CHAPTER II

LITERATURE REVIEW

Carbamates

The use of carbamates as insecticides began in the 1950s with their development as insect repellants. Recently, a considerable number of carbamate analogues were synthesized and widely used as insecticides in crop production (Hayes and Laws, 1991).

The Asia-Pacific region accounts for approximately 16% of the total world market for pesticides. Currently, many developing countries are steadily increasing their demand for imported chemicals used in agriculture (Forget, 1991).

Carbamate insecticides are the major type of insecticides widely used in Thailand and other agricultural countries. They are classified as anticholinesterase insecticides. The pharmacological action of these insecticides is ascribed to an accumulation of acetylcholine through cholinesterase (ChE) inhibition, which will be discussed later. The acute lethal profile in mammals administered a ChE inhibitor is characterized by a centrally mediated increase in blood pressure followed by respiratory failure. The primary cause of death is respiratory arrest produced by central respiratory failure, bronchoconstriction, obstruction of the airway due to excess secretions, and/ or neuromuscular blockade. Details of the cause vary with animal species, ChE inhibitors used, or dosage administered (Futagawa et al., 1997; Sinhaseni and Samatiwat, 1998; Futagawa et al., 2000).

Acetylcholine (Vander, Sherman, and Luciano, 1994; Hayes and Laws, 1991)

Acetylcholine (ACh) is a major neurotransmitter in the peripheral nervous system (PNS) and is also present in the brain. In the brain (central nervous system; CNS), ACh is used by the respiratory center to control respiration rate and breathing. At synaptic junctions of the autonomic nervous system, ACh is synthesized from choline and acetyl coenzyme A in the cytoplasm of synaptic terminals and is stored in synaptic vesicles. After its release, it activates receptors on the postsynaptic membrane, the concentration of ACh at the postsynaptic membrane is reduced by an

enzyme called acetylcholinesterase (AchE). This enzyme, located on the pre- and postsynaptic membranes, rapidly hydrolyzes ACh to choline and acetic acid. The choline is then rapidly transported back into the axon terminals where it is re-used in the synthesis of new ACh. Extracellular ACh concentrations can also be reduced by simple diffusion away from the site of release and being metabolized in the blood.

In both sympathetic and parasympathetic divisions of the autonomic nervous system, the major neurotransmitter released between pre- and post-ganglionic fibers is ACh. Likewise, the major neurotransmitter between the postganglionic fiber and the effector cell is also ACh in the parasympathetic division. However, in the sympathetic division, the major transmitter between the postganglionic fiber and the effector cell is norepinephrine (NE).

In the somatic nervous system, the somatic portion of the efferent division of the peripheral nervous system is made up of all the nerve fibers going from the CNS to skeletal-muscle cells. The cell bodies of such neurons are located in groups in the brainstem or spinal cord. Their large-diameter, myelinated axons leave the central nervous system and pass without forming any synaptic network innervation on to skeletal-muscle cells. Excitation of these neurons, which are often called motor neurons, leads to the contraction of skeletal-muscle cells via the neurotransmitter ACh. The axon terminals of a motor neuron contain membrane-bound vesicles resembling the vesicles found at synaptic junctions between two neurons. The vesicles contain the chemical transmitter ACh. When an action potential in a motor neuron arrives at an axon terminal, it depolarizes the nerve plasma membrane, opening voltage-sensitive calcium channels and thus allowing calcium ions to diffuse into the axon terminal from the extracellular fluid. This calcium triggers the release, by exocytosis, of ACh from the vesicles into the extracellular cleft separating the axon terminal and the motor end plate. The ACh diffuses across this cleft and binds to receptor sites [of the nicotinic type] on the motor end plate. The binding of ACh opens ion channels in the receptor complex. Both sodium and potassium ions can pass through these channels. Because of the differences in electrochemical gradients across the plasma membrane, more sodium moves in than potassium out, producing a local depolarizing of the motor end plate. The adjacent plasma membrane is then depolarized, initiating an action potential

in the muscle plasma membrane. This action potential is then propagated over the surface of the muscle fiber.

In addition to receptor sites for ACh, the motor end plate contains the AChE enzyme at its surface. The ACh-mediated signal transduction of motor neurons is controlled by AChE as this enzyme breaks down ACh and returns the end plate to its resting potential.

There are several types of receptors for each neurotransmitter. For ACh, there are two types of receptors classified: nicotinic receptors and muscarinic receptors. The great majority of ACh receptors on autonomic postganglionic neurons are nicotinic receptors. In contrast, the ACh receptors on smooth-muscle, cardiac-muscle, and gland cells are muscarinic receptors. Whereas the cholinergic receptors on the neuromuscular junctions of skeletal-muscle fibers, innervated by the somatic motor neurons are nicotinic receptors.

Nicotinic Receptor

Nicotinic acetylcholine receptor (nAChR) is the receptor present on the postganglionic neurons in the autonomic ganglia and on the postsynaptic membranes of skeletal muscle cells that binds ACh molecules released from the endplate of a motor neuron. The nAChR is an integral membrane protein. It is a pentamer protein consisting of five subunits. Each receptor contains two copies of a subunit called α, and one copy each of a β, γ, and δ subunit. The receptor acts as a site for the binding of both ACh and a channel for the passage of cations. The binding of ACh to the receptor causes a conformational change that opens an ion channel within the protein complex. The inward flux of Na^+ ions through the channel can then lead to a depolarization of the membrane and the activation of the muscle cell. The ion channel consists of a narrow pore lined by a wall composed of five alpha-helical segments, one from each of the surrounding subunits (Vander et al., 1994; Karp, 1999).

Muscarinic Receptor

Muscarinic acetylcholine receptors (mAChRs) belong to a group of seven-helix transmembrane-spanning receptors that mediate cellular responses by activating heterotrimeric G proteins. As a subgroup of the G protein-coupled receptor superfamily, muscarinic receptors are linked to a striking variety of effector systems depending on the cell type (Chang et al., 1997; Karp, 1999; Matsui et al., 2000). These receptors are distributed widely in both central and peripheral nervous systems (i.e. on smooth muscle, cardiac muscle, and gland cells) and play an important role in numerous physiological processes. Some of these include learning and memory, adjusting the amount of light that impinge on the retina, and regulating various organs innervated by autonomic nerves (e.g. gastrointestinal tract, heart, trachea and exocrine glands) (Vander et al., 1994; Matsui et al. 2000).

The mAChRs consist of five subtypes (M_1-M_5) that are encoded by five distinct genes. However, precise physiological functions for each subtype have not been elucidated. Recently, it is known that the M_1, M_3, and M_5 receptors are usually coupled to the $G_{q/11}$ protein, which activates phospholipase C, whereas the M_2 and M_4 receptors are mainly coupled to the $G_{i/o}$ protein, which inhibits adenylate cyclase activity (Matsui et al., 2000).

Activation of M_1, M_3, and M_5 receptors typically increases inositol 1,4,5-triphosphate (IP_3) and diacylglycerol levels, while activation of M_2 and M_4 receptors can either raise or lower cAMP levels, depending on the system (Chang et al., 1997). In addition, it is evident that M_2- and M_4-receptor activation can also modulate ion channel activity: Hanf et al. (1993) have reported that activation of M_2 receptors can open K^+ channels and block Ca^{2+} channels in cardiac cells; whereas M_4 receptors couple to blockade dihydropyridine-sensitive Ca^{2+} channels in neuronal cells (Bernheim, Mathie, and Hille, 1992).

Carbamates are esters of carbamic acid. The basic structure of the carbamate esters is shown in Figure 1.

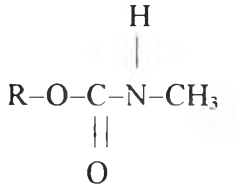

Figure 1 The basic structure of the carbamate esters. R is an aryl or alkyl group (Klaassen, 1996).

The first pesticidal carbamic acid esters were synthesized in the 1930s and were marketed as fungicides. Since these aliphatic esters possessed poor insecticidal activity, interest lay dormant until the mid-1950s when renewed interest in insecticides having anticholinesterase activity but reduced mammalian toxicity led to the synthesis of several potent aryl esters of methylcarbamic acid (Klaassen, 1996).

Carbamate insecticides are reversible inhibitors of nervous tissue AChE, and they are rapidly biotransformed *in vivo* (Klaassen, 1996). Despite the extensive toxicological research demonstrating that the pesticidal carbamate esters are "relatively safe" chemicals producing only transient, short-term toxicity following acute administration, carbamate insecticide toxicity has been reported in humans and fatalities have occurred (Hayes, 1982; Ecobichon, 1994).

Carbamates produce carbamylation of the ester site of the cholinesterase enzyme, therefore prevent AChE from hydrolyzing acetylcholine. However, the carbamylated enzyme complex is unstable and the spontaneous reactivation of cholinesterase occurs (Hayes and Laws, 1991; Klaassen, 1996).

Inhibition and Reactivation of Acetylcholinesterase

Carbamic acid esters, which attach to the reactive site of the AChE, undergo hydrolysis in two stages: the first stage is the removal of the "R" substituent (an aryl or alkyl group) with the formation of a carbamylated enzyme; the second stage is the decarbamylation of the inhibited enzyme with the generation of free and active enzyme (Figure 2) (Klaassen, 1996).

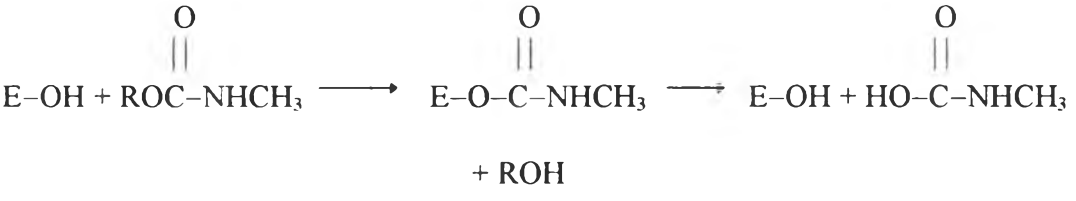

Figure 2 The interaction between carbamate esters and acetylcholinesterase enzyme. A carbamate ester interacts with the serine hydroxyl group in the active site of the acetylcholinesterase enzyme (E–OH) (Klaassen, 1996).

The enzymatic process can be represented schematically as in Figure 3. First, a reversible carbamate-acetylcholinesterase complex is formed, followed by a nonreversible carbamylation reaction with the enzyme; and finally, decarbamylation frees the enzyme and reactivates the original acetylcholinesterase. Carbamylation therefore appears to be reversible from the point of view of the enzyme; however, it loses its anticholinesterase potency in the process (Hayes and Laws, 1991).

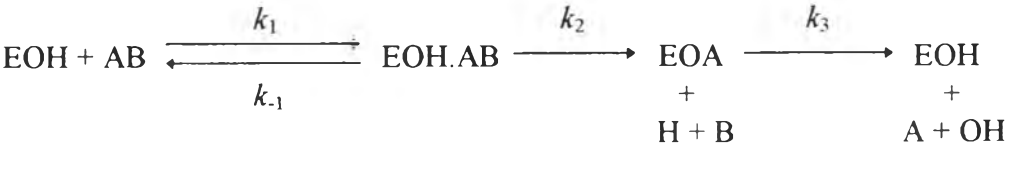

Figure 3 Enzymatic process of carbamates. AB, carbamate; EOH, acetylcholinesterase; EOA, carbamylated enzyme; B, alcohol, oxime, or phenol; and A, carbarmate moiety (Hayes and Laws, 1991).

For *N*-methylcarbamate esters in general, EOH·AB is formed almost instantly; kinetic values for the affinity constant K_a ($K_a = k^{-1}/k_1$) is small. Furthermore, because carbamylated cholinesterase are unstable, the degree of inhibition at a steady state depends on the rate constants for both inhibition and spontaneous reactivation (Hayes and Laws, 1991). Both k_2 (acylation constant) and k_3 (deacylation constant) are normally slow. It is shown that the k_3 is thousands of times slower with carbamate esters than with ACh (Ecobichon, 1979; Klaassen, 1996).

Effects of Carbamates on Nervous System (Hayes and Laws, 1991)

As mentioned before, in normal nervous system functions, a burst of the neurotransmitter ACh is released from a nerve cell terminal, diffuses across the synaptic cleft and transmits a nerve impulse to a specific cholinergic receptor. To end stimulation and restore the sensitivity of the receptor to new transmitter, ACh at the receptor must continually be eliminated by the enzyme AChE. By inhibiting AChE, carbamates allow ACh to accumulate at cholinergic junctions.

Accumulation of ACh can affect both CNS (e.g. effects on respiration rate and breathing) and PNS. In somatic nervous system of the PNS, when nicotinic receptors are overstimulated at the skeletal neuromuscular junctions, *nicotinic effects* (e.g. muscle fasciculation and muscle cramps) occur. Whereas the parasympathetic overstimulation of muscarinic receptors produces *muscarinic effects*, which include slowing of the heart, constriction of the pupil, urination, lacrimation, salivation, and defecation. Signs and symptoms of carbamate insecticide poisoning are summarized in Table 1.

Table 1 Signs and symptoms of anticholinesterase insecticide poisoning. (Ecobichon and Joy, 1982)

Nervous tissue and receptors affected	Site affected	Manifestrations
Parasympathetic autonomic (muscarinic receptors) postganglionic nerve fibers	Exocrine glands	- Increased salivation, lacrimation, perspiration
	Eyes	- Miosis (pinpoint and nonreactive), ptosis, blurring of vision, conjunctival injection, "bloody tears"
	Gastrointestinal tract	- Nausea, vomiting, abdominal tightness, swelling and cramps, diarrhea, tenesmus, fecal incontinence
	Respiratory tract	- Excessive bronchial secretions, rhinorrhea, wheezing, edema, tightness in chest, bronchospasms, bronchoconstriction, cough, bradypnea, dyspnea
	Cardiovascular system	- Bradycardia, decrease in blood pressure
	Bladder	- Urinary frequency and incontinence
Parasympathetic and sympathetic autonomic fibers (nicotinic receptors)	Cardiovascular system	- Tachycardia, pallor, increase in blood pressure
Somatic motor nerve fibers (nicotinic receptors)	Skeletal muscles	- Muscle fasciculations (eyelids, fine facial muscles), cramps, diminished tendon reflexes, generalized muscle weakness in peripheral and respiratory muscles, paralysis, flaccid or rigid tone - Restlessness, generalized motor activity, reaction to acoustic stimuli, tremulousness, emotional lability, ataxia

Table 1 (continued) Signs and symptoms of anticholinesterase insecticide poisoning. (Ecobichon and Joy, 1982)

Nervous tissue and receptors affected	Site affected	Manifestrations
Brain (acetylcholine receptors)	Central nervous system	- Drowsiness, lethargy, fatigue, mental confusion, inability to concentrate, headache, pressure in head, generalized weakness - Coma with absence of reflexes, tremors, Cheyne-Stokes respiration, dyspnea, convulsions, depression of respiratory centers, cyanosis

Effects of Carbamates on Immune Function

The immune suppression from pesticides can be particularly significant in many developing countries where infectious diseases cause nearly half of all deaths (UNIDO, 1996). Inhibition of acetylcholinesterase may affect the immune response, because the autonomic nervous system directly innervates thymus, spleen, lymph node, bone marrow, and other lymphoid tissues (Ballantyne and Marrs, 1992). Recent studies revealed that carbamates could affect the immune system:

Ladics et al. (1994) examined the immunotoxicological effects of the methyl-carbamate pesticide carbaryl in rats. Following inhalation exposures, dose-dependent decreases in thymus weights, spleen cell number, antibody forming cells/spleen, antibody forming cells/10^6 splenocytes, and serum levels of sheep red blood cells-specific immunoglobulin M (IgM) antibody were observed. Significant decreases of 33, 57, and 22% in spleen cell number, antibody forming cells/spleen, and thymus weight, respectively, were found at the 335 mg/m^3 exposure level. Rats exposed orally to 25 mg/kg carbaryl had a 34% decrease in white blood cell (WBC) counts. However, dermal exposure to carbaryl revealed no significant toxicological effects.

In addition, oral exposure of chickens to cabaryl resulted in acute and sometimes prolonged suppression of germinal centers and antibody production. Moreover, carbaryl exposure can suppress granulocyte phagocytosis, which may last for up to 9 months (Klaassen, 1996).

Epidemiological studies have demonstrated an association between use of carbamate insecticides, including carbaryl, and increased incidence of allergic asthma in farmers. In this study, rats were gavaged for 2 weeks with 0, 2, 10 or 50 mg/kg/day of carbaryl and were sensitized with a subcutaneous injection of house dust mite (HDM) allergens in aluminium hydroxide adjuvant 3 days after the beginning of carbaryl exposure and challenged with antigen via the trachea at day 15, a day after the final carbaryl ingestion. Two days after challenge, antigen-specific cell proliferation in pulmonary lymph nodes was significantly higher in the 50 mg/kg group than in controls, while antigen-specific splenocyte proliferation was decreased in groups dosed with 2, 10, and 50 mg/kg carbaryl. Total protein and lymphocyte number in bronchoalveolar lavage (BAL) fluid were also increased in the 50 mg/kg group. By 7 days after challenge, immune-mediated pulmonary inflammation (eosinophils), antigen-specific IgE level in serum, and antigen-specific IgE and IgA levels in BAL fluid were significantly elevated in the 50 mg/kg group. While the number of BAL macrophage were decreased in groups dosed with 10 and 50 mg/kg carbaryl. The results suggested that carbaryl may cause systemic immune suppression, while enhancing pulmonary allergic responses to house dust mite antigen. These studies have shown that spleen, the biggest peripheral lymphoid organ, is a major target organ or carbamate insecticide (Dong, 1998).

Pruett et al. (1992) evaluated the immunotoxicity of sodium methyldithiocarbamate, a chemical widely used for the control of weeds, fungi, and nematodes in soil. These investigators observed decreased thymus weight, depletion of the $CD4^{+}/CD8^{-}$ population of thymocytes, and profound suppression of natural killer (NK) cell activity in mice following both oral and dermal exposure.

Effects of Carbamates on Cytoskeleton

There are many evidences reported that carbamates could disrupt cytoskeleton and result in cellular function disturbances.

Cabaryl, carbamate insecticide, has been reported to be a spindle poison, tending to arrest mitosis in metaphase, especially at cytotoxic concentration (Onfelt, 1983; Onfelt and Klasterska, 1983; Onfelt and Klasterska, 1984).

Nakai et al. (1995) have revealed that carbendazim (methyl 2-benzimidazole carbamate; MBC) has been reported to disrupt the microtubules of Sertoli cells in rats, and the cells exposed to carbendazim are found unable to maintain their shape due to the loss of their cytoskeleton.

McManus and Trombetta (1995) reported that the cytoskeleton appears to be an important cellular target for injury by diethyldithiocarbamate (DDC) exposure. Cultured rat hippocampal astroglia treated with 35 μg/ml DDC showed alterations of the cytoskeleton. The study demonstrated that the microtubular protein, beta-tubulin, appeared to have an altered mobility while the major intermediate filament protein, glial fibrillary acidic protein (GFAP), was decreased.

The herbicide CIPC [N-(3-chlorophenyl)carbarmate] has been shown to disrupt microtubule organization in plants, apparently by interfering with the function of the microtubule organizing center. A study of Holy (1998) examined the effects of CIPC as a cytoskeletal disrupting agent on fertilization and early development. Fertilized sea urchin eggs were cultured in the presence of CIPC. It was found that *Lytechinus pictus* embryos were sensitive to micromolar amounts of CIPC, and that a characteristic set of cytoskeletal and developmental deficits was produced as a result of exposure to this herbicide. The study indicated that CIPC treatment resulted in unequal cell divisions, and skeletal spindle formation in these embryos was abnormal. Therefore, CIPC may pose a significant health risk during mammalian embryogenesis (Holy, 1998).

Methomyl

Methomyl, a carbamate insecticide, is widely used in Thailand and many agricultural countries for crop protection. It is known as an acetylcholinesterase inhibitor and its toxic effects are mostly cholinergic effects.

Methomyl is classified as a highly hazardous (class IB) carbamate insecticide by World Health Organization (WHO, 1986).

Chemical Name and Structure

Methomyl is S-methyl-N-[(methlcarbamoy)oxy]thioacetimidate. Its structure is shown in Figure 4.

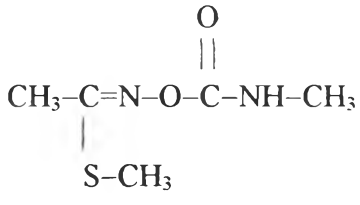

Figure 4 Chemical structure of methomyl. (IPCS, 1996)

Synonyms

The common name methomyl (ANSI, BSI, ISO, JMAF) is in general used. Other names are netomil and mesomile. Trade names include Lannate®, Lanox®, Methavin®, and Nudrin®. Code designation has included DX 1179, OMS 1196, SD 14999, and WK 18236. The CAS registry number is 16752-77-5 (IPCS, 1996).

Physical and Chemical Properties

Methomyl has the empirical formula $C_5H_{10}N_2O_2S$ and a molecular weight of 162.23. The pure material is a white crystalline solid with a slight sulfurous odor. The melting point is 78-79 °C, and the vapor pressure is 5 x 10 mm Hg at 25 °C. The solubility of methomyl at 25 °C is 3% in toluene, 5.8% in water, 22% in isopropanol, 42% in ethanol, 73% in acetone, and 100% in methanol. Methomyl is stable in solid form and in aqueous at pH 7.0 of less it decomposes repidly in alkaline solution and in moist soils (IPCS, 1996).

History and Formulation

Introduced in 1966, methomyl is used as a contact and stomach insecticide for broad spectrum control of pests of vegetables, soybeans, cotton, other field crops, some fruit crops, and ornamentals. Methomyl is formulated as water-soluble powders and liquids (IPCS, 1996).

Absorption, Distribution, Metabolism, and Excretion

The absorption, distribution, metabolism and excretion of methomyl after oral administration to rats are very rapid, the processes being completed within a few days. When rats were given 5 mg ^{14}C-methomyl/kg orally, approximately 53% of the radioactivity was excreted in urine over 7 days, 45% of the dose being excreted in the first 6 hours. Faecal excretion contributed only 2-3% over 7 days. The other major path of elimination (over 5 days) was via expired air as carbon dioxide (CO_2 22% of dose) and acetonitrile (12% of dose) within 5 days. Of this, 18% of the dose was expired as CO_2 within 6 hours and 10% as acetonitrile in 24 hours. Overall, most of the radiolabelled dose (80%) had been eliminated in 24 hours with an estimated half-life of 5 hours. After 7 days, 8-9% of the ^{14}C dose remained in the tissues and carcass, which was incorporated into endogenous constituents. The highest concentration of radioactivity was in the blood (representing 2% of the dose) (Hawkins et al., 1991; IPCS, 1996).

The single oral dose given to rats (5 mg/kg) produced mild clinical signs of cholinesterase inhibition which disappeared within 2 hours of dosing (Hawkins et al., 1991).

One hour after the dermal applications of ^{14}C-methomyl to mice, 2.9% of the dose was present in blood, 5% in liver, and 56% in the remaining carcass. After 8 hours, the distribution was 6.1% in blood, 3.3% in liver, 3.8% in the gastrointestinal tract and smaller amounts (<1%) in other individual tissues. The remaining carcass contained 15% of the original dose (Shah et al., 1981).

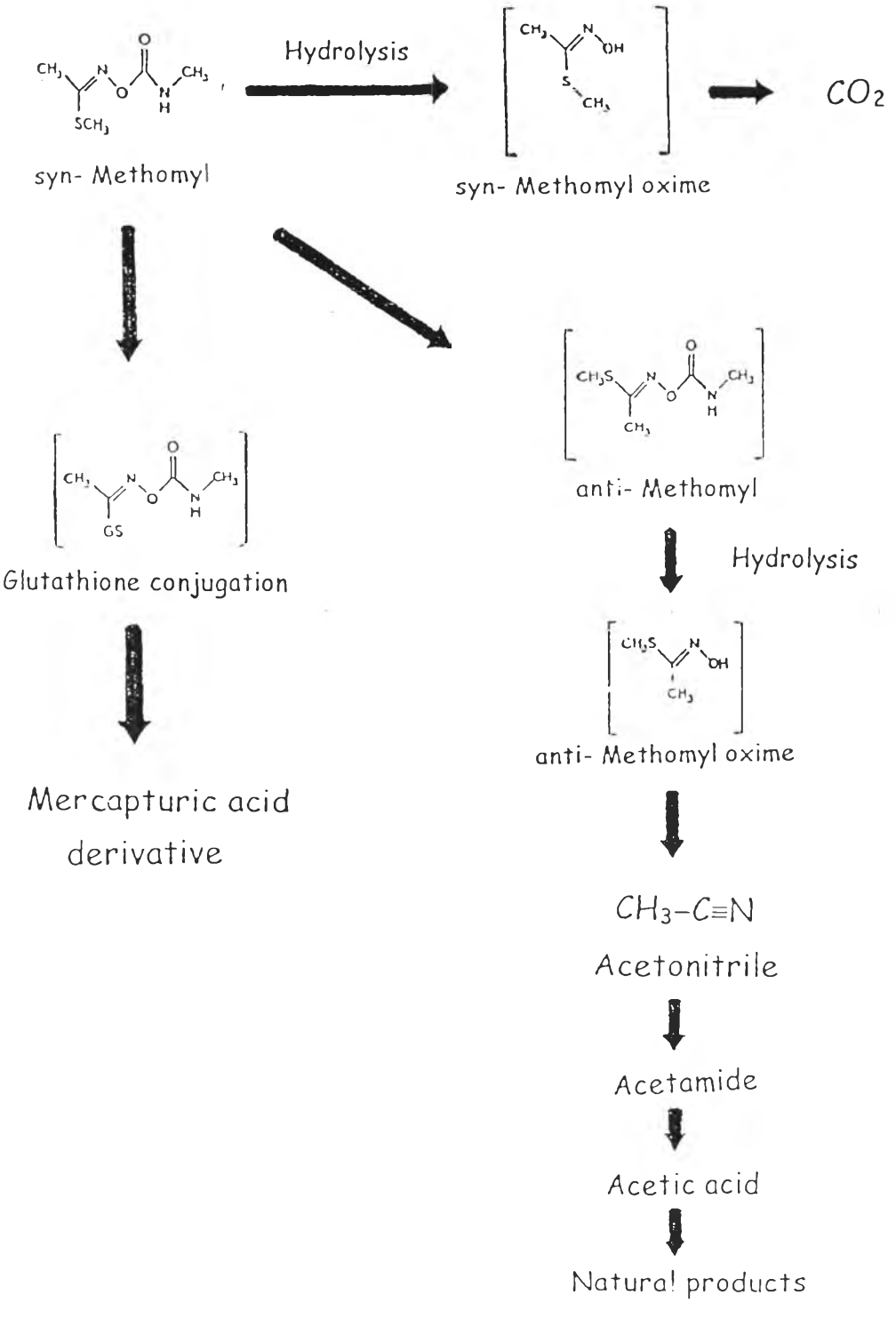

Figure 5 Metabolic pathways of methomyl in mammals. (IPCS, 1996)

Metabolic Pathways of Methomyl in Mammals

As shown in Figure 5, metabolic pathways of methomyl are as follows:

1. Displacement of the *S*-methomyl moiety by glutathione and enzymatic transformation to give the mercapturic acid derivative.

2. Hydrolysis of methomyl to give *S*-methyl-*N*-hydroxyacetimidate (MHTA) which is rapidly broken down to carbon dioxide.

3. Conversion of the *syn*-isomer of methomyl (the insecticidal form) to its *anti*-isomer which then undergoes a hydrolysis, rearrangement and elimination reaction to form acetonitrile. Acetonitrile may undergo further reactions to give the products incorporated into natural body constituent such as fatty acids, neutral lipids and glycerol.

Toxicological Studies

Toxicity to Humans

1) Accidental and Suicidal Poisoning

Three fishermen in Jamaica became critically ill within 5 minutes of eating a meat including roti, an unleavened bread made from flour, water and salt. Symptoms were perspiration, visual disturbances, trembling, vomiting, and defecation, progressing to convulsions and coma. The men were taken to a hospital about 3 hours later and pronounced dead on arrival. Postmortem examination revealed congestion of the stomach lining, lungs, trachea, and bronchi. One of two other men who had shared the meal showed generalized twitching, fasciculation, and severe bronchospasms, but recovered within 2 hours after treatment. Poisoning was traced to a small, unlabeled bag of pure methomyl power found in a tin in the fisherman hut; the powder was probably used instead of salt in preparing the roti, which contained methomyl at about 11,000 ppm. The lethal doses were estimated at 12-15 mg/kg (Liddle et al., 1979).

A 31-year-old Japanese housewife committed suicide by taking an insecticide containing methomyl in food, which was also eaten by her three chidren. All were found dead except a 9-year-old son, who survived. Autopsies of the woman and her 6-year-old son revealed congestion of the stomach lining and lungs, with edema and hemorrhaging of tissues due to acute circulatory failures. Methomyl doses were estimated at 55 mg/kg to the mother and 13 mg/kg to the child (Araki et al., 1982).

Noda (1984) reported the course of therapy and symptoms for a patient who had attempted suicide by ingesting about 2.25 g of methomyl. At 6 hours postingestion, methomyl was present in blood at 1.61 ppm and in the urine at 1.91 ppm; at 15 hours, levels were 0.04 ppm in the blood and 0.25 ppm in the urine; and at 22 hours, methomyl was not detectable in the samples. Volf and Hanus (1984) reported a case in which methomyl was detected in coffee that caused cholinergic symptoms.

2) Adverse Effects of Occupational Exposure

Dr. Keith T. Maddy, in an unpublished manuscript, reported that there were more than 225 poisonings in 1972 and 1973 (some serious, but none fatal) following exposure to dry formulations of methomyl, primarily by inhalation of powder. After the compound was reformulated and marketed as a liquid concentrate, cases decreased to fewer than 10 per year. Cases reported from Australia were unusually associated with formulation (Simpson and Penney, 1974; Simpson and Bermingham,1977). Cases reported from a tobacco-growing area of Japan occurred primarily on the day of application and often involved rates of application higher than those recommended (Kudo, 1975). Among Japanese tea-growers reporting acute symptoms (fatigue, headache, and profuse perspiration) following pesticide use, 42% named methomyl as one of the chemicals used (Fujita, 1985).

In plant manufacturing method and propanil, 11 of 102 workers had been hospitalized for occupationally acquired illness. Workers involved in packing methomyl reported the highest rate of hospitalization following chemical exposure (27%) and the highest frequency of cholinergic symptoms, including miosis, blurred vision, nausea or vomiting, muscle weakness, fatigue and increased salivation. No precise dose-response relationship could be determined, and reliable cholinesterase measurements were not made (Morse et al., 1979).

Toxicity to Laboratory Animals

The acute toxicity of methomyl is summarized in Table 2. Signs of acute are typical of acetylcholinesterase inhibiton. Methomyl did not cause skin irritation in rabbits or guinea pigs; mild erythema was seen in some individuals. In rabbits, methomyl produced mild eye irritation (conjunctivitis, irritic injection, and mucous

membrane irritation). Occular administration also caused miosis and other systemic cholinergic effects, less pronounced when the eye was washed. Methomyl did not cause a skin sensitization reaction in guinea pigs (Du Pont. 1986).

Acute dermal administration of methomyl (in acetone) to rats decreased acetycholinesterase activity in plasma, but not erythrocytes,' at 24 and 72 hours postdosing. After 24 hours, activity was reduced 46% at 99 mg/kg and 66% at 357 mg/kg. In a three-week study with rabbits, dermal exposure to methomyl at 200 mg/kg/day produced clinical cholinergic signs, but did not affect organ weight of histopathology (Du Pont, 1986).

Methomyl was administered orally to goats at 0.95 mg/kg/day for 4 days; at 48 hours after the last dose, acetycholinesterase activity was reduced by up to 28% in the plasma and 20% in the erythrocytes. Recovery was complete within 7 days (Osman et al., 1983).

Table 2 Acute toxicity of technical grade methomyl in laboratory animals.

Species	Sex	LD_{50} (mg/kg)	Vehicle	References
rat (oral)	both	12 - 48	various	Dashiell (1984) Union Carbide (1984) Du Pont (1986) Gaines (1986)
rat (dermal)	male	>1000 - >2400	various	Du Pont (1986) Gaines (1986)
rabbit (dermal)	both	556 - >1500	various	Union Carbide (1984) Du Pont (1986)

In a 29-day study, rats given methomyl at 800 ppm in the diet showed slight plasma and erythrocyte acetylcholinesterase depression on day 7-28; after 29 days, brain acetylcholinesterase activity was reduced by 12% in males and 15% in females. At dosage of 100 ppm and higher, slight brain acetylcholiesterase depression was also

seen in females after 29 days. Acetycholinesterase activity was unffected by dosage of up to 400 ppm.

Methomyl in the diet of rats for 90 days decreased food consumption and body weight at mean dosage of 18.83 mg/kg/day to males and 23.95 mg/kg/day to females. At 18.83 mg/kg/day, males also showed erythroid hyperplasia of the bone marrow. At mean of up to 20 69 mg/kg/day to males and 23.95 mg/kg/day to females there were no death or other effects on behavioral, clinical, hematological, serological, and uroloigical observation. A no-effect level for this study, based on effects seen in males, was 50 ppm in the diet, equivalent to 3.56 mg/kg/day. In other 90-day feeding studies, methomyl had no effect on mortality, body or organ weight, food consumption, gross or microscopic pathology, or a wide variety of clinical parameters at dosages of up to 20 mg/kg/day to mice or 400 ppm to dogs (14.68 mg/kg/day for males and 12.5 mg/kg/day for females). In a 5-month study, transient blood acetycholinesterase depression of 25-40% was seen in rats fed methomyl at 800 ppm; onset and return to normal were faster in males than in females. No effects on blood acetylcholinesterase were observed at dietary dosages of up to 400 ppm (Du Pont, 1986).

In a two-year study with dogs, methomyl in the diet at 1000 ppm (32.12 mg/kg/day for males and 32.67 mg/kg/day for females) resulted in clinical signs of acetycholinesterase inhibition, increased mortality, slight to moderate anemia, evidence of compensatory hematopoiesis in the spleen and bone marrow, epithelial swelling of kidney tubules, and minimal to slight bile-duct proliferation. At 400 ppm (10.93 mg/kg/day for males and 13.90 mg/kg/day for females), accumulation of pigment was noted in the spleen and kidneys. No effects were seen on body weight, food consumption, blood acetylcholinesterase activity, or serological or urological parameters. A no-effect level was 100 ppm (2.94 mg/kg/day for males and 2.31 mg/kg/day for females) (Du Pont, 1986).

In twenty-two- and twenty-four-month studies with rats, methomyl at 400 ppm (19.9 mg/kg/day for males and 26.2 mg/kg/day for females) reduced erythrocyte counts, hemoglobin values, and hematocrits in females and affected food consumption and body weights. Females receiving dosages of 200 ppm (11.18 mg/kg/day) and higher showed a dose-related decrease in hemoglobin values. Acetylcholinesterase activity was not affected. Effects on organs at 400 ppm were increased relative testis

weight and tubular lesions in kidneys; at 200 ppm and higher, spleens of females showed increased extramedullary hematopoiesis; and higher, relative liver weights were increased in females. Exposure had no significant effect on mortality, nor any other effects on organs, tissues, clinical parameters, or other indices of toxicity. A no-effect level was considered to be 100 ppm (4.83 mg/kg/day for males and 10.6 mg/kg/day for females) (Du Pont, 1986).

Other Findings

Lannate 20, formulation of methomyl, was evaluated for its effects on the germ cells of Swiss albino male mice, by the sperm morphology assay and meiotic chromosome preparations. The three sublethal concentrations of 20, 40 and 60 mg/kg body weight administered by the oral intubation method produced significant results ($p<0.01$), thus indicating that Lannate 20 is mutagenic in mice (Hemavathy and Krishnamurthy, 1987).

Methomyl and methomyl-containing technical formulation "Lannate 25" were tested on whole blood human lymphocyte cultures. Both products induced dose-dependent increase in chromosomal abberations and micronuclei. Lannate 25 induced DNA damage as measured by the alkaline elution assay and hydroxylation of guanine at the C8 position. However, sister chromatid exchange were not increased significantly with either product. Overall, the technical formalation was more active than the pure compound, when compared at similar concentrations of active principle (Bonatti et al., 1994).

Methomyl and Lannate 25 were also tested for DNA damage *in vivo*. Swiss CDI mice were treated intraperitoneally with test substances and the DNA damage was detected in liver and kidney cells by the alkaline elution assay and hydroxylation of guanine at the C8 position. This study concluded that methomyl could induce genotoxicity through the formation of active oxygen species (Bolognesi et al., 1994).

The genotoxicity of methomyl was additionally reported in a study of Amer, Fahmy, and Donya (1996). The results showed that methomyl caused chromosomal abberations in mouse spleen cells within 24 hours after an intraperitoneal injection of methomyl at 1 mg/kg body weight.

Methomyl was evaluated for its ability to induce micronuclei *in vitro* using cultured Chinese hamster ovary (CHO) cells, and *in vivo* in mouse bone marrow erythrocytes. *In vitro*, methomyl at 2, 4, 6, 8, 16, and 32 μg/ml induced a significant dose-dependent increase in micronucleated binucleate cells and when administered i.p., methomyl at 1, 3, 6, mg/kg induced a significant increase in micronucleated reticulocytes (MNRETs) in peripheral blood at all three sample times with a single treatment, and in the 48 hours sample following three treatments (Wei, Chao and Hong, 1997).

Klotz et al. (1997) used an *in vitro* assay to examine capacity of methomyl to modulate human estrogen and human progesterone receptors. Methomyl alone weakly activated estrogen- or progesterone-responsive reporter genes in breast and endometrial cells. Methomyl also decreased estradiol- or progesterone-induced reporter gene activity in the breast and endometrial cancer cells. In the whole cell competition binding assays, methomyl demonstrated a limited capacity to displace radiolabeled estrogen or progesterone from estrogen or progesterone receptors. They suggested that methomyl may function through a mechanism separated from ligand-binding and, therefore, may act as a general endocrine modulator in mammalian cells.

Methomyl has also been reported causing toxicity in plant. Previous studies showed that Texas male sterile maize, which expressed high levels of URF13 protein in its inner mitochondrial membrane, was susceptible to methomyl. The interaction of methomyl with this protein produced hydrophilic pores with diameter of 0.8-1.5 nm in the inner mitochondrial membrane leading to rapid swelling of the mitochondria, stimulation of NADH oxidation, inhibition of malate-driven respiration, uncoupling of oxidative phosphorylation, and the leakage of small molecules and ions (Rhoads et al., 1994; Chaumont et al., 1995). The URF13 is reported as a 13 KDa tetrameric protein with a central core of four-alpha-helical bundle that can undergo a conformational change after interaction with methomyl. More recently, it is known that this protein could possibly interfere with activity of mitochondrial aldehyde dehydrogenase (mtALDH) enzyme, which is required to inhibit mitochondrial PT pore generation (Irwin, Gaspers, and Thomas, 2002). Therefore, methomyl may interfere with energy metabolism via the metabolic pathways of ALDH and NADH, and induce PT pore generation followed by cell death.

Toxicity of methomyl independent on the acetylcholinesterase enzyme inhibition has been reported. Saiyed et al. (1992) observed ECG changes in the occupationally-exposed subjects and suggested that these changes were probably directly related to methomyl rather than its toxicity through cholinesterase inhibition. This study is supported by the study of Futagawa et al. (1997) and Futagawa et al. (2000), which showed that the developed depressor response from a decrease in cardiac contractility and/ or vascular resistance observed in rabbits treated with methyl carbamates was insensitive to atropine. Therefore, the lethality was probably mediated through the direct inhibitory effects of methyl carbamates on cardiac and vascular smooth muscle contraction.

In Thailand, it was reported that levels of lactate dehydrogenase (LDH) type 4 isozyme in blood was increased among tangerine growers exposed to 90% SP formulation of methomyl (Sinhaseni et al., 1993). In addition, it was also observed in laboratory animals that rats orally treated with single doses of 3-7 mg/kg of methomyl showed significant increase in serum LDH, especially in LDH_3 and LDH_4 isozymes, on day 1 after dosing (Lohitnavy and Sinhaseni, 1998).

Methomyl could induce splenotoxicity as shown in a study of Lohitnavy and Sinhaseni (1998). The spleen cell viability was significantly reduced in rats treated with an acute dose of 6 and 8 mg/kg of methomyl on day 1 and 3. This methomyl-induced splenotoxicity was protected by pretreating rats with N-acetylcysteine (NAC), a free redical scavenger. The study suggested that methomyl might generate oxidative stress, which consequently contributed to the splenotoxicity.

Suramana et al. (2001) reported that the wave numbers of FTIR spectra at amide I and amide II in both methomyl and colchicine exposed rats shifted in a dose response manner when compared with the control ($p<0.05$). The amide I and II shifted in these regions have been proposed to be the result of alpha-helix protein conformational change (Rice-Evans and Diplock, 1991; Wong et al., 1995). Mitomycin C, a DNA-crosslinking agent, at toxic dose 1 mg/kg i.p. (Moore et al., 1995) did not show this pattern. Moreover, all exposed rats showed increase in the absorbance ratios that were related to vibrational mode of the phosphodiester group in nucleic acid ($p<0.05$).

Acetonitrile

Acetonitrile is predominantly used as a solvent in the manufacture of pharmaceuticals, for spinning fibers and for casting and molding of plastic materials, in lithium batteries, for the extraction of fatty acids from animal and vegetable oils, and in chemical laboratories for the detection of materials such as pesticide residues. It is also used in dyeing textiles and in coating compositions as a stabilizer for chlorinated solvents and in perfume production as a chemical intermediate. Acetonitrile is classified a hazardous chemical class III (U.S. Environmental Protection Agency, [USEPA], 1985).

Chemical Formula

CH_3CN

Chemical Name

Methyl Cyanide

Synonyms

Cyanomethane, Ethanenitrile, and Ethyl nitrile

Physical and Chemical Properties

Acetonitrile has the chemical formula C_2H_3N and the molecular weight of 41.05. It is a clear colorless liquid with a sweet ethereal odor (Budavari, 1989; USEPA, 1985; USEPA, 1993a). It has an odor threshold of 170 ppm. (Amoore and Hautala, 1983). The boiling point is 81.6 °C, the melting point is -46 °C, and the vapor pressure is 74 mm Hg at 20 °C. Acetonitrile is very soluble in water (Budavari, 1989) with an octanol/water partition coefficient (log K_{ow}) of -0.34 (USEPA, 1985). Acetonitrile is stable under ordinary conditions of use and storage. Acetonitrile is quite flammable (Budavari, 1989), burning may produce fumes of cyanide, carbon monoxide, carbon dioxide, nitrogen oxides and sulfur oxides.

Health Hazard Information

In most cases, cyanide poisoning causes a deceptively healthy pink to red skin color. However, if a physical injury or lack of oxygen is involved, the skin color may be bluish. Reddening of the eyes and pupil dilation are symptoms of cyanide poisoning. Cyanosis (blue discoloration of the skin) tends to be associated with severe cyanide poisoning.

Acute Effects

1) Inhalation

Effects of overexposure are often delayed, possibly due to the slow formation of cyanide anions in the body. These cyanide anions prevent the body from using oxygen and can lead to internal asphyxiation. Concentrations up to 500 ppm acetonitrile through inhalation exposure cause early symptoms including nose and throat irritation, flushing of the face, and chest tightness. Higher concentrations may produce headache, nausea, vomiting, respiratory depression, weakness, blood changes, thyroid changes, irregular heart beat, abdominal pain, convulsions, shock, unconsciousness and death, depending on concentration and time of exposure (U.S. Department of Health and Human Services, 1993a; USEPA 1985; USEPA, 1987). Acetonitrile is considered toxic in humans when ingested or through skin contact (U.S. Department of Health and Human Services, 1993a).

2) Ingestion

Gastric irritation may occur. Other symptoms parallel those from inhalation exposure (U.S. Department of Health and Human Services, 1993a).

3) Skin contact

Acetonitrile may cause irritation and may be absorbed through skin with health effects to parallel those of inhalation.

4) Eye contact

Splashes may cause eye irritation with redness and pain.

The LD_{50} test in laboratory animals following acute toxicity of acetonitrile has shown that acetonitrile causes high to moderate acute toxicity from oral exposure, while the inhalation LC_{50} test has shown the chemical to have moderate acute toxicity as the following (U.S. Department of Health and Human Services, 1993b):

Oral rat:	LD_{50}	2460	mg/kg
skin rabbit:	LD_{50}	1250	µL/kg
inhalation rat:	LC_{50}	7551	ppm/ 8h

Chronic Effects (Noncancer)

Chronic (long-term) inhalation exposure to acetonitrile results in cyanide poisoning from metabolic release of cyanide after absorption. The major effects consist of those on the central nervous system (CNS), such as headaches, numbness, and tremor. Cyanide poisoning can also be produced through the ingestion of acetonitrile or from contact with the skin (U.S. Department of Health and Human Services, 1993a; USEPA, 1985; USEPA, 1987). In addition, application of acetonitrile to the skin may produce dermatitis (U.S. Department of Health and Human Services, 1993a). It is also found that long term exposures may affect liver, kidneys, and central nervous system.

Animal studies have shown that different species vary widely in susceptibility to acetonitrile by various routes (U.S. Department of Health and Human Services, 1993a). The reference dose (RfD) for acetonitrile is 0.006 mg/kg/day based on decreased red blood cell counts and hematocrit, and hepatic lesions in mice (USEPA, 1993b). Recently, the Environmental Protection Agency (EPA) has provided the reference concentration (RfC) for acetonitrile at 0.05 mg/m^3 (USEPA, 1993a; USEPA, 1993b; USEPA, 1994).

Workers using cyanide should have preplacement and periodic medical exams. Those with history of central nervous system, heart or lung diseases, or liver, kidney, or thyroid problems may be more susceptible to the effects of this substance.

Reproductive and Developmental Effects

Although no information is available on the reproductive or developmental effects of acetonitrile in humans, animal studies appear to suggest that acetonitrile may cause developmental and reproductive effects such as a decrease in average fetal body weight and a significant increase in the number of malformed offspring (USEPA, 1985; USEPA, 1987; USEPA, 1993b).

Cancer Risk

No pertinent data concerning the carcinogenicity of acetonitrile in humans or animals were located. However, the National Toxicology Program (NTP) is currently conducting a 2-year carcinogenesis inhalation study on acetonitrile using rats and mice (Clayton and Clayton, 1981; U.S. Department of Health and Human Services, 1993b; USEPA, 1985; USEPA, 1987; USEPA, 1993b).

Regulation of the Cell Division Cycle and Cell Death

The fundamental processes performed by any single cell are carefully regulated by purely intracellular mechanisms. The total activities of every individual cell in the body fall into two categories: 1) Each cell performs for itself all those fundamental basic cellular processes–movement of materials across its membrane extraction of energy, protein synthesis, and so on–that represent the minimal requirements for maintaining its individual integrity and life; and 2) each cell simultaneously performs one or more specialized activities that, in concert with the activities performed by the other cells of its tissue or organ system, contribute to the survival of the organism by helping maintain the stable internal environment (the extracellular fluid surrounding each cell) required by all cells. A multicellular organism can survive only as long as it is able to maintain the composition of its internal environment in a state compatible with the survival of its individual cell. It is from this fluid that the cells receive oxygen and nutrients and into which they excrete wastes (Vander et al., 1994).

The concept that the composition of the internal environment is maintained relatively constant is known as *homeostasis*. Changes do occur but the magnitudes of these changes are small and are kept within narrow limits. The activities of the cells, tissues, and organs must be regulated and integrated with each other in such a way that any change in the extracellular fluid initiates a reaction to minimize the change. A collection of body components that functions to maintain a physical or chemical property of the internal environment relatively constant is termed a *homeostatic control system* (Vander et al., 1994).

The description of how sweating is brought about in response to increased heat generation is an example of a homeostatic control system. Another example is when the concentration of oxygen in the blood significantly decreases below normal, the nervous system detects the changes and increases its output to the skeletal muscles responsible for breathing movements. The result is a compulsatory increase in oxygen uptake by the body and a restoration of normal internal oxygen concentration.

Thus an understanding of cellular physiology requires knowledge of the intracellular mechanisms to maintain the cells in homeostasis (Vander et al., 1994).

Cell division and cell death are processes controled by intracellular mechanisms. The consequence of a loss of proliferation control in a cell is cancer, whereas a low cell proliferation may contribute significantly to degenerative diseases. Therefore, in an organism made up of multiple cell types and tissues, it is most important that cell proliferation is tightly regulated (Normal and Lodwick, 1999).

Cell Cycle

Cell division is important for growth, development, repair and replacement of dead cells. The balance of cell proliferation and cell death determines the life cycle of the cell (Normal and Lodwick, 1999).

Phases of the Cell Cycle

The process of cell division is cyclical and unidirectional. The cell cycle can be divided into two major phases: *interphase* and *M (mitosis) phase*. Cells spend the majority of time in interphase, only a small percentage of cells are observed to be in the M phase. Whereas M phase usually lasts only 30 to 60 minutes, interphase may extend for hours, days, weeks, or longer, depending on the cell type and the conditions. Interphase is divided into G_1 (and G_0), S, and G_2 phases; while M phase includes the process of mitosis and cytokinesis (Karp, 1999). (See Figure 6)

G_1 (Gap 1) phase is characterized by gene expression and protein synthesis. This is the only part of the cell cycle regulated primarily by extracellular stimuli (e.g. mitogens and adhesion). This phase enables the cell to grow and to produce all the necessary proteins for DNA synthesis. This phase of the cell cycle is the most variable in duration (minutes to months). In non-dividing tissues, cells withdraw from the cell cycle into a ***resting state (G_0)***, but can re-enter G_1 upon stimulation.

S (Synthesis) phase, the cell replicates its DNA and has two complete sets of DNA. This allows the cell to divide into two daughter cells, each with a complete copy of DNA.

G_2 (Gap 2) phase, the cell undergoes growth and protein synthesis. The cell needs enough proteins synthesized for two cells.

M (Mitosis) phase is a continuous dynamic process, where *mitosis*, the division of nuclear material, and *cytokinesis*, the process of cytoplasmic division, occur. The

result of mitotic cell division is the production of two daughter cells that contain a complete copy of the genomic DNA of the parent cell and the cell cycle has been completed (Normal and Lodwick, 1999).

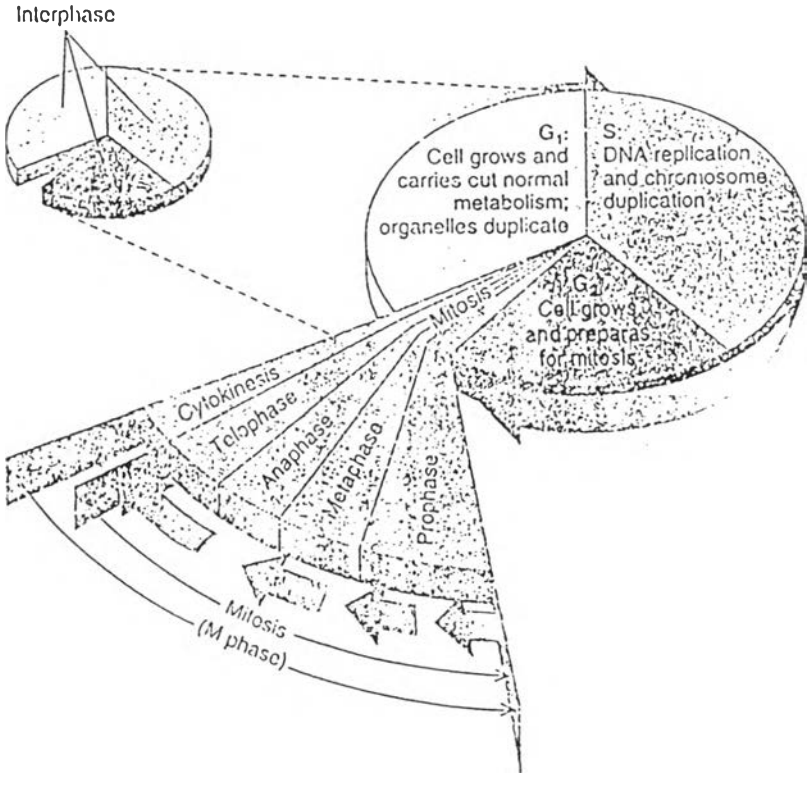

Figure 6 Cell division cycle. This diagram of the cell cycle indicates the phases through which a cell passes from one division to the next. The cell cycle can be divided into two phases: interphase and M phase (Karp, 1999).

G_0 to G_1 Transition (Mitogenesis)

Proliferation of quiescent cells (G_0) can be induced by mitogens in a cell-type-specific manner. Mitogens include growth factors, hormones and cell contact. Signaling pathways activated by mitogens lead to protein expression (includes some cyclins), the expression of responsive genes (e.g. by *myc*, *fos*, *jun* genes), and the progression of the cell through G_1 and past a 'restriction point' (mammalian cells) or START (yeast), where the cell becomes committed to a complete division cycle. Once

past the restriction point, cells no longer require the presence of growth factors and are unresponsive to anti-mitogenic signals, such as transforming growth factor beta (TGF-β) (Normal and Lodwick, 1999).

Regulation of the Cell Cycle

Progression through the cell cycle is controlled at three 'checkpoints' between the stages of the cycle: restriction point, mitosis entry and mitosis exit. There are a number of proteins that regulate and control the cell cycle. These proteins are: 1) cyclins and cyclin-dependent protein kinases (CDKs); 2) CDK inhibitors; and 3) tumor suppressor gene products, which will be discussed later (Normal and Lodwick, 1999). (See Figure 7)

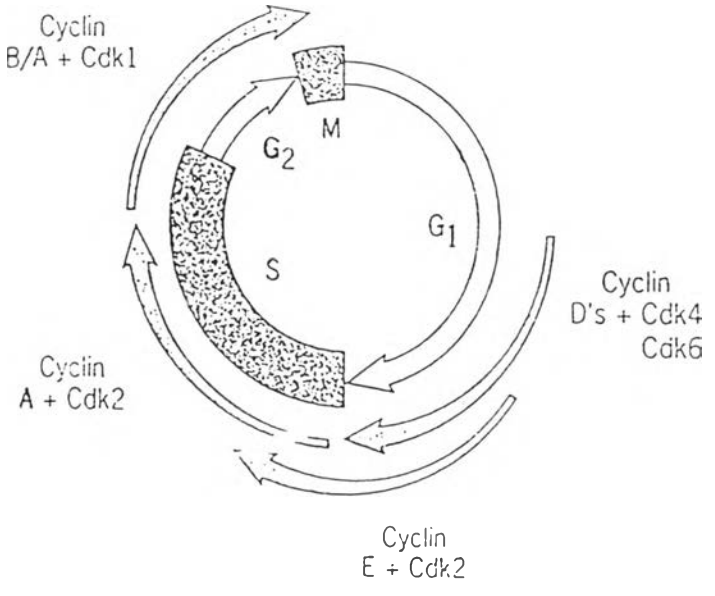

Figure 7 Regulation of the cell cycle. Combinations between various cyclins and cyclin-dependent kinases are used to control the cell cycle activities at different stages (Karp, 1999).

1) Cyclins and cyclin-dependent protein kinases

Transition between stages is triggered by increased activity of specific CDKs. Levels of CDK proteins remain relatively constant throughout the cell cycle but their activity is regulated at different stages. CDKs are activated primarily by the binding of specific cyclins. The levels of the different cyclins rise and fall at different points in the cell cycle and produce temporal activation of their specific CDKs. Each CDK is presumed to phosphorylate and modulate the activity of a subset of cellular target

proteins specific for progression through individual transitions within the cell cycle (Normal and Lodwick, 1999). Successive waves of synthesis and degradation of different cyclins probably play a key role in driving the cell from one stage to the next. The pairing between cyclins and CDKs is highly specific, and only certain combinations are found. For example, a complex formed between cyclin E and CDK2 is active at the G_1-S transition, whereas a complex between cyclin B and CDK1 is active at the G_2-M transition. Cell cycle activities during G_1 are accomplished primarily by CDKs associated with the D-type cyclins (D1, D2, and D3). Among the substrates for these CDKs are transcription factors that activate genes required for DNA replication. The G_1-S transition, which includes the initiation of replication, is driven by the activity of the cyclin E-CDK2 complex. The transition from G_2 to M is driven by the activity of cyclin A-CDK1 and cyclin B-CDK1 complexes, which are thought to phosphorylate such diverse substrates as cytoskeletal proteins, histones, and proteins of the nuclear envelope. Destruction of cyclin B is a key factor in the inactivation of CDK1 and the transition of the cell from mitosis back into G_1 (Karp, 1999). For example, the synthesis of cyclin B is relatively constant and concentrations rise in a linear manner until a threshold is reached when Cdc2 is activated and the cell preceeds into mitosis. At mitosis the rate of cyclin B breakdown is accelerated and the stimuation of Cdc2 is terminated. The levels of other cyclins, e.g. cyclin E, are more closely controlled at the level of expression and mRNA for these species is only present during limited phases of the cell cycle. For example, the S-phase CDK (CDK2/ cyclin D, E or A) may phosphorylate proteins involved in DNA replication, while mitosis-promoting factor (MPF) (Cdc2 (CDK1)/ cyclin B) may phosphorylate proteins during mitosis such as the nuclear lamins, histones and cytoskeletal proteins involved in chromosome condensation, assembly of the mitotic spindle, dissolution of the nuclear membrane and cell detachment (Normal and Lodwick, 1999).

CDKs are also subject to complex modulation in response to several intracellular signalling pathways. This serves to integrate intra- and extracellular signals reflecting nutritional status and intercellular communication. Whether or not a cell proceeds to the next stage of the cell cycle, therefore, depends on the integration of multiple factors at the level of the CDK (Normal and Lodwick, 1999).

2) CDK inhibitors

p21 and p27 are proteins that inhibit the cyclin-CDK complex that drive the cell cycle. In addition to their role in checkpoint control, the CDK inhibitors are active in cell differentiation and also appear to prevent uncontrolled growth that leads to the development of cancer (Karp, 1999).

3) Tumor suppressor gene products

The tumor suppressor genes encode proteins which are normally involved in the regulation of normal cell division and differentiation. The important tumor suppressor gene products are:

p53. p53 is a DNA-binding protein involved in regulating the expression of genes involved in cell cycle arrest (e.g. *p21WAF1/CIP1*) and apoptosis (e.g. *Bax*). p53 recognizes when there is an irreversibly damaged DNA in the cell and either arrests the cell cycle in G_1 or if all else fails, triggers cell removal by apoptosis (Normal and Lodwick, 1999).

Rb. Most of the substrates for CDKs remain unknown. However, one mechanism at the G_1-S checkpoint may involve the hyperphosphorylation of negative cell cycle regulators, such as the retinoblastoma tumour suppressor gene product (pRb), to overcome their suppression of passage from G_1. In normal quiescent cells and early G_1, pRb is found in a hypophosphorylated form, which actively binds several cytoplasmic proteins, including transcription factors of the E2F family, which go on to activate transcription of the S-phase genes. Therefore, in G_1, pRb appears to act as a cell cycle suppressor by binding members of the E2F transcription factor family. pRb is absent or inactive in retinoblastoma and several other tumours suggesting that, in its absence, passage through the G_1-S checkpoint goes unchecked (Normal and Lodwick, 1999).

Cell Cycle Arrest

The cell cycle may be arrested at two points (G_1-S and G_2-M) as a result of DNA damage. Mechanisms of cell cycle arrest are not well characterized but appear often to involve inhibition of CDKs as part of their action. The tumor suppressor gene product p53 is also reported important in arresting the cell cycle at G_1 phase. The expression of p53 is normally low but it is increased dramatically when cell DNA is

damaged, therefore, it may stimulate DNA repair indirectly. This leads to the suggestion that p53 is a 'guardian of the genome' (Normal and Lodwick, 1999). In addition, many studies show that cAMP plays an important role in G_1-cell cycle arrest via the increase in the CDK inhibitors, p27 (Xaus et al., 1999; Stuart et al., 2000).

Cytokines and Cell Cycle

Cytokines are polypeptide products of many cell types that modulate the function of other cell types. Cytokines can act on the same cell that produces them (autocrine effect), on other cells in the immediate vicinity (paracrine effect), or systemically (endocrine effect). Although historically associated with cellular immune responses, in many cases, cytokines are also growth factors influencing cell division cycle, and regulating cell growth and differentiation (Kumar, Cotran, and Robbins, 1997).

Cell Death

As the cell encounters physiologic stresses or pathologic stimuli, it can undergo adaptation, achieving a new steady state and preserving viability. If the adaptive capability of a cell is exceeded, cell injury develops. Up to a point, cell injury is reversible; however, with severe or persistent stress, the cell suffers irreversible injury and ultimately dies.

Cell death can be morphologically and biochemically distinguished and divided into two forms: necrosis and apoptosis. (See Figure 8)

1. **Necrosis** or accidental cell death occurs after severe and sudden injury. It is characterized by the swelling of organelles and a breakdown of the integrity of the plasma membrane, which results in the leakage of cellular contents and an inflammatory response (Normal and Lodwick, 1999).

2. **Apoptosis** or programmed cell death occurs in response to physiological triggers in development (tissue remodelling), defence, homeostasis and ageing. Apoptotic cells initially shrink and lose microvilli and cell junctions. Organelles maintain their structure but the plasma membrane becomes highly convoluted and the cell breaks down into small apoptotic bodies (membrane-enclosed vesicles), which are quickly phagocytosed by macrophages. This occurs without leakage of cellular

constituents and, hence, without an inflammatory response. Characteristics of apoptotic cells include DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly into apoptotic bodies. *In vivo*, as mentioned above, this process culminates with the engulfment of apoptotic bodies by other cells, preventing complications that would result from a release of intracellular contents. These changes can be completed within 30 to 60 minutes (Thornberry and Lazebnik, 1998; Normal and Lodwick, 1999).

Apoptosis is a major form of cell death that is used to remove excess, damaged or infected cells throughout life. It is important in normal cell turnover, the immune system, embryonic development, metamorphosis and hormone dependent atrophy, and also in chemical-induced cell death (Arends and Wyllie 1991; Ellis et al., 1991; Cohen et al., 1992). Apoptosis is important in eliminating cells containing damaged DNA that may contribute to the initial development of cancer and in suppressing the neoplastic signals in others. Failure of apoptosis in these situations could contribute to cancers. Moreover, failure to deplete self-reactive T cells by apoptosis may be important in the development of autoimmune disease (Normal and Lodwick, 1999; Bratton and Cohen, 2001).

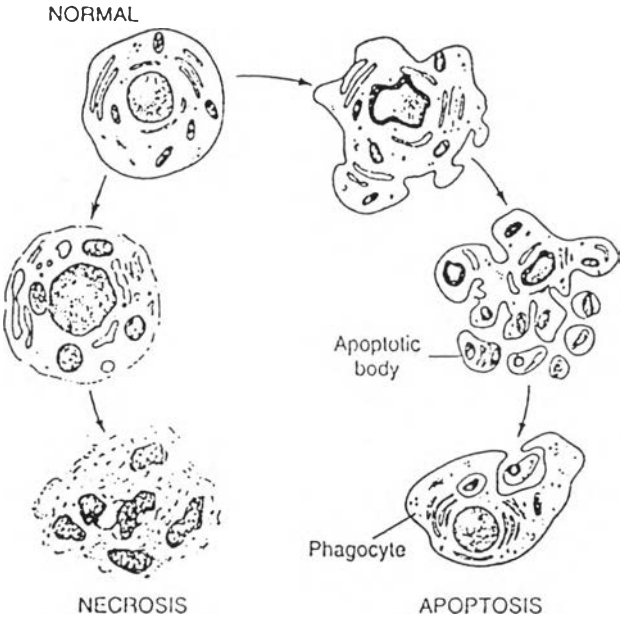

Figure 8 Cell death. The sequential ultrastructural changes seen are different between necrosis and apoptosis. Signs of necrosis include chromatin clumping, organellar swelling, and eventual membrane damage. In apoptosis, the initial changes consist of nuclear chromatin condensation and fragmentation, followed by cytoplasmic budding and phagocytosis of the extruded apoptotic bodies (Kumar et al., 1997).

Possible Mechanisms of Programmed Cell Death

Mechanisms of programmed cell death or apoptosis in cells remain to be elucidated but are clearly very varied. Signals for apoptosis in one cell type can induce cell proliferation and differentiation in other cell types. It is found that raised intracellular calcium concentrations ($[Ca^{2+}]_i$ and increased synthesis of tumour suppressor proteins (e.g. p53), are commonly but not universally implicated in apoptosis. Whether implicated gene products induce cell proliferation or apoptosis probably depends on their associations with other proteins and reflects the complexity and fine balance of the regulation of the cell cycle.

Apoptotic cell death occurs in two phases: first a commitment to cell death, followed by an execution phase characterized by dramatic stereotypic morphological changes in cell structure (Kamens et al., 1995), suggesting the presence in different cells of a common execution machinery (Jacobson, Burne, and Raff, 1994). Apoptosis, as mentioned above, is characterized by condensation and fragmentation of nuclear chromatin, compaction of cytoplasmic organelles, dilatation of the endoplasmic reticulum (frequently in a subplasmalemmal distribution), a decrease in cell volume and alterations to the plasma membrane resulting in the recognition and phagocytosis of apoptotic cells, so preventing an inflammatory response (Arends and Wyllie, 1991). The nuclear alterations, which are the pre-eminent ultrastructural changes of apoptosis, are often associated with internucleosomal cleavage of DNA (Wyllie, 1980), recognized as a 'DNA ladder' on conventional agarose gel electrophoresis and long considered as a biochemical as a biochemical hallmark of apoptosis (Brown, Sun, and Cohen, 1993; Oberhammer et al., 1993; Cohen et al., 1994). Internucleosomal cleavage of DNA now appears to be a relatively late event in the apoptotic process. Nevertheless, its measurement is simple and it is often used as a major criterion to determine whether a cell is apoptotic (Cohen et al., 1992b; Tomei, Shapiro, and Cope, 1993).

Apoptosis process requires specialized machinery. The central component of this machinery is a proteolytic system involving an activation of a family of proteases called caspases. These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell (Normal and Lodwick, 1999; Bratton and Cohen, 2001).

Caspases

Caspases are intracellular cysteine proteases, which are highly conserved in mammals, the worm *Caenorhabditis elegans*, and the fly *Drosophila melanogaster*. They are primarily responsible for the stereotypic morphological and biochemical changes that are associated with apoptosis. Exposure of cells to chemicals and radiation, as well as loss of trophic stimuli, perturb cellular homeostasis and signal the cell to undergo apoptosis by stimulating the formation of unique and/ or common caspase-activating complexes (Bratton and Cohen, 2001).

Caspases are among the most specific of proteases, with an unusual and absolute requirement for cleavage after aspartic acid. It is found that cleavage of proteins by caspases is not only specific, but also highly efficient and effective. The system keeps the effector protease inactive but is able to rapidly activate large amounts of it in response to minute quantities of an appropriate inducer. In apoptosis, caspases function in both cell disassembly (effectors) and in initiating this disassembly in response to proapoptotic signals (initiators) (Thornberry and Lazebnik, 1998).

How caspases contribute to this process is not fully understood. One role of caspases is to inactivate proteins that protect living cells from apoptosis. A clear example is the cleavage of I^{CAD}/DFF45, an inhibitor of the nuclease responsible for DNA fragmentation. Other negative regulators of apoptosis cleaved by caspases are Bcl-2 proteins, which will be discussed later. It appears that cleavage not only inactivates these proteins, but also produces fragments that promotes apoptosis (Thornberry and Lazebnik, 1998).

In addition, caspases contribute to apoptosis through direct disassembly of cell structures by the destruction of nuclear lamina, which is a rigid structure that underlies the nuclear membrane and is involved in chromatin organization, leading to chromatin condensation (Thornberry and Lazebnik, 1998).

Caspases also reorganize cell structures indirectly by cleaving several proteins involved in cytoskeleton regulation, including gelsolin, focal adhesion kinase (FAK), and p21-activated kinase 2 (PAK2). Cleavage of these proteins results in deregulation of their activity and hence cellular structure reorganization (Thornberry and Lazebnik, 1998).

Therefore, caspases participate in apoptosis of the cell by cutting off contacts with surrounding cells, reorganizing the cytoskeleton, destroying DNA, disrupting the nuclear structure, inducing the cell to display signals that mark it for phagocytosis, and disintegrating the cell into apoptotic bodies (Thornberry and Lazebnik, 1998).

***Activation of caspases* (Thornberry and Lazebnik, 1998)**

Recently, it is clearly known that the activation of initiator caspases requires binding to their specific cofactors, a mechanism commonly observed with proteases. For instance, activation of procaspase-8 requires association with its cofactor FADD (Fas-associated protein with death domain) through the DED (death recruitment domain). Activation of caspase-9 also requires cytochrome *c* and deoxyadenosine triphosphate.

Previous observations reveal that procaspases have low but detectable activity and dimerization is required for their activation. Therefore, it is proposed that procaspases that are overexpressed in cells and artificially cross-linked will become active. This proposed model states that caspases are latent in the cell because they exist at low concentrations as monomers. The cofactors serve to bring two or more caspase precursors in close proximity, allowing for intermolecular autoproteolytic activation. However, it is not clear whether or not this proposed model is correct; several questions remain unanswered. For example, it is not clear that initiator caspase precursors are indeed monomers in living cells.

There is another model proposed postulates that caspase precursors are present in cells in a conformation or a complex that prevents autocatalysis. The cofactors facilitate activation by changing the conformation of the precursors either directly or by removing an inhibitor.

Compartmentalization of caspases and their cofactors is likely to be another way of regulating caspase activation. For example, cytochrome *c* released from mitochondria is required for caspase-9 activation. Another example is provided by the finding that caspase-8 is activated when recruited to the Fas receptor complex.

At present, very little is known about the regulation of the interaction between procaspases and their cofactors. Therefore, obtaining the structures of caspases and

determining where caspases and their cofactors are in cells will likely contribute to a better understanding of caspase regulation.

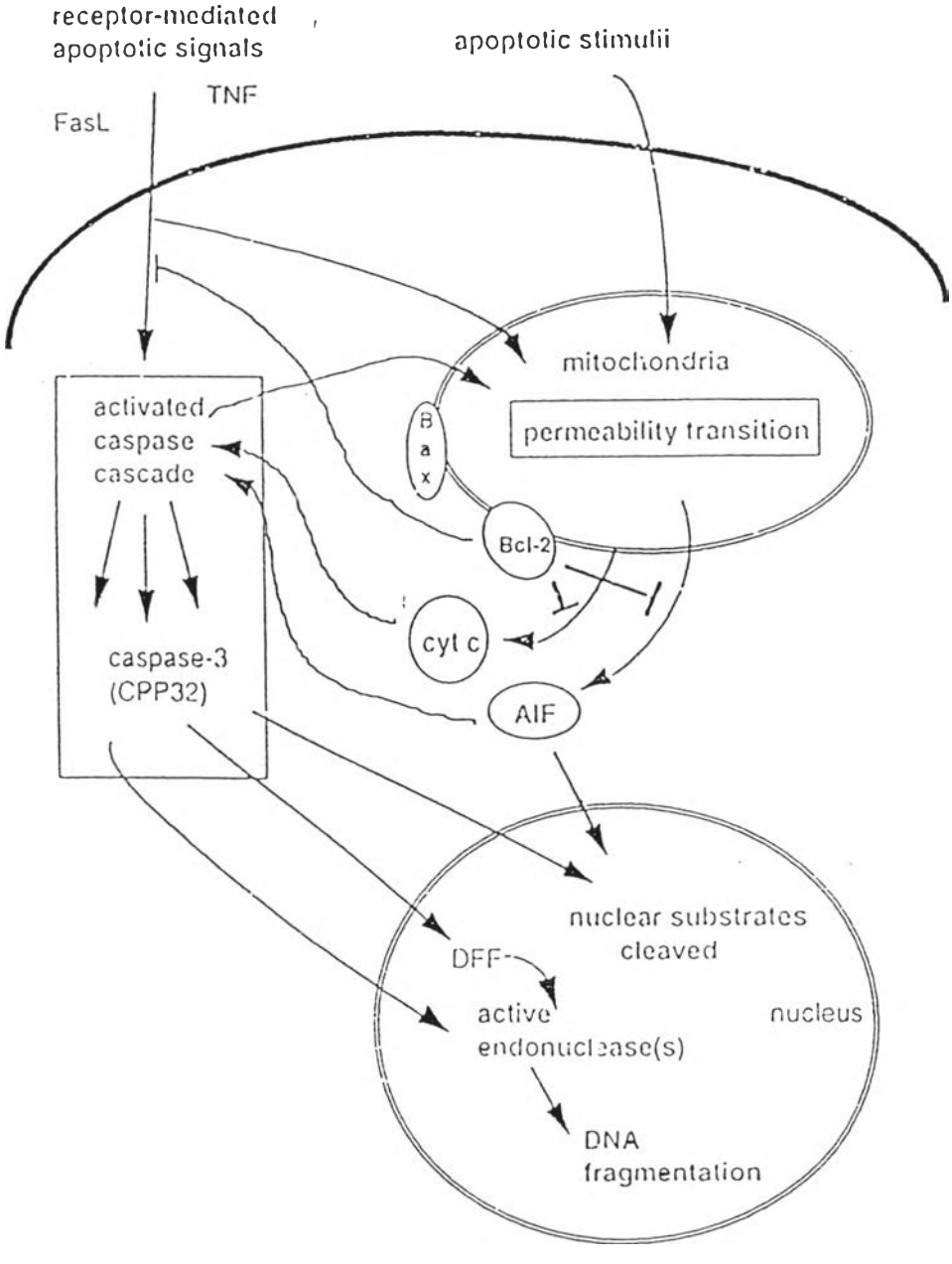

Figure 9 Model of apoptotic process. Mitochondrial and nuclear events are linked with the action of the caspases and the Bcl-2 family. AIF, apoptosis-inducing factor; DFF, DNA fragmentation factor; cyt c, cytochrome *c* (Shield and Mirkes, 1998).

In addition to the extensive studies of apoptotic cell death in animals, it is recently found that apoptosis can also occur in plants. In plants, apoptosis of single cells or small groups of cells occurs during normal development such as sex determination, gamete development, embryogenesis, formation of fluid-conducting channels called vessels and tracheids, and the hypersensitive response to pathogen infection (Jabs, 1999; Balk and Leaver, 2001). There is little evidence that plant mitochondria are involved in apoptosis. In a wide range of plant species, anther and/or pollen development is disrupted in a class of mutants termed CMS (cytoplasmic male sterility), which is associated with mutations in the mitochondrial genome. It is shown that the PET-CMS cytoplasm in sunflower causes premature apoptosis of the tapetal cells, which then extends to other anther tissues. In addition, immunocytochemical analysis revealed that cytochrome *c* was released partially from the mitochondria into the cytosol of tapetal cells before the gross morphological changes associated with apoptosis. The decrease in cytochrome *c* content in mitochondria isolated from male sterile florets precedeed a decrease in the integrity of the outer mitochondrial membrane and respiratory control ratio (Balk, Leaver, and McCabe, 1999; Balk and Leaver, 2001). Although little is known about apoptosis in plants, Balk and Leaver (2001) suggested that plant mitochondria, like mammalian mitochondria, play a key role in the induction of apoptosis. However, the association of mitochondria with apoptosis in plant cells still needs to be further investigated.

Mitochondria

Mitochondria are oval-shaped organelles containing the respiratory assembly, the enzymes of the citric acid cycle, and the enzymes of fatty acid oxidation. Mitochondria have two membrane systems: an outer membrane and an extensive, highly folded inner membrane. The inner membrane is folded into a series of internal ridges called cristae. Hence, there are two compartments in mitochondria: 1) the intermembrane space between the outer and inner membranes; and 2) the matrix, which is bounded by the inner membrane. Oxidative phosphorylation (see Appendix A) takes place in the inner mitochondrial membrane, in contrast with most of the reactions of the citric acid cycle and fatty acid oxidation, which occur in the matrix (Stryer, 1995).

The outer membrane is quite permeable to most small molecules and ions because it contains many copies of porin, a transmembrane protein with a large pore. In contrast, the inner membrane is intrinsically impermeable to nearly all ions and polar molecules. A large family of transporters shuttles metabolites such as ATP and citrate across the inner mitochondrial membrane. In normal condition, the membrane potential is negative on the matrix side and positive on the cytosolic side. Figure 10 shows the linkage between glycolysis pathway and mitochondria.

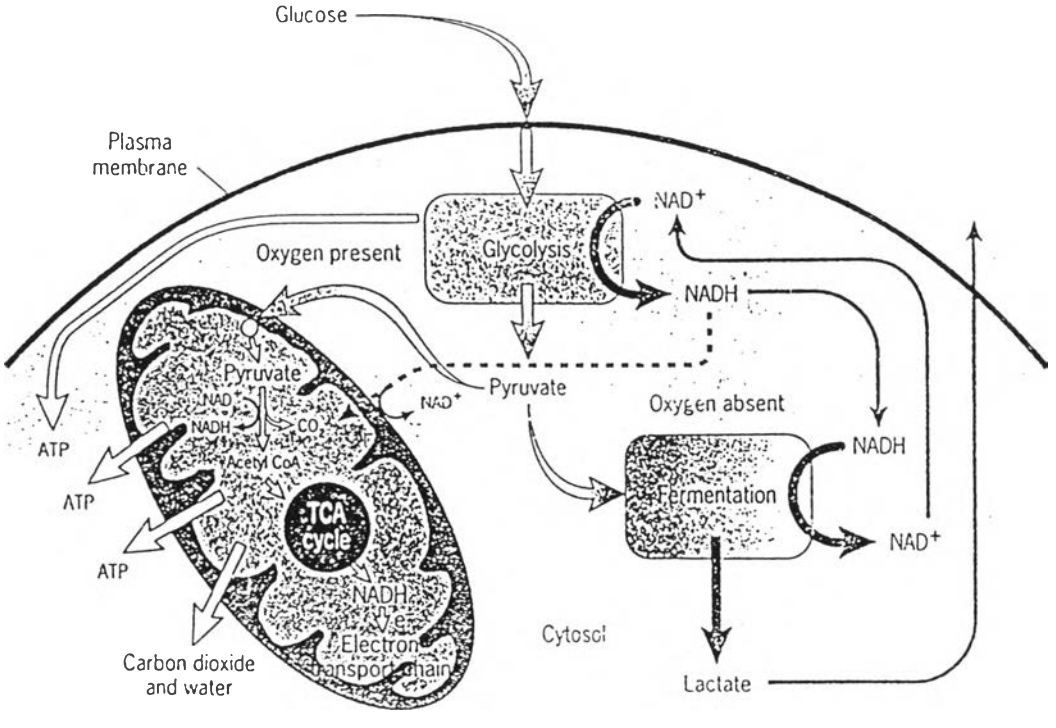

Figure 10 Glycolysis pathway and mitochondria. Glycolysis generates pyruvate and NADH in the cytosol. In the absent of oxygen, the pyruvate is reduced by NADH to lactate. In the presence of oxygen, the pyruvate moves into the mitochondrial matrix, where it is decarboxylated and linked to coenzyme A (CoA) and TCA cycle. The electrons in the generated NADH and FADH2 molecules are passed along the electron transport chain embedded in the inner mitochondrial membrane to molecular oxygen. The energy released during electron transport is used in the ATP formation (Karp, 1999).

Glycolysis is the sequence of reactions that converts glucose into pyruvate with the concomittant production of a relatively small amount of ATP (Figure 11). In aerobic organisms, glycolysis is the prelude to the citric acid cycle and the electron transport chain of mitochondria, which together harvest most of the energy contined in glucose. Under aerobic conditions, pyruvate enters mitochondria, where it is completely oxidized to carbon dioxide and water. If the supply of oxygen is insufficient, pyruvate is converted into lactate (Stryer, 1995).

The NADH and FADH2 formed in glycolysis, fatty acid oxidation, and the citric acid cycle are energy-riched molecules because each contains a pair of electrons having a high transfer potential. When these electrons are donated to molecular oxygen, a large amount of free energy is liberated, which can be used to generate ATP (Stryer, 1995) as shown in the Appendix A section.

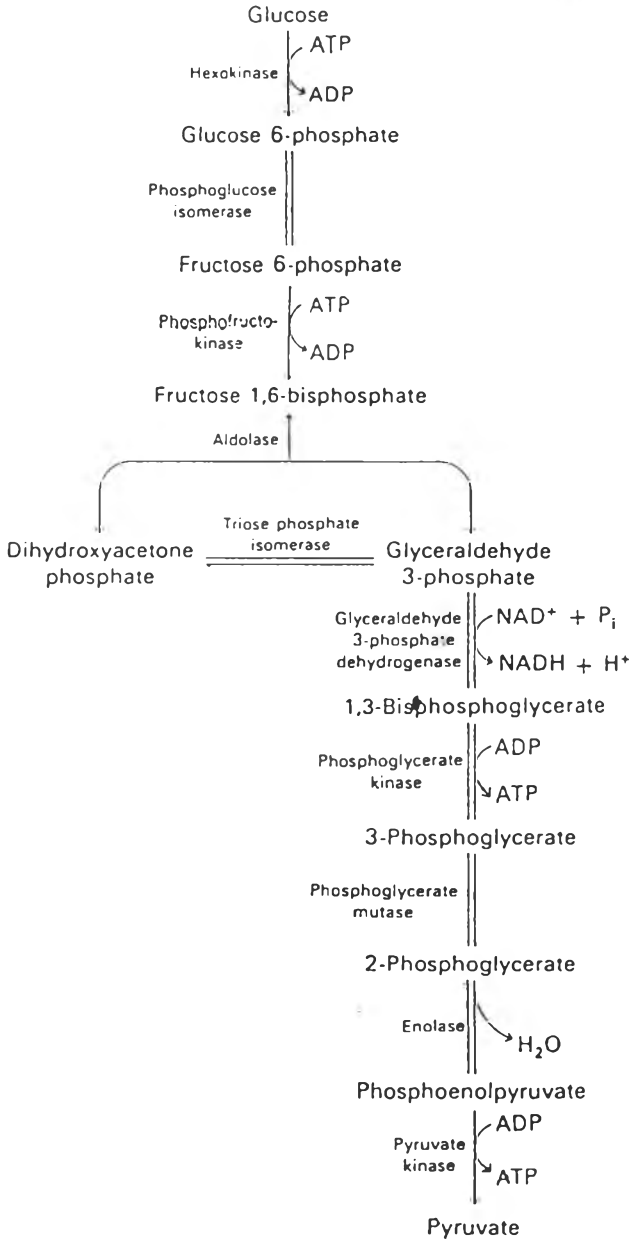

Figure 11 Glycolysis pathway.
(Stryer, 1995)

Role of Mitochondria in Apoptosis

A variety of key events in apoptosis focus on mitochondria, including the release of caspase activators (such as cytochrome *c*), changes in electron transport, loss of mitochondria transmembrane potential, alteration of cellular oxidant-reduction, and participation of pro- and antiapoptotic Bcl-2 family proteins. The different signals that converge on mitochondria to trigger or inhibit these events and their downstream effects delineate several major pathways in physiological cell death (Green and Reed, 1998).

Mitochondria and Permeability Transition Pore

In many apoptosis scenarios, the mitochondrial inner transmembrane potential ($\Delta\Psi m$) collapses, indicating the opening of a large conductance channel known as the mitochondrial permeability transition (PT) pore. The structure and composition of the PT pore remain partially defined, but its constituents include both inner membrane proteins, such as the adenine nucleotide translocator (ANT); and outer membrane proteins, such as porin (voltage-dependent anion channel; VDAC), which operate in concert, presumably at inner and outer membrane contact sites, and create a channel through which small molecules ($\leq$ 1.5 kDa) can pass. Opening of this nonselective channel in the inner membrane space of mitochondria dissipates the H^+ gradient across the inner membrane and uncouples the respiratory chain. Perhaps more importantly, PT pore opening results in a volume dysregulation of mitochondria due to the hyperosmolality of the matrix, which causes the matrix space to expand. Because the inner membrane with its folded cristae possesses a larger surface area than the outer membrane, this matrix volume expansion can eventually cause outer membrane rupture, releasing caspase-activating proteins (e.g. cytochrome *c* and AIF) located within the intermembrane space into the cytosol (Green and Reed, 1998; Bratton and Cohen, 2001).

Inhibitors of PT pore opening appear to block apoptosis in some systems, thus providing support for the idea that the PT pore is central to apoptotic precesses. For example, Bcl-2 can prevent the PT pore, whereas the ANT-activator attractyloside and Bax induce both apoptosis and PT pore. In addition, other stimuli that affect the PT pore directly, such as oxidants and pathological elevations in cytosolic Ca^{2+}, can induce

rupture of the outer membrane of mitochondria and release of caspase-activating proteins. Thus, in many cases, the PT pore appears to be the mastermind that orchestrates apoptosis.

However, some studies have provided evidence that cytochrome *c* release and caspase activation can occur before any detectable loss of $\Delta\Psi m$, implying that PT pore opening may occur downstream of apoptosome-mediated caspase activation. The ability of caspases to induce PT pore opening, which in turn can induce caspase activation (by release of cytochrome *c* and AIF), creates opportunities for a feedforward amplification loop and complicates attempts to dissect the normal sequence of cell death events.

The release of cytochrome *c* before or in the absence of a drop in $\Delta\Psi m$ in some cells suggests that different regulatory events control permeability of the inner and outer mitochondrial membranes. This event can be explained that a rapid opening and closing of the PT pore at its reversible low conductance state may allow a repeated, respiration-driven reestablishment of $\Delta\Psi m$ so that outer membrane disruption and cytochrome *c* release can occur before $\Delta\Psi m$ collapse.

Furthermore, another mechanism of outer membrane disruption has recently been suggested, and it involves hyperpolarization of the mitochondrial inner membrane. How the hyperpolarization occurs is obscure; nevertheless, increased export of protons into the intermembrane space may result in protonation of weak acids. These can then freely diffuse across the inner membrane and are trapped when the protons are lost (and exported). As these metabolites accumulate, osmolality increases and water enters, resulting in matrix space expansion and eventually rupture of the outer membrane, thereby releasing all contents of the intermembrane space (Green and Reed, 1998).

The hypothesis that disruption of mitochondrial metabolism can regulate cytochrome *c* release is based on the suggestion that mitochondrial metabolism is altered during apoptosis. Normally, VDAC and ANT coordinately shuttle ADP into the matrix from the cytosol in exchange for ATP. Following physiological withdrawal of growth factors, however, VDAC takes on a closed conformation, limiting the uptake of complex anions across the outer mitochondrial membrane. Because ANT continues to transfer ATP and ADP across the inner mitochondrial membrane, normal $\Delta\Psi m$ is

maintained until all of the available ADP in the matrix has been consumed. It is proposed that if the electron transport chain continues to operate in the absence of ADP, it might lead to an increase in $\Delta\Psi m$ or 'hyperpolarization' and enhanced generation of reactive oxygen species. Both these factors might stress the inner mitochondrial membrane and lead to an increase in the influx of water into the matrix, followed by swelling and rupture of the outer mitochondrial membrane. In contrast to the PT pore hypothesis, in this model Bcl-2 and/ or Bcl-XL are proposed to maintain VDAC in an open conformation to facilitate ATP and ADP exchange across the outer mitochondrial membrane, and thus inhibit mitochondrial hyperpolarization, cytochrome *c* release and apoptosis (Bratton and Cohen, 2001).

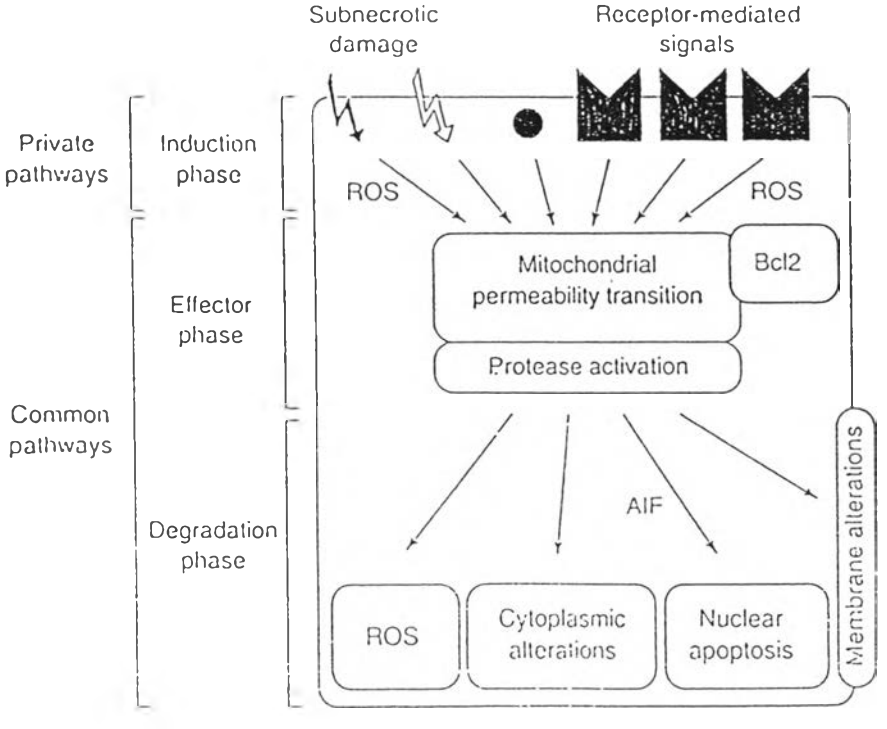

Figure 12 Mitochondrial permeability transition pore and apoptosis regulation. As a consequence of PT, mitochondria release an AIF (apoptosis-inducing factor) that causes signs of nuclear apoptosis in a cell-free system. In addition, PT causes the mitochondrial generation of ROSs, and rapid expression of phosphatidyl serine residues in the outer plasma membrane leaflet. PT is probably linked to the activation of specific proteases, enhancement of ROS generation, cytoplasmic changes (depletion of reduced glutathione and increase in cytosolic calcium), and nuclear apoptosis. These events are all secondary to PT pore, however not linked to each other in a cause-effect relationship (Kroemer, Zamzami, and Susin, 1997).

Bcl-2 (Schendel et al., 1997)

As mentioned before, Bcl-2 proteins are one of apoptosis-regulating proteins. These proteins appear to block a distal step in a common pathway for apoptosis and programmed cell death, with some functioning as suppressors (Bcl-2, Bcl-XL, Mcl-1, Ced-9, BHRF-1, E1b-19 kDa, A1, ASFV-5HL, Bcl-W, and NR13) and others as promoters (Bax, Bcl-XS, Bak, Bad, Bik, and Bid) of cell death. In many cases, these proteins can interact with each other in a complex network of homodimers and heterodimers. Aberrant expression of Bcl-2 and some of its homologs has been described in association with several disease states characterized by either excessive accumulation of cells or inappropriate cell death, including cancer, autoimmunity, ischemic diseases (stroke, myocardial infarction), HIV-associated immunodeficiency, and some neurodegenerative diseases.

Bcl-2 and most of its homologs contain a stretch of hydrophobic residues near their carboxyl termini that anchor them in intracellular membranes, primarily the outer mitochondrial membrane, nuclear envelope, and parts of the endoplasmic reticulum. These membrane locations, coupled with evidence that Bcl-2 can regulate Ca^{2+} fluxes and protein translocation across membranes, has prompted speculations that Bcl-2 family proteins may be involved in some aspect of either ion or protein transport. In addition, the present biophysical evidence indicates that the antiapoptotic protein Bcl-2 forms channels in membranes.

Bcl-2 is particularly abundant at the contact sites that adjoin the inner and outer mitochondrial membranes where a substantial proton gradient exists, hence pH is suggested as a channel modulator. Another potential regulator of Bcl-2's channel activity besides pH is proteins that have been reported to bind to Bcl-2. Thus, interactions with proteins such as BAG-1, which enhance Bcl-2's function as an antiapoptotic protein, may promote pore formation of Bcl-2.

Many questions remain unanswered about the relationship between of Bcl-2's channel activity and apoptosis suppression, for example, what is Bcl-2 intended to transport across membranes in cells? However, the preliminary data suggest that Bcl-2 allows transport of an ion or a protein across membranes in a direction that is cytoprotective.

p53 and apoptosis (Bratton and Cohen, 2001)

As previously mentioned, DNA damage, induced by irradiation, drugs or environmental toxicants, frequently induces cell death by apoptosis. However, cells have mechanisms involved with initial recognition and assessment of the damage, followed by an appropriate response that involves either DNA repair or cell death. The nuclear protein p53 is one of key sensors of DNA damage, it integrates numerous signals that are crucial to the control of life and death of the cell. The p53 is activated, in part, by various signals that induce its dissociation from its inhibitor. Once activated, p53 binds to specific sequences in DNA and initiates the trascription of many genes involved in genetic stability, cell-cycle inhibition, and apoptosis. For instance, p53 induces or maintains growth arrest through increased expression of cell-cycle regulators, such as p21WAF1/CIP1, an inhibitor of CDKs.

A recent study suggests that p53 relocalizes to mitochondria during p53-dependent apoptosis, preceding changes in mitochondrial membrane potential and cytochrome *c* release. Therefore, the possibility exists that p53 might also induce apoptosis through transcriptionally independent mechanisms.

Mitochondria are pivotal in controlling cell life and death. At least following three general mechanisms are known, and their effects may be interrelated:

1) Disruption of electron transport, oxidative phosphorylation, and ATP production

Disruption of electron transport has been recognized as an early feature of cell death. For example, γ-irradiation induces apoptosis in thymocytes and a disruption in the electron transport chain, probably at the cytochrome b-c_1/ cytochrome *c* step, is observed. Ceramide (a second messenger implicated in apoptosis signaling) disrupts electron transport at the same step in cells as well as in isolated mitochondria. In addition, ligation of Fas also leads to a disruption in cytochrome *c* function in electron transport.

One consequence of the loss of electron transport should be a drop in ATP production. Although such a drop has been observed during apoptosis, it often occurs relatively late in the process. Thus, although loss of mitochondrial ATP production can kill a cell, it is unlikely that this is a mechanism for induction of apoptosis.

2) Release of caspase-activating proteins (Green and Reed, 1998)

The importance of mitochondria in apoptosis has been suggested by many studies. It is shown that the cytochrome *c* released from mitochondria initiates formation of the "apoptosome," which is composed of cytochrome *c*, apoptotic protease-activating factor-1 (Apaf-1), and procaspase-9. The apoptosome in turn activates caspases-9, which then processes and sequentially activates other caspases (e.g. caspase-3 and -7) to orchestrate the biochemical execution of cells. Moreover, another caspase-activating protein found to be released from mitochondria is AIF, which is later revealed as a caspase enzyme.

However, the consequences of cytochrome *c* release may depend on the cell type. In those cells where cytochrome *c* is available in excess, caspases can be activated via mechanism involving Apaf-1 and enough cytochrome *c* may remain docked by its high-affinity binding sites to cytochrome b-c_1 and cytochrome *c* oxidase to maintain electron transport. In this case, oxygen consumption and ATP production may continue unabated while caspases attack on cytosolic and nuclear substrates, resulting in apoptosis. Alternatively, in cells that contain large quantities of endogenous caspase inhibitors, release of cytochrome *c* may fail to induce caspase-dependent apoptosis and instead the eventual loss of electron chain transport, generation of oxygen free radicals, and decreased production of ATP may drive the cell toward a necrotic demise.

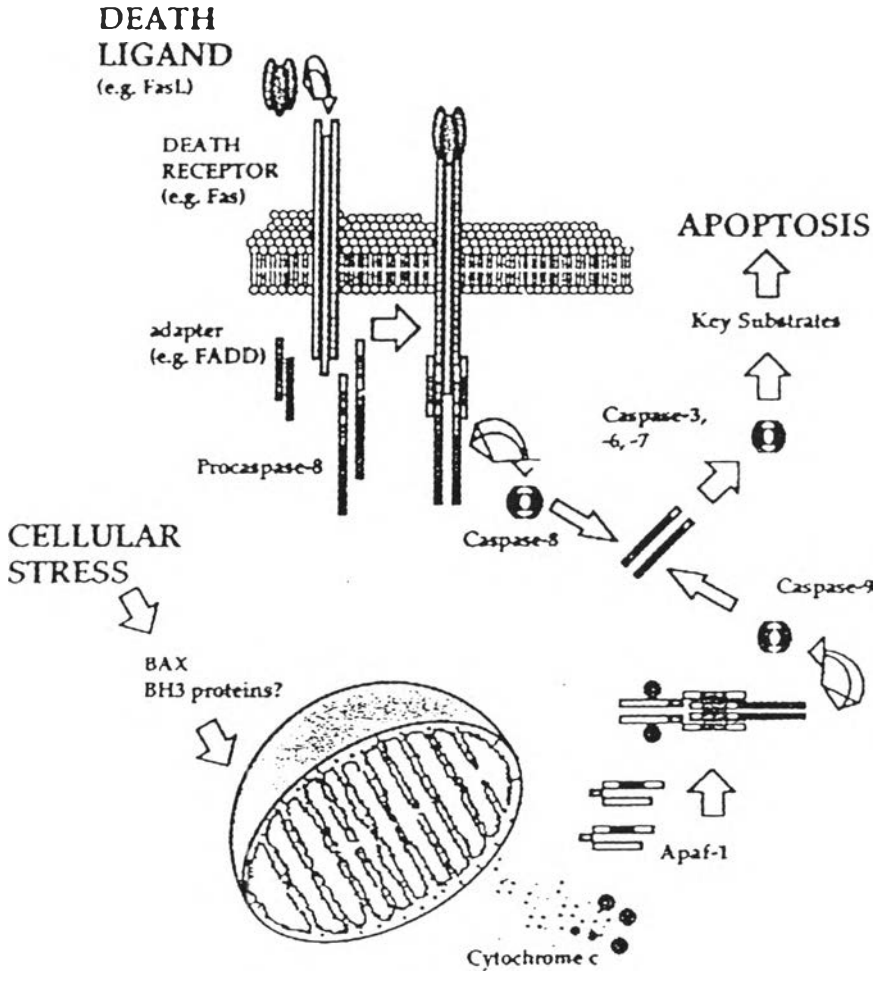

Figure 13 Caspase activation and apoptosis. The downstream caspases can be activated by two independent apoptosis pathways: (i) ligation of death receptors by their ligands; and (ii) Apaf-1 and the cytochrome *c* released from cellular stress-triggered mitochondria (Green and Reed, 1998).

3) Reactive oxygen species and alteration of cellular redox potential

Mitochondria are the major source of superoxide anion production in cells. During transfer of electrons to molecular oxygen, and estimated 1 to 5% of electrons in the respiratory chain lose their way, most participating in formation of O_2^-. Anything that decreases the coupling efficiently of electron chain transport can therefore increase production of superoxides.

Superoxides and lipid peroxidation are shown increased during apoptosis induced by myriad stimuli. Although generation of reactive oxygen species (ROSs) may be a relatively late event (occurring after cells have embarked on a process of caspase activation), their role in apoptosis cannot be excluded.

Reactive Oxygen Species

Reactive oxygen species (ROSs) have long been known as potent mediators of apoptosis in various animal systems and in plant systems. It is clear that antioxidants and antioxidative enzymes can protect cells under various death-promoting condition. Recent findings suggest that ROSs have two roles in the apoptotic process: (i) ROSs participate in diverse signal-specific pathways during the induction phase of apoptosis and serve as inducers of PT pore; and (ii) ROSs are one of several apoptogenic consequences of PT pore translating to the structural changes of the degradation phase of apoptosis. Recent findings indicate that the prototypic regulators of mammalian cell death, Bcl-2 and its homologues, exert their anti- or proapoptotic function by direct regulation of the PT pore opening probability or by modulation of the ion permeability of the mitochondrial membranes (Jabs, 1999). (See also Figure 13)

Oxidative Stress

Oxidative Stress is a general term used to describe a state of damage caused by ROSs. This damage can affect a specific molecule or the entire organism. The ROSs, such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist in all aerobic organisms. There are many different sources of ROSs that can cause oxidative damage to an organism. Most ROSs come from endogenous sources as by-products of normal and essential reactions, such as

energy generation from mitochondria or the detoxification reactions involving the liver cytochome P-450 system. Exogenous sources include bacterial, fungal or viral infections.

However, biologic systems have defensive mechanisms to either block free radical formation or scavenge them once they have formed as shown in Figure 14 (Kumar et al., 1997).

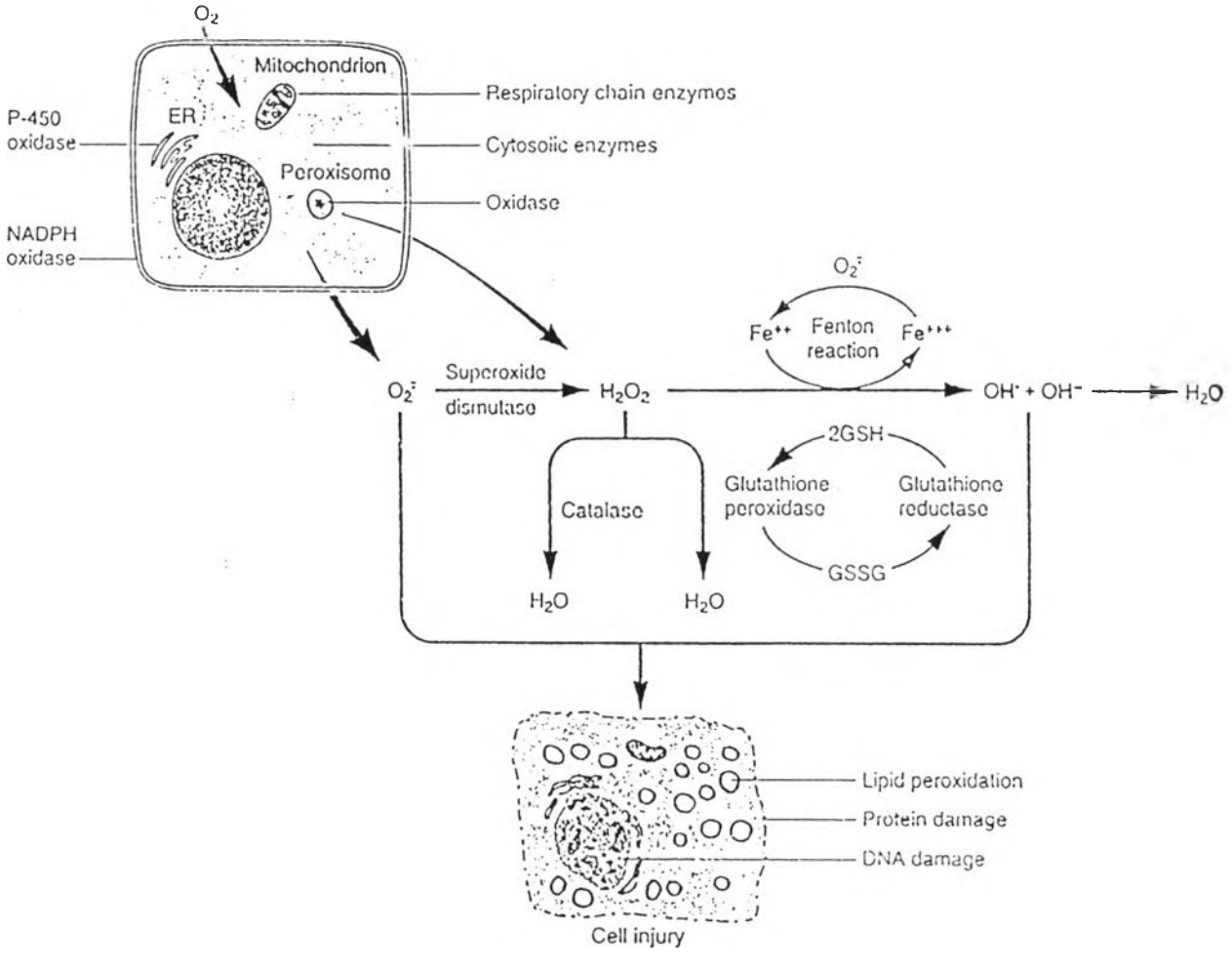

Figure 14 Formation of ROSs and antioxidant mechanisms in biologic systems. GSH, reduced glutathione; GSSG, oxidized glutathione; NADPH, reduced form of nicotinamide-adenine dinucleotide phosphate (Kumar et al., 1997).

From Figure 14, oxygen (O_2) is converted to superoxide ($O_2^{\cdot-}$) by oxidative enzymes in the endoplasmic reticulum (ER), mitochondria, plasma membrane, peroxisomes, and cytosol. $O_2^{\cdot-}$ is converted to H_2O_2 by superoxide dismutase (SOD) and then to $OH^{\cdot}$ by the Cu^{2+}/Fe^{2+} catalyzed Fenton reaction. H_2O_2 is also derived directly from oxidases in peroxisomes. The resultant free radicals can damage lipid (peroxidation), proteins, and DNA. Note that $O_2^{\cdot-}$ catalyzes the reduction of Fe^{3+} to Fe^{2+}, thus enhancing $OH^{\cdot}$ generation by the Fenton reaction. The major antioxidant enzymes are SOD, catalase, and glutathione peroxidase.

To study about what happens to a cell when it encounters oxidative stress conditions, it is recommended that mammalian red blood cell provides a valuable model system. Since mature mammalian erythrocytes are non-nucleated and devoid of organelles, they represent one of the simplest cell types for biochemical study (Stern, 1985). (The oxidative stress in erythrocytes is described in the Appendix B.)

Cell Signaling

Cells need to communicate with their neighbors, monitor the conditions in their environment, and respond appropriately to different stimuli that impinge on their surface. Apparently, cells carry out these interactions by a phenomenon known as *cell signaling*, in which information is relayed across the plasma membrane to the cell interior and often to the cell nucleus.

In most systems, cell signaling includes:

1. Recognition of the stimulus at the outer surface of the plasma membrane by a specific receptor embedded in the membrane.
2. Transfer of a signal across the plasma membrane to its cytoplasmic surface.
3. Transmission of the signal to specific effector molecules on the inner surface of the membrane or within the cytoplasm that trigger the cellular response.
4. Cessation of the response as a result of the destruction or inactivation of the signaling molecule, combined with a decrease in the level of the extracellular stimulus.

However, not all information is transmitted from the extracellular space to the cell interior by way of a cell-surface receptor. Steroid hormones, for example, act on target cells by diffusing through the plasma membrane and binding to a cytoplasmic

receptor. Conversely, a few extracellular agents can evoke cellular response directly without having to initiate a signal inside the cell. The neurotransmitter acetylcholine acts by such a mechanism when it binds to a receptor on a skeletal muscle cell, opening an ion channel that lies within the receptor itself (Karp, 1999).

Depending on the type of cell and stimulus, the response may involve a change in gene expression, an alteration of the activity of metabolic enzymes, a reconfiguration of the cytoskeleton, a change in ion permeability, the activation of DNA synthesis, or even the death of the cell.

It is known that cell signaling can affect virtually every aspect of cell structure and function, and is also intimately involved in the regulation of cell growth and division. Therefore, the study of cell signaling is important in understanding how a cell can lose growth control and develop into a malignant tumor.

Signal transduction is a complex process in which information is passed along signaling pathways that consist of a series of distinct proteins. Each protein in the pathway typically acts by altering the conformation of the next protein in the series, an event that activates or inhibits that protein. There are different types of signal transduction pathways: G protein-coupled receptors, receptor tyrosine kinases, and signaling originated from contacts between the cell surface and the substratum (Karp, 1999; Cooper, 2000). These signaling pathways are not linear pathways leading directly from a receptor at the cell surface to an end target. However, in fact, signaling pathways in the cell are much more complex as described below:

- Convergent signaling pathway: signals from a variety of unrelated receptors can converge to activate a common effector.

- Divergent signaling pathway: signals from the same ligand can diverge to activate a variety of different effectors and lead to diverse cellular responses.

- Crosstalk between signaling pathways: signals can be passed back and forth between different pathways.

Mitogen-Activated Protein Kinase (MAPK) Pathways

The MAPK pathways refers to cascades of protein kinases that are highly conserved in evolution and play central roles in signal transduction in all eukaryotic cells, ranging from yeasts to humans. Recently, identified MAPK family members include extracellular signal-regulated kinase (ERK), c-JUN NH2-terminal protein kinase or stress-activated protein kinase (JNK/SAPK), and p38 MAPK. The central elements in the pathways are a family of protein-serine/threonine kinases that are activated in response to a variety of growth factors and other signaling molecules (Figure 15). In yeasts, MAPK pathways control a variety of cellular responses, including mating, cell shape, and sporulation. In higher eukaryotes (including *C. elegans*, *Drosphila*, frogs, and mammals), the MAPK pathways are important for the control of cell growth, differentiation, and cell death (Cooper, 2000). The dynamic balance between and the integration of these pathways are important in determining whether a cell survives or undergoes apoptosis (Xia et al., 1995; Chen, Woodruff, and Mayo, 2000).

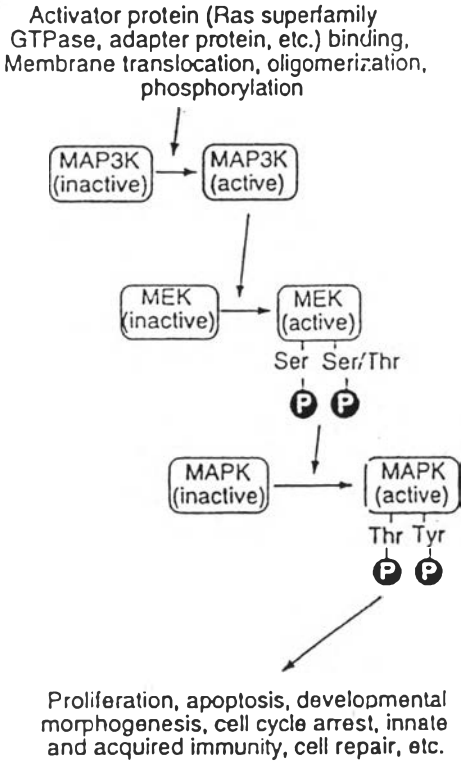

Figure 15 The mitogen-activated protein kinase (MAPK) core signaling module. Divergent inputs feed into core MAPK-kinase-kinase (MAP3K) → MAPK-kinase (MEK) → MAPK core pathways which then recruit appropriate responses (Kyriakis and Avruch, 2001).

Nonreceptor Protein-Tyrosine Kinases

Nonreceptor protein-tyrosine kinases are soluble intracellular protein-tyrosine kinases which are non-covalently associated with receptors. They are intermediate conductors of diverse intracellular signal pathways. Many of them are associated with transmembrane receptors, such as hormone receptors, cytokine receptors, and growth factor receptors. There are various classes of nonreceptor protein-tyrosine kinases grouped according to their structural and functional properties with the Src protein as a typical representative of them. In recent years, a considerable body of evidence has been obtained and indicates roles of nonreceptor protein-tyrosine kinases in different cellular processes, such as cell migration, activation of MAPK cascades, induction of DNA synthesis, regulation of the G_2/M transition, and prevention of apoptosis (McCubrey et al., 1993; Frisch and Francis, 1994; Altun-Gultekin and Wagner, 1996; Taylor and Shalloway, 1996; Broome and Hunter, 1997; Klemke et al., 1997; Schlaepfer, Broome, and Hunter, 1997). The nonreceptor protein-tyrosine kinase Src has been now also implicated in the signal switching from G protein-coupled receptor to the MAPK pathway (Fan et al., 2001).

The nonreceptor protein-tyrosine kinases are activated by means of association of receptors with extracellular ligands or cell adhesion components at particular phases of the cell cycle (Wilks, 1993; Shawver, Strawn, and Ullrich , 1995; Taniguchi, 1995). They are mostly required for signaling from cytokine receptors (e.g. interleukin-2 and interleukin-6) and from some polypeptide hormone receptors (e.g. growth hormone).

The first step in signaling from cytokine receptors is thought to be ligand-induced receptor dimerization and cross-phosphorylation of the associated nonreceptor protein-tyrosine kinases. These activated kinases then phosphorylate the receptor, providing phosphotyrosine-binding sites for the recruitment of downstream signaling molecules that contain SH2 domains.

It is well-defined that the Janus kinase (JAK) family members are the nonreceptor protein-tyrosine kinases required for signaling from cytokine receptors. The JAK family kinases play a critical role in coupling these cytokine receptors to the tyrosine phosphorylation of intracellular targets as discussed below (Xu et al., 1998; Cooper, 2000).

The JAK/STAT Pathway

The MAPK pathway provides an indirect connection between the cell surface and the nucleus, in which a cascade of protein kinases ultimately leads to transcription factor phosphorylation. An alternative pathway, known as the JAK/STAT pathway, provides a much more immediate connection because the protein-tyrosine phosphorylation directly affects transcription factor localization and function.

The key elements in this pathway are the STAT (signal transducers and activators of transcription) proteins. These proteins are a family of transcription factors that contain SH2 domains. They are inactive in unstimulated cells, where they are localized to the cytoplasm. Stimulation of cytokine receptors leads to recruitment of STAT proteins, which bind cytoplasmic domains of receptor polypeptides. Following their association with activated receptors, the STAT proteins are phosphorylated by members of the JAK family of nonreceptors. Tyrosine phosphorylation promotes the dimerization of STAT proteins, which then translocate to the nucleus, where they stimulate transcription of their target genes. (See Figure 16)

Further studies have shown that STAT proteins are also activated downstream of growth factor receptors, where their phosphorylation may be catalyzed either by the receptors themselves or by associated non-receptor tyrosine kinases. The STAT transcription factors thus serve as direct links between both cytokines and growth factor receptors on the cell surface and regulation of gene expression in the nucleus (Cooper, 2000).

Interleukin-6

Interleukin-6 (IL-6) is a multifunctional cytokine having pivotal roles in the immune response, inflammation, hematopoiesis, nervous system, cardiovascular system, and endocrine system. It is a well-known autocrine and paracrine that acts on various cells: IL-6 induces the differentiation of B cells to antibody producing plasma cells, T-cell growth and differentiation, the differentiation of myeloid leukemic cell lines into macrophages, megakaryocyte maturation, the neural differentiation of PC12 cells, the development of osteoclasts, and acute-phase protein synthesis in hepatocytes. Although IL-6 is involved in many biological activities, it is critical in some biological

reactions: the acute-phase response, the mucosal IgA response, the fever response, and estrogen deficiency-induced bone loss. IL-6 also acts as a growth factor for multiple myeloma (MM) plasma cells, keratinocytes, mesangial cells, renal cell carcinoma, and Kaposi's sarcoma, and promotes the growth of hematopoietic stem cells (Chauhan et al., 1997; Xu et al., 1998).

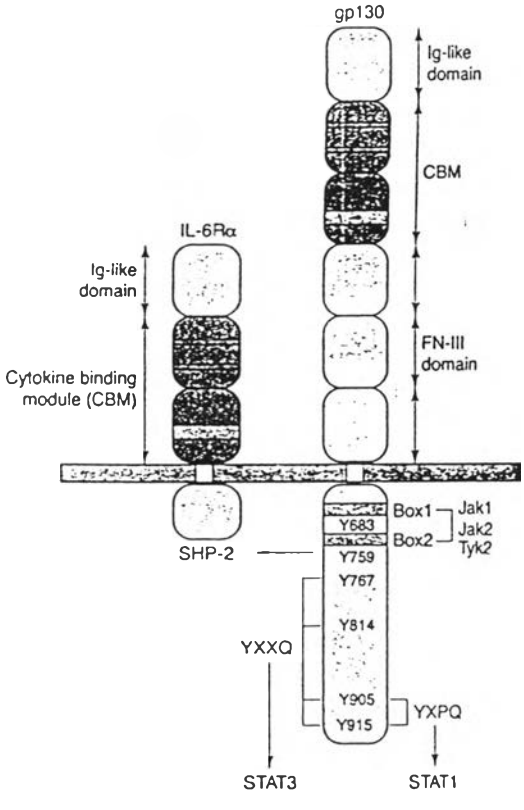

Figure 16 IL-6 receptor system. (Oppenheim et al., 1999, vol. 2)

The IL-6 receptor system consists of an 80 kDa IL-6 binding molecule termed IL-6Ralpha (IL-6Rα) or gp180, and a signal transducer, gp130 (Taga et al., 1989; Hibi et al., 1990). The cytoplasmic domain of IL-6Rα is not necessary for signal transduction; however, the gp130 which is a membrane-associated glycoprotein, functions as a signal transducing subunit of the IL-6R system (Taga et al., 1989; Sugita et al., 1990) (Figure 16). The binding of IL-6 to its receptor triggers the association of IL-6Rα with the gp130, then homodimerization of the gp130 occurs. This results in the signal transduction via various pathways involved in the IL-6-regulation of cell growth, survival, and differentiation (as shown in Figure 17). These pathways are: 1) activation of the intracytoplasmic Janus family of tyrosine kinases (JAKs) followed by downstream signaling via tyrosine phosphorylation of the signal transducers and activators of transcription factors (STAT); 2) Ras/MAPK (mitogen-activated protein kinase) pathway; 3) Src family tyrosine kinases; and 4) phosphatidylinositol-3-kinase (PI-3 kinase) pathway.

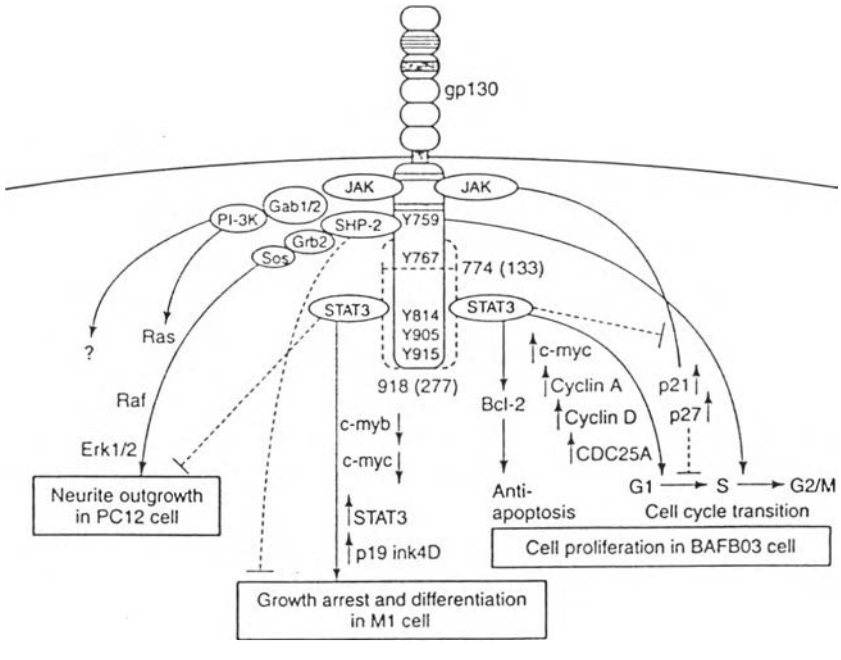

Figure 17 Signal transduction pathways involved with the IL-6 receptor. Distinct cytoplasmic regions of gp130 are involved in different signal transduction pathways (Oppenheim et al., 1999, vol. 1)

At present, it is found that the G_1-S cell cycle transition is being controlled by STAT3 of the JAK/STAT pathway, and the regulation of kinase cascades involving MAPKs is implicated as important regulatory pathways of apoptosis (Taga et al., 1989; Fukada et al., 1996; Urashima et al., 1997; Xu et al., 1998; Chauhan et al., 2000). Moreover, the interplay among these pathways are also found critically involved in the biological activities of IL-6.

Many studies show that IL-6 is capable of inhibiting MM plasma cell apoptosis as well as stimulating some MM cell types to proliferate. Chauhan et al. (1997) showed that SAPK and p38 MAPK were activated by anti-*fas* during apoptosis of MM plasma cells, and IL-6 was able to protect MM cells against anti-*fas*-induced apoptosis by inhibition of anti-*fas*-induced SAPK activation. In similar fashion, Xu et al. (1998) also found that JNK activity and subsequent c-*jun* transactivation induced during MM cell apoptosis was inhibited by protective concentrations of IL-6. The data showed that IL-6 protected MM cells from anti-*fas* through its inhibition of JNK activation and subsequent c-*jun* activity. Although the anti-apoptotic concentrations of IL-6 could inhibit JNK/SAPK activation, it was however shown having no effect on the activation of p38 MAPK (Chauhan et al., 1997). It is now evident that IL-6 mediates growth of MM cells via the Ras dependent MAPK cascade and phosphorylation of pRb, a cell cycle regulatory protein (Chauhan et al., 1997; Urashima et al., 1997; Xu et al., 1998).

In addition to its effect on inhibition of apoptosis in MM cells, IL-6 has also been reported to inhibit apoptosis in other cell types, e.g. thymic lymphoma cell lines, normal T cells, and renal cell carcinoma (RCC) (Van Snick et al. 1996; Takeda et al., 1998; Steiner et al., 1999). Narimatsu et al. (2001) reported that *stat3* gene activation induced by IL-6 is an important antiapoptotic signal in T cells of the spleen, especially in the red pulp region.

Calcium and Intracellular Signaling

Calcium (Ca^{2+}) is an extremely common second messenger. Recently, it is known that the inositol 1,4,5-triphosphate (IP_3)-mediated release of Ca^{2+} from the endoplasmic reticulum (ER) is not the only mechanism by which the intracellular Ca^{2+} levels ($[Ca^{2+}]i$) can be increased. In many cells, the transient increase in $[Ca^{2+}]i$ resulting from production of IP_3 is followed by a more sustained increase resulting from

extracellular Ca^{2+} entry. The resulting increases in $[Ca^{2+}]i$ then trigger the further release of Ca^{2+} from intracellular stores by activating distinct Ca^{2+} channels known as ryanodine receptors (Cooper, 2000).

The increase of cytosolic Ca^{2+} levels can affect the activities of a variety of target proteins, including protein kinases and phosphatases. Many studies show that alterations in $[Ca^{2+}]i$ homeostasis by increasing $[Ca^{2+}]i$ are associated with mitochondrial damage and apoptosis. McConkey and Orrenius (1997) reported that Ca^{2+} chelators and inhibitors of the endoplasmic reticular Ca^{2+}-ATPase could inhibit caspase activation, DNA fragmentation, and cell death. The depletion of $[Ca^{2+}]i$ from the ER, thought to be an early event in the apoptotic pathway, has been shown to be sufficient to induce apoptosis by causing the subsequent depletion of Ca^{2+} from mitochondrial stores. In addition, anti-apoptotic members of the Bcl-2 family have been shown to regulate Ca^{2+} compartmentalization in both ER and mitochondria by unclear mechanism (Baffy et al., 1993; He et al., 1997). One possible model is that Bcl-2 prevents the opening of Bax-induced channels, preventing Ca^{2+} efflux from the mitochondria. Therefore, outflow from intracellular stores and influx of Ca^{2+} across the plasma membrane could result in a sustained Ca^{2+} increase that acts as a signal for apoptosis (Baffy et al., 1993; He et al., 1997).

Sulfhydryl Groups and Roles in Intracellular Signaling

Sulfhydryl groups (-SH) are found as major components of enzymes and proteins. Reduced glutathione (GSH) is another example of an important peptide containing sulfhydryl group. GSH is very important in a variety of cellular processes: protection cells from oxidative damage, activation of sulfhydryl requiring enzymes; action as a co-enzyme; participation in the γ-glutamyl cycle; formation of mixed disulfides with proteins containing internal disulfide bonds; and control of microtubule formation. An increase in oxidised glutathione (GSSG) leads to microtubule disassembly which is involved during cell division; therefore, compounds that affect glutathione can possibly affect the cell division cycle. It is believed that reagents reacting with sulfhydryl groups are potent cell poisons (Zmuda and Friedenson, 1983).

Mitochondrial GSH plays and important role in the maintenance of cell functions and viability by metabolism of oxygen free radicals generated by the

respiratory chain. Loss of GSH, especially mitochondrial GSH, is associated with increased production of reactive oxygen species and cell toxicity. In the absence of GSH, pyruvate can increase cell injury by damaging the mitochondria, presumably as a consequence of enhanced electron flow and reactive oxygen production by the respiratory chain (Mari, Bai, and Cederbaum, 2002).

Lavrentiadou et al. (2001) have reported that the depletion of GSH can induce elevation of ceramide levels and apoptosis. In addition, Liu, Shen, and Ong (2001) showed that an intracellular thiol depletion could induce mitochondrial PT pore, subsequent increase in ROS generation, and eventually apoptotic cell death. However, pretreatment of cells with N-acetylcysteine (NAC), the GSH synthesis precursor, conferred complete protection against $\Delta\Psi m$ loss, ROS generation and apoptosis.

Thiol oxidation and crosslinking can cause a covalent modification of a critical cysteine residue at ANT, an important protein that controls the opening of PT pore, and lead to the pore opening and apoptosis (Belzacq et al., 2001; Moreno et al., 2001).

In addition, chemicals reacting with the -SH groups can cause a $\Delta\Psi m$ collapse and calcium release from mitochondria, which can be followed by cell death (Rizzuto, Pitton, and Azzone, 1987).

Spleen

The spleen is a specialized connective tissue located in the left hypochondriac, between the fundus of the stomach and the diaphragm. Anatomically, the spleen is positioned between the systemic circulation, from which it receives its arterial supply through the celiac trunk, and the portal circulation, into which the splenic vein drains. The spleen is composed of a three-dimensional reticular meshwork that serves as a filter in the path of blood circulation. It selectively clears the blood of cells, microorganisms, and antigens in distinctive stromal meshes–termed filtration beds. These beds are supplied by unique open-ended arteries and drain by veins and lymphatic vessels. In humans, the spleen does not have a major reservoir function under normal conditions, so the volume of blood present at any one time is relatively small. Depending on the species, 1 to 10 percent of the cardiac output flows through the spleen. In humans, the spleen receives 6 percent of the cardiac output, or about 300 ml/min. Functionally the spleen is a secondary lymphoid organ, which is the site where lymphocyte and antigen-presenting cell interaction occur with subsequent dissemination of the immune response (Henry and Symmers, 1992; Cuschieri and Forbes, 1994).

Functions of the Spleen (Cuschieri and Forbes, 1994)

The spleen functions in both the immune and the hematopoietic system as described below:

Immune System Functions of the Spleen:

1. Proliferation of lymphocytes
2. Production of humoral antibodies
3. Removal of macromolecular antigens from the blood

Hematopoietic Functions of the Spleen:

1. Formation of blood cells during fetal life
2. Removal and destruction of senile, damaged, and abnormal red blood cells and platelets
3. Retrieval of the iron from red cell hemoglobin
4. Storage of blood, especially red blood cells, in some species

The spleen is subdivided into the red pulp and the white pulp. Between the red and white pulp, there is a distinguishable marginal zone (MZ), which is more prominent in rats than in mice or humans, and which provides an important interface between the lymphoid and red pulp systems. Splenic arterial blood circulates through the trabecular vassels into the white pulp, then travels into the red pulp, before entering the venous system (Figure 18, 19).

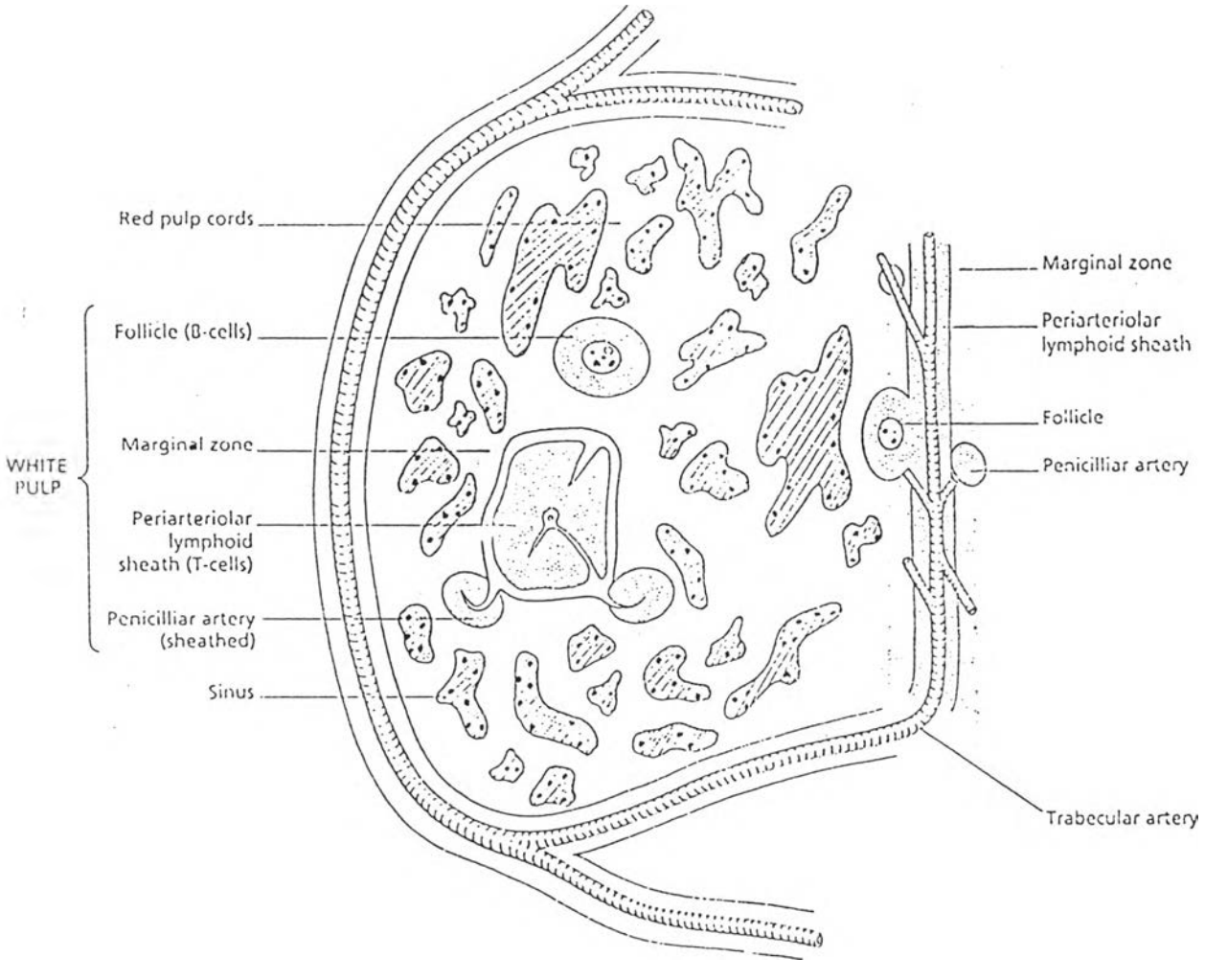

Figure 18 Histological appearance of red and white pulp. (Cuschieri and Forbes, 1994)

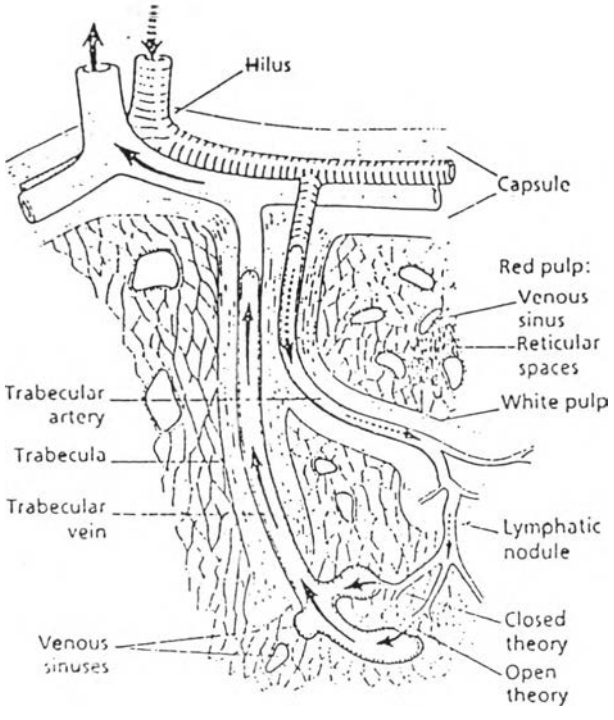

Figure 19 Splenic circulation. (Cuschieri and Forbes, 1994)

The Red Pulp

The red pulp of the spleen occupies about 80% of splenic parenchyma, it consists of numerous sinuses lined by an endothelial cell layer with red pulp cords intervening. The red pulp contains both the cells which are entering from the arterial system and the cells leaving via the venous sinuses (Table 3). The sinuses are specialized vessels composed of elongated endothelial cells, whose longitudinal axis runs parallel to the direction of the vessel. The outer sinus wall receives structural support from reticular fibers of the red pulp cords. The sinuses contain multiple interendothelial pores or slits through which pass normal erythrocytes that have already traversed the cords. These cells are supported by a distinctive predominantly circumferential basal lamina. This basal lamina contains pinocytic vesicles associated with the endothelial cells, as well as intracellular filaments which may play a role in cell stabilization as well as in controlling the flow of blood-borne cells through the inter-endothelial spaces. These endothelial cell filaments, referred to as "stress fibers", are reported to be composed of thin actin-like filaments and thick myosin-like filaments. They concluded that these stress fibers anchor to cell-to-extracellular matrix contacts

can create tension that might allow the endothelium to resist in the fluid shear forces of blood flow (Drenckhahn and Wagner, 1986). Groom (1987) have suggested that these fibres may play a role in the retention of red blood cells.

As mentioned above, the role of the red pulp is primarily that of blood filtration, i.e., removal of particulate materials, macromolecular antigens, and aged or damaged blood cells and platelets from the circulating blood. Senescent, or abnormal red cells are broken down by the lysosomes of the macrophages embedded in the reticular meshwork of the red pulp. In the process, cytokine mediators may be released, both locally and into the circulation, and these mediators may in turn influence immune function (Henry and Symmers, 1992). The iron of the hemoglobin is retrieved and stored as ferritin for future recycling. The heme portion of the molecule is broken down to billirubin, which is transported to liver via the portal system and there conjugated to glucuronic acid. This is then secreted into the bile, to which it gives a characteristic color (Cuschieri and Forbes, 1994).

The mechanism by which macrophages recognize old or abnormal blood cells is not yet elucidated. It has been proposed that as red cells age, they become more rigid and are then more easily trapped in the meshes of the red pulp. It has also been suggested that the immune system may respond to changes in the surface of erythrocytes and tagpathologically modified cells with opsonizing antibody. Investigators have recently described changes in the glycosylation of surface proteins in aging erythrocytes that might provide a clue to the mechanism by which they are recognized by macrophages (Cuschieri and Forbes, 1994).

The red pulp cords consist of a three-dimensional meshwork of reticular fibers on a basement membrane covered by reticular cells to which are attached the cells of the red pulp including macrophages, erythrocytes, platelets, and lymphoid cells. The majority of capillaries are opening into these cords, and from here blood flows into the venous sinuses. Alternatively, capillary blood flow may take a more direct route, opening straight into venous sinuses. There has been much controversy regarding the existence of each of these pathways in man. Finally, Schmidt, MacDonald, and Groom (1988) identified the presence of both circuits in freshly-isolated human spleens, without coexisting pathology. However, the direct arteriovenous connections are extremely rare in man.

The White Pulp

The white pulp contains lymphoid tissue and makes up the remaining 20%. The white pulp region has a distinctive distribution around the arteries where it forms the so-called peri-arteriolar lymphoid sheath (PALS). This region is composed of T cells and a dendritic cell population. Towards the periphery of the sheath is a B cell zone where, after appropriate stimulation, germinal centres are formed. The stromal structure of the white pulp allows for lymphocyte activation.

The entry of the lymphocyte population is directly by the blood. Proliferation of lymphocytes and differentiation of effector lymphocytes and plasma cells, as well as secretion of humoral antibodies, occur in the white pulp of the spleen (Henry and Symmers, 1992).

The immunologically competent white pulp is rather prominent. Lymphocytes and their accessory cells, including macrophages and antigen-presenting cells, are concentrated in white pulp, moving in and out under control, ready to react immunologically (Cuschieri and Forbes, 1994).

The white pulp is comprised of three main compartments: a central arteriole surrounded by a PALS, follicles, and the MZ surrounding the PALS are both T- and B- lymphocyte areas (Table 3). The T cells are located proximal to the central arteriole and the B cells beyond this zone. The B lymphocytes are organized into primary and secondary follicles. Primary follicles refer to unstimulated follicles, while secondary follicles refer to "stimulated follicles" with germinal centres. Follicular dendritic cells and phagocytic macrophages are also found in the primary follicles (Henry and Symmers, 1992; Cuschieri and Forbes, 1994).

The Marginal Zone

The marginal zone (MZ) is generally described as a layer surrounding the PALS and B cell follicles, predominantly composed of intermediate-sized lymphocytes. The lymphoid tissue comprising the white pulp is separated form red pulp by the MZ. The MZ is the site of entry of lymphocytes into the splenetic lymphoid areas. The most conspicuous anatomical feature of the MZ is the presence of a sinus in which part of the arterial blood stream opens. It is not a vessel, but a cleftlike space in which many capillaries terminate. The endothelial cells of the capillaries are continuous with the

cells that line the sinuses. Between the endothelial cells and the subjacent white pulp is a basement membrane which is interrupted or fenestrated, thus providing a passage for cells from the MZ into the white pulp and vice versa.

The MZ also contains B cells and specialized macrophages (Table 3). The latter cells, together with follicular dendritic cells, present antigen to B cells and initiate the formation of germinal centres (Kraal, 1992; Cuschieri and Forbes, 1994).

The situation in humans has been a bit controversial. Although the presence of a MZ has been described in the human spleen (Saitoh, Kamiyama, and Hatakeyama, 1982), the presence of a distinct marginal sinus has been debated. Recently, however, Schmidt et al. (1988) clearly demonstrated the presence of a marginal sinus, using scanning electron microscopy.

Table 3 Cellular composition of the spleen. (Cuschieri and Forbes, 1994)

Tissue type/subtype	Cell type
Red pulp	
Cords	Macrophages
	Reticular cells
Sinuses	Lymphocytes
	Interendothelial cells
White pulp	
PALS	T lymphocytes (central)
	B lymphocytes (peripheral)
Follicles	Dendritic cells
	Tingible body macrophages
	B lymphocytes
Marginal Zone	B lymphocytes
	MZ macrophages

Cytology of the Spleen

T lymphocytes

T cells or T lymphocytes can be divided into two main classes, which are distinguished by their expression of the cell-surface glycoproteins CD4 and CD8. T cells that express the CD8 glycoprotein, called CD8 T cells or cytotoxic T cells, have a cytotoxic function. Effector CD8 T cells kill cells infected with viruses or other intracellular pathogens. This prevents the replication of the pathogen and the spread of infection. A CD8 T cell recognizes only cells bearing its corresponding peptide antigen presented by an MHC (major histocompatibility complex) class I molecule. On the other hand, T cells that express the CD4 glycoprotein are called CD4 T cells. They secrete cytokines that help activate other types of immune-system cells, and respond to peptide antigens presented by MHC class II antigens (Cuscheri and Forbes, 1994).

The antigen receptor on T cells is commonly referred to T cell receptor (TcR). TcRs are highly variable and diverse in their antigen specificity. Each clone of T cells expresses a single species of antigen receptor and thus different clones possess different and unique antigen specificities (Parham, 2000). Functionally, it has been suggested that the CD3 group of molecules marker may act as a signal transduction chain for the TcR, and thus a schematic representation of the TcR often includes these additional molecules (Henry and Symmers, 1992). The CD8 and CD4 molecules are also called T cell co-receptors, because of their roles in T cell recognition (Parham, 2000).

The CD4 T cells can be further subdivided according to the cytokines they secrete and the cells they assist. Cells that secrete cytokines that mainly activate macrophages are called T_{H1} cells (where the 'H' stands for helper), whereas cells that chiefly help B cells to make antibodies are called T_{H2} cells. Both types of helper cells produce responses that facilitate the elimination of extracellular pathogens (Parham, 2000).

Whereas T_{H2} cells work within secondary lymphoid tissues, CD8 cytotoxic T cells and T_{H1} cells must travel to the site of infection in order to function (Parham, 2000). CD4 (and probably CD8) is also involved in signaling to T cells, which is associated with a tyrosine kinase (Henry and Symmers, 1992).

In the spleen, T lymphocytes are found around the central of the PALS. Similar to B cells, T cells first localize in the MZ and then migrate to the PALS. Nieuwenhuis

and Ford (1976) have reported that B and T cells may have a common pathway of entry into the spleen via the MZ, as indicated by the similar initial localization of both cell populations in the periphery of the PALS. T cells preferentially locate to the smallest PALS, suggesting a predominantly hilar movement. Although B cells migrate towards the hilum, they remain in the peripheral PALS. Studies by Mitchell (1973) have further elucidated in the pathway of exit of T cells from the spleen and suggested that T cells leave the PALS to return to the red pulp via MZ bridging channels. Nieuwenhuis and Ford (1976) speculate that initial contact between antigen and lymphocytes occurs in the MZ, which is both the common site of B and T cells and the site of antigen localization. The antigen presentation to T lymphocytes induces T cell proliferation, which is also a prerequisite for activation of antigen-reactive B cells. More recently, homing receptors were described on lymphoid cells (Pabst and Fritz, 1986). Adherence to splenic vessels or emigration into splenic parenchyma may depend on different lymphocyte surface structures (Pabst, 1988).

B lymphocytes

B cells or B lymphocytes mature in the bone marrow and then are carried by the blood to the peripheral lymphoid tissues. These lymphocytes are dedicated to making immunoglobulins and antibodies. Each B cell is programmed to make a single type of immunoglobulin. The cell-surface form of this immunoglobulin serves as the B cell receptor for specific antigen. Associated in the membrane with the immunoglobulin are the signal transduction molecules Igα and Igβ. A complex of the CD19, TAPA-1 (CD81), and CR2 (or CD21) polypeptides which associates with the B cell receptor can augment its response to specific antigen (Vander et al., 1994; Parham, 2000).

Interaction of antigen with surface immunoglobulin is communicated to the interior of the naive B cell by the proteins Igα and Igβ, which are associated with IgM in the B cell membrane to form the functional B cell receptor. Like the CD3 polypeptides of the T cell receptor complex, the cytoplasmic tails of Igα and Igβ contain immunoreceptor tyrosine-based activation motifs (ITAMs) with which the tyrosine kinases associate. The ITAMs become phosphorylated on tyrosine residues, and the active tyrosine kinases and the phosphorylated receptor tails then initiate

intracellular signaling pathways that lead to changes in gene expression in the nucleus (Parham, 2000).

Crosslinking of the B cell receptor by antigen generates a signal that is necessary but not sufficient to activate a naive B cell. The additional signals required are delivered in several ways. One set of signals is delivered when B cell receptor becomes crosslinked with a cell-surface protein complex known as the B cell co-receptor, causing the two receptors to cluster together. The B cell co-receptor is a complex of at least three proteins: 1) complement receptor 2 (CR2 or CD21), which binds to complement components deposited on a pathogen; 2) the protein CD19, which acts as the signaling chain of the receptor; and 3) the protein CD81 (TAPA-1), whose function is not yet known. Co-ligation of the B cell receptor and the co-receptor brings the Igα-bound tyrosine kinase Lyn into close proximity with the CD19 cytoplasmic tail, which it phosphorylates. The phosphorylated CD19 can then bind intracellular signaling molecules which generate signals that synergize with those generated by the B cell receptor complex. Simultaneous ligation of the B cell receptor and co-receptor increases the signals by 1000 to 10000 folds (Parham, 2000).

Even the combined effects of B cell receptor and co-receptor, signals are generally insufficient to activate a naive B cell. This requires additional signals provided by CD4 helper T cells, the effector cells produced upon antigen-activation of naive CD4 T cells. However, whether a B cell needs T cell help or not depends on the nature of the antigen (Parham, 2000).

In the spleen, B cells are found in the PALS-associated nodules and marginal sinus. The majority of B cells express MHC class II (DR) antigens. They possess C3b and C3d receptors which are expressed in the activated state and possibly involved in homing or initial localization of B cells in the marginal zone of spleen. The Fc receptors (FcRII) are also present. CD19, CD20 and CD22 are the most commonly-used markers for human B cells.

The B cells are first found to localize in the marginal zone along with T cells, then finally settle in the corona (area of small lymphocytes surrounding germinal centers of secondary follicle), follicles, and MZ. They are fundamental to the humoral immune response. Foreign antigen presentation initiates a specific clone of B cells to transform into antibody producing plasma cells. Although this may occur independent of T cell

cooperation, T-helper (CD4+) cells usually exert a regulatory by releasing B cell growth factor, IL-4, B cell differential factor (BCDF), and IL-2. Resultant specific antibodies bind antigen and generally activate complement. This may lead to immediate destruction of the antigen or its opsonization to enhance subsequent uptake by macrophages and neutorphils. B cells have capacity to function as antigen-presenting cells. They appear to require an activation step, in particular lipoplysaccharide (LPS) activation. As yet, it is unclear whether these cells can induce T cell proliferation by way of antigen presentation in a resident state (Krieger et al.,1985).

Pabst (1988) has shown a higher incidence of newly formed B cells in the spleen compared to T cells. Resultant lymphocytes may remain in the spleen or migrate to other lymphoid (thymic medulla) and non-lymphoid (gut, liver and lung) organs. These cells may be precursors of antibody producing cells. Those that migrate to the bone marrow are found to mature plasma cells.

Macrophages

Macrophages are a heterogeneous population of cells that develop from peripheral blood monocytes, enter a particular tissue in an apparently random fashion, and whose function is then gaveled by that tissue. Macrophage functions include antimicrobial and tumorical properties, as well as presentation of foreign antigen through CD4+ cell interaction. Macrophages also participate in the acute-phase response through release of inflammatory mediators such as tumor necrosis factor (TNF), IL-1, and IL-6. Macrophage function is regulated by macrophage activating and inhibiting factors, such as interferon-gamma, prostaglandin E_2 (PGE_2), and IL-4.

Splenic macrophages appear to have a dual origin: 55% of splenic macrophages is derived from monocyte influx, and 45% by local production. Studies have shown that the spleen contains a large number of macrophages, which have been identified in the marginal zone, red pulp and marginal centers of the white pulp (Buckley et al., 1987). The majority of macrophages were located in the red pulp. MZ macrophages were found to be Fc+, CR3+; the majority of red pulp macrophages were also Fc+, but CR-; and white pulp-germinal center macrophages, referred to as tingible body macrophages, lacked both Fc and CR3 receptors. MZ macrophage were larger and morphologically distinct compared with other splenic macrophage populations. MZ

macrophages play an important role in antigen presentation (Humphrey and Grennan, 1981). They were also noted to have adherent B cells when freshly isolated, which may be factor in determining B cell traffic. Functionally, MZ macrophages have the ability to phagocyte neutral polysaccharides. This may be related to an exclusive receptor on MZ macrophage for a common sugar determinant. These findings may have important clinical relevance in relation to postsplenectomy pneumococcal sepsis (Buckley et al., 1987). It is possible that splenectomy, by removing the the specialized MZ macrophages, eliminates significant antigen presentation of capsular polysaccharide to B cells, resulting in pneumococcal sepsis.

Splenic functions carried out by splenic macrophage include filtration and phagocytosis, enhancement of phagocytosis through synthesis of complement components, and foreign antigen presentation. Phagocytosis and subsequent breakdown of red blood cells is primarily carried out by red-pulp macrophages.

On reaching the spleen, different antigens move selectively to different areas where specific functional subsets of antigen-presenting cells are located, and are taken up by these cells where they can persist for long periods of time. T-independent polysaccharide antigens preferentially associate with MZ macrophages which present it to a subclass of B cells without T cells involvement, while T-dependent antigens (antigen-antibody complexes) attach to follicular dendritic cells (Parham, 2000).

Dendritic Cells

These lymphoid cells are characterized by tissue processes and are classified by tissue location and cell surface antigens (Buckley, 1987). In spleen, these cells are grouped as follicular dendritic cells (FDC) or B cell associated dendritic cells. They are functionally known as "non-phagocytes antigen-presenting cells".

Nossal et al. (1966) described these cells as "antigen-retaining dendritic reticular cells". They are located in the follicular center and histological appearances induce irregular-shaped nuclei, with the bulk of the cytoplasm located in the peripheral processes. These cells contain few lysosomes and are functionally characterized by the ability to bind immune complexes. Follicular dendritic cells are located in the germinal centers or secondary follicles of the white pulp, where they intimately communicate with B cells. Follicular dendritic cells, along with MZ macrophages present processed

antigen to B cells, and may also be involved in B cell activation. As these cells are not phagocytes, foreign antigen is probably processed at the cell surface or the following pinocytosis. Kaye, Chain, and Feldmann (1985) have demonstrated the ability of nonphagocytic dendritic cells to process and present whole mycobacterium as foreign antigen to T cells. This antigen processing cells may also be involved in graft rejection.

Plasma Cells

The final outcome of B cell activation is the proliferation and differentiation of the splenic B cell into plasma cells in the outer PALS to secrete antibodies (Cuschieri and Forbes, 1994). The morphological effects of activation are striking: the small resting B cell, which in appearance is all nucleus and no cytoplasm, gives rise to plasma cells committed to antibody secretion and whose large active cytoplasm packed with rough endoplasmic reticulum is testimony to this function (Vander et al., 1994; Parham, 2000). Each B cell always displays copies of the particular antibody on its plasma membrane, therefore the plasma cells derived from a particular B cell can secrete only one particular antibody. These secreted antibodies then travel all over the body to reach antigens of the kind that stimulate a particular immune response. They combine with antigens and guide an attack that eliminates the antigens or the cells bearing them. Antibody-mediated responses are also called humoral responses (Vander et al., 1994).

Microcirculatory Pathways Through the Spleen

According to Groom (1987), 90% of blood flowing through the spleen passes through a rapid channel network, while the remaining 10% travels more slowly via the red pulp reticular mesh. The rapid pathway involves the passage of blood directly into open-ended venous sinuses at the MZ as well as direct arteriovenous anastomoses (closed circulatory pathway). The open-ended venous sinuses that originate in the MZ allow blood to bypass the interendothelial spaces (IES). The sow (open circulatory theory) pathways consist of long circulatory channels from the ellipsoid sheaths and arterial capillary terminations in the reticular meshwork of red pulp. The terminations occur either as a scapulae of perforated endothelium or in a split funnel pattern, opening into the cords. Red blood cells (RBCs) can be retained in two specific sites

associated with the circuit. Firstly, they may attach to the reticular fibers and macrophages in the red-pulp cords, and secondary, they may be retained at the inter endothelial junctions of the venous sinuses. Trapping of RBCs by reticular fibres and macrophages with subsequent phagocytosis appears related to changes in RBCs surface properties, while IES retention may depend on the bulk property of erythrocyte (Groom, 1987; Cuschieri and Forbes, 1994).

Adhesion Molecules (Parham, 2000)

Cell adhesion molecules (CAMs) are cell-surface proteins that enable cells to bind to each other. Contact between cells is initiated by cell adhesion molecules on the T cell surface, which bind to complementary adhesion molecules on the surfaces of other cells. Although adhesion molecules work independently of antigen, they are sufficiently specific in their interactions with other adhesion molecules to direct the T cell to the appropriate cell type. T cell adhesion molecules that bind to high endothelial cells direct naive circulating T cells to leave the blood within a secondary lymphoid tissue, whereas other T cell adhesion molecules interact with adhesion molecules on antigen-presenting cells to set up the initial cell-cell contacts needed to test the interactions between T cell receptors and peptide:MHC complexes. Adhesion molecules also stabilize the interaction between an effector T cell and its target cell.

The entry of recirculating T cells and B cells into secondary lymphoid tissues is directed by selectins on the lymphocyte surface. L-selectin expressed by the naive T cell binds to the carbohydrate portions of vascular addressins, which are present on the surface of the endothelial cells of blood vessels. Two vascular addressins, CD34 and GlyCAM-1, are expressed on the surface of high endothelial venules in lymph nodes and direct naive T cells to leave the blood and enter a node. MAdCAM-1 is expressed on the endothelium of cappillaries in mucosal tissue, and guides lymphocytes into the lymphoid areas of the gut and other mucosal tissues. The interaction between L-selectin and vascular addressin is not in itself sufficient to enable lymphocytes to pass through the endothelium and into lymphoid tissue. This is achieved by strong interactions between integrins on the surface of the lymphocyte and immunoglobulin superfamily members on the surface of the endothelial cells. All T cells express an integrin known as lymphocyte function-associated antigen-1 (LFA-1). The principal

function of LFA-1 is to bind to the immunoglobulin superfamily intercellular adhesion molecules (ICAMs). Vascular endothelium expresses ICAM-1 and ICAM-2; binding of these to LFA-1 on a naive T cell enables the T cell to squeeze through the endothelium and enter the cortex of the lymphoid tissue. Small chemoattractant proteins, or chemokines, which are also bound to the endothelium, activate the integrin LFA-1 on the lymphocyte surface, enabling it to bind tightly to ICAM-1 on the endothelial cell.

The T cell's LFA-1 binds to ICAM-1 or ICAM-2 on the antigen-presenting cells, whereas the LFA-1 of the antigen-presenting cell binds to a third kind of ICAM–ICAM3–on the T cell surface. Adhesion is strengthened by interaction between two other members of the immunoglobulin superfamily, CD2 on the T cell and LFA-3 on the antigen-presenting cell. These transitory cell-cell interactions enable the T cell receptor to screen the peptide:MHC complexes on the surface of the antigen-presenting cell for ones that engage the receptor and activate the T cell.

When a naive T cell encounters a specific peptide:MHC complex, a signal is delivered through the T cell receptor. This induces a change in the conformation of the T cell's LFA-1 molecules that increase their affinity for ICAMs. The interaction of the T cell with the antigen-presenting cell is stabilized and can last for several days, during which time the T cell proliferates, and its progeny, while also remaining in contact with the antigen-presenting cell, differentiate into effector cells.

Innate Immune Responses

Innate immune responses are those the body mounts immediately without requiring previous contact with the microbe. Thus, they provide the body with a first line of defense. Innate immune responses are characterized by their lack of specificity; the same defensive activities are effective regardless of the pathogen presenting the threat (Karp, 1999). Innate immunity uses general molecular recognition mechanisms to detect the presence of bacteria and viruses, and it does not lead to long-term immunity to that particular pathogen (Parham, 2000).

Innate immunity is initiated by phagocytic cells that recognize common components of pathogen's cell surfaces. These phagocytic cells, called phagocytes, are of two kinds: 1) the macrophages resident in tissues; and 2) the neutrophils, which

circulate in the blood and enter tissues but only when they become infected. Macrophages and neutrophils bear cell-surface receptors that bind to common carbohydrate constituents of surfaces of pathogens, which are not components of human cells. This binding induces engulfment, killing, and degradation of the pathogen (Parham, 2000).

Macrophages resident in the infected tissues are generally the first phagocytic cells to encounter an invading bacterium. As part of their response to the presence of pathogens, macrophages secrete soluble proteins, called cytokines, which recruit other cells of the immune system, such as neutrophils, into the infected area. They also secrete a variety of other substances that act as pathogen-killing agents and as inducers of inflammation (Parham, 2000).

Neutrophils are specialized to phagocytose and kill pathogens. They begin to act soon after an infection arises and are the principal phagocytic cell in infected and inflamed tissues. They are rapidly mobilized to enter sites of infection and can work in the anaerobic conditions that often prevail in damaged tissue. Neutrophils are short-lived and die at the site of infection, forming pus (Parham, 2000).

The molecules released by phagocytes when they encounter infection induce a local state of inflammation. Cytokines induce the local dilation of blood capillaries and increase in the blood flow, which can cause the skin to warm and redden. Vascular dilation introduces gaps between the endothelial cells, causing more blood plasma to leak into the connective tissue. Such expansion of the local fluid volume causes edema or swelling putting pressure on nerve endings and causing pain. In addition, cytokines can change the adhesive properties of the vascular endothelium, signaling neutrophils, monocytes/ macrophages, and other white blood cells to bind to it and migrate out of the blood into the inflamed tissue. Infiltration of cells into the inflamed tissue increases the swelling, and some of the molecules they release contribute to the pain (Parham, 2000).

In addition, innate immunity is also mediated by soluble proteins in the blood. The plasma that leaks into infected and inflamed tissue carries with it plasma proteins. Among these are soluble recognition molecules, which are made mainly in the liver and travel freely throughout the body, involved in innate immunity. One such recognition molecule, the mannose-binding protein initiates a series of enzymatic reactions

involving a set of plasma proteins collectively called complement. The cascade of enzymatic reactions triggered by bacterial recognition is described as complement activation and leads to the covalent binding of complement proteins to bacterial cell surfaces. Complement activation is a general effector mechanism of the immune system and can also be triggered by the presence of bacterial cells alone (Parham, 2000).

Complement proteins bound to pathogens engage specific complement receptors on the surface of phagocytes, triggering the engulfment and destruction of the complement-coated pathogen. Soluble fragments of complement formed during the cascade of complement reactions recruit additional phagocytes into sites of complement activation, thus enhancing the destruction of the pathogen (Parham, 2000).

In an infection with extracellular bacteria, macrophages within the infected tissue are the first cells to respond. In addition to their phagocytic function, stimulated macrophages secrete a battery of cytokines and other molecules that recruit effector cells, especially neutrophils, into the infected area. There they develop a state of inflammation within the tissue. Inflammation describes the local accumulation of fluid accompanied by swelling, reddening, and pain. These effects stem from changes induced in the local blood capillaries that lead to an increase in their diameter (a process called dilation), reduction in the rate of blood flow, and increased permeability of the blood vessel wall. The increased supply of blood to the region causes the local redness and heat associated within inflammation. The increased permeability of the blood vessels allows the movement of fluid, plasma proteins, and white blood cells from the blood capillaries into the adjoining connective tissues, causing the swelling and pain. Prominent cytokines produced by activated macrophages are IL-1, IL-6, IL-8, IL-12, and tumor necrosis factor-alpha (TNF-α). These cytokines have potent effects that can be localized to the infected tissue or manifested systemically throughout the body (Parham, 2000).

Other molecules released by macrophages are plasminogen activator, phospholipase and other enzymes, prostaglandins, oxygen radicals, peroxide radicals, peroxides, nitric oxide, leukotrienes, and platelet-activating factor (PAF), which all contribute to inflammation and tissue damage. Molecules involved in the induction of inflammation are known generally as inflammatory mediators (Parham, 2000).

The Migration of Leukocytes into Tissues is Induced by Inflammatory Mediators (Parham, 2000)

Within the blood capillaries, white blood cells move more slowly than in the larger vessels, particularly in the region close to the endothelial wall. Inflammatory mediators stimulate the endothelium of the capillaries in infected tissue to express adhesion molecules and MHC class II molecules. This is known as the activation of the vascular endothelium. Inflammatory mediators also induce the expression of complementary adhesion molecules on circulating monocytes and neutrophils. These changes enable monocytes and neutrophils in the blood to bind to activated vascular endothelium within an infected site and then to squeeze through the spaces between endothelial cells and enter the infected tissue macrophages. The release of inflammatory mediators by the increasing numbers of macrophages and neutrophils steadily increase the inflammation within the infected tissue.

At various stages in their maturation, activation, or function, all types of white blood cell leave the blood and home to particular target tissues. In all cases, the white blood cell is directed to its destination by the combinations of adhesion molecules expressed by the vascular endothelium in the tissue and by the white blood cell. The process by which leukocytes migrate out of blood capillaries and into tissues is called extravasation and it occurs in four steps. The first step is an interaction between circulating leukocytes and blood vessel walls that slows the leukocytes down. This interaction is mediated by selectins, adhesion molecules that bind to particular types of carbohydrate. In healthy tissue, vascular endothelial cells contain granules, known as Weibel-Palade bodies, which contain P-selectin. On exposure to inflammatory mediators, including leukotriene LTB4, complement fragment C5a, and histamine, the P-selectin in the Weibel-Palade bodies is mobilized and within a few minutes is delivered to the cell surface. A second selectin, E-selectin, is also expressed on the endothelial cell surface a few hours after exposure to LPS or TNF-α. The two selectins bind to the carbohydrates of cell-surface glycoproteins on the leukocyte. These reversible interactions allow the leukocytes to adhere to the blood vessel walls and to 'roll' slowly along them by forming new adhesive interactions at the front of the cell while breaking them at the back.

The second step in extravasation depends on interactions between the integrins LFA-1 and CR3 on the phagocyte and adhesion molecules on the endothelium, for example ICAM-1, whose expression is also induced by TNF-α. Under normal conditions, LFA-1 and CR3 interact only weakly with endothelial adhesion molecules, but exposure to chemoattractant cytokines such as IL-8, produced by cells in the inflamed tissues, induces conformational changes in the LFA-1 and CR3 on a rolling leukocyte that strengthen their adhesion. As a result, the leukocyte holds tightly to the endothelium and stops rolling.

In the third step, the leukocyte crosses the blood vessel wall. LFA-1 and CR3 contribute to this movement, as does adhesion involving the immunoglobulin superfamily protein CD31, which is expressed by both leukocytes and endothelial cells at their junctions with one another. The leukocyte squeezes between neighboring endothelial cells, a maneuver known as diapedesis, and reaches the basement membrane, a part of the extracellular matrix. If then crosses the basement membrane by secreting proteases that break down the membrane. The fourth and final step in extravasation is movement of the leukocyte toward the center of infection in the tissue. This migration is accomplished along gradients of chemoattractants such as IL-8, which have their source within the infected site.

Chemoattractant Cytokines Recruit Phagocytes to Infected Tissues (Parham, 2000)

The chemoattractant cytokines, or chemokines, comprise a family of proteins that recruit effector cells into sites of infection. IL-8, which attracts neutrophils from the blood into sites of infection and inflammation, is one example. The chemokines are a family of small soluble proteins with similar structures, and are secreted by a variety of cell types in response to tissue damage or infection. Two major subfamilies of chemokines are defined according to the position of certain cysteine residues in the amino-acid sequence and the type of cells that they attract: 1) CC chemokines, having two adjacent cysteine residues, can attract monocytes, effector T cells, and memory T cells; and 2) CXC chemokines, having motif with two cysteines separated by another amino acid, can attract neutrophils and naive T cells.

In recruiting neutrophils from the blood, IL-8 serves two purposes. First, it converts the rolling adhesion of leukocytes to vascular endothelium into a stable binding. Second, it directs neutrophil migration along a gradient of the chemokine that increases in concentration towards the infection. The gradient is set up by the binding of chemokines emanating from the infected area to proteoglycan molecules of the extracellular matrix and the endothelial cell surfaces. This means that the chemokines are immobilized on a solid substrate along which the white blood cells can migrate. White blood cells sense the chemokine gradient through chemokine receptors on their surface.

Cytokines Released by Macrophages Cause a Rise in Body Temperature and Also Activate the Acute-Phase Response (Parham, 2000)

The cytokines IL-1, IL-6, and TNF-alpha produced by macrophages have various effects, some of which have already been mentioned. Another systemic effect of these inflammatory cytokines is to cause the rise in body temperature that is called fever. The cytokines and other mediators act on temperature control sites in the hypothalamus, and on muscle and fat cells, altering energy mobilization to generate heat. In this context, such molecules are called 'endogenous pyrogens' to distinguish them from the 'exogenous' products of pathogens that also raise the body temperature. On balance, a raised body temperature helps the immune system in the fight against infection, because most bacterial and viral pathogens grow better at temperatures lower than those of the human body, and adaptive immunity becomes more potent at higher temperatures. At elevated temperatures, bacterial and viral replication is decreased, whereas processing of antigen is enhanced. In addition, human cells become more resistant to the deleterious effects of TNF-alpha at raised temperatures.

A second systemic effect of TNF-alpha, IL-1, and IL-6 is to change the spectrum of soluble plasma proteins secreted by hepatocytes in the liver. The changes induced by acute infection are collectively called the acute-phase response. Those proteins whose synthesis and secretion is increased during the acute-phase response are called acute-phase proteins.

Two of the acute-phase proteins-C-reactive protein and mannose-binding protein-enhance the fixation of complement at pathogen surfaces. C-reactive protein is a pentamer of identical subunits and a member of the pentraxin family of proteins. C-reactive protein binds to the phosphorylcholine component of lipopolysaccharides in bacterial and fungal cell walls, but not to the phosphorylcholine present in the phospholipids of human cell membranes. In binding to bacteria, C-reactive protein acts as an opsonin, which can then bind C1q, initiating the classical pathway of complement fixation. The interaction of C-reactive protein with C1q involves the stalks of the C1q molecule, whereas the interaction of antibodies with C1q involves the globular heads. Despite these differences in the initial binding mechanism, the same sequence of complement reactions occurs when either C-reactive protein or antibody interacts with a pathogen.

The second acute-phase protein that can activate the complement system is mannose-binding protein (MBP), also called mannan-binding lectin (MBL). It binds to mannose-containing carbohydrates of bacteria and yeast and is a calcium-dependent lectin. In its overall structure and domain organization, the MBP molecule is similar to C1q, although there are no obvious similarities in their amino-acid sequences. MBP resembles a bunch of flowers in which the carbohydrate-recognition domains form the flowers. Each flower contains three binding sites and each MBP molecules has five or six flowers, giving MBP either 15 or 18 potential sites for attachment to a pathogen's surface. Even relatively weak individual interactions with a carbohydrate structure can be developed into high-avidity interactions through the use of multipoint attachments. Although some carbohydrates of human cells contain mannose, they do not bind MBP because their geometry does not permit multipoint attachment to MBP.

Like C1q, MBP activates a proteolytic enzyme complex (MBP-associated serine protease or MASP) that cleaves C4 and C2 and thus initiates complement activation. This pathway is known as the lectin-mediated pathway of complement activation. MBP also serves as an opsonin that facilitates the uptake of bacteria by monocytes in the blood. These cells do not express the macrophage mannose receptor but have receptors that can bind to MBP molecules coating a bacterial surface. MBP is a member of a protein family called the collectins; the family also includes the

pulmonary surfactant proteins A and D (SP-A and SP-D), which work in the lungs to opsonize pathogens such as *Pneumocystis carinii*.

In the absence of infection, C-reactive protein and MBP are present at low levels in plasma; their levels increase during the acute-phase response. Both proteins bind to structures that are commonly found on pathogens but not on human cells, and initiate complement activation and fixation by a pathway that is almost identical to the classical pathway used by antibodies. Such comparisons reveal how the antibodies of the adaptive immune response have a role analogous to that of C-reactive protein and MBP in innate immunity and serve to expand the range of pathogen-recognition molecules. Furthermore, nearly all of the complement components used in the classical and lectin pathways are structurally and functionally related to components of the alternative pathway, which was probably the first sequence of complement reactions to evolve.

Adaptive Immune Responses

In contrast to innate immunity, the adaptive immune responses focus specifically on the pathogen at hand and lead to a condition of long-lived protection called adaptive immunity to that pathogen alone, and no other. Infection with the measles virus, for example, results in immunity to measles but not to mumps, an infection caused by a different virus (Parham, 2000). Adaptive immunity requires a lag period during which the immune system gears up for an attack against a foreign antigen. There are two broad categories of adaptive immunity: humoral immunity, which is carried out by antobodies; and cell-mediated immunity, which is carried out by cells such as T cells and macrophages (Karp, 1999). Through the activation of phagocytes, the production of cytokines, and the creation of a state of inflammation, innate immunity sets the scene for the development of the adaptive immune response to pathogens, to which an individual has never been previously exposed. Such infections stimulate a primary immune response, involving the coordinated activation of naive T lymphocytes and B lymphocytes and the production of effector cells and molecules including antibodies specific against the pathogen. These effectors help to clear the pathogen from the body and prevent re-infection in the short term, while the pathogen is still infecting others in

the population. Usually, an adaptive immune response also leads to long-lasting immunity against the pathogen (Parham, 2000).

The primary adaptive immune response terminated most infections that elude the innate immune response. However, within the population suffering from an infectious disease, some individuals succumb to its effects before the immune system can gain the upper hand. Those at greatest risk are the young, the old, the malnourished, and those already suffering from other diseases (Parham, 2000).

On the other hand, the immunity due to a secondary immune response is absolutely specific for the pathogen that provoked the primary response. The individual therefore appears immune specific to that disease. Potency of the secondary immune responses is sufficient to repel the pathogen before there is any detectable symptom of disease. However, highly mutable RNA viruses, such as influenza, which change antigenically from year to year, might not be recognized by antibodies and memory cells developed against a previous strain (Parham, 2000).

T Cell Activation (Parham, 2000)

Extracellular pathogens and other antigens are carried by the flow of lymph and blood from the infected site to the secondary lymphoid tissues (e.g. lymph nodes and spleen), where macrophages process and present them to T cells. Macrophages have been activated to become professional antigen-presenting cells as one of the consequences of the innate response. Pathogens also reach secondary lymphoid tissue by carriage within cells specialized for this purpose. Dendritic cells transport both viral infections and endocytosed extracellular antigens from epithelial tissues into lymph nodes and tonsils whereas, in the gut, M cells deliver antigens from the gut lumen to Peyer's patches and other lymphoid tissues lining the gut wall. Infections of the skin drain through the lymphatics to nearby lymph nodes, blood-borne pathogens are trapped in the spleen, and infections of the gastrointestinal and respiratory tracts are dealt with by the lymphoid tissue that line their mucosal surfaces.

Secondary lymphoid tissues provide places where naive T cells can encounter specific antigen. The dendritic cells express a chemokine, DC-CK, that specifically attracts naive T cells. T cells arriving from the blood scan the surface of dendritic cells and macrophages for specific peptide:MHC complexes. The small subset of T cells that

are specific for the antigens in the node are activated on engagement of their T cell receptors; proliferation and differentiation begins and eventually leads to large clones of antigen-specific effector T cells.

Virus-specific cytotoxic T cells are almost always produced during viral infection, as a result of the presentation of viral peptides to naive CD8 T cells by professional antigen-presenting cells in secondary lymphoid tissues. Furthermore, in addition to their cytotoxic function, CD8 T cells also secrete cytokines (e.g. IL-10 and TGF-β), which can influence the immune response. In virus infections, both cytotoxic T cells and antibodies help to clear the infection, and antibodies are of particular importance for long-lived protective immunity.

The decision for CD4 T cells to become either T_{H1} or T_{H2} effector cells is made only during the initial response of naive CD4 T cells to antigen. To determine whether CD4 T cell populations will help principally macrophages or B cells, a major influence is the cytokine environment experienced by the activated CD4 T cell during its initial burst of cell division.

The IL-12, which is mainly the product of dendritic cells and macrophages at the early stage of infection, drives the differentiation of T_{H1} cells. In addition, IL-12 stimulates NK cells to produce IFN-γ, which in turn activates macrophages and stimulates T_{H1} differentiation. IL-12 and IFN-γ also function to prevent the development of T_{H2} cells. Conversely, differentiation of T_{H2} cells is promoted by IL-6 and IL-4 and these cells in turn activate B cells and their production of antibodies. Further favoring a T_{H2} response is the property of IL-4 to inhibit the generation of T_{H1} cells. In addition, mast cells activated during innate immune responses might also be a source of the IL-4 that drives T_{H2} development.

Moreover, the commitment of the CD4 T cell response toward a T_{H1} or T_{H2} phenotype probably depends on the way that pathogens interact with immature dendritic cells, macrophages, and NK cells during the early phases of an infection, and the mix of cytokines that is synthesized at that time. In this manner, the course of the innate immune response early in infection, therefore determines the strategy taken by the adaptive immune response.

The direction of CD4 T cell differentiation in the primary immune response is also influenced by the abundance and density of the peptide: MHC complexes at the surface of professional antigen-presenting cells and their affinity for T cell receptors. Peptides that are abundantly presented, or have strong affinity for T cell receptors, tend to stimulate T_{H1} responses, whereas peptides that are at low abundance, or have weak affinity for T cell receptors, tend to stimulate T_{H2} responses.

Furthermore, the cytokines produced by one type of effector CD4 T cell can also inhibit the differentiation and/or the function of the other type. The IL-10 produced by T_{H2} cells prevents the development of a T_{H1} response, conversely, the IFN-γ produced by T_{H1} cells prevents the activation of T_{H2} cells.

Homing of T Cells to Sites of Infection (Parham, 2000)

The activation of naive T cells and their differentiation into effector T cells takes 4-5 days. In the process, their complement of cell-surface adhesion molecules changes, enabling them to leave the secondary lymphoid tissue and travel to the site of infection. The expression of L-selectin, which enables naive T cells to enter lymph nodes, is lost and the expression of other adhesion molecules is increased. A crucial changes is the increasd expression on effector T cells of the integrin VLA-4, which binds to VCAM-1 adhesion molecules induced on vascular endothelium by inflammatory cytokines.

The differential expression of adhesion molecules is also thought to direct subsets of effector T cells to distinct anatomical sites. Homing to Peyer's patches and to the lamina propria of the gut involves simultaneous binding of L-selectin and LPAM-1 (the integrin $\alpha_4\beta_7$) on the T cell to the MAdCAM-1 adhesion molecule on the vascular endothelium. T cells that home to gut epithelium express the integrin $\alpha_E\beta_7$ and bind to E-cadherin on gut epithelial cells. In contrast, T cells that home to the skin enpress the cutaneous lymphocyte antigen (CLA) and are thought to bind E-selectin on vascular endothelium.

In general, effector T cells enter all tissues in small numbers, whether the tissue is infected or not. Such uninvited visits to an infected site could provide the opportunity for effector T cells to meet specific antigen and produce cytokines such as TNF-α, which activates local endothelial cells and facilitate the entry of additional effector T cells. In this manner, the entry of one or a few effector T cells into an infected site could initiate the inflammatory response required to make the site a destination for the rest of the effector T cell population. However, for uninfected tissues that effector T cells enter in a non-specific manner, the effector T cells either die in the tissues by apoptosis or leave in the draining lymphatics to return to the blood.

Antibody Responses (Parham, 2000)

Whereas effector CD8 T cells and T_{H1} cells work mainly at sites of infection, effector T_{H2} cells activate B cells within the secondary lymphoid tissues, from whence secreted antibodies then travel in blood or lymph or across epithelia to the sites of infection. Early in an infection, as previously mentioned, pathogen-derived antigens are presented by the dendritic cells and macrophages in the draining lymph node. Clones of antigen-specific T cells are expanded within the T cell areas of the node and some differentiate into effector T_{H2} cells. Naive B cells also recirculate through the blood and lymph, entering lymph nodes in the T cell areas, where they bind and endocytose specific antigen by capturing it within their cell-surface immunoglobulin. The endocytosed antigen is then processed by the B cells and presented on MHC class II molecules to the effector T_{H2} cells, which in response secrete the cytokines necessary for B cell proliferation and differentiation.

Antigen-specific B cells in contact with T_{H2} cells proliferate in the T cell area to form a primary focus. Some of the B cells activated in these primary foci migrate to the medullary cords of the lymph nodes. The corresponding B cells in the spleen move to those parts of the red pulp directly adjoining the T cell zones of the white pulp. In these sites, B cell numbers increase; the cells divide and differentiate into short-lived antibody-producing plasma cells that produce a first wave of pathogen-specific antibody. Most of these plasma cells die by apoptosis after 2-3 days. Other activated B cells migrate to the follicles, where they proliferate further, forming a germinal center in which the B cells undergo somatic hypermutation.

The antibodies produced by the first wave of plasma cells form immune complexes with antigen and activate complement. Some of the immune complexes bind to follicular dendritic cells in the lymphoid follicles. The variant antibodies produced by the mutated B cells are tested against the antigen in the immune complexes collected on the follicular dendritic cells. B cells having the highest-affinity antigen receptors compete most effectively for antigen and will be selected to survive, to be helped by CD4 T cells, and to become plasma cells. They secrete a second wave of antibody with a higher overall affinity for antigen than the antibody of the first wave.

It is found that about 10% of the activated B cells in germinal centers leave as immature plasma cells called plasmablasts, and migrate to distant sites where they differentiate into plasma cells that live on average for about a month. The remaining 90% die in the germinal center, having failed to capture antigen and cognate T cell help.

Plamablasts originating in the follicles of Peyer's patches and mesenteric lymph nodes migrate to the lamina propria of the gut and other epithelia. Those originating in peripheral lymph nodes migrate to the medullary cords or the bone marrow, and those originating in the spleen migrate to the bone marrow. The germinal centers are present for only 3-4 weeks after the supply of antigen is turned off. After this time, small amounts of antigen are retained on follicular dendritic cells and these continue to stimulate the proliferation of B cells in follicles. These B cells are likely to be the precursors of pathogen-specific plasma cells that can be found in the mucosa and bone marrow over peroids of months to years. They are thus the source of the antibodies that provide protective immunity.