จุฬาลงกรณ์มหาวิทยาลัย

ทุนวิจัย

กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

ผลกระทบของตะกั่วต่อเซลล์เม็ดเลือดแดงตัวอ่อนของคน

โดย

สุกัญญา สุนทรส

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27 กันยายน 2545

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณ คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล สำหรับสถานที่ห้องปฏิบัติการเลี้ยงเซลล์เม็ดเลือดแดง ขอขอบคุณคณะแพทยศาสตร์ ศิริราชพยาบาล สำหรับเครื่อง Flow Cytometer และขอขอบคุณกองทุนวิจัยรัชดาภิเษกสมโภช จุฬาลงกรณ์มหาวิทยาลัย สำหรับความสนับสนุนด้านงบประมาณ

ชื่อโครงการวิจัย ผลกระทบของตะกั่วต่อเซลล์เม็ดเลือดแดงตัวอ่อนของคน (Effect of Lead on Human Erythroid Precursor Cells)

ชื่อผู้วิจัย สุกัญญา สุนทรส, เวณิกา เบ็ญจพงษ์, อานนท์ บุญยรัตเวช และ โกวิทย์ พัฒนปัญญาสัตย์

เดือนและปีที่ทำวิจัยเสร็จ 2545

บทคัดย่อ

การวิจัยนี้เป็นการศึกษาความเป็นพิษของตะกั่วในเซลล์เม็ดเลือดแดง(ตัว)อ่อนของ ความผิดปกติในการเจริญของเซลล์เม็ดเลือดแดง(ตัว)อ่อนเหล่านี้อาจมีความเกี่ยวข้องกับการเกิดโรคโลหิตจางเนื่องจากพิษของตะกั่ว เซลล์เม็ดเลือดแดง(ตัว)อ่อนของคนซึ่งใช้เป็นแบบจำลองในการศึกษาเตรียมขึ้นโดยเทคนิคการเลี้ยงเซลล์แบบ Two-phase liquid culture (TPLC) เซลล์เม็ดเลือดแดง(ตัว)อ่อนที่เลี้ยงโดยวิธีนี้จะมีความบริสุทธิ์สูง (> 90%) และมีจำนวนมาก ($30.46\pm19.48\times10^6$ เซลล์ต่อเลือด 1 ยูนิต)

เซลล์เม็ดเลือดแดง(ตัว)อ่อนระยะแรกที่ได้จากการเลี้ยงเซลล์ในวันที่ 5 ของเฟสที่ 2 ถูกนำมาเลี้ยงในสภาวะที่มีตะกั่ว การศึกษาทางสัณฐานวิทยาจากการดูด้วยกล้องจุลทรรศน์พบว่าตะกั่วมีผลทำให้เกิดการตายของเซลล์เม็ดเลือดแดง(ตัว)อ่อนโดยไปทำลายเซลล์ทั้งในลักษณะของ cell cytolysis และ apoptosis ทั้งนี้ขึ้นอยู่กับเวลาและความเข้มข้นของตะกั่ว กล่าวคือ เมื่อความเข้มข้นของตะกั่วอะซิเตทมากกว่า 1 ส่วนในล้านส่วน จะมีผลต่อความสามารถในการมีชีวิตอยู่ของเซลล์อย่างเห็นได้ชัด การศึกษาการเกิด apoptosis โดยใช้ flow cytometric analysis ติดตามสารเรืองแสงที่ติดฉลากบน annexin V ซึ่งสามารถจับ phosphatidylserine ที่ผิวนอกของเซลล์ที่เกิด apoptosis พบว่าตะกั่วสามารถทำให้เซลล์เม็ดเลือดแดง(ตัว)อ่อนเข้าสู่การตายแบบ apoptosis การเกิด apoptosis เกิดขึ้นเมื่อความเข้มข้นของตะกั่วอะซิเตทมากกว่า 1 ส่วนในล้านส่วน โดยขึ้นอยู่กับเวลาและความเข้มข้นของตะกั่ว การค้นพบนี้นำไปสู่มุมมองใหม่ของการเกิดโรคโลหิตจางเนื่องจากพิษของตะกั่วนั่นคือนอกจากความบกพร่องของการสังเคราะห์ฮีโมโกลบินและการทำให้เซลล์เม็ดเลือดแดง(แก่)มีอายุสั้นแล้ว การยับยั้งการมีชีวิตอยู่ของเซลล์เม็ดเลือดแดง(ตัว)อ่อนโดยการทำให้เกิดการตายแบบ apoptosis อาจเป็นอีกสาเหตุหนึ่งของการเกิดโรคโลหิตจางเนื่องจากพิษของตะกั่ว

Project Title Effect of Lead on Human Erythroid Precursor Cells

Name of the Investigators Suganya Soontaros, Wenika Benjapong, Ahnond Bunyaratvej and Kovit Pattanapanyasat

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Abstract

The mechanism of lead toxicity in human erythroid precursor cells (EPCs) is the main aim of this research. Abnormal development of EPCs, the immature red blood cells, may involve with lead induced anemia. Human EPCs, the model in this study, were prepared by two-phase liquid culture (TPLC) technique. Highly purified EPCs (>90%) and substantial numbers of the cells (30.46 $\pm$ 19.48x10^6 cells/blood unit) were obtained from this technique.

By using TPLC system, the early stage of EPCs obtained on day 7 of secondary phase were cultured in the presence of lead acetate. Morphological study showed that lead could inhibit EPC survival by inducing the cell cytolysis and apoptosis. The inhibition was time and dose-dependent. Marked effect of lead on EPC survival was at lead acetate concentration $\geq$1 ppm. Flow cytometric analysis was used to detect apoptotic cells by monitoring the binding of fluorescence labeled annexin V to phosphatidylserine on the outer membrane of apoptotic cells. The study showed that lead could induce apoptosis in EPCs in time and dose-dependent manner at lead concentration $\geq$1 ppm. These findings suggest new aspect of lead induced anemia besides the impairment of hemoglobin synthesis and shortened life span of erythrocytes, lead induced apoptosis in human EPCs resulting in the inhibition of EPC survival may be another mechanism of lead induced anemia.

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CHAPTER I

INTRODUCTION

1. Lead

1.1. Physical and Chemical Properties (Reilly,1991; Lewis,1996; Piomelli,1998)

Lead (Pb) is one of the heavy metal with a density of 11.34 and the atomic weight is 207.19. Its melting point is 327.43 °C and boiling point is 1740 °C. It is soluble in nitric acid and acetic acid. Oxidative states are 0,+2, and +4. In inorganic compound lead is usually in state +2. Lead has a strong affinity for the sulfhydryl groups of cysteine, the amino group of lysine, the carboxyl group of glutamic and aspartic acid, and the hydroxyl group of tyrosine. Lead binds to proteins, modifies their tertiary structure, and inactivates enzyme properties.

1.2. Lead Contamination

Lead is the important toxic metal that leads to a public health problem in many countries including Thailand. Since lead is a ubiquitous metal in our environment and is utilized in many industries including agriculture all over the world, a large number of articles on this metal and its harmful effect have been being increased around the world. Recently (1998), at Kanjanaburi province of Thailand, high blood lead level (20-40 μg/dl) was detected in Klity villagers who lived near the creek with lead contamination from mining. Drawing water and eating fish from the creek for years induced lead poisoning in the villagers.

Nowadays, the use of lead compounds, both organic and inorganic, is extensively and continuously increasing. These range from an additive in food and drink to a material for making containers; from a poisonous substance (pesticide) to a medicinal drug; from a stabilizer for plastics to an antiknock additive in gasoline; from

printing ink to paint manufacturing including glazing of ceramics, etc (Castellino, 1995). As a result of these maltifarious uses, lead is a ubiquitous environmental contaminant that causes harmful effect in human.

Lead contamination occurs widely throughout the environment and has been found in soil, water and air (Stubbs, 1973; Carelli et al., 1995). The major non -industrial sources of lead contamination for people are food and water (Flegal et al., 1990). Nowadays, as Thailand has planned for the development and expansion of industries, there is no doubt that the use of lead, the basic material in industries, will be increased. Due to the extensive use of lead, lead contamination in soil (ปรีญาพร สุวรรณเกษ, 2535 และ นภวัส บัวสรวง, 2536), water (นิตยา มหาผล, 2534) and air (Supat Wangwongwatana, 1997) have occurred in many areas of Thailand, especially in the capital and industry areas. Lead contamination in environment is one of the major cause of lead in human diet. From the report of the department of medical sciences (ทวีศักดิ์ บุณยโชติมงคล และ คณะ, 2531), in 563 samples of 5 kinds of thai foods, lead content higher than 1.00 mg/kg (Thai standard of lead in food: An Annoucement of Ministry of Public Health, No. 98/1986 and CODEX 1985) was found in 97 samples. In addition, from the report of Napawas Buasruang, it was found that the vegetative plant from the industrial zone of Samuthprakan had high lead content, the average was 29.09 mg/kg dry weight (2.00-454.00 mg/kg dry weight). Recently, the animals in Klity creek (lead contamination creek) were analysed and found that all of them had lead content higher than the indicated allowance value. The maximum lead content was 451.8 mg/kg (ชุติมา นุ่นมัน, 2543).

1.3. **Lead Toxicity** (Waldron, 1980; Goldfrank et al., 1990; Shibamoto et al., 1993; Goyer, 1996)

Lead is a toxic metal without any function in human body. Because of its affinity for sulfhydryl groups, it damages a multitude of enzymes and essential cellular structure (such as mitochondria). The organ systems most affected by lead are hematopoietic, nervous and renal system. Gastrointestinal, cardiovascular and reproductive systems also be affected. Lead poisoning was defined as a blood lead level greater than or equal to 10 μg/dl (Table 1). Lead has toxicity at low doses towards neurobehavioral

Table 1 : Lowest observed effect levels for induced health effects and blood lead concentration (μg/dl) (Goldfrank, 1990; Goyer, 1996).

EFFECT	CHILDREN	ADULTS
Heme effects		
Anemia	80-100	80-100
U-ALA	40	40
B-EPP	15	15
ALA inhibition	10	10
Py-5-N inhibition	10	-
Neuro effects		
Encephalopathy (overt)	80-100	100-112
Hearing deficit	20	-
IQ deficit	10-15	-
In vivo effects	10-15	-
Peripheral neuropathy	40	40
Possible decreased nerve conduction	-	25-50
Renal effects		
Nepharopathy	40	60
Blood pressure (male)	-	30
Reproductive effects		
Impaired spermatogenesis and oogenesis	-	25-50
Impaired cognitive development-neonate	-	<25

NOTE : U-ALA = Urine aminolaevulinic acid

EPP = Erythrocyte protoporphyrin

ALAD = Aminolaevulinic acid dehydratase

Py-5-N = Pyrimidine-5-nucleosidase

development and hematological conditions been recognized. The world health organization (WHO) provides a guideline value for lead in blood should be below 20μg/dl for adult (WHO, 1987). A blood lead concentration of 10 μg/dl is considered the maximum level not associated with any know adverse effect in children. The blood lead concentrations actually measured in the general population often exceeds the guideline value of 10 μg/dl. For occupationally people exposed to lead, blood lead levels were much higher.

Lead has a multiplicity of biochemical and physiological effect especially in infant and children. The most common, though not universal, consequence of lead poisoning is anemia and this is produced by interfere with heme synthesis (Hernberg et al, 1970; Rossi et al., 1993), globin synthesis (Kassenaar et al, 1957; Ali et al., 1977) and the induction of erythrocyte membrane defects (Waldron, 1964; Valentino et al., 1982; Grabowska et al., 1996). The anemia observed in chronic lead poisoning is generally of moderate severity, with the number of red cells very occasionally dropping and hemoglobin level below 8 g/dl. Moreover, it is usually normocytic and only slightly hypochromic. There are increased numbers of reticulocytes with basophilic stippling.

Toxic effect of lead on the nervous system occurs in both the central nervous system (CNS) and the peripheral nervous system (PNS). In the CNS, lead causes edema and has a direct cytotoxic effect result in the symptom of lead encephalophathy. Lead encephalophathy is rare in adults but it occurs more frequently in children. Decreased nerve condition, lower IQ , increased psychomotor activity and learning disorders have all been report in children expose to lead. Peripheral neuropathy of lead poisoning involves considerable loss of motor function leads to the symptom of wrist drop or foot drop.

The kidney represents one of the major target organs in human lead exposure. Acute lead nephropathy is a reversible tubular defect and produces fanconi syndrome. Chronic lead nephropathy is a irreversible tubular interstitial nephritis which may follow an acute and massive lead poisoning or a prolonged moderate lead exposure. This condition may end in renal failure. It also may be associated with gout and hypertention.

Gastrointestinal problem such as abdominal colic, vomiting and constipation occur. Liver function abnormalities have been reported and probably present as a toxic hepatitis. In the heart, lead causes swelling of the myocardial fibers, which results in myocarditis and eventual fibrosis. Lead exert spermatotoxic effects on the male resulting in infertility. Lead does cross the placenta, thereby affecting the fetus. High lead content in placenta and fetal membranes has been associated with premature rupture of membranes and preterm delivery. Further, low level exposure of lead is known to induce inflammatory response and modulates immune functions (Borella and Giardino, 1991)

Inorganic lead compounds are classified as possibly carcinogenic to human by the international agency for research on cancer (IRAC, 1987). Evidence for carcinogenicity is adequate in animals but inadequate in human. Epidemiological evidence is not conclusive to implicate lead as a human carcinogen. However, the findings are not entirely negative. The general opinion at this time is that exposure to lead compounds may be contribute to higher incidences of human cancer and that therefore cannot be ruled out as a carcinogen (Cohen et al., 1990; Hartwig, 1994). In short, studies on the genetic toxicology of lead compounds reach the conclusion that lead-induced mutation. Lead may not be a result of direct damage to DNA but may occur via indirect mechanisms including disturbances in enzyme function in DNA synthesis and/or repair, or in DNA helical structure (Frenkel and Middleton, 1987; Zelikoff et al. 1988; Beyersmann, 1994). Although inorganic lead compounds exhibit only a weak mutagenic and induce DNA strand breaks only at toxic concentrations (Hartwig, 1990; Hartwig, 1995; Ariza, Bijur, and Williams, 1998) possibly via reactive oxygen species (Yang et al., 1999). However under certain condition, lead may be comutagenic and mitogenic (Zelikoff et al., 1988 ; Calabrese and Baldwin, 1992 ; Roy and Rossman, 1992).

2. Lead and Erythropoietic system

All of the blood cells are produced from a single type of cell, the pluripotent hematopoietic stem cell (PSC), in bone marrow. The processes involved in production of all the various cell of the blood are collectively called **hematopoiesis**. A single pluripotent

hematopoietic stem cell now know as the spleen colony-forming unit(CFU-S). The CFU-S give rise to other progenitor cells that are more committed to a give lineage, myeloid and lymphoid progenitor. The myeloid progenitor will develop into erythrocytes, granulocytes, macrophages and platelets, while the lymphoid progenitor will develop into B-lymphocytes (plasma cells) and T-lymphocytes (Bagby, 1994; Bondurant and Koury, 1998). The entire process by which red cells are produced in the bone marrow is called **erythropoiesis** (Figure 1). Erythropoiesis is a multistep process involving the differentiation of PSC through the lineage-committed burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) progenitor cells, which give rise to a series of erythroid precursor cells, eventually leading to the formation of mature erythrocytes (Dessypris, 1998). During this process, the sequential formation of proerythroblasts, basophilic, polychromatophilic and orthochromatic erythroblasts is positively regulated by erythropoietin (EPO), a glycoprotein hormone produced by the kidney in response to tissue hypoxia. EPO displays multiple positive effects on early erythroblasts, including increased proliferation, progression through maturation, stimulation of hemoglobin synthesis and protection from apoptosis (Krantz, 1991; Kelley et al., 1993; Adamson, 1994).

In human, the most primitive single lineage committed erythroid progenitors are named BFU-E because *in vitro* and in the presence of growth factors, it produces a burst consisting of thousands of nucleated red cells. As the progenitor cell matures, its capacity to produce progeny diminishes until it reaches a stage of the CFU-E, at whichtime it only produces a small colony consisting of between 30 and 60 nucleated red cells.

The erythroid precursor cell compartment is a term of erythroid cell including the five maturation stages of proerythroblast, basophilic erythroblast, polychromatophilic erythroblast, orthochromatic erythroblast, and reticulocyte. These cells can defined by morphological criteria. The earliest morphologically recognizable erythroid cell is the proerythroblast.The cell divides and matures through various stages that involve nuclear condensation and extrusion and hemoglobin accumulation. During the maturation, 3 to 4 mitotic divisions occur between proerythroblast and polychromatophilic erythroblast.

BONE MARROW

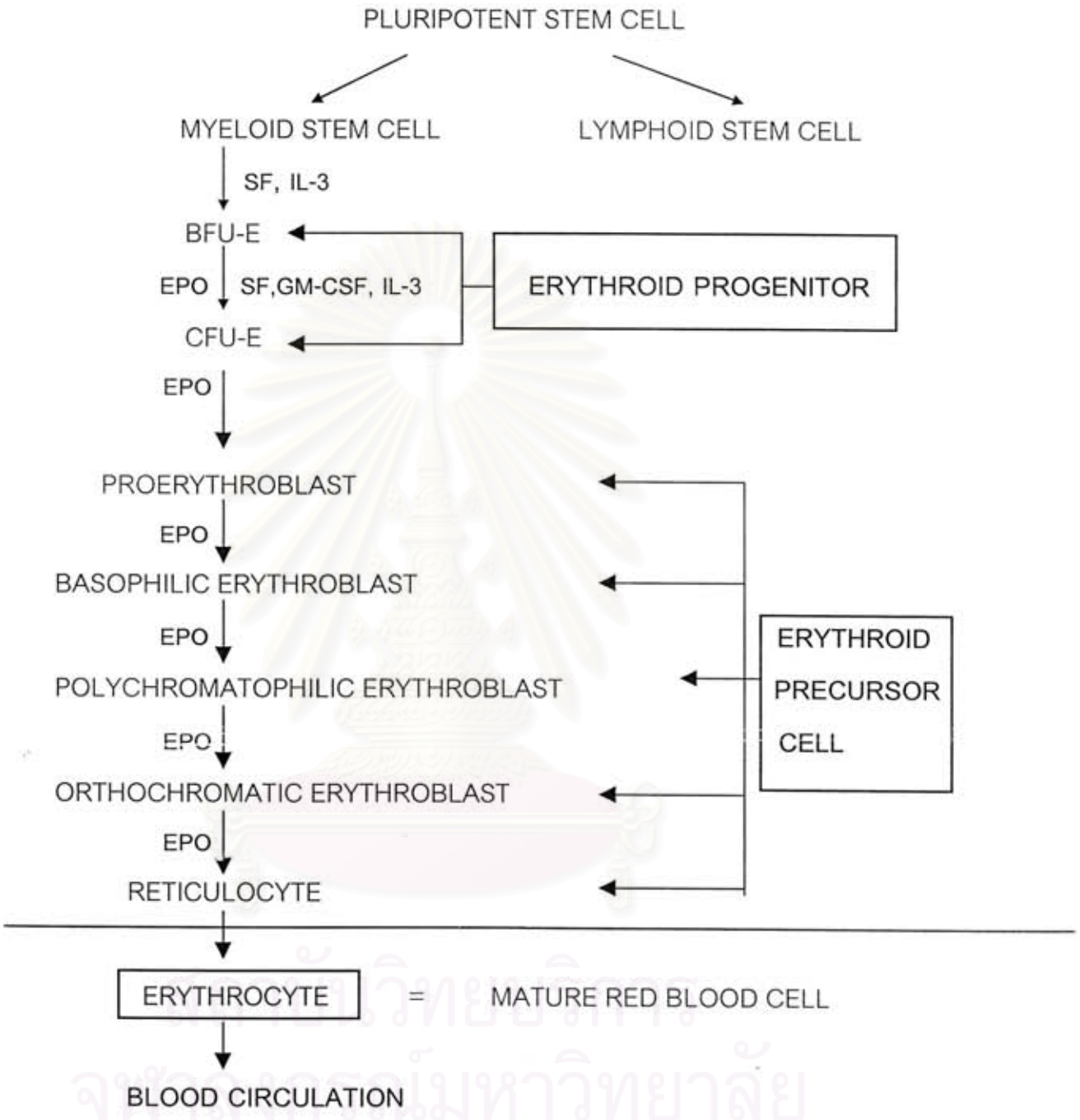


Figure 1 : Red blood cell development.

When the cell reaches the orthochromatic erythroblast stage, the nucleus is extremely condensed and can not synthesize DNA, therefore, cannot divide. After approximately one more day, the nucleus is extruded. Soon after denucleation, the nucleus is engulfed by a macrophage. The cell may remain with in the marrow, as a reticulocyte, for 2 to 3 days before it is released into the peripheral blood and develops into mature red blood cell (erythrocyte) with in 1 to 2 days (Hunter, 1993; Bull and Gorius, 1995; Bondurant and Koury, 1998).

It has long been known that lead poisoning causes adverse effects on the erythropoietic system. Anemia is the most common erythrologic effect induced by lead. Several effects of chronic inorganic lead poisoning on the bone marrow and peripheral blood have been suspected and proved (Albahary, 1972). Some are as follow.

2.1. Effect of Lead on Hemoglobin Synthesis

The interference of Hb synthesis is one of the important mechanisms of lead poisoning anemia. Hb synthesis starts and ends inside the mitochondria ; intermediate steps take place in the cytoplasm. At the cellular level lead interacts with sulfhydryl groups, leading to the interference of the enzyme action necessary for Hb synthesis (Hernberg and Nikkanen, 1970; Goldfrank et al., 1990; Rossi et al., 1993; Piomelli, 1998). Lead interferes at several points in the heme synthetic pathway (Figure 2). The two most important steps affected by lead are those catalyzed by δ-aminolaevulinic acid dehydratase (δ-ALAD) and ferrochelatase. δ-ALAD is a cytosolic enzyme. Direct binding of lead to the sulfhydryl groups of δ-ALAD results in its inhibition, leading to the δ-ALA accumulation. The last step of heme synthesis is catalyzed by ferrochelatase, located in the inner matrix of the mitochondria. The inhibition by lead of the step catalyzed by this enzyme, the insertion of iron into the protoporphyrin ring, results in the accumulation of the latter compound. Since lead limits the intracellular delivery of iron to the site of ferrochelatase, and the surrogate metal zinc is inserted into protoporphyrin by ferrochelatase as in iron deficiency so that zinc protoporphyrin accumulate in the maturing erythrocyte (Labbe et al., 1987). Moreover, lead also inhibits the activity of the other enzymes rich in sulfhydryl groups in several steps of heme synthesis such as

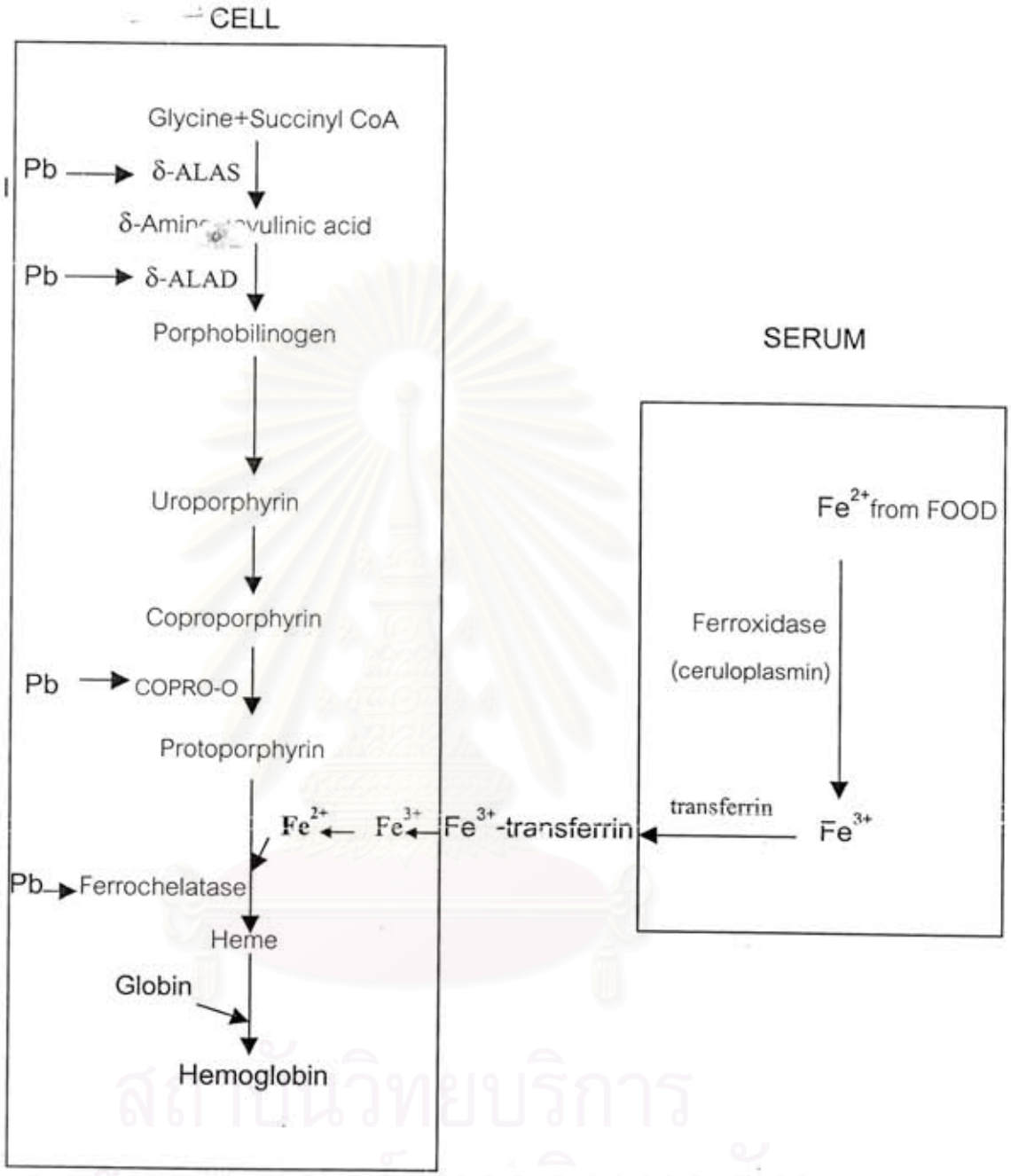


Figure 2 : Scheme of heme synthesis showing sites where lead has an effect Red arrow (→) indicates those enzymes blocked by lead (Goldfrank, 1990; Goyer,1996).

δ-aminolaevulinic acid synthetase(δ-ALAS) and coproporphyrinogen oxidase(COPRO-O) (Waldron, 1966).

The effects of lead on Hb synthesis are not only limited to an overall decresse in heme production but are also seen in the synthesis of globin (Kassenaar et al., 1957; White and Harvey, 1972; Ali and Quinlan, 1977). It inhibits the incorporation of tritiated leucine into globin chains. A markered decrease occurs in the synthesis of α chain than of β chain.

2.2. Effect of Lead on Red Blood Cell Survival

Shortened erythrocyte life span is also another important cause of lead-induced anemia. Lead interferes with the sodium-potassium ATPase pump mechanism leading to cellular loss of K^+, and also attaches to red blood cell membranes causing increased fragility and decreased membrane fluidity thus reducing red cell survival (Karai et al., 1982; Valentino et al., 1982; Grabowska and Guminska, 1996). Lead also impairs the enzyme pyrimidine- 5'-nucleotidase activity, resulting in the accumulation of nucleotides that inhibit pentose phosphate shunt (Lachant et al., 1984) and promote hemolysis as in the genetically determined deficiency of the enzyme (Paglia et al., 1977). The inhibitory effect of lead on the acyltransferase enzyme changed lipid structure of red blood cell membrane, resulting in enhanced osmotic resistance and an increase in hemolysis (Cook et al., 1987). Lead is also enhanced auto-oxidation of Hb with the production of free reactive oxygen radicals such as O_2^- and $H_2O_2^-$, giving rise to an increase in the peroxidation rate of the membrane lipids. Finally, the inhibition of glutathione activity might also reduce red blood cell defenses against oxidative processes and cellular aging, thus accelerating their destruction (Fabri and Castellino, 1995).

2.3. Lead and Bone Marrow Alteration

Another factor is partly responsible for this anemia may be the relative inefficiency of bone marrow, temporarily unable to renew itself after the loss due to hemolysis. The toxic effects of lead poisoning appear earlier in bone marrow than in the blood stream. Nearly fifty times as much lead is found in bone marrow as in peripheral blood (Waldron,1966). The mitochondria and the ribosomes of erythroblasts and the

reticulocytes are known to be damaged, leading to the formation of basophilic stippled cells and abnormalities of heme synthesis. The hemolysis in lead poisoning appears to depend chiefly on the fragility of the reticulocytes and the stippled cells, but the other red cells are involved too. Moreover, the erythroblastic maturation is also altered (Taketani et al., 1985; Osterode et al., 1999). The bone marrow eventually takes on a typical hyperstimulated because of high hemolysis or ineffective erythropoiesis (Waldron, 1966; Berk et al., 1970; Albahary, 1972). However, with prolonged lead exposure, erythroid hypoplasia may occur (Leikin and Eng, 1963).

As the mechanism of lead interference on Hb synthesis is rather clear, but it is uncertain on how lead affects the maturation of erythroid cells. Recently studies in human hematopoitic progenitor cells demonstrate that lead can inhibit both myeloid and erythroid colony formation in a dose-dependent manner. However, lead influences erythroid cells to a greater extent than that upon the myeloid cells (Boucher et al., 1998; Osterode et al., 1999; Van Den Heuvel et al., 1999). The mechanism leading to the reduction of these cells is not clear. It may be due to the effect of lead on the mechanical fragility of cell membrane leading to cell hemolysis, or the other pathways such as the production of apoptosis in these cells.

3. Apoptosis

3.1. The Induction of Apoptosis (Thompson, 1995; Israels et al., 1999)

Apoptosis and necrosis are two primary processes of cell death. Cell death by necrosis usually follows major pathological acute injury such as hypoxia, hyperthermia, viral invasion, exposure to various exogenous toxins, or attack by complement. Necrosis is characterized by early mitochondrial swelling and failure, dysfunction of the plasma membrane with loss of homeostasis, cell swelling, and rupture. The loss of cell membrane integrity with release of cell contents, including proteases and lysozymes, induces an inflammatory response are also the characterization of necrosis. Incontrast, apoptosis, also known as programmed cell death, is the mechanism by which cells are "silently" removed under normal conditions when they reach the end of their life span.

The activation of apoptosis is regulated by many different signals that may originate from both the intracellular and the extracellular milieu. These include lineage information, deprivation of growth factors or cytokines (e.g.,erythropoietin) and cellular damage inflicted by ionizing radiation , chemotherapeutic drugs, oxidants, free radicals or viral infection.

Apoptotic cell death characterized by controlled autodigestion of the cell. Cells appear to initiate their own apoptotic death through the activation of endogenous proteases (the caspase mechanism) (figure 3). This results in cytoskeletal disruption, cell shrinkage, and membrane blebbing. Apoptosis also involves characteristic changes within the nucleus. The nucleus undergoes condensation as endonucleases are activated and begin to degrade nuclear DNA. Apoptosis is also characterized by a loss of mitochondrial function. The dying cell maintains its plasma membrane integrity.

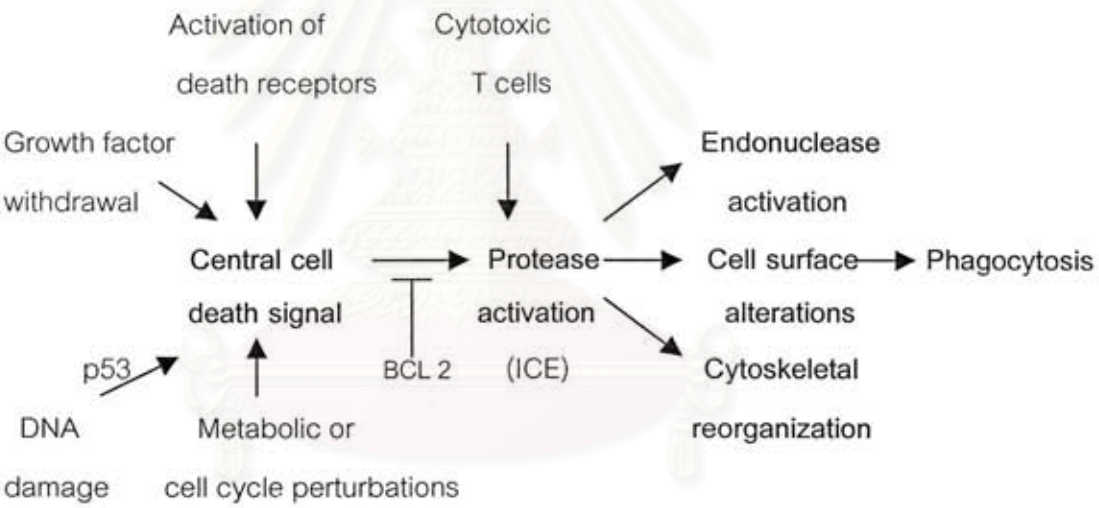


Figure 3: A hypothetical model for the regulation of apoptotic cell death. As diagrammed, the major end point of apoptotic cell death is removal of the dying cell by phagocytosis. Both the death repressor BCL 2 and ICE (interleukin converting enzyme) are members of larger gene families (Thompson, 1995:1457).

However, there is altertions in the plasma membrane of apoptosis cell. The cell is accompanied by a loss of membrane phospholipid asymmetry, resulting in the exposure

of phosphatidylserine (PS) at the cell surface. Appearance of PS on the outer leaflet of the plasma membrane plays an important role in the recognition and removeal of apoptotic cells by phagocytic cells. Finally, there is disruption of the cytoskeletal architecture, the cell shrinks and then fragments into a cluster of membrane-enclosed "apoptotic bodies" that are rapidly ingested by adjacent macrophages or other neighboring phagocytic cells. The rapid phagocytosis of apoptotic cells may prevent the potential tissue damage resulting from the lysis of these cells. Therefore, this process progresses without concomitant induction of an inflammatory response.

Apoptotic process may be set in motion by: A) genes responding to DNA damage; B) death signals received at the cell membrane (Fas ligand); or C) proteolytic enzymes entering directly into the cell (granzymes). The final events, evidenced by the changes in cell structure and disassembly, are the work of specific proteases (caspases). Although the death signal may be regulated by gene expression, the process can be set in motion by diverse stimuli such as genotoxic damage or cytotoxic damage.

In gene regulation of apoptosis: cell injury resulting in genotoxic events activates p53, a transcription regulatory gene. The p53 protein can induce cell cycle arrest in G1, allowing time for repair. In the event that DNA damage is more severe and non-reparable, p53 perform its alternate role of moving the cell into apoptosis through the Bax/Bcl 2 pathway. A high expression of the Bax group promotes apoptosis, while a high expression of the Bcl 2 group inhibits apoptosis. Upon receipt of the apoptotic signal, Bax proteins migrate and bind to the mitochondrial membrane, resulting in the release of cytochrome c binding to Apaf 1 (apoptotic protease-activating factor) is necessary for the subsequent activation of caspase mechanism, responsible for the DNA fragmentation and cytological changes characteristic of apoptosis.

In cytotoxic regulation of apoptosis: This event is initiated through the granzyme system and the Fas/Fas ligand system. The granzyme system is operative in removing pathogen-infected cells and tumor cells. Perforins and granzyme are secretory protein of cytotoxin lymphocytes and natural killer cells. Upon receptor-mediated binding to a target cell, these proteins are inserted into the cells and induce the apoptosis process. Fas-Fas ligand; the alternative non-secretory mechanism of apoptosis is through

activation of death receptors expressed on the cell membrane. Fas (CD 95), a cell-surface receptor and a member of the tumor necrosis factor receptor (TNF-R) family, is a transducer of the apoptotic signal. Fas ligand (FasL) is a member of the TNF family. FasL, by binding to and cross-linking the Fas receptor, set the apoptotic process in motion.

3.2. Apoptosis in Erythropoietic System

Both erythropoietic cell production and elimination are regulated by apoptosis. The maintenance of the erythropoietic stem cells (BFU-E and CFU-E) is dependent upon the presence of erythropoietin (EPO); withdrawal of EPO results in apoptosis of these red cell precursors (Koury and Boudurant, 1990; Kelley et al., 1994). In addition, the sequential formation of proerythroblasts, basophilic, polychromatophilic, and orthochromatic erythroblast is positively regulated by EPO. EPO also serves to prevent apoptosis in early erythroblasts (Kelley et al, 1993). Circulating EPO will become attached to its receptors, initiating a cascade of cytoplasmic phosphorylation terminating in a gene-activating signal (Youssoufian et al., 1993; Haseyama et al., 1999). This signal may promote cellular multiplication or merely act as a survival factor preventing apoptosis of newly formed progenitor cells and proerythroblast (Koury and Bondurant, 1990; Gregoli and Bondurant, 1997). Because of the low expression of antiapoptotic genes, immature erythroblasts are particularly vulnerable in the absence of EPO, which has been shown to repress apoptosis through the induction of Bcl-XL, a member of the Bcl 2 family involved in protection from cell death in a number of systems (Gregoli and Bondurant, 1997; Chao and Korsmeyer, 1998). Released Bcl-XL may then suppress cell death pathway that involve the activity of APO 1, cytochrome c and the caspase protease cascade (Downward, 1998).

By analyzing the DNA cleavage of highly purified human colony-forming unit-erythroids (CFU-Es) generate from purified peripheral blood burst forming unit-erythroids (BFU-Es), it was shown that EPO, insulin-like growth factor-I (IGF-I) and stem cell factor (SCF), each reduced apoptosis of these cells (Muta and Krantz, 1993).

Several other regulatory systems, including the interaction of death receptors with their ligands, may be also involved in the physiological inhibition of erythropoiesis. There are the reports concerning the involvement of Fas and FasL in the apoptosis during erythroid differentiation (Dai et al., 1998; Maria, 1999). Fas is a major member of death receptors family. Fas is rapidly upregulated in early erythroblasts and expressed at high levels through terminal maturation. Molecular crosslinking of Fas by its ligand (FasL) or by agonistic antibodies results in the sequential triggering of caspases responsible for induction apoptotic cell death.

Cytokines or inflammatory factors able to increase Fas sensitivity in immature erythroblasts are likely to alter the balance between EPO and Fas/FasL, with deleterious effects on erythropoiesis. This may account for the potent erythroid suppression induced by tumor necrosis factor-α (TNF-α) and interferon- γ (IFN-γ), which has been shown to upregulate both Fas and its apoptotic mechinery (Maciejewski et al., 1995; Ossina et al., 1997).

Furthermore, recent study (Nagata and Todokoro, 1999) indicates that apoptosis during erythroid differentiation involves in C-Jun amino terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38. The p38 and JNK/SAPK cascades are primarily activated by various environmental stress : osmotic shock, UV radiation, heat shock, X-ray radiation, hydrogen peroxide, and protein synthesis inhibitors and by the proinflammatory cytokines, TNF-α and IL-1. These cellular stresses and proinflammatory cytokines induce apoptotic cell death (Verheij et al., 1996). Stimulation of Fas also induces activation of p38 and JNK/SAPK (Goillot et al., 1997; Juo et al., 1997). In erythroid differentiation, activation of p38 and JNK/SAPK is required for both cell differentiation and apoptosis, and the duration of their activation may determine the cell fate, cell differentiation and apoptosis. Activation for a short time cause erythroid differentiation although its prolonged activation induced apoptosis (Nagata and Todokoro, 1999).

3.3. Induction of Apoptosis by Lead

The review summarizes current studies have been found that lead can induce apoptosis in various cells, such as human lung epithelial cells (Singh et al., 1999), human neuronal and glial cells (Scortegana and Hanbauer, 1997), rat cerebellar neurons (Oberto et al., 1996) rat rod and bipolar cells (Fox et al., 1997 ; He et al., 2000), and rat liver cells (Dini et al.,1999; Ruzittu et al., 1999).

There are many reports indicate that lead can generate reactive oxygen species and oxidative damage resulting in the induction of oxidative stress and stimulation of mitogenic signals (Stohs and Bagchi, 1995; Bondy and Guo, 1996; Skoczylnska, 1997; Sieg and Billings, 1997; Adonaylo and Oteiza, 1999). In addition, recent study (Ramesh et al., 1999) have shown that lead is also found to upregulate the related kinase such as mitogen activated protein kinase (MEK) and JNK (also known as stress-activated protein kinase) in a dose and time-dependent manner. Therefore, it is possible that lead may be able to induce apoptosis in erythropoietic system by the activation of apoptotic caspase mechanism through the stimulation of mitogenic signals or death signals.

THE AIMS

Lead toxicity on erythropoietic system is one of the major causes of anemia in lead-exposed people. Previous studies of lead toxicity on erythropoietic system were performed in animal and human bone marrow or human erythrocytes. However it is difficult and dangerous to aspirate the bone marrow from human. Moreover, erythrocytes are not an appropriate model in this study because they cannot synthesize hemoglobin. Lead toxicity study in this research was thus performed on hemoglobin synthesizing erythroid precursor cells, prepared from erythroid progenitor cells (BFU-Es) in human peripheral blood mononuclear cells by the two phase liquid culture (TPLC) procedure (Fibach et al., 1991). Therefore, the first aim of this research is to prepare human erythroid precursor cells by *in vitro* technique in sufficient amount, to use as the model for the study of lead toxicity

Since erythroid precursor cells are responsible for the continuous production of red blood cells and hemoglobin synthesis, any effects on erythroid precursor cell development may cause an inadequate red blood cell production or lower Hb content, resulting in anemia. Human erythroid precursor cells are located in bone marrow. Lead is relatively higher concentration in bone marrow than peripheral blood (Westerman et al.,1965; Waldron,1966; Berry,1975) and this may result in the exposure of the erythroid precursor cells to high local concentration of this metal, thereby leading to a deleterious effect of lead on these cells. Finally, the second aim of this research is to study the effect of lead on the development of human erythroid precursor cells and its involvement on apoptosis of the cells. The result may help elucidating the mechanism of lead toxicity on erythroid precursor cell and finally give a better understanding on lead induced anemia.

RESEARCH METHODOLOGY

1. Preparation of Human Erythroid Precursor Cells

Human erythroid precursor cells serving as the model throughout this study were prepared by two-phase liquid culture (TPLC) system from human erythroid progenitor cells (BFU-Es) in the peripheral blood (Fibach et al, 1991). The procedure was divided into two phase. The first phase, in the presence of a conditioned medium containing burst-promoting activity, allowed the BFU-Es to proliferate and differentiate to CFU-Es. In the second phase, CFU-Es undergone partial erythroid maturation by the stimulation of specific growth factor, EPO. This procedure provided substantial numbers of erythroid precursor cells for the following studies.

2. The Study of Lead Toxicity on Human Erythroid Precursor Cells.

To understand the mechanism of lead toxicity, morphological assessing under light microscope and flow cytometric analysis were used to examine cell development and apoptosis of erythroid precursor cells after lead exposure. The combination of flow cytometry which is a very powerful technique in analytical cytology and the development of monoclonal antibodies provides us with the excellent tools for the analysis of human hemopoietic cells.

2.1. Study Effect of Lead on Erythroid Precursor Cell Development

After early stage of erythroid precursor cell development, the cells were continued to culture in the same medium with or without lead acetate at various concentrations for different times. Two cell surface markers; glycophorin A and transferrin receptor, were used to define the maturation of erythroid precursor cells. Flow cytometry was used to determine the expression of these cell surface markers. The cells expressed both glycophorin A and transferrin receptor were identified as erythroid precursor cells. Morphological observation under light microscope was also used to identify erythroid precursor cell maturation. The viability of cells was determined by

trypan blue exclusion assay using a hemocytometer and observed under phase contrast microscope.

2.2. Evaluation of Lead Induced Apoptosis in Erythroid Precursor Cells

After cultured the cells in the medium with or without lead, the apoptosis of erythroid precursor cells could be detected by monitoring the binding of fluorescein-labeled annexin V to phosphatidylserine (PS) on the outer leaflet of apoptotic cell membrane by using flow cytometric analysis. Annexin V for flow cytometric detection of PS expression is the simple and rapid method for apoptotic detection. The method uses the binding of fluorescein isothiocyanate-labeled annexin V to PS, which is exposed on the surface of apoptotic cells but not on viable cells. The method allows for easy quantification of apoptosis. Since, annexin V binds to PS with high specificity, staining with annexin V can be used as a specific marker for apoptosis in the early phase where the cell membrane is still intact.

THE EXPECTED OUTCOME

1. The method of human erythroid precursor cells preparation by *in vitro* technique. The cells obtained serve as the model to study the mechanism of lead toxicity.
2. A better understanding on the mechanism of lead toxicity on human erythroid precursor cell development in order to educidate the effect of lead on erythropoietic system
3. The knowledge from this research may further give a clear picture on lead induced anemia.

CHAPTER III

METHODS

1. Erythroid Precursor Cells Cultures

Based on the finding of Clarke and Housman (1977) that the peripheral blood of normal human contained a significant number of committed erythroid stem cells of high proliferative capacity, the cultures of human erythroid precursor cells prepared in this study were derived from the committed erythroid stem cells isolated from normal human peripheral blood by the two-phase liquid culture procedure (Fibach et al., 1991).

1.1. Reagent Preparation

1.1.1. Alpha-mineral essential medium (α-MEM), pH 7.4

α-MEM, pH 7.4 was prepared by dissolving 10.1 g α-MEM and 2.2 g. $NaHCO_3$ in 1 liter of distilled water and diluting to a desired volume. The pH of the medium was adjusted to 0.2-0.3 below the final working pH (pH 7.4). The solution was immediately sterilized by membrane filtration.

1.1.2. 45% Percoll solution

Fourty five percent of Percoll solution was prepared by mixing Percoll reagent (containing 10% of 10xPBS) with 1xPBS in the ratio of 1:1 (v/v).

1.1.3. 200 IU Recombinant-human erythropoietin (r-HuEPO)

Two hundred unit per milliliter of r-HuEPO was prepared by diluting 0.5 ml 2000 IU r-HuEPO with 10 ml α-MEM containing 5% BSA and sterilizing immediately by membrane filtration.

1.1.4. 1 mg/ml Cyclosporin A

One milligram per milliliter of Cyclosporin A was prepared by diluting 0.2 ml Cyclosporin A 50 mg/ml with 10 ml 2% FBS in α-MEM.

1.1.5. 0.15 M L-glutamine

One hundred and fifty mM of L-glutamine was prepared by dissolving 2.19 g. L-glutamine in 100 ml distilled water and sterilizing immediately by membrane filtration.

1.1.6. 1×10^{-3} M Dexamethasone

One mM of Dexamethasone was prepared by dissolving 51.64 g. dexamethasone with 100 ml absoluted ethanol and sterilizing immediately by membrane filtration.

1.1.7. 10^{-2} M β-Mercaptoethanol

Ten mM of β-Mercaptoethanol was prepared by diluting 0.0697 ml β-Mercaptoethanol in 100 ml α-MEM and sterilizing immediately by membrane filtration.

1.2. Blood Sample Preparation

Platelet-rich buffy coats were prepared from freshly collected whole blood units from normal donors at Thai Red Cross Society (TRCS). The collection bag of whole blood was centrifuged at 5,108 xg for 10 minutes. All blood units were screened for Anti-HIV, HIV-Ag, Anti-HCV, VDRL and HBsAg.

1.3. Two-Phase Liquid Culture

Before isolation of mononuclear cells, the platelets suspended in platelet-rich buffy coat were removed by centrifugation with two volumes of 1xPBS at 400 xg for 5 minutes. After removal of the upper layer containing platelets, the lower layer containing buffy coat was transferred and layered on a gradient of Ficoll-Hypaque (density = 1.077 g/ml) in 50 ml tube. Equal volume of ficoll and buffy coat was used to optimize mononuclear cells recovery. The solution was then centrifuged at 600 xg for 30 minutes, afterwhich the interphase layer of mononuclear cells was collected (Figure 4). The

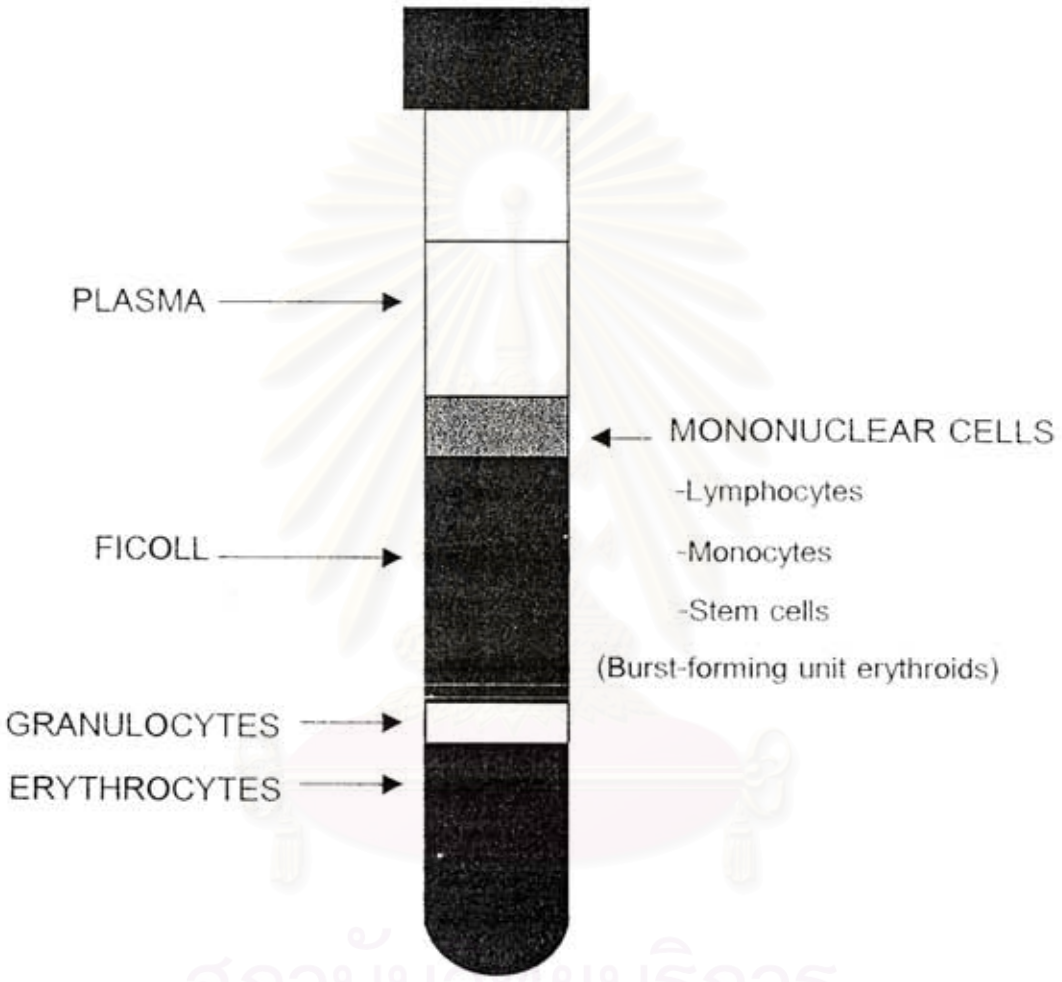


Figure **4** : Blood fraction derived from buffy coat separation process. The interphase white layer of mononuclear cells was collected.

mononuclear cells fraction containing lymphocytes, committed erythroid stem cells, and other stem cells were washed with α-MEM at low speed (250 xg) for 5 minutes to remove the remained platelet. After that, the cells were cultured into three 75 cm^2 flasks at a density of 5×10^6 MNC/ml, in primary liquid medium containing α-MEM supplemented with 10% FBS, 1 μg/ml cyclosporin A and 10% conditioned medium (CM) collected from culture of the 5637 bladder-carcinoma cell line. This CM contains a variety of hemopoietic growth factors, not including erythropoietin (EPO). The culture was incubated at 37°C in an atmosphere of 5% CO_2 in air with extrahumidity.

Following seven days in this primary culture, the nonadherent cells were harvested and washed with α-MEM. After that, the cells were recultured in secondary liquid medium composed of α-MEM, 30% FBS, 10% BSA, 1×10^{-5} M β-ME, 1.5 mM L-glutamine, 1×10^{-6} M dexamethasone, 1 μg/ml cyclosporin A and 1 U/ml r-HuEPO. This secondary culture was also incubated at 37°C in an atmosphere of 5% CO_2 in air with extrahumidity.

After a 5-day of incubation in secondary culture, lymphocytes were removed by the following steps. The cells were harvested, spun down, and the medium was saved. The cells were suspended in a small volume of culture medium and layered on 45% Percoll solution (density = 1.0585 g/ml) and centrifuged at 600 xg for 20 minutes. The interphase layer containing proerythroblasts and trace amount of monocytes was collected, washed with α-MEM, and resuspended in the save medium. The incubation was continued. During incubation, phase contrast microscope was used to observe the maturation of erythroblasts in the culture flask. In addition, the portion of cell suspension (0.2 ml) was collected during incubation and transferred to determine cell morphology under light microscope. Flow cytometry was also used to analyse erythroblast maturation in this culture.

1.4. Morphological Assessment

The morphology of cells were observed under light microscope after the cells were stained with Wright's stain. The cell suspension was placed on cytospin chamber and cytocentrifuged onto a glass slide at 1,000 rpm for 5 minutes using Cytospin 3.

After cytocentrifugation, the cells were stained with Wright's stain for 5 minutes. Without removing the stain, an equal volume of distilled water was added to the slide. The two solutions were mixed and left standing for 4 minutes, afterwhich the slide was rinsed with distilled water. The stained cells on glass slide were counted and identified for their characteristic morphological appearance.

1.5. Evaluation of Erythroid Maturation by Flow Cytometry

Since the development of transferrin receptor and glycophorin A corresponded to erythroid maturation, staining of these two markers followed by fluorescent analysis with flow cytometry (Loken et al, 1987) would define the maturation of the cell.

Flow cytometric analysis

Flow cytometric analyzer was used to analyse cell surface antigen of erythroid precursor cells by the technique of immunofluorescence. The procedure was as following : At day 8 and day 12 of secondary culture, the cells were harvested and washed with 1%FBS / α-MEM, pH 7.4. Then, the cells were resuspended in small volume of incubation buffer and stained with RPE-conjugated monoclonal mouse anti-human glycophorin A and FITC-conjugated monoclonal mouse anti-human transferrin receptor (CD 71) and then incubated in dark at 4°C for 15 minutes. After that, the stained cells were pelleted and washed once with incubation buffer (at least five folds that staining volume). Then, the cells were resuspended in the buffer and analysed by flow cytometry using FACScan (Becton Dickinson) equipped with 15-mW Argon ion laser emitting at 488 nm blue line for excitation of fluorochromes. The emission photon was detected using appropriate filters for FITC into FL-1 channel and for RPE into FL-2 channel. The data were collected for 10,000 events and then analysed by Cell Quest 3.1 Software (Becton Dickinson). The cells that were positive for both glycophorin A and transferrin receptor were exclusively of the erythroid precursor cells.

2. Effect of Lead on Human Erythroid Precursor Cells

2.1. Evaluation of Erythroid Precursor Cell Development

Two cell surface markers, glycophorin A and transferrin receptor, were used to define the maturation of erythroid precursor cells exposed to lead. Flow cytometry was used to determine the expression of these cell surface markers and morphological observation under light microscope was used to identify erythroid precursor cell maturation.

Flow cytometric analysis

At day 7 in secondary culture, the cells were subcultured with a concentration of $1x10^5$ cells/ml in 10 ml of the same secondary medium supplemented with various lead acetate concentrations. After incubation for one and five days, respectively, the cells were collected, washed to remove cell debris, and stained with RPE-conjugated monoclonal mouse anti-human glycophorin A and FITC- conjugated monoclonal mouse anti-human transferrin receptor (CD 71) as previously described (1.4). The expression of both cell surface markers were analysed by flow cytometry using FACScan. The cells expressed both glycopholin A and transferrin receptor were identified as erythroid precursor cells.

Morphology study and cell counting

After incubation with various lead acetate concentrations for one and five days, respectively, 0.2 ml of cultured cells were collected and divided to two portions. The first 0.1 ml portion was taken to assess cell morphology by the method described in 1.3. The second portion of 0.1 ml was taken for cell count (the method described in 2.4).

2.2. Evaluation of Apoptosis by Flow Cytometry

A loss of membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine (PS) on the surface of the cell is one of apoptotic characteristics. The

detection of PS on the outer leaflet of apoptotic cell membranes was performed by using annexin V-biotin in conjugation with streptavindin-fluorescein. Annexin V preferentially binds to negatively charged phospholipid like PS (Andree, 1990). The apoptosis of erythroid precursor cells could be quantified with fluorescein-labeled annexin V.

Detection of apoptosis with annexin V-biotin

After incubation with lead, the cells were collected, washed, and stained for both cell surface markers as described to identify the erythroid precursor cells. PS on the outer membrane was also stained with biotin-labeled annexin V. The procedure was as follows:the culture cells were resuspended with 100 µl incubation buffer supplemented with annexin V-biotin, RPE-conjugated monoclonal mouse anti-human glycophorin A and FITC- conjugated monoclonal mouse anti-human transferrin receptor. The suspension was incubated in the dark at 4°C for 15 minutes. After that, the stained cells were washed once with incubation buffer to remove excess labeling reagent and stained again with RPE/Cy5-conjugated streptavindin and then incubated in the dark at 4°C for 20 minutes. The cells were subsequently washed and resuspended in 0.3 ml incubation buffer. The fluorescence stained cells were then analysed by flow cytometry for triple labelled cells. The controlled sample without annexin V-biotin was prepared to set the background correction. The population of cells labeled with annexin V above background was determined as annexin V-positive cells. Flow cytometric detection was performed as described in 6.1.

3. Statistical Analysis

Statistical analysis was performed using ANOVA with repeated measurement and multiple comparison by Bonferroni Test.

CHAPTER IV

RESULTS

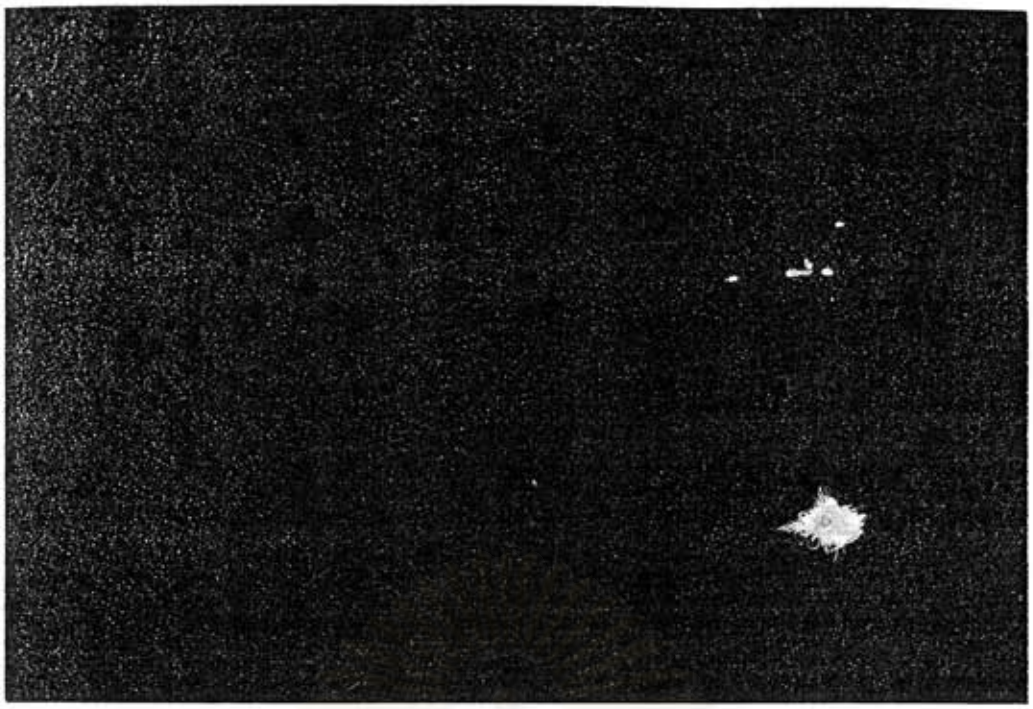
1. Two-Phase Liquid Culture of Erythroid Precursor Cells

1.1. Mononuclear Cells Separation

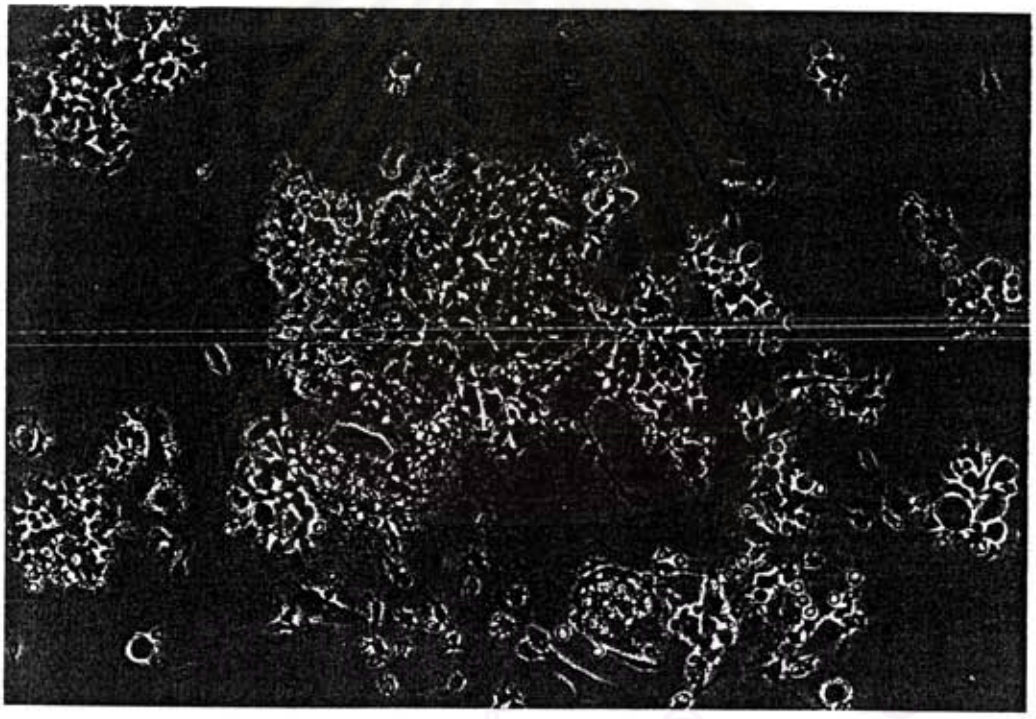
The isolation of mononuclear cells from buffy coat of normal people by centrifugation on Ficoll-Hypaque (density = 1.077 g/ml) provided high cell number collection. Using this procedure, mononuclear cell yield reached 300-500 $\times 10^6$ cells per blood unit.

1.2. Primary Phase (EPO-independent phase) Culture

After seven days in primary culture, the observation under phase contrast microscope showed the appearance of colony forming cells (Figure 5A). These colonies seemed to be CFU-Es, which are present in human bone marrow but are not normally found in peripheral blood (Ogawa, M. et al., 1977). Since no CFU-Es were present in the original peripheral blood cell population, the appearance of CFU-Es after 1 week in this culture could be explained by proliferation and differentiation of BFU-Es. In addition, after the nonadherent cells were harvested, it showed many types of cells adhered to the plastic surface (Figure 5B.). Most of adherent cells seemed to be monocytes, which differentiated into macrophages, and endothelial cells. Morphology of the nonadherent cells at day 7 of primary culture observed under light microscope (Figure 8A) showed that the cultured cells composed of many lymphocytes, macrophages, myeloblasts, and unidentified blasts including CFU-Es. The latter could not be identified because they looked like small lymphocytes. Moreover, small amounts of proerythroblasts, the first stage of erythroid precursor cells, were also observed (Table 2). Further proliferation and differentiation of these CFU-E-like progenitors into erythroid precursor cells could also occur in EPO-dependent phase.

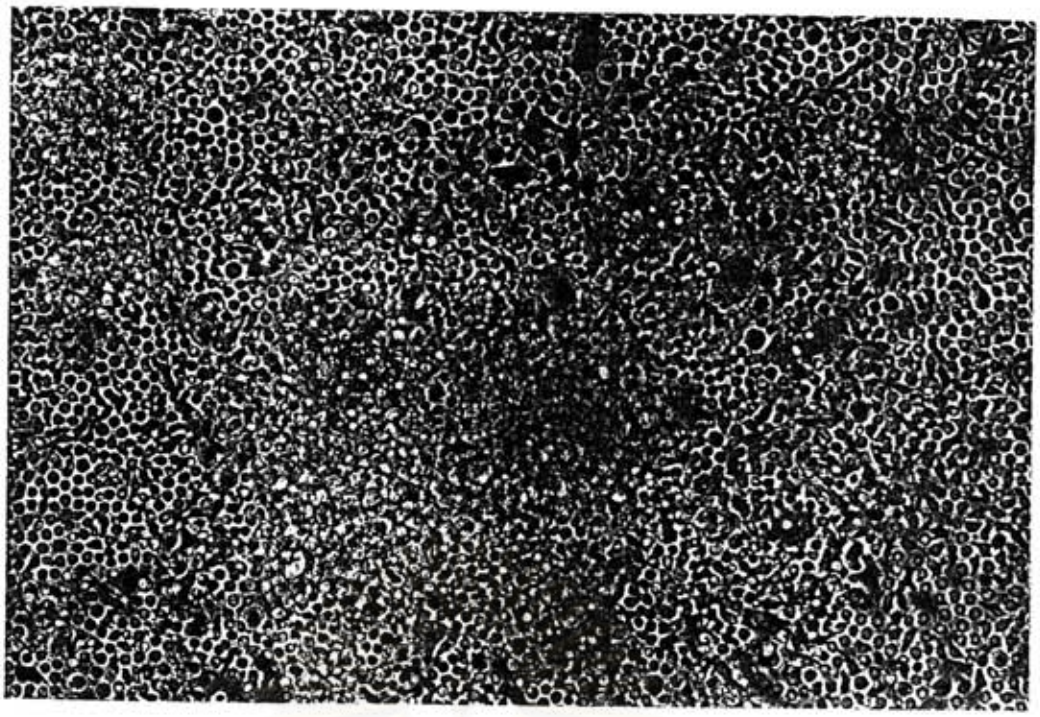


A : Nonadherent cells (100 × magnification)

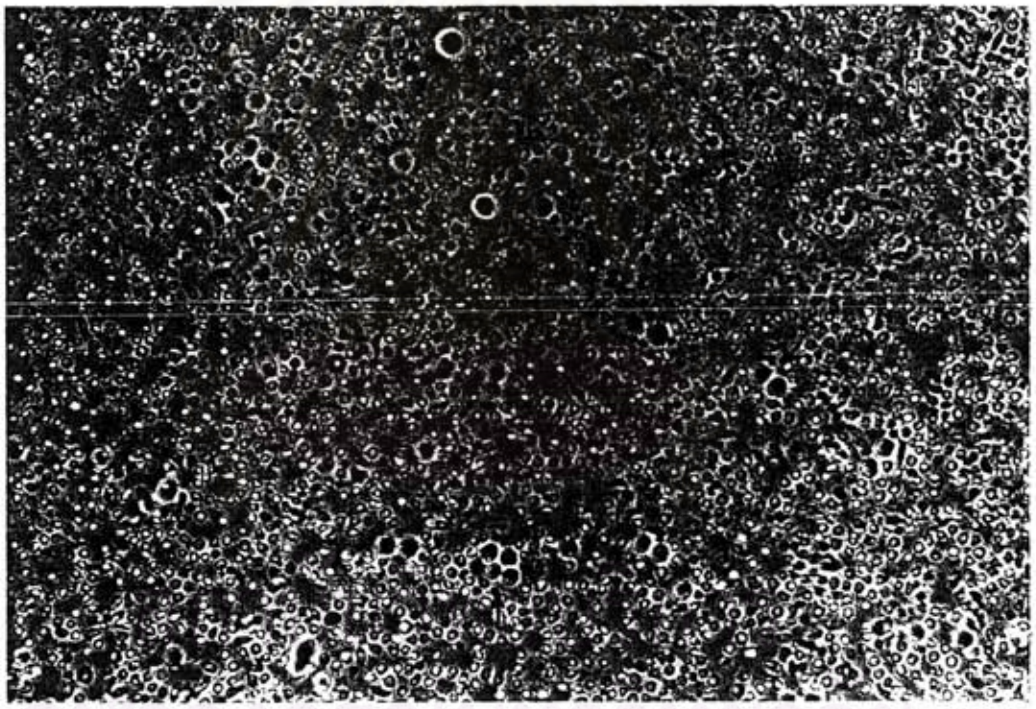


B : Adherent cells (400 × magnification)

Figure 5 : Phase contrast microscopy of cultured cells at day 7 in primary culture. A lot of colony forming cells (arrow) were observed in this culture (A). The adherent cells consisted of macrophages and endothelial cells (B).

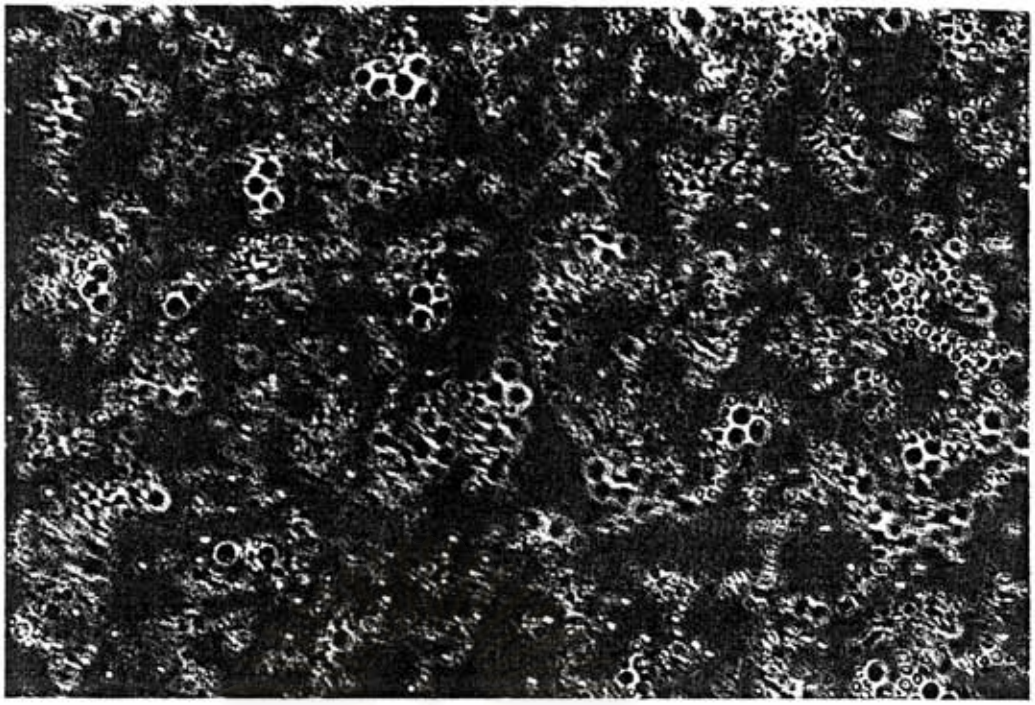


A : day 0 (400 × magnification)

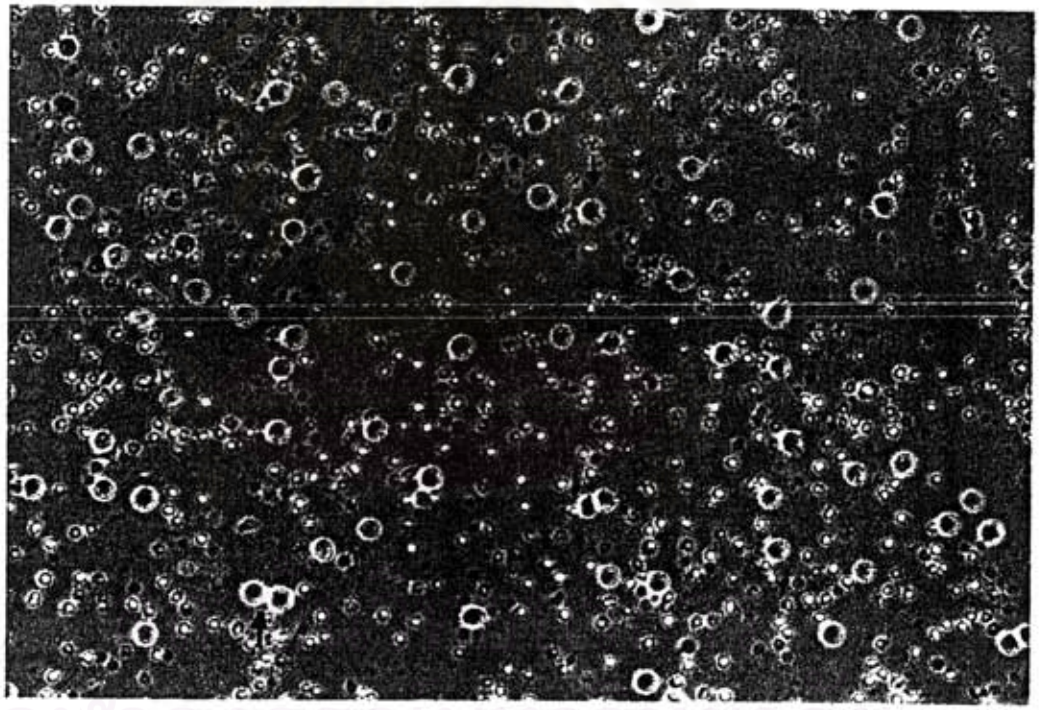


B : day 3 (400 × magnification)

Figure 6 : Phase contrast microscopy of cultured cells at day 0 (A) and day 3 (B) in secondary culture. At the beginning of secondary phase, many small lymphocytes (arrow-head) were observed. The cells became dead and fragmented into cell debris, whereas small amounts of proerythroblasts (arrow) were also observed and increased considerably.



A : Before lymphocyte separation (400 × magnification)



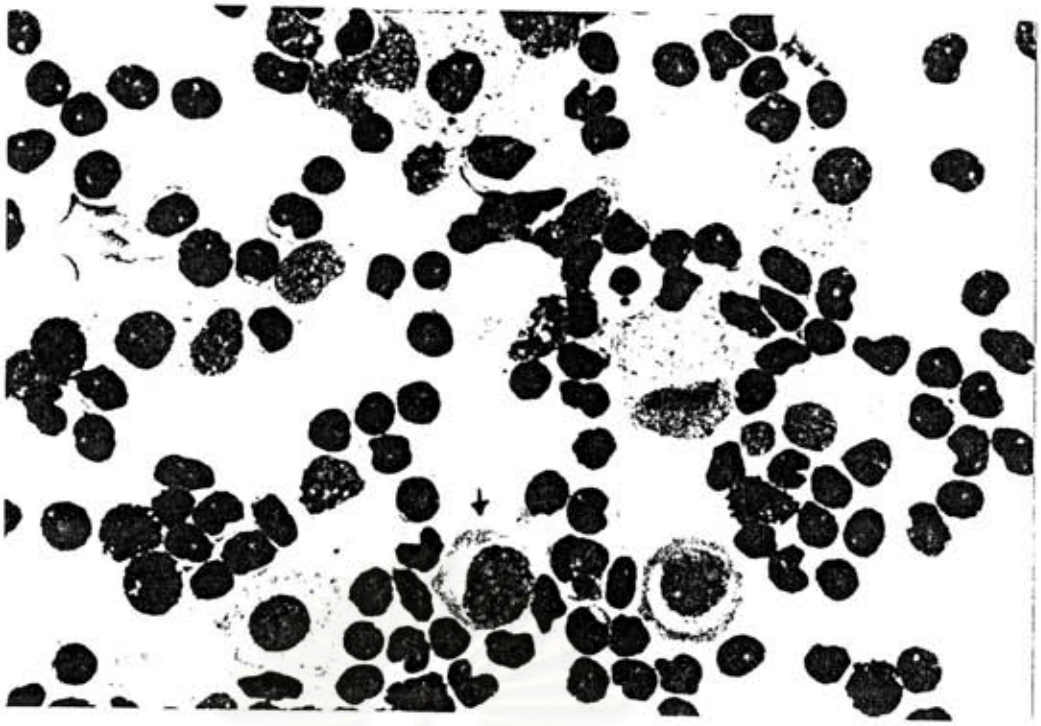
B : After lymphocyte separation (400 × magnification)

Figure 7 : Phase contrast microscopy of cultured cells at day 5 in secondary culture before (A) and after (B) removal of lymphocytes. A lot of proerythroblast colonies (arrow) were observed in this culture but the majority of cultured cells were lymphocytes(A). After separation, the bulk of cells were proerythrocytes.

1.3. Secondary Phase (EPO-dependent phase) Culture

After five days in EPO supplemented medium, the observation under phase contrast microscope showed the appearance of many proerythroblast colonies. However, a lot of small lymphocytes were also present (Figure 7A). Most of the lymphocytes could be later removed from this population by separation on percoll, with the basis of lower buoyant density of erythroid precursor cells (proerythroblasts and early erythroblasts). Figure 7B showed that after the separation, the majority of the culture consisted of proerythroblasts.

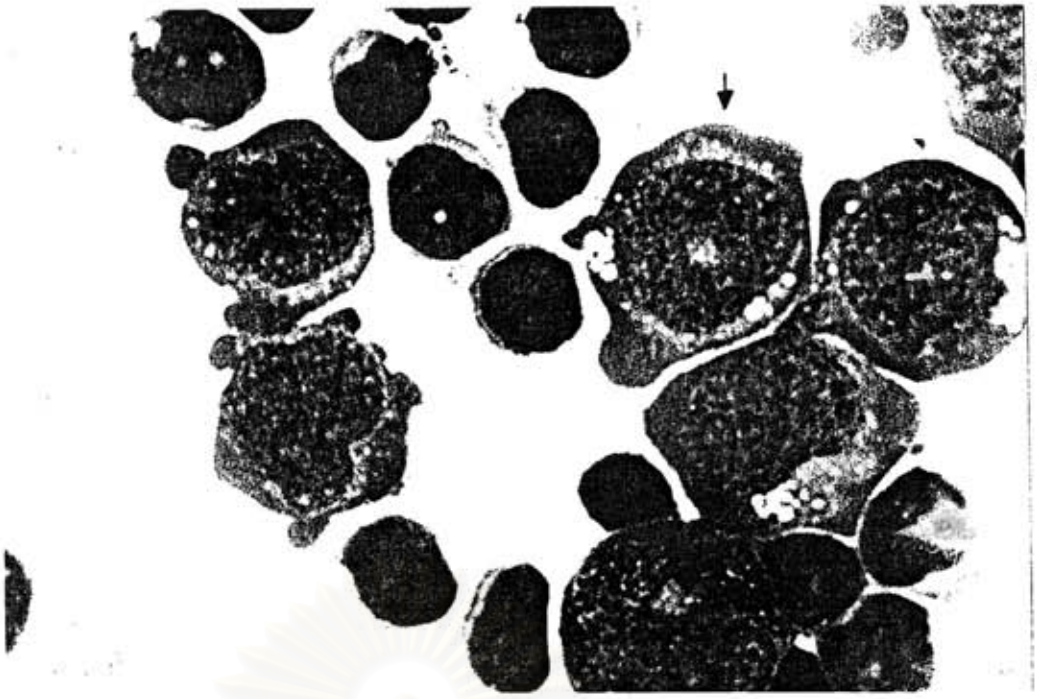
In addition, the observation of cell morphology under light microscope showed that after three days in EPO-supplemented medium, proerythroblast colonies appeared (Figure 8B) with considerable degree of proliferation. As a consequence, many proerythroblast colonies were observed in day 5 of secondary culture (Figure 9A). After the removal of lymphocytes, the bulk of cultured cells consisted of proerythorblasts which rapidly proliferated and maturated into the next stage of erythroid precursor cells. As a result, the majority of cultured cells at day 8 were erythroid precursor cells consisted of proerythroblasts, basophilic and polychromatophilic erythroblasts (Figure 9B). After that, basophilic erythroblasts rapidly proliferated and maturated into polychromatophilic erythroblasts. So that a lot of polychromatophilic erythroblasts appeared at day10 in secondary culture (Figure 10A). Polychromatophilic erythroblasts continued to proliferate and maturate into the last stage of erythroid precursor cells which still contained nucleus, orthochromatic erythroblasts. Therefore, many orthochromatic erythroblasts were observed at day12 in secondary culture (Figure 10B). After 14 days in this medium, the number of cells declined and the erythroid precursor cells started to disintegrate (Figure 11).



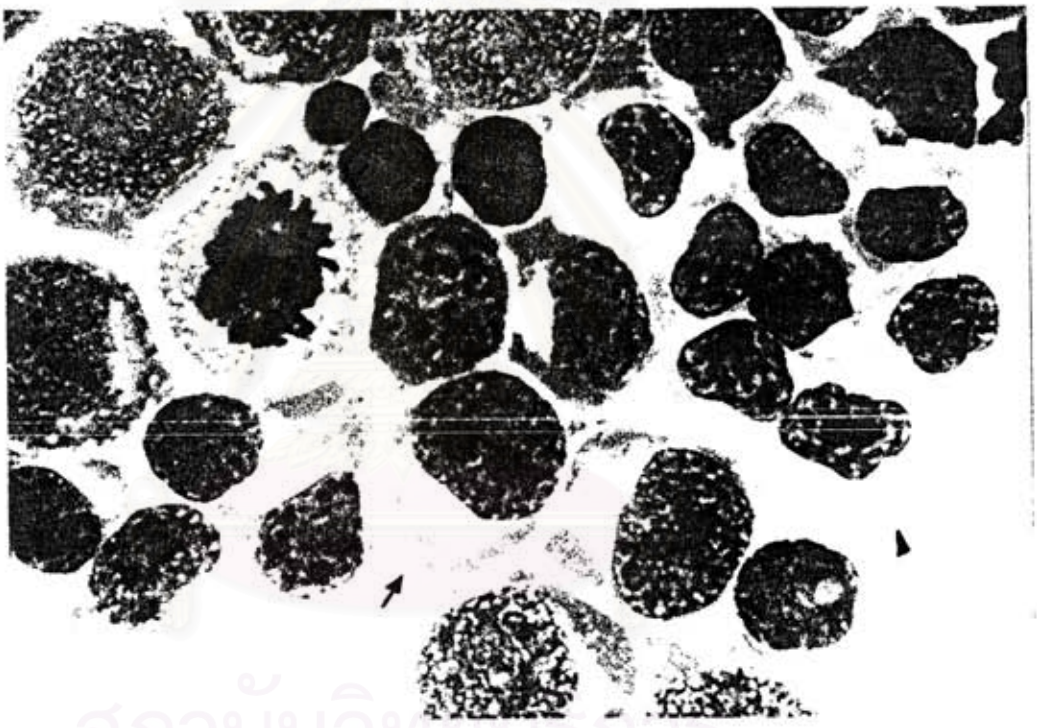
A : day 7 in 1° culture

B : day 3 in 2° culture

Figure 8 : Light microscopy of cultured cells at day 7 in 1° culture (A) and day 3 in 2° culture(B). After seven days in 1° phase, the culture consisted of large amounts of lymphocytes and many blastic cells including CFU-Es. Small amounts of proerythroblasts (arrow) were also observed. After three days in EPO supplemented medium, proerythroblast colonies (arrow) appeared whereas lymphocytes became dead (arrowhead), 400 × magnification.

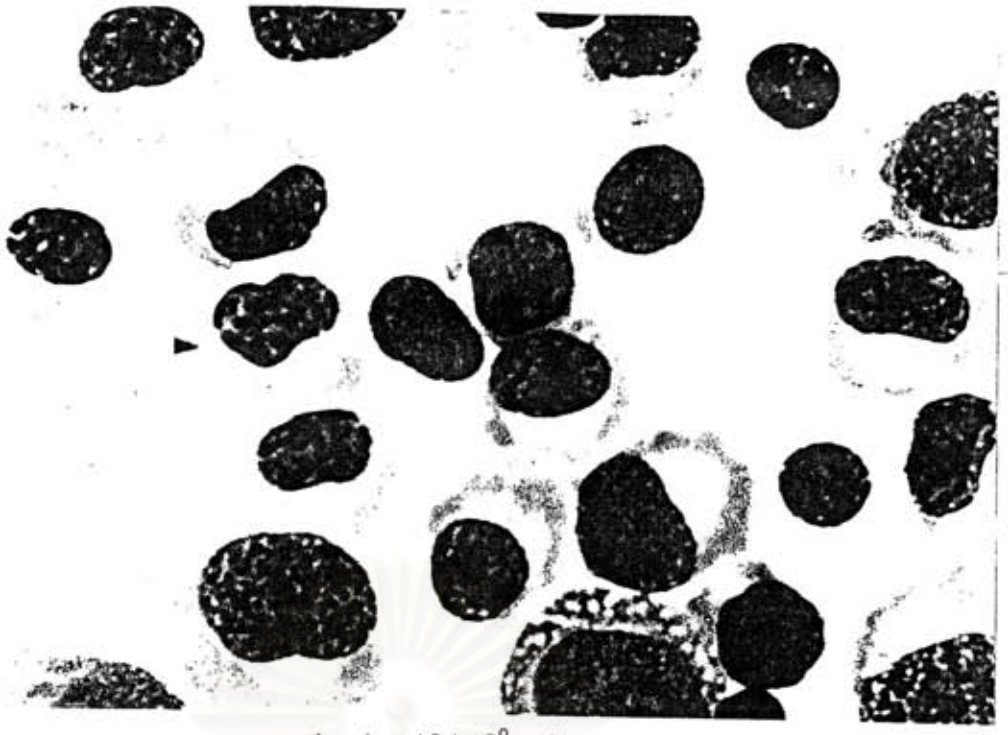


A : day 5 in 2° culture



B : day 8 in 2° culture

Figure 9 : Light microscopy of cultured cells at day 5 (A) and day 8 (B) in 2° culture. At day 5 in 2° phase, the culture consisted of many proerythroblast colonies (arrow). After that, they grewn with high proliferation and maturation. Therefore, at day 8 in 2° phase, the majority of the culture consisted of erythroid precursor cells in the stage of proerythroblasts, basophilic (arrow) and polychromatophilic erythroblasts(arrow head),1,000 x magnification.



A : day 10 in 2° culture

B : day 12 in 2° culture

Figure 10 : Light microscopy of cultured cells at day 10 (A) and day 12 (B) in 2° culture. At day 10 in 2° phase, the bulk of erythroid population were polychromatophilic erythroblasts (arrow head). After that, the cells were rapidly proliferated and maturated into orthochromatic erythroblasts. Therefore, at day 12 in 2° phase, the majority of the culture consisted of erythroid precursor cells in the last stage with still contained nucleus, the orthochromatic erythroblasts (arrow), 1.000 × magnification.

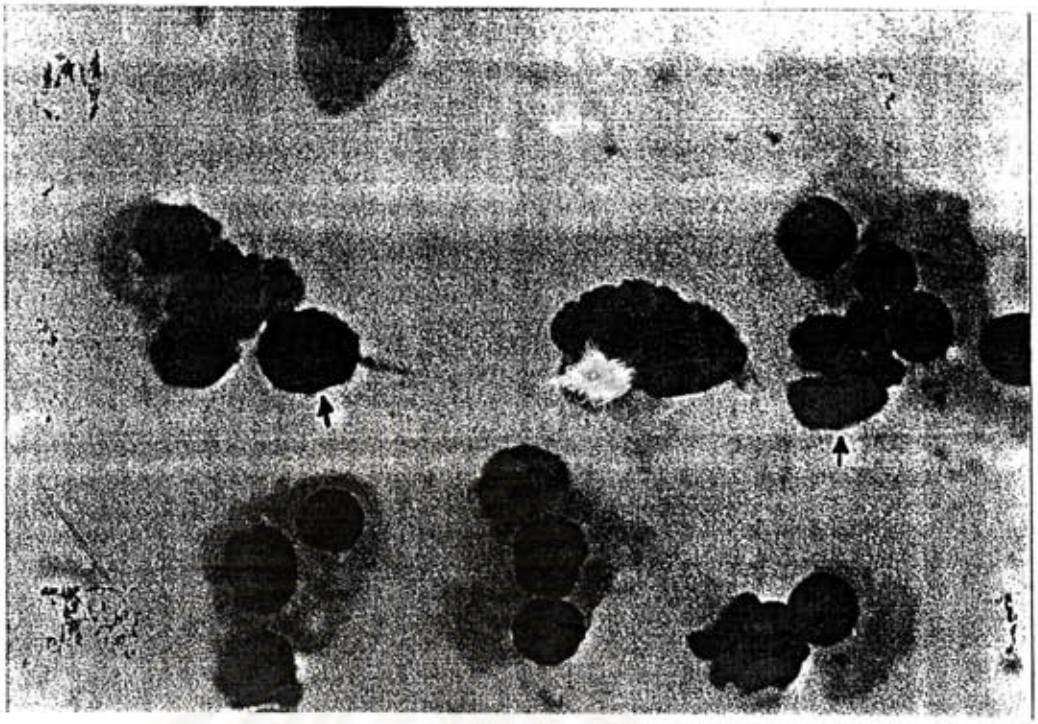


Figure 11 : Light microscopy of cultured cells at day 14 in 2° culture. Many of dead cells (arrows) appeared in the cultured after 14 days in this phase,1,000 × magnification.

Table 2 showed the percentage of each cell type in two phase liquid culture. The data indicated that, in the presence of EPO, the percentage of myeloid and lymphoid cells decreased whereas the percentage of erythroid precursor cells increased considerably and reached a maximum at day 12 in secondary culture.

Therefore, the cells of day12 in culture supplemented with EPO were used for further experiment: At this stage more than 90% of the cells were proliferating erythroid precursor cells and more than 75% of erythroid population were at the stage of orthochromatic and polychromatophilic erythroblasts. Less than 6% was the contaminating lymphocytes. The total yield of erythroid precursor cells was 30.46 ± 19.48) x10^6 cells per blood unit.

Table 2 : Percentage of each type of blood cells in two phase liquid culture at various days.

Cell types	EPO-independent phase		EPO-dependent phase			
	day 0 of 1° culture	day 7 of 1° culture	day 5 of 2° culture	day 8 of 2° culture	day 10 of 2° culture	day 12 of 2° culture
Proerythroblast	0	2	19	28	8	5
Basophilic erythroblast	0	0	2	37	12	10
Polychromatophilic erythroblast	0	0	0	18	47	24
Orthochromatic erythroblast	0	0	0	0	20	52
Myeloid cell	5	6	3	2	0	0
Macrophage	5	7	7	5	5	4
Lymphocyte and unidentified blast	90	85	69	10	8	5

1.4. Flow Cytometric Analysis

Since the maturation of erythroid precursor cells was characterized by the increase in glycophorin A and relative decrease in transferrin receptor, the concept of analysis was based on the quantitation of these two surface markers.

Flow cytometric analysis of erythroid maturation at eight and twelve days in secondary culture was shown in Table 3 and Figure 12. These data showed that at day 8 and 12, the expression of transferrin receptor decreased slightly (81.17% to 76.83%), whereas the expression of glycophorin A increased considerably (45.58% to 75.25%). The forward light scatter (FSC), demonstrating of the cell size (Figure 12 and 13), showed that the cells at the late stage of maturation (day 12) had smaller FSC as compared to those at earlier stage (day 8). The expression of both glycophorin A and transferrin receptor on the same cell was shown to be specific for erythroid precursor cells. Using the combined detection of transferrin receptor and glycophorin A, the major population of erythroblasts were present at day 12 of secondary culture representing the mature nucleated red cell. It increased from 41.56% in day 8 to 70.01% in day 12. This result indicated ,the most of cell population at day 12 of secondary culture consisted of erythroid precursor cells. The result corresponded to that of cell count from morphological observation under light microscope (Table 2).

Table 3 : **Flow cytometric analysis of erythroid precursor cells at day 8 and day 12 in secondary culture.** It showed glycophorin A and / or transferrin receptor (CD 71) expression.

subject number	Day in 2° culture	%Erythroid surface markers		
		Glycophorin A (+)	CD 71 (+)	Glycophorin A (+) / CD 71 (+)
1	8	30.80	65.27	34.50
	12	62.44	57.05	55.70
2	8	32.18	86.32	30.97
	12	85.61	71.52	65.35
3	8	37.77	73.39	36.64
	12	77.07	86.55	80.36
4	8	52.00	94.28	35.81
	12	77.24	91.42	69.95
5	8	55.50	74.96	53.98
	12	66.67	72.02	70.11
6	8	58.03	94.47	57.42
	12	79.43	90.43	68.63
7	8	52.81	79.52	41.86
	12	78.24	68.94	79.99
mean $\pm$ SD	8	45.58 $\pm$ 11.59	81.17 $\pm$ 11.03	41.56 $\pm$ 10.23
mean $\pm$ SD	12	75.25 $\pm$ 7.95	76.83 $\pm$ 12.89	70.01 $\pm$ 8.52

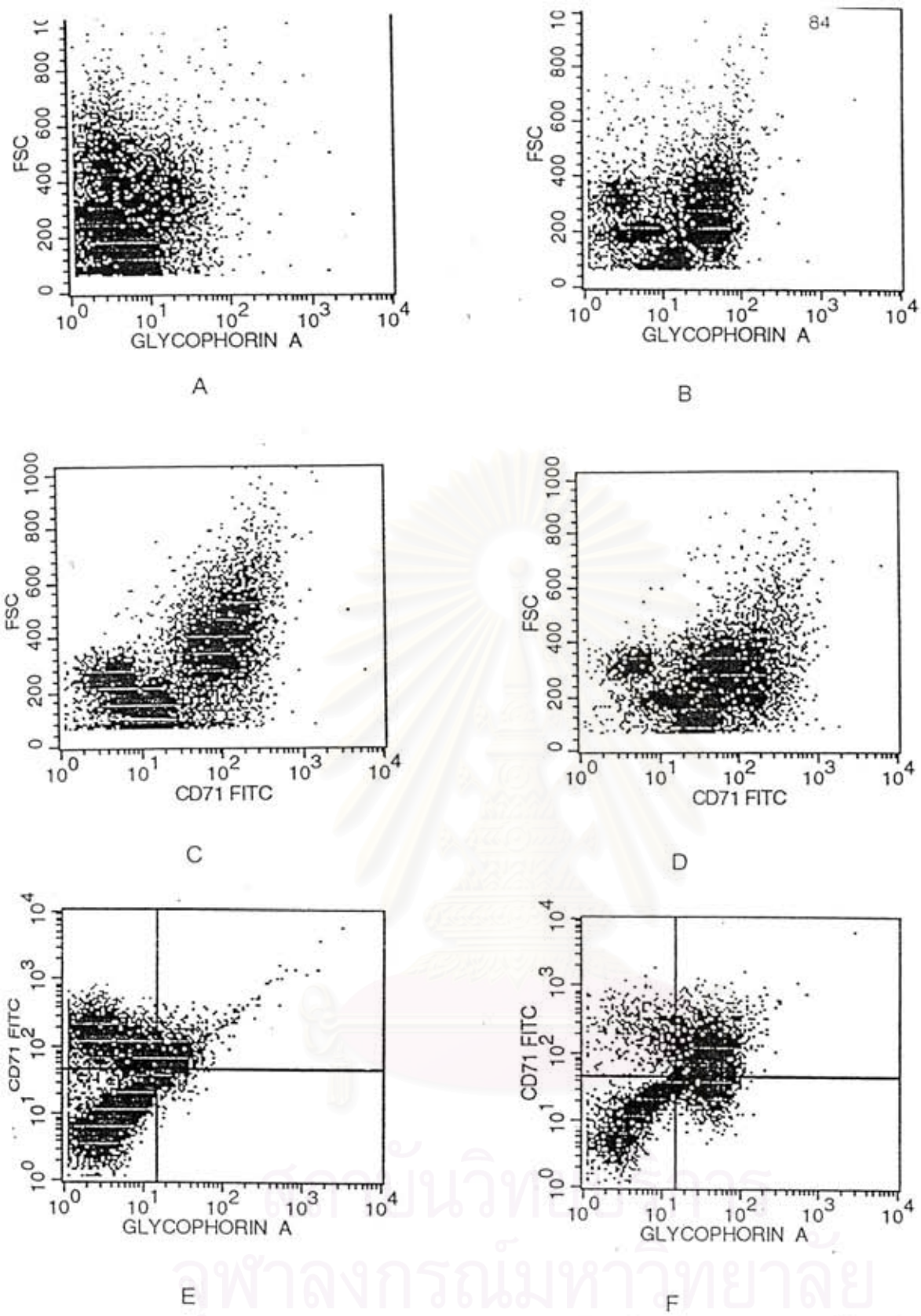


Figure 12 : Flow cytometric analysis of erythroid precursor cell maturation.

The expression of erythroid surface markers, transferrin receptor (CD 71) and glycophorin A, was displayed at day 8 (A,C,E) and day 12 (B,D,F) of the culture. The more maturation was indicated by higher glycophorin A expression and lower transferrin receptor expression.

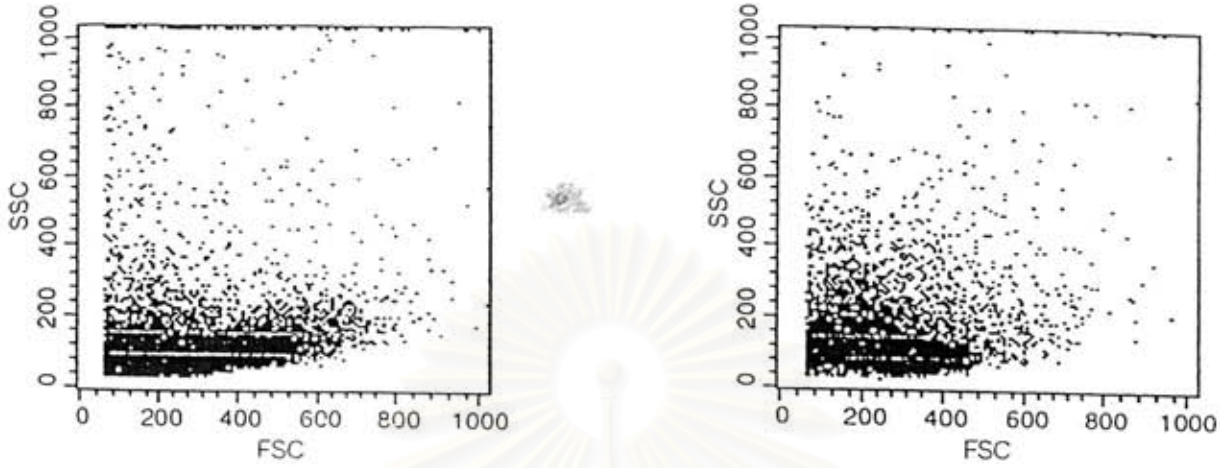


Figure 13 : Correlation between forward scatter (FSC) and side scatter (SSC) of the cells at day 8 (A) and day 12 (B) of the culture. The cell size was detected by FSC and granularity of cell was detected by SSC. At late stage of maturation (day 12), most of the cells displayed lower FSC as compared to the early stage (day 8).

2. Effect of Lead on Human Erythroid Precursor Cells

2.1. Effect of Lead on Erythroid Precursor Cells Development

After seven days in secondary medium containing EPO, the culture generated cells that predominantly consisted of erythroid precursor cells. The cells induced with EPO were subsequently cultured for one and five days in the presence of various lead concentrations. The number of viable cells (trypan blue negative cells) was measured. As shown in Figure 14, the number of viable cells decreased slightly at 0.5 ppm lead acetate concentration. The decreased was significant at high concentration of lead ($\geq$ 1.0 ppm). This indicated that, the survival of erythroid precursor cells was markedly inhibited by lead in a dose-dependent manner at lead acetate concentration $\geq$ 1.0 ppm (Table 4).

Percentage of viable cell number which was comparable to the control (0.0ppm Pb) after lead exposure for one and five days was shown in Figure 15. The decrease of cell viability was noted after one day exposure. At lead acetate concentration $\geq$ 1.0 ppm, the percentage of viable cell number was decreased after one day exposure (from 100% to 74.28, 48.27, and 39.84% at lead acetate concentration of 1.0, 2.0, and 4.0 ppm, respectively) and the decrease was more pronounced after five days exposure (from 100% to 40.64, 34.14, and 16.91% at lead acetate concentration of 1.0, 2.0, and 4.0 ppm, respectively). At lower lead acetate concentration (0.5 ppm), the marked decrease of cell viability could also be detected but with longer incubation time (from 100% to 63.52%). This indicated that, the decrease in erythroid precursor cells viability by lead was also time-dependent.

The study of cell morphology (Figure 16-21) showed that in the presence of lead, the cells could still maturate and reach the last stage of erythroid precursor cells which still contained nucleus (orthochromatic erythroblasts). At low lead acetate concentration (Pb $\leq$0.5 ppm), the survival of erythroid precursor cells was not inhibited by lead, the cultured cells consisted of many viable cells in the stage of proerythroblast

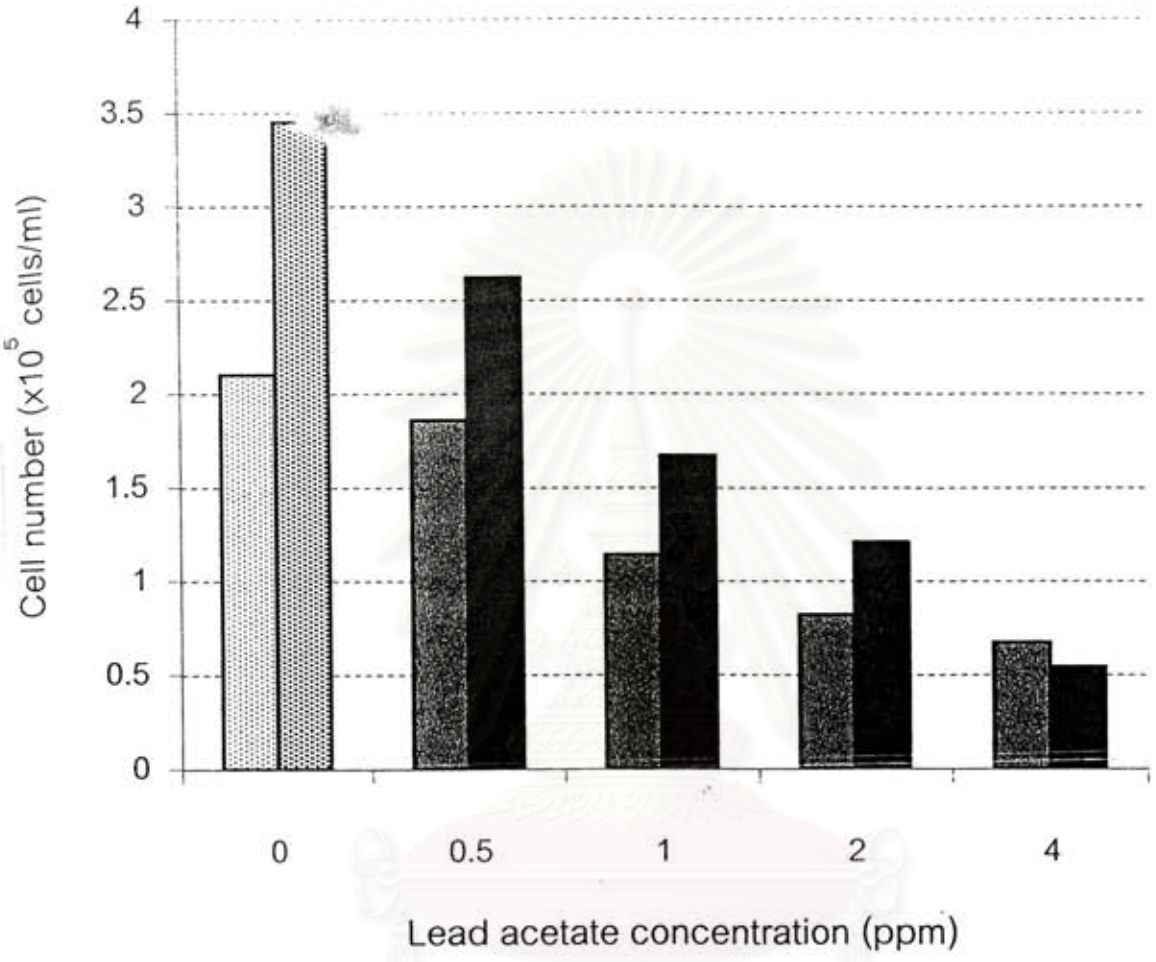


Figure 14: The number of viable cells after exposed to various concentrations of lead acetate when compare to the control (▨) for one(■) and five(■) days.

Table 4 : Effect of lead on the number of viable cells. Cell viability on day 1 and day 5 of lead incubation was determined by the trypan blue exclusion assay.

Pb concentration (ppm)	Viable cell number ($\times 10^5$ cells/ml)		%Viable cells	
	Day 1	Day 5	Day 1	Day 5
0.0 (control)	2.10 ± 1.70	3.45 ± 3.72	100.00	100.00
0.5	1.86 ± 1.40	2.62 ± 3.30	91.19 ± 5.92	63.52 ± 17.21
1.0	1.14 ± 0.45	1.67 ± 2.04	74.28 ± 40.51	40.64 ± 11.12
2.0	0.82 ± 0.41	1.21 ± 1.39	48.27 ± 22.77	34.14 ± 3.60
4.0	0.67 ± 0.30	0.54 ± 0.51	39.84 ± 14.90	16.91 ± 2.87

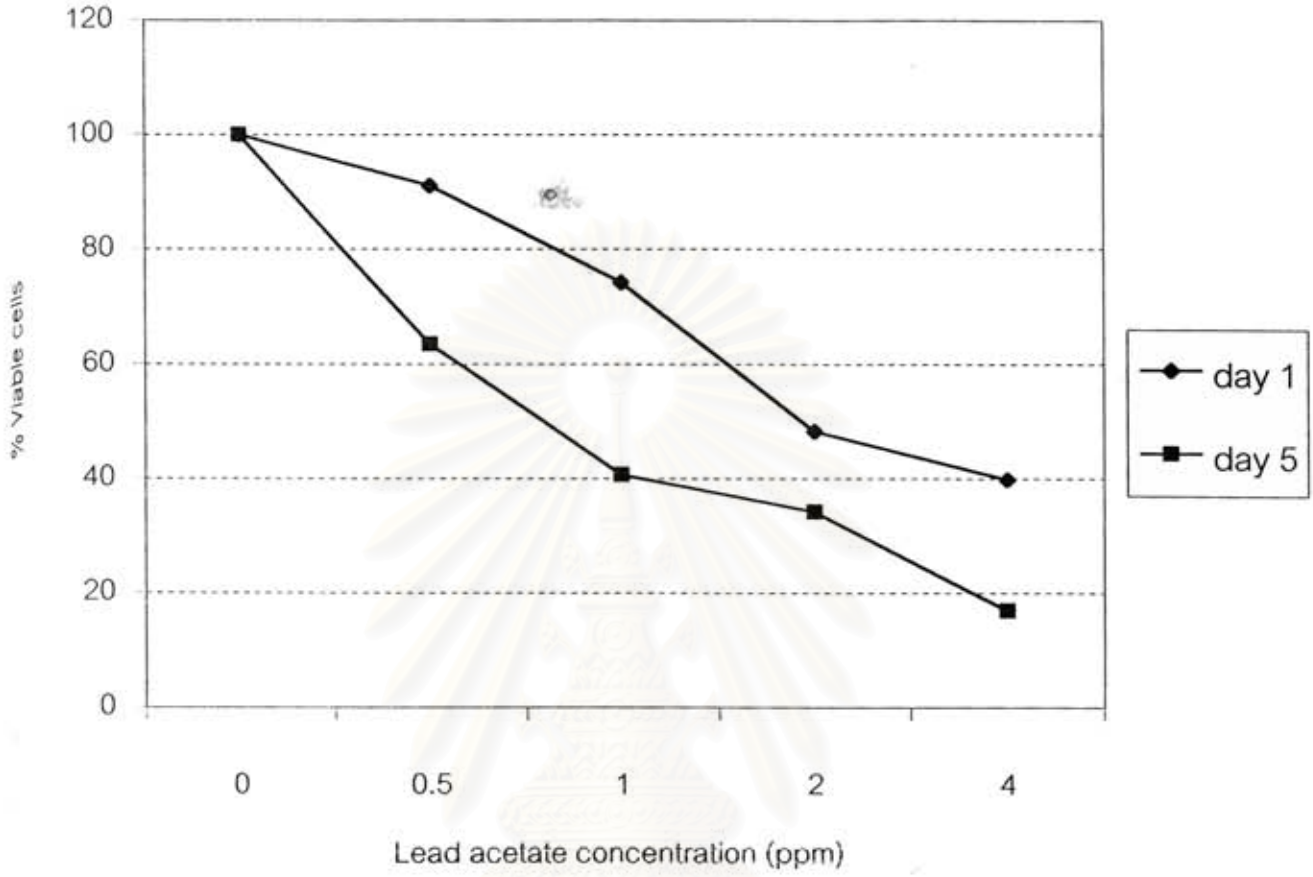
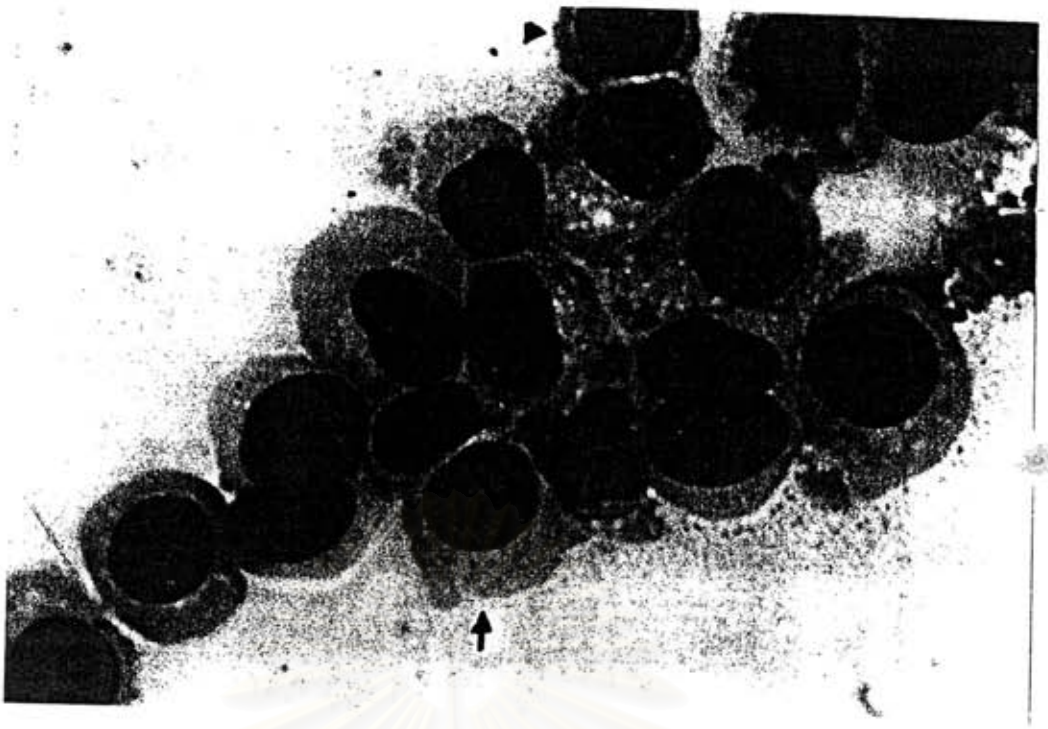


Figure 15: Effect of lead on viable cell number for one and five day-incubation periods.

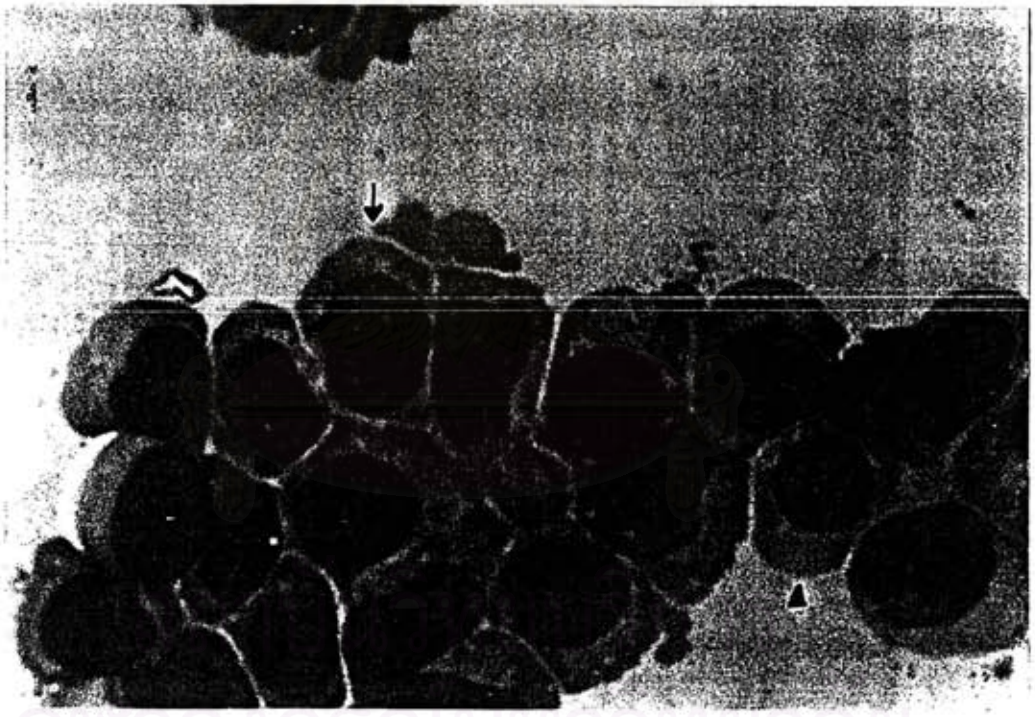
A : 0.0 ppm Pb / day 1

B : 0.1 ppm Pb / day 1

Figure 16 : Light microscopy of cultured cells at day 8 in 2^0 culture after exposed to 0.0 (A) and 0.1 (B) ppm lead acetate for one day (1000 X magnification).
In the absence(A) and presence(B) of lead, the culture consisted of a large number of viable cells, most of erythroid population were proerythroblasts (arrow) and basophilic erythroblasts (arrow head).



A : 0.1 ppm Pb / day 5



B : 0.3 ppm Pb / day 5

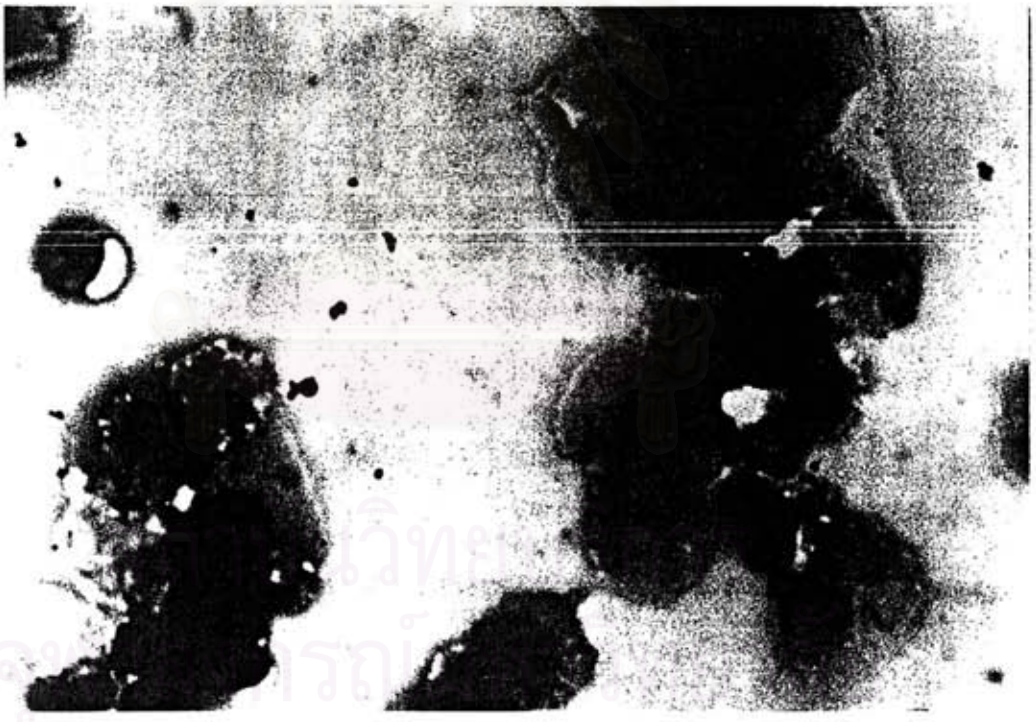
Figure 17 : Light microscopy of cultured cells at day 12 in 2^0 culture after exposed to 0.1 (A) and 0.3 (B) ppm lead acetate for five days (1000 X magnification). In the absence(A) and presence(B) of lead, the culture consisted of a large number of viable cells, most of erythroid population were polychromatophilic(arrow) and orthochromatic erythroblasts (arrow head).

A : 1.0 ppm Pb / day 1

B : 1.0 ppm Pb / day 5

Figure 18 : Light microscopy of cultured cells exposed to 1.0 ppm lead acetate for one and five days (1000 X magnification). In lead containing medium, many abnormal erythroid precursor cells with cytoplasmic blebbing (arrow) were observed. However, apoptotic cells (arrow head) were also observed.

A : 3.0 ppm Pb / day 1



B : 3.0 ppm Pb / day 5

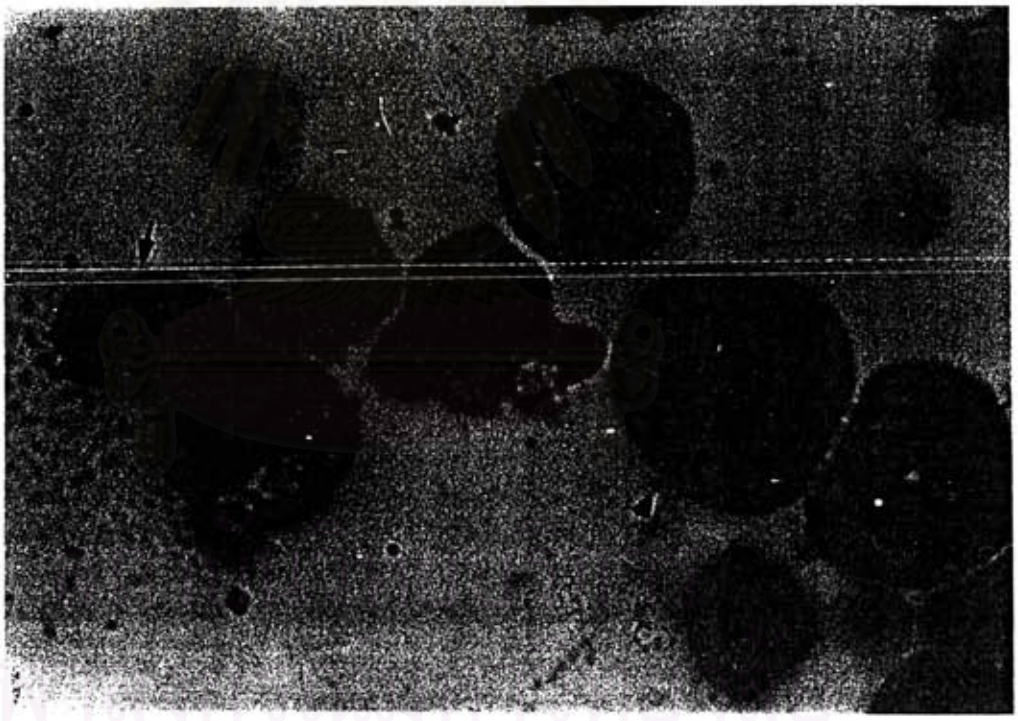
Figure 19 : Light microscopy of cultured cells exposed to 3.0 ppm lead acetate for one and five days (1000 X magnification). In lead containing medium, many abnormal erythroid precursor cells.

A : 0.0 ppm Pb / day 5

B : 2.0 ppm Pb / day 5

Figure 20 : Light microscopy of cultured cells at day 12 in 2^0 culture after exposed to 0.0 (A) and 2.0 (B) ppm lead acetate for five days(1000 X magnification). In the absence(A) and presence(B) of lead, the late stage of erythroid precursor cells, polychromatophilic (arrow) and orthochromatic erythroblasts(arrow head) were also observed in the both conditions.

A : 2.0 ppm Pb / day 1



B : 4.0 ppm Pb / day 1

Figure 21 : Light microscopy of cultured cells at day 8 in 2^0 culture after exposed to 2.0 (A) and 4.0 (B) ppm lead acetate for one day (1000 X magnification). In lead containing medium , many dead cells (arrow) were observed wherease the mitotic cells (arrow head) were also observed.

and basophilic erythroblast at day 8 of secondary culture (Figure 16) and in the stage of polychromatophilic and orthochromatic erythroblast at day 12 of secondary culture (Figure 17). Whereas a considerable number of dead cells were detected when lead acetate of concentration ≥ 1.0 ppm was added to the medium (Figure 18 - 21). Most of them were cytolytic cells, apoptotic cells were also observed at a less extent. Therefore, cytoplasmic damage may be a major cause of cell death caused by lead.

In addition, flow cytometric analysis detected the expression of glycophorin A and transferrin receptor on day 8 and day 12 in secondary culture (Table 5). This showed that during maturation, in the presence or absence of lead, the expression of transferrin receptor decreased slightly (Figure 22), whereas the expression of glycophorin A increased considerably (Figure 23). Moreover, at the same day, the percentage of erythroid precursor cells (the cells expressed both of glycophorin A and transferrin receptor) in the absence and presence of lead were nearly the same (Figure 24). Although, the inhibition of cell viability by lead was dose-dependent but the maturation of erythroid precursor cells still proceeded.

2.2. Lead Induced Apoptosis in Erythroid Precursor Cells

Morphological study showed that, eventhough the decrease in erythroid precursor cells viability with lead exposure caused by cytoplasmic damage, the apoptotic cells were also observed. This implied that an internal suicide program (apoptosis) may also serve as a mechanism of cell death by lead. Flow cytometric analysis was used to detect apoptotic cells by monitoring the binding of fluorescence-labeled annexin V to phosphatidylserine on the outer membrane of apoptotic cells. According to the staining of two erythroid surface markers, glycophorin A and transferrin receptor (CD 71), the cells expressed both of erythroid surface markers were identified as erythroid precursor cells and gated to detect apoptotic cells. Total count was fixed at 10,000 events, the percent of annexin V-positive cells represented apoptotic cells (Figure 25).

Table 5 : **Flow cytometric analysis of erythroid precursor cells after lead exposure for one and five days.** It showed percentage of erythroid apoptosis (mean ± SD) and percentage of erythroid surface marker (mean ± SD) ; glycophorin A and / or transferrin receptor (CD 71) expression.

Day of exposure	[Pb] (ppm)	% Apoptosis AnV(+),GlyA(+), CD71(+)	%Erythroid surface marker		
			GlyA(+)	CD71(+)	GlyA(+),CD71(+)
Day 1	0.0	15.96 ± 6.39	40.65 ± 10.22	84.66 ± 10.54	34.37 ± 2.96
	0.5	17.18 ± 4.95	44.71 ± 7.91	86.98 ± 10.09	37.05 ± 7.03
	1.0	22.73 ± 0.94	46.76 ± 5.44	84.74 ± 9.45	37.61 ± 7.71
	2.0	24.93 ± 5.87	46.99 ± 9.61	89.64 ± 6.15	41.30 ± 10.67
	4.0	32.97 ± 4.73	45.16 ± 5.39	83.16 ± 10.84	35.33 ± 4.79
Day 5	0.0	19.89 ± 3.53	75.05 ± 11.80	83.16 ± 10.37	71.89 ± 7.69
	0.5	19.61 ± 4.52	73.90 ± 10.74	82.08 ± 9.75	73.69 ± 9.32
	1.0	25.47 ± 4.60	75.14 ± 7.45	83.39 ± 7.70	70.29 ± 8.40
	2.0	32.81 ± 6.63	72.85 ± 8.94	86.50 ± 2.19	74.77 ± 3.78
	4.0	44.18 ± 9.06	71.05 ± 11.40	81.21 ± 4.08	75.62 ± 6.32

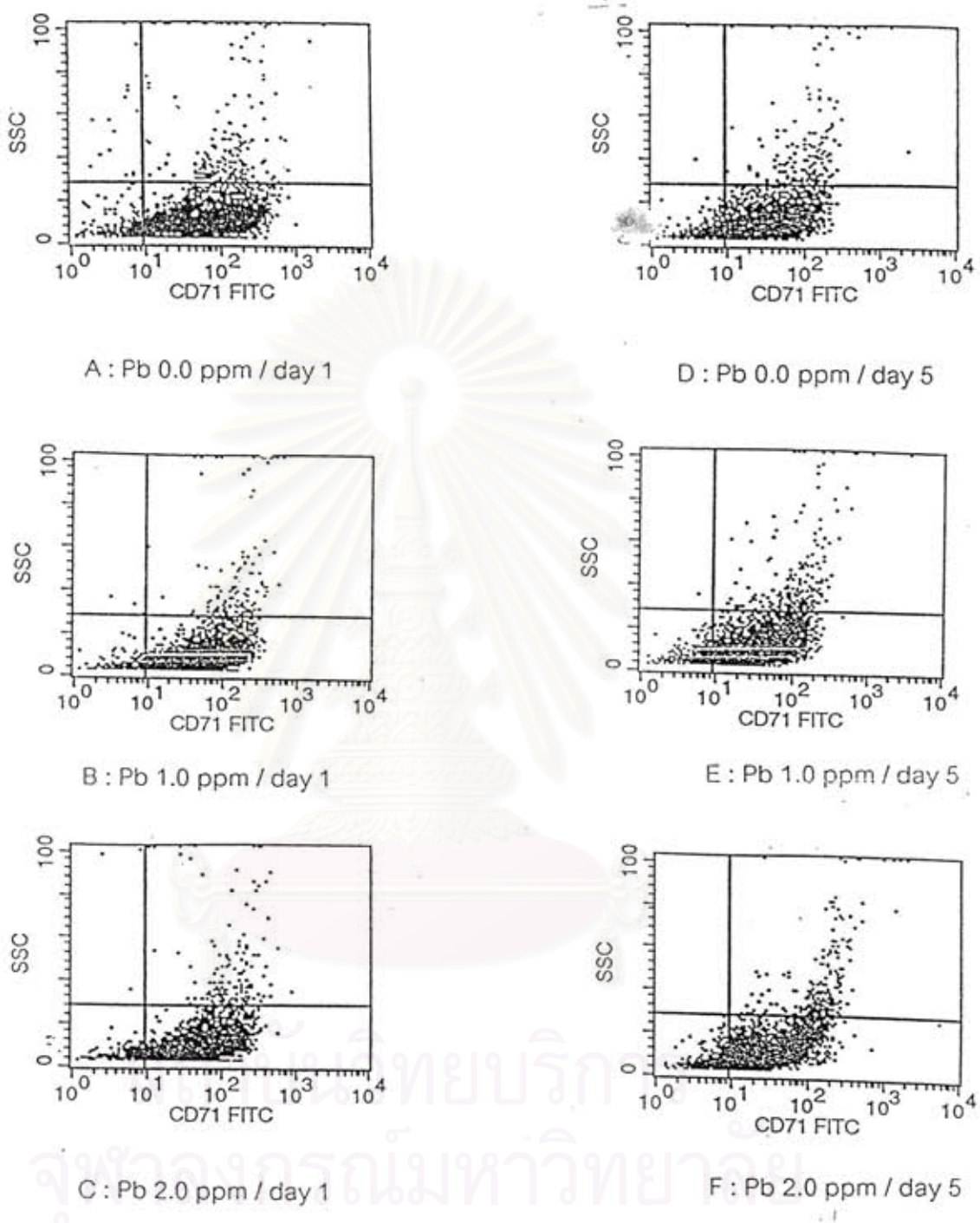


Figure 22 : Flow cytometric analysis of transferrin receptor (CD 71) expression on the cultured cells after exposed to 0.0, 1.0, and 2.0 ppm lead acetate for one (A-C) and five (D-F) days.

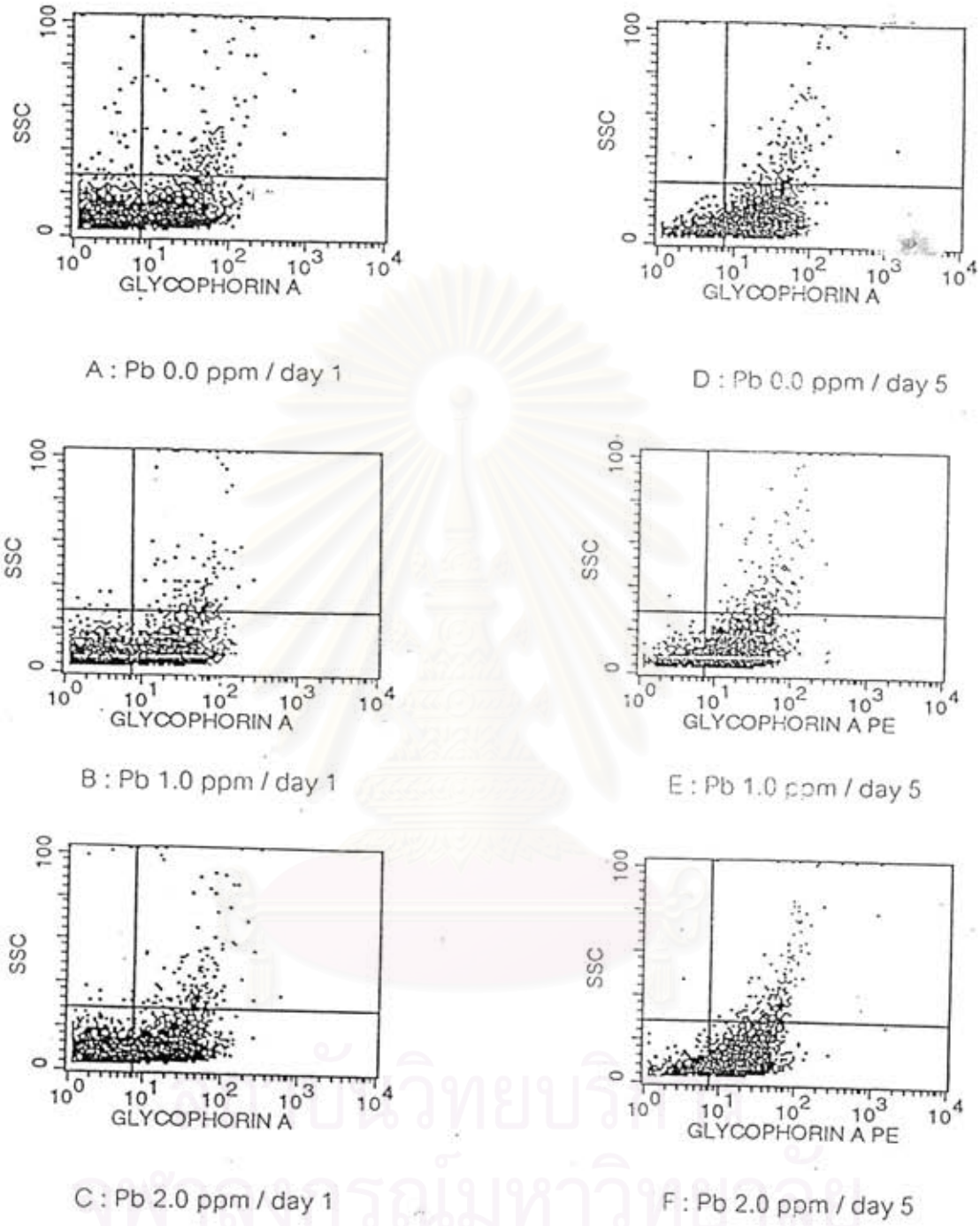
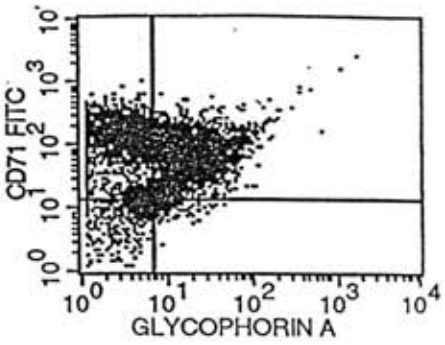
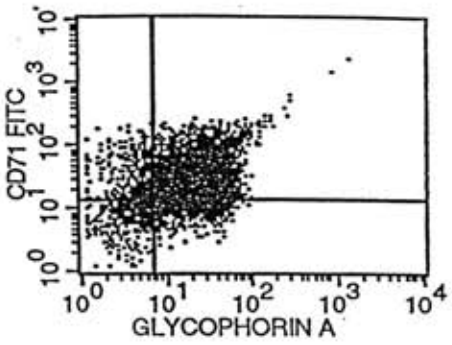


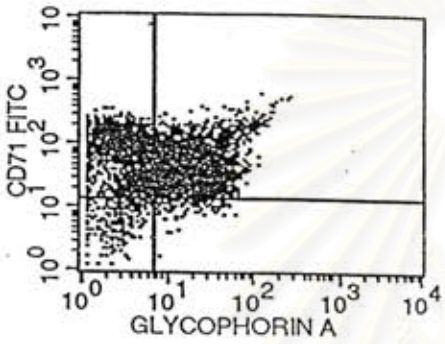
Figure 23 : Flow cytometric analysis of glycophorin A expression on the cultured cells after exposed to 0.0, 1.0, and 2.0 ppm lead acetate for one (A-C) and five (D-F) days.



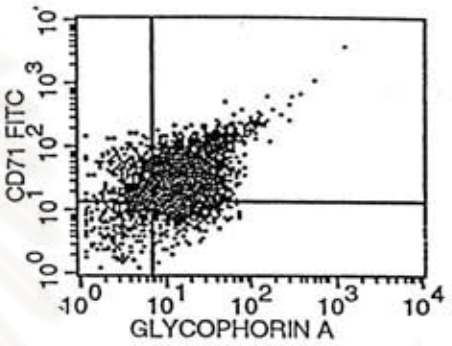
A : Pb 0.0 ppm / day 1



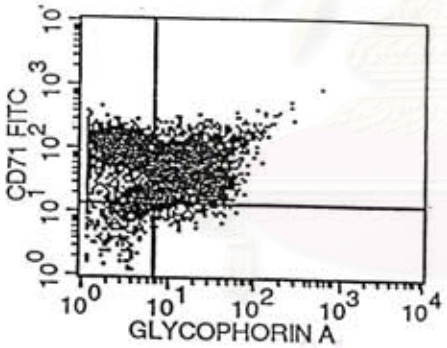
D : Pb 0.0 ppm / day 5



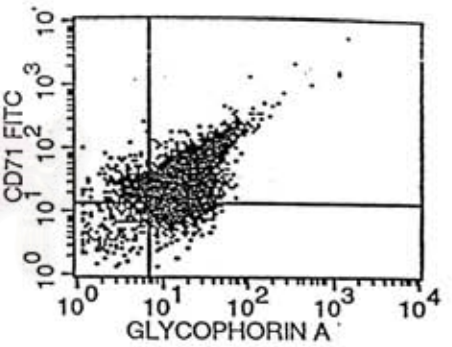
B : Pb 1.0 ppm / day 1



E : Pb 1.0 ppm / day 5



C : Pb 2.0 ppm / day 1



F : Pb 2.0 ppm / day 5

Figure 24 : Correlation between glycophorin A and transferrin receptor (CD 71) expression on the cultured cells after exposed to 0.0, 1.0, and 2.0 ppm lead acetate for one (A-C) and five (D-F) days. The cells that were positive for both glycophorin A and transferrin receptor were identified as erythroid precursor cells.

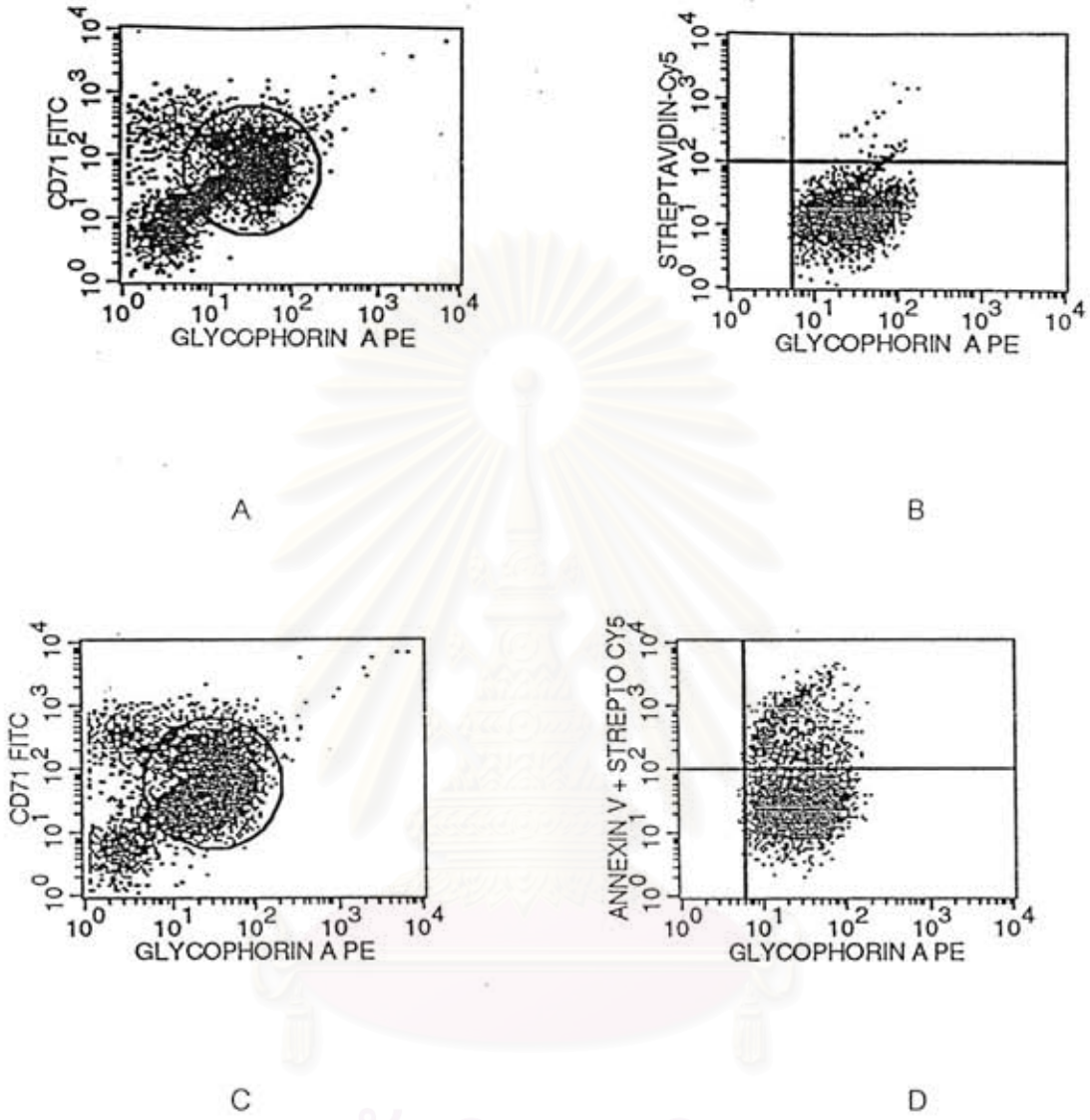


Figure 25 : Flow cytometric analysis of erythroid precursor cells at day 12 of secondary phase. The erythroid precursor cells positively stained for glycophorin A and transferrin receptor (A and C) were gated and analysed for annexin V-staining (B and D).

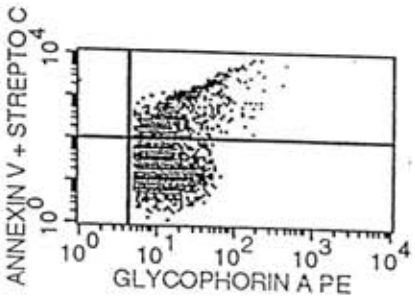
A and B = The controll sample without annexin V-staining.

C and D = The sample with annexin V-staining.

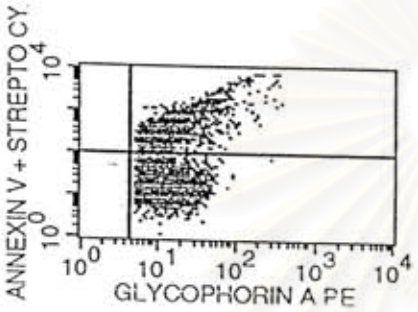
The scattergrams of apoptosis in erythroid precursor cells after exposed to various lead concentrations were shown in Figure 26-27. After lead exposure for one and five days, the percent of annexin V–positive cells was nearly unchanged at 0.5 ppm lead acetate concentration but increased considerably at lead acetate concentration ≥ 1.0 ppm (Table5). This indicated that, the apoptosis of erythroid precursor cells was induced by lead in dose-dependtdent manner at lead acetate concentration ≥ 1.0 ppm (Table 5).

Time-course of the lead effect on apoptosis in erythroid precursor cells (Figure 28) showed that, after lead acetate exposure at concentration 0.0 and 0.5 ppm, the percent of apoptotic cells (annexin V–positive cells) increased slightly with time. Eventhough the percent of apoptotic cells in 0.0 and 0.5 ppm lead acetate was nearly the same, after the exposure to lead acetate at concentration ≥ 1.0 ppm, the apoptosis increased considerably with time and higher than the control. Therefore, the effect of lead on apoptosis in erythroid precursor cells after lead acetate exposure at concentration ≥ 1.0 ppm was time and dose-dependtdent.

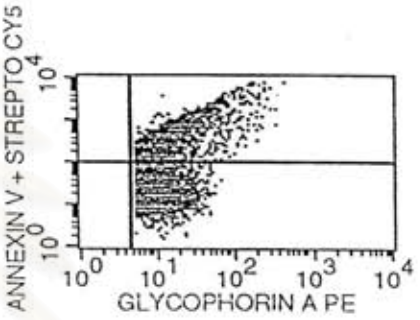
In addition, the correlation between the percent of apoptotic cells and viable cell number was also shown (Figure 29). After exposure to low lead acetate concentration (0.5 ppm), both the percent of apoptotic cells and viable cell number after lead exposure for one and five days were nearly the same. However, at higher concentration of lead acetate (≥ 1.0 ppm), the percent of apoptotic cells markedly increased while viable cell number decreased. Therefore, apoptosis of erythroid precursor cells was induced by lead may be one of the mechanism of cell death resulting in the decrease of viable cells.



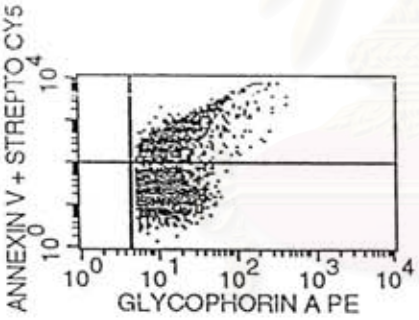
A : Pb 0.0 ppm



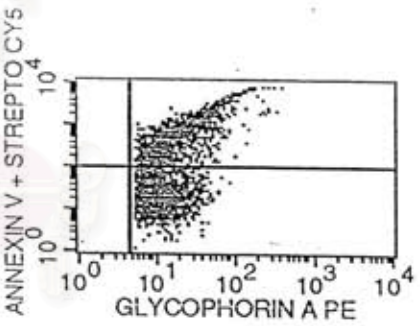
B : Pb 0.5 ppm



C : Pb 1.0 ppm



D : Pb 2.0 ppm



E : Pb 4.0 ppm

Figure 26 : Apoptosis of erythroid precursor cells exposed to 0.0 (A), 0.5 (B), 1.0 (C), 2.0 (D), and 4.0 (E) ppm lead acetate for one day. After staining, the cells were analysed by flow cytometry. The erythroid precursor cells that expressed both glycophorin A and transferrin receptor (CD 71) were gated and analysed for apoptosis by the appearance of annexin V-binding cells.

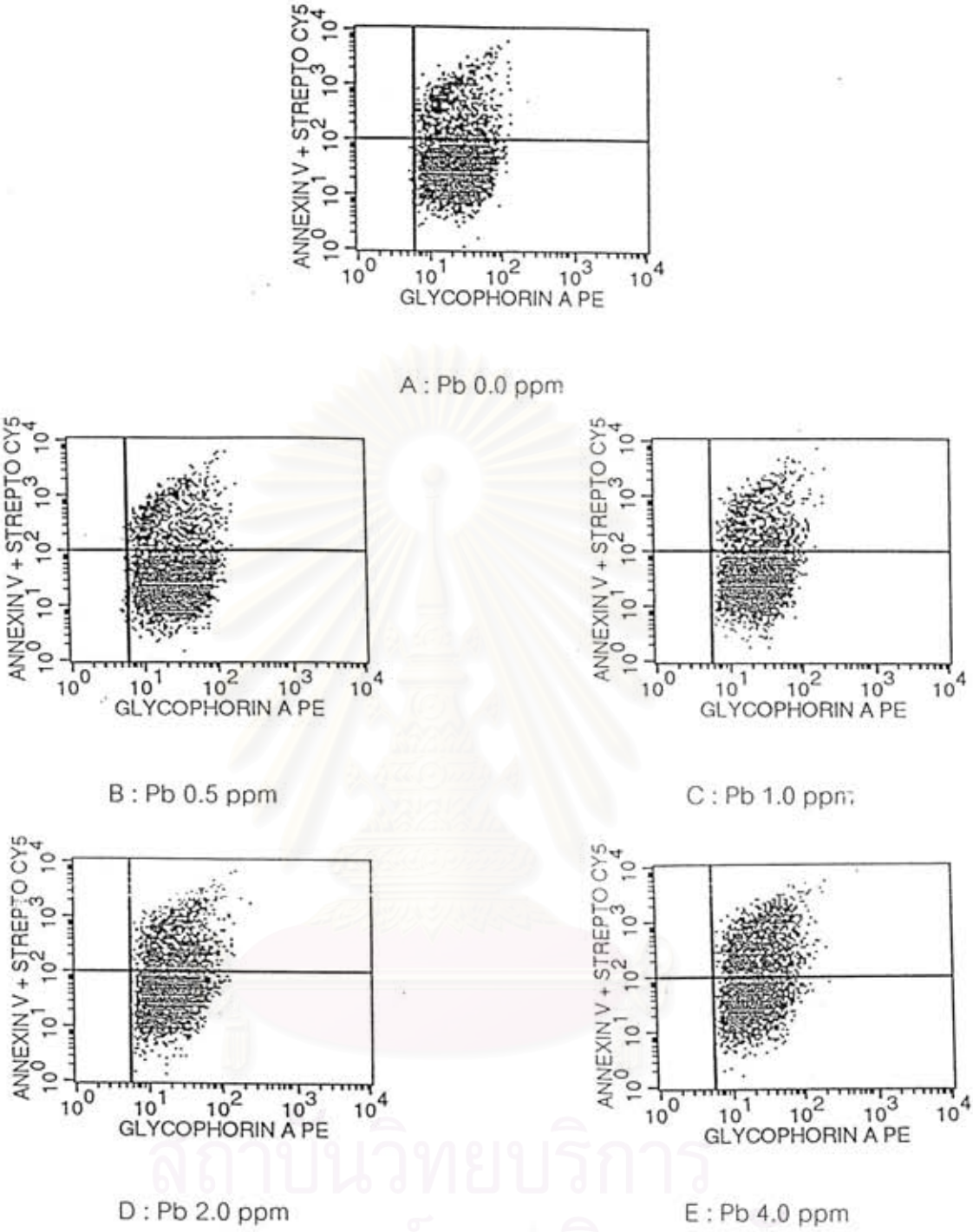


Figure 27 : Apoptosis of erythroid precursor cells exposed to 0.0 (A), 0.5 (B), 1.0 (C), 2.0 (D), and 4.0 (E) ppm lead acetate for five days. After staining, the cells were analysed by flow cytometry. The erythroid precursor cells that expressed both glycophorin A and transferrin receptor (CD 71) were gated and analysed for apoptosis by the appearance of annexin V-binding cells.

Table 6 : Flow cytometric analysis of erythroid precursor cells at day 1 and day 5 after various lead concentrations exposure. It showed the percentage of erythroid apoptosis (annexin V–positive cells of CD71 and glycophorin A) and the number of viable cells.

[Pb] (ppm)	Day 1		Day 5	
	cell number ($\times 10^5$ cells/ml)	% Apoptosis An V(+), GlyA(+), CD71(+)	cell number ($\times 10^5$ cells/ml)	% Apoptosis An V(+), GlyA(+), CD71(+)
0.0	2.10 ± 1.70	15.96 ± 6.39	3.45 ± 3.72	19.89 ± 3.53
0.5	1.86 ± 1.40	17.18 ± 4.95	2.62 ± 3.30	19.61 ± 4.52
1.0	1.14 ± 0.45	22.73 ± 0.94	1.67 ± 2.04	25.47 ± 4.60
2.0	0.82 ± 0.41	24.93 ± 5.87	1.21 ± 1.39	32.81 ± 6.63
4.0	0.67 ± 0.30	32.97 ± 4.73	0.54 ± 0.51	44.18 ± 9.06

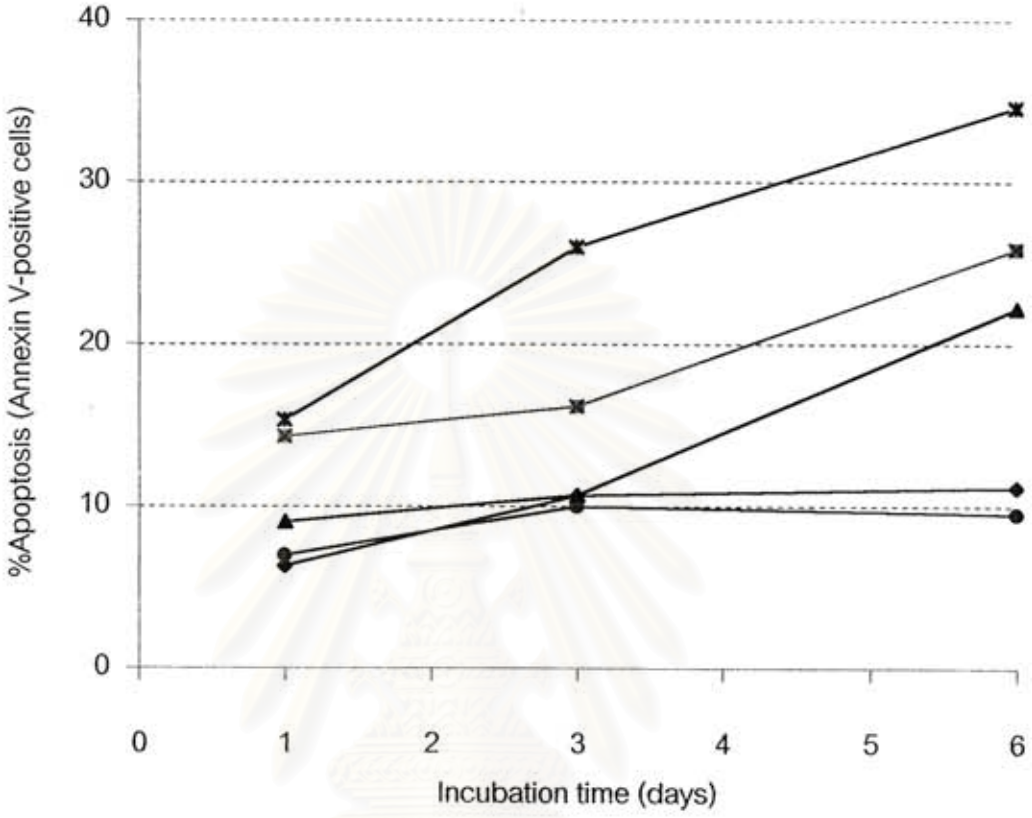


Figure 28: Time-course of lead effect on apoptosis of erythroid precursor cells. The exposures were with —◆— 0.0 ppm, —◆— 0.5 ppm, —▲— 1.0 ppm, —✱— 2.0 ppm and —✱— 4.0 ppm, lead acetate for one, three and six days.

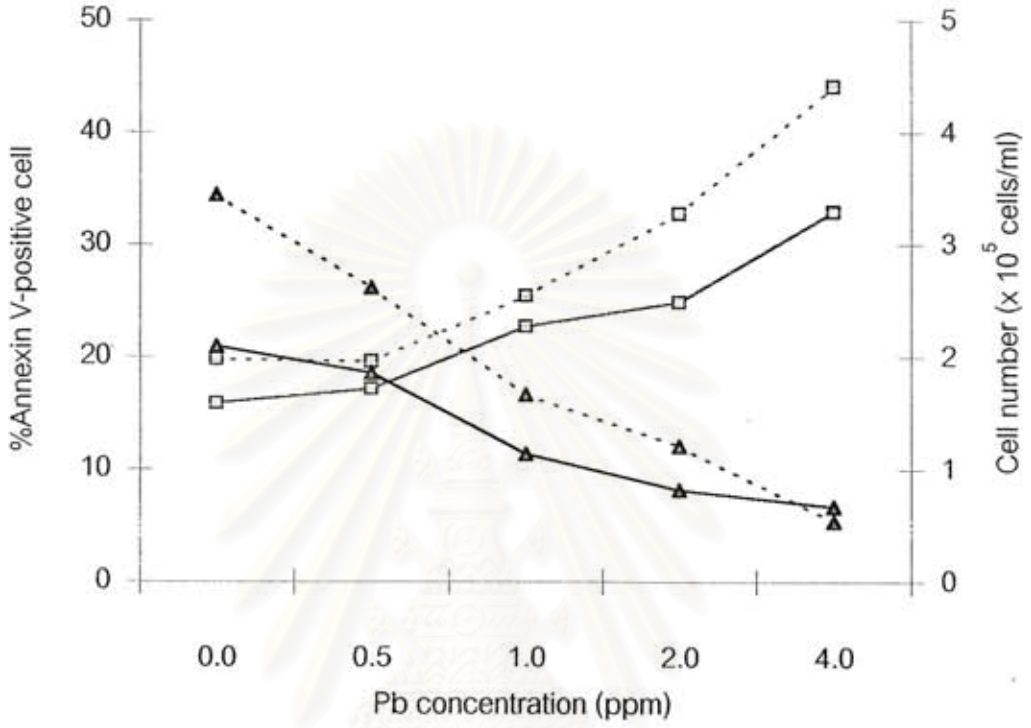


Figure 29: Apoptosis and cell viability of erythroid precursor cells during culture at various concentrations of lead for one (———) day and five (– – – –) days . After incubation with moderate concentration of lead (0.5 - 4.0 ppm), the percentage of annexin V-positive cells (▣) increased with lead concentration whereas the number of viable cells (trypan blue-negative cells) (▲) decreased.

CHAPTER V

DISCUSSION

1. Development of Human Erythroid Precursor Cells in TPLC

Since the peripheral blood of normal human contained homogeneous population of early erythroid progenitor cells, BFU-Es (Clarke and Housman, 1977), the white blood cell fraction (buffy coat) separated from whole blood unit of normal donor at TRCS was used as the starting material in this study. The development of human erythroid precursor cells from BFU-Es could be accomplished in TPLC. BFU-Es were easily separated from the buffy coat by ficoll-hypaque density gradient centrifugation. Because of their lower buoyant density, lymphocytes, monocytes and other mononuclear cells were packed on the ficoll solution while granulocytes and erythrocytes, the higher buoyant density cells, were sedimented through the solution. Therefore, the harvested mononuclear cell fraction contained a high proportion of lymphocytes, monocytes and also BFU-Es which looked like small lymphocytes.

In primary phase, mononuclear cells were cultured in the medium supplemented with FBS and conditioned medium derived from the 5637 human bladder carcinoma cell line cultures. The conditioned medium provided erythroid burst-promoting activity (BPA), the important growth factor for erythroid progenitor development (Iscove,1977; Mayers, et al., 1984; Lipton and Nathan, 1987). Therefore, during primary phase, early erythroid progenitor cells (BFU-Es) proliferated and differentiated into late erythroid progenitor cells (CFU-Es). Moreover, the growth factors presented in conditioned medium, such as granulocyte-monocyte colony stimulating factor (GM-CSF), could stimulate myeloid progenitors (Burgess and Metcalf, 1980). So that, granulocytes and macrophages were also developed. However, EPO was absent in the conditioned medium.

Under one week in EPO-independent phase, many colonies of CFU-Es were developed from BFU-Es by the stimulation of specific growth factors from the

conditioned medium. However, small amounts of early erythroid precursor cells, proerythroblasts, could develope from CFU-Es in this phase. These cells may be stimulated by trace amounts of EPO in FBS that were supplemented in primary medium. Since monocytes and macrophages were adherent cells, they were easily separated from the non-adherent CFU-Es by harvesting. Lymphocytes, the interference of erythroid precursor cell development, were depleted by addition of the lymphotoxic drug (cyclosporin A) this drug could inhibit the activation and proliferation of lymphoid cells (Borel and Ryffel, 1985). After seven days in primary phase, the non-adherent cells containing a high proportion of CFU-Es and lymphocytes were harvested leaving the monocytes and macrophages behind. Hemopoietic growth factors from primary medium were removed by the washing process before recultured in secondary medium.

In secondary phase, CFU-Es were cultured in the medium supplemented with the specific growth factor for erythroid precursor cell development, EPO (Liboi et al., 1993; Adamson, 1994; Kirby et al., 1996). Since the development of CEU-Es into erythroid precursor cells required EPO (Eaves, 1978), during EPO-dependent phase, erythroid progenitor cells (CFU-Es) could proliferate and mature into erythroid precursor cells. The colonies of proerythroblast, the first stage of erythroid precursor cell, were developed after three days in this phase. By the stimulation of EPO, proerythroblasts continued to proliferate and mature into the next stage of erythroid precursor cells (Craber and Krantz, 1978).

Although lymphotoxic drug was added, the culture still contained large amounts of lymphocytes. In order to decrease the interference of lymphocytes and collect pure population of erythroid precursor cells at early maturation stage, 45% percoll was used to separate lymphocytes. After the development of large amounts of proerythroblasts, most of the lymphocytes were removed by percoll density gradient centrifugation. Lymphocytes, the higher buoyant density cells, were sedimented through the solution leaving the lower buoyant density proerythroblasts on the percoll solution. Without the interference of lymphocytes, erythroid precursor cells could develop with high proliferation and maturation. After twelve days in secondary phase, by the stimulation of EPO, the cells reached maximum number and maturation of the last stage of erythroid

precursor cells which still contained nucleus (orthochromatic erythroblasts). In the absence of the appropriate growth factors, myeloid progenitors largely disappeared. Therefore, at day 12 of secondary phase, more than 90% of the cell population were erythroid precursor cells.

Human erythroid precursor cell development in TPLC could be demonstrated with flow cytometry by analyzing the expression of two cell surface markers, glycophorin A and transferrin receptor. In the stage of erythroid precursor cell, the cell expressed both of glycophorin A and transferrin receptor. Glycophorin A appeared only on the erythroid lineage (Langlois et al.,1985; Loken et al.,1987). This antigen appeared at the early stage of erythroid precursor cell (proerythroblast) just after the late erythroid progenitor cell (CFU-E) and rapidly reached maximum expression by the intermediate stage of erythroid precursor cell. During further maturation, the amount remained constant (Figure 2). Transferrin receptor began to express at the early erythroid progenitor cell (BFU-E) and reached maximum before glycophorin A expression. The expression of transferrin receptor progressively declined during further maturation and disappeared at the late reticulocyte stage (Loken et al., 1987).

With cell surface antigen expression and light scattering characteristic, one could demonstrate the progressive change during cell maturation. This study showed that, after eight days in EPO-dependent phase (Figure 12 and 13), most of cells displayed anti-glycophorin A from intermediate to dim, CD 71 bright, and forward light scattering (FSC) from intermediate to large. This indicated that, the culture contained predominantly early erythroid precursor cells. In this stage, the cells were larger compared to the later stages and expressed intermediate to high levels of glycophorin A and high level of transferrin receptor. This result corresponded to that from morphological analysis, which demonstrated that most of the cells in this population were proerythroblasts and basophilic erythroblasts (Table 2). After twelve days, most of the cells displayed anti-glycophorin A bright, CD 71 from intermediate to bright, and FSC from low to intermediate. This indicated that, after twelve days, the culture contained predominantly late erythroid precursor cells. These cells expressed high levels of glycophorin A and transferrin receptor. However, during maturation, the

expression of transferrin receptor decresed gradually while glycophorin A expression increased markedly. Since the cell size in late erythroid precursor cell was smaller than the early erythroid precursor cell, the cells in the late stage displayed lower FSC. This result corresponded to the morphological analysis which demonstrated that most of cells in this population were polychromatophilic and orthochromatic erythroblasts (Table 2).

Therefore, after cultured in EPO-dependent phase, the cells could develop from the early to the late erythroid precursor cells. Glycophorin A expression served as a good marker in monitoring the development of erythroid precursor cell. However, since the cultured cells could not be developed to reticulocytes in this culture, most of the cells at day 12 of the secondary phase still contained a high level of transferrin receptor and was suitable for the consequent study on the uptake of lead.

2. Effect of Lead on Human Erythroid Precursor Cells

2.1. Effect of Lead on Erythroid Precursor Cell Development

It has long been known that lead disturbs the Hb synthesis in erythroid precursor cells which leads to anemia but it is unclear that how lead affects the cell development. By using human erythroid precursor cell cultured *in vitro*, it was found that although lead caused the decrease of erythroid precursor cell survival, the cells could still mature to the stage of orthochromatic erythroblast. The result is similar to the effect of lead on Friend leukemia cells and rat bone marrow cells (Taketani et al., 1985). It is possible that, at lead acetate concentrations used in this experiment (0.1-4.0ppm), the inhibition of Hb synthesis is incomplete and the amount of Hb remained is sufficient to allow cells undergo the maturation process.

However, the viable cell number of human erythroid precursor cells in lead contained medium was markedly reduced at lead acetate concentration ≥ 1.0 ppm and the inhibition was dose and time-dependent. The suppressive effects of lead on erythroid precursor cell survival may be explained, at least in part, by the effect of this metal on the amino acid sulfhydryl (SH) groups. These groups are found in many enzymes involved in the maintenance of membrane related function including osmotic resistance and mechanical fragility of cells. There are many reports indicate that lead can produce membrane damage in immature and mature red blood cells both in the marrow and in the peripheral blood resulting in the cell cytolysis or hemolysis (Westerman et al., 1965; Waldron, 1966; Albahary, 1972; White and Selhi, 1975; Lachant, Tomoda and Tanaka, 1984).

The morphological study in this experiment indicated the presence of many cytolytic cells in erythroid precursor cell culture after lead exposure at lead acetate concentration ≥ 1.0 ppm (Figure 18-21). The mechanism of lead induced cytolysis on these cells may be due to its effect on the inhibition of ATPase, leading to cellular loss of K^+ (White and Selhi, 1975). The second possible mechanism may be the impairment of pyrimidine 5′-nucleotidase activity which causes accumulation of nucleotides leading to

the inhibition of the pentose phosphate shunt (Lachant et al,1984) and promote cytolysis (Paglia et al., 1977). Other pathways which decrease osmotic resistance or increase mechanical fragility of the cells may also be responsible the cytolysis.

6.2. Lead Induced Apoptosis in Erythroid Precursor Cells

The morphological study in this experiment also observed apoptotic cells in erythroid precursor cell culture after lead exposure. In addition, flow cytometric study could also detect apoptotic cells in the erythroid population. The apoptosis of erythroid precursor cells was induced by lead in time and dose dependent at lead acetate concentration $\geq$ 1.0 ppm (Figure 28). These finding leads to the conclusion that lead induced apoptosis may be another supressive effect of lead on erythroid precursor cell survival. Although the molecular mechanisms underlying the apoptosis induced by lead in these cells are uncertain, three possible triggering mechanisms have been suggested.

The first mechanism may be the genotoxicity of lead. Genotoxic damage including DNA single or double-strand breaks or nucleotide deprivation can activate a cascade beginning with the DNA-binding transcription factor p53 whose targets induce either growth arrest or entry of the cell into the apoptotic pathway. Since, lead can induce genotoxic damage by interaction with DNA repair processes leading to an enhancement of genotoxicity in combination with a variety DNA damaging agents (Zelikoff et al., 1988; Roy and Rossman, 1992; Hartwig, 1994) and DNA strand breaks at toxic concentrations (Hartwig, 1995; Ariza et al., 1998). It is possible that, apoptosis in erythroid precursor cells may be induced by the genotoxic effect of lead on its DNA. Cell injury resulting in genotoxic effect of lead may activate p53, a transcription regulatory gene. The p53 protein product is a regulator of DNA transcription, it binds directly to DNA, recognizes DNA damage. If cellular damage is considered reparable, p53-induced cell cycle arrest allows time for DNA repair. With more extensive damage, to prevent the cell with an impaired DNA sequence from proliferating as a defective or malignant clone, p53 moves the cell into apoptotic pathway.

Therefore, apoptosis may occur in the cell that receives high extensive cellular damage such as high dose of lead or prolonged lead exposure that lead to DNA damage is more severe and non-repairable, p53 performs its alternate role of moving the cell into apoptosis.

The second mechanism may be the direct disturbance of lead on mitochondria. The mitochondria are among the cellular structures that are particularly sensitive to lead. Studies using ^{210}Pb and ^{203}Pb have shown the tendency of lead to accumulate in the mitochondria (Castellino and Aloj, 1969; Barltrop et al., 1974). Electron microscopic study of erythroblasts in lead poisoning patients shows many grossly swollen mitochondria in the cells (Jensen, Moreno, and Bessis, 1965). Mitochondria are now considered major players in the apoptosis process of mammalian cells (Susin, Zamzami, and Kroemer, 1998). The opening of the mitochondrial permeability transition pore (PTP) lead to apoptogenic protein release, including cytochrome c or apoptosis inducing factors (AIF), then cause caspase activation which cleave downstream death substrates and activate endonucleases that cleave genomic DNA into fragments resulting in the apoptotic nuclear morphology (Figure 30). The mitochondrial PTP, a megachannel in the inner mitochondrial membrane, is opened by a variety of apoptotic inducers such as elevated matrix Ca^{2+}, pro-oxidants and thiol-reactive agents (Susin et al., 1998; Ichas and Mazat, 1998; Bernardi, 1999). In addition, from the report of He et al.(2000) shown that calcium (Ca^{2+}) and/or lead (Pb^{2+}) induced mitochondrial depolarization, swelling and cytochrome c release resulting in rod cell apoptosis. The result suggested that $Ca^{2+}$and Pb^{2+} bound to the internal metal (Me^{2+}) binding site of the PTP and induced its opening lead to cytochrome c release and subsequently activation of caspase-9 and caspase-3 which induce apoptotic cell death.

Since the mitochondria of erythroid precursor cells are known to be disturbed by lead (Albahary, 1972). In addition, lead is reported to alter Ca^{2+} homeostasis and this alteration have often been associated with mitochondrial mechanisms (Rosen and Pounds, 1989; Simons, 1993b). A disruption of Ca^{2+} homeostasis with an increase of cytosolic Ca^{2+} level lead to the mitochondrial PTP opening (Bernardi and Vassanelli, 1992; Zoratti and Szabo, 1995; Macho et al., 1996). Thus, it is possible that lead may

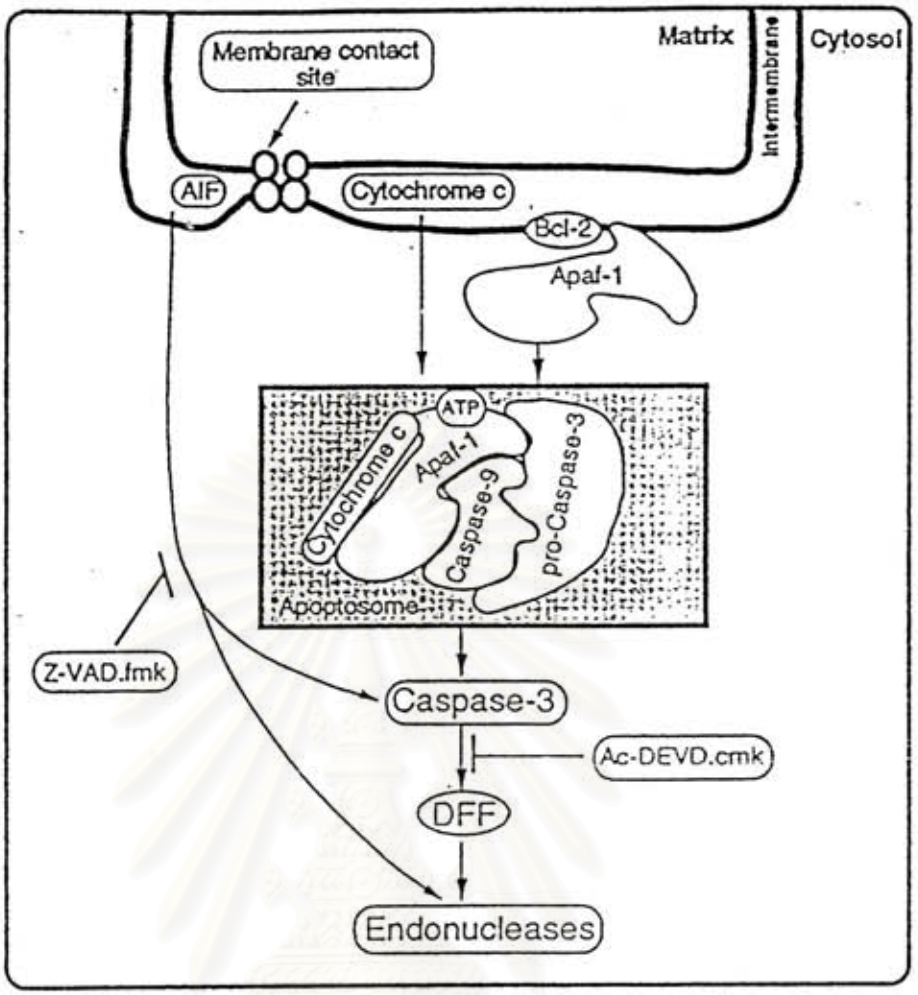


Figure 30 : Caspase and nuclease activation after release of soluble proteins from mitochondria. Several different factors are released from mitochondria during apoptosis : AIF (which is associated with the intermembrane space), cytochrome c (in the intermembrane space) and Apaf-1 (associated with the mitochondrial surface, probably with Bcl-2). Whereas AIF suffices to proteolytically activate caspase-3 and nuclear endonucleases, cytochrome c has to interact with additional proteins in the so - called 'apoptosome'. The apoptosome implies the participation of cytochrome c, Apaf-1 (which binds ATP) and Apaf-3 (caspase-9), which together activate pro-caspase-3. Caspase-3 cleaves and activate DNA fragmentation factor (DFF), which then activates endonucleases (Susin et al., 1998: 160).

produce apoptosis in erythroid precursor cells by the induction of Ca^{2+} overload. After that $Pb^{2+}$and Ca^{2+} may have an additive effect on the mitochondrial PTP. The possible mechanism may be the binding of $Pb^{2+}$and Ca^{2+} to the matrix Me^{2+} binding site of the mitochondrial PTP and subsequently open the PTP, which initiate the cytochrome c-caspase cascade of apoptosis in the cells.

The last mechanism may be the indirect effect of lead on apoptosis production through the generation of reactive oxygen species (ROS). It appears that several pro-apoptotic signal transduction pathways can induce the mitochondrial PTP (Figure 31). Change in cellular redox potentials due to an enhanced generation of ROS, depletion of non-oxidized glutathione, or depletion of NAD(P)H suffice to induce or facilitate the PTP (Zoratti and Scabo, 1995; Bernardi, 1996; Bernardi and Petronilli, 1996). There are many reports indicate that lead can deplete glutathione and generate ROS such as superoxide ion, hydrogen peroxide and hydrogen radical (Stohs and Bagchi, 1995; Sandhril and GILL, 1995; Bondy and Guo, 1996; Skocazylnska, 1997; Ariza, Bijur, and Williams, 1998; Hunaiti and Soud, 2000). Moreover, lead also upregulate the related kinases including mitogen activate protein kinase (MAK) and c-Jun amino terminal kinase (JNK) (Romesh et al., 1995). JNK (also knows as stress-activated protein kinase) and p38 subgroups of MAK have been suggested to play a critical role in signal transduction of apoptotic cell death in erythroid precursor cells (Nagata and Todokoro, 1999). JNK and p38 are also activated by ROS such as hydrogen peroxide (Kyriakis et al., 1994; Wang and Ron, 1996).

Thus, the generation of ROS may be another possible mechanism of lead induced apoptosis in human erythroid precursor cells. It is possible that lead induced apoptosis may be triggered by the generation of ROS which induce the PTP opening and induce apoptosis through the activation of the protein kinases,JNK and MAK.

However, JNK and p38 are required for both cell differentiation and apoptosis of erythroid precursor cells and the duration of their activation may determine the cell fate. Short time activation caused erythroid differentiation while prolonged activation induced apoptosis (Nagata and Todokoro, 1999). In this research also showed that the duration

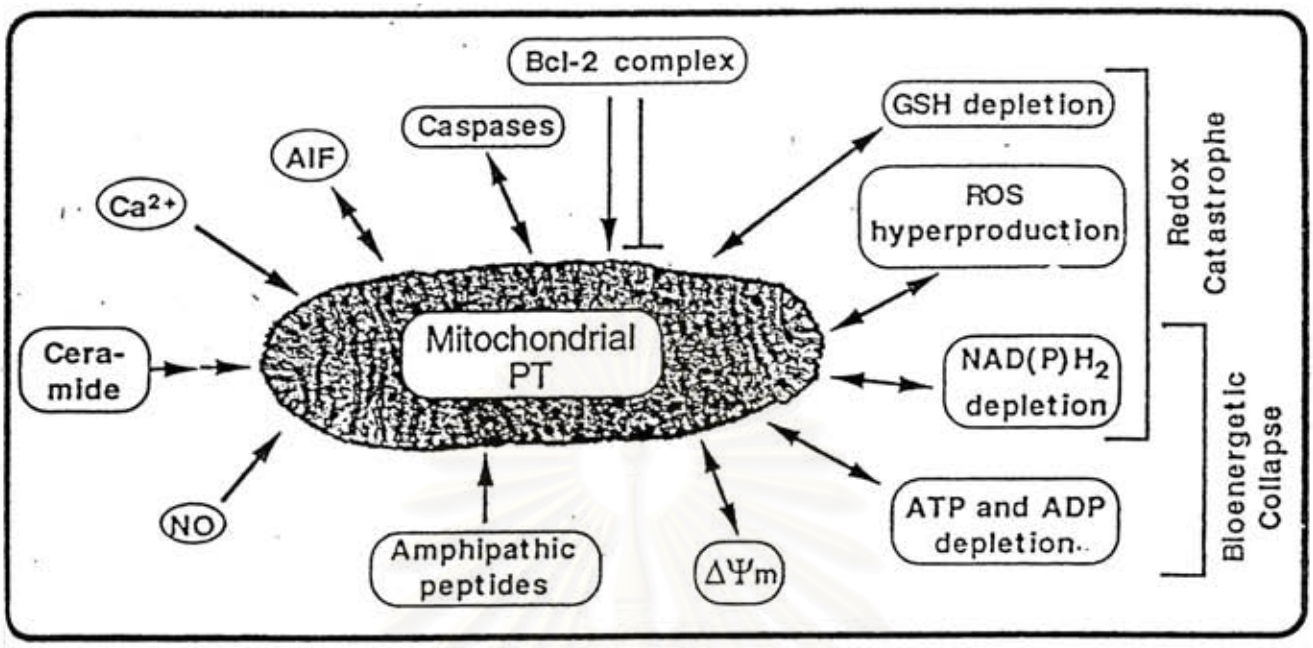


Figure 31 : Inducers of permeability transition. Different signal transduction pathways can promote the activation of caspases, increases in cytosolic Ca^{2+} level, nitric oxide, amphipathic peptides and ceramide, which can provoke PTP either in a direct or an indirect fashion. In addition, changes in the composition of the Bcl-2 complex,such as hyperexpression of the Bcl-2 antagonists Bax or Bak,may induce PTP.Major changes in the cellular redox balance or in bioenergenic parameters can also trigger PTP. Note that PTP is a self-amplifying process in the sense that several consequences of PTP themselves can provoke PTP (two-headed arrows) (Susin et al., 1999: 156).

time of lead exposure had influenced on apoptotic production in erythroid precursor cells. Prolonged incubation with lead increased apoptotic cells in the erythroid culture. It may be explained that the prolong and persistent in lead exposure may overcome a certain threshold level to trigger activation of the factors required for apoptosis such as caspase family and specific DNases and/or to induce inhibition of apoptosis inhibitors such as Bcl2 and $BclX_L$ family, which finally lead to apoptotic cell death. Therefore, the duration of lead exposure may be one of the important factors for apoptotic production by this metal.

Therefore, the inhibition of erythroid precursor cell survival during maturation by lead may be involved in lead induced apoptosis in the cells. Besides the impairement of Hb synthesis, the lead induced apoptosis in erythroid precursor cells may be another mechanism of lead induced anemia.

CHAPTER VI

CONCLUSION

1. Development of Human Erythroid Precursor Cell in TPLC

Since normal human bone marrow contains heterogeneous mixture of erythroid precursor cells and isolation of a large number of stage-specific cells is difficult, a TPLC system was then used to prepare human erythroid precursor cells in this study. This system yields sufficient numbers of late erythroblasts and hence is useful for studying lead transport and toxicity. The procedure is divided into an EPO-independent phase and an EPO-dependent phase. The mononuclear cells were cultured in the first phase in the presence of a conditioned medium containing burst-promoting activity. After 6 to 7 days in primary culture, CFU-Es were developed from BFU-Es. These cells were harvested and cultured in the second phase in the presence of EPO, the specific growth factor for erythroid precursor cell development, CFU-Es developed into proerythroblasts and these cells continued to proliferate and mature into the next stage of erythroid precursor cells. After 12 days in secondary culture, the cells reached maximum number and maturation of the late stage of erythroid precursor cells which still contained nucleus (orthochromatic erythroblasts). At this time, most of cells were the late erythroid precursor cells, polychromatophilic and orthochromatic erythroblasts. These cells expressed high levels of glycophorin A and transferrin receptor. This procedure provided highly purified erythroid precursor cells (>90%) and substantial numbers of the cells ($30.46\pm19.48\times10^{6}$ cells/blood unit).

Moreover, the TPLC system has several characteristic features, such as (1) sample from human specimen, (2) use of erythroid progennitor cells in peripheral blood, and (3) provide reasonable number of erythroid precursor cells with good maturation. Thus, it is suitable procedure for the preparation of human erythroid precursor cells.

2. Effect of Lead on Human Erythroid Precursor Cells

By using TPLC system, BFU-Es could differentiate into erythroid precursor cells. At day 7 in secondary phase, the culture generated the early stage of erythroid precursor cells (proerythroblasts and basophilic erythroblasts). These cells were cultured in the presence of lead. Morphological study showed that lead could inhibit erythroid precursor cell survival by inducing the cell cytolysis and apoptosis. The inhibition was dose and time-dependent manner. Marked effect of lead on erythroid precursor cell survival occurred at lead acetate concentration ≥1 ppm. However, at lead acetate concentration used in this study (0.5-4.0 ppm), the cells still matured to the stage of orthochromatic erythroblasts.

Flow cytometric analysis was used to detect apoptotic cells by monitoring the binding of fluorescence labeled annexin V to phosphatidylserine on the outer membrane of apoptotic cells. This study demonstrated that lead could induce apoptosis in erythroid precursor cells in time and dose-dependent manner at lead concentration ≥1 ppm.

The results from this study suggest a new aspect of lead induced anemia besides the impairment of Hb synthesis and shortened life span of erythrocytes, lead induced apoptosis in human erythroid precursor cells resulting in the inhibition of erythroid precursor cell survival may be another mechanism of lead induced anemia.

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