การนำเข้าและความเป็นพิษของตะกั่วในเซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ของคน

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LEAD UPTAKE AND TOXICITY IN HUMAN ERYTHROID PRECURSOR CELLS

WENIKA BENJAPONG

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เวณิกา เบ็ญจพงษ์ : การนำเข้าและความเป็นพิษของตะกั่วในเซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ของคน (LEAD UPTAKE AND TOXICITY IN HUMAN ERYTHROID PRECURSOR CELLS) อ. ที่ปรึกษา : ผศ. ดร. สุกัญญา สุนทรส, อ. ที่ปรึกษาร่วม : ศ. ดร. อานนท์ บุณยะรัตเวช, อ. ที่ปรึกษาร่วม : รศ. ดร. ทรงศักดิ์ ศรีอนุชาติ จำนวนหน้า 202 หน้า. ISBN 974-13-0349-1

การวิจัยนี้เป็นการศึกษากลไกการนำเข้าและความเป็นพิษของตะกั่วในเซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ของคน ความผิดปกติในการเจริญของเซลล์เม็ดเลือดแดงระยะอิริดทรอยด์เหล่านี้อาจมีความเกี่ยวข้องกับการเกิดโรคโลหิตจางเนื่องจากพิษของตะกั่ว เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ของคนซึ่งใช้เป็นแบบจำลองในการศึกษาถูกเตรียมขึ้นมาโดยเทคนิคการเลี้ยงเซลล์แบบ Two-phase liquid culture (TPLC) แซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ที่เลี้ยงโดยวิธีนี้จะมีความบริสุทธิ์สูง (>90%) และมีจำนวนมาก ($30.46\pm19.48\times10^6$ เซลล์ต่อเลือด 1 ยูนิต) เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ที่พบในวันที่ 12 ของเฟสที่ 2 ถูกนำมาใช้ในการศึกษาการนำตะกั่วเข้าสู่เซลล์ใน 1%FBS/α-MEM (pH7.4) ที่ 37°ซ โดยเปรียบเทียบกับเซลล์เม็ดเลือดแดง(แก่) พบว่าตะกั่วสามารถเข้าสู่เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์อย่างรวดเร็วและมีค่าสูงสุดในเวลา 30 นาที ปริมาณตะกั่วที่เข้าสู่เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์มีค่าสูงกว่าเซลล์เม็ดเลือดแดง(แก่) ประมาณ 3-4 เท่า อัตราการเข้าสู่เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์เพิ่มขึ้นเมื่อความเข้มข้นของตะกั่วภายนอกและเวลาที่สัมผัสกับตะกั่วเพิ่มขึ้น ทรานส์เฟอร์รินซึ่งเป็นพลาสมาโปรตีนที่ทำหน้าที่ขนส่งเหล็กสามารถช่วยขนส่งตะกั่วเข้าสู่เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์แต่ไม่ช่วยการขนส่งตะกั่วในเซลล์เม็ดเลือดแดง(แก่) แสดงว่านอกจากเหล็กแล้วตะกั่วก็อาจจะจับกับทรานส์เฟอร์รินและถูกนำเข้าสู่เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ผ่านทางกระบวนการ receptor-mediated endocytosis เช่นเดียวกับเหล็ก อย่างไรก็ตามทรานส์เฟอร์รินเพิ่มการขนส่งตะกั่วเข้าสู่เซลล์เพียง 26% (จาก 3.28 เป็น 4.08 นาโนกรัมต่อ 10^5 เซลล์) ดังนั้นการขนส่งตะกั่วเข้าสู่เซลล์ผ่านทางทรานส์เฟอร์รินอาจไม่ใช่กลไกหลักของการขนส่งตะกั่วเข้าสู่เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ จากการศึกษาผลของสารยับยั้ง microtubule (colchicine และ vinblastine) ซึ่งสามารถยับยั้งกระบวนการ endocytosis ของ ทรานส์เฟอร์รินและสารยับยั้ง anion transport (4,4'-diisothiocyanostilbene-2,2'-disulfonate) พบว่าสารยับยั้งเหล่านี้สามารถลดปริมาณตะกั่วที่เข้าสู่เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ได้ แสดงว่าอย่างน้อยต้องมีสองกลไกที่เกี่ยวข้องกับการขนส่งตะกั่วเข้าสู่เซลล์เหล่านี้ กลไกแรกอาจเป็น transferrin receptor-mediated endocytosis ส่วนกลไกที่สองอาจจะเป็น anion exchanger system อย่างไรก็ตามการยับยั้งตะกั่วเข้าสู่เซลล์โดยสารยับยั้ง anion transport เกิดขึ้นเพียงเล็กน้อยเท่านั้น แสดงว่ากระบวนการนี้อาจไม่ใช่กลไกหลักของการนำตะกั่วเข้าสู่เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์

เซลล์เม็ดเลือดแดงระยะอิริดทรอยด์ที่ได้จากการเลี้ยงเซลล์ในวันที่ 5 ของเฟสที่ 2 ถูกนำมาเลี้ยงในสภาวะที่มีตะกั่ว การศึกษาทางสัณฐานวิทยาจากการดูด้วยกล้องจุลทรรศน์พบว่าตะกั่วมีผลทำให้เกิดการตายของเซลล์เม็ดเลือดแดงระยะอิริดทรอยด์โดยไปทำลายเซลล์ทั้งในลักษณะของ cell cytolysis และ apoptosis ขึ้นอยู่กับเวลาและความเข้มข้นของตะกั่วพบว่าเมื่อความเข้มข้นของตะกั่วอะซิเตทมากกว่า 1 ส่วนในล้านส่วน จะมีผลต่อความสามารถในการมีชีวิตอยู่ของเซลล์อย่างเห็นได้ชัด การศึกษาการเกิด apoptosis โดยใช้ flow cytometric analysis ติดตามสารเรืองแสงที่ติดฉลากบน annexin V ซึ่งจับ phosphatidylserine ที่ผิวนอกของเซลล์ที่เกิด apoptosis พบว่าตะกั่วสามารถทำให้เซลล์เม็ดเลือดแดงเหล่านี้เข้าสู่การตายแบบ apoptosis การเกิด apoptosis เกิดขึ้นเมื่อความเข้มข้นของตะกั่วอะซิเตทมากกว่า 1 ส่วนในล้านส่วน โดยขึ้นอยู่กับเวลาและความเข้มข้นของตะกั่ว การค้นพบนี้นำไปสู่มุมมองใหม่ของการเกิดโรคโลหิตจางเนื่องจากพิษของตะกั่วนั่นคือนอกจากความบกพร่องของการสังเคราะห์ฮีโมโกลบินและการทำให้เซลล์เม็ดเลือดแดง(แก่)มีอายุสั้นแล้ว การยับยั้งการมีชีวิตอยู่ของเซลล์เม็ดเลือดแดงระยะอิริดทรอยด์โดยการทำให้เกิดการตายแบบ apoptosis อาจเป็นอีกสาเหตุหนึ่งของการเกิดโรคโลหิตจางเนื่องจากพิษตะกั่ว

หลักสูตร... วิทยาศาสตร์ชีวภาพ... ลายมือชื่อนิสิต.....

สาขาวิชา...วิทยาศาสตร์ชีวภาพ... ลายมือชื่ออาจารย์ที่ปรึกษา.....

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

ABSTRACT

C827719 : MAJOR BIOLOGICAL SCIENCES

KEY WORD: LEAD / UPTAKE / ERYTHROID PRECURSOR CELL / TRANSFERRIN / ENDOCYTOSIS/ APOPTOSIS

WENIKA BENJAPONG : LEAD UPTAKE AND TOXICITY IN HUMAN ERYTHROID PRECURSOR CELLS. THESIS ADVISOR : ASIST. PROF. SUGANYA SOONTAROS, Ph.D., THESIS COADVISOR : PROF. AHNOND BUNYARATVEJ, Ph.D., THESIS COADVISOR : ASSOC. PROF. SONGSAK SRIANUJATA, Ph.D., 202 pp. ISBN 974-13-0349-1

The mechanism of lead uptaket and toxicity in human erythroid precursor cells (EPCs) are the main aims 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 ($30.46 \pm 19.48 \times 10^6$ cells/blood unit) were obtained from this technique. The late stage of EPCs obtained in day 12 of secondary phase were used for lead uptake study. The study was compared between human EPCs and erythrocytes in 1%FBS/α-MEM (pH 7.4) at 37°C, and showed that lead was rapidly incorporated into EPCs and reached the maximum value at 30 minutes. The lead content in EPCs was about 3-4 folds higher than that in erythrocytes. The rate of lead uptake into EPCs increased with extracellular lead concentration and time. Transferrin (Tf), plasma iron transport protein, could enhance lead uptake in EPCs but not erythrocytes. These findings indicate that besides iron, Tf may bind and deliver lead to EPCs through the receptor-mediated endocytosis. However, the increase of lead content by Tf in EPCs was only 26% (from 3.23 to 4.08 ng/10^5cells). Thus, the mechanism involved with Tf may not be the major route of lead transport into these cells. The study of inhibitory effect of microtubule inhibitors which prevent Tf endocytosis (colchicine and vinblastine) and anion transport inhibitor (4,4'-Diisothiocyanostilbene-2,2'-disulfonate) showed that these inhibitors could inhibit lead uptake in EPCs. This suggests that at least two mechanisms have been involved in lead uptake in these cells. The first may be through Tf receptor-mediated endocytosis. The second route may be the anion exchanger system. However, the anion transport inhibitor provided a slight inhibition of lead uptake, suggesting that this may not be the major route of lead uptake in EPCs.

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 manner. 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. The findings in this study 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.

Department.....Biological Sciences.... Student's signature.....

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ABBREVIATION

Ab	Antibody
Ag	Antigen
ALA	Aminolaevulinic acid
ALA-D	Aminolaevulinic acid dehydratase
Apo-Tf	Apo-Transferrin
ATP	Adenosine triphosphate
BFU-E	Burst-forming unit-erythroid
Ca	Calcium
Ca^{2+}-ATPase	Calcium- adenosine triphosphatase
CFU-E	Colony-forming unit-erythroid
CFU-S	Spleen colony-forming unit
CO_2	Carbondioxide
COPRO-O	Coproporphyrinogen oxidase
DIDS	4,4'-Diisothiocyanatostilbene-2,2'-disulfonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EPC	Erythroid precursor cell
EPO	Erythropoietin
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FITC	Fluorescein isothiocyanate
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
GM-CSF	Granulocyte-macrophage colony stimulating factor
Hb	Hemoglobin
IL	Interleukin
IGF	Insulin-like growth factor
JNK	C-Jun amino terminal kinase

Ka	An association equilibrium constant
Kd	An dissociation equilibrium constant
MEK	Mitogen activated protein kinase
α-MEM	Alpha-minimal essential medium
ml	Millilitre
μl	Microlitre (10^{-6} litre)
μM	Micromolar (10^{-6} molar)
μg	Microgram (10^{-6} gram)
ng	Nanogram (10^{-9} gram)
Pb	Lead
PBS	Phosphate buffered saline
ppm	part per million (μg/ml)
PS	Phosphatidylserine
PSC	Pluripotent hematopoietic stem cell
PTP	Permeability transition pore
Py-5-N	Pyrimidine-5-nucleosidase
RNA	Ribonucleic acid
RBC	Red blood cell
R-PE	R-phycoerythrin
Tf	Transferrin
TNF	Tumor necrosis factor
TPLC	Two-Phase Liquid Culture
SAPK	Stress-activated protein kinase
SCF	Stem cell factor
SF	Stell factor
UV	Ultraviolet
WBC	White blood cell

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 element number 82 in group IVB of the periodic table. 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. Most salt of lead (II), lead oxides and lead sulfide are only slightly soluble in water, with the exception of lead acetate, lead chlorate and lead chloride.

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 นภาวัส 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 และคณะ, 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 Metabolism (Smith, 1976; Relly, 1991; Castellino, 1995; Goyer, 1996)

At present, the major sources of human exposure to lead affecting the general population are food, drinking water and other beverages, while the main risk of lead

absorption in occupation exposure comes from inhalation of lead aerosols. Moreover, organic lead compound (such as alkyl lead that has escaped by evaporation from automobile fuel system) can be easily absorbed through the skin. Sources of exposure lead are listed in table 1.

Table 1: Sources of lead exposure (Gossel and Bricker, 1990: 173)

Environmental	Household	Persons at high industrial risk
Water	Crayons and toy	Miners
Air	Paper and clothes	Smelters
Soil	Dirt and sand	Automobile finishers
Food	Paint flakes	Storage battery workers
	Furniture	Sheet metal workers
	Wallpaper	Spray painters
	Lead-glazed dishes, cup, glasses, etc	

The amount of lead absorption is influenced by several factors: the routes of intake; the amount of element in the specific media; the physiochemical characteristics of lead compounds; and specific host factors such as age, sex, physical condition, and dietary deficiencies.

Various factors can influence the degree of ingested lead absorption through the transportation across the gut. Organic lead compounds are readily absorbed from the gastrointestinal tract (>90%) while inorganic lead compounds are poorly absorbed. Gastrointestinal lead absorption is higher in infant and decreases inversely proportion of age. The amount of inorganic lead absorption is about 40-50% in infant and children (Alexander et al., 1974; Zieler et al., 1978) and about 5-15% in adult (Kehoe, 1964; Rabinowitz et al., 1976). Several dietary factors can affect the level of lead absorption. Dietery deficiencies of copper, zinc, calcium, iron and protein, and increase in dietary

fat cause an increase in absorption of lead (Mahaffey, 1981). Most of the ingested lead pass directly to the faeces, to be excreted with no consequence for the human metabolism.

Lead absorption by the lungs also depends on a number of factors in addition to concentration. These include volume of air respired per day and the particle size of lead. A fine aerosol of inorganic lead salts are in a form that is readily inhaled into alveolar region of the lung (about 90%). Only a part of inhaled lead is deposited, the remainder being exhaled. About 40-50% of retained lead are absorbed through aveoli into blood (Waldron, 1980).

Very little inorganic lead is absorbed through the skin but organic lead absorbed very well, as it is through lung and gut (Laug et al., 1948).

After absorption, lead is transported to the target organs and tissues by blood. Most of lead in blood is in the red blood cells, while small fractions remain in the plasma. Plasma ligands are not well defined, but it has been suggested that plasma may contain diffusible fractions of lead in equilibium with soft tissue or end organ binding sites for lead. Lead is distributed to form exchangeable compartments which are blood and soft tissues and a storage compartment that is the skeleton. Lead substitutes for calcium in bone and becomes incorporated into the hydroxyapatite crystal. Nearly 90% of the human total body burden of lead is found in bone. Most of the remaining are found in kidneys and liver and, in decreasing order, in other soft tissues such as pancreas, lung, spleen, heart, muscular and brain. Inorganic lead does not normally cross blood–brain barrier but organic lead does. An age-related increase in tissue lead levels is seen only in the bone and aorta, while in many soft tissue lead concentration decreases with age.

Finally, once lead is absorbed, it is very slowly excreted. Absorbed lead is excreted primary in urine (75%) and faeces (16%). Other excretory pathway are gastrointestinal secretions, hair, nails and sweat. Lead also finds its way out of the body in milk.

1.4. 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 2). Lead has toxicity at low doses towards neurobehavioral 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 people occupationally exposed to lead, blood levels were much higher (table 3).

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

Table 2 : 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

Table 3 : Average blood lead levels in different population groups surveyed in Bangkok between 1985 and 1991.

Population groups	Average blood lead level (μg/dl)
General adult population in Bangkok	16.2-19.6
General adult population in the rural areas	8.6
Children up to 12 years of age in Bangkok	11.2-18.8
Children up to 12 years of age outside the municipality in the rural areas	4.4- 8.2
Traffic policemen in Bangkok	14.8-25.7
Bus drivers in Bangkok	11.8-21.4

Source : The office of occupational and environmental medicine, Department of Medicine, Ministry of public health.

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. The distribution of lead in peripheral blood

A comparison of the data derived from animal and human studies lead to a three-compartment model of lead distribution in the body including : blood, soft tissue and bone tissue. In human, blood is a compartment with a rapid exchange of lead. Lead in human blood have a mean life of 36±5 days (Rabinowitz, Wetherill, and Kopple, 1973, 1976). Blood lead concentrations are dependent upon the dose absorbed, the route of entry into the body and the time elapsed since exposure (Castellino, 1995).

2.1. Lead in Human Erythrocytes

The lead present in peripheral blood is mainly bound to the erythrocytes (94-99%) with a small fraction retained in plasma (1-6%) and this distribution ratio (erythrocytes/plasma) varies according to the dose and the time of lead exposure (Barltrop and Smith, 1975; Ong and Lee, 1980; Desilva, 1981; Manton and Cook, 1984; Barton, 1989).

Most of lead in human erythrocytes is in cytoplasm with only a small percentage in the membrane fraction (Bruenger, Stevens, and Stover, 1973; Barton, 1989; Sugawara et al., 1990). Within cytoplasm, lead bind mainly to hemoglobin (Hb) (Baltrop and Smith, 1971; Bruenger et al., 1973; Raghavan, Culver, and Gonick, 1980) but also to lower molecular weight cytoplasmic components (Kaplan et al.,1975; Ong and lee, 1980). Lead binds to Hb with a stable link and it can only be released under certain favorable condition such as with pH or temperature variations (Allison and Cecil, 1958; Baltrop and Smith, 1975; Ong and lee, 1980a; Barton, 1989; Sugawara et al., 1990). In contrast, lead links to the external surface of erythrocyte membrane with a weak link and it can be detached by EDTA (Fabri and Castellino, 1995).

The type of link formed inside erythrocytes is considerable toxicological significance since only free or easily detachable blood lead is available for transfer to other organs where it can carry out its toxic effect. The interaction of lead with cytosolic proteins in erythrocytes may represent a fundamental mechanism by which lead exerts toxicity. Most of lead in the erythrocytes bind to cytosolic lead-binding protein and small amount of the intracellular lead is free (Raghavan, Culver, and Gonick, 1980; Simons, 1986a; Sugawara et al., 1990). Excess intracellular lead bind not only to Hb, but also to the enzymes, with consequent supression of their activities (Mark, 1985; Goering, 1993; Wetmur,1994; Bergdahl et al., 1997). Metallothionein, low molecular weight protein, is another major lead-binding protein found in erythrocytes of lead exposed workers. This protein may act to sequester excess lead into a nonbioavailable form and hence protects against lead toxicity (Lolin and O'Gorman, 1988; Church et al., 1993).

The erythrocyte membrane rapidly accumulate 10-20% of total erythrocytic lead (Bruenger et al., 1973; Kaplan et al.,1975; Ong and lee, 1980a; Barton, 1989), and that there are some membrane groups with which lead links and from which it can be transferred to the inside of cells. Some lead is bound to the abundant phosphate groups of phospholipid on the cell surface (~2%) (Bruenger et al., 1973), while another part is linked to sulfhydryl groups (~2.5%) (Barton, 1989) and carboxyl groups (~9.5%) (Ong and lee, 1980a). Thus, phosphate, sulfhydryl and perhaps carboxyl groups are probably important for lead binding and/or uptake by erythrocytes.

2.2. Lead Uptake in Human Red Blood Cells

Lead can enter through erythrocyte membrane by passive transport according to a concentration gradient, whereas an active pump is needed for the reverse process of lead elimination from erythrocytes (Simons, 1984, 1986a, 1993). Most of lead (>90%) rapidly move across the erythrocyte membrane via. the anion exchanger (Simons, 1986a, 1986b). The abundance of the anion channel (~1 million/cell) in these cells may explain why erythrocytes quickly accumulate lead after ingestion.

Previous studies have also shown that erythrocyte anion exchanger is the main route of the uptake of zinc, probably in the form of $[Zn(HCO_3)_2Cl]^-$ anionic complex (Alda and Garay, 1989) and copper, probably in the form of $[Cu(OH_2)Cl]^-$ or $[Cu(OH_2)HCO_3]^-$ anionic complex (Alda and Garay, 1990). Therefore, the anion carrier may play an important role for the uptake of the elements able to give complex with bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), and/or chloride (Cl^-) insignificant amounts under physiological condition. However, it is not clear how this transport mechanism allow a cation such as lead (Pb^{2+}) to enter the cells. It is probably that Pb^{2+} pairs with a carbonate ion and enter erythrocytes in the form of $PbCO_3$. Exchange of $PbCO_3$ for a monovalent anion may be the best explanation for Pb^{2+} transport into erythrocytes, further experiments are needed. According to this hypothesis, $PbCO_3$ by itself exchanges with an anion (Figure 1). Another hypothesis is $PbCO_3$ form a ternary complex with another anion, and this anion complex is exchanged with an anion, such as Cl^- (Simons, 1986b). Since anion transport inhibitor (DIDS) dose not completely block

lead uptake into human erythrocytes in serum, there may also be another lead influx pathway (Simons, 1993a).

Although lead is rapidly taken up into erythrocytes by anion exchanger, the cells will be expected to pump the metal out through the action of the Ca^{2+}-ATPase located on the plasma membrene (Baltrop and Smith, 1971; Simons, 1988,1993). The calcium pump is a primary active transport mechanism which utilizes the energy to transport calcium (Ca^{2+}) out of cell. Pb^{2+} can replace Ca^{2+} in stimulating the Ca^{2+}-ATPase from human erythrocyte membrane (Pfleger and Wolf, 1975) resulting in the extrusion of Pb^{2+} from the cells by the Ca^{2+} pump (Simons, 1984,1988).

However, in erythrocytes, lead levels remain relatively high. A likely explanation is that Pb^{2+}, once inside the cell, binds to intracellular constituents (Bruenger et al., 1973; Baltrop and Smith, 1971) and is unavailable to the Ca^{2+}-ATPase for extrusion. There are three proteins in erythrocytes including metallothionein (Church et al., 1993), ALAD (Wetmur, 1994), and hemoglobin (Bruenger et al., 1973) have been suggested to bind Pb^{2+} at exposed thiol groups.

Lead receptors may be different from those in other metals : for example, although calcium links with the same proteins in erythrocyte membrane (Ca^{2+}-ATPase), it does not interfere with lead uptake (Kaplan et al., 1975). On the contrary, by acting through the calcium pump (Simons, 1988) endocellular calcium impedes lead release.

Iron also interferes with lead kinetics, ferrous and ferric ions inhibit lead uptake in both *in vitro* human erythrocytes (Barton et al., 1978) and *in vitro* and *in vivo* rabbit erythrocytes (Kaplan et al., 1975). While lead can inhibits iron uptake in both *in vitro* and *in vivo* human reticulocytes (Jandl et al., 1960; Boyett and Butterworth, 1962) and *in vitro* and *in vivo* rabbit reticulocytes (Sroczynski and Piekarski, 1959; Qian and Morgan, 1990; Qian et al., 1997). The fact that reticulocytes unlike mature red blood cells (erythrocytes), have receptor for transferrins through which they take up iron. Since small quantities of lead can bind to plasma transferrin, this may serve as another route

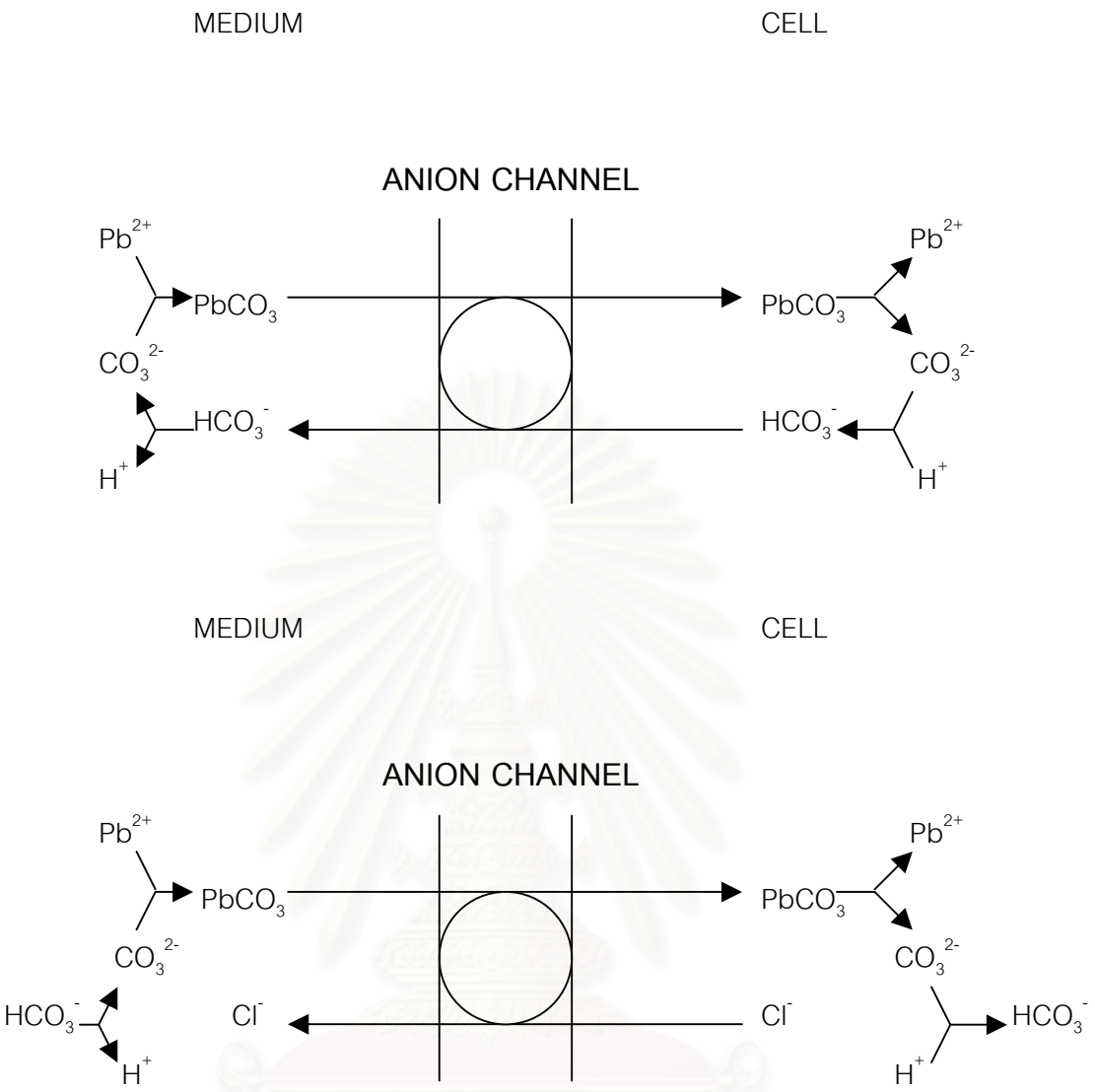

Figure 1 : Consequences of $PbCO_3$ formation and its exchange with internal anion (HCO_3^- or Cl^-). Net transport will proceed in the direction shown as long as a $PbCO_3$ gradient from medium to cell is maintained. Although only anions are transported across the anion channel, both exchange modes couple the transport of Pb^{2+} to an oppositely directed displacement of H^+ equivalents. (Modified from : Becker and Duhm,1978: 165).

of lead uptake, espacially in immature red blood cells (erythroid precursor cells), and may partly explain the toxicity of lead induced anemia.

2.3. Lead in Human Blood Plasma

The chemical state of lead in blood plasma and interstitial fluid is considerable interest. Because lead is transferred to these fluids after its absorption in the gut or lungs and whatever intermediate storage sites may be involved, is carried by them to its site of action or to kidneys for excretion. Relatively little is known of the distribution of lead in the plasma. More than 99% of lead in plasma binds to the proteins or components in the plasma and the remaining is the free inorganic ion (Al-Modhefer, Bradbury and Simons, 1991). The concentration of the free form is likely to a very small fraction of the total, but this concentration may be of special importance in determining the flux across membranes, binding and toxicity.

The plasma lead pool is assumed to be comprised of four possible states: as part of a metalloprotein, bound with high affinity and only slowly dissociating; lossely bound to serum proteins such as albumin and globulin; complexed to ligands of low molecular weight components such as amino acids and other carboxylic acid; and as the free inorganic ion (Pb^{2+}).

In the *in vitro* study by Ong and Lee (1980a, 1980b), it was found that lead distribution in the plasma by arbitrarily dividing the latter into albumin and globulin and most of lead (88%) was bound to the albumin while the remaining was bound to the globulin. Albumin binds as well as many other metal ions non-specifically (Gurd and Murrays, 1954). While, Al-Modhefer et al.(1991) found that about 60% of the binding of lead in serum bound to the thiol compounds, mainly cysteine and the remaining 40% appeared to be due to protein, maining albumin.

In addition, there are several specific metal-binding proteins in human serum which are capable of binding to lead including ceruloplasmin (Gercken and Barnes, 1991; Rosawan Srivoravit, 1996; Marasri Ujjin, 1999) and transferrin (Kochen and Greener, 1975; Suphitcha Mangkalee, 1994). In human blood serum, both copper and

lead can bind to copper carier protein named ceruloplasmin (Gercken and Barnes, 1991). Moreover, lead can replace copper in the molecule of ceruloplasmin resulting in the decrease of its oxidase activity (Rosawan Srivoravit, 1996). In addition, transferrin the iron carier protein has been reported to bind lead (Kochen and Greener, 1975) and lead also replaces iron in the molecule of transferrin (Suphitcha Mangkalee, 1994). Therefore, competition for mutual binding sites on transferrin can explain the inhibitory effect of iron on erythrocyte lead uptake. Furthermore, it is possible that this plasma protein may act as a "lead transporting protein" that carries lead to the target cells containing its receptor resulting in lead toxicity on the cells.

3. Transferrin (Simon, 1992; Harford et al., 1994; Ponka, 1997)

Transferrin (Tf) is the protein whose primary function is to bind iron and transport it through the blood. The body of an adult contains about 14 g of transferrin, of which approximately half is found in the blood plasma. Under normal physiological condition serum transferrin is about 30% saturated with iron. Human serum transferrin is a single polypeptide chain glycoprotein with a molecular weight about 80,000 which have the capacity to bind two atoms of ferric iron (Fe^{3+}) in association with the binding of an anion. Transferrin is the only physiologic source of iron for heme synthesis in erythroid cells.

The transferrin polypeptide has 679 amino acid. By weight it consists of 6% carbohydrate. The protein folds into two globular lobes, N-terminal and C-terminal lobe, joined by a short connecting sequence of amino acids. Each lobe contains a deep cleft which partially splits each lobe into two dissimilar regions. These regions are called domain I and domain II.The cleft between these two domains contains the amino acid residues whose side chains comprise the iron-binding sites within each lobe(Figure 2A). Binding of iron to transferrin is accompanied by binding of anion, physiologically carbonate or bicarbonate.The distinguishing feature of the transferrin is that a synergistic bicarbonate anion is bound along with the metal ion to form a stable Fe^{3+}- CO_3- Tf ternary complex.The protein's three-dimensional folding provides a pocket in which the metal- and anion-binding residues reside (Figure 2B). The anion is thought

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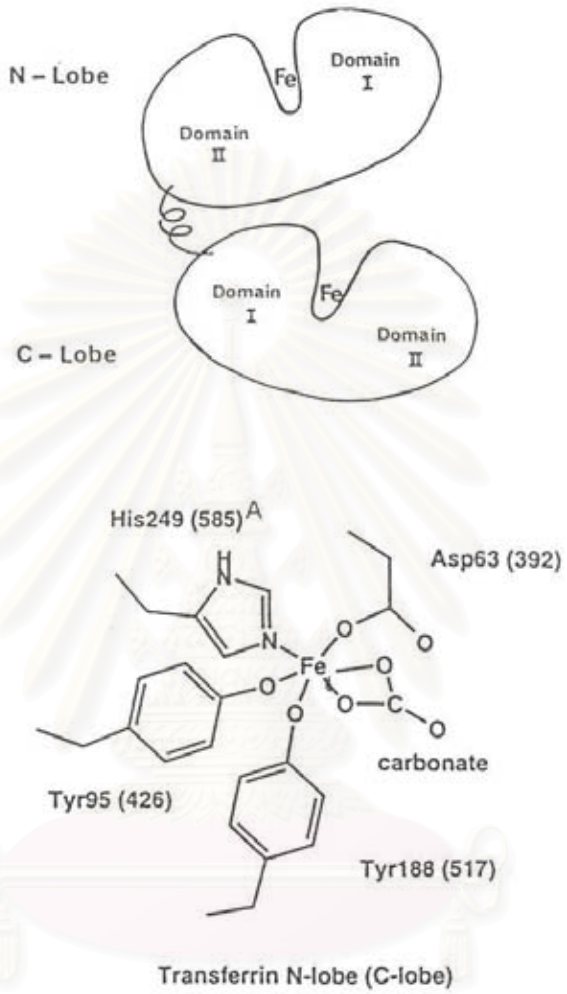

Figure 2 : The structure of human transferrin : A. The two lobes and the two domains within each lobe (separated by the iron-binding site) (Simon, 1992: 77). B. The proposed structure for the iron-binding sites of human transferrin, showing the amino acid residues thought to be involved and their positions within the polypeptide chain (residue numbers are for the N-lope with the C-lope in brackets) (Baker, 1994: 388).

to interact at arginine 124. Initial binding of the iron may occur through the three ligands located in domain II (two tyrosines, one histidine). Finally, to complete the process, the iron binds to the aspartic acid residue from domain I as this domain closes over the iron site. Conformational changes in the protein may be induced as a consequence of iron binding and formation of these interactions. Binding of iron is associated with the release of several protons.

Although transferrin has a very high affinity for ferric iron (Ka~10^{-20}M), its affinity for ferrous iron is much less. Release of ferric iron from transferrin occurs predominantly, if not exclusively, in the intracellular environment. At least five mechanisms have been proposed as participating in the release of ferric iron from the protein : (1) protonation of the protein's iron-binding ligands, (2) reduction of bound ferric iron to ferrous iron, (3) a primary attack on the anion, (4) competition between transferrin and a strong chelator, and (5) an influenence on iron binding by the binding of ferric transferrin to transferrin receptor.

Iron on transferrin molecule is required for Hb synthesis in erythroid precursor cells. The cells can take up iron via the transferrin receptor mediated endocytosis. Transferrin receptors (TfR) on the outer surface of the plasma membrane bind diferric-Tf with high affinity. The TfR is depicted as a disulfide linked homodimer with two molecules of diferric-Tf bound. One Tf molecule can bind to each subunit of the receptor. Binding of Tf to the receptor is a simple physiochemical process independent of cell metabolism. However, near 37^{o}C, the transferrin-iron-receptor complex is rapidly endocytosed by cells. At physiological pH (7.4) the affinity of the receptor for transferrin depends upon the degree of iron binding. The transferrin-iron-receptor complex is clustered and localized in chathrin-coated pit, which eventually bud off to form coated vesicle, variously called endosome or endocytic vesicle. After 15 to 45 minutes, the evolving endosome fuses with a lysosome, thereby delivering the internalized contents for hydrolytic degradation. Hence, endosome is acidified to pH about 5-6 through the action of ATP-dependent proton pump. Endosomal acidification weakens binding of iron to Tf and produces conformational changes in both Tf and TfR, strengthening their

association. Iron release may also be facilitated by a plasma membrane oxidoreductase.

After iron is released from Tf, it possible transport across endosomal membrane by the transmembrane transport system (by a carrier or channel). The apo-Tf-TfR complex is recycled back to the plasma membrane, where apo-Tf is discharged, thereby completing an elegant and efficient cycle (Figure 3). Iron, after it release from endosome, very likely enters the labile intermediate pool from which it is available for mitochondrial heme synthesis, for the insertion into iron-dependent proteins and enzymes, and for storage in ferritin.

Transferrin has also the capacity to bind a variety of metal ions in addition to the ferric ion (Fe^{3+}). Many studies have reported that transferrin binds a large number of divalent, trivalent and tetravalent metal ions including cadmium (Cd^{2+}) (Harris and Madsen, 1988), copper (Cu^{2+}) (Zweier and Aisen, 1977), chromium (Cr^{2+}) (Tan and Woodworth, 1969), manganese (Mn^{2+}) (Chua et al., 1996), nickel (Ni^{2+}) (Harris, 1986), zinc (Zn^{2+}) (Moutafchiev and Sirakov, 1998), aluminum (Al^{3+}) (Martin et al., 1987 ; Aramini, Saponja, and Vogel, 1996), gadolinium (Gd^{3+}) (Zak and Aisen, 1988), terbium (Tb^{3+}) (Meares and Ledbetter, 1977), plutonium (Pu^{4+}) (Duffield et al., 1984), etc.

Lead is another metal that is able to bind Tf. Since TfRs are prefusely present in erythroid precursor cells at all stage (300-800,000 per cell) and its density gradually declines with erythroid precursor cell maturation and after the reticulocyte stage, they appear to be shed off as small lipid vesicles (Sherke and Huh, 1992). It is possible that transferrin may be responsible for lead transport into the cells possessing TfR such as erythroid precursor cells.

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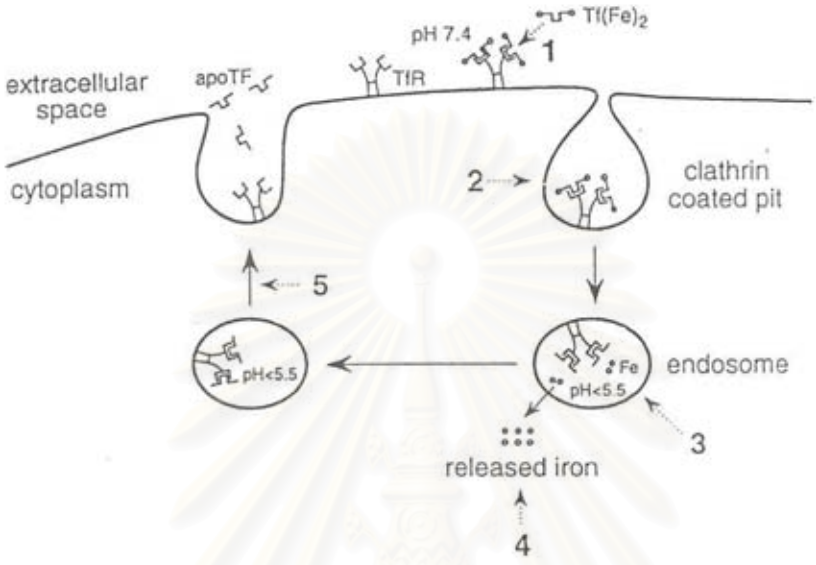

Figure 3 : Fe-transferrin cycle. (1) Transferrin attaches to specific receptors on the cell surface. (2) By a temperature- and energy-dependent process, the transferrin-receptor complexes are internalized by the cells enclosed within endocytic vesicles. (3) Iron is released from the transferrin within the endocytic vesicles by a temperature- and energy-dependent process which involves endosomal acidification. (4) Iron is transported to intracellular sites of utilization and/or storage in ferritin. Only in erythroid cells does evidence exist for a specific targeting of iron toward mitochondria. (5) The iron-free apotransferrin, which remains attached to the receptor at pH~5.5, returns to the cell surface, where the apotransferrin is released from the cells. (Ponka, 1997: 8)

4. Erythropoiesis

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** (Figure 4). 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 5). 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).

4.1. Erythroid Progenitor Cells (Erslev, 1997; Dessypris, 1998)

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

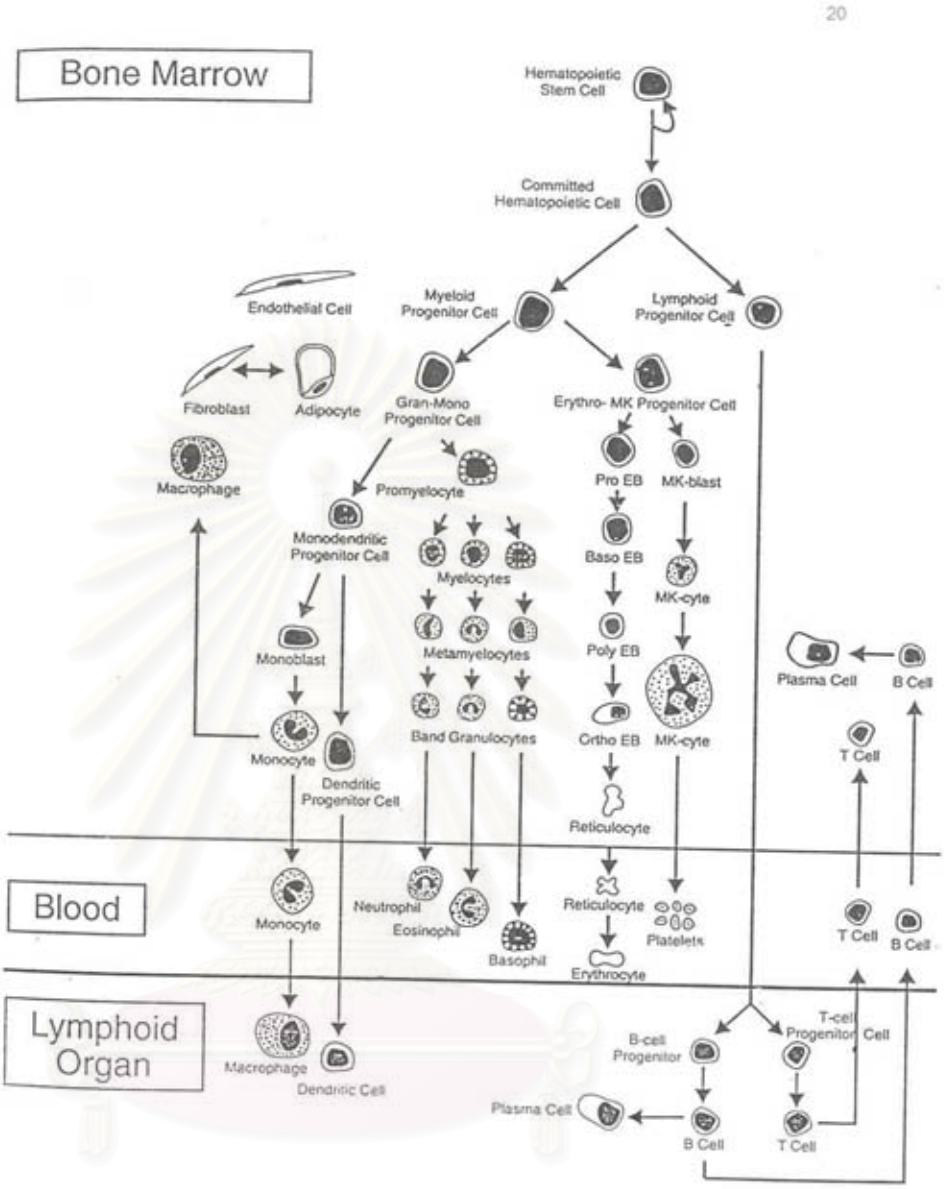

Figure 4 : The hematopoietic system. The processes involved in production of all the various cells of the blood (Bondurant and Koury,1998: 147).

BONE MARROW

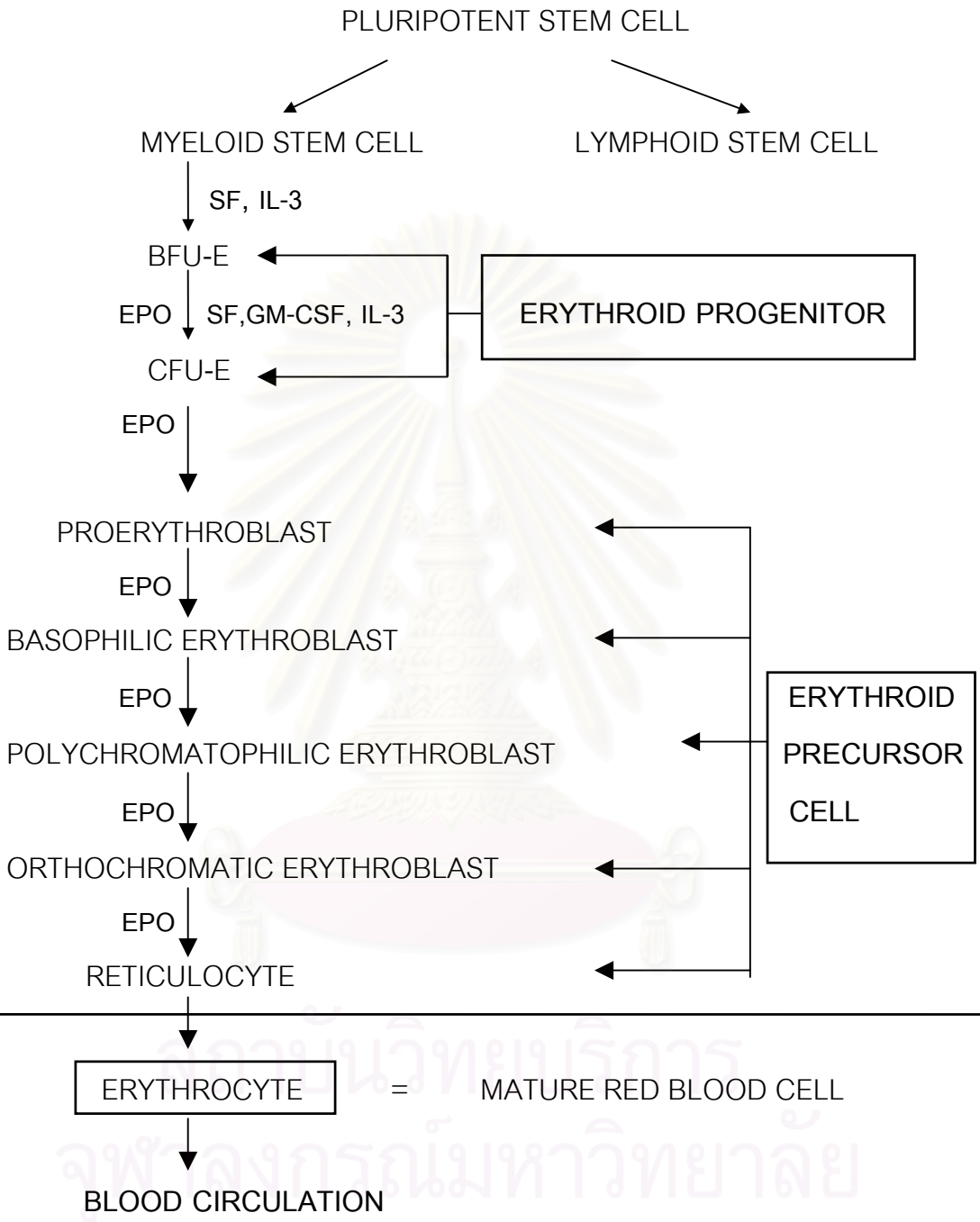

Figure 5 : Red blood cell development.

whichtime it only produces a small colony consisting of between 30 and 60 nucleated red cells.

The early stages of BFU-E proliferation and differentiation are EPO-independent, but depend on interleukin-3 (IL-3) and steel factor (SF), and dependence on EPO develops at a stage between BEU-E and CFU-E. BEU-Es give rise in 6 to 7 days to CFU-Es in response to the combination of EPO and other growth factors such as SF, granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-3. BFU-Es are found in bone marrow and also detected in the peripheral blood at a concentration of 0.02 to 0.05% of light-density(<1.077) mononuclear blood cells.

The CFU-E is a rapidly dividing cell which is highly responsive to small concentration of EPO and give rise in 5 to 8 days in human bone marrow to colonies of hemoglobinized erythroblasts. The CFU-E is the most EPO-sensitive cell, carries the highest density of EPO receptors on its surface, and is absolutely dependent on EPO for its survival.

4.2. Erythroid Precursor Cells (Hunter, 1993; Bull and Gorius, 1995; Bondurant and Koury, 1998)

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. 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.

Stages of erythroid precursor cell maturation

Proerythroblast

Proerythroblast is a large cell with a diameter of 20-25 μm and irregularly rounded or slightly oval. The nucleus occupies approximately 80% of its area and contains fine chromatin delicately distributed in small clumps. One or several nucleoli are present. Polyribosomes arranged in group of 2 to 6 are numerous in the cytoplasm that gives the cytoplasm of these cells its characteristic intense basophilia. At this stage only small amounts of hemoglobin are present.

Basophilic erythroblast

Basophilic erythroblast is similar to the proerythroblast except that the nucleoli are no longer visible and the cell is smaller (16-18 μm in diameter). The nucleus is round and occupies ¾ of the cell area. Condensation of chromatin begins in this stage. The nuclear structure may assume a wheel-spoke arrangement. The ribosomes reach their maximum number during this stage, and as a consequent the cytoplasm is deeply basophilic.

Polychromatophilic erythroblast

Cells at this stage are smaller (12-15 μm in diameter). The nucleus is round and occupies less than ½ of the cell area. Increasing condensation of nuclear chromatin is observed. Irregular mass of chromatin are formed, which may stain very deeply. The cytoplasm changes from blue to pink as hemoglobin dilutes the polyribosome content. The maximum number of mitochondria is found in the early phases of this stage, but as hemoglobin become more plentiful, mitochondria decreases in number.

Orthochromatic erythroblast

This cell is the smallest of the erythroblast series (10-15 μm in diameter). The nucleus remains round and occupies approximately ¼ of the cell area and is eccentric. The cytoplasm has almost its full complement of hemoglobin. The cell stains slightly different from a mature erythrocyte. However, because of the residual mono and polyribosomes it is always show a grayish tinge of polychromatophilia. In this stage, the nucleus undergoes pyknotic degeneration, the chromatin becomes greatly condensed, and the nucleus shrinks. Finally the nucleus is extruded.

Reticulocyte

After the nucleus has been extruded, the cell is known as a reticulocyte. The cell is larger than mature erythrocyte, perhaps 20% greater in volume. This cell contains certain cytoplasmic organelles such as ribosomes, mitochondria, and golgi complex. Supravital staining with brilliant cresyl blue or new methylene blue produces aggregates of ribosomes and other cytoplasmic organelles.These artifactual aggregates stain deep blue and, arranged in reticular strands, give the reticulocytes its name.

During maturation, the specific surface markers, namely transferrin receptor (CD 71) and glycophorin A are changed (Figure 6). Transferrin receptor begins to be expressed at the BFU-E stage and disappeared at the late reticulocyte stage, while glycophorin A begins to be expressed on morphologically recognizable erythroid precursors just after the CFU-E stage. In contrast to transferrin receptor, which is progressively lost during the maturation process, glycophorin A has been maximally expressed on the cell surface, it remains at constant quantities to the mature erythrocyte stage (Loken et al., 1987).

Two events may decrease the theoretic yield of erythroid cells. One of these is the death of the cell before or shortly after its release from the marrow (ineffective erythropoiesis). The second is a skipped cell division, a phenomenon that results in a large hemoglobin-poor cell. Hemoglobin synthesis occurs in early erythroid precursor cell and successively increased in concentration with increasing maturation (Table 4).

Hemoglobin synthesis continues throughout the orthochromatic stage and persists at a very low rate in the reticulocyte after denucleation. In contrast, DNA and RNA synthesis decrease with increasing maturation. By the orthochromatic stage, the nucleus is completely inactive, unable to synthesize either DNA or RNA (Figure 7). Mature red blood cells, lacking ribosomes, cannot synthesize hemoglobin.

Table 4 : The hemoglobin content and lifespan of human erythroid precursor Cells. (อานนท์ บุณยะรัตเวช, 2535: 31)

Erythroid stage	Life span (hr.)	Hb content (pg/cell)
Proerythroblast	30	0-14.4
Basophilic erythroblast	12.4-95.0	7.2-25.2
Polychromatophilic erythroblast	8.8-3.7	12.6-27.0
Orthochromatic erythroblast	19	13.5-24.5
Reticulocyte	72	24.5-29.5

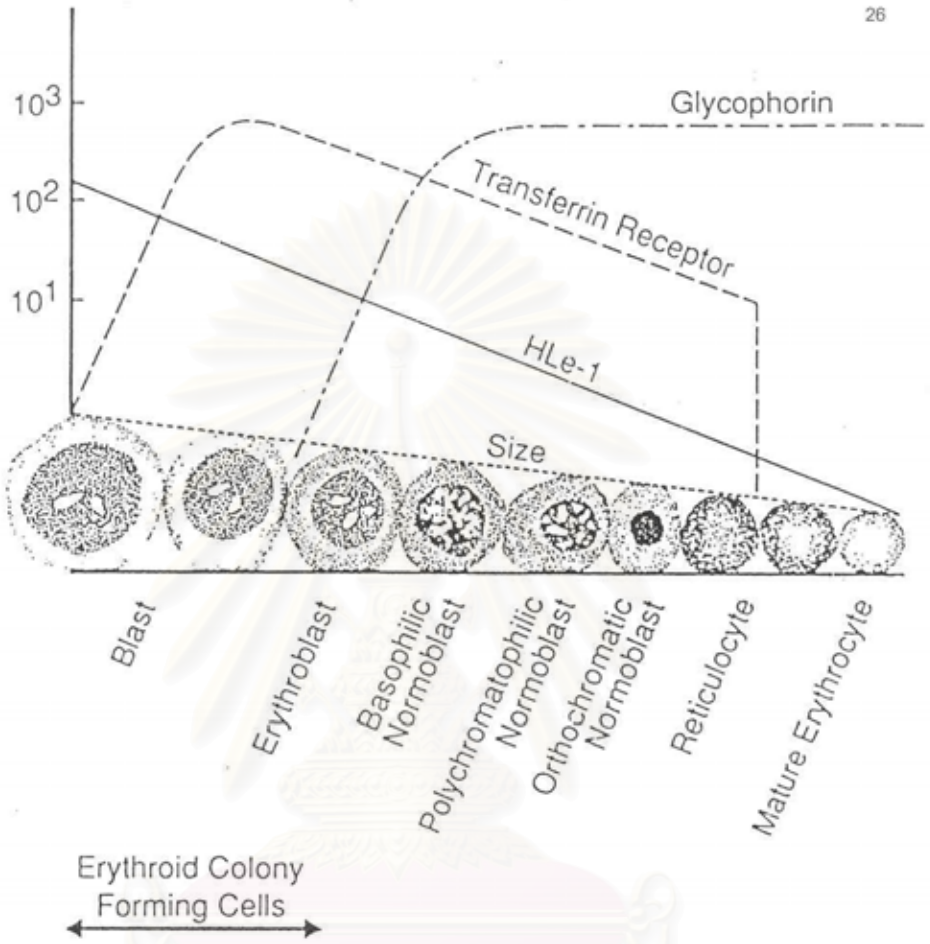

Figure 6 : **Schematic representation summarizing the characteristics of erythroid cells as they mature.** The relative quantities of the cell surface antigens are depicted in the upper portion of the diagram. These antigenic characteristics can be correlated with cell size, morphology, nucleic acid content, and erythroid colony-forming ability (Loken et al., 1987: 261).

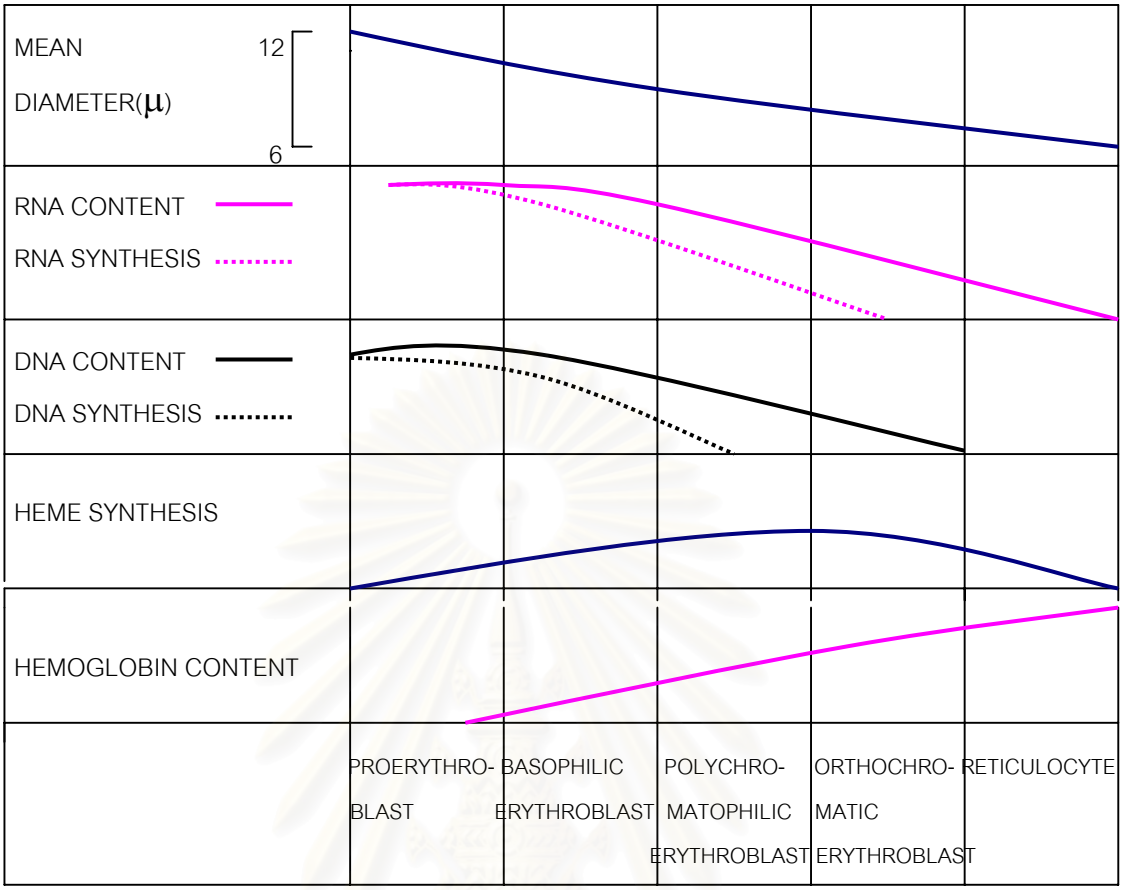

Figure 7: Schematic representation of DNA, RNA, and hemoglobin during erythroblastic maturation (Miwa et al.,1981)

5. Lead and Erythropoietic system

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.

5.1. 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 8). 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 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

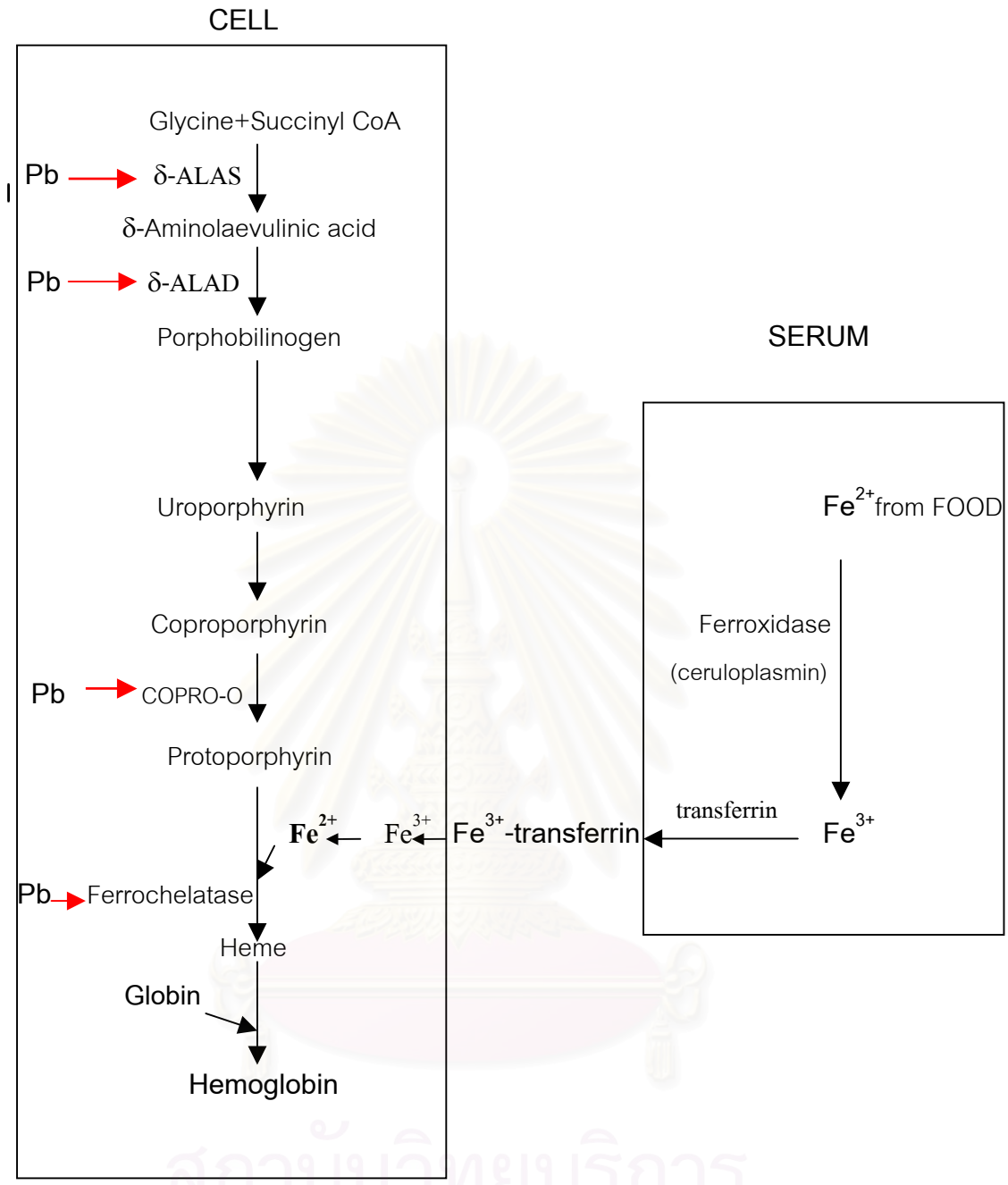

Figure 8 : Scheme of heme synthesis showing sites where lead has an effect

Red arrow (→) indicates those enzymes blocked by lead (Goldfrank, 1990; Goyer,1996).

leucine into globin chains. A markered decrease occurs in the synthesis of α chain than of β chain.

5.2. 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).

5.3. 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.

6. Apoptosis

6.1. 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 9). 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.

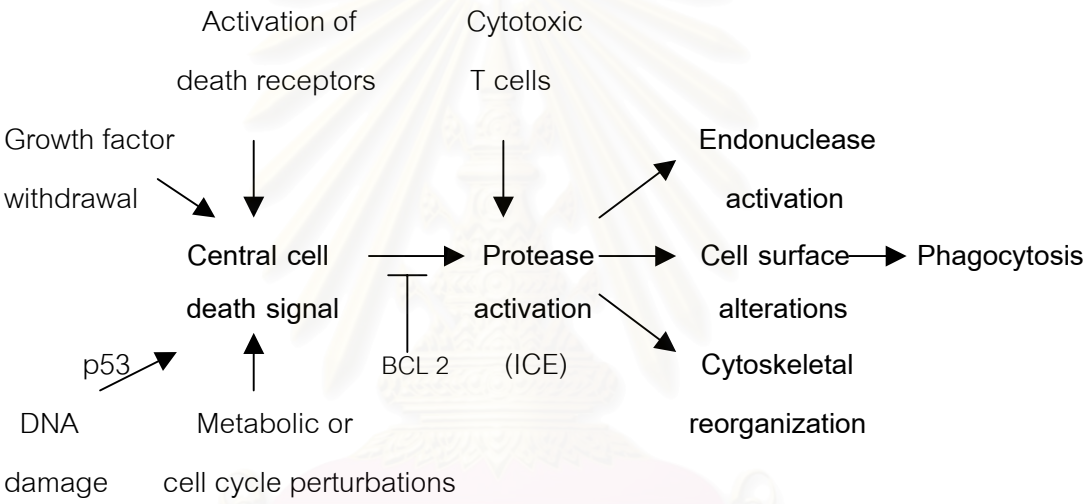

Figure 9: A hypothetical model for the regulation of apoptotic cell death. As diagrammed, the major end point of apoptotic cell death is removal of the dying cell by phagocytosis. Both the death repressor BCL 2 and ICE(interleukin converting enzyme) are members of larger gene families (Thompson, 1995:1457).

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 (figure 10).

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

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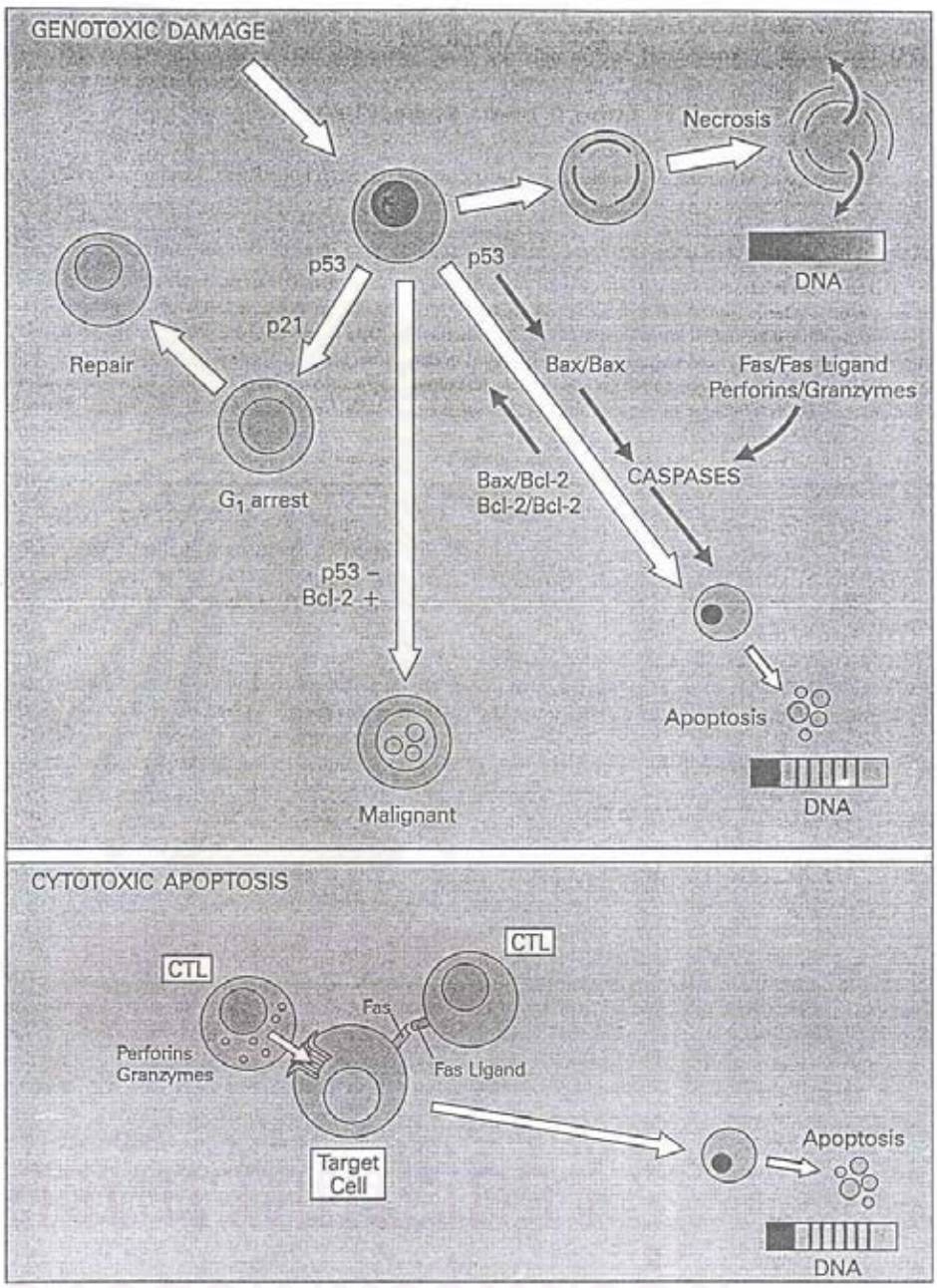

Figure 10 : Pathways to apoptosis (Israels and Israels,1999: 307)

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.

6.2. 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).

6.3. 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 OF THESIS

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 uptake and 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. However, it is not clear about the mechanism of lead transport into human erythroid precursor cells. Since lead can replace iron on transferrin, the iron transport protein, this protein may be responsible for lead transport to the organs possessing its receptor. Moreover all stages of erythroid precursor cells possess transferrin receptors on their plasma membrane, it is then possible that transferrin may be an important factor for lead transport into human erythroid precursor cells. Therefore the second aim of this research is to study lead transport mechanism in these cells with the emphasis on transferrin. This may lead to a better understanding on the mechanism of lead uptake in human erythroid precursor cells.

Finally, the third 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 Uptake in Human Erythroid Precursor Cells

2.1. The Role of Transferrin on Lead Transport

To investigate the role of transferrin on lead transport, lead content in the cells preincubated in the buffer containing lead acetate with or without transferrin was measured by the standard addition technique with a graphite-furnace atomic absorption spectrometry (GFAAS). The GFAAS is a suitable technique for determining low lead content sample with high accuracy and sensitivity. The technique requires a small volume of the solution, using an elctrothermally heated graphite tube to atomize the metal. After atomization, the lead atoms then absorbed the light emitted a lead cathode lamp. The degree of light absorption depended on the amount of lead present in graphite tube.

2.2. Effect of Microtubule Inhibitors

Two microtubule inhibitors (colchicine and vinblastine) which are capable of preventing transferrin endocytosis were chosen for studying the mechanism of lead uptake via. transferrin receptor-mediated endocytosis. Lead content in the cells preincubated in the buffer containing lead acetate and transferrin with or without the inhibitors was determined by GFAAS.

2.3.Effect of Anion Transport Inhibitor

Since, lead is known to move across the erythrocyte membrane through the anion exchanger system (Simons, 1986b), this mechanism may be involved in lead transport into erythroid precursor cells which possess the anion exchange proteins. The anion transport inhibitor (DIDS) which is capable of inhibiting lead uptake in erythrocytes was chosen for studying the involvement of this system to lead uptake. Lead content in the cells preincubated in the buffer containing lead acetate with or without anion transport inhibitor was determined by GFAAS.

3. 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.

3.1. 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.

3.2. 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 transport and toxicity.

2. A better understanding on the mechanism of lead uptake in human erythroid precursor cells leading to acknowledge the mechanism of lead toxicity in these cells.
3. 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
4. The knowledge from this research may further give a clear picture on lead induced anemia.

CHAPTER II

LITERATURE REVIEW

1. Lead Uptake in Human Red Blood Cells

The study of lead uptake in human red blood cells have only been studied *in vitro* and mainly in mature red blood cells (erythrocytes), only little have been done in immature red blood cells (erythroid cells).

Many observations indicate that the rate of lead uptake in human erythrocytes is dependent on some factors in blood plasma.

The uptake of lead into human erythrocytes has been studied by Simons (1986a, 1986b), using lead buffers, he found that more than 90% of lead uptake occurred by the anion exchanger system and the transport was strongly stimulated by bicarbonate ion (HCO_3^-). In the presence of HCO_3^-, the rate was stimulated in the order $ClO_4^-<NO_3^-$ and $CH_3CO_2^-<F^-<Cl^-<Br^-<I^-$. Exchange of $PbCO_3$ for a monovalent anion best fits the experimental data, although transport of a ternary $PbCO_3$-anion$^-$ complex is a possibility.

One can conclude from the above finding that, HCO_3^- and the second anions are the important factors in plasma for lead transport into human erythrocytes. However, anion exchanger system is not only the route of lead uptake in human erythrocytes.

From the study of Simons (1993a) in human erythrocytes, it was found that DIDS, the anion transport inhibitor, did not completely block ^{203}Pb uptake for fresh erythrocytes in serum. This result suggests that there may existing another lead influx pathway. Small lead influxes and effluxes were seen in earlier work in the presence of DIDS (Simons,1986a).

Furthermore, there are some metal carrier proteins in human plasma which are able to bind lead such as ceruloplasmin (copper carrier protein) and transferrin (iron carrier protein). These proteins may also serve as other factors for lead transport.

Rosawan Srivoravit (1996) studied in human blood serum and found that besides copper, ceruloplasmin could bind lead resulting in copper release and the decrease of its oxidase activity. Copper was released from serum protein upon lead binding and the ratio of 0.31 atom of lead bound to 1 atom of copper released.

Suphicha Mangkalee (1994) also reported that lead was observed to decrease the concentration of protein-bound iron in human serum. Higher concentration of lead cannot completely replace iron, since the decrease in mole ratio of Fe:Tf molecule is from 1.73:1 to 0.56:1. This finding also suggests that transferrin may involve in lead transport.

Besides lead, transferrin has been shown to transport several metals in serum. It is also an important carrier of zinc (Zn^{2+}) (Evans and Winter, 1975), manganese (Mn^{2+}) (Chua et al., 1996), americium (Am^{3+}) (Bruenger et al., 1969), aluminum (Al^{3+}) (Rahman et al., 1985) and plutonium (Pu^{4+}) (Duffield et al., 1984).

From the study of Chua et al. (1996) in rabbit reticulocytes and erythrocytes showed that the uptake of transferrin-bound manganese occurred only with reticulocytes and depended on receptor-mediated endocytosis of Mn-Tf.

Therefore, it is possible that transferrin may also be responsible for lead transport into erythroid precursor cells through transferrin receptor-mediated endocytosis. The competition between lead and iron for transferrin molecule may decreases the iron uptake into the cells.

2. Effect of Lead on Transferrin Function

Lead has also been shown to inhibit the uptake of iron into erythroid cells (Jandl et al., 1959; Allen and Jandl, 1960; Boyett and Butterworth, 1960; Lothe and Falbe-Hansen, 1963) and its toxicity may be involved with transferrin function (Kohno, Taketani, and Tokonaga, 1993; Quian et al., 1997).

Quian et al. (1997) reported that the inhibitory effect of lead on iron uptake in rabbit reticulocytes might occur in intracellular process rather than in membrane binding step probably inhibiting translocation of iron across the endosomal membrene.

Kohno et al. (1993) also found that iron uptake in human erythroleukemia (K562) cells was inhibited by lead and correlated with a decrease concentration of surface transferrin receptors with no change in receptor binding affinity. Since the biosynthesis of the receptor was unaffected by lead treatment, the down-regulation of surface transferrin receptors in lead-treated cells might be due to a redistribution rather than an actual loss of receptors from the cells.

Furthermore, Cark et al. (1988) observed that microcytic anemia in children with lead poisoning might result from a coexisting iron deficiency. In a study of 75 children with lead poisoning, microcytic anemia was present only in children which there was a coexisting iron deficiency as indicated by a transferrin saturation <16%.

In addition, Mahaffey (1981) and Barton et al. (1978) reported that the intestinal absorption of ingested lead was greater in iron deficient subjects and these appeared to be a competition between iron and lead for intestinal absorbance.

Although the mechanisms for intestinal absorption of iron and lead are not well understood, evidence indicates that transferrin and its receptor may be directly or indirectly involved.

From the study of Anderson et al. (1990), it was shown that intestinal transferrin receptor levels vary inversely with body iron stores and directly with changes in iron absorption in adult rats.

These observations may indicate that lead uptake and toxicity may be involved with transferrin function. It is possible that transferrin may transport lead instead of iron into the cell, leading to the reduction of iron uptake. The inhibitory effect of lead on iron uptake may be another explanation for lead impairs heme synthesis besides the inhibition on heme-synthesizing enzymes. If so, transferrin serves as a key clue to introduce lead toxicity on the cell or organ possessing its receptor.

3. Effect of Lead on Human Red Blood Cells

It has long been known that lead apparently interferes with the normal production of human red blood cells, probably through a combination of various mechanisms. Hb synthesis is markedly abnormal in patients with lead poisoning. There are many reports indicate that several enzymes of heme synthesis are inhibited by lead. In addition, lead also impairs globin synthesis and induce quantitative alteration in the synthesis of globin subunits

In 1972, White and Harvey observed a fall in the α-chain synthesis compared to that of β-chains, with a concomitant drop in the α-β ratio, in lead poisoning patients.

In 1977, Ali and Quinlan studied in reticulocyte-enriched cell of patients who have elevated reticulocytes with normoblastic erythropoiesis and found out that lead had an inhibitory effect on globin synthesis at a concentration of 20 μg/dl and this effect was dose-dependent.

In 1980, Telisman, Kersanc, and Prpic-Majic studied in occupational exposure to inorganic lead, and found out that ALA-D was the first enzyme on heme synthesis to be affected by lead (even at blood lead level 10-20 μg/dl). It became apparent before Hb was seen to be reduced.

In 1992, the study in rabbit by Zereba and Chmielnicka showed a significant inhibition of ALA-D activity in blood, bone marrow and liver by lead.

The observation of Rossi, Taketani, and Garcia-Wedd (1993) in lead exposure workers was the increase of urinary coproporphyrin excretion and the accumulation of zinc protoporphyrin in red blood cells. The study of the same group demonstrated that the accumulation of these metabolites resulted from lead inhibition of the two mitochondrial enzymes of heme synthesis, coproporphyrinogen oxidase (COPRO-O) and ferrochelatase.

Hernfandez, Gutiferrez-Ruiz, and Garcifa-Vargas (1998) studied in HepG6 cells, human hepatoma cell line, and found that lead caused COPRO-O inhibition in the cells.

Due to the difficulty and danger caused by the aspiration of erythroid precursor cells (Hb-synthetic cells) from human bone marrow, the studies of lead effect on Hb synthesis in human are always performed in human erythrocytes or reticulocytes from peripheral blood of the patients having high reticulocytes elevation. Many observations and experiments on lead toxicity in the Hb synthetic cells have also performed in the animal bone marrow or hematopoietic cell line.

In 1978, Kusell, O'Cheskey, and Gerschenson studied the effect of lead on cultured cell proliferation in rat liver cell line (RLC-GAI). They found that lead reversibly inhibited the growth of these cells after 6 days of exposure to the metal. In addition, Lake and Gerschenson (1978) showed that heme synthesis in RLC-GAI was also inhibited by lead in a dose- dependent manner.

In 1984, Lutton et al. studied the toxic effect of lead on rat bone marrow cells, the result indicated that lead acetate (at the concentration of 0.1-100 μM) caused the decrease in CFU-E number of rat bone marrow cells after *in vitro* exposure.

In 1985, Taketani et al. studied the effect of lead on the differentiation of Friend leukemia cell line and rat bone marrow cells. They found that lead induced a lag in onset of maturation and decreased ALA-D activity. In Friend leukemia cell line, the percentage

of Hb containing cells decreased slightly at lead concentration of 500 μM while the lower concentration of lead (100 μM) could lyse most of the cells in rat bone marrow cells.

Since the previous studies in animal and cell line indicate that lead can affect on the development of Hb synthetic cells, it is possible that lead may affect on human erythroid differentiation and proliferation.

In 1998, Boucher et al. studied in human erythroid progenitor cells and found that lead acetate (10^2—10^3 μM) disturbed *in vitro* human BFU-E/CFU-E proliferation and differentiation.

Recent study (1999) of Van Den Heuvel, Leppens, and Schoeters in erythroid progenitor cells (BFU-Es) from human umbilical cord blood and murine bone marrow showed that *in vitro* lead exposure caused a dose-dependent depression of erythroid and myeloid colony numbers. However, lead influenced erythroid cells to a greater extent than myeloid cells. In addition, lead was 10-15 times more toxic to human hematopoietic cells than to murine bone marrow cells.

In addition, from the *in vivo* study of Osterode, Barnas, and Geissler (1999) in the patients with high blood lead level (0.796-4.4 μM). It was shown that in subjects exposed to lead with blood lead level ≥2.896 μM, BFU-Es were significantly reduced and the reduction was dose dependent.

From *in vitro* and *in vivo* studies in human indicate that lead can affect on proliferation and differentiation of human erythroid progenitor cells. Since erythroid precursor cells, the Hb synthetic cells, develop from erythroid progenitor cells, it is possible that lead may also affect the human erythroid precursor cell proliferation and maturation. Abnormal or inadequate maturation of these cells may be a sequence leading to the anemia in lead—intoxicated patients.

Although apoptosis is a morphologically distinct form of programmed cell death that normally plays a major role during cell development, many reports have shown that lead can induce apoptosis in various cells of animal and human.

In 1997, Scortegana and Hanbauer studied in rat embryonic mesencephalic primary cultures contained neuronal and glia cells, and found that in serum free medium, Pb^{+2} elicited mainly necrosis and apoptosis in maximally 13% of the cells in culture.

In 1997, Fox, Campbell, and Blocker observed the exhibition of rod and bipolar apoptotic cell death in the retina of developing and adult rats exposed to lead.

In 1999, Ruzittu et al. found that after the *in vivo* administration of lead nitrate, the rat sinusoidal liver cells were very active in internalizing apoptosis.

In 1999, Singh et al. showed that when human lung small airway epithelial (HSAE) cells were exposed to lead chromate, the cells underwent dose-dependent apoptosis. The formation of Cr-DNA adducts and DNA- associated Pb in lead chromate-treated HSAE cells were also observed. They suggested that lead chromate-induced apoptosis may be a mechanism to eliminate cells with chromium- and/or lead-damaged DNA.

In 2000, He et al. found that $[Ca^{2+}]$ and/or $[Pb^{2+}]$ were localized to photoreceptors of retina in lead-treated rat and produced rod-selective apoptosis. Ca^{2+} and Pb^{2+} also induced mitochondrial depolarization, swelling, and cytochrome c release. They suggested that Ca^{2+} and Pb^{2+} bound to the internal metal (Me^{2+}) binding site of the mitochondrial permeability transition pore (PTP) and subsequently opened the PTP, which initiated the cytochrome c-caspase cascade of apoptosis in rods.

Many reports indicate that lead can induce apoptosis in various cells of animal and human. It is possible that erythropoietic cells including erythroid precursor cells may also serve as another target of lead and since these cells are responsible for Hb synthesis and red blood cell production, this may be another mechanism of lead

induced anemia. However, no report on the effect of lead on the erythroid precursor cells regarding the apoptosis in available at present, the statement is not yet confirmed. This research will therefore investigate toxic effect of lead on the maturation and proliferation of human erythroid precursor cells and also upon their apoptosis.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Chemicals

1.1. Alpha minimal essential medium (α-MEM); Gibco BRL

1.2. Iscove's modifided dulbecco's medium (IMDM); Gibco BRL

1.3. Fetal bovine serum (FBS); Hyclone

1.4. Bovine serum albumin (BSA); Boehringer

1.5. β-Mercaptoethanol (β-ME); Sigma

1.6. L-Glutamine; Gibco BRL

1.7. Dexamethasone; Sigma

1.8. Recombinant-human erythropoietin (r-HuEPO); EPREX-2000 ; Cilag

1.9. Percoll reagent (density 1.129 g/ml); Pharmacia

1.10. Ficoll-Hypaque : HISTOPAQUE-1077; Sigma

1.11. 5637-Human bladder-carcinoma cell line; ATCC, Rockville, Maryland, USA

1.12. Cyclosporin A; Sandoz

1.13. Fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse anti-human transferrin receptor, CD 71 (CD 71-FITC); Dako

1.14.R-phycoerythrin(R-PE)-conjugated monoclonal mouse anti-human glycophorin A (glycophorin A-PE); Dako

1.15.Annexin V-biotin; Boehringer

1.16.Streptavidin Cy 5; Dako

1.17. Streptavidin Cy 5/R-PE; Dako

1.18.Phosphate buffer saline (PBS), pH 7.4

1.19.N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES); Sigma

1.20.Calcium chloride ($CaCl_2$); Merck

1.21.Potassium chloride (KCl); CARLO

1.22.Potassium hydroxide (KOH); Merck

1.23.Potassium dihydrogen phosphate (KH_2PO_4); CARLO

1.24.Disodium hydrogen phosphate (Na_2HPO_4); CARLO

1.25.Sodium bicarbonate ($NaHCO_3$); CARLO

1.26.Sodium chloride (NaCl); BDH

1.27.Sodium hydroxide (NaOH); Merck

1.28.Glucose ($C_6H_{12}O_6$); M&B

1.29.Lead acetate $(CH_3COOH)_2Pb.3H_2O$; BDH

1.30.Lead standard solution; Merck

1.31.Triton X-100; Merck

1.32.Nitric acid (HNO_3); Merck

1.33.Ethylenediamine tetraacetic acid (EDTA); Fluka

1.34.Colchicine; Fluka

1.35.Vinblastine; Sigma

1.36.4,4'-Diisothiocyanostilbene-2,2'-disulfonate (DIDS); Sigma

1.37.Trypan blue dye; Gibco BRL

1.38.Apo-transferrin; Sigma

2. Instruments

2.1. Flow cytometric analyzer: FACScan; Becton Dickinson

2.2. Graphite-Furnace Atomic Absorption Spectrometer (GFAAS): Varian spectr AA-30 Zeeman graphite tube atomizer

2.3. CO_2-incubator; Heraeus instrument

2.4. Laminar flow hood; Gelman Sciences BH 2000 Series

2.5. Centrifuge; Kubota 5100

2.6. Cytospin 3; Shandon

2.7. Light microscope; Nikon Alphaphot YS

2.8. Phase contrast microscope; Nikon phase contrast-2 ELWD 0.3

2.9. pH-meter; Orion, Model SA 720

2.10.Autocave; Tomy autocave SS-320

2.11.Hot air oven; Venticell

2.12.Hemacytometer; American optical

2.13.Water bath-incubator; Memmert

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. The blood seperation were shown in Figure 11. 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 12). The 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

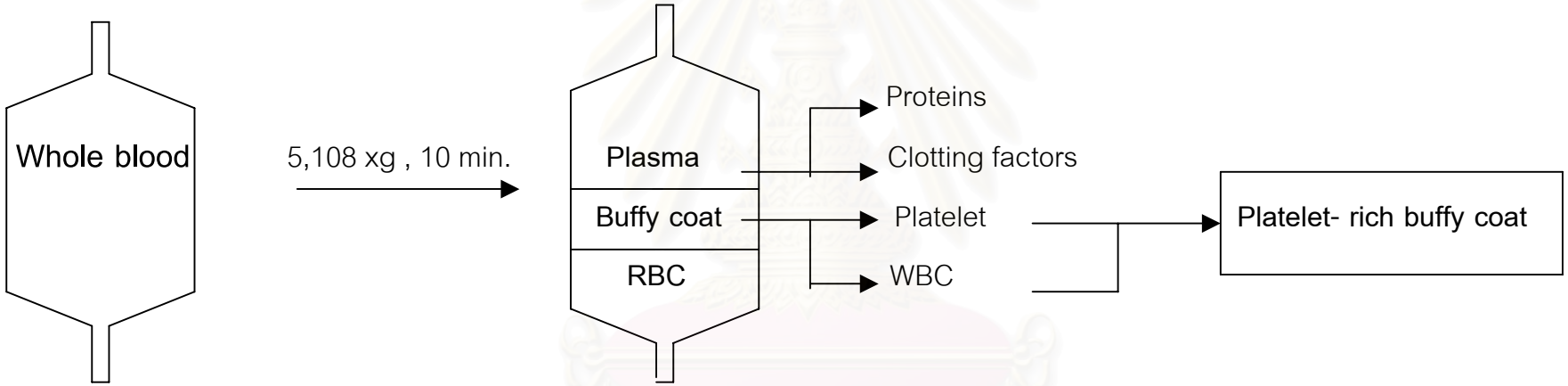

Figure 11 : Schematic representation of blood compositions derived from whole blood separation process

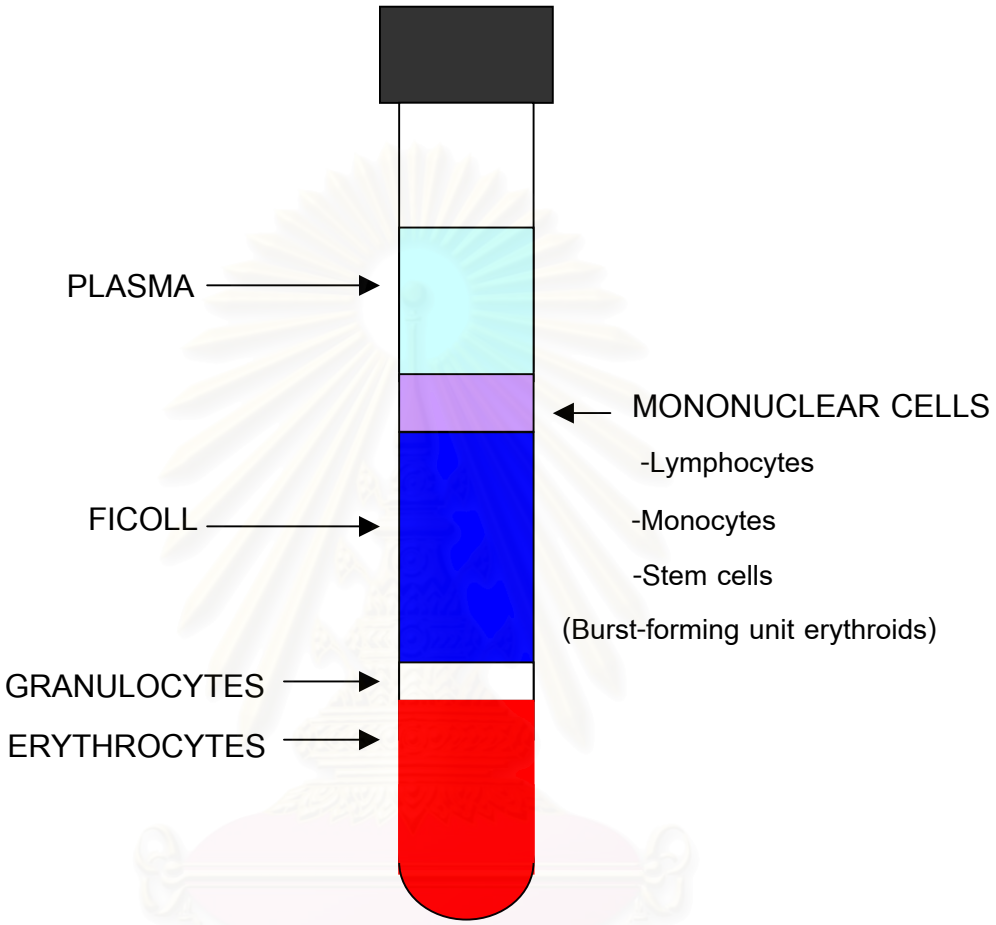

Figure 12 : Blood fraction derived from buffy coat separation process. The interphase white layer of mononuclear cells was collected.

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. Lead Uptake by Human Erythrocytes Suspended in Different Buffers

In order to receive the suiTable medium for cell suspension in the study of lead uptake by human red blood cells, three different buffers namely buffer A (145 mM KCl, 5 mM Glucose and 15 mM HEPES/KOH, pH 7.4), buffer B (5 mM $CaCl_2$, 140 mM NaCl, 5 mM Glucose and 10 mM HEPES/KOH, pH 7.4) and α-MEM (pH 7.4) were tested as incubation buffer.

2.1. Sample Preparation

Erythrocytes were separated from heparinized whole blood obtained from healthy donors by centrifugation at 600 xg for 10 minutes. After the centrifugation, plasma and white blood cells were discarded. Erythrocytes were washed once with 20 volume of incubation buffer.

2.2. The Study of Lead Uptake by Erythrocytes in Different Buffers

Erythrocytes (~1×10^7 cells) were suspended in three incubation buffers, pH 7.4 containing 12 ppm lead acetate. The first incubation buffer contained 145 mM KCl, 5 mM Glucose and 15 mM HEPES/KOH, pH 7.4. The second incubation buffer contained 5 mM $CaCl_2$, 140 mM NaCl, 5 mM Glucose and 10 mM HEPES/KOH, pH 7.4. The third incubation buffer was α-MEM composed of Minimum Essential Medium (Alpha medium) and 2.2 g/liter $NaHCO_3$ (pH 7.4). The cells were separately incubated in these buffers containing lead at 37°C in an atmosphere of 5% CO_2 in air with extrahumidity. After incubation for various times, the suspensions were centrifuged at 600 xg for 10 minutes, the cell pellets were washed three times with EDTA washing medium,(1 mM EDTA in incubation buffer, pH 7.4) to remove lead from the cell surface. The cells were then resuspended into 1 ml of incubation buffer, and divided into three portions. The first portion (0.1 ml) was counted for the cell viability by trypan blue exclusion assay. The second portion (0.1 ml) was assessed for cell morphology under light microscope. The remaining was diluted with 0.1% triton x-100/0.01 N HNO_3 solution and further subjected for lead measurement by GFAAS. The control was performed

by incubating the cells in the absence of lead. Lead uptake was calculated after subtraction of the control. The results were expressed in ng lead/10^5 cells.

2.3. The Study of Lead Uptake by Erythrocytes in 1% FBS / α-MEM

Erythrocytes (~1×10^7 cells) were suspended with 1% FBS in α-MEM (pH 7.4) containing lead acetate at different concentrations and incubated for 30 minutes at 37°C in an atmosphere of 5% CO_2 in air with extrahumidity. After incubation, the cells were washed three times with 1 mM EDTA in 1% FBS/α-MEM (pH 7.4), and resuspended into 1 ml with 1% FBS/α-MEM . The suspension was divided into three portions for measuring the number of cells, assessing cell morphology, and determining lead content by the method described in 2.2.

2.4. Determination of Cell Viability and Cell Counting

The viability of cell was determined by trypan blue exclusion assay using a hemocytometer and observed under phase contrast microscope. Trypan blue exclusion assay is a rapid and reliable method that relies on the breakdown of membrane integrity, allow the uptake of a dye that is normally membrane impermeant (Mather, 1998). These procedures were done as follow : The cell suspension was diluted to 1:1 (v/v) with 0.25% trypan blue dye. Viable cells could exclude trypan blue dye while dead cells would take up the dye, because of the breakdown of membrane intigrity. Therefore, the live cells were unstained and clear, whereas the dead cells were stained in blue. After staining, the cell suspension was placed on the "V" shape of the hemocytometer chamber and then covered with the coverslip. The chamber was placed on the stage of phase contrast microscope and counted for the number of cells; those excluded the dye for the viable cell and those stained in blue for the dead cell. The observation under phage contrast microscope was with a 10 × objective, focus on one of the 25 smaller squares bounded on all slides by three parallel lines. The cells were counted at least 10 cells/mm^2. If the number of cells fewer than 100 cells in the square, counted one or more additional squares. The number of cells were calculated as cells/ml by multiplying the number of cells counted in 1-mm square by 10^4

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2.5. Determination of Lead Content by Graphite-Furnace Atomic Absorption Spectrometry (GFAAS)

GFAAS is suiTable technique for determining low lead content sample, using an electrothermally heated graphite tube. A small volume of the solution was placed in the graphite tube. Predetermined temperatures for drying, ashing, and atomization were achieved by passing an electric current through the graphite. After atomization, the lead atoms then absorbed the light emitted a lead cathode lamp. The degree of light absorption depended on the amount of lead present in graphite tube.

To prevent metal contamination, all of laboratory wares were cleaned by submerging in 20% HNO_3 (v/v) overnight. After that, the wares were rinsed twice with distilled water and once with deionized water, dried in hot air oven and kept in the closed clean container until use.

Lead content in the cells was determined by using a Varioan Spectr AA-30 with a Zeeman background corrector. Pure nitrogen was used as a sheath and purge gas. The determination of samples against the reference standard solution were carried out by the standard addition technique. The instrumental conditions for lead determination were given in Table 5.

Table 5 : Instrumental conditions for the determination of lead by GFAAS.

Wavelength	283.3	nm
lamp current	5.0	mA
Slit width	0.5	nm
Background	on	
Measurement	Peak height	

FURNACE PARAMETER

Step no.	Temperature (°C)	Time (sec)	Gas flow (L/min.)	Read command
1	85	10	3	No
2	110	15	3	No
3	150	20	3	No
4	400	10	3	No
5	650	5	3	No
6	700	5	0	No
7	2300	1	0	Yes
8	2300	2	0	Yes
9	2500	2	3	No

2.75% w/v of $NH_4H_2PO_4$ was used as matrix modifier for lead. The autosampler was programmed to take up 20 μl of sample and 5 μl of modifier.

3. Lead Uptake by Erythroid Precursor Cells

3.1. Sample Preparation

Erythroid precursor cells were harvested from the culture at day 12 of secondary medium. After the centrifugation at 250 xg for 10 minutes, the supernatant was removed and the cell pellets were washed once with α-MEM by centrifugation at 250 xg for 5 minutes.

3.2. The study of Lead Uptake by Erythroid Precursor Cells Suspended in α-MEM

Erythroid precursor cells(~ 4×10^6 cells) were suspended in α-MEM (pH 7.4) containing 12 ppm lead acetate and incubated for various times at 37°C in an atmosphere of 5% CO_2 in air with extrahumidity. Before and after incubation, the cells were counted and observed for the viability by trypan blue exclusion assay. After incubation, the cells were washed for three times with 1 mM EDTA in α-MEM (pH 7.4), resuspended in 1 ml of α-MEM, and divided into three portions for measuring the number of cells , assessing cell morphology and determining lead content by the method described in 2.2.

3.3. The Study of Lead Uptake by Erythroid Precursor Cells Suspended in 1% FBS/ α-MEM

To avoid hemolysis of erythroid precursor cells after washing the cells, fetal bovine serum (FBS) was used to preserve cells from hemolysis during centrifugation. Therefore, 1% FBS in α-MEM (pH 7.4) was used on the incubation buffer and EDTA washing medium.

3.3.1. Lead Uptake at Various Times

Erythroid precursor cells (~ 4×10^6 cells) were suspended with 1% FBS in α-MEM (pH 7.4) containing lead acetate and incubated at 37°C for various times in an atmosphere of 5% CO_2 in

air with extrahumidity. Before and after incubation, the cells were counted and determined for their viability by trypan blue exclusion assay. After incubation, the cells were washed for three times with 1 mM EDTA in 1% FBS/α-MEM (pH 7.4). The cells were then resuspended in 1 ml of 1% FBS/α-MEM, and divided into three portions for measuring the number of cells, assessing cell morphology and determining lead content by the method described in 2.2.

3.3.2. Lead Uptake at Different Extracellular Lead Concentrations

Erythroid precursor cells (~ 4×10^6 cells) were suspended with 1% FBS in α-MEM (pH 7.4) containing lead acetate at different concentrations and incubated at 37°C in an atmosphere of 5% CO_2 in air with extrahumidity. The suiTable incubation time that showed in the previous experiment (30 min.) was used in this experiment. After incubation, the cells were washed and divided into three portions for measuring the number of cells, assessing cell morphology and determining lead content by the method described in 2.2.

4. The Role of Transferrin on Lead Transport into Erythroid Precursor Cells

4.1. Sample Preparation

Erythroid precursor cells from eight erythroid cultures obtained from mononuclear cells of eight healthy people were used in this study. The cells were harvested from the cultures at day 12 of secondary medium and prepared by the method as described in 3.1.

4.2. The Study of Transferrin and Lead Transport

Erythroid precursor cells were suspended with 1% FBS in α-MEM (pH 7.4) containing 10 ppm lead acetate with or without 1.67 mg/ml apo-transferrin. The cell were incubated for 30 minutes at 37°C in an atmosphere of 5% CO_2 in air with extrahumidity. After incubation,the cells were washed for three times with 1mM EDTA in 1%FBS/α-MEM (pH 7.4). The cells were then resuspended in 1 ml of 1% FBS/α-MEM, and divided into three portions for measuring the number of cells, assessing cell morphology and determining lead content by the method described in 2.2.

5. Effect of Inhibitor on Lead Uptake in Human Erythroid Precursor Cells

5.1. The Study of Microtubule Inhibitors

5.1.1.Reagent Preparation

Stock solution of 20 mM colchicine : 20 mM colchicine was prepared by dissolving 0.08 g colchicine in absoluted ethanol with the final volume of 10 ml. The working solution (2 mM colchicine) was prepared by diluting 1 ml of 20 mM colchicine with 9 ml α-MEM (pH 7.4).

Stock solution of 10 mM vinblastine : 10 mM vinblastine was prepared by dissolving 1 mg vinblastine in α-MEM(pH 7.4) with the final volume of 10 ml. The working solution (1 mM vinblastin) was prepared by diluting 1 ml of 10 mM vinblastine with 9 ml α-MEM (pH 7.4).

5.1.2.Treatment with Microtubule Inhibitors

After culture in EPO supplemented medium for 12 days, the cells were in log phase of growth at $1x10^5$ cells/ml. Microtubule inhibitors (colchicine and vinblastine) was added to the cultured cells at a final concentration of 10, 25, 50, and 100 μM for colchicine or 40, 100, and 200 μM for vinblastine. Control cell was incubated without microtubule inhibitors. Incubation with various microtubule inhibitor concentrations was done for one hour at 37^{o}C in an atmosphere of 5%CO_2 in air with extrahumidity. The cells were then collected and washed once with 1%FBS/α-MEM (pH 7.4) and then incubated at 37^{o}C for 30 minutes in 1%FBS/α-MEM (pH 7.4) containing 10 ppm lead acetate with 1.67 mg/ml apo-transferrin. After incubation, the cells were washed and taken to measure the number of viable cells, assess cell morphology and determine for lead content by the method described in 2.2.

5.2. The Study of Anion-Transport Inhibitor

5.2.1.Reagent Preparation

Stock solution of 10mM DIDS : 10 mM DIDS was prepared by dissolving 0.05 g DIDS in α-MEM (pH 7.4) with the final volume of 10 ml.

5.2.2.Treatment with Anion-Transport Inhibitor

After 12 days in EPO supplemented medium, the cells were harvested and washed with 1%FBS/α-MEM (pH 7.4) and suspended in 1%FBS/α-MEM (pH 7.4) containing 10 ppm lead acetate with 10, 100, 1,000, 2,000, and 3,000 μM DIDS (anion-transport inhibitor). Control cell was incubated without DIDS. The cells were then incubated at 37°C for 30 minutes in an atmosphere of 5%CO_2 in air with extrahumidity. After incubation, the cells were washed and taken to measure the number of viable cells, assess cell morphology and determine for lead content by the method described in 2.2.

6. Effect of Lead on Human Erythroid Precursor Cells

6.1. 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).

6.2. 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 followes: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.

7. 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 13A). 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 13B.). 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 16A) 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 6). Further proliferation and differentiation of these CFU-E-like progenitors into erythroid precursor cells could also occur in EPO-dependent phase.

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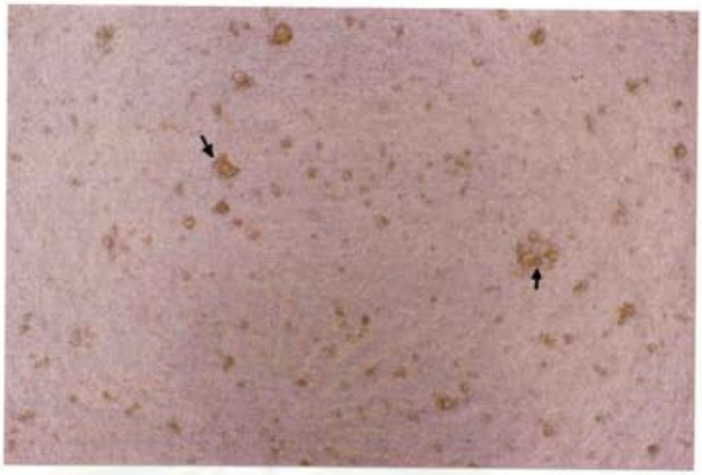
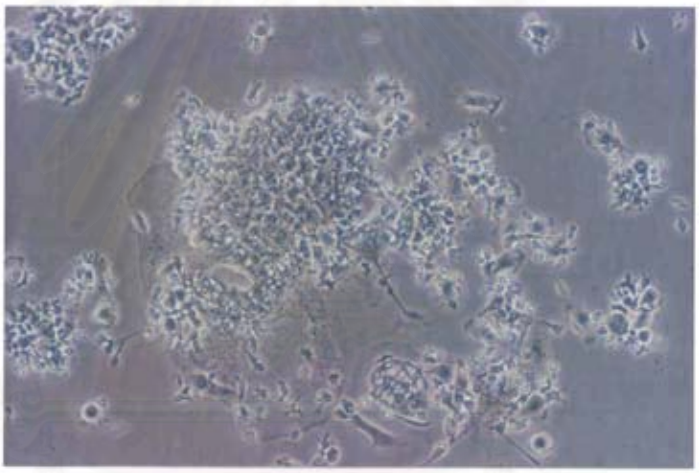


A : Nonadherent cells (100 × magnification)



B : Adherent cells (400 × magnification)

Figure 13 : 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).

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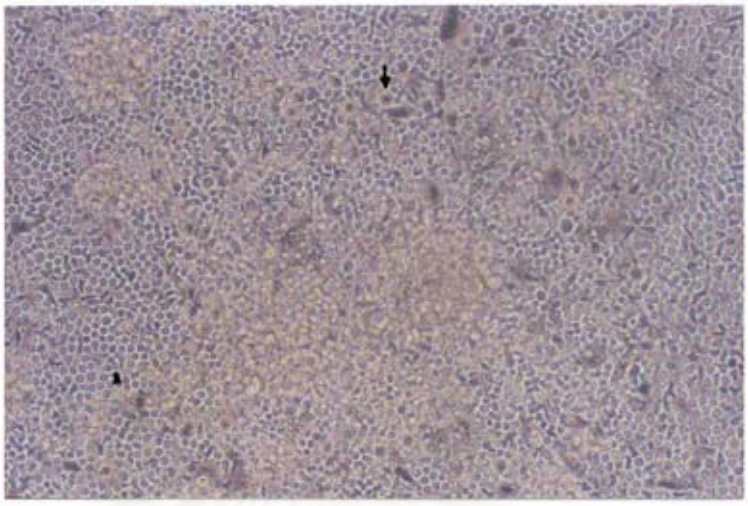
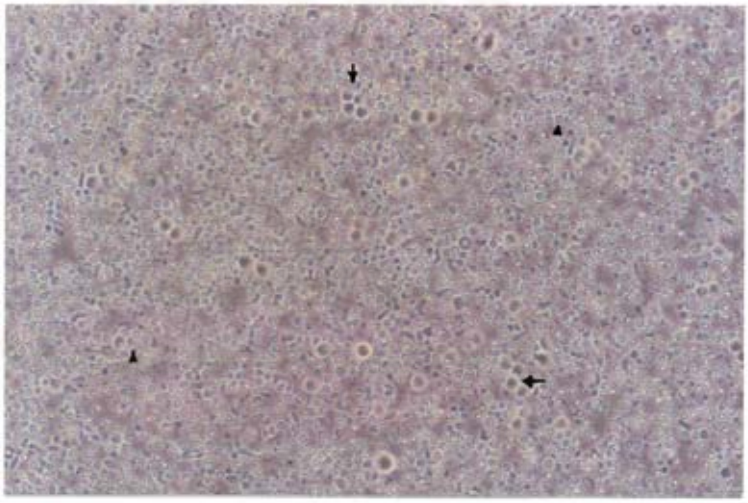


A : day 0 (400 × magnification)



B : day 3 (400 × magnification)

Figure 14 : 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.

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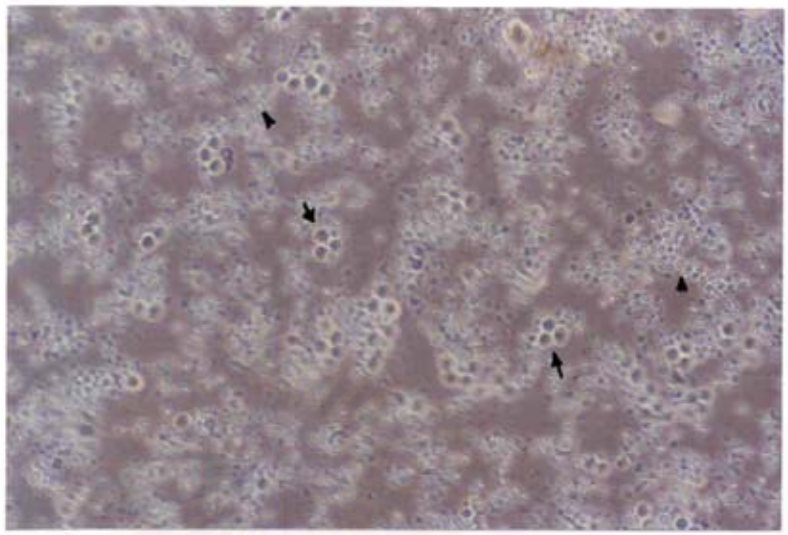
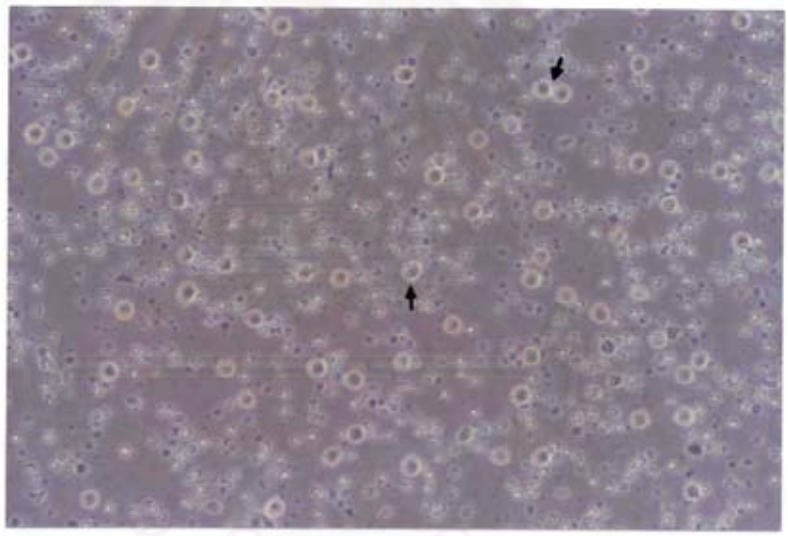


A : Before lymphocyte separation (400 × magnification)



B : After lymphocyte separation (400 × magnification)

Figure 15 : 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 15A). 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 15B 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 16B) with considerable degree of proliferation. As a consequence, many proerythroblast colonies were observed in day 5 of secondary culture (Figure 17A). 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 17B). 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 18A). 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 18B). After 14 days in this medium, the number of cells declined and the erythroid precursor cells started to disintegrate (Figure 19).

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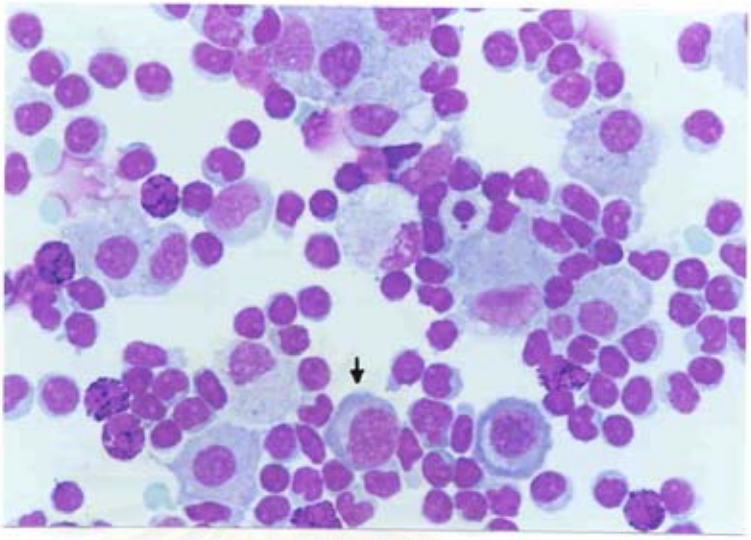
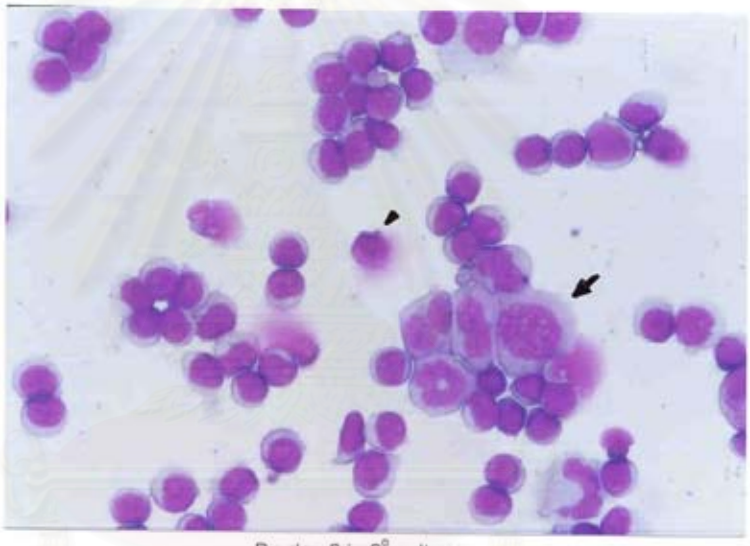


A : day 7 in 1° culture



B : day 3 in 2° culture

Figure 16 : 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.

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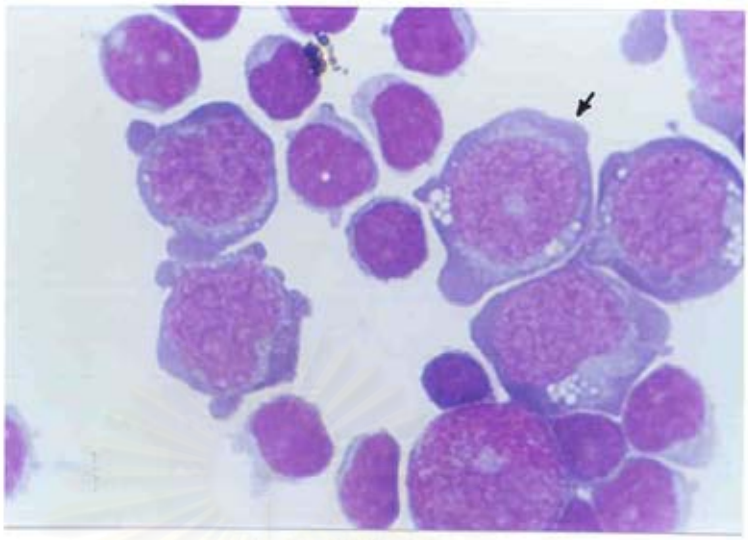
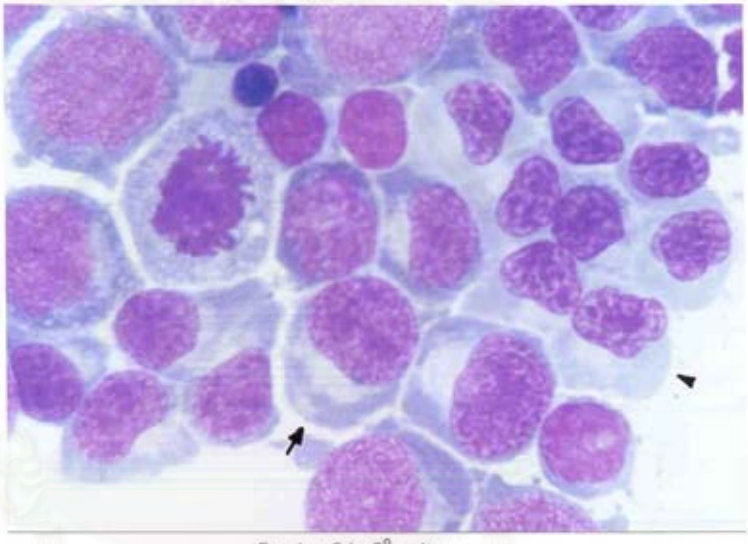


A : day 5 in 2° culture



B : day 8 in 2° culture

Figure 17 : 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.

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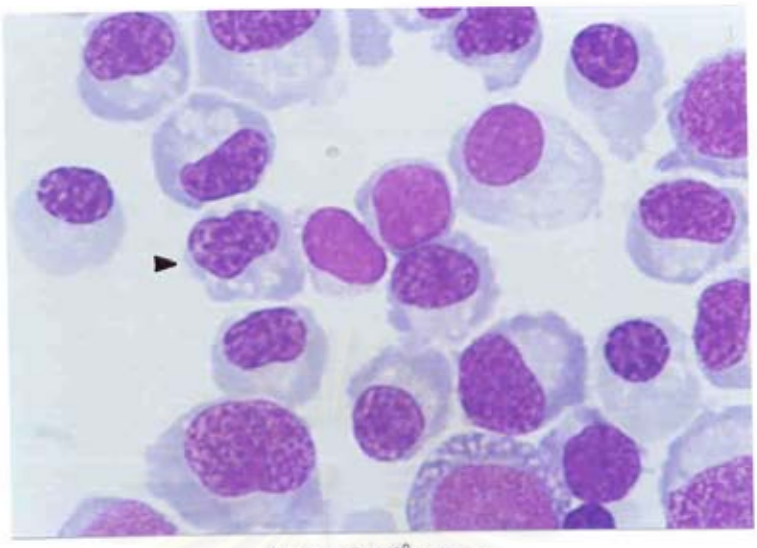
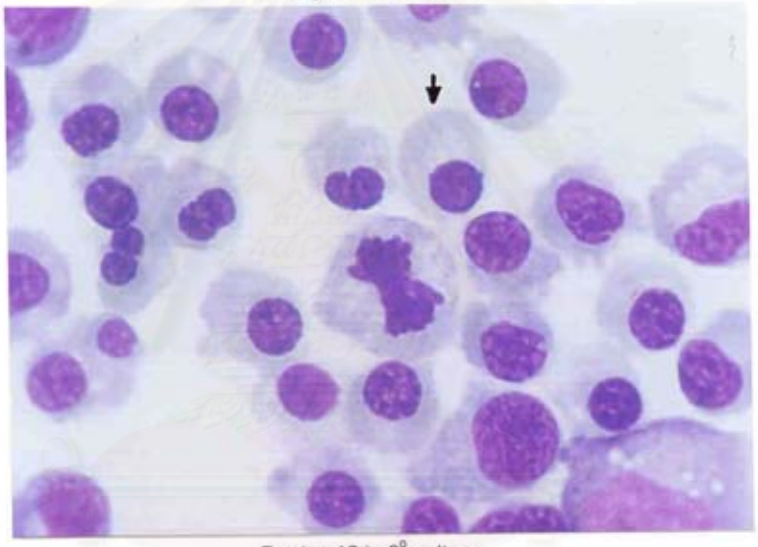


A : day 10 in 2° culture



B : day 12 in 2° culture

Figure 18 : 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.

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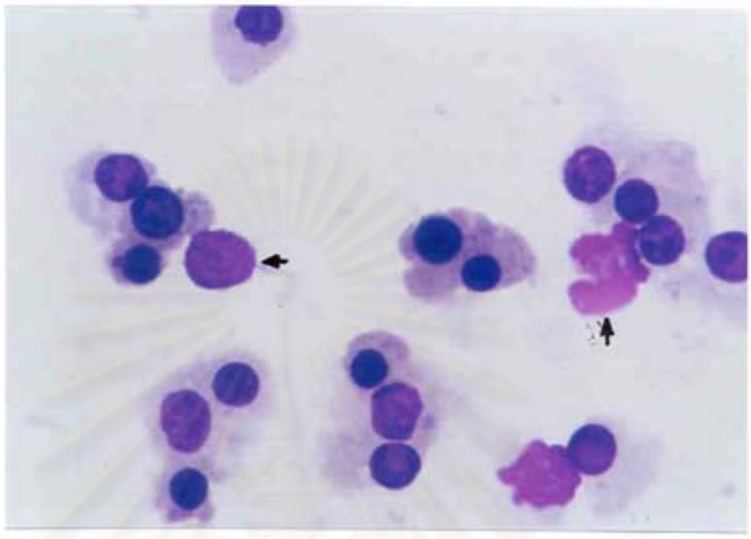

Figure 19 : Light microscopy of cultured cells at day 14 in 2^{0} culture. Many of dead cells (arrows) appeared in the cultured after 14 days in this phase,1,000 × magnification.

Table 6 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 6 : 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 (Figure 6), 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 7 and Figure 20. 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 20 and 21), 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 6).

Table 7 : 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^{o} 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 ± SD	8	45.58 ± 11.59	81.17 ± 11.03	41.56 ± 10.23
mean ± SD	12	75.25 ± 7.95	76.83 ± 12.89	70.01 ± 8.52

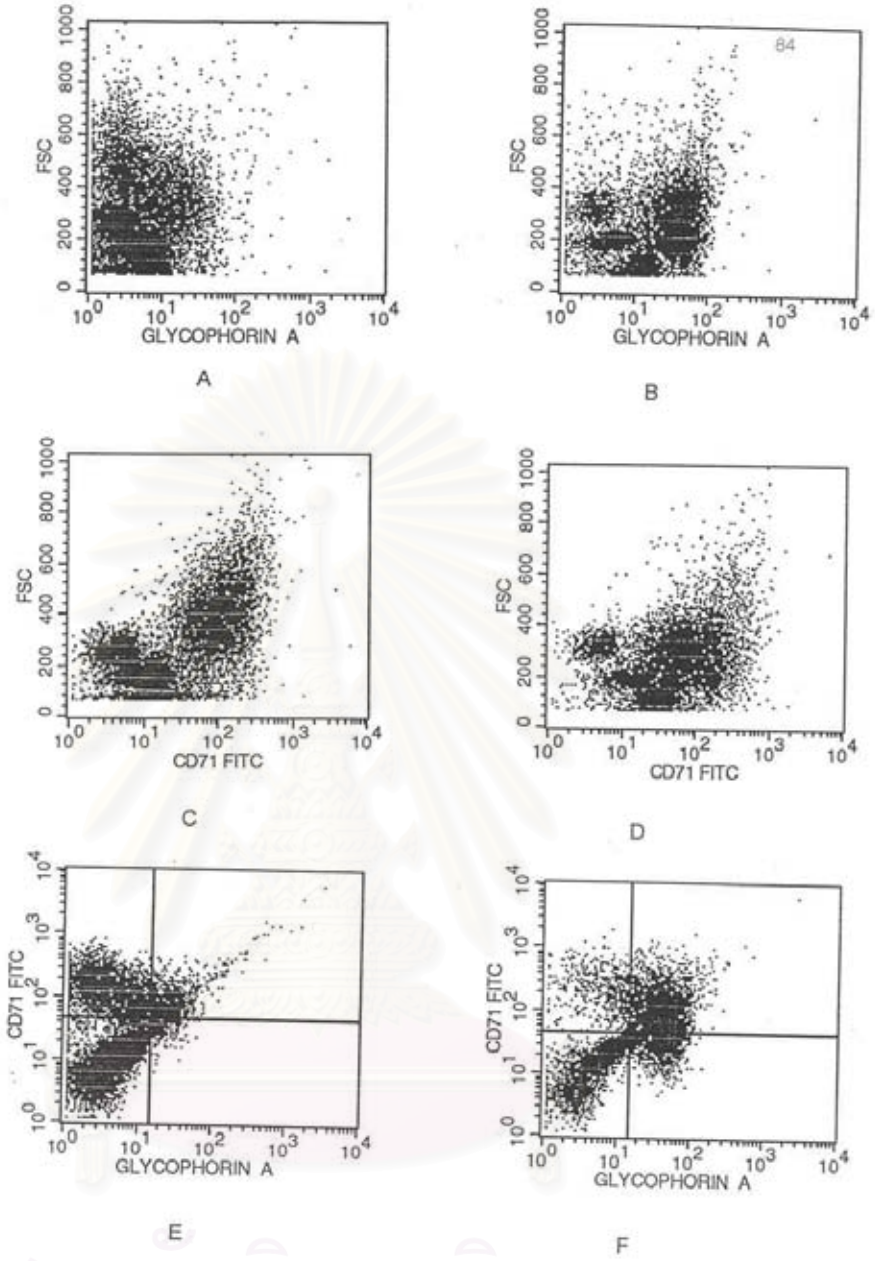

Figure 20 : Flow cytometric analysis of erythroid precursor cell maturation.

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 lesser transferrin receptor expression.

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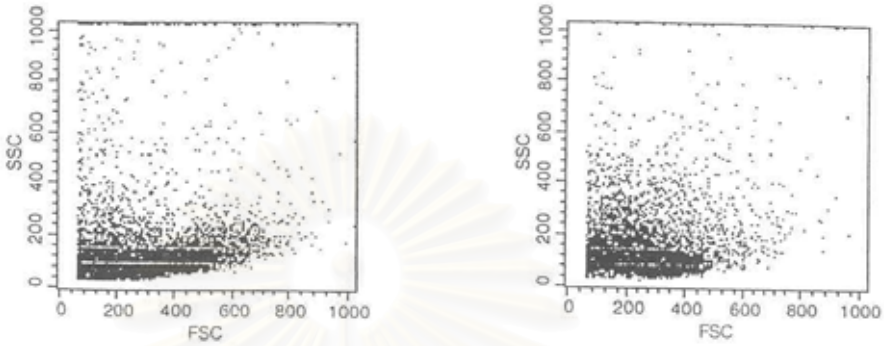

Figure 21 : Correlation between forward scatter (FSC) and side scatter (SSC) of the cells at day 8 (A) and day 12 (B) of the culture. The cell size was detected by FSC and granularity of cell was detected by SSC. At late stage of maturation (day 12), most of the cells displayed lower FSC as compared to the early stage (day 8).

2. Lead Uptake by Human Erythrocytes

2.1. Lead Uptake by Erythrocytes Suspended in Different Buffers

Lead content in erythrocytes incubated with α-MEM (pH 7.4) containing lead acetate (12 ppm) was higher than that incubated with other buffers at every incubation time (Figure 22). In α-MEM, lead was rapidly taken up into the cells, and the rate of lead uptake was the highest when compared to that in other buffers. In α-MEM, the rate of lead uptake reached the steady state within 15 minutes while those in other buffers could not reach the steady state in 90 minutes. However, the rate of lead uptake into erythrocytes in buffer containing 5 mM $CaCl_2$, 140 mM NaCl, 5 mM Glucose and 10 mM HEPES/KOH, pH 7.4 (buffer B) was higher than that in buffer containing 145 mM KCl, 5 mM Glucose and 15 mM HEPES/KOH, pH 7.4 (buffer A). Therefore, α-MEM seemed to be a suiTable incubation buffer for the study of lead uptake by human erythrocytes.

2.2. Viability of Erythrocytes Exposed to Lead in Different Buffers

Possible effects of lead on the viability of erythrocytes exposed to lead in three incubation buffers were determined by trypan blue exclusion assay. The ratio of live and dead cells in erythrocyte population that exposed to lead in three incubation buffers were shown in Table 8. These results showed that all experiments had low dead cells in their population (less than 6%). The number of viable cells were also nearly the same in these three conditions.

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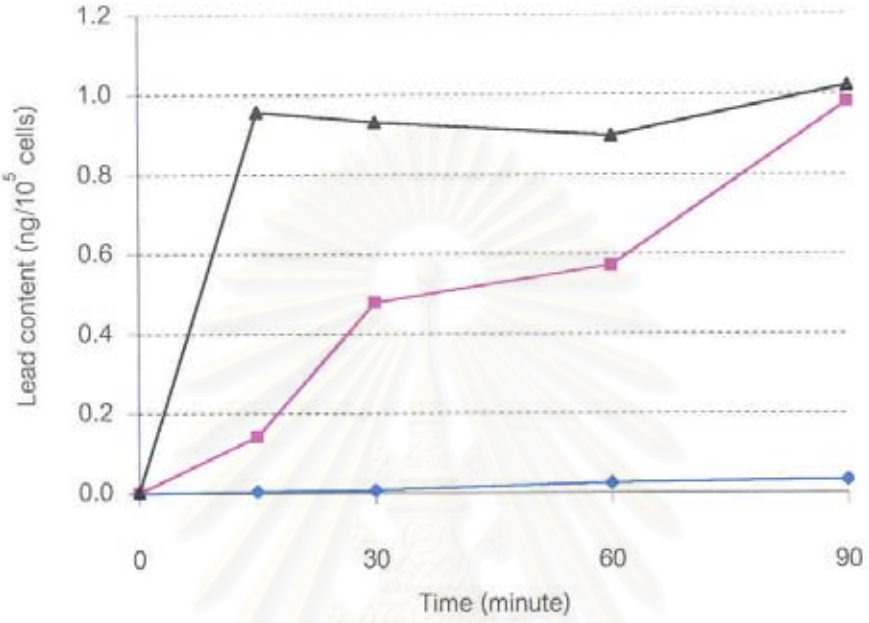

Figure 22 : Time course of lead uptake into erythrocytes in different buffers. Human erythrocytes exposed to 12 ppm lead acetate in buffer A , buffer B and α-MEM . The cells were incubated in these incubation buffers containing lead for 15, 30, 60 and 90 minutes at 37°C. After washing the cells for three times with 1 mM EDTA in incubation buffer, lead content of the cells was measured by GFAAS.

Table 8 : The percentage of live and dead cells in erythrocytes population that exposed to lead in three incubation buffers. After washing with 1 mM EDTA in incubation buffer, the viability of cells was determined by trypan blue exclusion assay and observed under phase contrast microscope.

Incubation time	number of cells (%)					
	Buffer A		Buffer B		α-MEM	
	Live cell	Dead cell	Live cell	Dead cell	Live cell	Dead cell
Control	96	4	97	3	99	1
15 min	95	5	97	3	98	2
30 min	96	4	96	4	99	1
60 min	95	5	96	4	98	2
90 min	95	5	95	5	98	2

3. Lead Uptake by Human Erythroid Precursor Cells

3.1. Lead Uptake by Human Erythroid Precursor Cells Suspended in α-MEM

3.1.1. Lead Uptake by Human Erythroid Precursor Cells and Erythrocytes

Lead content in human erythroid precursor cells and erythrocytes incubated at 37°C with α-MEM containing 12 ppm lead acetate for various times was shown in Figure 23. These data showed that in α-MEM, the lead content in erythroid precursor cells was higher than that in erythrocytes at every incubation time. Moreover, lead was also rapidly incorporated into erythroid precursor cells and reached the steady state within 15 minutes. However, the lead content further decreased as evidenced at 60 minutes incubation time.

3.1.2. Viability of Erythroid Precursor Cells Exposed to Lead in α-MEM

The ratios of live and dead cells in the population of erythroid precursor cells and erythrocytes exposed to 12 ppm lead acetate in α-MEM (pH 7.4) were shown in Table 9. These data showed that dead cells in erythroid precursor cells population were higher than that in erythrocytes ranging from 19-30% for the erythroid precursor cells and from 1-2% for the erythrocytes. After washing with 1 mM EDTA in α-MEM, The cell number of erythroid precursor cells decreased to 20.9 and 23.1% in controll and lead treatment sample when compared to the number of cells before incubation for 30 minutes (Table 10). In addition, the study of cell morphology showed the presence of a lot of hemolytic cells in erythroid population (Figure 24) as compared to only a few in erythrocyte population (Figure 25). Therefore, α-MEM seemed an unsuitable medium for study of lead uptake by erythroid precursor cells.

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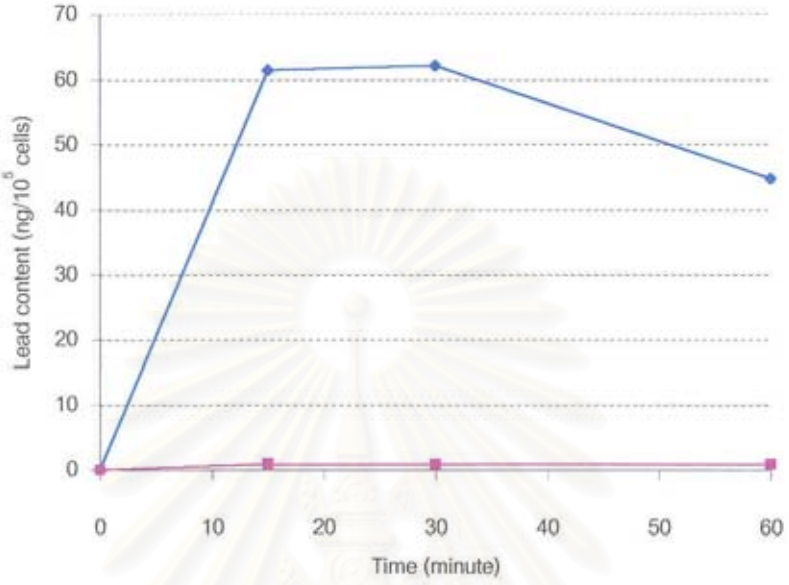

Figure 23 : Time course of lead uptake in erythroid precursor cells and erythrocytes exposed to 12 ppm lead acetate in α-MEM (pH 7.4) for various times at 37 °C. After washing the cells for three times with 1 mM EDTA in α-MEM, lead content of the cells was measured by GFAAS.

Table 9 : The percentage of live and dead cells in the population of erythroid precursor cells and erythrocytes exposed to lead in α-MEM. After washing with 1 mM EDTA in α-MEM, the viability of cells was determined by trypan blue exclusion assay and observed under phase contrast microscope.

Incubation time	Number of cells (%)			
	Erythroid precursor cell		Erythrocyte	
	Live cell	Dead cell	Live cell	Dead cell
Control	80	20	99	1
15 min	81	19	98	2
30 min	78	22	99	1
60 min	70	30	98	2

Table 10 : The number of erythroid precursor cells exposed to lead for 30 minutes in α-MEM (pH 7.4) before incubation, after incubation and after washing with 1 mM EDTA in α-MEM.

Incubation time	Before incubation		After incubation		After washing	
	Live cell number		Live cell number		Live cell number	
	cells/ml	(%)	cells/ml	(%)	cells/ml	(%)
Control (30 min)	12.9×10^5	100	9.7×10^5	75.2	2.7×10^5	20.9
C + Pb (30 min)	13.0×10^5	100	10.1×10^5	77.7	3.0×10^5	23.1

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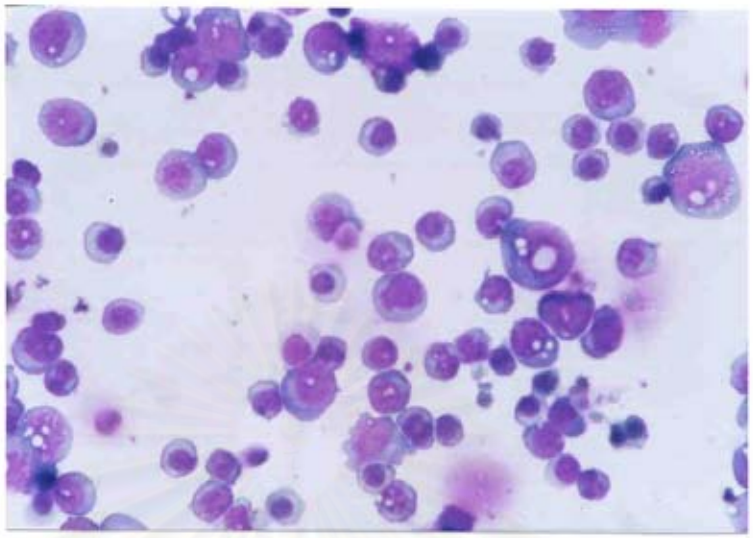
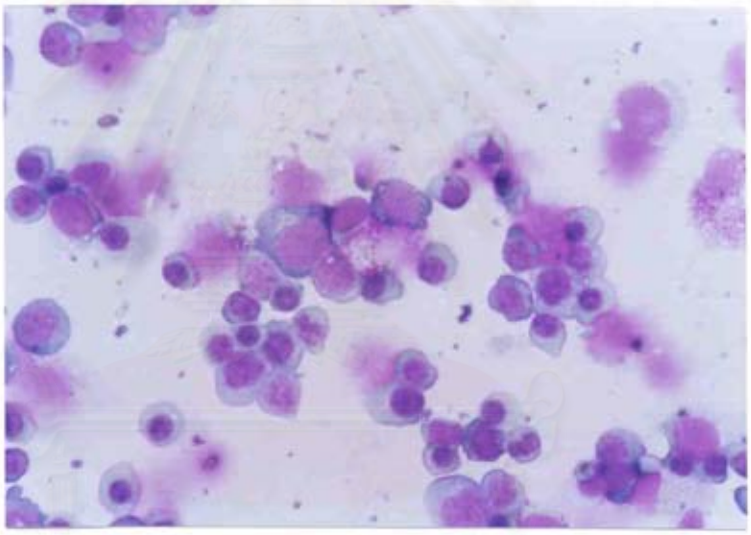


A : Control (-Pb)



B : Cell + Pb (15 min)

Figure 24 : Cell morphology of erythroid precursor cells exposed to 12 ppm lead acetate in α-MEM (pH 7.4) for various times at 37°C. After washing with 1 mM EDTA in α-MEM, the cells were stained with Wright's stain and observed under light microscope (400 X magnification). The cells were incubated in α-MEM, pH 7.4 : A = without lead for 30 minutes , B = with lead for 15 minutes.

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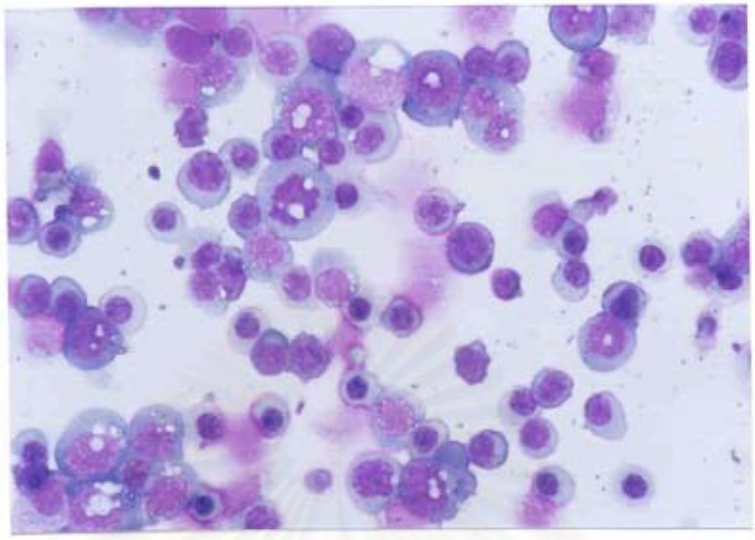
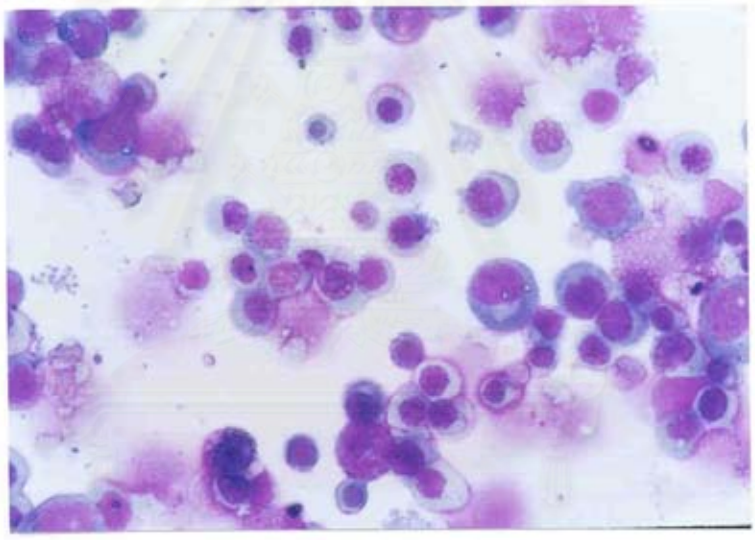


C : Cell + Pb (30 min)



D : Cell + Pb (60 min)

Figure 24 : (Continue) Cell morphology of erythroid precursor cells exposed to 12 ppm lead acetate in α-MEM (pH 7.4) for various times at 37°C. After washing with 1 mM EDTA in α-MEM, the cells were stained with Wright's stain and observed under light microscope (400 X magnification).The cells were incubated in α-MEM, pH 7.4 : C = with lead for 30 minutes, D = with lead for 60 minutes

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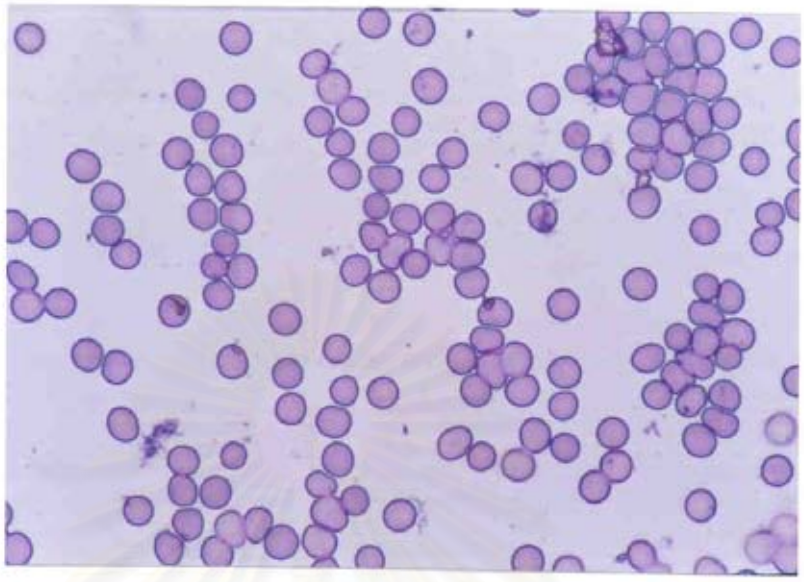
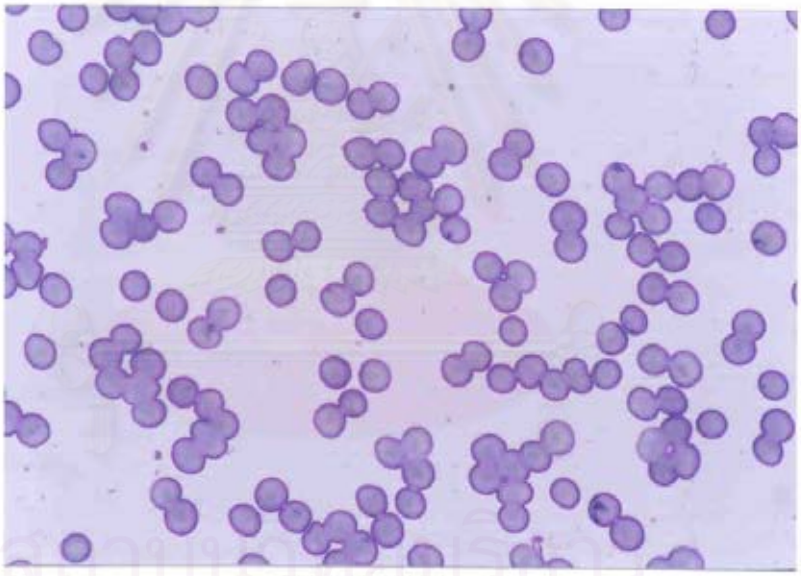


A : Control (-Pb)



B : Cell + Pb (15 min)

Figure 25 : Cell morphology of erythrocytes exposed to 12 ppm lead acetate in α-MEM (pH 7.4) for various times at 37°C. After washing with 1 mM EDTA in α-MEM, the cells were stained with Wright's stain and observed under light microscope (400 X magnification). The cells were incubated in α-MEM, pH 7.4 : A = without lead for 30 minutes , B = with lead for 15 minutes.

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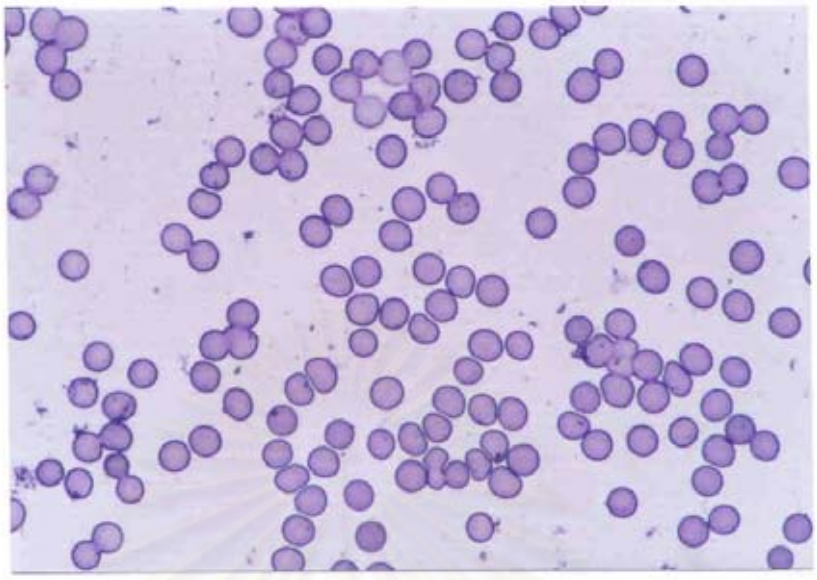
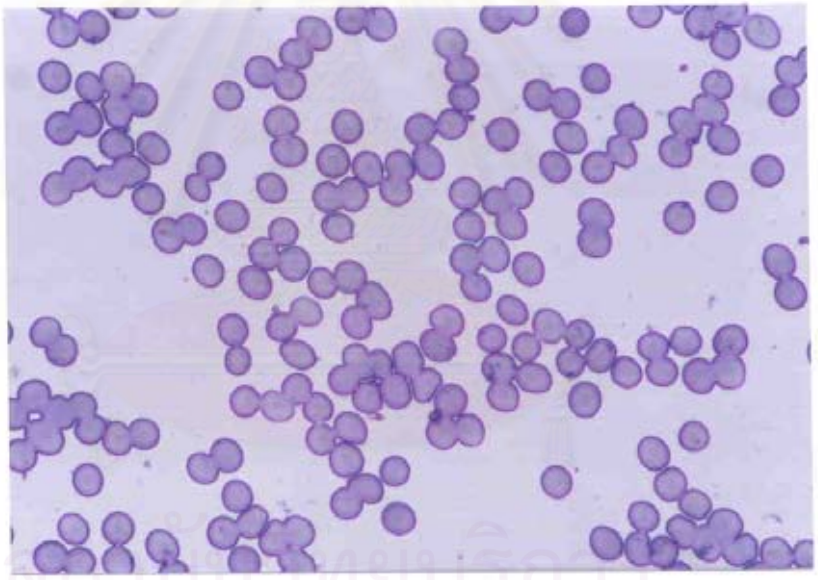


C : Cell + Pb (30 min)



D : Cell + Pb (60 min)

Figure 25 : (Continue) Cell morphology of erythrocytes exposed to 12 ppm lead acetate in α-MEM (pH 7.4) for various times at 37°C. After washing with 1 mM EDTA in α-MEM, the cells were stained with Wright's stain and observed under light microscope (400 X magnification). The cells were incubated in α-MEM, pH 7.4 : C = with lead for 30 minutes, D = with lead for 60 minutes.

3.2. Lead Uptake by Human Erythroid Precursor Cells Suspended in 1% FBS/α-MEM

3.2.1. Viability of Erythroid Precursor Cells Exposed to Lead in 1% FBS/α-MEM

The ratios of live and dead cells in erythroid population exposed to 12 ppm lead acetate in 1% FBS/α-MEM (pH 7.4) as compared to that in α-MEM (pH 7.4) were shown in Table 11. This data showed that when 1% FBS was added to the α-MEM, the dead cells in erythroid population occurred only 3-9%. Therefore, 1% FBS seemed to be factor needed to stabilize erythroid precursor cells during lead uptake study. Moreover, the FBS requirement was also tested during each step of the study (Table 12). This indicated that, 1% FBS could preserve the cells both during incubation and washing. However, prolong incubation to 60 minutes decreased the number of cells. In addition, the study of cell morphology showed that the majority of cell population were viable cells (Figure 26). Therefore, 1% FBS/α-MEM (pH 7.4) seemed to be a suitable medium for the study of lead transport into erythroid precursor cells.

3.2.2. Time Dependence of Lead Uptake by Erythroid Precursor Cells

The result in Figure 27 showed that when erythroid precursor cells were incubated with 1% FBS/α-MEM (pH 7.4) containing 10 and 12 ppm lead acetate for various times at 37°C, lead incorporated into the cells and reached the maximum value at 30 minutes. However, prolong incubation to 60 minutes decreased the lead contents. Therefore, 30 minutes incubation time was chosen for the study of lead uptake by erythroid precursor cells in 1% FBS/α-MEM (pH 7.4)

3.2.3. Concentration Dependence of Lead Uptake by Erythroid Precursor Cells

The results from the study of lead uptake by erythroid precursor cells and erythrocytes at different lead concentrations were shown in Figure 28. This data showed that both cells were incubated with 1% FBS/α-MEM (pH 7.4) containing lead acetate at different concentrations for 30 minutes at 37°C, lead content in both cells increased with

Table 11 : The percentage of live and dead cells in erythroid precursor cells population that exposed to lead in 1%FEB/α-MEM (pH 7.4) and in α-MEM (pH 7.4). After washing, cell viability was determined by trypan blue exclusion assay and observed under phase contrast microscope.

Incubation time	Number of cells (%)			
	1%FEB/α-MEM		α-MEM	
	Live cell	Dead cell	Live cell	Dead cell
Control	97	3	80	20
15 min	95	2	81	19
30 min	97	3	78	22
60 min	91	9	70	30

Table 12 : The number of erythroid precursor cells exposed to lead for 30 and 60 minutes in 1%FEB/α-MEM(pH 7.4) before incubation, after incubation and after washing with 1mM EDTA in 1%FEB/ α-MEM.

Incubation time	Before incubation		After incubation		After washing	
	Live cell number		Live cell number		Live cell number	
	cells/ml	(%)	cells/ml	(%)	cells/ml	(%)
Control (30 min)	99×10^5	100	90×10^5	87	72×10^5	73
C + Pb (30 min)	94×10^5	100	88×10^5	88	70×10^5	74
Control (60 min)	95×10^5	100	75×10^5	79	58×10^5	61
C + Pb (60 min)	97×10^5	100	78×10^5	80	58×10^5	60

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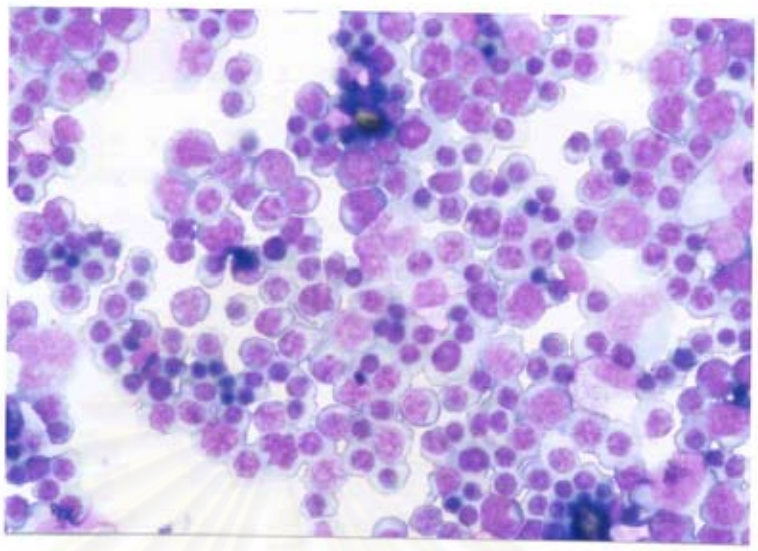
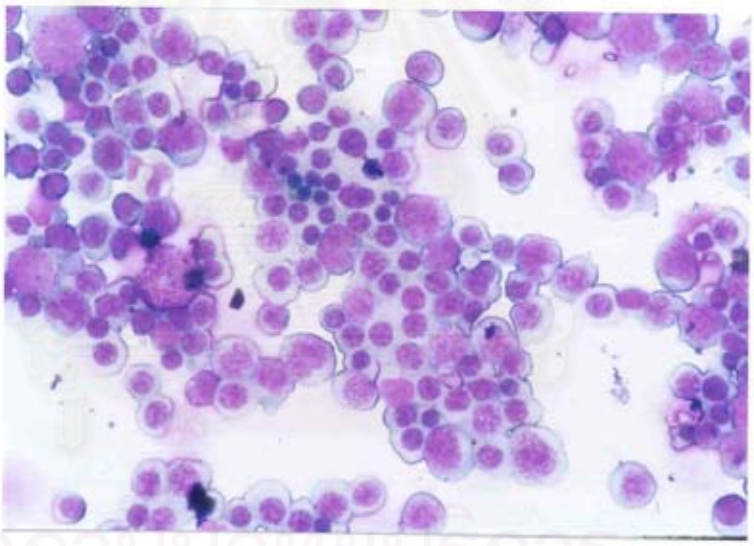


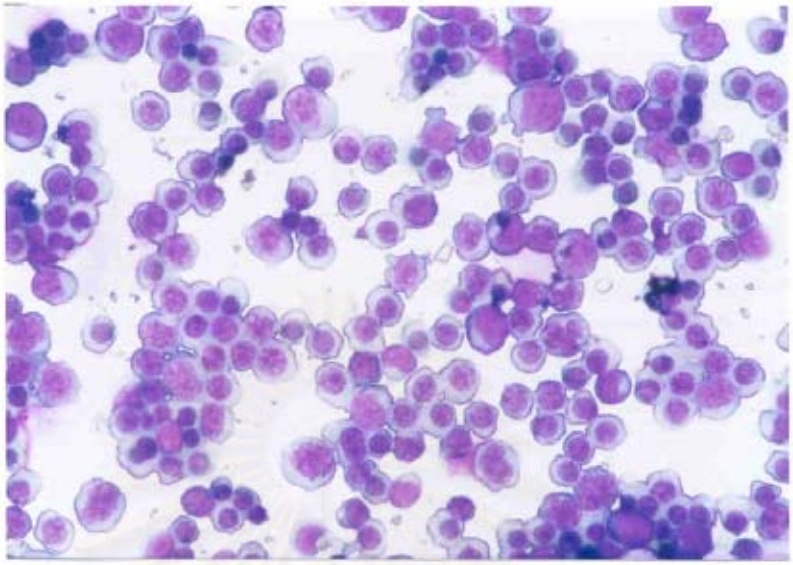
A : Control (-Pb)



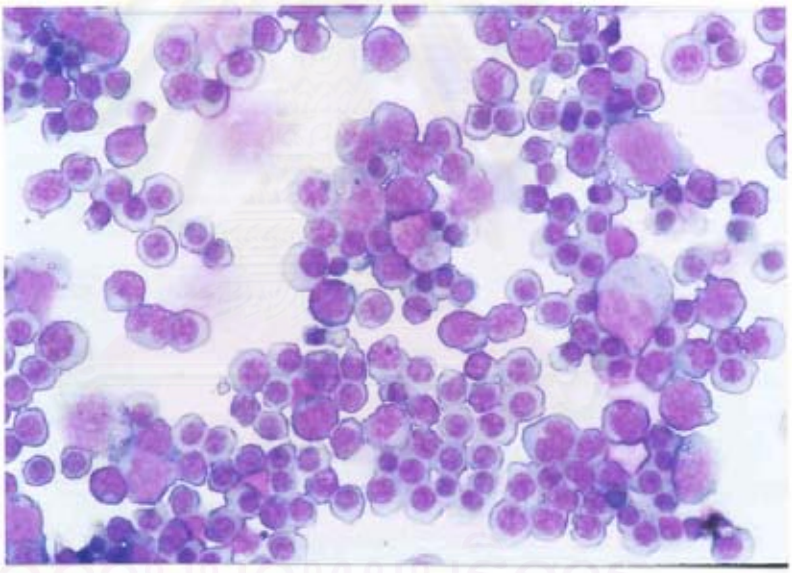
B : Cell + Pb (15 min)

Figure 26 : Cell morphology of erythroid precursor cells exposed to 12 ppm lead acetate in 1%FBS/α-MEM (pH 7.4) for various times at 37°C. After washing with 1 mM EDTA in α-MEM, the cells were stained with Wright's stain and observed under light microscope (400 X magnification). The cells were incubated in 1%FBS/α-MEM, pH 7.4: A=without lead for 30 minutes, B=with lead for 15 minutes.

99



C : Cell + Pb (30 min)



D : Cell + Pb (60 min)

Figure 26 : (Continue) Cell morphology of erythroid precursor cells exposed to 12 ppm lead acetate in 1%FBS/α-MEM (pH 7.4) for various times at 37°C. After washing with 1 mM EDTA in α-MEM, the cells were stained with Wright's stain and observed under light microscope (400 X magnification). The cells were incubated in 1%FBS/α-MEM, pH 7.4: C = with lead for 30 minutes, D = with lead for 60 minutes.

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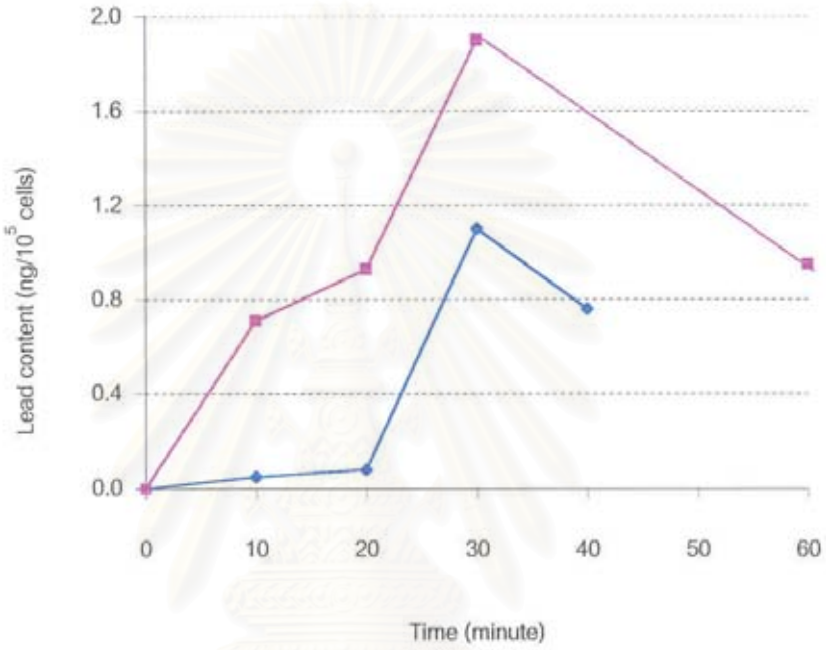

Figure 27 : Time dependence of lead uptake by erythroid precursor cells.
The cells were suspended in 1% FBS/α-MEM (pH 7.4) containing 10 ppm and 12 ppm lead acetate and incubated at 37 °C. The cell suspensions were taken up at the indicated time and immediately washed for three times with 1 mM EDTA in 1% FBS/α-MEM (pH 7.4) . Lead content of the cells was determined by GFAAS.

101

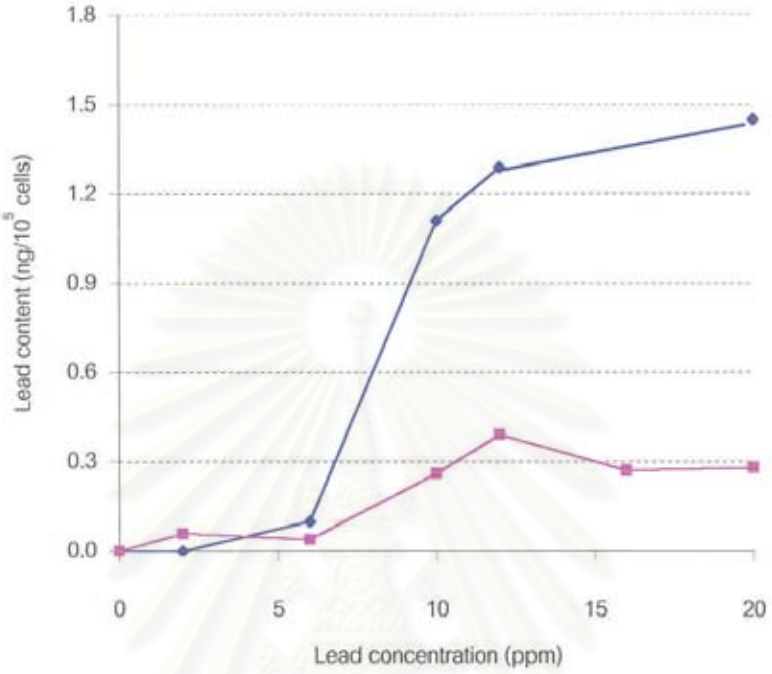

Figure 28 : Concentration dependence of lead uptake by erythroid precursor cells and erythrocytes. The cells were suspened in 1%FBS/α-MEM (pH 7.4) containing different concentrations of lead acetate and incubated for 30 minutes at 37 °C. After washing the cells for three times with 1 mM EDTA in 1%FBS/α-MEM (pH 7.4), lead content of the cells was measured by GFAAS.

extracellular lead concentration. In addition, lead content in erythroid precursor cells was about 3-4 folds higher than that in erythrocytes.

4. The Role of Transferrin on Lead Transport into Human Erythroid Precursor Cells

4.1. Lead Content in Erythroid Precursor Cells Exposed to Lead with or without Transferrin

Lead content in erythroid precursor cells from 8 erythroid cultures incubated in lead containing buffer (1%FBS/α- MEM, pH 7.4) with or without transferrin were shown in Table 13. This data, although varied from each culture, suggested that transferrin increased the uptake. Calculation of the data from 8 cultures gave the average increase from 3.23 to 4.08 ng/10^5 cells, of 26%. The graphical comparison of lead content of each group was presented in Figure 29, it is clearly shown that lead content in the cells incubated with lead and apo-transferrin were higher than that incubated with lead alone. The average of lead content in erythroid precursor cells that incubated with 10 ppm lead acetate plus 1.67 mg /ml apo-transferrin was significantly greater than the cells that incubated with or without 10 ppm lead acetate with $p<0.05$. Therefore, apo-transferrin seemed to enhance lead uptake in erythroid precursor cells.

4.2. Viability of Erythroid Precursor Cells Exposed to Lead with or without Transferrin

A comparison of the number of erythroid precursor cells that were incubated in 1%FBS/α-MEM, pH 7.4 without lead, with 10 ppm lead acetate and with 10 ppm lead acetate plus 1.67 mg/ml apo-transferrin was shown in Table 14. The average of the number of cells suspended in 1%FBS/α-MEM (controlled groups) was 37.19 x10^5 cells with a range of 12.77 x 10^5 - 70.40 x 10^5 cells. The average of the number of cells suspended in 1%FBS/α-MEM containing 10 ppm lead acetate was 36.42 x10^5 cells with a range of 13.27 x10^5 – 72.00 x10^5 cells. The average of the number of cells suspended in 1%FBS/α-MEM containing 10 ppm lead acetate plus 1.67 mg/ml apo-transferrin was 36.54 x10^5 cells with a range of 12.70 x10^5 – 74.40 x10^5 cells. There were no significant difference in three groups ($p<0.05$). The number of viable cells were nearly the same in

Table 13 : Comparison of lead content in erythroid precursor cells incubated in 1%FEB/α-MEM (pH 7.4) without lead (cell), with 10 ppm lead acetate (cell+Pb) and with 10 ppm lead acetate plus 1.67 mg/ml apo-transferrin(cell+Pb+apo-Tf). The cells were incubated at 37°C for 30 minutes, After washing the cells for three times with 1 mM EDTA in 1%FEB/ α-MEM, lead content of the cells was measured by GFAAS.

Sample No.	Lead content (ng/10^5 cells)		
	cell	cell + Pb	cell + Pb + apo-Tf
1	0.68	2.13	2.77
2	3.70	4.72	6.83
3	4.56	5.14	5.33
4	0.27	1.13	2.89
5	0.90	2.55	3.25
6	0.13	1.31	1.20
7	1.10	2.73	3.71
8	4.55	6.10	6.66
Mean ± SD	1.99 ± 1.93	3.23 ± 1.86	4.08 ± 2.00

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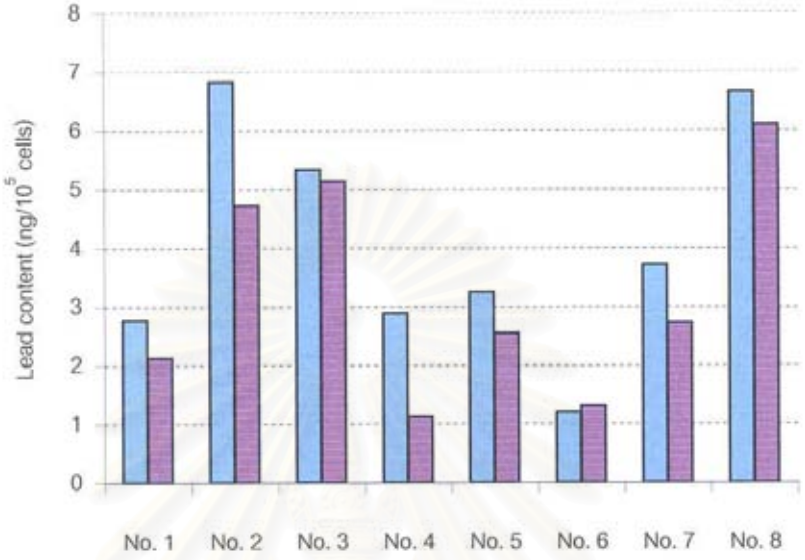

Figure 29 : Comparison of lead content in erythroid precursor cells between the cells were incubated in 1% FBS/α-MEM (pH 7.4) containing 10 ppm lead acetate ☐ with transferrin or ☐ without transferrin for 30 minutes at 37 °C.

Table 14 : Comparison of the number of erythroid precursor cells incubated in 1% FEB/α-MEM (pH 7.4) without lead (cell), with 10 ppm lead acetate (cell+Pb) and with 10 ppm lead acetate plus 1.67 mg/ml apo-transferrin (cell+Pb+apo-Tf). After washing, cell viability was determined by trypan blue exclusion assay and observed under phase contrast microscope.

Sample No.	Number of cells ($\times 10^5$ cells)		
	cell	cell + Pb	cell + Pb + apo-Tf
1	12.77	13.27	12.70
2	18.30	18.80	18.45
3	13.20	12.60	11.00
4	36.60	38.00	35.60
5	44.80	40.40	42.80
6	32.40	26.50	29.10
7	69.05	69.75	68.30
8	70.40	72.00	74.40
Mean $\pm$ SD	37.19 $\pm$ 23.08	36.42 $\pm$ 23.63	36.54 $\pm$ 24.16

these three conditions (Figure 30). This suggested that in this condition, lead and/or transferrin could not affect the viability of the cells.

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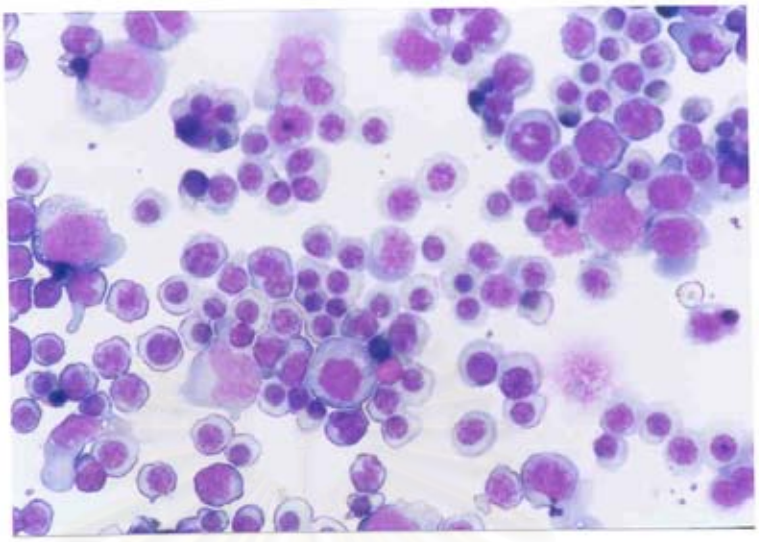
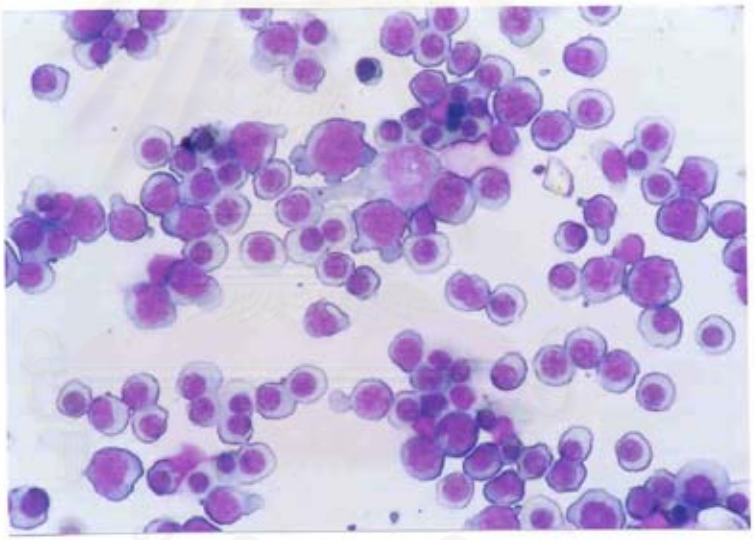


A : Control (-Pb)



B : Cell + Pb

Figure 30 : Cell morphology of erythroid precursor cells exposed to 10 ppm lead acetate in 1%FBS/α-MEM with or without 1.67 mg/ml apo-transferrin for 30 minutes at 37°C. After washing, the cells were stained with Wright's stain and observed under light microscope (400 X magnification). The cells were suspended: A = with 1%FBS/ α-MEM , B = with 10 ppm lead acetate in 1%FBS/ α-MEM.

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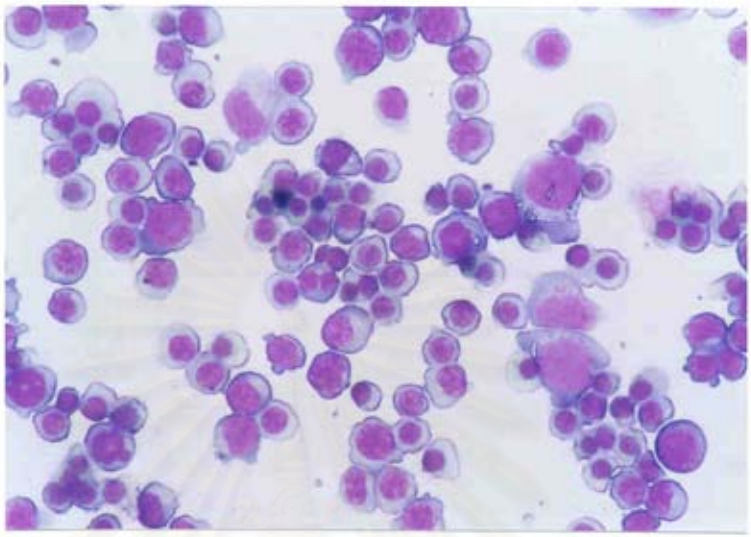

C : Cell + Pb + apo-Tf

Figure 30 :(continue) Cell morphology of erythroid precursor cells exposed to 10 ppm lead acetate in 1%FBS/α-MEM with or without 1.67 mg/ml apo-transferrin for 30 minutes at 37°C. After washing, the cells were stained with Wright's stain and observed under light microscope (400 x magnification). The cells were suspended: C = with 10 ppm lead acetate plus 1.67 mg/ml apo-transferrin in 1%FBS/ α-MEM.

A : Control (-Pb)

5. Effect of Inhibitors on Lead Uptake in Human Erythroid Precursor Cells

5.1. Effect of Microtubule Inhibitors on Lead Uptake

Lead uptake by human erythroid precursor cells which were pre-incubated with the microtubule inhibitors for one hour at 37°C was shown in Table 15 and Figure 31. In the presence of 1.67 mg/ml apo-transferrin, colchicine (up to 100 μM) caused a small degree of lead uptake inhibition (from 100% to 84.12 and 86.13% at 50 and 100 μM, respectively). The percentage of lead uptake in the presence of colchicine and apo-transferrin was slightly higher than in the absence of apo-transferrin (73.64%). Vinblastine was more efficient than colchicine in lead uptake inhibition (from 100% to 32.30, 37.73, and 37.73% at 40, 100, and 200 μM, respectively). The percentage of lead uptake in the presence of vinblastine and transferrin was much lower than in the absence of apo-transferrin (73.44%).

5.2. Effect of Anion-Transport Inhibitors on Lead Uptake

Lead uptake by human erythroid precursor cells which incubated with anion-transport inhibitor (DIDS) was shown in Table 16 and Figure 32. At low concentration (10 and 100 μM), DIDS did not inhibit lead uptake of erythroid precursor cells. But lead uptake decreased slightly at 1,000 μM (from 100% to 85.55%). However, high concentration of DIDS increased lead uptake markedly (from 100% to 221.28 and 251.68% at 2,000 and 3,000 μM, respectively).

Table 15 : Effect of microtubule inhibitors on lead uptake (% control value) by human erythroid precursor cells.

Inhibitors (μM)	% Lead uptake					
	with apo-transferrin					without apo-transferrin
	0	10	25	50	100	0
Colchicine	100.00	104.35	98.46	84.12	86.13	73.64
Vinblastine	0	40	100	200		0
	100.00	32.30	37.73	37.73		73.44

Table 16 : Effect of anion-transport inhibitors on lead uptake (% control value) by human erythroid precursor cells.

Inhibitor (μM)	% Lead uptake					
	0	10	100	1,000	2,000	3,000
DIDS	100.00	134.96	111.02	85.55	221.20	251.68

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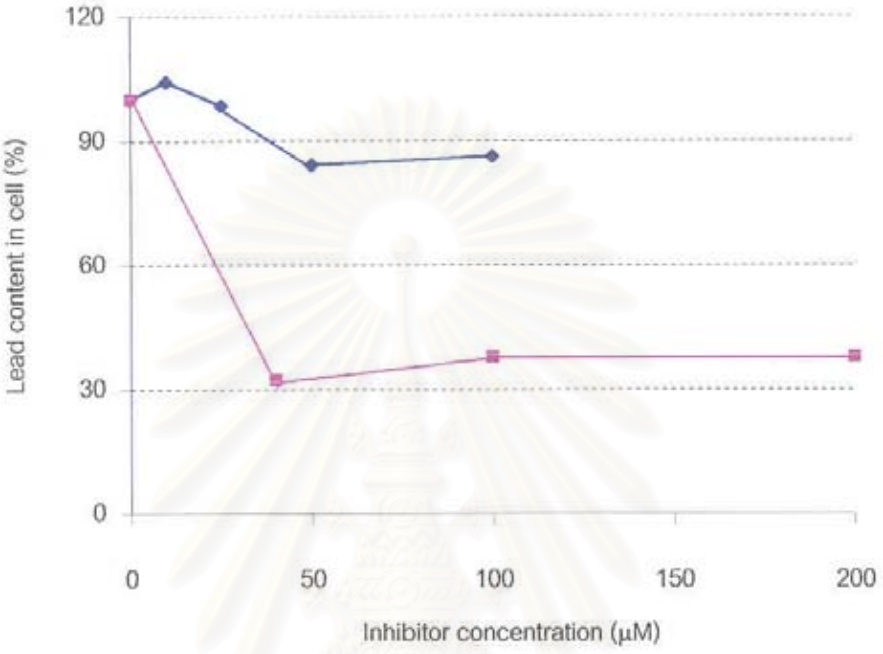

Figure 31 : Effect of microtubule inhibitors, —◆— colchicine and —■— vinblastine , on lead uptake with transferrin (% control value) by human erythroid precursor cells. The cultured cells were suspended in various concentrations of inhibitor in secondary medium for one hour at 37 °C. After that, the cells were harvested, washed and incubated in 1% FBS/α-MEM (pH7.4) containing 10 ppm lead acetate with 1.67 mg/ml transferrin for 30 minutes at 37 °C. Duplicate samples were processed to measure lead content in cell.

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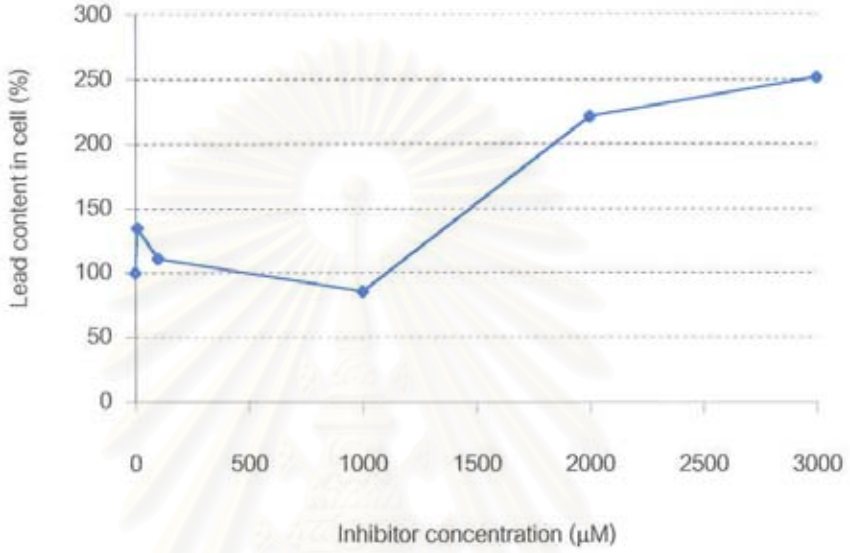

Figure 32 : Effect of anion-transport inhibitor (DIDS) on lead uptake by human erythroid precursor cells. The cultured cells were incubated in 1% FBS/α-MEM (pH 7.4) containing 10 ppm lead acetate and various concentrations of anion-transport inhibitor at 37 °C for 30 min. Duplicate samples were processed to measure lead content in cell.

6. Effect of Lead on Human Erythroid Precursor Cells

6.1. 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 33, the number of viable cells decreased slightly at 0.5 ppm lead acetate concentration. The decreased was significant at high concentration of lead (≥ 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 ≥ 1.0 ppm (Table 17).

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 34. The decrease of cell viability was noted after one day exposure. At lead acetate concentration ≥ 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 35-40) 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 ≤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

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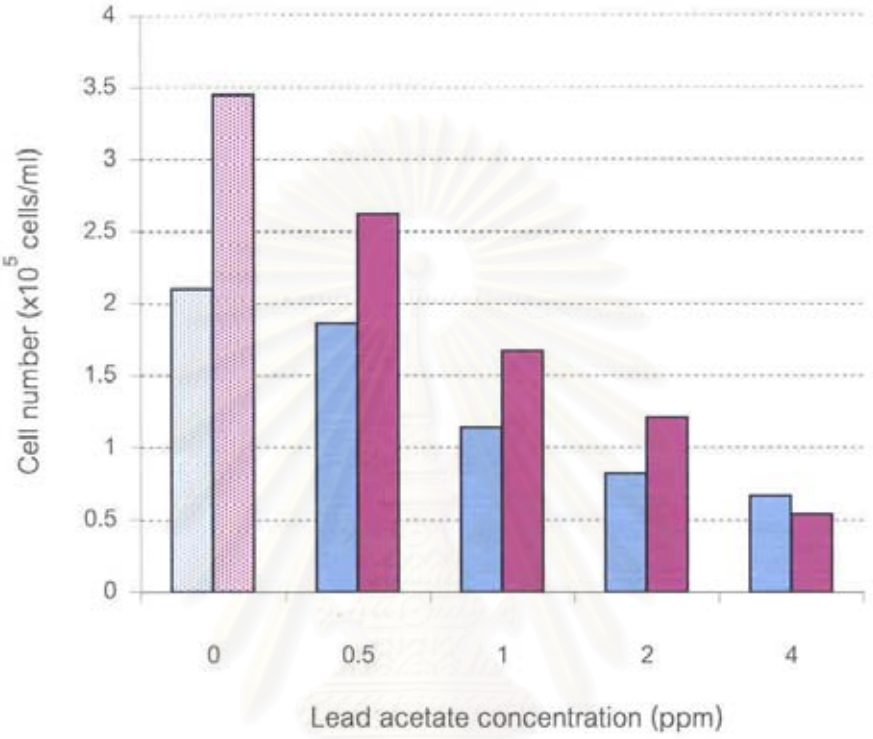

Figure 33: The number of viable cells after exposed to various concentrations of lead acetate when compare to the control (▨) for one(■) and five(■) days.

Table 17 : 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

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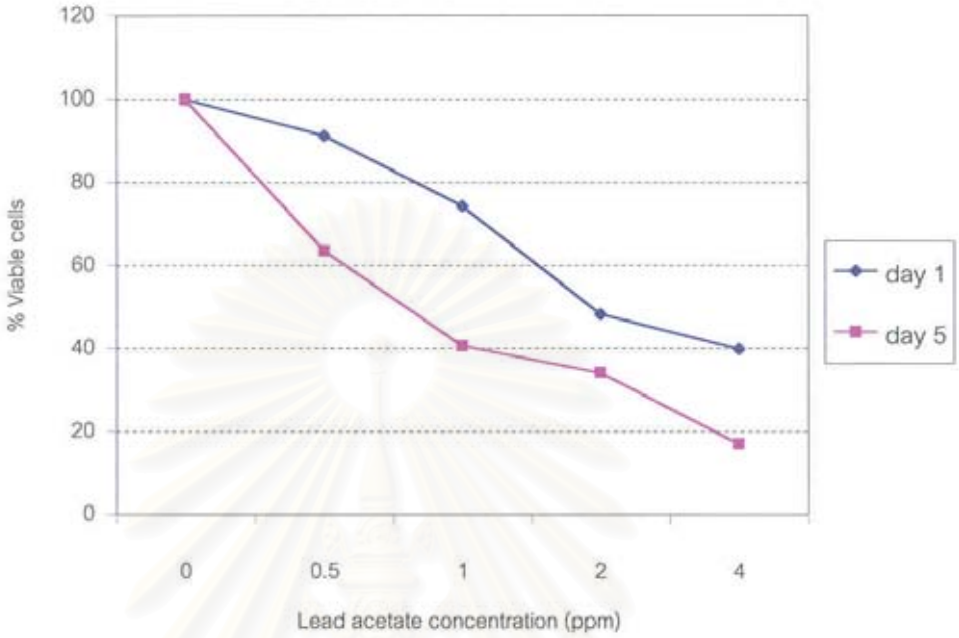

Figure 34: Effect of lead on viable cell number for one and five day-incubation periods.

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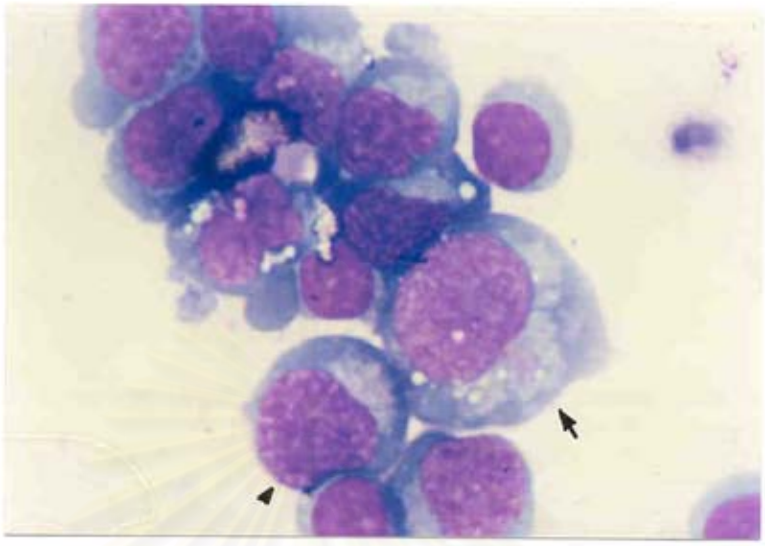
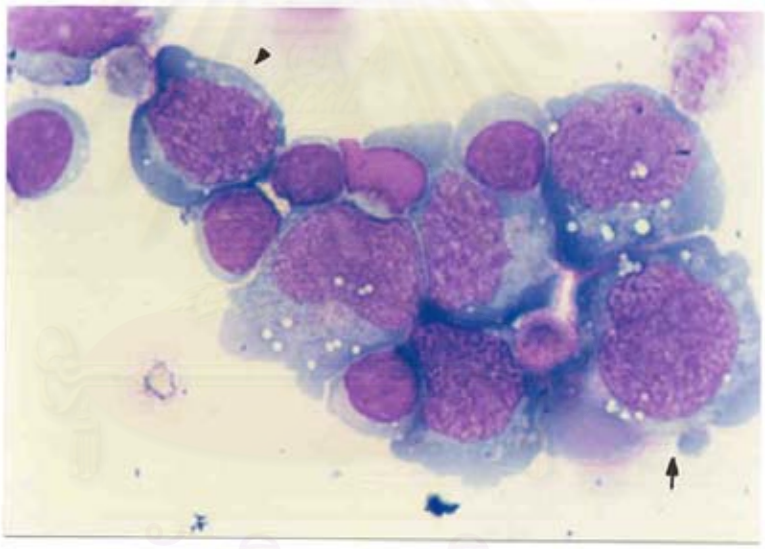


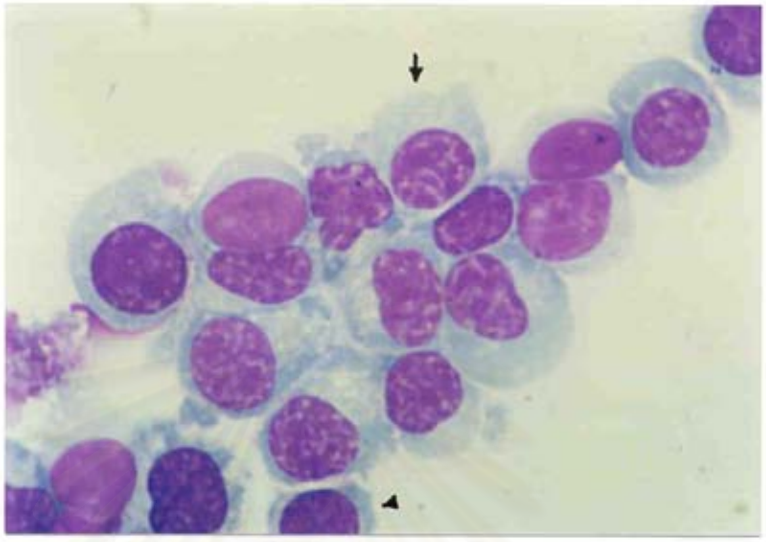
A : 0.0 ppm Pb / day 1



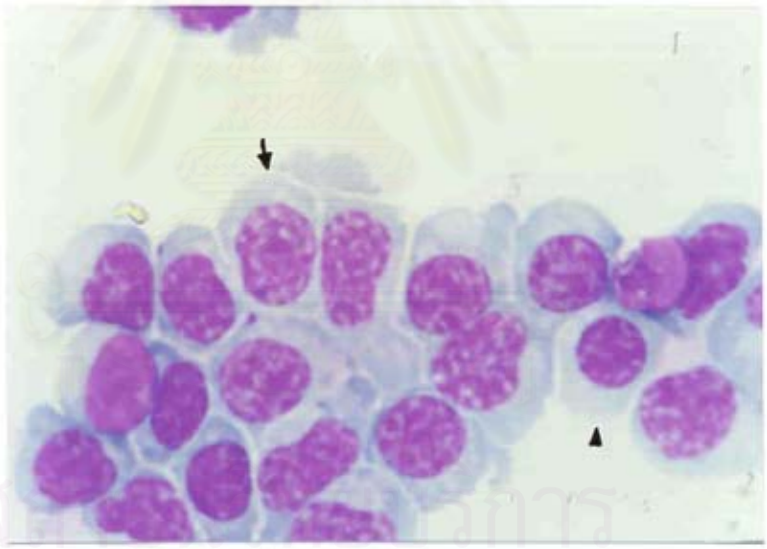
B : 0.1 ppm Pb / day 1

Figure 35 : Light microscopy of cultured cells at day 8 in 2^{δ} 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).

119



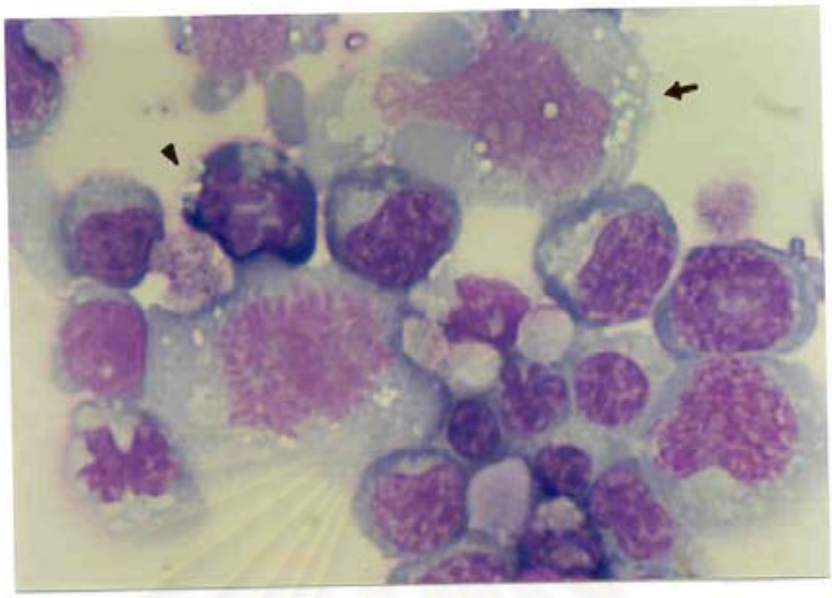
A : 0.1 ppm Pb / day 5



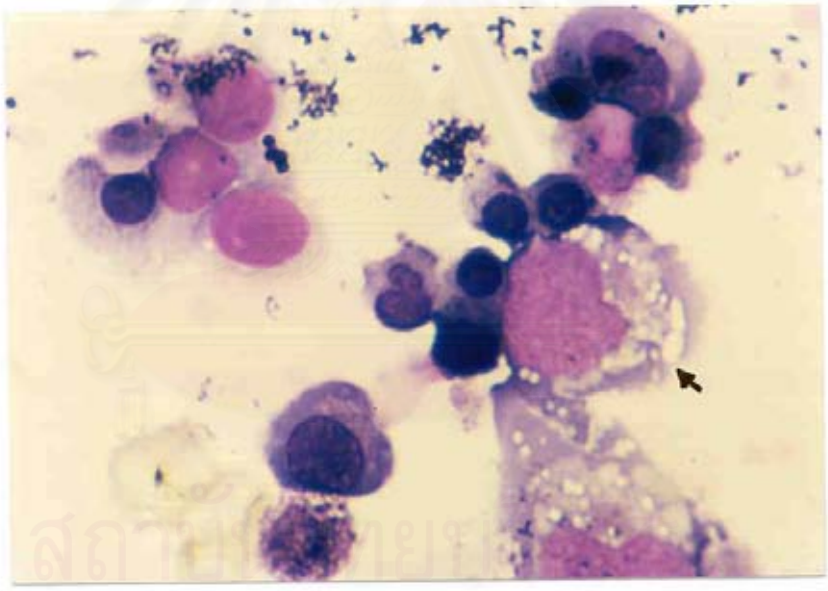
B : 0.3 ppm Pb / day 5

Figure 36 : Light microscopy of cultured cells at day 12 in 2° 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).

120



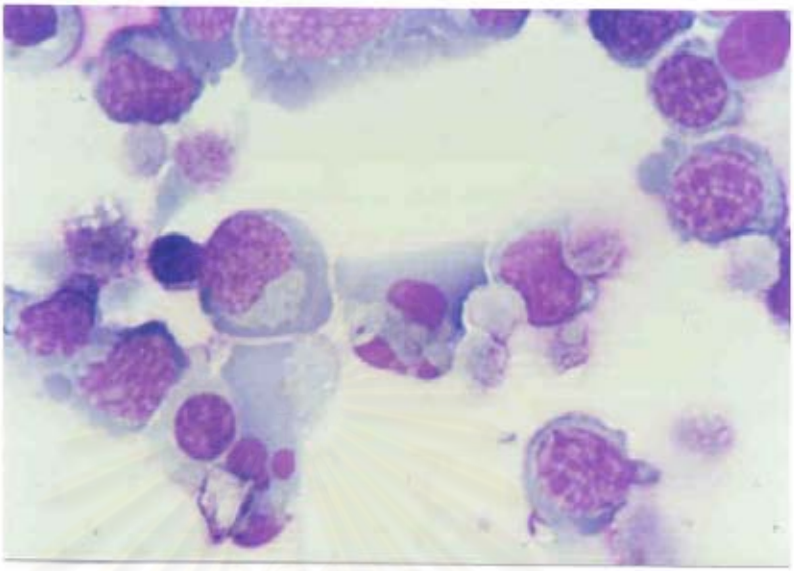
A : 1.0 ppm Pb / day 1



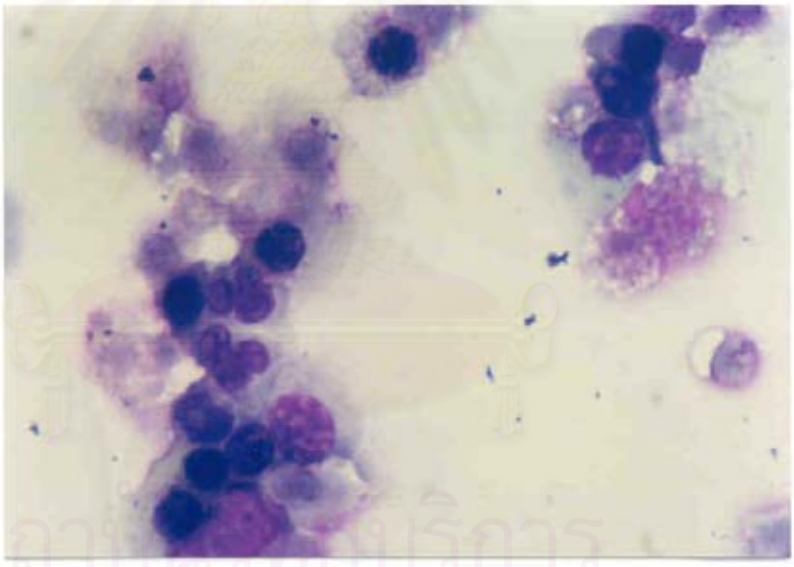
B : 1.0 ppm Pb / day 5

Figure 37 : 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.

121



A : 3.0 ppm Pb / day 1



B : 3.0 ppm Pb / day 5

Figure 38 : 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.

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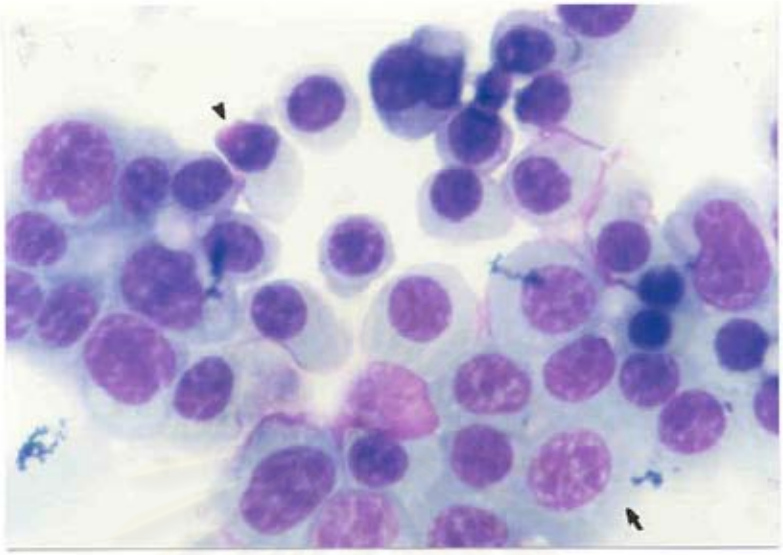
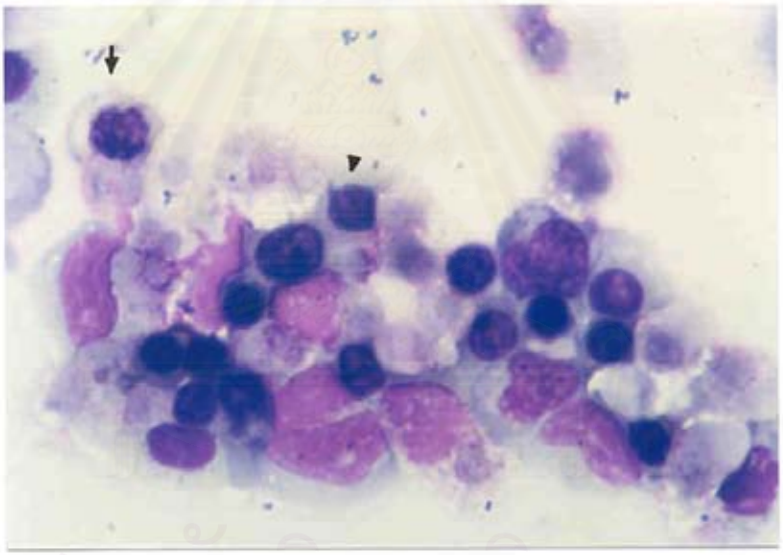


A : 0.0 ppm Pb / day 5



B : 2.0 ppm Pb / day 5

Figure 39 : 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.

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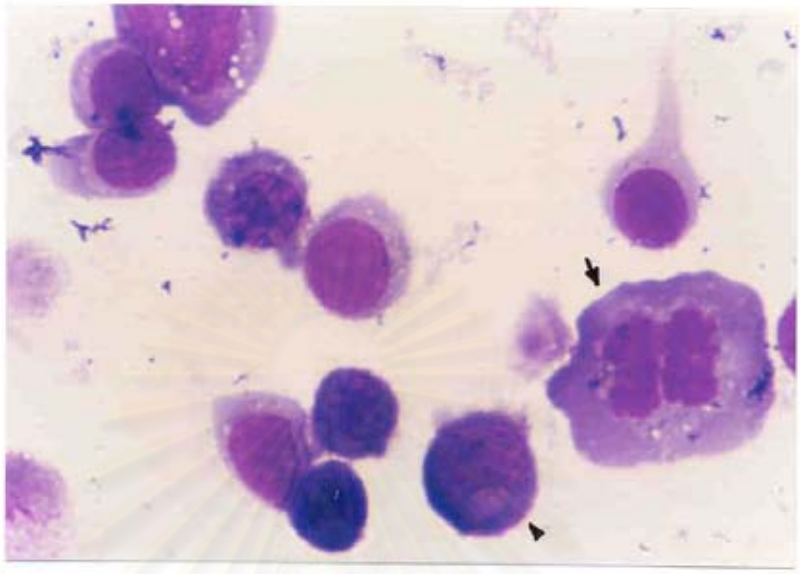
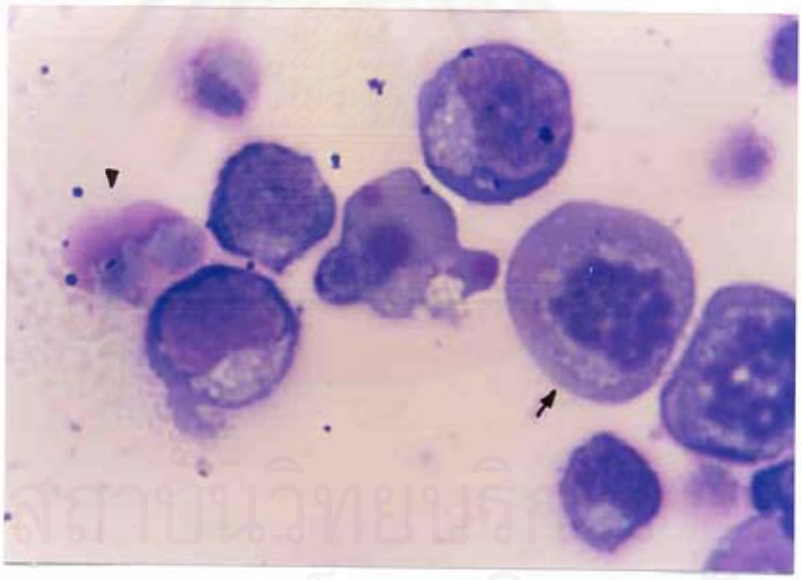


A : 2.0 ppm Pb / day 1



B : 4.0 ppm Pb / day 1

Figure 40 : 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.

tured cells at day 12 in 2^0 culture after exposed to

and basophilic erythroblast at day 8 of secondary culture (Figure 35) and in the stage of polychromatophilic and orthochromatic erythroblast at day 12 of secondary culture (Figure 36). Whereas, a considerable number of dead cells were detected when lead acetate of concentration ≥ 1.0 ppm was added to the medium (Figure 37 - 40). 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 18). This showed that during maturation, in the presence or absence of lead, the expression of transferrin receptor decreased slightly (Figure 41), whereas the expression of glycophorin A increased considerably (Figure 42). 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 43). Although, the inhibition of cell viability by lead was dose-dependent but the maturation of erythroid precursor cells still proceeded.

6.2. 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 44).

Table 18 : 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 Pb 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

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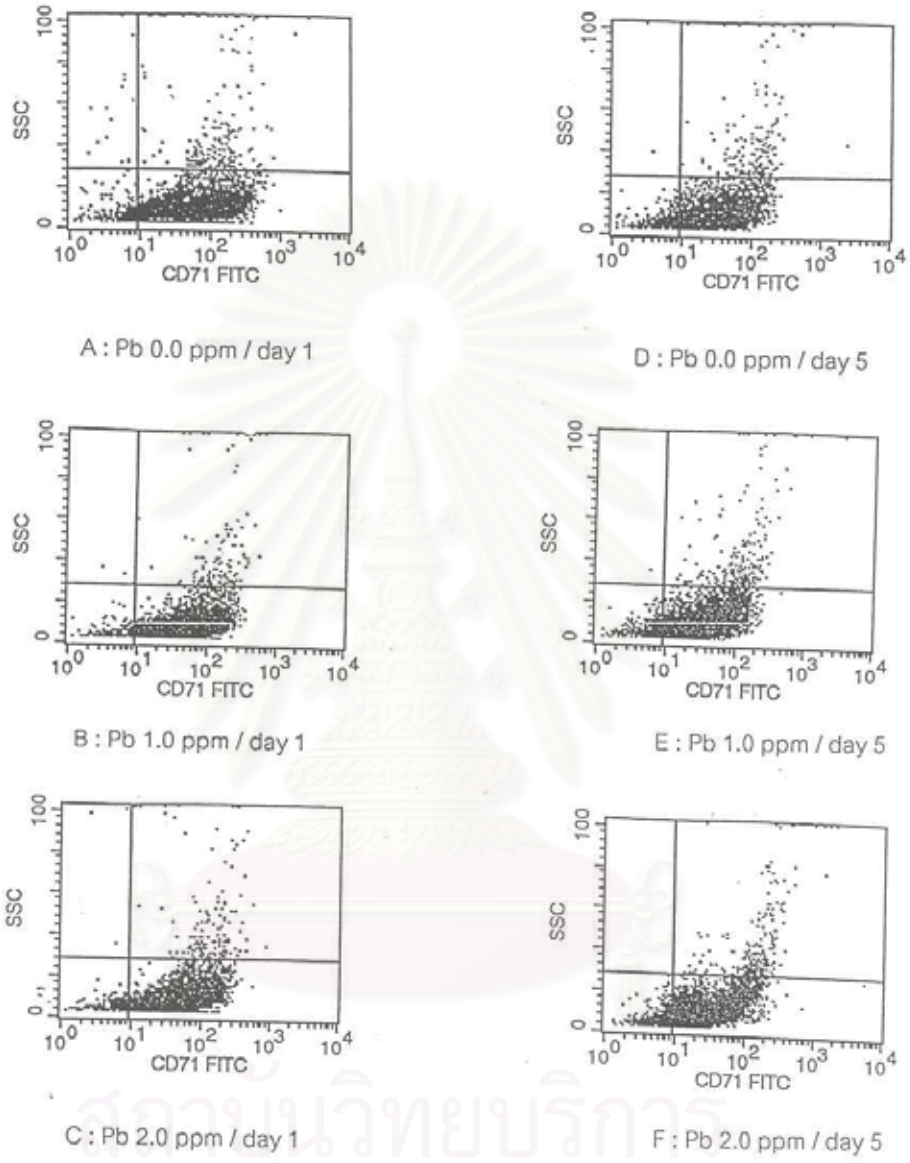
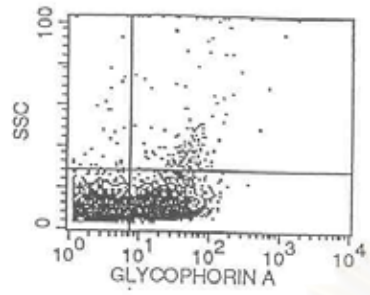
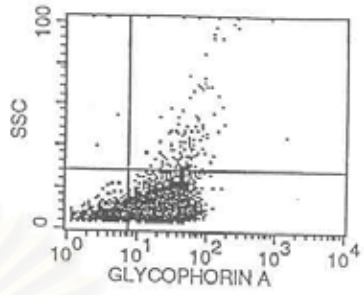


Figure 41 : Flow cytometric analysis of transferrin receptor (CD 71) expression on the cultured cells after exposed to 0.0, 1.0, and 2.0 ppm lead acetate for one (A-C) and five (D-F) days.

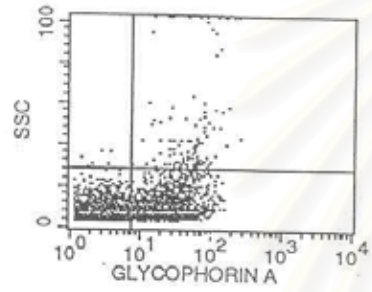
127



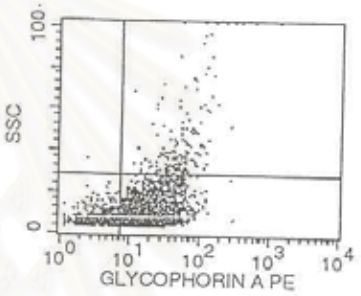
A : Pb 0.0 ppm / day 1



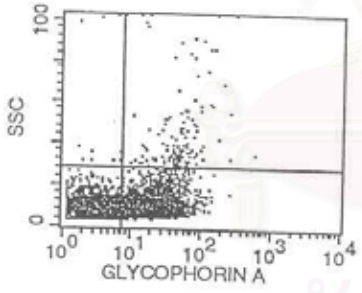
D : Pb 0.0 ppm / day 5



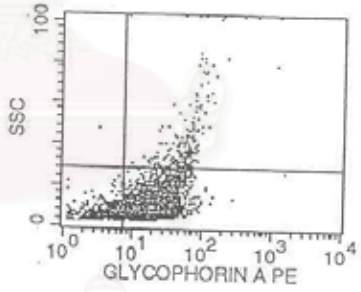
B : Pb 1.0 ppm / day 1



E : Pb 1.0 ppm / day 5



C : Pb 2.0 ppm / day 1



F : Pb 2.0 ppm / day 5

Figure 42 : 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.

128

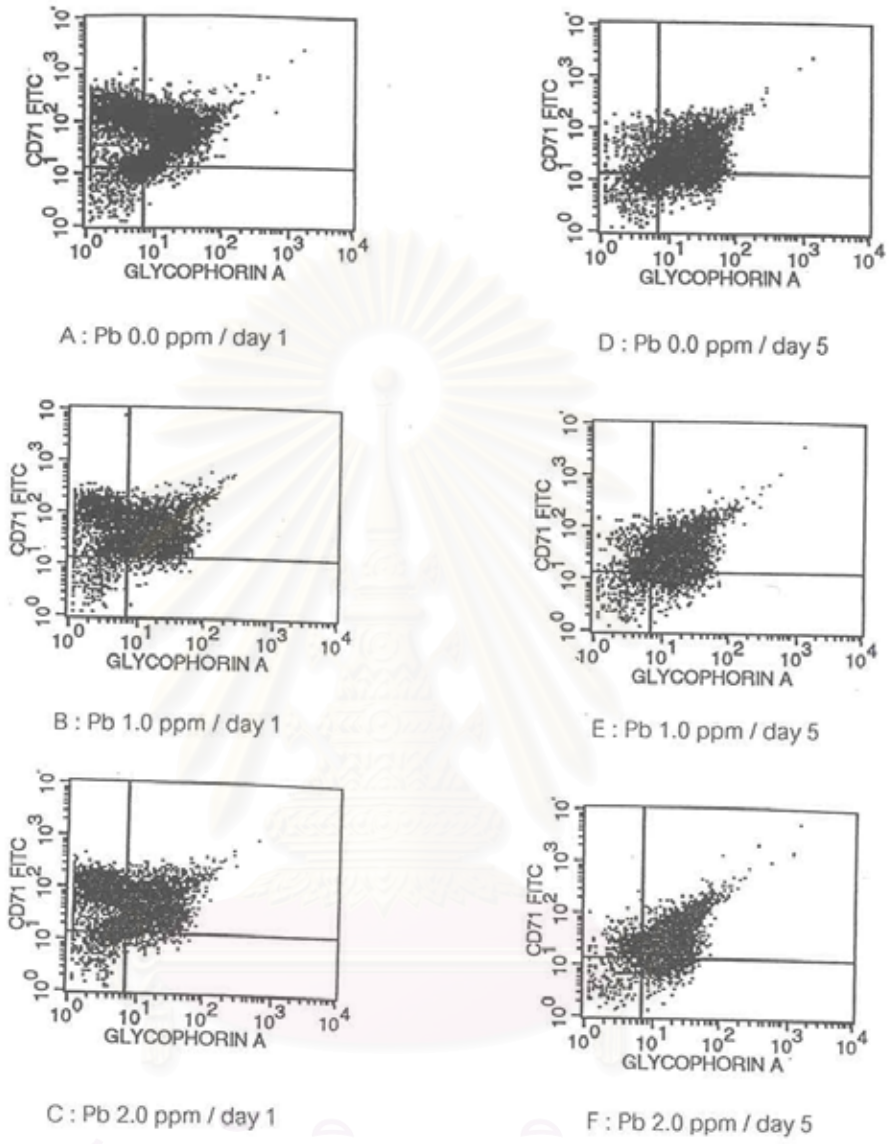


Figure 43 : Correlation between glycophorin A and transferrin receptor (CD 71) expression on the cultured cells after exposed to 0.0, 1.0, and 2.0 ppm lead acetate for one (A-C) and five (D-F) days. The cells that were positive for both glycophorin A and transferrin receptor were identified as erythroid precursor cells.

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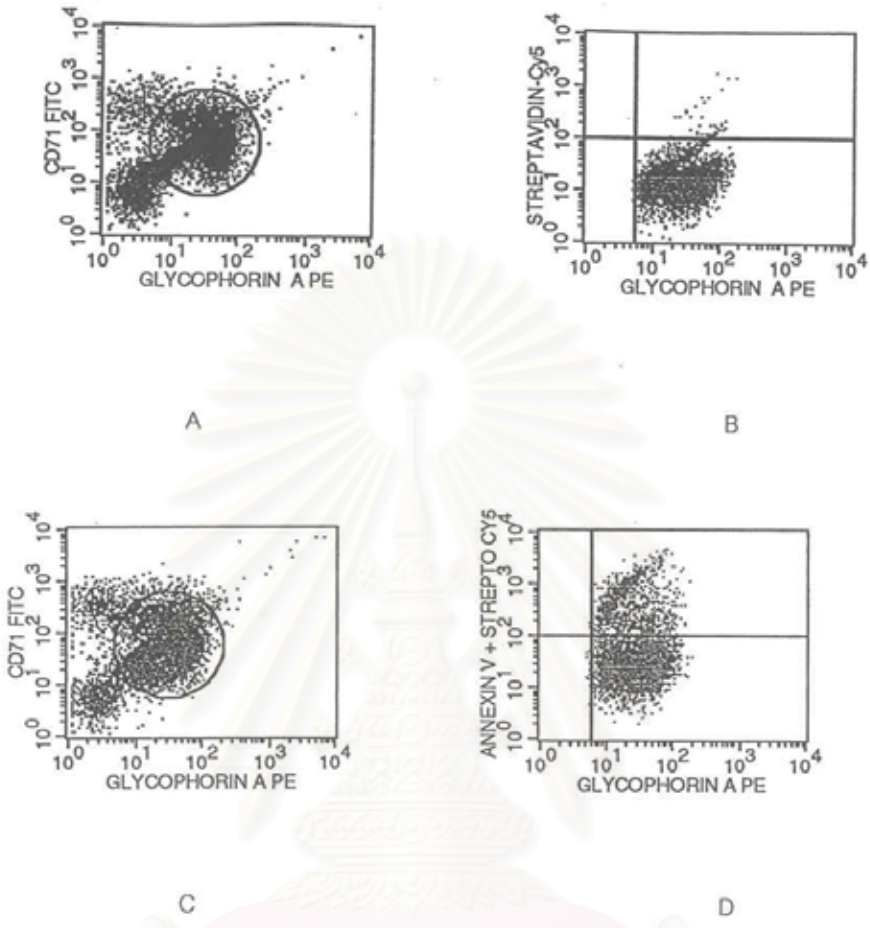

Figure 44 : Flow cytometric analysis of erythroid precursor cells at day 12 of secondary phase. The erythroid precursor cells positively stained for glycophorin A and transferrin receptor (A and C) were gated and analysed for annexin V-staining (B and D).

A and B = The controlled 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 45-46. 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 (Table19). This indicated that, the apoptosis of erythroid precursor cells was induced by lead in dose-depentdent manner at lead acetate concentration ≥ 1.0 ppm (Table 18).

Time-course of the lead effect on apoptosis in erythroid precursor cells (Figure 47) 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-depentdent.

In addition, the correlation between the percent of apoptotic cells and viable cell number was also shown (Figure 48). 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.

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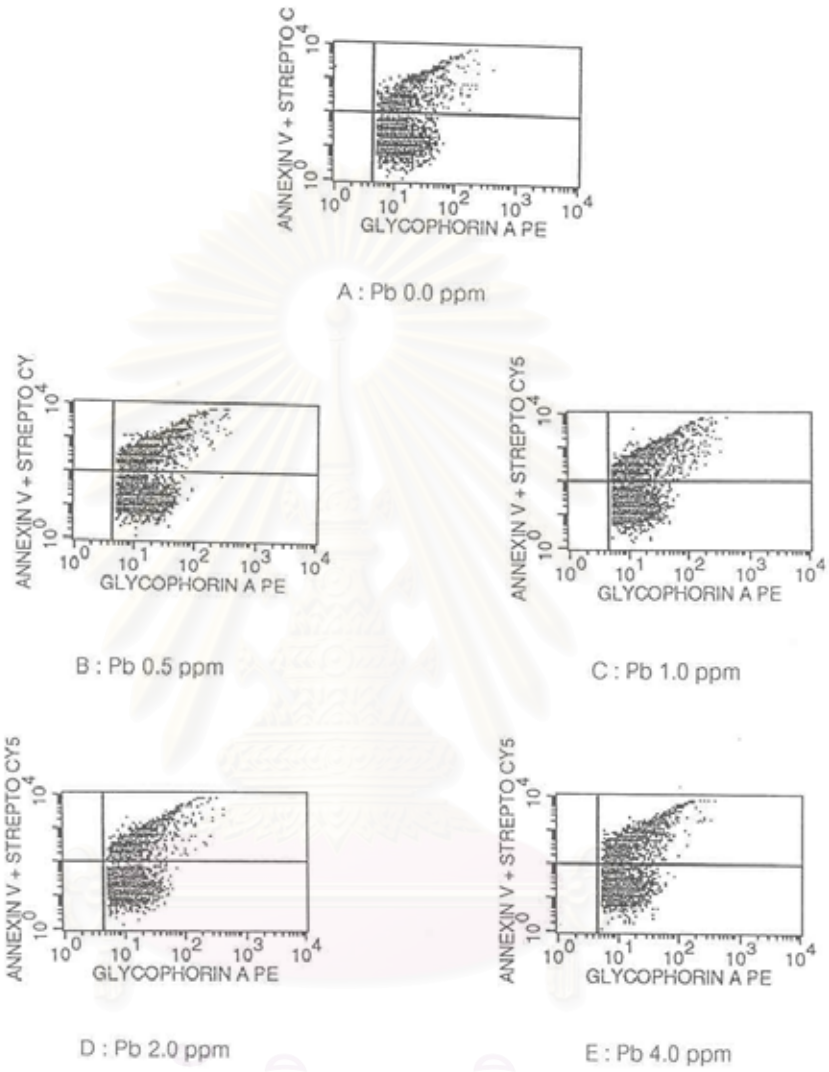

Figure 45 : Apoptosis of erythroid precursor cells exposed to 0.0 (A), 0.5 (B), 1.0 (C), 2.0 (D), and 4.0 (E) ppm lead acetate for one day. After staining, the cells were analysed by flow cytometry. The erythroid precursor cells that expressed both glycophorin A and transferrin receptor (CD 71) were gated and analysed for apoptosis by the appearance of annexin V-binding cells.

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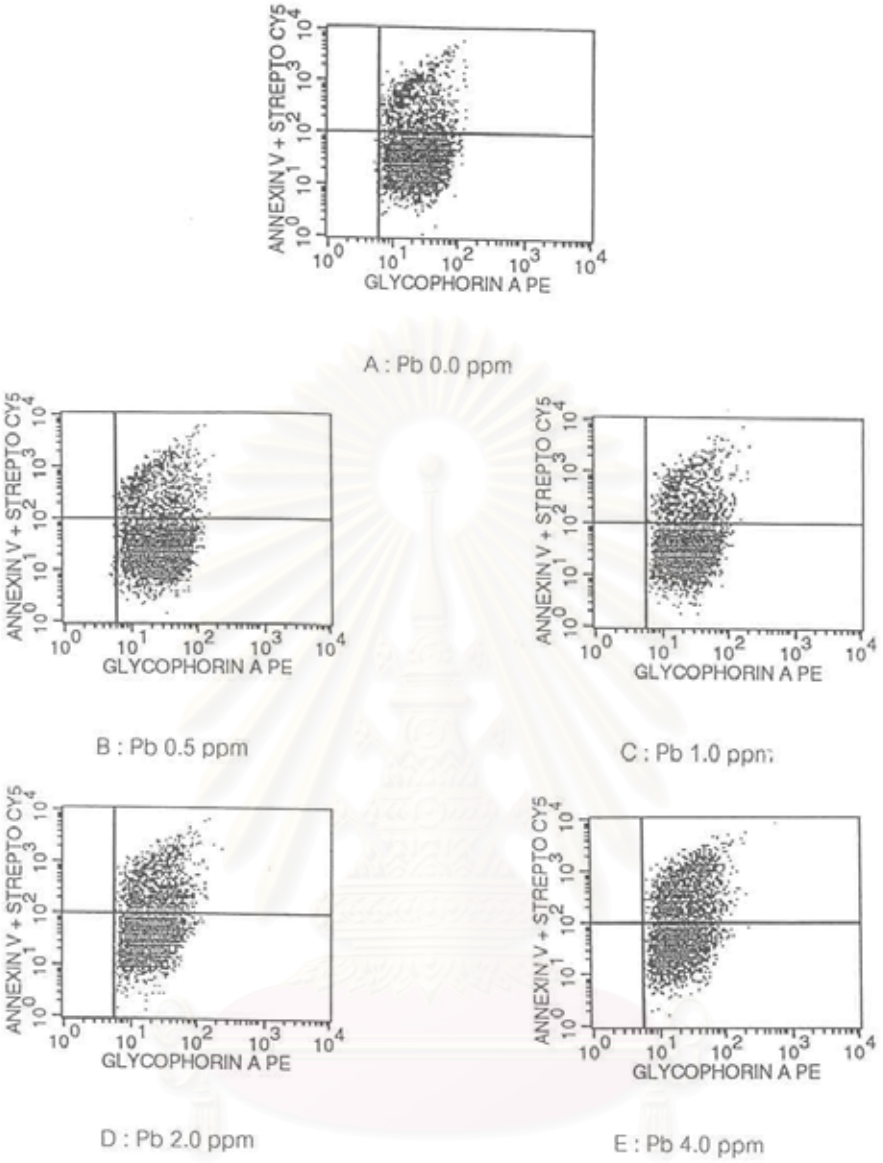

Figure 46 : Apoptosis of erythroid precursor cells exposed to 0.0 (A), 0.5 (B), 1.0 (C), 2.0 (D), and 4.0 (E) ppm lead acetate for five days. After staining, the cells were analysed by flow cytometry. The erythroid precursor cells that expressed both glycophorin A and transferrin receptor (CD 71) were gated and analysed for apoptosis by the appearance of annexin V-binding cells.

Table 19 : 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

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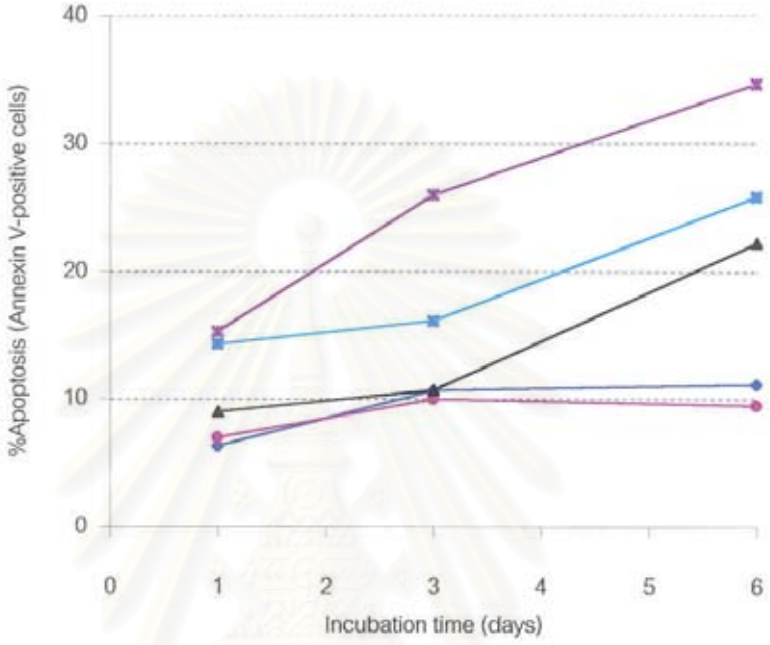

Figure 47 : Time-course of lead effect on apoptosis of erythroid precursor cells. The exposures were with 0.0 ppm, 0.5 ppm, 1.0 ppm , 2.0 ppm and 4.0 ppm , lead acetate for one, three and six days.

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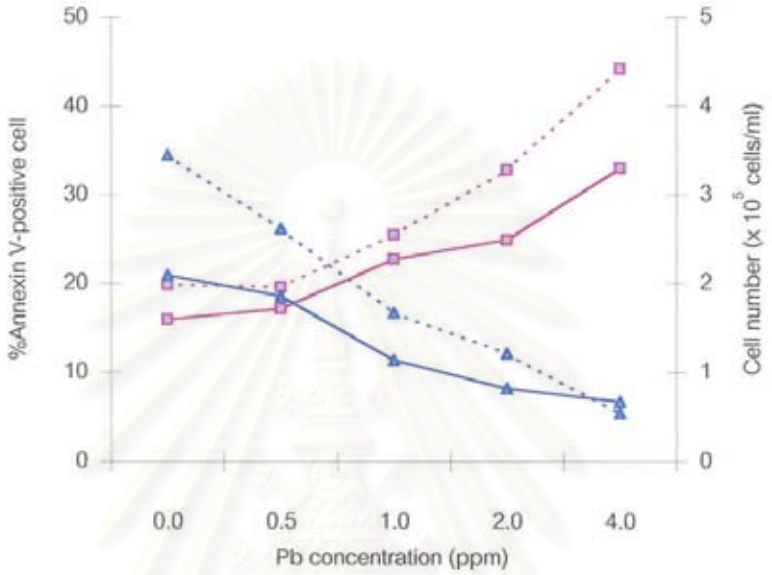

Figure 48 : Apoptosis and cell viability of erythroid precursor cells during culture at various concentrations of lead for one (———) day and five (———) days . After incubation with moderate concentration of lead (0.5 - 4.0 ppm), the percentage of annexin V-positive cells (■) increased with lead concentration 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 6). 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 20 and 21), 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 6). 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 6).

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 Buffer on Lead Uptake by Human Erythrocytes

When human erythrocytes were suspended in different buffers, the rate of lead uptake in α-MEM was highest when compared to that in other buffers and reached the steady state within 15 minutes (Figure 22). Whereas in the other buffers, it could not reach the steady state. The steady state represents a balance between influx and extrusion of lead. It seems possible that essential factors required for lead uptake are present in α-MEM but may be insufficient in the other buffers.

Since the anion exchanger is the main route of lead uptake into erythrocytes, the anion carriers including bicarbonate, carbonate and/or the second anions (such as chloride, bromide, iodide and nitrate) may play an important role for the uptake of lead, probably in the form of lead carbonate ($PbCO_3$) or a ternary $PbCO_3$-anion$^-$ complex (Simons, 1986a, 1986b). The possible important factors for lead uptake into erythrocytes including bicarbonate and halides salts (such as NaCl, KCl, $MgCl_2$, and $CaCl_2$) are contained in α-MEM (Appendix 1) while the other buffers contain only halide salts. This result agrees with the study of Simons (1993a), he found that bicarbonate enhanced lead uptake into human erythrocytes in buffer containing KCl.

Moreover, in α-MEM, lead was rapidly incorporated into human erythrocytes. The result was similar to the study in plasma that reported by Sugawara et al. (1990). In addition, the result of viability study also showed that there were low dead cells (<2%) in erythrocyte population when suspended in α-MEM containing lead (Table 8). So that, α-MEM which was used as the medium for the culture of erythroid precursor cells was also chosen for the study of lead transport into human erythroid precursor cells.

3. Lead Uptake by Human Erythroid Precursor Cells

3.1. Lead Uptake by Erythroid Precursor Cells Suspended in α-MEM

In α-MEM, lead was rapidly incorporated into erythroid precursor cells and reached the steady state within 15 minutes (Figure 23). However, lead content decreased after prolong incubation (60 minutes) which may be due to the increases of lead extrusion rate. A lot of dead cells (~20%) were observed in erythroid precursor cell population both with or without lead, and the number of dead cells increased with incubation time (Table 9). It indicated that α-MEM was unsuitable for erythroid precursor cell survival in prolong incubation.

Although after incubation in α-MEM, lead content in erythroid precursor cells was significantly higher than that in erythrocytes, but higher cytolytic cells were also observed in erythroid precursor cell population. It is possible that, unsuitable factor(s) in α-MEM may induce the impairment of erythroid precursor cell membrane resulting in the high incorporation of lead into these cells. Furthermore, prolong incubation in α-MEM may also enhance lead extrusion, possible by destroying membrane integrity where eventually loss of its homeostasis.

This result suggests that erythroid precursor cells are less stable than erythrocytes. Other factor(s) may be needed to stabilize the former cells during the uptake study.

3.2. Lead Uptake by Erythroid Precursor Cells Suspended in 1%FBS/α-MEM

3.2.1. Viability of Erythroid Precursor Cells Exposed to Lead in 1%FBS/α-MEM

To avoid the unsuitable condition induced cytolysis of erythroid precursor cells, 1%FBS was added to the α-MEM and served as incubation buffer instead of α-MEM alone. The result showed that when 1%FBS was added to the α-MEM, the dead cells in erythroid precursor cell population occurred only 3-9% (Table 11). 1%FBS could

stabilize erythroid precursor cells during lead uptake study. However, prolong incubation to 60 minutes seemed to be unsuitable condition for this study. Because in this incubation time, dead cells became increase (Table 11) and cell loss in each step of the study also became increase (Table 12).

The effect of 1%FBS/α-MEM was more pronounced on the washing step than the incubation step (Figure 10 and 12). It is possible that the major effect of FBS to maintain erythroid precursor cell survival may be the role of FBS which protect the cells from cytolysis after the step of washing with high speed centrifugation.

3.2.2. Time Dependence of Lead Uptake by Erythroid Precursor Cells

When erythroid precursor cells were incubated in 1%FBS/α-MEM (pH 7.4) containing lead at 37°C, lead was rapidly incorporated into the cells and reached the maximum value at 30 minutes (Figure 27). The rate of lead uptake into the cells increased with time. This data suggests that the rate at which lead enter the cells is greater than the rate at which it is extruded from the cells. It is possible that once lead enters the erythroid precursor cells, most of lead may be trapped by intracellular cation binding proteins which could not be extruded from the cells. The nature of the labile lead-binding in erythroid precursor cells has not yet to be determined. In erythrocytes, three proteins including metallothionein (Church et al., 1993), ALAD (Wetmur, 1994), and hemoglobin (Simons, 1986a; Sugawara et al., 1990) have been suggested to bind lead at exposed thiol groups. Thus, these proteins may also be responsible for lead binding in erythroid precursor cells.

However, the decrease in lead content in prolong incubation may be due to the intensive impairment of erythroid precursor cell membrane untill it could not maintain homeostasis. This impairment may also cause the cell cytolysis resulting in the increase of cell death (Table 11 and 12). Therefore, 30 minutes was chosen as the suitable incubation time for further lead uptake study. After incubation in 1%FBS/α-MEM (pH 7.4), only 3% of dead cells were observed at this time (Table 11).

3.2.3. Concentration Dependence of Lead Uptake by Erythroid Precursor Cells

After incubation in 1%FBS/α-MEM (pH 7.4) containing different lead concentrations for 30 minutes, the rate of lead uptake into erythroid precursor cells and erythrocytes increased with extracellular lead concentration which was saturated at about 12 ppm of lead acetate concentration (Figure 28). In addition, lead content in erythroid precursor cells was 4 times higher than erythrocytes ; the maximum value was 1.45 ng/10^5cells in erythroid precursor cells and 0.39 ng/10^5cells in erythrocytes.

The value of lead content in erythrocytes suspended in 1%FBS/α-MEM is comparable to the study in plasma (Sugawara et al., 1990). Thus, 1%FBS/α-MEM is the suitable incubation buffer for lead uptake study.

Since lead content in erythroid precursor cells suspended in 1%FBS/α-MEM containing 10 ppm lead acetate was in the vicinity to its maximum value while the other concentrations below 10 ppm was very far from its maximum value, the concentration of lead acetate at 10 ppm was chosen for the study of lead uptake into human erythroid precursor cells.

The higher lead level in erythroid precursor cells than erythrocytes may be due to their different size or their different mechanism of uptake. There is no report about the mechanism of lead transport into human erythroid precursor cells but many reports of Simons have observed that the major mechanism of lead transport into human erythrocytes is the anion transport system (Simons, 1987a,1987b,1993a). Simons found that over 90% of lead ion (Pb^{2+}) moved across the erythrocyte membrane through the anion channels.

The mechanism of metal transport into human erythroid precursor cells may be different from erythrocytes. The previous study on rabbit reticulocytes by Chua et al. (1996) could identify three saturable manganese transport mechanisms, two for manganese ion (Mn^{2+}) with high and low affininity processes and one for transferrin-bound manganese (Mn-Tf). They also found that high affinity Mn^{2+}transport occurred in reticulocytes but not erythrocytes. It was inhibited by metabolic inhibitors and several

metal ions. While low affinity Mn^{2+}transport occurred in erythrocytes as well as in reticulocytes. It was inhibited by ion transport inhibitors. The uptake of transferrin-bound manganese occurred only with reticulocytes and depended on receptor-mediated endocytosis of Mn-Tf. The characteristics of the three saturable manganese transport mechanisms were similar to corresponding mechanism of iron by erythroid precursor cells, suggesting that the two metals are transported by the same mechanisms. It is possible that high affinity manganese transport is a surface representation of the process responsible for the transport of manganese across the endosomal membrane after its release from transferrin. Low affinity transport probably occurs by the previously decribed Na^{+}-Mg^{2+} antipore.

The same as manganese, lead is divalent metal which is able to bind transferrin in the form of transferrin-bound lead (Pb-Tf) (Suphitcha Mangkalee, 1994). In addition, it also inhibits iron uptake into erythroid precursor cells (Jandl et al., 1959; Boyett and Butterworth, 1962; Kohno et al., 1993; Quian et al., 1997). It is possible that lead and iron may be transported by the same mechanism. Therefore, the mechanism of lead transport into erythroid precursor cells may occur in the other different pathways from that occurs in erythrocytes. One of these mechanisms may be the receptor-mediated endocytosis of Pb-Tf.

4. The Role of Transferrin on Lead Transport into Human Erythroid Precursor Cells

Transferrin is known to act as a general ligand for many metal ions of different charge and size beside the physiological iron ions (Fe^{3+}). Lead ion (Pb^{2+}) is one of the metal ions which can bind to transferrin molecule. The study of Suphitcha Mangkalee in 1994 demonstrated that the binding of lead to transferrin molecule caused the release of iron from this protein. Although the normal substrate for transferrin is ferric ion (Fe^{3+}), the protein is only about 30% saturated with iron in normal serum (Chasteen, 1983), leaving large amount of the apo-form, a considerable binding capacity for other metal ions. With this reason, transferrin may serve as the serum transport agent for the metals, capable of binding to it, such as lead.

Transferrin is the principal iron transport protein in the developing erythroid precursor cells which require a large amount of iron for hemoglobin synthesis and cell proliferation. The extraordinary requirement is supplied by the rapid influx of transferrin-bound iron through the receptor-mediated endocytosis. Relatively high levels of transferrin receptor are found on the surface of the erythroid cells. The result from this study suggests that beside iron, transferrin may also deliver lead to these cells. Since lead content in erythroid precursor cells incubated with lead and transferrin was higher than that incubated with lead alone (Figure 29). Transferrin through its receptor seems to be another route of lead transport into erythroid precursor cells. It is possible that when apo-transferrin is reacted with lead acetate at physiological pH (pH7.4), the binding of lead ion (Pb^{2+}) occurs at the metal binding site of transferrin with the same mechanism of transferrin-bound iron, the transferrin-bound lead (Pb-Tf) may enter into these cells though the surface transferrin receptor.

Similar to iron binding, divalent metal ions including Cd^{2+}(Harris and Madsen, 1988), Mn^{2+}(Harris and Chen, 1994), Ni^{2+}(Harris, 1986) and Zn^{2+}(Harris, 1983) can bind to C-terminal and N-terminal binding sites of transferrin molecule at physiological pH in the presence of bicarbonate. However, the conditional binding constants of transferrin and these metal ions are lower than ferric ion (Fe^{3+}) but higher than ferrous ion (Fe^{2+})

(Table 20). It indicates that the binding of these metal ions to transferrin molecule is weaker than the ferric ion, which further suggests that these metal ions are not competitive with Fe^{3+} in binding to transferrin. Different from these metal ions, small amount of lead can replace iron in transferrin molecule (Suphitcha Mangkalee, 1994). It is possible that lead, a divalent metal ion, may also bind to transferrin molecule at C-terminal and/or N-terminal binding sites. The interpretation is lead binding may weaken the binding of iron to transferrin, and cause the release of iron from transferrin molecule. However, it is not clear about the competitive binding between lead and iron in the metal binding site of transferrin molecule. It is possible that the binding of lead in transferrin molecule may induce a conformational change in the protein resulting in iron released. Thus, transferrin may deliver lead instead of iron into erythroid precursor cells. This may explain the lead inhibition of iron uptake into these cells observed in the previous studies (Jandl et al., 1960; Boyett and Butterworth, 1962).

Table 20 : Conditional binding constants for metal-ion binding to transferrin

Metal ion	Log K1	Log K2	References
Fe^{3+}	22.7	22.1	Martin et al., 1987
Fe^{2+}	3.2	2.5	Harris, 1986
Cd^{2+}	6.0	4.9	Harris and Madsen, 1988
Mn^{2+}	4.1	3.0	Harris and Chen, 1994
Ni^{2+}	4.1	3.2	Harris, 1986
Zn^{2+}	7.8	6.4	Harris, 1983

This study also found that transferrin enhanced lead transport occurred in erythroid precursor cells but not erythrocytes. In erythrocytes, transferrin seems to decrease lead transport (Appendix 2). It is possible that, after binding to transferrin, Pb-Tf cannot enter across erythrocyte membrane because of the disappearance of transferrin receptor in these cells after maturation. This suggests that the uptake of Pb-Tf

seems to occur only with erythroid precursor cells but not erythrocytes and may be depended on receptor-mediated endocytosis of Pb-Tf.

Whilst lead has not yet been shown to have an essential role in normal body metabolism, the ability of transferrin to bind and deliver the element to the cells that express transferrin receptor such as erythroid precursor cells has important implication in the aetiology of lead toxicity. An area of considerable interest at the present time is the possible role of lead in the pathogenesis of a variety of hematopoietic disorders. The possible association between plasma transferrin and the accumulation of lead within erythroid precursor cells may involve in lead induced anemia.

Although transferrin is the major route of iron transport into erythroid precursor cells, it may not be the major route of lead transport into the cells. The result from this study showed that in the presence of transferrin, the average of lead content in these cells increased only 26% of that in the absence (Table 13). This suggests that there are the other routes of lead transport into erythroid precursor cells, of which anion exchanger transport system as in erythrocytes may be the one.

5. Effect of Inhibitors on Lead Uptake in Human Erythroid Precursor Cells

5.1. Effect of Microtubule Inhibitors

The uptake of transferrin-bound iron (Fe-Tf) by erythroid precursor cells involves receptor-mediated endocytosis of Fe-Tf followed by release of iron from transferrin within intracellular vesicle and exocytosis of the apo-transferrin (Morgan, 1981). Furthermore, transferrin also binds the other metals, such as aluminum and manganese, and transport them into the cells in a process involving receptor-mediated endocytosis (Cochran et al., 1991; Chua et al., 1996). The same as these metals, transferrin may transport lead into erythroid precursor cells by the same mechanism.

The mechanism of receptor-mediated endocytosis including receptor internalization and vesicle movement within the cytoplasm appears to require an intact network of cytoplasmic microtubules (De Brabander et al., 1988; Gruenberg et al., 1989). Endocytic vesicles are transported to the cell interior by the dynein motor (Vallee et al., 1989), whereas exocytic vesicles are transported back to the cell surface by the kinesin motor (Vale et al., 1986; Sheetz, 1989). Both of these molecular motors require intact cytoplasmic microtubules and are inhibited by microtubule depolymerizing agents (Vallee et al., 1989; Vale et al., 1986; Sheetz, 1989; Schroer and Sheetz, 1989). These agents interfere with normal assembly and disassembly of microtubules resulting in microtubule depolymerization. Microtubule inhibitors such as colchicine and vinblastine have been found to inhibit transferrin and iron uptake in erythroid precursor cells, possibly because they play a role in endocytosis and exocytosis (Hemmaphardh, Kailis, and Morgan, 1974; Thatte et al., 1994). Thus, in this study, the possible involvement of the receptor-mediated endocytosis in lead uptake mechanism was investigated by using agents which interfere the function of these cellular components such as colchicine and vinblastine. Colchicine and vinblastine irreversibly disrupt microtubules (Structures in appendix 3). Colchicine combines specifically with tubulin heterodimers and blocks the addition of further tubulin subunits to the plus end of microtubules. Presumably, the capped microtubules then disassemble from their minus ends. Vinblastine links heterodimers into large semicrystalline aggregates. The linkage

removes the heterodimers from the cytoplasmic pool and pushes the heterodimer-microtubule equilibrium in the direction of microtubule disassembly (Wofe, 1993).

The result from this study showed that both microtubule inhibitors could inhibit lead uptake in erythroid precursor cells ; vinblastine caused a marked inhibition of lead uptake while colchicine caused a small degree of inhibitor (Table 15). However, there was no marked difference between the lead uptake in the presence of colchicine and in the absence of apo-transferrin. Many previous studies reported that these inhibitors could prevent transferrin receptor-mediated endocytosis in a wide variety of cell types including immature erythroid cells (Hemmaphardh et al., 1974; Morgan and Iacopetta, 1987; Thatte et al., 1994). Colchicine and vinblastine immobilize cell surface transferrin receptor by causing microtubule depolymerization (Thatte et al., 1994). Possibly microtubules play a role in the movement of endocytic vesicles in nucleated erythroid cells (Iacopetta, Morgan, and Yeoh, 1983). Disruption of microtubule function could impede this movement in a non-specific manner. It is possible that the inhibitory effect of these inhibitors on lead uptake may occure by their action on transferrin endocytosis. This observation suggests that transferrin may transport lead into human erythroid precursor cells by the mechanism of receptor-mediated endocytosis.

This study also found that there was marked difference between the lead uptake in the presence of vinblastine and in the absence of transferrin. It indicated that not only transferrin endocytosis, vinblastine also inhibited the other mechanisms which involved in lead uptake, in agreement with the study in iron uptake of Hemmaplardh et al. (1974). The latter investigators found that the inhibitory effect of vinblastine was not confined to the uptake of transferrin, the agent also inhibited other mechanisms of iron uptake including citrate-bound iron. It is possible that the inhibitory action of vinblastine is not only mediated via the impairment of transferrin endocytosis. Its action may lead to the alternation of structural component of cell membranes resulting in disturbance of the other lead uptake mechanisms. This action may be due to non-specific interactions with membrane lipids (Seeman, Chau-Wong, and Moyyen, 1973) or to a more specific action on spectrin (Jacob, 1975).

5.2. Effect of Anion Transport Inhibitor

The anion exchange protein (band 3), a transmembrane glycoprotein with a molecular weight of 100,000, is the most abundant protein of human erythrocyte membrane. It acts as anion transporter and serve as the binding site of the skeleton (Tanner,1993). This protein is responsible for the exchange of bicarbonate and chloride across erythrocyte membrane (Jenning, 1985; Knauf, 1987). The transport process facilitated by band 3 is electrically neutral. One negatively charged bicarbonate ion is exchanged for one negatively charged chloride ion. No energy is required for the process. Furthermore, it is also the major transport mechanism for lead uptake, probably in the form of lead carbonate ($PbCO_3$) or the ternary $PbCO_3$-anion$^-$ complex (Simons, 1986a, 1986b, 1993a). Since the synthesis of this protein is initiated at the proerythroblast stage and increases up to the late erythroblast stage (Hanspal and Palek, 1992). It is possible that, the same as erythrocytes, the anion exchanger may play a role in lead uptake in the late stage of erythroid precursor cells.

In this study DIDS, the anion transport inhibitor, was used to study the involvement of the anion exchanger mechanism in lead uptake in human erythroid precursor cells. This inhibitor can bind tightly and irreversibly to band 3 (Cabantchik and Rothstein, 1974). The result showed that in the absence of transferrin, about 15% of lead uptake by erythroid precursor cells was inhibited by 1,000 μM DIDS. It indicated that, not only the mechanism of transferrin receptor-mediated endocytosis, the anion exchanger may serve as another lead uptake mechanism in erythroid precursor cells. However, higher inhibition (~60%) of lead uptake has occurred in human erythrocytes suspended in serum containing the same concentration of DIDS (Simons, 1993a). It is possible that the role of anion exchanger on lead uptake in human red blood cells may depend on amount of the anion exchange protein which assemble on their plasma membrane. This suggests that the anion exchanger is the major lead uptake mechanism in erythrocytes but not in erythroid precursor cells, probably due to lower expression of the anion exchange protein in the latter cell membrane (Hanspal and Palek, 1992).

Although lead uptake slightly decreased at 1,000 μM DIDS, it markedly increased at higher concentration. The increase may not relevant to the transport mechanism but may be due to the destruction of cell membrane. Because of incomplete membrane assembly and remodeling (Chasis et al., 1989), erythroid precursor cell membrane may be easily disrupt by high dose of DIDS.

However, both microtubule inhibitors and anion transport inhibitor could not completely block lead uptake in these cells, suggesting that there may be another lead uptake mechanism.

6. Effect of Lead on Human Erythroid Precursor Cells

6.1. 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 37-40). 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 ≥ 1.0 ppm (Figure 47). 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 49). 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

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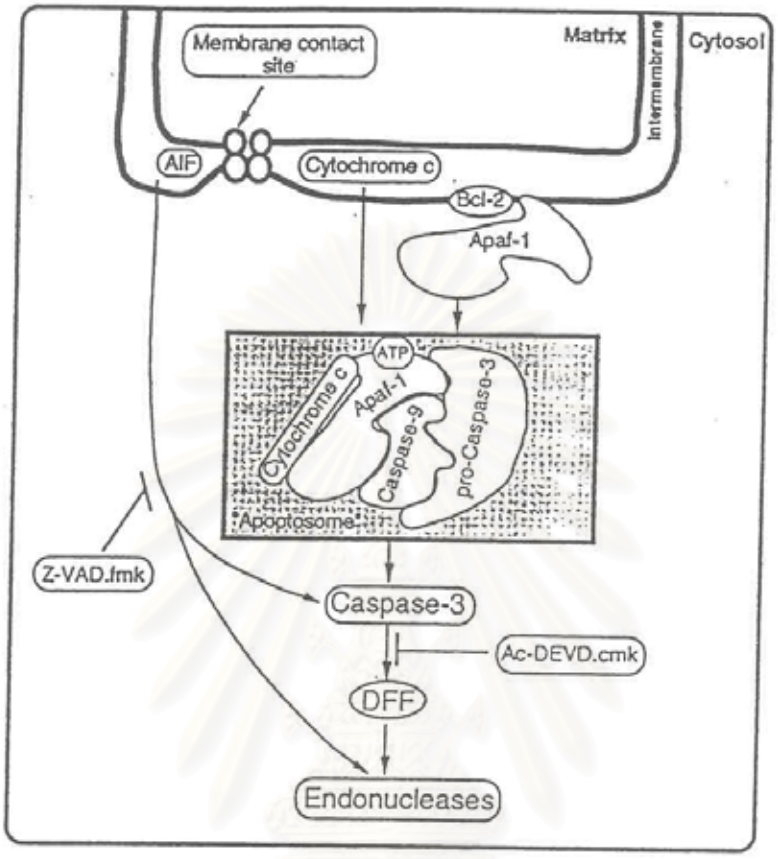

Figure 49 : Caspase and nuclease activation after release of soluble proteins from mitochondria. Several different factors are released from mitochondria during apoptosis : AIF (which is associated with the intermembrane space), cytochrome c (in the intermembrane space) and Apaf-1 (associated with the 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 50). 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

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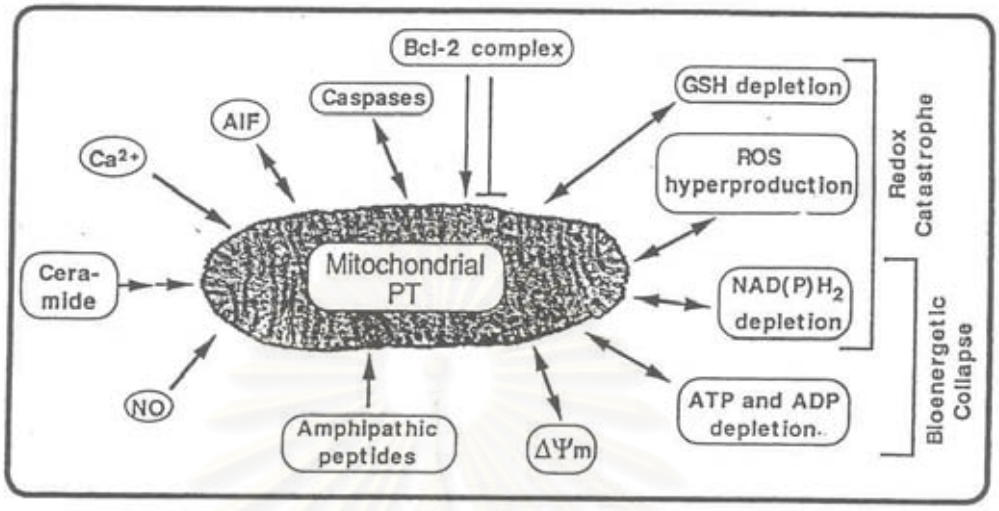


Figure 50 : Inducers of permeability transition. Different signal transduction pathways can promote the activation of caspases, increases in cytosolic Ca^{2+} level, nitric oxide, amphipathic peptides and ceramide, which can provoke PTP either in a direct or an indirect fashion. In addition, changes 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. The Study of Lead Uptake in Human Erythroid Precursor Cells

From the TPLC system, The late erythroid precursor cells at day 12 of secondary culture which still contained high levels of transferrin receptors were used to study lead uptake. 1%FBS/α-MEM (pH 7.4) was chosen as incubation medium for this study because it could maintain erythroid precursor cell survival during lead uptake study, probably by protecting the cells from cytolysis after the step of washing with high speed centrifugation.

In 1%FBS/α-MEM (pH 7.4), lead was rapidly incorporated into human erythroid precursor cells and reached the maximum value at 30 minutes, and lead content was higher than that in erythrocytes, probably due to their different size or their different mechanism of uptake. The rate of lead uptake into erythroid precursor cells increased with extracellular lead concentration and time. However, prolong incubation may cause the intensive impairment of erythroid precursor cell membrane. The loss of homeostasis may result in the decrease in lead content.

Transferrin could enhance lead transport into erythroid precursor cells but not erythrocytes, suggesting that transferrin may bind and deliver lead to erythroid precursor cells through the receptor-mediated endocytosis. The result corresponds to the high levels of transferrin receptors on erythroid precursor cells and the disappearance in erythrocytes. However, in the presence of transferrin, the lead content in erythroid precursor cells was slightly increased (only 26%), suggesting that the mechanism involved in transferrin may not be the major route of lead uptake in these cells. Another route of lead uptake in these cells may involve with anion exchanger system.

Microtubule inhibitors (colchicine and vinblastine) and anion transport inhibitor (DIDS) were used to study the mechanism of lead uptake in erythroid precursor cells. This study suggested that at least two mechanisms may be responsible for the uptake of lead across plasma membrane of erythroid precursor cells. One logical possibility is that lead follows the same route as iron, binding to the transferrin in plasma, and taken up

into the cells by transferrin receptor-mediated endocytosis. The second route may be the anion exchanger system. However the anion transport inhibitor provided a slight inhibition of lead uptake, suggesting that the anion exchanger system is not the major route of lead uptake mechanism.

Moreover, both microtubule inhibitors and anion transport inhibitor could not completely block lead uptake in erythroid precursor cells, suggesting that not only transferrin receptor-mediated endocytosis and anion exchanger system, there may be another mechanism of lead uptake in these cells. Additional studies are required for a better understanding of this mechanism.

3. 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.

REFERENCES

ภาษาไทย

ชุติมา นุ่นมัน. 30 กันยายน 2543. ชาวบ้านคลิตี้ อีกหนึ่งตัวอย่างความล้มเหลวในการแก้ไขปัญหามลพิษ. มติชนรายวัน: 6-7.

ทวีศักดิ์ บุณยโชติมงคล และ คณะ. 2531. โลหะหนักในอาหารไทย. โภชนาการสาร 22: 368-401.

นิตยา มหาผล. 2534. มลพิษทางน้ำ. ใน การประชุมวิชาการพิษวิทยา 2534 เรื่องมลพิษสิ่งแวดล้อม : น้ำ และ อากาศ, หน้า 37-42. 20-22 พฤศจิกายน 2534 ณ กระทรวงวิทยาศาสตร์และเทคโนโลยี และการพลังงาน กรุงเทพฯ.

นภวัส บัวสรวง. 2536. การปนเปื้อนของตะกั่วในพืชบางชนิดจากเขตอุตสาหกรรมในจังหวัดสมุทรปราการ. วิทยานิพนธ์ปริญญาวิทยาศาสตรมหาบัณฑิต สหสาขาวิชาวิทยาศาสตร์สภาวะแวดล้อม บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย.

ปรีญาพร สุวรรณเกษ. 2535. การปนเปื้อนของสารตะกั่วในดินริมถนนบริเวณกรุงเทพมหานครและปริมณฑล. วิทยานิพนธ์ปริญญาวิทยาศาสตรมหาบัณฑิต สหสาขาวิชาวิทยาศาสตร์สภาวะแวดล้อม บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย.

อานนท์ บุณยะรัตเวช. 2535. การสร้างเม็ดเลือดแดง. ใน โลหิตวิทยาเม็ดเลือดแดง, หน้า 23-32. กรุงเทพฯ : ห้างหุ้นส่วนจำกัด ฟันนี่พับบลิชชิ่ง.

ภาษาอังกฤษ

Adamson, J.W. 1994. The relationship of erythropoietic and iron metabolism to red blood cell production in human. Semin. Oncol. 21 (Suppl 3): 9-15

Adonaylo, V.N., and Oteiza, P.T. 1999. Lead intoxication: Antioxidant defenses and oxidative damage in rat brain. Toxicology 135: 77-85.

Albahary, C. 1972. Lead and hemopoiesis: The mechanism and consequences of the erythropathy of occupational lead poisoning. Am. J. Med. 25: 367-378.

Alda, J.Q., and Garay, R. 1989. Evidence for a major route for zinc uptake in human red blood cells: $[Zn(HCO_3)_2Cl^-]$ influx through the $[Cl^-/ HCO_3^-]$ anion exchanger. J. Cell. Physiol. 138: 316-323.

Alda, J.Q., and Garay, R. 1990. Chloride (or bicarbonate)-dependent copper uptake through the anion exchanger in human red blood cells. Am. J. Physiol. 259: 570-576.

Alexander, F.W.; Clayton, B.E.; and Delves, H.T. 1974. Mineral and trace-metal balances in children receiving normal and synthesis diets. Q.G. Med. 43: 89-111.

Ali, M.A.M., and Quinlan, A. Effect of lead on globin synthesis *in vitro*. Am.J. Clin. Pathol. 67: 77-79.

Allen, D.W., and Jandl, J.H. 1960. Kinetic of intracellular iron in rabbit reticulocytes. Blood 15: 71-81.

Allison, A.C., and Cecil, R. 1958. The thiol groups of normal adult human hemoglobin. Biochem. J. 69: 27-34.

Al-modhefer, A.J.A.; Bradbury, M.W.B.; and Simons, T.J.B. 1991. Observations on the chemical nature of lead in human blood serum. Clin. Sci. 81: 823-829.

Anderson, G.J.; Powell, L.W.; and Halliday, J.W. 1990. Transferrin receptor distribution and regulation in the rat small intestine: Effect of iron stores and erythropoiesis. Gastroenterology 98: 576-585.

Andree, H.A.M., et al. 1990. Binding of vascular anticoagulant α (VAC α) to planar phospholipid bilayer. J. Biol. Chem. 265: 4923-4928.

Aramini, J.M.; Saponja, J.A.; and Vogel, H.J. 1996. Spectroscopic studies of the interaction of aluminum (III) with transferrin. Coord. Chem. Rev. 149: 193-229.

Ariza, M.E.; Bijur, G.H.; and Williams, M.V. 1998. Lead and mercury mutagenesis: Role of H_2O_2, superoxide dismutase, and xanthine oxidase. Environ. Mol. Mutagen. 31: 352-361.

Bagby, G.C. 1994. Hematopoiesis. In G.Stamatoyannopoulos; A.E. Nienhuis; P.W. Majererus; and H. Varmus (eds.), The molecular basic of blood diseases. 2nd ed., pp 71-103. Philadelphia: WB. Saunders company.

Baker, E.N. 1994. Structure and reactivity of transferrin. Adv. In. Org. Chem. 41: 389-463.

Barltrop, D., and Smith, A. 1971. Interaction of lead with erythrocytes. Experientia 27: 92-93.

Barltrop, D., and Smith, A. 1975. Kinetics of lead interaction with human erythrocytes. Postgrad. Med. J. 51: 770-773.

Barltrop, D., et al. 1974. Subcellular distribution of lead in the rat. J. Lab. Clin. Med. 77: 705-712.

Barton, J.C.; Conrad, M.E.; Nuby,S.; and Harrison, L. 1978. Effects of iron on the absorption and retention of lead. J. Lab. Clin. Med. 92: 536-547.

Barton, J.C. 1989. Retention of radiolead by human erythrocytes *in vitro*. Toxicol. Appl. Pharmacol. 99: 314-322.

Barry, P.S.I. 1975. A comparison of concentration of lead in human tissue. Br. J. Ind. Med. 32: 119-139.

Becker, F., and Duhm, J. 1978. Evidence for anionic cation transport of lithium, sodium and potassium across the human erythrocyte membrane induced by divalent anions J. Physiol. 282: 149-168.

Bergdahl, I.D.; Grubb, A.; Schutz, A.; Deshick, R.J.; Wetmur, J.G.; Sassa, S.; and Skerfving, S. 1997. Lead binding to delta-aminolevulinate dehydratase (ALAD) in human erythrocytes. Pharmaco. Toxico. 81: 153-158.

Bernardi, P. 1996. The permeability transition pore: Control points of a cyclosporin A-sensitive mitochondrial channel involved in cell death. Biochim. Biophys. Acta. 1275: 5-9.

Bernardi, P., and Petronilli, V. 1996. The permeability transition pore as a mitochondrial calcium release channel: A critical appraisal. J. Bioenerg. Biomembr. 28: 131-138.

Bernardi, P. 1999. Mitochondrial transport of cations: Channels, exchangers, and permeability transition. Physiol. Rev. 79: 1127-1155.

Bernardi, P.; Vassanelli, P.; Veronese, R.; Colonna, I.; and Zoratti, J. 1992. Modulation of the mitochondrial permeability transition pore: Effect of protons and divalent cations. J. Biol. Chem. 267: 2934-2939.

Bessis, M.C., and Breton-Gorius, J. 1959. Ferritin and Ferruginous micelles in normal erythroblasts and hypochromic hypersideremic anemias. Blood 14: 423-427.

Beyersmann, D. 1994. Interaction in metal carcinogenicity. Toxicol. Lett. 72: 333-338.

Bondy, S.C., and Guo, S.X. 1996. Lead potentiates iron induced formation of reactive oxygen species. Toxicol. Lett. 87: 109.

Bondurant, M.C., and Koury, M.J. 1998. Origin and development of blood cells. In G.R. Lee; J. Foerster; J. Lukens; F. Paraskevas; J.P. Greer; and G.M. Rodgers (eds.), Wintrobe' clinical hematology. Vol.1. 10th ed., pp 56-71. London: A waverly company.

Borel, J.F., and Ryffel, B. 1985. The mechanism of action of cyclosporin A continuing puzzle. In R. Schnidler (ed.), Cyclosporin in autoimmune diseases, pp 24-32. Heidelberg: Springler-Verlag.

Borella, P., and Giardino, A. 1991. Lead and cadmium at very low doses effect *in vitro* immune response of human lymphocytes. Environ. Res. 55: 167-177.

Boucher, A.; Rio, B.; Guinard-Flament, J.; and Parent-Massin, D. 1998. *In vitro* effects of lead acetate on human erythroblastic progenitor. Toxico. Lett. 95: 127-132.

Boyett, J.D., and Butterworth, C.E. 1962. Lead poisoning and hemoglobin synthesis: report of a study of fifteen patients with chronic lead intoxication. Am. J. Med. 32: 884-890.

Bruenger, F.W.; Stevens, W; and Stover, B.J. 1969. Americium-24 in the blood: *In vivo* and *in vitro* observations. Radiat. Res. 37: 349-360.

Bruenger, F.W.; Stevens, W; and Stover, B.J. 1973. The association of ^{210}Pb with constituents of erythrocytes. Health Phys. 25: 37-42.

Bull, B.S., and Gorius, J.B. 1995. Morphology of the erythron. In E. Beutler; M.A. Lichtman; B.S. Coller; and T.J. Kipps (eds.), Williams: Hematology. 5th ed., pp 349-361. New York: Megraw Hill.

Burgess, A.W., and Metcalf, D. 1980. The nature and action of granulocyte-macrophage colony stimulating factor. Blood 56: 947-958.

Cabantchik, Z.I., and Rothstein, A. 1974. Membrane protein related to anion permeability of human red blood cells. I. Localization of disulfonic stilbene binding sites involved in permeation. J. Membr. Biol. 15: 207-226.

Calabrese, E.J., and Baldwin, L.A. 1992. Lead-induced cell proliferation and organ specific tumorigenicity. Drug. Metab. Rev. 24: 409-416.

Carelli, G.; Sannolo, N.; De Lorenzo, G.; and Castellino, N. 1995. Lead exposure. In N. Castellino; N. Sannolo; and P. Castellino (eds.), Inorganic lead exposure: Metabolism and intoxication, pp 13-111. London: Lewis publishers.

Castellino, N., and Aloj. S. 1969. Intercellular distribution of lead in the liver and kidney of the rat. Br. J. Ind. Med. 26: 139-143.

Castellino, N., and Castellino, P. 1995. Lead metabolism. In N. Castellino; P. Castellino; and N. Sannolo (eds.), Inorganic lead exposure : Metabolism and intoxication, pp 115-202. London: Lewis publishers.

Chao, D.T., and Korsmeyer, S.J. 1998. BCL-2 family: Regulators of cell death. Annu. Rev. Immunol. 16: 395-417.

Chasteen, N.D. 1983. Transferrin: A perspective. Adv. Inorg. Biochem. 5: 201-203.

Chua, A.C.G.; Stonell, L.M.; Savigni, D.L.; and Morgan, E.H. 1996. Mechanisms of manganese transport in rabbit erythroid cells. J. Physiol. (London). 493: 99-112.

Church, H.J.; Philip Day, J.; Braithwaite, R.A.; and Brown, S.S. 1993. Binding of lead to a metallothionein-like protein in human erythrocytes. J. Inorg. Biochem. 49: 55-68.

Clarke, B.J., and Housman, D. 1977. Characterization of an erythroid precursor cell of high proliferative capacity in normal peripheral blood. Proc. Natl. Acad. Sci. USA. 74: 1105-1109.

Clark, M.; Royal, J.; and Seeler, R. 1988. Interaction of iron deficiency and lead and the hematologic findings in children with severe lead poisoning. Pediatrics 81: 247-254.

Cochran, M.; Chawtur, V.; Jones, M.E.; and Marshall, E.A. 1991. Iron uptake by human reticulocytes at physiologic and sub-physiologic concentrations of iron transferrin: The effect of interaction with aluminum transferrin. Blood 77: 2347-2353.

Cohen, M.; Latta, D.; Coogan, T.; and Costa, M. Mechanisms of metal carcinogenesis: The reactions of metals with nucleic acid. In E.S. Foulkes. (ed.), Biological effects of heavy metal. Vol. 2: Metal carcinogenesis, pp 19-76. Boston: CRC Press.

Cook, L.R.; Stons, S.J.; Angle, C.R.; Hickman, T.I.; and Maxell, R.C. 1987. Erythrocyte membrane microviscosity and phospholipid composition in lead workers. Br. J. Ind. Med. 44: 841-844.

Czak, D., and Aisen, P. 1988. Spectroscopic and thermodynamic studies on the binding of gadolinium (III) to human serum transferrin. Biochemistry 27: 1075-1080.

Dai, C.H.; Price, J.O.; Brunner, T.; and Krantz, S.B. 1998. Fas ligand is present in human erythroid colony-forming cells and interacts with Fas induced by interferon γ to produce erythroid cell apoptosis. Blood 91: 1235-1242.

De Brabander, M.; Nuydens, R.; Gurts, H.; and Hopkins, C.R. 1988. Dynamic behaviour of the transferrin receptor followed in living epidermoid carcinoma (A431) cells with nanovid microscopy. Cell. Motil. Cytoskeleton 9: 30-47.

Desilva, P.E. 1981. Determination of lead in plasma and studies on its relationship to lead in erythrocytes Br. J. Ind. Med. 38: 209-217.

Dessypris, E.N. 1998. Erythropoiesis. In G.R. Lee; J. Lukens; F. Paraskevas; J.P. Greer; and G.M. Rodgers (eds.), Wintrobe' clinical hematology. Vol. 1. 10th ed., pp 169-191. London: A waverly company.

Dini, L., et al. 1999. Citrate carrier and lipogenic enzyme activities in lead nitrate induced proliferative and apoptotic phase in rat liver. Biochem. Mol. Biol. Int. 47: 607-614.

Downward, J. 1998. Mechanism and consequences of activation of protein kinase B/AKT. Curr. Opin. Cell. Biol. 10: 262-267.

Duffield, J.R.; May, P.M.; and Williums, D.R. 1984. Computer stimulation of metal ion equilibrium in biofluids. IV. Plutonium specification in human blood plasma and chelation therapy using polyaminopolycarboxylic acid. J. Inorg. Biochem. 20: 199-214.

Eaves, C.J., and Eaves, A.C. 1978. Erythropoietin (Epo) dose-response curves for three classes of erythroid progenitors in normal human marrow and in patients with polycythemia vera. Blood 52: 1196-1210.

Eguchi, Y.; Shimizu, S.; and Trujimoto, Y. 1997. Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer. Res. 57: 1835-1840.

Erslve, A.J. 1997. Clinical erythrokinetics: A critical review. Blood 11: 160-167.

Evans, G.W., and Winter, T.W. 1975. Zinc transport by transferrin in rat portal blood plasma. Biochem. Biophys. Rec. Comm. 66: 1218-1224.

Fabri, G., and Castellino, N. 1995. Lead and the erythropoietic system. In N. Castellino; P. Castellino; and N. Sannolo (eds.), Inorganic lead exposure : Metabolism and intoxication, pp 369-402. London: Lewis publishers.

Fibach, E.; Manor, D.; Treves, A.; and Rachmilewitz, E.A. 1991. Growth of human normal erythroid progenitors in liquid culture: A comparison with colony growth in semisolid culture. Inter. J. Cell. Cloning. 9 : 57-64.

Flegal, A.R.; Smith, D.R.; and Elias, R.W. 1990. Lead contamination in food. In J.O. Nriagu, and M.S. Simmons (eds.), Food contamination from environment source, pp 85-202. Newyork: John Wiley & Sons, Inc.

Frenkel, G.D., and Middleton, C. 1987. Effect of lead acetate on DNA and RNA synthesis by intact Hela cells, isolated nuclei and purified polymerase. Biochem. Pharmacol. 36: 265-268.

Gercken, B., and Barnes, R. 1991. Determination of lead and other trace element species in blood by size exclusion chromatography and inductively coupled plasma/ mass spectrometry. Anal. Chem. 63: 283-287.

Goering, P.L. 1993. Lead protein interactions as a basic for lead toxicity. Neurotoxicology 14: 45-60.

Goillot, E., et al. 1997. Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway. Proc. Natl. Acad. Sci. USA. 94: 3302-3307.

Goldfrank, L.R.; Osborn, H.; and Hartnett, L. 1990. Lead In L.R. Goldfrank; N.E. Flomenbaum; R.S. Weisman; and M.A. Howland (eds.), Goldfrank's toxicologic emergenies. 4th ed., pp 627-640. New Jersey: Prentice-Hall International Inc.

Gossel, T.A., and Bricker, J.D. 1990. Lead. In Principle of clinical toxicology. 2nd ed., pp 173-181. London: CRC Press.

Goyer, R.A. 1996. Toxic effect of metals. In C.D. Klassen; M.O. Amdur; and J. Doull (eds.), Casarett & Doull's toxicology: The basic science of poison. 5th ed., pp 691-736. New York: McGraw-Hill.

Goyer, R.A., and Krull, R. 1969. Ultrastructural transformation in mitochondria isolated from kidneys of normal and lead intoxicated rats. J. Cell. Biol. 41: 393-400.

Graber, S.E., and Krantz, S.B. 1978. Erythropoietin and the control of red cell production. Ann. Rev. Med. 29: 51-66.

Grabowska, M., and Guminska, M. 1996. The effect of lead on lactate formation, ATP level and membrane ATPase activity in human erythrocytes in vitro. Int. J. Occup. Med. Environ. Health 9: 265-274.

Gregoli, P.A., and Bondurant, M.C. 1997. The role of Bel-X_L and apopain in the control of erythropoiesis by erythropoietin. Blood 2: 630-640.

Gruenberg, J.; Griffiths, G.; and Howell, K.E. 1989. Characterization of the early endosome and putative endocytic carrier vesicles *in vivo* and with an assay of vesicle fusion *in vitro*. J. Cell. Biol. 108: 1301-1316.

Gurd, F.R.N., and Murray, G.R.Jr. 1954. Preparation and properties of serum and plasma protein. XXXIX. The interaction of human serum albumin with plumbous ions. J. Am. Chem. Soc. 76: 187-190.

Hanspal, M., and Palek, J. 1992. Biogenesis of normal and abnormal red blood cell membrane skeleton. Semin. Hematol. 29: 305-319.

Harford, J.B.; Rouault, T.A.; Huebers, H.A.; and Klausner, R.D. 1994. Molecular mechanisms of iron metabolism. In G.Stamatoyannopoulos; A.E. Nienhuis; P.W. Majererus; and H. Varmus (eds.), The molecular basic of blood diseases. 2nd ed., pp 351-377. Philadelphia: WB. Saunders company.

Harris, W.R. 1983. Thermodynamic binding constants of the zinc-human serum transferrin. Biochemistry 22: 3920-3926.

Harris, W.R. 1986. Estimation of the ferrous-transferrin binding constants based on thermodynamic studies of nickel (II)-transferrin. J. Inorg. Biochem. 27: 41-52.

Harris, W.R., and Madsen, L.J. 1988. Equilibrium studies on the binding of cadmium (II) to human serum transferrin. Biochemistry 27: 284-288.

Harris, W.R., and Chen, Y. 1994. Electron paramagnetic resonance and difference ultraviolet studies of Mn^{2+} binding to serum transferrin. J. Inorg. Biochem. 54: 1-19.

Hartwig, A.; Schlepegrell, R.; and Beyersmann, D. 1990. Indirect mechanism of lead induced genotoxicity in culture mammalian cells. Mutat Res. 241: 75-82.

Hartwig, A. 1994. Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: A review. Environ. Health. Persect. 102 (Suppl. 3): 45-50.

Hartwig, A. 1995. Current aspects in metal genotoxicity. Biometal 8: 3-11.

Haseyama, Y., et al. 1999. Phosphatidylinositol 3-kinase is involved in the protection of primary cultured human erythroid precursor cells from apoptosis. Blood 94: 1568-1577.

He, L.H.; Poblenz, A.T.; Medrano, C.J.; and Fox, D.A. 2000. Lead and calcium produce rod photoreceptor cell apoptosis by opening the mitochondrial permeability transition pore. J. Biol. Chem. 275: 12175-12184.

Hemmaplardh, D.H.; Kailis, S.G.; and Morgan, E.H. 1974. The effect of inhibitors of microtubule and microfilament function on transferrin and iron uptake by rabbit reticulocytes and bone marrow cells. Br. J. Haematol. 28: 53-65.

Hernberg, S., and Nikkanen G. 1970. Enzyme inhibition by lead under normal urban condition. Lancet 1: 63-64.

Hernfandez, E.; Gutiferrez-Ruiz, M.C.; and Garcifa Vorgas, G. 1998. Effect of acute lead treatment on coproporphyrinogen oxidase activity in Hep G2 cells. Toxicology 126: 163-171.

Holtzman, D., and Shenshu, J. 1976. Early effects of inorganic lead on immature rat brain mitochondrial respiration. Pediatr. Res. 10: 70- 75.

Hunaiti, A.A., and Soud, M. 2000. Effect of lead concentration on the level of glutathione, glutathione S-transferase, reductase and peroxidase in human blood. Sci. Total. Environ. 248: 45-50.

Hunter, R.C. 1993. Hematopoiesis. In B.A. Brown; R.C. Hunter; A. O'Hare; and G. Erimj (eds.), Hematology: Principle and procedures. 6th ed., pp 35-82. Philadelphia: Williams & Wilkins.

Iacopetta, B.J.; Morgan, E.H.; and Yeoh, G.C.T. 1983. Receptor-mediated endocytosis of transferrin by developing erythroid cells from the fetal rat liver. J. Histochem. Cytochem. 31: 316-344.

IARC, 1987. Monographs on the evaluation of carcinogenic risk of chemicals to humans. IARC. (Suppl.7) , Lyon, France.

Ichas, F., and Mazat, J.P. 1998. From calcium signaling to cell death: Two conformation for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim. Biophys. Acta. 1366: 33-50.

Iscove, N.N. 1977. The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse bone marrow. Cell. Tissue. Kinetic 10: 323-334.

Israels, E.D., et al. 1999. Apoptosis. Stem Cell 17: 306-313.

Jacob, H.S. 1975. Effect of drugs on red cell membranes: Insights into normal red cell shape. J. Clin. Pathol. 28: 40-45.

Jandl, J.H.; Inman, J.K.; Simmons, R.L.; and Allen, D.W. 1959. Transfer of iron from serum iron-binding protein to human reticulocytes. J. Clin. Invest. 38: 161-170.

Jennings, M.L. 1985. Kinetic and mechanism of anion transport in red blood cells. Annu. Rev. Physiol. 47: 519-533.

Jensen, W.N.; Moreno, G.D.; and Bessil, M. 1965. An electron microscopic description of basophilic stippling in red cells. Blood 25: 933-938.

Juo, P., et al. 1997. Fas activation of the p 38 mitogen activated protein kinase signalling pathway requires ICE/ CED-3 family proteases. Mol. Cell. Biol. 17: 24-35.

Kaplan, M.L.; Jones, A.G.; Davis, M.A.; and Kopito, L. 1975. Inhibitory effect of iron on the uptake of lead by erythrocytes. Life. Sci. 16: 1545-1554.

Karai, I.; Fukumoto, K.; and Horiguchi, S. 1982. Effect of lead *in vitro* on water metabolism and osmotic fragility of human erythrocytes. Br. J. Ind. Med. 39: 295-299.

Kassenaar, A.; Morell, H.; and London, I.M. 1957. The incorporation of glycine into globin and the synthesis of heme in duck erythrocytes. J. Biol. Chem. 299: 423-435.

Kehoe, R.A. 1964. Normal metabolism of lead. Arch. Environ. Health 8: 232-235.

Kelley, L.L., et al. 1993. Survival of death of individual proerythroblasts results from differing erythropoietic sensitivities: A mechanism for controlled rate of erythrocyte production. Blood 82: 2340-2352.

Kelley, L.L., et al. 1994. Apoptosis in erythroid progenitor deprived of erythropoietin occurs during the G1 and S phases of the cell cycle without growth arrest or stabilization of wild type p 53. Mol. Cell. Biol. 14: 4183-4192.

Kirby, S.; Cook, D.; Walton, W.; and Smithies, O. 1996. Proliferation of multipotent hematopoietic cells controlled by truncated erythropoietin receptor transgene. Proc. Natl. Acad. Sci. USA. 13: 9402-9407.

Knauf, P.A. 1987. Anion transport in erythrocytes. In T.E. Andreoli; J.F. Hoffman; and D.D. Fanestil (eds.), Membrane Physiology, pp 533-774. New York: Plenum.

Kochen, J.A., and Greener, Y. 1975. Lead binding by transferrin. Pediatr. Res. 323.

Kohno, H.; Taketani, S.; and Tokunaga, R. 1993. The effect of lead on iron uptake from transferrin in human erythroleukemia (K562) cells. Biometals 6: 77-83.

Koury, M. J., and Bondurant, M.C. 1990. Erythropoietic retard DNA breakdown and prevents programmed cell death in erythroid precursor cells. Science 248: 378-381.

Krantz, S.B. 1991. Erythropoietin. Blood 77: 419-434.

Kroemer, B.; Dallaporta, M.; and Resche-Rigon. M. 1998. The mitochindrial death/life regulator in apoptosis and necrosis. Annu. Rev. Physiol. 60: 619-642.

Kusell, M.; O'Cheskey, S; and Gerschenson, I.E. 1978. Cellular and molecular toxicity of lead: I. Effect of lead on culture cell proliferation. J. Toxicol. Environ. Health 4: 503-513.

Kyriakis, J.M., et al. 1994. The stress-activated protein kinase subfamily of c-Jun kinase. Nature 369: 156-160.

Labbe, R.F.; Rettmer, R.L.; Shah, A.G.; and Turnlund, J.R. 1987. Zinc protoporphyrin: Past, Present and Future. Ann. NY. Acad. Sci. 514: 7-14.

Lachant, N.A.; Tomoda, A.; and Tanaka, K.R. 1984. Inhibition of the pentose phosphate shunt by lead: A potential mechanism for hemolysis in lead poisoing. Blood 63: 518-524.

Lake, L., and Gerschenson, L.E. 1978. Cellular and molecular toxicology of lead: III. Effect of lead on heme synthesis. J. Toxicol. Environ. Health 4: 527-540.

Langlois, R.G.; Bigbee, W.L.; and Hensen, R.H. 1985. Flow cytometric characterization of normal and variant cells with monoclonal antibodies specific for glycophorin A. J. Immunol. 134: 4009-4017.

Laug, E.P., and Kunze, F.M. 1948. The penetration of lead through the skin. J. Ind. Hyg. Toxicol. 30: 256-259.

Leikin, S., and Eng, G. 1963. Erythrokinetic studies of the anemia of lead poisoning. Pediatrics 31: 996-1002.

Leist, M.; Single, B.; Castoldi, A.F.; Kuhnle, S.; and Nicotera, P. 1997. Intracellular adenosine triphosphate (ATP) concentration: A switch in the decision between apoptosis and necrosis. J. Ext.Med.185: 1481-1486.

Lewis, R.J. 1996. SAX'S Dangerous properties of industrial materials. 9th ed., pp 2027-2202. USA: Van Nostrand Reinhold. A Division of International Thomson Publishing, Ins.

Liboli, E.; Carroll, M.; D'Andrea, A.; and Mathey-Prevot, B. 1993. Erythropoietin receptor signals both proliferation and erythroid-specific differentiation. Proc. Natl. Acad. Sci. USA. 90: 11351-11355.

Lipton, J., and Nathan, D. 1983. Cell-cell interactions in regulation of erythropoiesis. Clin. Haematol. 46: 91-102.

Loken, M.R., et al. 1987. Flow cytometric analysis of human bone marrow: I Normal erythroid development. Blood 69: 255-263.

Lolin, Y., and O' gorman, P. 1988. An intra-erythrocytic low molecular weight lead-binding protein in acute and chronic lead exposure and its possible protective role in lead toxicity. Ann. Clin. Biochem. 25:688-697.

Lothe, K., and Falbe-Hansen, I. 1963. Incorporation of ^{59}Fe into erythrocytes nonhaem iron and haemoglobin in anaemic and lead-poisoned rabbits. Clin. Sci. 24: 47-54.

Lutton, J.D.; Ibraham, N.G.; Friedland, M.; and Levere, R.D. 1984. The toxic Effects of heavy metal on rat bone marrow in vitro erythropoiesis: Protective role of hemin and zinc. Environ. Res. 35: 97-103.

Macho, A., et al. 1997. Glutathione depletion is an early and calcium elevation is a late event of thymocyte apoptosis. J. Immunol. 19: 4612-4619.

Maciejewski, J.P.; Selleri, C.; and Young, N.S. 1995. Fas antigen expression on CD 34^{+} human marrow cells is induced by interferon gramma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoietic suppression in vitro. Blood 85: 3183-3190.

Mahaffey, K.R. 1981. Nutrition factors in lead poisoning. Nutrition. Rev. 39: 353-365.

Manton, W.I., and Cook, J.D. 1984. High accuracy (stable isotope dilution) measurements of lead in serum and cerebrospinal fluid. Br. J. Ind. Med. 41: 313-319.

Marasri Ujjin. 1999. Kinetics of lead or copper binding on apoceruloplasmin. Master' Thesis, Department of Biochemistry, Graduate School, Chulalongkorn University.

Maria, R.D., et al. 1999. Apoptotic role of Fas/ Fas ligand system in the regulation of erythropoiesis. Blood 93: 769-803.

Mark, G.S. 1985. Exposure to toxic agents: the heme biosynthesis pathway and hemoproteins as indicator. CRC. Crit. Rev. Toxicol.15: 151-179.

Martin, R.B.; Savory, J.; Brown, S.; Bertholf, R.L.; and Wills, M.R. 1987. Transferrin binding of Al^{3+} and Fe^{3+}. Clin. Chem. 33: 405-407.

Mather, J.P., and Roberts, P.E. 1998. Standard cell culture techniques. In Introduction to cell and tissue culture: Theory and technique, pp 63-87. New York: Plenum press.

Mayer, C.D.; Katz, F.E.; Joshi, G.; and Millar, J.L. 1984. A cell line secretion stimulating factors for CFU-GEMM culture. Blood 64: 152-155.

Meares, C.F., and Ledbetter, J.E. 1977. Energy transfer between terbium and iron bound to transferrin: Reinvestigation of the distance between metal-binding sites. Biochemistry 16: 5178-5180.

Miwa, S., et al. 1981. Recent advance in hematology: Red cell series. Nippon Ketsueki Gakkai Zasshi 44: 1277-1281.

Morgan, E.H. 1981. Transferrin: Biochemistry, physiology and clinical significance. Mol. Asp. Med. 4: 1-123.

Morgan, E.H., and Iacopetta, B. J. 1986. Vinblastine but not other microtubule inhibitors block transferrin endocytosis and iron uptake by reticulocytes. Clin. Exp. Pharmacol. Physiol. 14: 119-126.

Moutafchiev, D.A., and Sirakov, L.M. 1998. Influence of ascorbic acid, sodium citrate, and sodium bicarbonate on the uptake of Fe-59-transferrin, Mn-54-transferrin, Zn-65-transferrin from lactating mouse mammary gland cells. Biol. Trace. Element. Res. 63:31-36.

Muta, K., and Krantz, S.B. 1993. Apoptosis of human erythroid colony-forming cells is decreased by stem cell factor and insulin-like growth factor I as well as erythropoietin. J. Cell. Physiol. 156: 264-271.

Nagata, Y., and Todokoro, K. 1999. Requirement of activation of JNK and p 38 for environmental stress-induced erythroid differentiation and apoptosis and of inhibition of ERK for apoptosis. Blood 94: 853-863.

Oberto, A.; Marks, N.; Evans, H.L.; and Guidotti, A. 1996. Lead (Pb^{++}) promotes apoptosis in newborn rat cerebellar neurons: Pathological implications. J. Pharm. Exp. Ther. 279: 435-442.

Ogawa, M.; Grush, O.C.; O' Dell, R.F. 1977. Circulation erythropoietic precursors assessed in culture: Characterization in normal men and patients with hemoglobinopathies. Blood 50: 1081-1091.

Ong, C.N., and Lee, W.R. 1980a. Distribution of lead-203 in human peripheral blood *in vitro*. Br. J. Ind. Med. 37: 78-84.

Ong, C.N., and Lee, W.R. 1980b. Interaction of calcium and lead in human erythrocytes. Br. J. Ind. Med. 37: 70-77.

Ossina, N.K., et al. 1997. Interferon-γ modulates a p 53-independent apoptotic pathway and apoptosis-related gene expression. J. Biol. Chem. 272: 16351-16357.

Osterode, W.; Barnas, U.; and Geissier, K. 1999. Dose dependent reduction of erythroid progenitor cells and inappropriate erythropoietin response in exposure to lead: New aspects of anemia induced by lead. Occup. Environ. Med. 56: 106-109.

Paglia, D.C.; Valentine, W.N.; and Dahlgren, J.G. 1975. Effects of low-level lead exposure on pyrimidine-5'-nucleotidase in the pathogenesis of lead-induced anemia. J. Clin. Invest. 56: 1164-1169.

Paglia, D.C.; Valentine, W.N.; and Fink, K. 1977. Further observations on erythrocyte pyrimidine-5'-nucleotidase deficiency and intracellular accumulation of pyrimidine nucleotides. J. Clin. Invest. 60: 1362-1366.

Pfleger, H., and Wolf, H.U. 1975. Activation of membrane-bound high-affinity calcium ion-sensitive adenosine triphosphatase of human erythrocytes by bivalent metal ions. Biochem. J. 147: 359-361.

Piomelli, S. 1998. Lead poisoning. In D.G. Nathan, and S.H. Orkin (eds.), Hematology of infancy and childhood. Vol. 1. 5th ed., pp 480-496. Philadelphia: W.B. company.

Ponka, P. 1997. Tissue-specific regulation of iron metabolism and heme synthesis: Distinct control mechanism in erythroid cells. Blood 89: 1-25.

Quian, Z.M., and Morgan, H. 1990. Effect of lead on the transport of transferrin-free and transferrin-bound iron into rabbit reticulocytes. Biochem. Pharmacol. 40: 1049-1054.

Quian, Z.M., and Tang, P.L. 1995. Mechanism of iron uptake by mammalian cells. Biochem. Biophy. Acta. 1269: 205-214.

Quian, Z.M.; Xiao, D.S.; Wang, Q.; Tang, P.L.; and Pu, Y.M. 1997. Inhibitory mechanism of lead on transferrin-bound iron uptake by rabbit reticulocytes: A fraction analysis. Mol. Cell. Biochem. 173: 89-94.

Rabinowitz, M.B.; Wetherill, G.W.; and Kopple, J.D. 1973. Lead metabolism in the normal humans: Stable isotope studies. Science 182:725-727.

Rabinowitz, M.B.; Wetherill, G.W.; and Kopple, J.D. 1976. Kinetics analysis of lead metabolism in health human. J. Clin. Invest. 58: 260-270.

Raghavan, S.R.V.; Culver, B.D.; and Gonick, H.C. 1980. Erythrocytes lead-binding protein after occupational exposure. Environ. Res. 22: 264-269.

Rahman, H.; Skillen, A.W.; Channon, S.M.; Ward, M.K.; and Kerr, D.N.S. 1985. Method of studying the binding of aluminum by serum protein. Clin. Chem. 31: 1969-1973.

Ramesh, G.T.; Manna, S.K.; Aggarwal, B.B.; and Jadhav, A.L. 1999. Lead activates nuclear transcription factor-KB, activator protein-1, and amino-terminal c-Jun kinase in pheochromacytoma cells. Toxicol. Appl. Pharmacol. 155: 280-286.

Reilly, C. 1991. Lead. In Metal contamination of food. 2nd ed., pp 105-129. London: Applied Science Publishers.

Rosawan Srivoravit.1996. Lead-binding by ceruloplasmin. Master' Thesis, Department of Biochemistry, Graduate School, Chulalongkorn University.

Rosen, J. F., and Pounds J.G. 1989. Quantitative interaction between Pb^{2+} and Ca^{2+} homeostasis in culture osteoclastic bone cells. Toxicol. App. Pharmaco. 98: 530-543.

Rossi, E.; Taketani, S.; and Garcia, W.P. 1993. Lead and the terminal mitochondrial enzymes of haem biosynthesis. Biomed. Chromato. 7: 1-6.

Roy, N.K., and Rossman, T.G. 1992. Mutagenesis and comutagenesis by lead compounds. Mutat. Res. 298: 97-103.

Ruzittu, M., et al. 1997. Modulation of cell surface expression of liver carbohydrate receptors during in vivo induction of apoptosis with lead nitrate. Cell. Tissue. Res. 298: 105-112.

Sandhir, R., and Grill, K.D. 1995. Effect of lead on lipid-peroxidation in liver of rat. Biol. Trace. Element. Res. 48: 91-97.

Scortegagna, M., and Hanbauer, I. 1997. The effect of lead exposure and serum deprivation on mesencephalic primary cultures. Neurotoxicology 18: 331-339.

Seeman, P.; Chau-Wong, M.; and Moyyen, S. 1973. Membrane expansion by vinblastine and strychnine. Nature. New. Biol. 241: 22- 27.

Sherke, S., and Huhn, D. 1992. Identification of CD 71 (transferrin receptor) expressing erythrocytes by multiparameter-flow-cytometry (MP-FCM): Correlation to the quantitation of reticulocytes as determined by conventional microscopy and by MP-FCM using a RNA-staining dye. Br.J. Haematol. 81: 432-439.

Shibamoto, T., and Bjeldances, L.F. 1993. Heavy metal. In Introduction to food toxicology, pp 126-140. New York: Academic Press Inc.

Sieg, D.J., and Billings, R.E. 1997. Lead/cytokine-mediated oxidative DNA damage in culture mouse hepatocytes. Toxicol. Appl. Pharmacol. 142: 106-115.

Singh, J., et al. 1999. Internalization of carcinogenic lead chromate particles by cultured normal human lung epithelial cells: Functional alterations and apoptotic cell death in the retina following developmental or adult lead exposure. Neurotoxicology 18: 645-664.

Simon, W. 1992. Transferrin structure and iron binding. In Transferrin: The iron carrier, pp 62-109. London: CRC Press.

Simons, T.J.B. 1984. Active transport of lead by human red blood cells. FEBS. Lett. 172: 250-254.

Simons, T.J.B. 1986a. Passive transport and binding of lead by human red blood cells. J. Physiol. 378: 267-286.

Simons, T.J.B. 1986b. The role of anion transport in the passive movement of lead across human red cell membrane. J. Physiol. 378: 287-312.

Simons, T.J.B. 1988. Active transport of lead by the calcium pump in human red cell ghosts. J. Physiol. 405: 105-113.

Simons, T.J.B. 1993a. Lead transport and binding by human erythrocytes *in vitro.* J. Physiol. 423: 307-313.

Simons, T.J.B. 1993b. Lead-calcium interactions in cellular lead toxicity. Neurotoxicology 14: 77-86.

Smit, J.L. 1976. Metabolism and toxicity of lead. In A.S. Prasad, and D. Oberleas (eds.), The nutrition foundation: Trace element in human health and disease (Essential and toxic elements), pp 443-451. New York: Academic Press Inc.

Skoczynska, A. 1997. Lipid peroxidation as a toxic mode of action for lead and cadmium. Med Pr. 48: 197-203.

Sroczynski, J., and Piekarski, B. 1959. Obraz bialek surowicy krwi krolikow wostrym zatruciu olowie. Postepy. Hig. Med. Dosw. 13: 781-785.

Stons, S.J., and Bagchi, D. 1995. Oxidative mechanism in the toxicity of metal ions Free. Rad. Biol. Med. 18: 321-336.

Stubbs., R.T. 1973. Source of lead in the environment. In P. Hepple (ed.), Lead in the environment, pp 1-7. England: Applied science Publishers Ltd.

Sugawara, E.; Nagamura, K.; Fukumura, A.; and Seki, Y. 1990. Uptake of lead by human red blood cells and intracellular distribution. Kitasato. Arch. Exp. Med. 63: 157-165.

Supat Wangwongwatana. 1997. Unleaded air in Bangkok : A story of success. Pollution control'97. 12-14 november 1997.

Suphitcha Mangkalee. 1994. Characterization of lead-binding protein in human serum. Master' Thesis, Department of Biochemistry, Graduate School, Chulalongkorn University.

Susin, S.A.; Zamzami, N.; and Kroemer, G. 1998. Mitochondria as regulators of apoptosis: Doubt no more. Biochim. Biophys. Acta. 1366: 151-165.

Taketani, S.; Kohno, H.; Kinoshita, S.; and Tokunaka, R. 1985. The effects of lead on differentiation of the friend leukemia cells and rat bone marrow cells. Toxico. Appl. Pharmacol. 77: 374-380.

Tan, A.T., and Woodworth, R.C. 1969. Ultraviolet difference spectral studies of conalbumin complexes with transition metal ions. Biochem. 8: 3711-3716.

Tanner, M.J.A. 1993. Molecular and cellular biology of the erythrocyte anion exchanger (AE1). Semin. Hematol. 30: 34-57.

Telisman, S.; Kersanc, A.; and Prpic-Majic, D. 1982. The relevance of arguments for excluding ALAD from the recommeded biological limit values in occupational exposure to inorganic lead (WHO 1980). Int. Arch. Occup. Environ. Health 50: 397-412.

Thatte, H.S.; Bridges, K.R.; and Golan, D.E. 1994. Microtubule inhibitors differentially affect translational movement, cell surface expression and endocytosis of transferrin receptors in K 562 cells J. Cell. Physiol. 160: 345-357.

Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267: 1456-1462.

Umemura, T.; Papayannopoulou, T.; and Stamatoyannopoulous, G. 1993. The mechanism of expansion of late erythroid progenitors during erythroid regeneration: Target cells and effects of erythropoietin and interleukin-3. Blood 73: 1993-1998.

Valentino, M.; Fiorini, R.M.; Curatola, G.; and Governa, M. 1982. Changes of membrane fluidity in erythrocytes of lead exposed workers. Int. Arch. Occup. Environ. Health 51: 105-112.

Van Den Heuvel, R.L.; Leppens, H.; and Schoeters, G.E.R. 1999. Lead and catechol hematotoxicity *in vitro* using human and murine hematopoietic progenitor cells. Cell. Biol. Toxicol. 15: 101-110.

Verheij, M., et al. 1996. Requirement for ceramide-initiated SAPK/ JNK signaling in stress-induced apoptosis. Nature 380: 75-79.

Waldron, H.A. 1966. The anemia of lead poisoning: A review. Br. J. Ind. Med. 23: 83-100.

Waldron, H.A. 1980. Lead. In H.A. Waldron (ed.), Metal in the environment, pp 156-157. London: Academic Press Inc.

Wang, X.Z., and Ron, D. 1996. Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD 153) by p 38 MAP kinase. Science 272: 1347-1349.

Wetmur, J.C. 1994. Influence of the common human delta-aminolevulinate dehydratase polymorphism on lead body burden. Environ. Health. Perspect. 102: 215-219.

Westerman, P., et al. 1965. Concentration of lead in bone in plumbism. New. Eng. J. Med. 273: 1246-1250.

White, J.M., and Harvey, D.R. 1972. Defective synthesis of α and β globin chains in lead poisoning. Nature 236: 71-73.

White, J.M., and Selhi, H.S. 1975. Lead and the red cell. Br. J. Haematol. 30: 133-138.

WHO. 1987. Air quality quidelines for Europe (European Series no 23). Geneva: World Health Organization 45-58: 242-261.

Yang, J.L.; Wang, L.C.; Chang, C.Y.; and Liu, T.Y. 1999. Singlet oxygen is the major species participating in the induction of DNA strand breakage and 8-hydroxyguanine adduct by lead acetate. Environ. Mol. Mutagen. 33: 194-201.

Youssoufian, H., et al. 1993. Structure, function and activation of the erythropoietin receptor. Blood 81: 2223-2236.

Zamzami, T.; Hirsch, B.; Dallaporta, P.X.; Petit, G.; and Kroemer, J. 1997. Mitochondrial implication in accidental and programmed cell death: Apoptosis and necrosis. J. Bioenerg. Biomembr. 29: 185-193.

Zelikoff, J.T.; Li, J.H.; Hartwig, A.; Wang, X.W.; Costa, M.; and Rossman, T.G. 1988. Genetic toxicology of lead compounds. Carcinogenesis 9: 1727-1732.

Zereba, G., and Chmielnicka, J. 1992. Disturbances in heme biosynthesis in rabbits after administration per os of low does of tin or lead. Biol. Trace. Element. Res. 34: 115-122.

Ziegler, E.E.; Edwards, B.B.; Jensen, R.L.; Mahaffey, K.R.; and Fomon, S.J. 1978. Absorption and retention of lead by infants. Pediatr. Res. 12 : 29-34.

Zoratti, M., and Szabo, I. 1995. The mitochondrial permeability transition. Biochim. Biophys. Acta. 1241: 139-176.

Zweier, J.L., and Aisen, P. 1977. Studies of transferrin with the use of Cu^{2+} as an electron paramagnetic resonance spectroscopic probe. J. Biol. Chem. 252: 6090-6096.

APPENDIX 1

THE COMPOSITION OF α-MEM

Component	mg/liter
NaCl	6800.0
KCl	400.0
$MgCl_2.6H_2O$	200.0
$CaCl_2$	200.0
$NaH_2PO_4.H_2O$	150.0
Choline	1.0
Folic acid	1.0
Inositol	2.0
Nicotinamide	1.0
Pantothenic acid	1.0
Pyridoxal HCl	1.0
Riboflavin	0.1
Thiamine HCl	1.0
L-Arginine	105.0
L-Cystine	24.0
L-Glutamine	292.0
L-Histidine	31.0
L-Isoleucine	52.0
L-leucine	52.0
L-Lycine	58.0
L-methionine	15.0
L-Phenylalanine	32.0
L-Threonine	48.0
L-Tryptophane	10.0

Component	mg/liter
L-Tyrosine	36.0
L-Valine	46.0
Glucose	1000.0
$NaHCO_3$	2000.0
Phenol red	10.0

APPENDIX 2

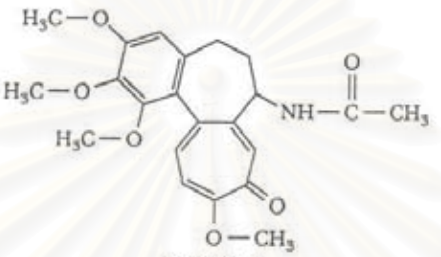
Table 21 : The percentage of lead content in erythrocytes after lead exposure with and without transferrin. Human erythrocytes were incubated in 1%FBS/α-MEM (pH 7.4) containing 20 ppm lead acetate at 37°C with and without various concentrations of apo-transferrin. After washing the cells for three times with 1mM EDTA in 1%FBS/α-MEM, lead content in the cells was determined by GFAAS.

Lead content (%)			
Without Tf	with 0.5mg/ml Tf (Pb:Tf = 2:0.25)*	with 2 mg/ml Tf (Pb:Tf = 2:1)*	with 3 mg/ml Tf (Pb:Tf = 2:1.5)*
100%	97.18%	84.76%	82.78%

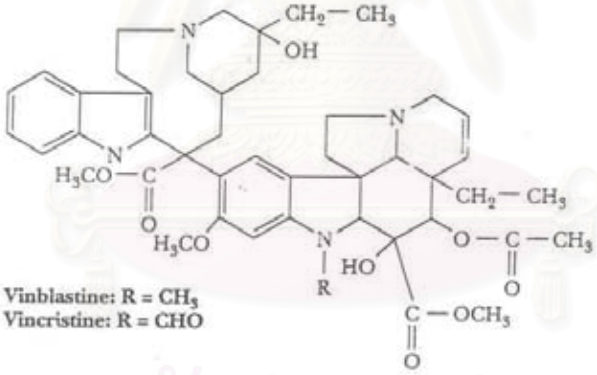
* Mole ratio of Pb:Tf (It is assumed that one molecule of transferrin (Tf) binds to two molecules of lead)

APPENDIX 3

The structure of colchicine, vinblastine and vincristine. These drugs interfere with normal assembly and disassembly of microtubules (Garrett and Grisham, 1995).



Colchicine



Vinblastine: R = CH_3
Vincristine: R = CHO

BIOGRAPHY

Miss Wenika Benjapong was born on September 20, 1962 in Bangkok, Thailand. She graduated with the Bachelor degree of Science in Biochemistry from Chulalongkorn University in 1984 and received her Master degree of Science in Nutrition from Mahidol University in 1987. Since that time, she has been working at the Institute of Nutrition, Mahidol University. Her research involve in food toxicology.