CHAPTER II

LITERATURE REVIEWS

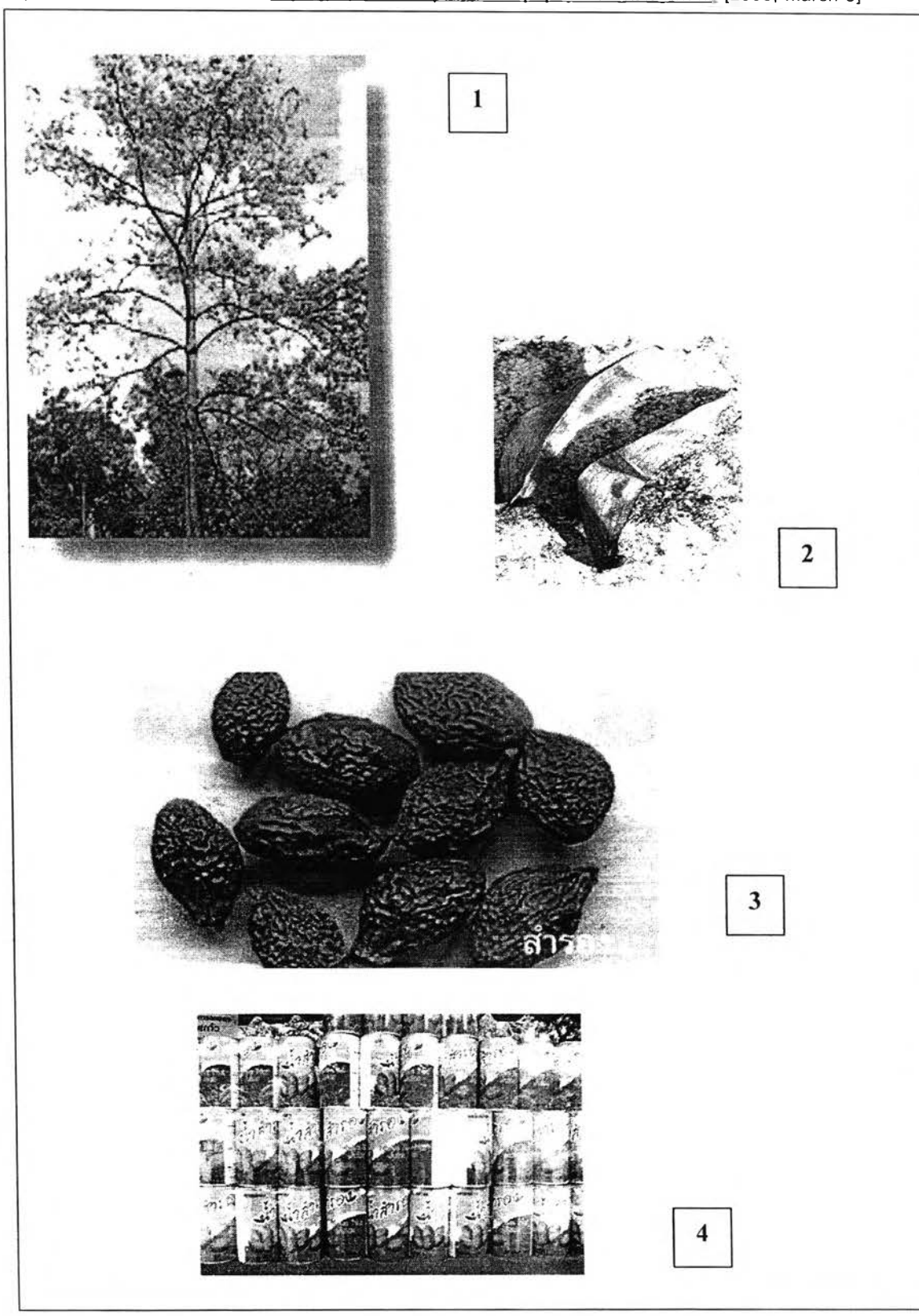
2.1 *Scaphium scaphigerum*

Scaphium scaphigerum (G.Don) Schott & Endl or *Scaphium macropodum* Beaumee. syn. *Sterculia lychnophora* Hence is a plant in a small genus of Sterculiaceae family. It is found in Thailand and southwards into Malaysia and known in Thai as Pung-Ta-Lai, Sam-Rong, Tai-Pao and Chong. *S. scaphigerum* is mostly grown in Chantaburi province. It is large, heavily buttressed tree, 30 – 40 m. in height and looks splendid with fragrant flowers on the bare twigs before the leaves appear (Figure 2.1).

The fruit of *S. scaphigerum* is in a boat shape. The dried fruit becomes brown and small ellipsoid. The fruit contains mucilaginous matters; bassorin 59%, a few of caffeine and theobromine (Thailand Institute of Scientific and Technological Research, 1991). The pericarp contains glucose, xilose, arabinose and rhamnose. When the fruit is macerated with water, it forms a bulky gelatinous jelly, which is sweetened and eaten as delicacy. Nowadays the fruits have been used for producing refreshing beverage, which is a commercial product of Chantaburi province. They are also used as traditional medicine for conjunctivitis curing in Thailand, for treatment of lung, kidney and bowel complaints in China, and for curing sprue and cough at Java.

It was previously reported that the water extract of these fruits contained histamine (Hayman *et al.*, 1988: 338) and sterculia polysaccharide PP III (Chen *et al.*, 1996: 39-41). There are very few studies about the pharmacological activities of *S. scaphigerum*. It had no anti-tumor activity (Itokawa *et al.*, 1990: 58-62) and no inhibitory effect on the platelet-activiting factor receptor binding (Jantan *et al.*, 1996: 86-89).

Figure2.1 *S. scaphigerum* Tree; 1, boat shape fruits; 2, Dried fruits; 3, Pung-ta-lai commercial products; 4 (Available from http://thaimedicinalplant.com/popup/Pungtalay.html, [2005, March 3]



2.2 Immune function

Human body has defense mechanisms which are composed of lymphoid tissues and a variety of cells and soluble molecules. The functions of the human immune system include discrimination between self and foreign antigens, the development of a memory response to antigens, recognition of neoplasms and the elimination of pathogens that invade the host. The immune function can be divided into two categories based on the rapidly and specificity of the response, known as nonspecific and specific immune response which act collaboratively to eliminate or inactivate any antigen (Figure 2.2).

The nonspecific (innate) immune responses are the body's first line defense mechanisms against a foreign antigen. They are not acquired and do not required specific recognition of antigens for initiating immune responses. Additionally, they are not enhanced response by subsequent exposures of the antigen structure but they can be enhanced by specific immune responses. The components of the innate immunity are physical barriers, phagocytic cells such as macrophages and neutrophils, complements and cytokine. On the other hand, the specific (adaptive) immune responses must be driven by antigens to go through different phases of activation, expansion (multiplication of cells) and differentiation in order to carry out their functions. They are provided by the coordinate activities of T and B lymphocytes which recognize antigens in different ways. B cells recognize the antigen itself, while T cells can only recognize an antigen when it is on the surface of another cell, bound to a specific molecule known as a major histocompatibility complex, or MHC molecule. The function of B lymphocytes is to produce antibodies while the function of T lymphocytes is to help immune responses (in the case of T helper cells) or to initiate the death of target cells (in the case of cytotoxic T cells).

The specific immune response has a "memory" that allows a quicker and stronger to attack antigen than innate immune response the next time that specific pathogen is encountered. Moreover macrophages bridge innate and adaptive immunity. After they have devoured foreign antigens or microbes as part of their role in innate

immunity, they assist B cells and T cells in adaptive responses by producing cytokines that regulate lymphocyte function or by presenting antigens bound on MHC molecules so that these antigens can be recognized by T cells. Furthermore, they increase other substances (called co-stimulatory molecules) on their cell surface that can generally enhance their interaction with T cells (Figure 2.3) (Zane, 2001; Goldsby *et al.*, 2003).

Figure 2.2 Nonspecific (innate) immunity and specific (adaptive) immunity (Available from: http://medic.med.uth.tmc.edu/edprog/immuntbl.htm, [2005, March 3]

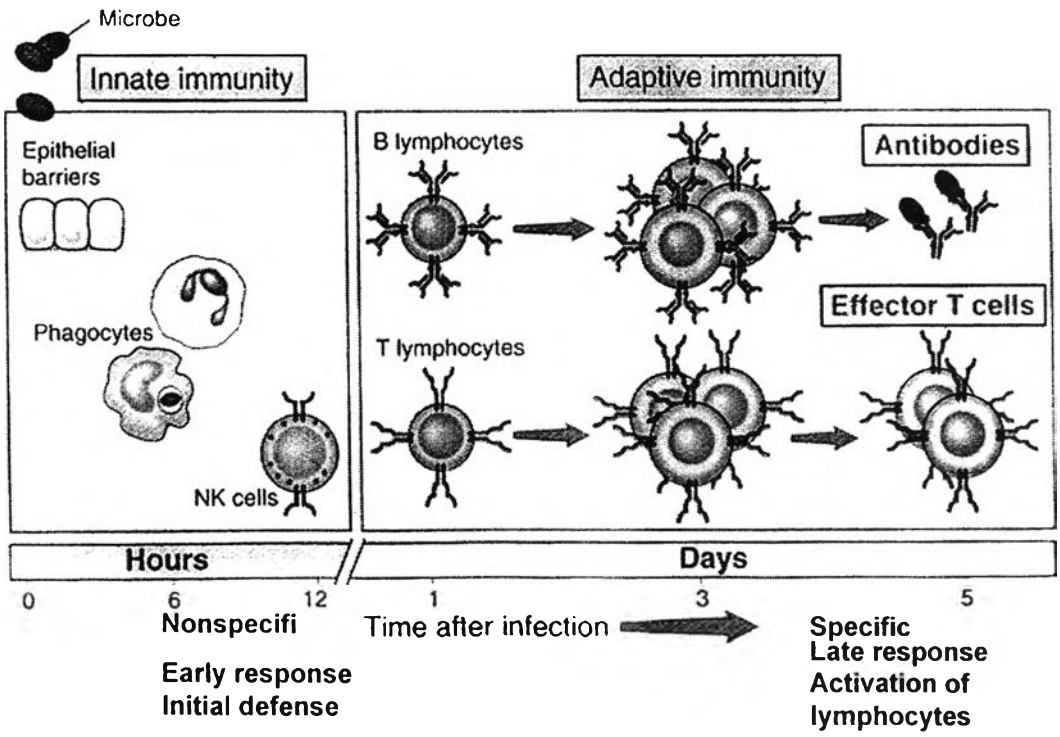
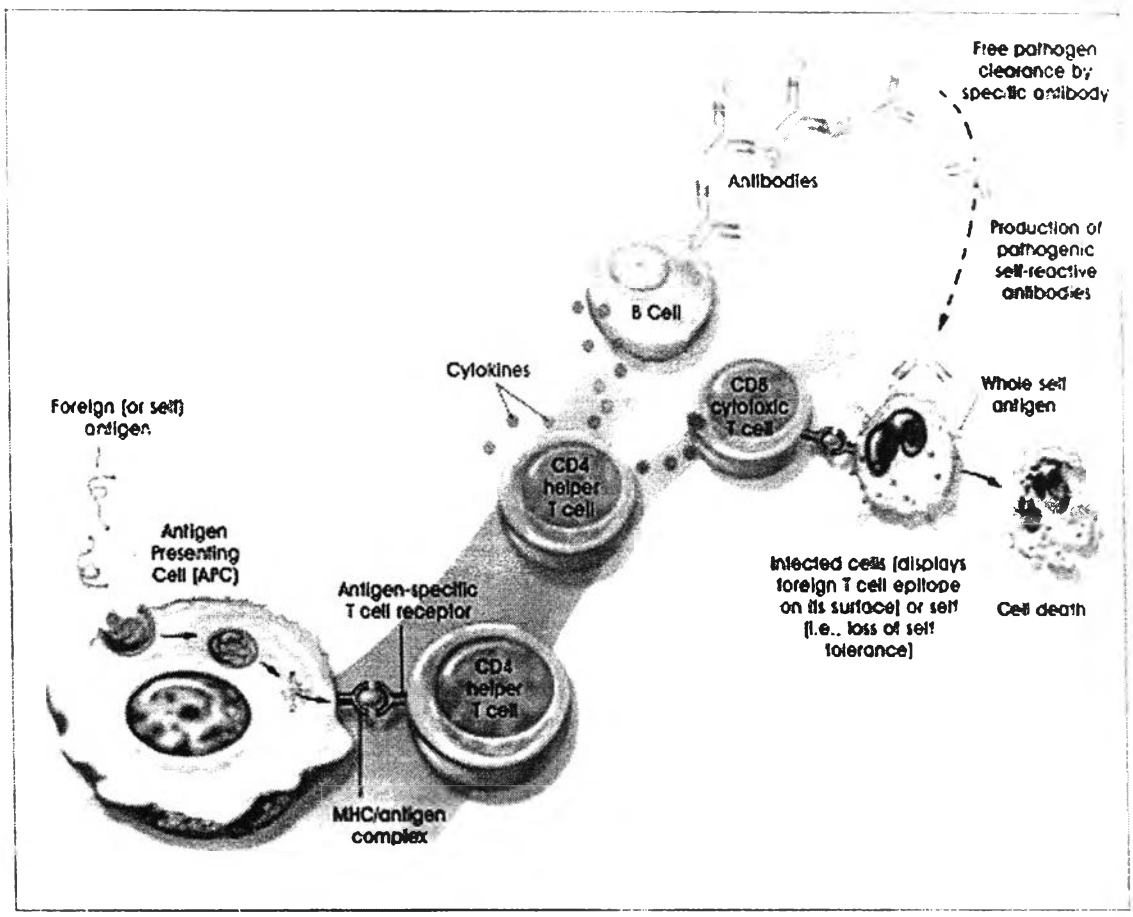


Figure 2.3 Summary of the immune response involving B and T cells (Available from: (Available from: http://stemcells.nih.gov/info/scireport/chapter6.asp [2005, March 3])



2.3 Immunostimulation

Immunostimlation comprises a prophylactic or therapeutic concept which aims at the stimulation of immune system. The immunostimulation is required in immunodeficiencies and some types of chronic infection. The stimulation of the immunity can be *in vitro* investigated as follow (Cochet *et al.*, 1998; Zane, 2001; Jame and John, 2003: 702-711).

- Nonspecific immunity:
  - Natural killer cell function
  - Polymorphonuclear neutrophils activity
  - Macrophage activation
  - Complement system
- Specific immunity:
  - lymphocyte proliferation
  - lymphocyte activation:
    - B cell: antibody production
    - T cell: cytokine production, cell surface marker expression, CTL functions

In this study, we are interested in studying immunostimulatory effect of *S. scaphigerum* on both types of immunity. Macrophage activation was chosen for evaluating nonspecific immune response and lymphocyte proliferation and activation was selected for determining specific immune response.

2.3.1 Lymphocyte stimulation

Lymphocyte stimulation is an ordered series of resting lymphocyte activation. When naive lymphocytes specifically recognize an antigen, the cell is activated before dividing into its progenies which can fully function as regulatory or effector lymphocytes.

2.3.1.1 Lymphocyte activation and proliferation

During the last ten years much has been learned about the molecular mechanisms regulating receptor-mediated signaling cascades in lymphocytes. The sequence of events in antigen receptor (e.g. the T-cell receptor, TCR or the B-cell receptor, BCR) signaling leading to activation involves regulation of a number of protein tyrosine kinases (PTKs) and the phosphorylation status of many of their substrates. Proximal signaling pathways involve PTKs of the Src, Syk, Csk and Tec families, adapter proteins and effector enzymes in a highly organized tyrosine-phosphorylation cascade.

The cytosolic enzyme phospholipase C-γ1 is stimulated by the PTK cascade. Activated PLCγ1 hydrolyses phosphatidyl-inositol-4, 5 bisphosphate (PIP2) thereby generating the second messenger diacylglycerol (DAG) and inositol trisphosphate (IP3). While DAG is mainly responsible for the activation of protein kinase C, a family of cytosolic enzymes that can phosphorylate other proteins at serine and threonine residue, IP3 releasing into the cytoplasm binds to specific membrane receptors and mediates rising of free calcium ions in intracellular which deluge into cytosol from organelle storage pools, reaching maximal concentrations within 1 minute after contacting with the activating stimulus. Both activated DAG and IP3 molecules are thought to be critical for initiating the subsequent event in activation (David, 2004: 314 – 320).

The rates of oxidative metabolism and overall protein and RNA synthesis in the lymphocyte are raised within the first hour after stimulation. The chromatin becomes less dense as previously silent genes are transcribed and the cell prepares to undergo mitosis. After 2-4 hours, specific proteins which are responsible to control cell proliferation, express in nucleus. At the same time, the morphologic cells shift to a process known as blast transformation: it's over all diameter increase to 15 – 30 μm as both nucleus and cytoplasm enlarge: the nuclear chromatin becomes loose and pale staining; and the cell acquires a prominent nucleolus (reflecting a high rate of RNA

synthesis). Within 8 – 12 hours, the changes are sufficiently marked that the cell can be recognized under the light microscope as lymphoblast, a lymphocyte poised to begin mitosis. DNA synthesis spends time around 18 - 24 hours after stimulation. The first cell division occurs 2 – 4 hours later depends on the conditions, and can be repeated five or more times in succession, at intervals of 6 hours. The effecter cells, produced as result of each division, mature completely within a few days and express the immune functions typical of their lineage for several days thereafter.

- *In vitro* measurement of lymphocyte stimulation

Lymphocyte stimulation can be investigated by measuring cell proliferation. *In vitro* studies, some mitogen such as lactins are used as stimuli for inducing lymphocyte proliferation. Phytohemagglutinin (PHA) is a mitogen commonly used for studying lymphocyte stimulation. MTT and ^{3}H-thymidine incorporation assays are approachable standard methods using worldwide to evaluate lymphocyte stimulation.

- Mitogenic activity by MTT assay

MTT can be used for measuring the cell proliferation and the reduction in cell viability. Yellow tetrazolium, MTT (3-(4,5-dimethylthiazolyl–2)-2,5–diphenyltetrazolium bromide), is reduced in metabolically active cells, in part by the action of succinic dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometer (Gerlier and Thomasset, 1986: 57-63; Weichert *et al.*, 1991: 139 - 144).

- Lymphocyte proliferation assay by ^{3}H-thymidine incorporation

The incorporation of radiolabelled tritiated thymidine (^{3}H-thymidine) into the DNA of dividing cells is commonly used for measuring cell proliferation. This assay requires cells to be incubated in the presence of the antigenic or mitogenic stimulus for 2 - 7

days, before the addition of ^{3}H-thymidine for 6 – 18 hours. The total amount of the radiolabeled thymidine incorporated into the cells is measured and the rate of DNA synthesis by the entire population of cells is then evaluated (Julian, 1998).

- Detection activated lymphocyte by measuring activated cell surface marker

Immune cells defend our bodies by acting as a coordinated team. This cellular defense team communicates in precise, highly regulated ways. Specific molecules on surfaces are mediated to activate the immune cells. Stimulation of lymphocytes leads to up regulation of various cell surface markers at various stages of cellular activation, such as CD69 (very early), CD71 or transferrin receptor (early), CD25 (late), and HLA-DR (very late). All of these markers are up regulated following T-cell activation and correlated well with cell proliferation (Caruso *et al.*, 1996: 71-76; Ferenczi *et al.*, 2000: 63-78; Reddy *et al.*, 2004: 127-142). CD69 is a disulfide – linked homodimeric type II transmembrane glycoprotein with a C-type lectin binding domain in the extracellular portion of the molecule. CD69 expression is induced *in vitro* on cells including T and B lymphocyte, Natural killer cells, murine macrophages, neutrophils and eosinophils while it is constitutively expressed on human monocytes, platelets and epidermal Langerhans cells. Moreover, certain results indicated that CD69 may be involved in the pathogenesis of such as acquired immunodeficiency syndrome (Marzio *et al.*, 1999: 565 -582; Harry and Mary, 1997: 217-222). In this study, evaluation of CD69, expressed on T cell activation in response to the water extract of S. scaphigerum was performed using antibodies against CD3 and CD69 molecules and detecting with flow cytometer (Simms and Ellis, 1996: 301-304; Cochet *et al.*, 1998)

2.3.2 Macrophage activation

The mononuclear phagocytic cells consist of monocytes in the blood circulation and macrophages in tissues. Monocytes are produced in the bone marrow. They are motile and phagocytic. They constitute up to 10% of the blood leucocytes. However, the

majorities of them leave the blood after a few hours and migrate into almost all tissues where they develop into macrophages. Macrophages are matured monocytes found in tissues. They play a key role in immunity by ingesting and processing antigens.

Macrophage activation results from the interaction of multiple cytokines and other factors. Lipopolysaccharide (LPS), the main component of the external membrane in gram negative bacteria, and interferon-gamma (IFN -γ), from Th1 cells, are the most frequently used as activating agents for investigating the processes caused by macrophage activation. Interaction with bacterial component triggers macrophages to release TNF-alpha which leads to produce other cytokines and various mediators including nitric oxide.

Macrophage activation can be evaluate by using various parameters, including phagocytosis, synthesis of a particular set of cytokines, releases of free radicals (NO, H_2O_2, $O_2^{\cdot}$), expression of enzyme and cell surface proteins (Amano and Noda, 1995: 425-428; Rostyslav *et al.*, 2001: 652-658; Tafalla *et al.*, 2002: 197–205; Manosroi *et al.*, 2003: 155-160). Phagocytosis and nitric oxide production were used for studying macrophage activities in this study.

2.3.2.1 Phagocytosis

The phagocytic response of innate immune cells such as macrophages is defied by the activation of complex signaling networks that are stimulated by microbial contact (David and Adrian, 2002: 825-852). Phagocyte–microbe contact is accompanied by intracellular signal that triggers cellular processes as diverse as cytoskeletal rearrangement, alterations in membrane trafficking, activation of microbial killing mechanisms, production of proinflammatory cytokines and chemokines, activation of apoptosis, and production of molecules required for efficient antigen presentation to the adaptive immune system (Steven and Sergio, 2002: 136-145) (Figure2.4).

- Measurement of phagocytic activity

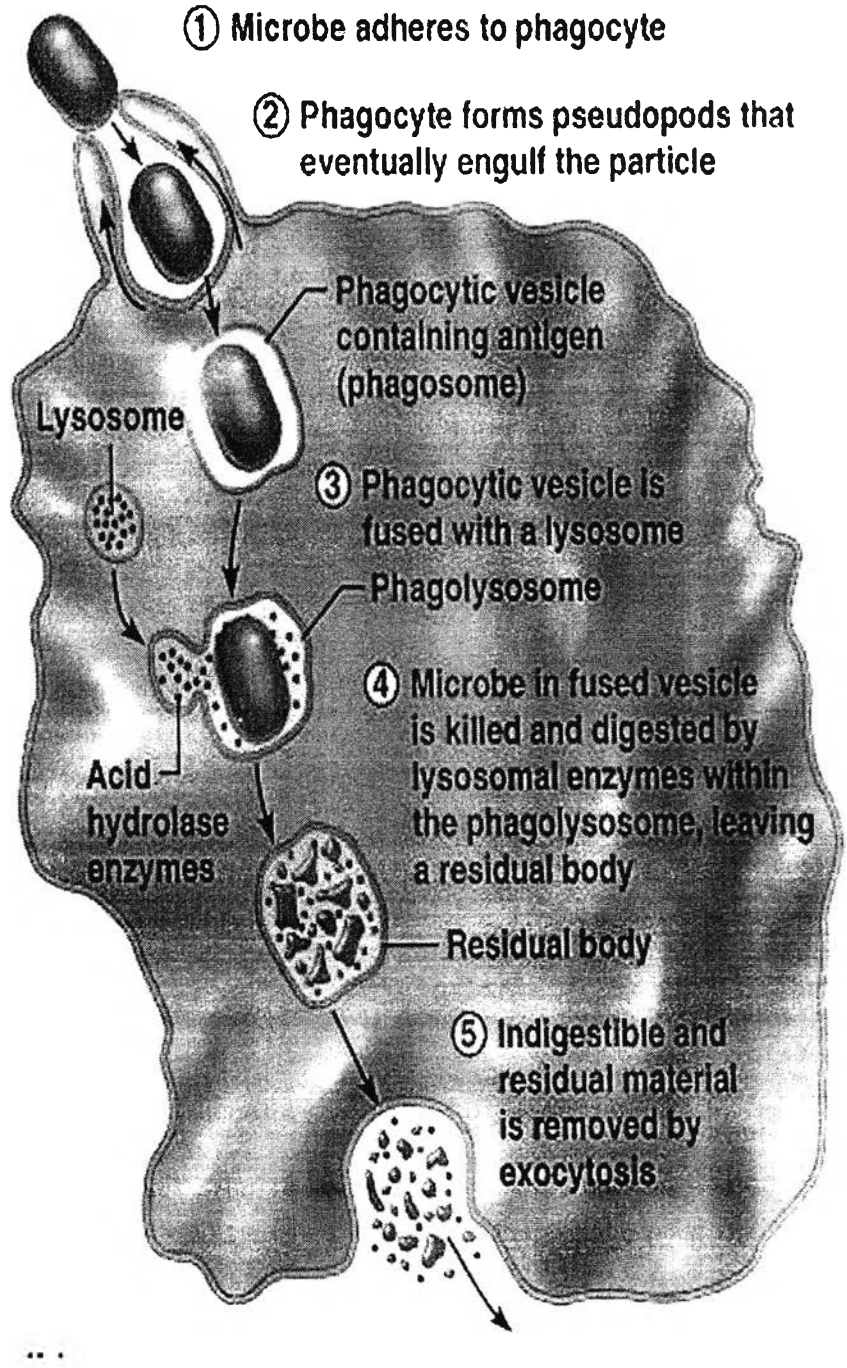
The commonly used method is zymosan phagocytosis. By this method, macrophages are stimulated with opsonized zymosan, a particulate stimulus that reflects the interaction of macrophage–microorganism during the phagocytic process, The phagocytosed zymosan can be detected by using nitro blue tatrazolium (NBT) as indicator (Chompoonuch Boonarkart , 2003).

- Zymosan phagocytosis assay

Zymosan is a *Saccharomyces cerevisiae* yeast cell wall particle that is made up primarily of α-mannan/mannoproteins and β-glucans. The macrophage mannose receptor, binding α-mannan, and nonphagocytic cell dectin-1 receptor, binding β-glucan have been demonstrated to mediate phagocytosis of yeast and zymosan (Alan *et al.*, 1990: 1785-1794; Gordon and Siamon, 2001: 36 - 37). Phagocytosis of zymosan through its glucan component has been shown to trigger the production of O_2-, nitric oxide (Amano and Noda, 1995: 425-428; Tafalla *et al.*, 2002: 197–205)

In this assay, NBT, added along with the zymosan, is used as the indicator of phagocytosis. The NBT is swept into phagocytic vacuole with zymosan. It is a soluble yellow redox dye. In the present of superoxide anions the dye is chemically reduced. The yield is a dark purple insoluble compound which can be extracted from the macrophage with dimethyl sulfoxide (DMSO). This dark purple from formazan can be quantified by spectrophotometer. (Manosroi *et al.*, 2003: 155-160)

Figure2.4 Phagocytosis and processing of exogenous antigen by macrophage (Available from http://faculty.washington.edu/kepeter/118/photos/non-specific_images.htm [2005, March 3]



2.3.2.2 Nitric oxide synthesis and function

Activated macrophages synthesize several of antimicrobial and cytotoxic substances to destroy phagocytosed microorganism. Many of these mediators are listed in table which Table 2.1. Nitric oxide (NO) radical from macrophage is increasingly recognized as an important mediator of physiological and pathology in the immune response. NO is concerned in the pathogenesis and control of infectious diseases, anti-tumor, autoimmune process and chronic degenerative diseases (Christian, 2001: 907-916).

Table 2.1 Mediators of antimicrobial and cytotoxic activity of macrophage

Oxygen – depending killing	Oxygen – independent killing
Reactive oxygen intermediates	Defensins
O_2^- (superoxide anion)	Tumor necrosis factor
OH- (hydroxyl radicals)	Lysozyme
H_2O_2 (hydrogen peroxide)	Hydrolytic enzymes
ClO- (hydrochloride anion)	
Reactive nitrogen intermediates	
NO (nitric oxide)	
NO_2 (nitrogen dioxide)	
HNO_2 (nitrous acid)	
$OONO^-$ (peroxynitrite)	
Other	
NH_2Cl (monochloramine)	

Nitric oxide is an uncharged molecule composed of seven electrons from nitrogen and eight electrons from oxygen. This combination results in the presence of an unpaired electron, which makes NO paramagnetic and a radical. The majority of biological molecules contain bonds filled with two electrons. NO generation yields nitrite (NO_2^-) and nitrate (NO_3^-) as its end products in aqueous systems and at air-liquid interfaces. The basis of many biological actions of NO is the activation of guanylyl cyclase through binding to the haem prosthetic group of the enzyme. Guanylyl cyclase

increases the production of cyclic GMP (cGMP). The immediate target of cGMP is cGMP-dependent protein kinase (PKG) (Ruth, 1995: 355-372).

Nitric oxide is a product from macrophages which are activated by cytokines, microbial components or both. It is derived from the amino acid L-arginine by the enzymatic activity of inducible nitric oxide synthase (iNOS or NOS2). It is also one of the major antimicrobial mechanisms of macrophages in innate immunity response. Macrophage NOS (NOS2, iNOS) is expressed at low levels under basal condition but induced by gamma-interferon and lipopolysaccharide. NOS2 has been documented in macrophages from human, horse, cow, goat, sheep, rat, mouse, and chicken.

An effect of nitric oxide from macrophage plays a fundamental role in the antimicrobial and anti-tumor. Sustained production of NO makes macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminthes, and tumor cells (John *et al.*, 1997). These reactions have wide implications for the physiologic and toxic effects of NO. NO acts against tumor cells on the contrary to antimicrobial intracellular actions. This property requires extracellular killing. In addition, production of reactive oxygen species or NO mediated tumor cell death can induce apoptosis of macrophage. This may be due to oxidative burst and accompanying cytochrome c release and caspase-3 activation (Xiantang *et al.*, 1997: 133-145; Jorge and Jonathan, 1998: 39–53; Jae B.P., 2003: 325-335). However, high rates of NO synthesis can also damage host tissues, especially if prolonged synthesis (as in chronic inflammations) occurs.

- Measurement of nitric oxide release

The method commonly used for determining the amount of NO is the Griess reaction. Activated macrophages can produce several nanograms per liter of NO in a culture. However, NO is not stable. It is a very reactive gas and readily trapped by heme, reactive oxygen or other biological materials. Nitric oxide is rapidly converted to

nitrite and nitrate in typical oxygenated aqueous solutions. So, end products, nitrite or nitrate, are used in the determination as an indicator of nitric oxide produced by activated macrophage (Amano and Noda, 1995: 425-428; Teri L. *et al.*, 2000: 43-58). The nitrite or nitrate specifically reacts with Griess reagent, showing a colorimetric change.

- Griess reaction assay

Griess reaction assay consists of two reagents; sulfanilamide and *N*-(1-naphthyl) ethylenediamine. These reagents are used for determining nitrite, which is an indicator of nitric oxide production. Sulfanilamide is quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to *N*-(1-naphthyl) ethylenediamine and formed an azo dye that can be determined by spectrophotometer.