host and the virulence and dose of the infectious agent will influence this relationship. Immunocompetence in a population can probably be represented as a bell-shaped curve, such that a portion of the population is highly susceptible to infection, a portion is highly resistant, and the remaining population falls somewhere in between (Figure 19.4). Genetics, age, nutritional status, and preexisting disease all contribute to the risk represented by this curve. In addition, the portion of the population at risk is determined by the dose and virulence of any infectious agent that might be encountered. The higher the dose and the virulence, the more people are at risk. Exposure to an immunosuppressive agent shifts the whole bell-shaped curve to the left, thus increasing the population at risk. Unfortunately, it is difficult to determine more quantitatively the relationship between small decrements in immune responsiveness and the degree of change in the population at risk.

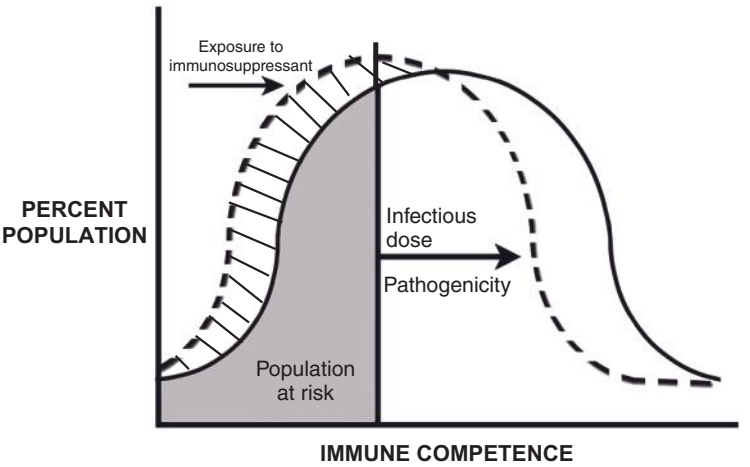

Figure 19.4 Adverse effect of immune suppression: Immune competence is represented by a bell-shaped curve. The shaded area represents the population at risk of infection, which increases or decreases depending on the dose and virulence of infectious agents that are encountered. Exposure to an immunosuppressant shifts the whole curve to the left, such that a larger population is at risk for any given infectious challenge.

19.4 CLASSIFICATION OF IMMUNE-MEDIATED INJURY (HYPERSENSITIVITY)

Under certain circumstances, immune responses can produce tissue damage. These deleterious reactions are collectively known as hypersensitivity or allergy. Hypersensitivity reactions have been divided into four types (originally proposed by Gell and Coombs) based on mechanism (Table 19.5). In all cases, the adverse effects of hypersensitivity develop in two stages: (1) Induction (sensitization) requires a sufficient or cumulative exposure dose of the sensitizing agent to induce immune responses that cause no obvious symptoms. (2) Elicitation occurs in sensitized individuals upon subsequent exposure to the antigen and results in adverse antigen-specific responses that include inflammation.

Type I hypersensitivity (sometimes referred to as atopy) is mediated by antigen-specific cytophilic antibody (usually IgE) that binds to mast cells and basophils. On subsequent exposure, the allergen binds to these cell-bound antibodies and cross-links IgE molecules, causing the release of mediators such as histamine and slow-reacting substance of anaphylaxis (SRS-A). These mediators cause vasodilation and leakage of fluid into the tissues, plus sensory nerve stimulation (leading to itching,

TABLE 19.5 Classification of Hypersensitivity Reactions

Type	Mechanisms		Example
	Induction (Initial Exposure to Antigen)	Elicitation (Re-Exposure to Antigen)	
I (immediate)	Clonal expansion of B cells; Cytophilic antibody (IgE) generated; binds to mast cells	Antigen binds to cell-bound antibody, cross-links receptors, causing release of mediators	Anaphylactic response to bee sting or food allergen
II (cytolytic)	Clonal expansion of B cells; IgM, IgG generated. Antigen binds to cell membrane	Anamnestic[a] Ig binds to cell-bound antigen, and in the presence of complement or activated macrophages, cell lysis occurs	Rh factor incompatability, Hemolytic anemia in reaction to drug treatment
III (Arthus)	Clonal expansion of B cells; IgM, IgG generated	Anamnestic Ig response; antigen antibody complexes form in some tissues leading to inflammation	Glomerular nephritis, Bacterial endocarditis, Farmer's lung
IV (delayed)	Clonal expansion of antigen-specific T cells occurs	T cells activated, release cytokines, activate macrophages, inflammation	Contact dermatitis

[a] Heightened response on re-exposure to antigen.

sneezing, and cough). Type I is also called immediate-type hypersensitivity because reactions occur within minutes after exposure of a previously sensitized individual to the offending antigen. Type I reactions include immediate asthmatic responses to allergen, allergic rhinitis (hay fever), atopic dermatitis (eczema), and acute urticaria (hives). The most severe form is systemic anaphylaxis (e.g., in response to a bee sting), which results in anaphylactic shock and, potentially, death.

Type II hypersensitivity is the result of antibody-mediated cytotoxicity that occurs when a foreign antigen or hapten is incorporated into the cell membrane and antibodies respond to this cell surface antigens. Antibodies bound to antigen on the cell surface activate the complement system and/or macrophages leading to lysis of the target cell. Frequently, blood cells are the targets, as in the case of an incompatible blood transfusion or Rh blood incompatibility between mother and child. The basement membrane of the kidney or lung may also be a target. Autoimmune diseases can result from drug treatments with penicillin, quinidine, quinine, or acetaminophen. Apparently, these drugs interact with the cell membrane such that the immune system detects "foreign" antigens on the cell surface. This type of autoimmune disease may also have unknown etiologies.

Type III reactions are the result of antigen–antibody (IgG) complexes that accumulate in tissues or the circulation, activate macrophages and the complement system, and trigger the influx of granulocytes and lymphocytes (inflammation). This is sometimes referred to as the Arthus reaction and includes postinfection sequelae, such as bacterial endocarditis. Another example, serum sickness, occurs when poorly catabolized foreign antigens are injected in large quantities, which can occur in response to antivenom treatment for snake bites or following treatments that involve monoclonal antibodies. Arthus reactions can also occur when inhaled antigens provoke an IgG rather than an IgE response. An example is Farmer's lung, a pneumonitis caused by molds, which appears to involve both type III and type IV responses. Unlike the preceding three types, Type IV or delayed-type hypersensitivity (DTH), involves T cells and macrophages, not antibodies. Activated T cells release cytokines that cause accumulation and activation of macrophages that in turn cause local damage. This type of reaction is very important in defense against intracellular infections such as tuberculosis, but is also responsible for contact hypersensitivity responses (allergic contact dermatitis) such as the response to poison ivy. Inhalation of beryllium can result in a range of pathologies including acute pneumonitis, tracheobronchitis, and chronic beryllium disease, all of which appear to be due to type IV beryllium-specific immune responses. The expression of type IV responses following challenge is delayed, occurring 24–48 h after exposure. Type IV responses can be further subdivided into three groups based on knowledge of T cell biology obtained since Gel and Coombs devised their classification. CD4 Th1 cells respond to soluble antigens presented by MHC II and activate macrophages to produce the classic tuberculin type reaction. CD4 Th2 cells also respond to soluble antigens presented by MHC II but activate eosinophils leading to inflammation typically seen in allergy and asthma. CD8 cells respond to cell-associated antigens presented by MHC I and are directly cytotoxic.

The different types of immune mediated injury are not mutually exclusive. More than one hypersensitivity mechanism may be involved in the response to a particular antigen. Also, the resulting pathology, particularly that caused by type III and IV reactions, may appear very similar although the mechanisms leading to the effect are different.

19.5 EFFECTS OF CHEMICALS ON ALLERGIC DISEASE

Xenobiotics can affect allergic disease in one of two ways. They can themselves act as antigens and elicit hypersensitivity responses, or they can enhance the development or expression of allergic responses to commonly encountered allergens, such as dust mite. Chemicals that act as allergens include certain proteins that can by themselves induce an immune response and low molecular weight chemicals (known as haptens) that are too small to induce a specific immune response, but may react with a protein to induce an immune response that is then hapten specific. Haptens have been associated with both allergic contact dermatitis (ACD), sometimes called contact hypersensitivity (CHS), and respiratory hypersensitivity. Systemic hypersensitivity, the most extreme manifestation of which is anaphylaxis, can also occur in response to low molecular weight compounds, most notably drugs. Proteins have been associated with respiratory hypersensitivity and systemic hypersensitivity responses such as food allergy. When a chemical is an allergen or a hapten, there are two doses of concern, the sensitizing dose and the elicitation dose. In general, the dose required for sensitization is greater than that required to elicit a response in a sensitized individual. However, the two are not completely independent of one another. When the sensitizing dose is high, the elicitation dose may be lower than when the sensitizing dose is low. Chemicals that enhance the development of allergic sensitization are referred to as adjuvants. Air pollutants have been associated both with enhanced sensitization and exacerbation of allergic respiratory symptoms.

19.5.1 ACD

ACD or CHS is one of the most common occupational health problems and hence is one of the most common problems associated with immunotoxicity. It is a type IV response that occurs as a result of dermal exposure to chemicals that are haptens. Following dermal exposure, the chemical reacts with host cell protein at the surface of the skin and is picked up by epidermal dendritic cells known as Langerhans cells. Cytokines released from the epidermal keratinocytes and from Langerhans cells cause maturation and mobilization of the Langerhans cells, which travel to the draining local lymph node and presents antigen to lymphocytes. Clonal expansion occurs enlarging the number of T lymphocytes specific for that allergen and generating memory cells that in addition to specificity for the allergen have the propensity to home to the skin. On reexposure to the chemical, these specific T cells are activated, proliferate, home rapidly to the site of exposure, and produce erythema and edema typical of a type IV response. This elicitation response is mediated by Th1 cells which produce various cytokines that contribute to the inflammatory response, CD8 cells which are directly cytotoxic to keratinocytes, and regulatory cells that serve to control the response. The reaction to poison ivy is the classic example.

Methods to assess chemicals (including drugs, pesticides, dyes, cosmetics, and household products) for potential to induce CHS are well established, and several protocols using guinea pigs have been in use since the 1930s. These protocols assess the actual disease end point, skin erythema and edema, following sensitization and challenge with the test agent. Two commonly used tests are the guinea pig maximization test and the Buehler occluded patch test. The sensitization procedure for the

maximization tests includes intradermal injection of the test chemical with an adjuvant (intended to enhance the sensitization process) as well as topical application. The Buehler test relies on topical sensitization alone. In both cases, after approximately 2 weeks, animals are challenged at a different site on the skin and erythema and edema are assessed 24–48 h later. This assessment is somewhat subjective, and these tests are fairly expensive. A chemical is considered to be a sensitizer if 30% (maximization) or 15% (Buehler) of the animals respond. Recently, a more economical, less subjective, test for CHS has been developed using mice. This test, the local lymph node assay (LLNA), assesses the proliferative response of lymphocytes in the draining lymph node following application of the agent to the ear and is based on our understanding of the immunologic mechanisms underlying CHS; that is, clonal expansion has to occur in the draining lymph node if there is to be allergic sensitization (Figure 19.5). In most cases, the LLNA is accepted as a stand-alone alternative to the guinea pig tests and is generally the assay of choice. Finally, structure activity approaches have recently been developed to identify contact sensitizers. This approach is based on the concept that the biologic mechanisms that determine a chemical's effect are related to its structure and hence, chemicals with similar structures will have similar effects. Computer models have been developed to compare the structure of an unknown chemical to structures in a database for known contact sensitizers. CHS lends itself to this approach because there is a large database of chemicals known to cause it, and there is a reasonable understanding of chemical characteristics that facilitate skin penetration, chemical reactivity with host proteins, and immune reactivity.

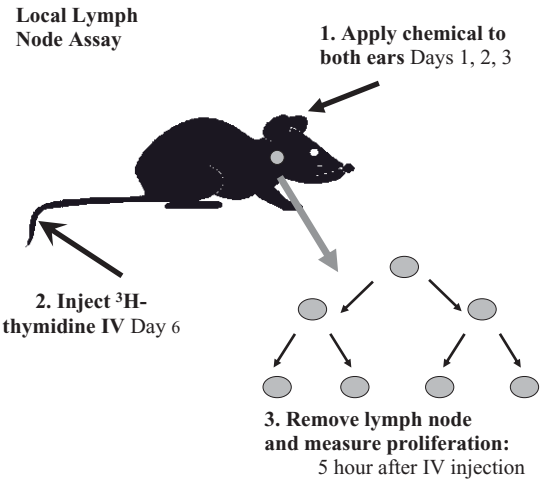

Figure 19.5 Assessing chemicals for potential contact sensitivity: In the local lymph node assay, the chemical in question is applied to both ears on three consecutive days. Control mice are treated with vehicle. Radioisotope is injected intravenously on day 6. The draining lymph nodes are removed 5 h later, and the proliferative response is measured by the incorporation of radio isotope. Results are frequently presented as a stimulation index (counts per minute [cpm] for the test chemical/cpm for control). Adapted from Sailstad, D. *Lab. Anim.* **31**:36, 2002.

Because nonspecific inflammatory responses also can occur following chemical exposure to the skin, a distinction must be made between an irritant and a sensitizer. An irritant is an agent that causes local inflammatory effects but induces no immunological memory. Therefore, on subsequent exposures, local inflammation will again result, but there is no enhancement of the magnitude of the response and no change in the dose required to induce the response. In immunologically mediated inflammation (hypersensitivity), there may be no response to a sensitizer during the induction stage, but responses to subsequent exposures are exacerbated. The dose required for elicitation is usually less than that required to achieve sensitization.

19.5.2 Respiratory Allergens

There is evidence that both occupational and environmental exposures to chemicals (both proteins and haptens) can result in the induction or exacerbation of respiratory allergies (Table 19.6). Of particular concern is the induction of allergic asthma. In sensitized asthmatic individuals, antigen challenge generally causes a type I (IgE-mediated) immediate hypersensitivity response with release of mediators responsible for bronchoconstriction. Between 2 and 8 h after the immediate response, asthmatics experience a more severe and prolonged (late phase) reaction that is characterized by mucus hypersecretion, bronchoconstriction, airway hyperresponsiveness to a variety of nonspecific stimuli (e.g., histamine, methacholine), and airway inflammation characterized by eosinophils. This later response is not mediated by IgE.

TABLE 19.6 Example of Chemicals Associated with Respiratory Allergy

Proteins
Enzymes
Latex
Animal dander
Dust mite
Molds
Cockroach
Microbial pesticides
Low molecular weight (<3000)-haptens
Toluene diisocyanate
Diphenylmethane diisocyanate
Phthalic anhydride
Trimellitic anhydride
Platinum salts
Reactive dyes
Adjuvants
Ozone
Nitrogen dioxide
Diesel exhaust
Residual oil fly ash

Although proteins are generally immunogens, not all proteins are allergens, and there is a range of potencies for those that are. There is also a strong genetic component associated with susceptibility to develop allergic reactions to proteins. Susceptible individuals are called atopic. There is at present no structural motif that can be used to characterize a protein as an allergen for hazard identification. Examples of occupational protein exposures associated with respiratory allergy and asthma include enzymes, latex, flour (both the grain itself and fungal contaminants), and animal dander. Environmental (mostly indoor) exposures including molds, spores, dust mite, animal dander, and cockroach have also been associated with this type of respiratory disease. Because this is a type I response, cytophilic antibodies (IgE) specific for the allergen are frequently used to identify proteins that may cause this effect. For example, in order to determine the etiology of occupational asthma in human subjects, the skin prick test is often used. Different proteins are injected under the skin to test for the presence of cytophilic antibodies in order to identify which proteins are causing a response in an individual. Serum may also be tested for protein-specific IgE. Because IgE can sometimes be detected in the absence of respiratory responses, a positive IgE test may be followed by an assessment of respiratory responses. Under very controlled situations, patients may be exposed via the respiratory route to suspect allergens (bronchoprovocation test) and respiratory function monitored to pinpoint the offending allergen. Guinea pigs and mice have been used to test proteins for potential allergenicity. Animals are usually sensitized by the respiratory route and monitored for the development of cytophilic antibody (IgG1 in guinea pigs; IgE in mice) as well as increased respiratory rate and other changes in pulmonary function. The guinea pig intratracheal test has been used to establish the relative potency of different detergent enzymes and establish safe occupational exposure levels. As the name implies, guinea pigs are sensitized by intratracheal exposure and induction of cytophilic antibodies are assessed. Dose responses obtained for new enzymes are compared to a reference enzyme for which safe exposure levels have been established. The relative potency of the new enzyme to this reference can be used to establish a safe exposure level for the new enzyme.

Exposure to certain low (<3000) molecular weight compounds (haptens) has also been associated with the development of occupational asthma. Highly reactive compounds such as the diisocyanates or acid anhydrides have the capacity to react with protein and induce an immune response. Toluene diisocyanate (TDI) and trimellitic anhydride are the compounds that have been most extensively studied in this regard. There is a great deal of interest in developing a test to screen chemicals for this type of effect in order to avoid induction of immune responses that could lead to occupational asthma. Although specific IgE antibodies have been detected in some individuals with TDI asthma, it has not been uniformly present, and some of these individuals exhibit the late phase but not the immediate response. Hence, unlike proteins, there is less certainty about the mechanisms underlying respiratory allergic responses to low molecular weight compounds. Structure activity approaches similar to those described for contact sensitizers have been developed, but this approach has limitations because the database of known respiratory sensitizers is small compared to contact sensitizers, and the underlying mechanisms are less well defined. At the other extreme, guinea pigs have been exposed by inhalation for a number of days, rested, and then challenged at a later date by inhalation with subsequent monitoring of respiratory responses. Although this approach has produced

a good model of TDI asthma, it is too cumbersome and expensive for routine testing. Because the capacity to interact with protein is a prerequisite to allergenicity, it has been suggested that testing for protein reactivity *in vitro* could provide an initial screening test for chemicals. Also, because it appears that respiratory sensitizers are a subset of chemicals that produce positive results in a contact sensitivity test, it has been suggested that the LLNA test be used as the first tier in screening chemicals for this effect. The problem then becomes separating chemicals that are strictly contact sensitizers from those that have the capacity to cause respiratory sensitization. Efforts have been made to determine whether differences in responses to dermal application of these chemicals could provide a means for making this distinction. One proposal is to assess total serum IgE following dermal exposure, assuming that respiratory sensitizers would produce a bigger IgE signal. Another approach has been to assess cytokine profiles in the draining lymph node following dermal exposure. Different subsets of Th cells have been associated with type I immediate (Th2) and type IV delayed (Th1) responses. These different populations of T cells are distinguished by different cytokine profile, and efforts are underway to use these differing profiles to distinguish respiratory from contact sensitizers. However, there is as yet no well-validated, well-accepted test to assess low molecular weight chemicals for the capacity to induce respiratory allergy. This remains a subject of research.

19.5.3 Adjuvants

An adjuvant is a compound administered in conjunction with an antigen that nonspecifically enhances the immune response to that antigen. Adjuvants are used in vaccines to promote immunogenicity. There is now growing concern that chemicals in our environment (particularly air pollutants) might act as adjuvants for allergic sensitization to common allergens such as dust mite and pollen. Laboratory rodents have been used to show that nitrogen dioxide, residual oil fly ash, and diesel exhaust enhance allergic sensitization and disease. Enhanced sensitization to an allergen has also been demonstrated in rhesus monkeys exposed to ozone and humans exposed to diesel exhaust. The significance of these findings in terms of enhanced burden of respiratory allergies in the human population is unclear. As in other areas of toxicology, simultaneous environmental exposures to agents that are not the agent of immediate concern can certainly influence outcomes. Adjuvancy is a concern that likely extends beyond air pollution and type I responses.

19.5.4 Systemic Hypersensitivity

All allergic responses are systemic in that sensitized immune cells can circulate throughout the body and can respond when challenge occurs at sites other than the original site of sensitization. However, for the allergic diseases described above, the response to challenge is usually localized around the site of challenge. Food allergy is an example of a more systemic response. IgE-mediated food allergies can cause symptoms in the skin, the upper and lower respiratory tract, as well as the gastrointestinal tract. Food allergens have been reported to be one of the leading causes of systemic anaphylaxis seen in emergency departments. Hymenoptera stings and administered drugs are the other common causes of anaphylactic reactions seen

in medical facilities. Immune-related problems are the largest single area of adverse events that are not detected by preclinical testing of drugs. Many of these events are dermal reactions associated with systemic administration of drugs although multiorgan reactions are a more worrisome occurrence. Good methods for assessing the potential for drugs to elicit such responses are not available.

Toxicologists have recently been drawn into the area of food allergy by advances in biotechnology and the need to assess the safety of genetically modified foods in terms of potential allergenicity. There is concern that insertion of a novel gene into a food crop (e.g., to increase yield or pest resistance) might inadvertently introduce a new allergen into the food supply. Food allergies are relatively rare, affecting approximately 5% of children and 2–3% of adults, and even in these individuals, most proteins are not allergens. However, when food allergy does occur, the consequences can be severe. Anaphylactic (life-threatening) reactions to peanuts provide the best example. Unfortunately, the mechanisms underlying food allergies and oral tolerance which protects most of people from developing reactions to the foreign proteins they eat are poorly understood. Also, the characteristics that make a protein a food allergen, and the characteristics that make an individual susceptible to food allergies are unclear. These are some of the issues that need to be resolved in order to develop appropriate safety assessment tools.

19.6 OTHER ISSUES: AUTOIMMUNITY AND THE DEVELOPING IMMUNE SYSTEM

Autoimmune diseases result from a breakdown of immunological tolerance leading to immune responses against self-molecules that involve activation of both innate and adaptive immune responses. Autoimmune disorders can affect virtually any site in the body, and present as a spectrum of diseases. Autoimmune diseases affect about 3% of the population and comprise a diverse array of both organ-specific (e.g., type I diabetes, thyroiditis) and systemic (systemic lupus erythematosus) diseases. Epidemiologic studies suggest associations with specific genetic loci, and environmental factors, including exposures to certain drugs, chemicals, and infectious agents. In many cases, women appear to be more vulnerable than men. Xenobiotics have the potential to affect the development, progression, or severity of autoimmune diseases. A variety of mechanisms could contribute to xenobiotic effects on the development and maintenance of immune tolerance or unmasking or modification of self-proteins. There is evidence that exposure to certain drugs, heavy metals, silica, asbestos, and endocrine disruptors are a concern in this regard. Although there are now a variety of animal models designed to mimic different types of autoimmune diseases, much of the information we have to date comes from associations based on human epidemiology. Current research includes both human and animals studies to determine the extent of risk and ways to assess and control it.

Finally, there is growing concern that the developing immune system may be particularly vulnerable to xenobiotic exposures and that perinatal and/or *in utero* exposures may have a lifelong impact on susceptibility to infectious, allergic, or autoimmune diseases. As in other areas of toxicology, tests designed to assess the risk of immunotoxicity for adults may not be sufficient to protect children, and research is currently underway to determine how best to meet this need.

Clearly, exposure to xenobiotics can have a number of effects on the immune system that in turn can affect an array of health outcomes. In some areas of immunotoxicology, significant progress has been made in terms of identifying and understanding the risks associated with xenobiotic exposure. In other areas, more research is needed.

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SAMPLE QUESTIONS

1. Innate (nonspecific) responses include
- **a.** activation of toll receptors
- **b.** clonal expansion and differentiation of plasma cells
- **c.** inflammation
- **d.** natural killer cell responses
- **e.** immunoglobulin production

2. T-helper (Th) cell subsets
- **a.** are distinguished by the cytokines they produce
- **b.** are mutually antagonistic
- **c.** provide "help" in the production of various classes of antibodies
- **d.** All of the above is true
- **e.** None of the above is true

3. Which of the following tests are the most commonly used to assess potential immunotoxic outcomes either allergenic or immune suppression
- **a.** host resistance to infectious challenge
- **b.** T cell cytotoxicity
- **c.** local lymph node assay
- **d.** cytokine profiling
- **e.** IgM response to sheep red blood cells

4. Mechanisms involved in immune suppression could include
- **a.** disruption of T cell development in the thymus
- **b.** upset in Th1/Th2 balance
- **c.** phagocytosis
- **d.** cytotoxicity
- **e.** lymphocyte proliferation

5. Which of the following have been associated with immune suppression in humans?
- **a.** poison ivy
- **b.** environmental tobacco smoke
- **c.** polychlorinated biphenyls (PCBs)
- **d.** diisocyanates
- **e.** arsenic

6. The consequences of immune suppression are influenced by
- **a.** pathogenicity of infectious agent
- **b.** degree of immune deficit
- **c.** exposure dose of infectious agent
- **d.** genetics of the host
- **e.** all of the above

7. Which of the following responses is mediated by IgE antibodies?
- **a.** Type I immediate-type hypersensitivity
- **b.** Type II hypersensitivity (cytolytic)
- **c.** Type III hypersensitivity (immune complex)
- **d.** Type IV delayed-type hypersensitivity
- **e.** None of the above

8. Which of the following responses are associated with asthma?
- **a.** IgM responses
- **b.** eosinophilic inflammation
- **c.** Th1 responses
- **d.** all of the above
- **e.** none of the above

9. Which of the following are associated with allergic contact dermatitis (contact hypersensitivity)?
- **a.** lymphocyte proliferation
- **b.** IgG antibody responses
- **c.** erythema and edema
- **d.** Th2 responses
- **e.** mobilization of Langerhans (dendritic) cells