LEUKOTRIENE PRODUCTION BY MACROPHAGE CELLS IRRADIATED WITH COBALT-60 GAMMA IRRADIATION

A DISSERTATION

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To the Dean for Graduate Studies and Research:

I am submitting herewith a dissertation written by Asghar Hajibeigi entitled "Leukotriene Production by Macrophage Cells Irradiated with Cobalt-60 Gamma Irradiation". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Radiation Biology.

Eugene W. Hupp, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted

Dean for Graduate Studies & Research

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"God is Kind to His servants. He gives sustenance to whomever He wants. He is All-powerful and Majestic." The Holy Quran 42:18

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ABSTRACT

LEUKOTRIENE PRODUCTION BYMACROPHAGE CELLS IRRADIATED WITH COBALT-60 GAMMA IRRADIATION

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Leukotriene biosynthesis was assessed in vitro following treatment of rat peritoneal macrophage cells with Ca-ionophore A23187 or Co-60 gamma irradiation. Pre-incubated macrophage cells cultured in plastic flasks under 5% CO_2 and 95% moisturized air at 37 °C were labeled with [^{14}C]arachidonic acid for 12 hours prior to administration of Ca-ionophore or various doses of gamma radiation(2 Gy - 32 Gy). Treatment was stopped after one hour incubation with addition of isopropanol to the culture medium. The arachidonic acid metabolites were extracted with diethyl ether, and analyzed by high performance liquid chromatography using a C_{18} reverse phase column. The eluted materials were detected using an in line radioactivity flow detector equipped with a dot matrix printer. The results indicated that both treatments caused quantitatively higher conversion of arachidonic acid to leukotriene C_4 and release of several other radiolabeled metabolites when compared to the control. However, in all treatments

including the control the amount of unmetabolized arachidonic acid released from cells in the culture medium was significantly higher than the released metabolites. The release of arachidonic acid from the cell membrane was quantitatively related to the presence of fetal bovine serum (FBS) in the culture medium during the treatment period. In the absence of FBS the stimulated macrophage cells produced a larger amount of arachidonate metabolites with a significant decrease in the release of free arachidonic acid compared to those that had FBS during the treatment period. In the presence of ibuprofen but no FBS, Ca-ionophore stimulated macrophage cells produced both qualitatively and quantitatively higher amounts of arachidonates including LTC_4 compared to other treatments.

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CHAPTER I

INTRODUCTION

The recognition of leukotrienes as a separate family of arachidonic acid metabolites began in 1979 when Murphy, Hammarstrom, and Samuelsson chemically characterized a component of slow-reacting substances (SRS) released from murine mastocytoma cells by the calcium ionophore A23187 (Murphy et al. 1979). Leukotrienes constitute a group of biologically active compounds derived from arachidonic acid through the 5-lipoxygenase pathway in various inflammatory cells (Davies et al. 1984; Needleman et al. 1986). Neutrophils, eosinophils, macrophages, mast cells, keratinocytes as well as lung, spleen, brain, heart and blood vessels are among cells, organs and tissues that have been recognized as major sources of leukotriene biosynthesis (Borgeat and Samuelsson 1979a ; Morris et al. 1980; Mencia-Huerta et al. 1983; Borgeat 1986). Because of their origin(leukocytes) the term leukotriene (LTs) has been proposed to represent a new family of non-cyclized eicosanoids, with one or two oxygen substituents and the common structural feature of a conjugated triene (Samuelsson et al. 1980). The conjugated triene is responsible for the characteristic UV absorption spectrum (Borgeat and Samuelsson 1979b). Structurally different

leukotrienes are distinguished by capital letters A, B, C, D, etc. (Samuelsson et al. 1980) and a numerical subscript indicates the number of double bonds in the molecule (Samuelsson and Hammarstrom 1980).

Arachidonic acid is a tetraunsaturated 20 carbon acid which is formed from dietary linoleic acid in mammals (Borgeat 1986). It is considered to be an integral component of the cell membrane and is present mainly in the form of esters: phospholipids, glycerides, and cholesterol esters (Kuehl and Egan 1980; Irvine 1982). A variety of different stimuli lead to liberation of arachidonic acid from the phospholipid fraction of the cell presumably mainly through hydrolysis via a calcium-dependent mechanism involving phospholipase A_2(Marcus 1985) or indirectly through the sequential action of phospholipase C and by diacyl glycerol lipase(Davies et al. 1984).

The capacity of the cells to synthesize arachidonic acid oxygenation products including leukotrienes, both in terms of the nature of the products and the extent of their synthesis are dependent upon cell type (Davies et al. 1984). Studies on the lipid composition of macrophage cell membrane have shown that 25% of the total fatty acid component of this cell type is arachidonic acid in rabbit, mouse and human, whereas the fatty acid complement of most other cell types rarely exeeds a few percent (Stossel et al. 1974). In vitro treatment of mouse peritoneal macrophages with unopsonized zymosan promoted the release of 40 to 50 percent of the arachidonic acid without loss

of cell viability (Scott et al. 1980). However, the release of arachidonic acid as the precursor of biologically active mediators from the phospholipid layer of cell membrane was inhibited when the cells were treated by anti-inflammatory steroids (Flower and Blackwell 1979; Hirata et al. 1980; Samuelsson 1983). Treatment of leukocytes with corticosteroids induces the synthesis of proteins which inhibit phospholipase A; these proteins, isolated from macrophages and neutrophils were named macrocortin and lipomodulin from macrophages and neutrophils respectively. The investigations also have indicated that these proteins may be identical (Oliw et al. 1983).

Other factors that induce or increase the release of arachidonic acid from various tissue and cells in different species have been reported. A rapid release of epidermal arachidonic acid following exposure of domestic pig skin to X-ray irradiation (300-1000 rads) has been demonstrated(Ziboh et al. 1982). A similar observation was reported when the effect of ionizing irradiation on bovine pulmonary artery endothelial cells in vitro was studied for ∝-aminoisobutyric acid uptake and prostacyclin (PGI_2) production (Friedman et al. 1986). The results indicated that the gamma irradiation (150-3000 rads) caused an increase in both --aminoisobutyric acid intake and PGI_2 synthesis and was shown to be dose dependent. It was concluded that the increased synthesis of PGI_2 was due to an increase in release of arachidonic acid from

plasma membrane (Friedman et al. 1986). Although the exact mechanisms responsible for the release of arachidonic acid are not known, it is possible that the ionizing irradiation induced the hydrolysis of arachidonic acid via the activation of phospholipase A, or by peroxidation of cell membrane lipids or by free radical formation in tissue (Ziboh et al. 1982; Friedman et al. 1986).

Newly released arachidonic acid is rapidly oxygenated by two enzymes in the cell, a particulate cyclooxygenase enzyme and cytoplasmic lipoxygenases (Marcus 1985). The cyclooxygenase pathway involves the oxygenation and cyclization reaction with arachidonic acid to form unstable cyclic endoperoxide intermediate prostaglandin G_2 (PGG_2) which is subsequently reduced by a hydroperoxidase to prostaglandin H_2 (PGH_2) as a second unstable intermediate cyclic endoperoxide compound (Ueno et al. 1982; Davies et al. 1984; Marcus 1985). Depending upon the enzymes present in a given cell type the PGH_2 is then a substrate for thromboxane synthetase to produce thromboxane A_2 (TXA_2) and PGI_2 synthetase, prostaglandin D_2 (PGD_2) isomerase, or prostaglandin E_2 (PGE_2) isomerase(Kuehl et al. 1980; Dewitt and Smith 1983; Marcus 1985).

Thromboxane A_2 and prostaglandins are unstable intermediates that are converted via non-enzymatic reactions respectively into thromboxane B_2

(TXB_2) and 6-Keto prostaglandin $F_{1\alpha}$ (PGE_{α}) with no biological activity (Dewitt and Smith 1983; Pace-Asciak and Granstrom 1983). PGI_2 is known as a vasodilator agent, an inhibitor of platelet aggregation, and inhibitor of bronchococonstriction, wheras TXA_2 has been reported to cause vasoconstriction, enhancement of platelet aggregation, and acts as a bronchoconstriction(Dewitt and Smith 1983).

Noting the wide range of biological effects of prostaglandins, it becomes apparent that these compounds could affect almost all body functions. It seems that there is hardly a single organ or function in the body that has not been shown to be influenced one way or other by prostaglandins (Oliw et al. 1983).

The extensive investigation of oxidative metabolism of polyunsaturated fatty acids lead to discovery of lipoxygenases almost four decades ago in plants (Borgeat et al. 1986). These enzymes were thought to be involved in introduction of an oxygen into polyunsaturated fatty acids (Borgeat 1986). The first true lipoxygenase enzyme of animal origin was isolated from blood platelets and guinea-pig lungs (Hamberg and Samuelsson 1974). The discovery of 12-s-hydroperoxy- 5,8,10,14-eicosatetraenoic acid (12-HPETE) in platelets was the first indication that the animal cells also catalyze lipoxygenase reactions (Hamberg and Samuelsson 1974). This compound

(12-HPETE) is considered to be an arachidonic acid metabolite synthesized by 12-lipoxygenase in platelet cells (Lagarde et al. 1984; Borgeat 1986; Needleman et al. 1986). It is then enzymatically metabolized through the glutathione peroxidase system to its hydroxy analogue 12-L- hydroxy-5,8,10,14-eicosatetraenoic acid(12-HETE) (Bryant et al. 1982; Chang et al. 1982; Davies et al. 1984). Platelet-derived 12-HETE may be utilized as a substrate to undergo a second oxygenation by neutrophil 5-lipoxygenase and reduced to 5(s),12(s)- dihydroxyeicosa- 6(trans),8(cis),10(trans),14(cis)-tetraenoic acid (5,12-diHETE) (Needleman et al. 1986). It has been suggested that these metabolites have chemotactic and chemokinetic properties toward leukocytes (Nugteren 1982).

The initial step in biosynthesis of leukotrienes is an oxygenation of arachidonic acid at carbon-5 by the 5-lipoxygenease to form 5-hydroperoxy eicosatetraenoic acid (5-HPETE) (Davies et al. 1984). Partially purified 5-lipoxygenase enzyme from guinea-pig polymorphonuclear leukocytes was found to be cytosolic, calcium dependent and stimulated by several nucleotides, ATP was the most effective (Ochi et al. 1983). It has been indicated that 5-HPETE may be enzymatically dehydrated by abstraction of one of the hydrogens at carbon-10 and elimination of a hydroxyl moeity from the hydroperoxy group to form unstable epoxide intermediate 5(S)-trans-oxido-7,9- trans-11,14-cis-eicosatetraenoic acid (LTA_4) (Borgeat and

Samuelsson 1979a; Davies et al. 1984; Ochi et al. 1983). Subsequently LTA_4 can be converted enzymatically by epoxide hydrolase into 5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid (LTB_4) (Borgeat and Samuelsson 1979c; Davies et al. 1984) or may be metabolized by glutathione transferase to introduce glutathione at carbon-6 to form a peptido-lipid conjugate (5S,6R)-5-hydroxy-6-S- glutathionyl- (E,E,Z,Z)-7,9,11,14- eicosatetraenoic acid (LTC_4) (Murphy et al. 1979; Davies et al. 1984; Needleman et al. 1986).

LTC_4 may be metabolized by enzymatic hydrolysis of its peptide chain through the loss of the -glutamyl residue into (5S,6R)-hydroxyl-6-S-cysteinylglycyl-(E,E,Z,Z)-7,9,11,14-eicosatetraenoic acid (LTD_4) (Orning et al.1980; Jubiz et al. 1982). Since the enzyme for biosynthesis of LTD_4 is located in the plasma membrane, LTC_4 is most likely transformed to LTD_4 as it passes through the plasma membrane to the outside of cell(Needleman et al. 1986). Further metabolism gives rise to the enzymatic transformatin of LTD_4 to (5S)-hydroxy-6-S-cysteinyl(E,E,Z,Z)-7,9,11,14- eicosatetraenoic acid (LTE_4) by removal of the glycine residue by a peptidase enzyme (Houglum et al. 1980; Bernstrom and Hammarstrom 1981). Such enzyme activity has been identified in polymorphonuclear leukocyte specific granules and human

plasma (Borgeat 1986) and the product is considered to be a naturally occuring part of native slow-reacting substances(Pace-Asciak and Granstrom 1983).

LTC_4, LTD_4 and to a lesser extent LTE_4 that act as biological mediators, are associated with early inflammation are known as slow-reacting substance of anaphylaxis (SRS-A) (Samuelsson 1983; Davies et al. 1984). These cysteine-containing leukotrienes are considered to be released with other mediators like histamine and chemotactic factors following interaction between immunoglobulin E(IgE) molecules bound to membrane receptors and antigens such as pollen (Samuelsson 1983).

Lipoxygenation of arachidonic acid with enzymes other than 5-lipoxygenase and 12-lipoxygenase has also been investigated. The15-lipoxygenase reaction occurs in polymorphonuclear leukocytes as well as other tissue cells of various species (Borgeat and Samuelsson 1979c). The enzyme has been isolated and partially purified from rabbit peritoneal neutrophils. It was noted that divalent metal cations such as calcium, magnesium and manganese greatly stimulated the crude but not the purified enzyme(Narumiya et al. 1981; Narumiya and Salmon 1982).

It has been suggested that this enzyme is involved in inactivation of slow-reacting substances of anaphylaxis (SRS-A) (Narumiya and Salmon 1982). The metabolic reaction of this enzyme is presumed to be the first step

in arachidonic acid conversion via oxygenation at carbon-15, giving rise to 15-hydroperoxyeicosatetraenoic acid (15-HPETE) (Maas et al. 1981). Nonenzymatic decomposition of purified 15-HPETE in the incubation mixture occurred and the decomposed products have been isolated and identified as 15-HETE, 15-keto-5,8,11,13-eicosatetraenoic acid, two cis/trans isomers of 13-hydroxy-14,15-epoxy-5,8,11- eicosatrienoic acid, and 11,14,15-trihydroxy-5,8,12-eicosatrienoic acid (Narumiya et al. 1981). The 15-HPETE or 15-HETE compounds may be metabolized by further oxygenation by 5-lipoxygenase activity into 5,15-dihydroxyeicosa-6,13(trans),8,11(cis)-tetraenoic acid (5,15-diHETE)(Maas et al. 1982). It is also of interest that in porcine and human blood granulocytes, 15-HPETE may be dehydrated to a 14,15-oxido-5,8,10,12- eicosatetraenoic acid analogous to LTA_4 (Maas et al. 1981). A similar product was produced when 15-HPETE was incubated with various hemoproteins (Hammarstrom 1983a).

The biological activities of the leukotrienes derived from arachidonic acid indicate that they are involved in contraction of smooth muscles, stimulation of vascular permeability, alteration and activation of leukocytes (Hammarstrom 1983b; Piper 1984). Budnitskaia (1986) has written a review article containing 81 references on work done in the USSR on the mediatory function of lipoxygenase systems affected by ionizing radiation. The emphasis has been on biologically active leukotrienes which take part in biological

processes involved in inflammatory and hypersensitive reactions under the effect of ionizing radiation.

The most effective chemotactic and chemokinetic agents within the lipoxygenase series of products of neutrophils and macrophages that have been identified in vivo and in vitro is LTB_4(Ford-Hutchinson et al. 1980; Hansson et al. 1983). Compared to other potent chemotactic agents, LTB_4 is considered almost as active as synthetic N-formylmethionylleucylphenyl-alanine(FMLP) at equimolar concentrations (Piper 1984). Its chemotactic potency for macrophages and neutrophils has been shown to be at concentrations of about 1 ng/ml, which is greater than any other known lipid chemotactic agent (Ford-Hutchinson et al. 1980). In addition to neutrophils and monocytes, LTB_4 is also considered as a potent chemotactic agent for eosinophils (Davies et al. 1984; Piper 1984). It also stimulates the expression of the component-derived peptide C3b receptor site on these cells (Davies et al. 1984). The effects of cysteine-containing leukotrienes (LTC_4, LTD_4 and LTE_4) on different biological systems have been investigated since discovery of SRS-A. It has been shown that these compounds are potent bronchoconstrictors in several species, including humans, with specific effects on the peripheral airways (Dahlen et al. 1980; Smedegard et al. 1982; Samuelsson et al.1980).

The present study was designed to extend the investigation of the metabolic activity of arachidonic acid as one of the integral components of cell membrane especially in mononuclear leukocytes (macrophages). This research relates the effects of different stimulating agents on macrophage cells obtained from the rat peritoneal cavity to release arachidonic acid as the major precursor of biologically active mediators such as leukotrienes. The specific objective of this project is to determine in vitro biosynthesis of leukotrienes by macrophages following exposure to Cobalt-60 gamma irradiation as a physico-chemical stimulating agent for the release of arachidonic acid from phospholipids of the cell.

CHAPTER II

LITERATURE REVIEW

A. Discovery, structure and nomenclature of leukotrienes:

In 1938 Feldberg and Kellaway(1938) injected cobra venom into guinea-pig perfused lungs and observed that the injection of venum was followed with the release of a substance into the perfusate which caused not only a rapid histamine-like contraction but also a delayed and slow contraction of isolated jejunum of the guinea-pig. The material that causes slow contraction of smooth muscle was referred to as slow reacting substance (SRS) and it was assumed to be lysocithin from lecithin or substances formed in the tissues from lipids other than lecithin(Feldberg and Kellaway 1938).

Two years later, Kellaway and Trethewie showed that a similar substance was released by immunological challenge of sensitized guinea-pig lungs that during anaphylatic shock produce contractions of the guinea-pig ileum (Brocklehurst 1953). They were, however, unable to separate its effect from that of histamine because anti- histamine drugs were not available, and their evidence for the release of SRS was based on the observation that this substance caused a more prolonged contraction of the gut compared with

histamine alone. Subsequently SRS was termed slow reacting substance of anaphylaxis(SRS-A) when isolated guinea-pig lung perfusate was assayed against histamine in the guinea-pig ileum with the use of mepyramine anti-histamine drug in the organ bath (Brocklehurst 1953, 1960). It was shown that the occurence of this substance is not dependent upon antigen types; ovalbumin, bovine and horse serum albumin, diphtheria toxoid, pollen grains, cat and horse scurf, and pneumococcus polysaccharide all are involved in release of histamine and the appearance of SRS-A(Brocklehurst 1960).

SRS-A has been considered as a mediator with an important role in pathophysiology of asthma since it was produced by lung of asthmatics. In vitro studies have shown that perfused segments of human lung treated with pollen extract released SRS-A, and this lead to prolonged smooth muscle contracting activity (Jakschik et al. 1977, Samuelsson et al. 1980). Since the discovery of SRS-A and its identification it has been suggested that this substance is released during allergic reactions.

In 1979 when Samuelsson and his colleagues, while studing the metabolism of arachidonic acid by using polymorphonuclear leukocyte from rabbit peritoneal cavity, discovered the lipoxygenase pathway converted arachidonic acid to peptidolipids. Initially an unstable epoxide with a conjugated triene was identified, and this compound was found to be a key intermediate for the biosynthesis of other other arachidonic acid

derivatives(Borgeat and Samuelsson 1979a,b; Samuelsson et al. 1980, Marx 1982, Hansson et al. 1983).

Quantitative studies on generation of SRS from different cells and species showed that non-immuniological sources could be used to produce enough of this bioactive material for structural elucidation (Hansson et al. 1983). Human polymorphonuclear leukocytes and murine mastocyloma cells treated with calcium ionophore A23187 and h-cysteine were produced SRS, and it was suggested that arachodonic acid was released through activation of a calcium- dependent phospholipase (Borgeat and Samuelsson 1979a; (Samuelsson et al. 1980, Marx 1982). Since all SRS compounds are generated from metabolism of arachidonic acid by lipoxygenase activity , and because their chemical identity was originally discovered in leukocytes the term leukotriene (LT) as a trivial name was proposed for this new type compound. (Samuleson et al. 1980, Hammarstrom 1983b).

During the initial studies of leukotriene production by polymorphonuclear leukocytes, one of the most important and fruitful observations that linked SRS with structural determination of leukotrienes was the ultraviolet(UV) spectrum. The UV spectrum of SRS-A indicated a conjugated triene unit in the SRS molecule (Borgeat and Samuelsson 1979b,c; Samuelsson et al. 1980). Thus, the first complete structure of a SRS compound was described as 5(S)-hydroxy- 6(R)-S-glutathionyl- 7,9-trans-11,14-cis-eicosatetraenoic acid

and this compound was given the trivial name leukotriene C (Marx 1982; Hansson et al. 1983). This compound was originally generated from murine mastocytoma cells, labelled with [^{3}H]arachidonic acid and [^{35}S]cysteine following Ca-ionophore A23187 stimulation. Both tritium and sulfur-35 were incorporated into the SRS compound(Samuelsson et al. 1980, Hansson et al. 1983, Hammarstrom 1983a). Based on structural resemblance of LTC with various members of arachidonic acid metabolities, a pathway has been proposed which involves the biosynthesis of leukotrienes that have been designated alphabetically leukotriene A_4(LTA_4) as an unstable epoxide intermediate, leukotriene B_4(LTB_4), leukotriene C_4(LTC_4), leukotriene D_4(LTD_4), etc(Samuelsson and Hammarstrom 1980). A numerical subscript has been used to describe the total number of double bonds in the molecule (Samuelsson and Hammarstrom 1980, Piper 1984). More studies concerning the chemical nature of SRS-A have indicated that sulfur-containing peptidolipids such as LTC_4, LTD_4 and to a lesser extent LTE_4 that act as important lipid mediators are involved in various pathophysiological disorders related to acute hypersensitivity reactions as is the SRS-A substance (Davies et al. 1984; Balazy and Murphy 1986). These cysteine containing leukotrienes have been shown to be released with other mediators like histamine and chemotactic factors following interaction between

immunoglobulin E(IgE) molecules bound to membrane receptors and antigens such as pollen (Samuelsson 1983).

B. Biosynthetic Pathways for Arachidonic Acid Oxygenation.

Arachidonic acid is a tetraunsaturated 20 carbon fatty acid that is present in large amounts as a major constituent of cell membrane (Davies et al. 1984, Borgeat 1986). Enzymatically the newly released arachidonic acid is rapidly oxygenated by two enzymes in the cell, a particulate cyclooxygenase enzyme and cytoplasmic lipoxygenase (Marcus 1985).

1. Cyclooxygenase Pathway:

The enzyme fatty acid cyclooxygenase that is involved in oxygenation and cyclization reactions of arachidonic acid to form intermediate compounds has been demonstrated in almost all animal tissues, particularly in seminal vesicles, lungs and the renal medulla (Oliw et al. 1983). This enzyme has been shown to be a membrane-associated enzyme (Samuelsson et al. 1975). The enzyme catalyzes a complex reaction by inserting two molecules of oxygen into arachidonate to yield 9-,11--peroxido-15(S)-hydroperoxyprosta-5-cis,13-trans-dienonic acid which is an unstable cyclic endoperoxide intermediate called prostaglandin G_2(PGG_2). This compound is subsequently reduced by hydroperoxidase activity to 9,11-peroxido-

15(S)-hydroxyprosta-5-cis,13- trans-dienoic acid(PGH_2) as a second unstable intermediate compound in the cyclooxygenase pathway (Uneo et al. 1982, Oliw et al. 1983, Needleman et al. 1986).

The metabolism of prostaglandin endoperoxides(PGG_2 and PGH_2) is dependent upon the enzymes present in a given cell type (Marcus 1985). Studies on the transformation of labeled arachidonic acid by human platelets induced by various agents have shown that three major products are formed from arachidonic acid(Samuelsson et al. 1978). Platelet thromboxane synthetase activity, associated with dense tubular membranes catalyzes the formation of 12-L-hydroxy- 5,8,10- heptadecatrienoic acid (HHT) and thromboxane A_2 (TXA_2) from PGG_2 and PGH_2(Samuelsson et al. 1978, Needleman et al. 1986). Some HHT may be formed nonenzymatically from the breakdown of PGH_2 (Needleman et al. 1986).

Furthermore, the prostaglandin endoperoxides were transformed to stable prostaglandins via enzymatic or nonenzymatic reactions (Oliw et al. 1983). The endperoxides are either isomerized to a ß-hydroxy ketone, such as prostaglandin E (PGE) and prostaglandin D(PGD), or to prostaglandin F(PGF) by endperoxide reductase activity(Oliw et al. 1983, Harris 1985, Needleman et al. 1986). The principal steps in biosynthesis of prostaglandins and thromboxanes via the cyclooxygenase pathway are shown in Fig.1.

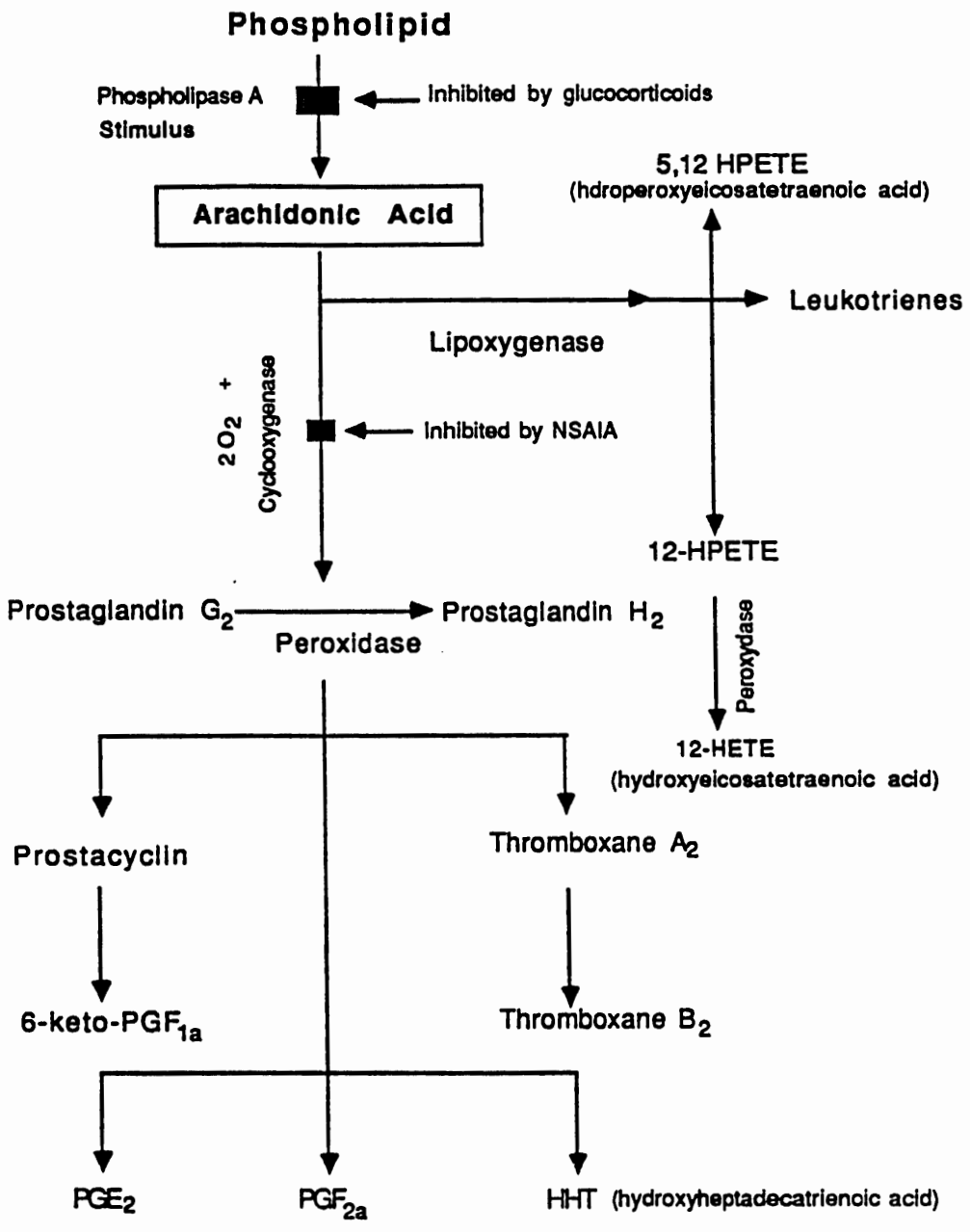


Fig. 1: Biosynthesis of biologically active mediators through the action of the cyclooxygenase or lipoxygenase enzymes on released arachidonic acid from phospholipids(modified from Ninnemann, J. L. 1984).

2. Lipoxygenase Pathway:

The extensive investigation of oxidative metabolism of polyunsaturated fatty acids lead to the discovery of lipoxygenases (Samuelsson et al. 1980) that had initially been recognized in plants (Borgeat 1986). A variety of oxygenated derivatives are formed during the reaction of lipoxygenases with unsaturated fatty acids(Hansson et al. 1983, Borgeat 1986). Hydroperoxy acids are considered to be the intermediate products of lipoxygenase and these are subsequently reduced to hydroxy acids, both enzymatically by peroxidases and through non-enzymatic decomposition (Hansson et al. 1983). The lipoxygenase enzymes that are involved in oxygenation of arachidonic acid are:

a. The 12-Lipoxygenase Products:

The first true lipoxygenase enzyme of animal origin was isolated from human blood platelets after incubation of [1-c^{14}]arachidonic acid. Compared to other arachidonic acid metabolites (PGG_2 and PGH_2) the least polar compound was found to be 12-L-hydroxy-5,8,10,14- eicosatetraenoic acid(12-HETE). It was concluded that this compound was formed from 12-L-hydroperoxy-5,8,10,14- eicosatetraenoic acid(HPETE) by a glutathione-dependent peroxidase(Hamberg and Samuelsson 1974, Hamberg et al. 1974, Chang et al. 1982). 12-HPETE production by platelet

cells via 12-lipoxygenase was the first indication that animal cells also have lipoxygenase activity(Nugteren 1975). Experiments with bovine and rat platelets showed most of the activity of 12-lipoxygenase to be cytosolic (Nugteren 1975). In another investigation this enzyme activity was found to be exclusively localized in a particulate fraction prepared from human platelet homogenates (Ho et al. 1977). In a study with human blood platelets it was suggested that this enzyme is bimodally distributed between cytosol and membranes (Lagarde et al. 1984, Needleman et al. 1986).

Recent investigation has indicated that platelet derived 12-HETE may be subjected to a second oxygenation by a leukocyte lipoxygenase enzyme other than 12-lipoxygenase (Wong et al. 1984), perhaps 5-lipoxygenase (Needleman et al. 1986). Incubation of [1-c^{14}]12-HETE with a suspension of human polymorphonuclear leukocytes (HPMNL) lead to the formation of 12-L,20-dihydroxy- 5,8,10,14- eicosatetraenoic acid(12,20-DHETE), however, no 12, 20-DHETE was formed when 12-HETE was incubated with washed platelets alone. Therefore it was concluded that platelet-leukocyte interaction may generate further metabolism of 12-HETE into biologically important compounds (Wong et al. 1984).

b. <u>The 15-Lipoxygenase Products</u>:

The reaction of the 15-lipoxygenase enzyme in polymorphonuclear

leukocytes as well as other tissue cells of various species has been studied (Borgeat and Samuelsson 1979a,b; Bryant et al. 1982, Serhan et al. 1984). Incubation of human polymorphonuclear leukocyte suspensions labelled with [1-c^{14}]arachidonic acid with Ca-ionopore A23187 lead to the formation of (15S)-hydroxy- 5,8,11,13-eicosatetraenoic acid(15-HETE), 12HETE and (5S)-hydroxy-6,8,11,14-eicosatetraenoic acid(Borgeat and Samuelsson 1979a). The most abundant arachidonate metabolite recovered from Ca-ionopphore A23187 stimulated human eosinophils was 15(S)-HETE, the reduced form of 15(S) HPETE the initial product of the enzymatic pathway (Turk et al. 1982). Since the primary oxygenation was restricted to carbon 15 of arachidonic acid, the enzyme was termed the arachidonic acid 15-lipoxygenase (Narumiya et al. 1981). Immunoprecipitation studies have shown that this enzyme is contained in reticulocytes and not in other tissues with lipoxygenase activity. This enzyme oxygenates fatty acid in phosphlipid as well as the unesterified substrate, and mitochondrial membranes are more readily oxidized than plasma membranes (Rapoport et al. 1979). Although the activity of arachidonic acid 15-lipoxygenase was recovered in the cytosol fraction, the actual subcellular localization is not clear (Narumiya et al. 1981).

When 15-lipoxygenase catalyses arachidonic acid to 15(S)-HPETE, which is then decomposed non-enzymatically to a mixture of 15-HETE, 15-keto-5,8,11,13- eicosatetraenoic acid, 13-hydroxy-14,15- epoxy-

5,8,11-eicosatrienoic acid, and 11,14,15-trihydroxy-5,8,12- eicosatrienoic acid(Narumiya et al. 1981). Similar decomposition products were observed and reported by Gardner and co-workers who found that when an isomeric mixture of linoleic acid hydroperoxides was decomposed homolytically by Fe^{2+} in an ethanol-water solution and nine species including keto, epoxy-hydroxy, and trihydroxy compounds were obtained (Gardner et al. 1974). Additional studies on the metabolism of 15-HPETE or its reduced product 15-HETE performed with rat peritoneal mononuclear leukocytes, in the presence of arachidonic acid lead to the production of a new dihydroxy acid, 5(S),15(S)- dihydroxy-6,13- trans-8,11- cis- eicosatetraenoic acid (5,15-diHETE)(Maas et al. 1982). It was shown that both 5-HETE and 15-HETE were formed in incubations of rat monocytes with arachidonic acid and thus could serve as substrates for conversion to 5,15-diHETE(Maas et al. 1982). Since both cellular C-5 and C-15 lipoxygenases are sterospecific, the formation of 5,15-diHETE may be catalyzed via enzymatic double oxygenation (Maas et al. 1982). Either 15-HPETE or 15-HETE may be oxygenated by the 5-lipoxygenase and then reduced to yield 5,15-diHETE or this compound may also be formed by the action of the 15-lipoxygenase on 5-HETE (Needleman et al. 1986).

c. The 5-Lipoxygenase Products:

inflammatory cells, [1-^{14}C]arachidonic acid and homo- -[1-C^{14}]-linolenic acid were added to suspension of polymorphonuclear leukocytes from the peritoneal cavity of rabbits. When the cells were stimulated with Ca-ionophore A23187, the formation of 5-L-hydroxy-6,8,11,14-eicosatetraenoic acid(5-HETE) was observed, and this lead to the discovery of a new family of arachidonic acid metabolites. The enzyme that initiated this reaction pathway was 5-lipoxygenase enzyme(Borgeat et al. 1976, Samuelsson et al. 1980; Sammuelsson 1983). The cytosolic activity of 5-lipoxygenase enzymes has been reported in neutrophils, eosinophils, monocytes, mast cells, and keratinocytes as well as lung, brain, spleen and heart (Soberman et al. 1986, Needleman et al. 1986). Partially purified 5-lipoxygenase from guinea-pig peritoneal polymorphonuclear leukocytes was found to be cytosolic and calcium dependent (Ochi et al. 1983), and the calcium-dependent reaction was stimulated by ATP and several nucleotides (Ochi et al. 1983, Furukawa et al. 1984, Sammuelsson et al. 1987).

The 5-lipoxygenase enzyme oxygenates the C-5 position of arachidonic acid to produce 5-hydroperoxy-6,8,11,14-eicosatetraeonic acid (5-HPETE) (Samuelsson 1983). The 5-lipoxygenases from all sources were shown to catalyze not only the formation of 5-HPETE from arachidonic acid, but also the subsequent conversion of 5-HPETE to LTA_4(Samuelsson et al. 1987).

d. Biosynthesis of Leukotriene A_4

A mechanism for the biosynthesis of LTA_4 is the conversion of arachidonic acid to the unstable epoxide(LTA_4) via 5-lipoxygenase enzyme (Hammarstrom 1983a). The structure for this intermediate epoxide, 5,6-oxido-7,9,11,14- eicosatetraenoic acid(LTA_4) was confirmed by the labelling of arachidonic acid in polymorphonuclear leukocyte suspensions with $^{18}O_2$. Clearly it was shown that a metabolite of arachidonic acid can undergo a facile reaction with nucleophiles to yield 5-hydroxy-12-O-alkyl derivatives (Borgeat and Samuelsson 1979a).

Leukotriene A_4 was found to be a short lived precursor to the other leukotrienes. LTA_4 reached maximum concentration about 45 sec after incubation of rabbit peritoneal polymorphonuclear leukocytes(Borgeat and Samuelsson 1979b), and in human peritoneal polymorphonuclear leukocytes the maximum was reached after 1.5 min incubation (Radmark et al. 1980a,b).

e. Biosynthesis of Lekotriene B_4

Leukotriene B4, or (5s),12(R)-dihydroxy-6,8,10,14- eicosatetraenoic acid was found in human(Borgeat and Samuelsson 1979a,b; Ford Hutchinson et al. 1980), guinea-pig (Bokoch and Reed 1981) and mouse leukocytes

(Humes et al 1982). Alveolar macrophages of adult male New Zealand rabbits treated with phagocytic stimulating agent (zymosan) or Ca-ionophore A23187 demonstrated an increase in LTB_4 biosynthesis when compared to resting cells (Hsueh and Sun 1982). Human alveolar macrophage cells cultured in vitro, and stimulated with Ca-ionophore A23187 produced LTB_4(Martin et al. 1984). LTB_4 was considered the predominant arachidonate lipoxygenase product when compared to other arachidonic acid metabolites(Martin et al. 1984).

The direct addition of synthetic LTA_4 to suspensions of human leukocytes or murine mastocytoma cells lead to the synthesis of LTB_4 (Radmark et al. 1980b, Radmark et al. 1980c). Cell-free plasma from mammals, including guinea-pigs, pigs, cows, sheep, rabbits, rats, dogs and humans contain a component with capablity of converting LTA_4 into LTB_4 (Fitzpatrick et al. 1983). The activity appeared to be enzymatic since it was stopped by heating at 56 ^{o}C or digestion of plasma with a proteolytic enzyme (Fitzpatrick et al. 1983). The enzymatic conversion of LTA_4 into LTB_4 was demonstrated with purified LTA_4-hydrolase from human leukocytes, or rat neutrophils and it was found that this enzyme is localized in the cytosolic fraction (Radmark et al. 1984). Several studies have shown that this enzyme

is rate-limiting in LTB_4 production (Sun and McGuire 1984). In an in vitro investigation of human neutrophils treated with Ca^{2+} ionophore A23187, maximum production of LTB_4 was evidenced after 2 minutes at which time it was converted to 20-hydroxyleukotriene B4 and or 20-carboxyleukotriene B_4 (Sun and McGuire 1984, Ford-Hutchinson and Evans 1986).

Although a considerable number of reports have been published on biosynthesis and biological activity of LTB_4, relatively little is known about the metabolic fate of this compound in the intact organism. However, at the cellular level, it has been shown that LTB_4 inactivated by human leukocytes to form a 5,12,20- trihydroxy-eicosatetraenoic acid (20-OH-LTB_4), as well as a 5,12-dihydroxy-1,20-dicarboxylic eicotetraenoic acid (COOH-LTB_4)(Hansson et al. 1981). Subsequently it was found that a soluble enzyme(LTB_4 20-hydroxylase) in neutrophils is responsible for hydroxylation of LTB_4 (Powell 1984, Soberman et al. 1985). Recent studies of LTB_4 metabolism of rodent hepatocytes indicate that the hydroxylation of LTB_4 is cytochrome P-450 enzyme dependent (Harper et al. 1986, Romano et al. 1987).

f. Biosynthesis of Peptidolipid Leukotrienes:

The peptidolipid leukotrienes constitute a group of arachidonate lipoxygenase products that contain a small peptide linked to the C-6 position on the arachidonate chain through a cysteinyl sulfur. The individual compounds in this group are LTC_4, LTD_4, LTE_4 and LTF_4. It has been reported that the LTC_4, LTD_4 and to a lesser extent LTE_4 were produced under pathophysiological disorders and identified as the major active consituents of slow reacting substance of anaphylaxis(Davies et al. 1984, Rokach and Fitzsimmons 1986).

Enzymatic formation of the peptidolipid leukotrienes has been initiated by addition of the tripeptide glutathione to the intermediate LTA_4 in human and murine mastocytoma leukocyte cultures(Radmark et al. 1980c). LTC_4 is then metabolized through the loss of glutamic acid and cysteine to LTD_4 and LTE_4, respectively (Jakschik et al. 1982). The structure of LTC_4 is (5S,6R)-5-hydroxy-6-S-glutathionyl-(E,E,Z,Z)-7,9,11,14-eicosatetraenoic acid and LTD_4 has the structure (5S,6R)-5-hydroxy-6-S-cysteinylglycyl-(E,E,Z,Z)-7,9,11,14-eicosatetraenoic acid(Borgeat and Samuelsson 1979a,b). Both of these compounds are formed by the same biosynthetic route; a combination of oxygenation of arachidonic acid by 5-lipoxygenase and further metabolism by

glutathione transferase and γ-glutamyltransferase, respectively (Borgeat and Samulesson 1979a,b; Morris et al. 1982). Both of these enzymes were found in significant quantities in guinea-pig lung and RBL-1 cells (Morris et al. 1982). An enzyme that is involved in the metabolic pathway of LTD_4 has been partially purified from porcine kidney. This enzyme converted LTD_4 to a less polar compound, 5S-hydroxy-6-S- cysteinyl-7,9- trans-11,14,cis-eicosatetraenoic acid, designated LTE_4 (Bernstrom and Hammarstrom 1981).

Following incubation of LTC_4 with highly purified --glutamyltrans-peptidase, the rate of LTC_4 conversion to LTD_4 was significantly more rapid when the reaction was carried out in presence of an amino acid mixture closely approximating the concentration of amino acids in blood plasma(Anderson et al. 1982). The conversion of LTD_4 to LTE_4 is catalyzed much more rapidly by renal dipeptidase than by renal aminopeptidase M (Anderson et al. 1982). The reversible conversion of LTD_4 to LTC_4 may be effectively catalyzed by γ-glutamyltranspeptidase in the presence of slightly lower concentrations of glutathione (Anderson et al. 1982). The incubation of LTE_4 with γ-glutamyltranspeptidase (γ-GT) results in the incorporation of glutamic acid into the molecule and formation of LTF_4.

The release of LTC_4 and other SRS-A products (LTD_4 and LTE_4) from mouse peritoneal macrophages (Rouzer et al. 1980) and mouse pulmonary macrophages in reponse to phagocytic stimulus (zymosan) (Rouzer et al. 1982) has been demonstrated. Following incubation of rat basophilic leukemia cells with arachidonic acid, glutathione and calcium a mixture of LTC_4, LTD_4 and dihydroxy acid isomers was found, and it was suggested that the enzymes responsible for this pathway appeared to be in the particulate fraction(Jakschik et al. 1982). Direct evidence for transcellular metabolism of LTA_4 into LTC_4 was reported when LTA_4 was incubated with confluent cultures of porcine aortic endothelium. Endothelial cells contain LTC_4 synthetase but they are unable to synthesize LTC_4 directly from endogenous or exogenous arachidonic acid when stimulated with ionophore A23187(Miller et al. 1985, Feinmark and Cannon 1986). The possible intercellular transfer of LTA_4 as a substrate for LTC_4 formation has been observed between polymorphonuclear leukocytes and endothlial (Feinmark and Cannon 1986) and platelet cells (Maclouf and Murphy 1988). Platelets were unable to synthesize LTC_4 directly from endogenous or exogenous arachidonic acid by 5-lipoxygenase, however, platelets could convert LTA_4 into LTC_4 (Pace-Asciak et al. 1986). Human neutrophils stimulated with the

calcium ionophore (5μM) produced little LTC_4, whereas the production of this compound was greatly increased when neutrophils were transfered into platelet suspensions (Maclauf and Murphy 1988). Experiments have demonstrated that platelets contain a glutathione S-transferase specific for the biosynthesis of LTC_4 from neutrophil-derived LTA_4 (Maclouf and Murphy 1988). Figure 2 shows the lipoxygenase pathway for leukotrienes synthesis.

g. Other Lipoxygenase Products: Lipoxins:

Lipoxins are recently discovered oxygenated derivatives of arachidonic acids and are the first natural products containing a fully conjugated tetraene. These new compounds were isolated for the first time from human leukocytes exposed to 15-HPETE(Serhan et al. 1984a,b). The structure of the major tetraene was established by physical and chemical methods and found to be 5,6,15- trihydroxy- 7,9,11,13- eicosatetraenoic acid designated lipoxin A(LXA) (Serhan et al. 1984a). In another study the second new member of the trihydroxytetraene series of compounds was isolated from human leukocytes culture exposed to 15-HPETE and calcium ionophore A23187. The chemical structure for this compound was shown to be 5-D,14,15,-L-trihydroxy-6,8,10,12-eicosatetraenoic acid and the trivial name lipoxin B(LXB) was proposed(Serhan et al. 1984b). On the basis of the biosynthetic pathway, the

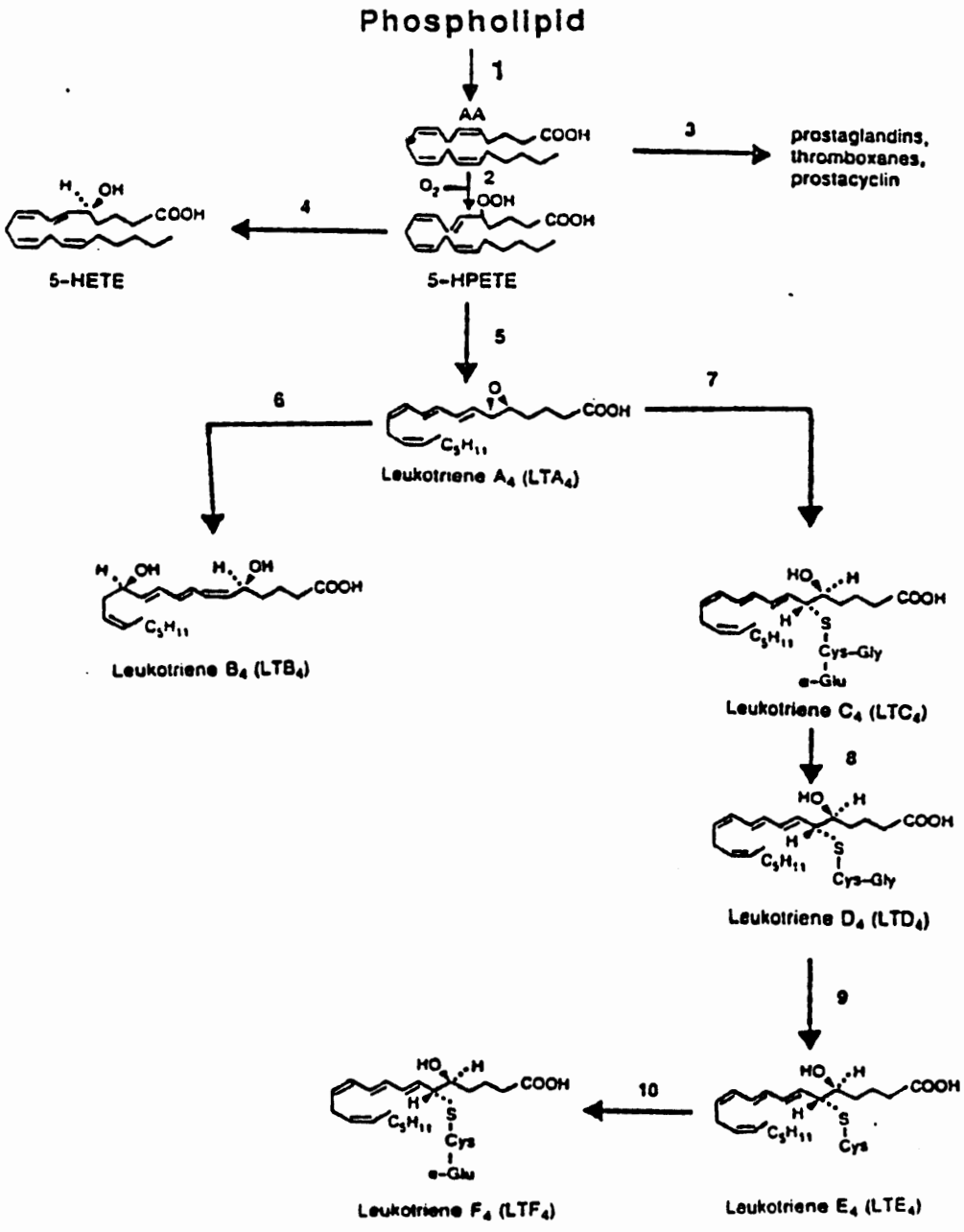


Fig. 2: Formation of leukotrienes via the 5-lipoxygenase pathway. (1) phospholipase; (2) 5-lipoxygenase; (3) cyclooxygenase; (4) hydroperoxidase; (5)Leukotriene A synthetase, dehydrase; (6)hydrolase, leukotriene B synthetase; (7)glutathione S-transferase, leukotriene C synthetase; (8) _glutamyl transpeptidase; (9)leukotriene D-aminopeptidase; (10)_glutamyl transpeptidase (adapted from Harris, D. N. 1985).

results indicated that exposure to 15-HPETE lead to both activation of 5-lipoxygenase and consumption of 15-HPETE for the formation of LXA and LXB (Serhan et al. 1984b, Adams et al 1985, Serhan et al. 1986a). Experiment with activated leukocytes revealed transformation of 15-HETE both to LXA and LXB. However, when 15-HETE was added to the cells in the absence of stimulus(either ionophore A23187 or fMet-Leu-Phe), it was not transformed to lipoxins, suggesting that leukocyte activation is required for lipoxins formation (Serhan et al. 1986a).

Further investigations on lipoxins production have indicated that with successive enzymatic oxidations at C15 and C5 of arachidonic acid to yield the 5,15-diHPETE analogous to LTA_4 formation, this intermediate could then undergo a stereospecific enzymatic dehydration to give a 5(S),6(S)-tetraene epoxide (Fitzsimmons et al. 1985). It was indicated that further enzymatic hydrolysis of the epoxide at C6 would lead to LXA, while non-enzymatical homoconjugate addition of water at C14 would produce LXB (Adams et al. 1985). By using an improved isolation procedure in the study of LXA biosynthesis, incubation of 15-HPETE with stimulated human leukocytes were lead to the formation of LXA. Three isomers of LXA were identified, (5S,6S,15S)-5,6,15- trihydroxy- 7,19,13-trans-11- cis- eicosatetraenoic acid (6S-LXA), (6S,6R,15S)-5,6,15-trihydroxy-7- 9,11,13-trans- eicosatetraenoic acid (11-trans-LXA), and (5S,6S,15S)- 5,6,15-trihydroxy- 7,9,11,13-trans-

eicosatetraenoic acid (6S-11-trans-LXA)(Serhan et al. 1986a). Similar experimentation with human leukocytes lead to the formation of two LXB isomers. These compounds were shown to be (5S,14R,15S)-5,14,15-trihydroxy- 6,8,10,12-trans-eicosatetraenoic acid (8-trans-LXB) and (5S,14S,15S)-5,14,15- tri-hydroxy- 6,8,10,12-trans- eicosatetraenoic acid designated (14S)-8-trans-LXB (Serhan et al. 1986b).

C. The Effects of Radiation on the Cellular Components of Biological Systems

Although it is generally accepted that many biological consequences of ionizing, as well as ultraviolet, irradiation induce damage to DNA, physical and biochemical alteration of the cellular membranes have been considered as a second important field of investigation, because these cellular components perform a decisive role in the functional organization of the cell (Konings and Drijver 1979, Wolters and Konings 1982). Several investigations have suggested that changes in membrane functions such as regulation of membrane permeability, membrane-bound enzyme activities, intracellular compartmentalization and intercellular communication arise from the alteration of membrane lipids as a result of radiation-induced lipid peroxidation (Wolters and Konings 1982; Yukawa et al. 1985). Both lipids and proteins which are considered as the main components of the biological membrane may be responsible for radiation-induced impairment of cell

function (Wolters and Konings 1982; George et al. 1983). From studies with model bilayer membranes (liposomes), it has been predicted that the polyunsaturated fatty acids are very radiosensitive and peroxidizable (Wolters and Konings 1982).

Studies have also indicated that radiation-induced phospholipid peroxidation could be prevented by naturally occuring membrane antioxidant ∝tocopherol (vitamin E)(Huijbers et al. 1979; Wolters and Konings 1982; George et al. 1983; Wolters and Konings 1984). There are antioxidant agents other than vitamin E such as water-soluble nicotinamide adenine dinucleotide(NADH), catechol, and cysteamine that protect membrane phospholipid against radiation induced peroxidation(Raleigh 1987). The cellular membranes are classified into two groups according to their composition, each with different radiosensitivity. Plasma membrane and the lysosomal membrane contain relatively much lipid, cholesterol, and carbohydrates but no vitamin E(Chow 1975). In contrast, the membranes of mitochondria and endoplasmic reticulum contain a high amount of protein and low amount of lipid, cholesterol, and carbohydrates, whereas vitamin E is present in this group of membranes(Lussier and Roy 1977). Because cholesterol and unsaturated fatty acids are considered as the main points of attack for the free radicals and peroxides formation, membranes such as plasma and lysosomal membrane rich in cholesterol and unsaturated fatty

acids are considered much more susceptible to ionizing irradiation (Huijbers et al. 1979). The modification of enzymatic activites and increase of malonaldehyde formation in mitochondrial fractions has been investigated (Kergonou et al. 1981, Yukawa et al. 1985). Following whole body gamma irradiation(8 Gy) of rats, the relationships between the enzymatic acitivities and increase of malonaldehyde concentration in liver mitochondrial fractions and increase of lipid peroxidation was studied (Kergonou et al. 1981).The results indicated that the increased malonaldehyde content is probably due to a higher production of toxic oxygentated species (O_2 and H_2O_2), by high activites of NADH -cytochrome C reductase and cytochrome oxidase enzymes and also alteration in defense mechanisms against these toxic oxygenated products, resulting from lowered superoxide dismutase and catalase activities (Kergonou et al. 1981). In addition, contents of arachidonic acid in phosphatidylcholine, phosphatidylinositol and phosphatidylethanol- amine were also markedly decreased in a radiation dose-dependent fashion. It was suggested that the decrease of arachidonic acid may reflect the radiation dose-dependent increase in the formation of malonaldhyde (Yukawa et al. 1985). The mechanism of the radiation- induced damage in the enzyme activites in the cellular and subcellular membranes was thought to be caused mainly by peroxidation of membrane lipids and not a direct damage by radiation to the enzyme molecule itself (Yukawa et al. 1985).

D. Radiation and Immune Response:

The effects of radiation on the immune response have been extensively investigated as an area of cellular immunity with respect to the number and definition of subpopulations of lymphocytes and their known cooperation in immune responses(Anderson 1976, Bernardo 1982). Total body X-irradiation with a wide range of 90 to 1190 R was found to alter the number and distribution of cells in the peritoneal cavity of unstimulated mice(Kornfeld and Greenman 1966, Swartz and Saluk 1981). After exposure, the peritoneal macrophages remained essentially unaltered in number or decreased gradually to about 50% of the control value. However, the number of lymphocytes in the peritoneal cavity decreased sharply within 24 hours and were considered as the most radiosensitive cells in the body(Kornfeld and Greenman 1966).

In a morphological and biochemical study of alveolar macrophages from X-irradiated mice(1800 R), electron micrographs of some cells revealed an increased accumulation of cytoplasmic inclusions and membrane-bound vacuoles that were partially filled with myelin figures, usually showing irregularly spaced membranes(Gross and Balis 1978). Considering the potential effect of radiation damage on macrophage function with respect to immune induction, it has been reported that the ability of macrophages to phagocytose a wide variety of other agents is quite resistant to irradiation

(Geiger and Gallily 1974). In various studies reported by Anderson (1976), doses of irradiation of around 600-800 R or in the Kilorad range of up to 10,000 R have been used without any demonstrable reduction in phagocytic activity. However, the radiosensitivity of several post-phagocytic macrophage functions including the ability to catabolize, degrade or retaining of antigen has been shown (Anderson 1976) .

In the course of a study of the biochemical effects of lung irradiation in mice received two doses of X-irradiation to the thorax, 600 R per dose, separated by two days, the total number of alveolor macrophages reached a minimum at two weeks and then returned to control levels at 6-10 weeks. It was suggested that the effect was due to damage to the dividing monocytes in the lung interstitium as the precursors of macrophages (Gross 1977).

E. Radiation and Arachidonic Acid Metabolism

The release of arachidonic acid from phospholipids catalyzed by phospholipase A_2, and possibly by phospholipase C, and 1,2-diacylglycerol-lipase has been well documented(Oliw et al. 1983; Davies et al. 1984; Marcus 1985; Lognonne et al. 1985). It has been shown that radiation can , possibly by generating free radicals or through activation of phospholipase A_2, liberate the arachidonic acid leading to the formation of

prostaglandins(Allen et al. 1981; Ziboh et al. 1982). The effect of a single total- body exposure to 0.5, 1.5, or 3.0 Gy Co-60 gamma radiation on changes in production of prostaglandin in guinea-pig parenchymal lung tissues was examined. Significant alterations in prostaglandin and thromboxane concentration were observed following irradiation in dose dependent manner. PGE_2, PGF_2, and TxB_2 levels were shown signficantly increased in animals receiving 3.0 Gy at 3 h post-exposure. However, by 24 h, tissue prostaglandin and thromboxane levels had returned to near control value (Steel and Catravas 1982). In an in vivo study of whole-body gamma irradiation (8.4 Gy) in rat, it was shown that the arachidonic acid metabolism in blood platelets into TxB_2 was increased at day 1 after irradiation and then gradually decreased to very low values from day 7 to 10. It was suggested that the conversion of exogenous arachidonic acid into TxB_2 was decreased because a lower incorporation of arachidonic acid into phospholipid membrane of platelets which must have resulted in structural and functional changes in these membrane after irradiation (Lognonne et al. 1985).

Prostacyclin (PGI_2) which is thought to play a critical role in several aspect of circulatory physiology, including the inhibition of platelet aggregation and the relaxation of vascular smooth muscle (Moncada et al. 1976; Bergofsky 1980) has been synthesized from cultured pulmonary artery

endothelial cells from calf when exposed to 100 and 3000 rads of gamma irradiation (Hahn et al. 1983). Increase in both PGI_2 synthesis and ∝aminoisobutyric acid uptake was found to be significant at doses of 600 to 3000 rad(Friedman et al. 1986).

The induced biosynthesis of cutaneous prostaglandins by irradiation (300-1000 rad), caused a rapid release of epidermal arachidonic acid and increased epidermal PGE_2 formation via the cyclooxygenase enzyme pathway. The results also indicated that after 24 h the epidermal PGE_2 concentration was decreased with a single dose of radiation higher than 1000 rad(Ziboh et al. 1982). The prodromal symptoms of the gastrointestinal syndrome which usually occur a few hours after total body exposure have been reported as nausea and vomiting, occasionally accompanied by diarrhea, producing watery stools and severe cramps (Maxfield et al. 1973). The relation between these prodromal symptoms and radiation exposure as well as the posssible role of prostaglandins were studied in rhesus monkeys as an animal model which appears closest to man in terms of brain organization and gastric function by Dubois and co-workers in 1987. After total body exposure of animals to 800 cGy Co-60 gamma irradiation at a rate of 500 cGy/min,there was an immediate significant increase in gastric juice concentration of PGE_2 and PGI_2. However, both of these metabolites

returned to pre-irradiation levels two days after exposure (Dubois et al. 1987).

The pathophysiological effects of UV radiation on skin and biosynthesis of prostaglandins-like compounds have been reported (Ziboh et al. 1982; De Leo et al. 1984). DeLeo and co-workers (1984), pre-labeled human keratinocytes obtained from normal breast skin with [^{3}H]arachidonic acid and then exposed them to UVB irradiation within a range of 600-1800 J/m$_{2}$. The major metabolites of the cyclooxygenase pathway produced by irradiated cells and identified by high-performance liquid chromatography (HPLC) were shown to be PGE_2- and PGF_2, -like material (De Leo et al. 1984).

Leukotrienes and other related compound production by peritoneal cells exposed to ultraviolet (Hardcastle and Minoui 1987) and ionizing irradiation (Steel et al. 1988) have been investigated. Peritoneal macrophages were isolated from mice and grown in plastic petri dishes. Macrophages prelabeled with [^{14}C]-arachidonic acid were UV- irradiated. The HPLC analysis and radioassay of extracts from treated cells indicated that ^{14}C-arachidonic acid incorporated into phospholipid membrane had been converted into LTB_4 and LTC_4 through the lipoxygenase pathway (Hardcastle and Minoui 1987). It recent studies, investigation of LTC_4 and PGE_2 production was followed by isolation of resident peritoneal cells from mice 2 h and 10 day postexposure to

a single dose (7, 10 or 12 Gy) of gamma irradiation (Steel et al. 1988). The reported results indicate that both PGE_2 and LTC_4 synthesis were quantitatively much higher, in response to Ca^{2+} ionophore A23187 stimulation, when compared to unirradiated peritoneal cells that were treated similarly with ionophore (Steel et al. 1988).

F. The Biochemistry of Arachidonic Acid Metabolism and Regulation

The release of prostaglandins, leukotrienes and other related compounds that are synthesized from a common precursor, arachidonic acid, is under several regulatory controls(Billah et al. 1980; Billah et al. 1981; Lapetina et al. 1981a; Bonney and Humes 1984; DeGeorge et al. 1987). Arachidonic acid does not exist free within cells but is esterified in position 2 of certain phospholipids (Bonney and Humes 1984). It has generally been accepted that its production is induced by a receptor-mediated activation of phospholipase A_2 activity (Bills et al. 1977, Billah et al. 1980, Bonney and Humes 1984). This has been confirmed following the study of phospholipid metabolism in the presence of various stimuli and has provided sufficient information supporting the existence of two pathways for liberation of arachidonic acid in different types of cells (Broekman et al. 1980). One pathway involves the activation of calcium-regulated phospholipase A_2 acting

on membrane to produce unesterified arachidonic acid (Nakamura and Ui 1985). Investigation has shown that phospholipase A_2 is optimally active at a neutral pH is highly sensitive to Ca^{2+}, and is inhibited by deoxycholate (Billah et al. 1981). Calcium is needed because it activates some enzymes and to maintain the functions of microtubles and microfilaments involved in transporting secretory granules to the cell peripheries (Billah et al. 1981). The other one is involved with combined action of phophatidylinositol (PI)-specific phospholipase C and diacylglyceride lipase (Broekman et al. 1980; Bonney and Humes 1984). The product of the phospholipase C action is 1,2-diacylglycerol(DAG), which can either be degraded by 1,2-diacylglycerol lipase to yield free fatty acid or be phosphorylated to phosphatidic acid by 1,2-diacylglycerol kinase (Billah et al. 1981, Dixon and Hokin 1984). The further breakdown of PI and formation of arachidonic acid may result from activation of phospholipase A_2 (Lapetina et al. 1981b). Deoxycholate treatment of horse platelets pre-labelled with [^{14}C]arachidonic acid produced selective conversion of [^{14}C]phosphatidylinositol [PI] to [^{14}C]1,2-diacyl-glycerol via phospholipase C with specific activity for PI (Billah et al. 1980).

Phorbol myristate acetate which is recognized as tumor-promoting and inflammatory agent in some cell types (Levine and Ohuchi 1978), has been shown to stimulate the release of arachidonic acid from peritoneal

macrophages in mice and increased synthesis of prostaglandins both in vivo and in tissue culture (Bonney et al. 1980). The phorbol myristate acetate that cause the increased synthesis of arachidonic acid metabolites could be inhibited by RNA and protein synthesis inhibitors (Bonney et al. 1980).

To investigate whether nonsteroidal anti-inflammatory drugs(NSAID's) affect the metabolism of arachidonic acid through lipoxygenase as well as cyclo-oxygenase in neutrophils, cells were isolated from the pleural cavity of male rats then pre-incubated in the presence of 100 μM aspirin. The inhibitory effects of aspirin were revealed with inhibition of 11-HETE and 15-HETE as well as prostaglandins (Siegel et al. 1980). Although the mechanism of action of aspirin and its analogous compounds(i.e., indomethacin) as prostaglandin biosynthesis inhibitors is not clear, they seem to block only the 11-lipoxygenase and to be ineffective as inhibitors of other lipoxygenase enzymes such as 5-lipoxygenase pathway for leukotriene production (Corey and Park 1982). Compared to NSAID's, Steroidal compounds(i.e., corticosteroids) reduce the availability of arachidonic acid (Kuehl and Egan 1980; Oliw et al. 1983). Fig. 3 shows the inhibitory action of both steroidal and nonsteroidal anti-inflammatory drugs on cellular metabolism of arachidonic acid.

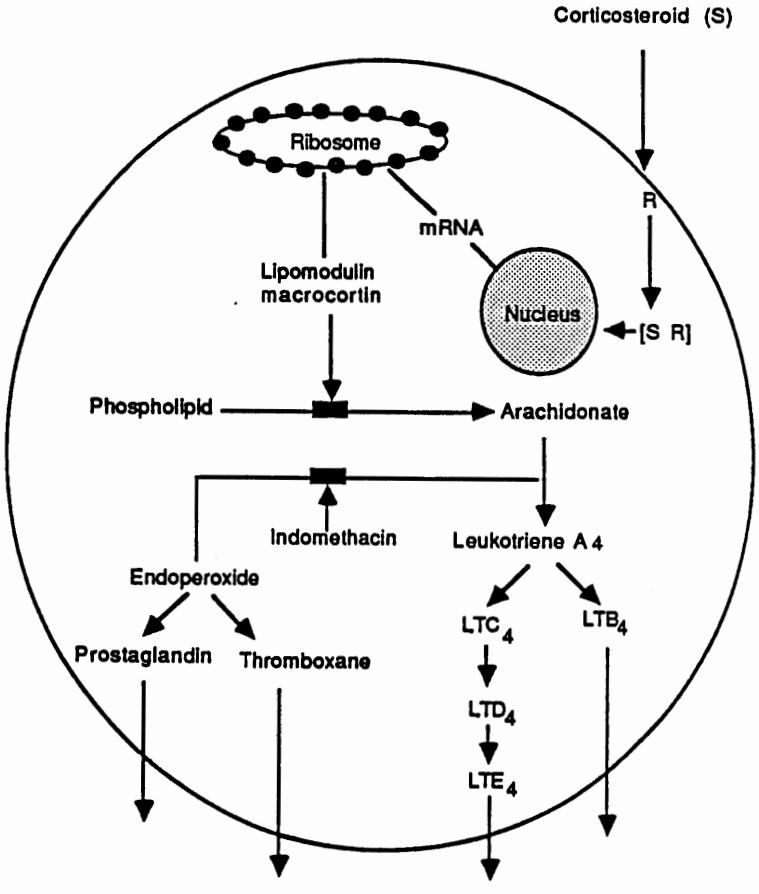


Fig. 3: Mechanism of action of corticosteroids and a nonsteroidal anti-inflammatory drug(indomethacin) on cellular metabolism of arachidonic acid. Treatment with corticosteroids induces synthesis of lipomodulin or macrocortin which inhibits the enzyme fatty acid cyclooxygenase and thus the formation of prostaglandins and thromboxanes (adapted from Oliw et al. 1983).

G. Biological Effects of Eicosanoids:

1. Prostaglandins

Around 1930 two gynecologists observed that human semen contained an unknown factor that could cause either strong contraction or relaxation of human uterine smooth muscle (Kurzrok and Lieb 1930). The biological effects and the chemical nature of this factor that was originally found in semen was characterized as an acidic lipid and named prostaglandin(Kurzrok and Lieb 1930). In these early studies, prostaglandin was observed to cause contraction of several different smooth muscles and lowered arterial blood pressure in rabbits (Kurzrok and Lieb 1930). When various types of prostaglandins became generally available for pharmacological studies,it soon became apparent that prostaglandins could affect almost all body functions. It seem that there is hardly an organ or a function in the body that has not been shown to be influenced one way or other by these compounds (Oliw et al. 1983). The relationships between chemical structures and biological activities of prostaglandins are complex. Two different members of the same group but differing only in the degree of unsaturation in the carbon chain may exert similar influences in one tissue and opposing in another, or one compound may exert opposite actions with different concentration (Oliw et al. 1983). Moreover, there have been marked species variations in sensitivites of the same compound, for example, $PGF_{2\alpha}$ has been found to

lower the blood pressure in rabbit and cat, wheras it acts as a weak pressor in man, rat and dog (Oliw et al. 1983, Martin 1984).

Prostaglandins have a wide range of biological effects on different target tissues of different species. In general prostaglandins can affect behavior through direct actions on individual neurons, on the hypothalamus, on the pituitary as well as the vasomotor and temperature-regulatory center (Norman and Litwack 1987). They may play important roles in the control of cerebrovascular tone and neuroglia functions(Moore et al. 1988). It has also been reported that prostaglandins act on anterior pituitary trophic hormone target tissues such as thyroid, adrenal, ovary and testis, on excocrine hormone targets such as pancreas and gastric mucosa, and on endocrine target tissues such as renal tubules, bone and adipocytes (Norman and Litwack 1987). Extensive studies concerning the possible physiological or pathophysiological roles of prostaglandins in the complex functions of the respiratory system and circulatory system(red blood cells,leukocytes and platelets) as well as smooth muscles of cardiovascular and reproductive systems have been reported (Samuelsson et al. 1975, O'Flaherty et al. 1979, Wedmore and Williams 1981, Oliw et al. 1983, Davies et al. 1984).

2. Leukotreienes

For many years arachidonic acid metabolites, including thromboxane A_2

and a multitude of lipoxygenase pathway products, were held to be synonymous with prostaglandins (Davies et al. 1984). Indeed, the study of biological and pharmacological effects of leukotrienes in broad spectrum was followed by the recognition of leukotrienes as a separate family of arachidonic acid metabolites by Murphy et al. (1979). Although the chemical characterization of leukotrienes was as products of leukocytes, it is now well recognized that leukotrienes can be produced by several other cellular elements including keratinocytes, blood vessels, heart, lung, spleen, and brain (Borgeat and Samuelsson 1979a; Samuelsson 1983; Mencia-Huerta et al. 1983). Furthermore, it is also commonly accepted that leukotrienes can be produced not only by immune stimuli(e.g. IgE) but their synthesis can be induced in response to several nonimmune stimuli, such as hormones, Ca^{2+} ionophore and endotoxins (Feuerstein and Hallenbeck 1987). Because of the wide range complexity of biological actions of leukotrienes and other lipoxygenase pathway products, the biological actions of the leukotrienes have been extensively studied.

The microcirculatory effects of leukotrienes in the skin have been reported to cause wheal formation, flare, induration, and pain, thus the suppression of their action by anti-inflammatory glucocorticosteroids suggested that leukotrienes might play an important role in the microcirculatory and cellular events of inflammatory responses (Feuerstein

and Hallenbeck 1987). It has been shown that LTB_4 is involved in the modulation of inflammatory reponse through four different mechanisms. These are induction of leukocyte accumulation (Smith et al. 1980), mediation of vascular permeability changes, modulation of pain responses (Rackham and Ford-Hutchinson 1983) and mediation of blood flow changes (Ford-Hutchinson 1985). Detection of LTB_4 in human inflammatory disease has suggested that the concentraiton of the leukotriene is higher in joint fluid from patient with rheumatoid arthritis or spondyloarthritis than in fluid from individuals with non-inflammatory joint problems. Moreover, following injection of an anti-inflammatory steroid drug into joints of six patients, a reduction in the number of leukocytes has been observed (Marx 1982). This phenomenon, may be co-related with depression of LTB_4 synthesis, because LTB_4 is one of the most potent known chemotactic factors for polymorphonuclear leukocytes (Ford-Hutchinsons et al. 1980).

LTC_4, LTD_4 and to a lesser extent LTE_4, known as SRS-A, are involved in early inflammatory responses (Samuelsson 1983). Skin inflammation and changes in vascular permeability in animal models were investigated in reponse to leukotriene treatment. LTC_4 or LTD_4 failed to cause increased vascular permeability in rabbit, however, administration of these compounds

as well as LTE_4 increased the vascular permeability of guinea-pig and rat skin(Peck et al. 1981, Davies et al. 1984).

It has also been demonstrated that SRS-A is active in the terminal vascular bed of the hamster cheek pouch, where they cause plasma leakage from post-capillary venules and vasoconstriction in the terminal arterioles (Piper 1983). It has been proposed that during cerebral ischemia, there is an increased synthesis of LTC_4 and that may play an important role in the pathogenesis of disruption of the blood-brain barrier during cerebral ischemia(Moskowitz et al. 1984). Therefore, a study was performed by Mayhan et al.(1986) to determine whether LTC_4 constricts cerebral arterioles and distrupts the blood-brain barrier in hamsters. By using intravital fluorescent microscopy and fluorescent-labeled tracers, they compared responses of vessels in the cerebrum with vessels in the cheek pouch. The results indicated that in the cheek pouch, LTC_4 produced a dose-related decrease in diameter of arterioles and increase in microvascular permeability. However, only a moderate constriction of cerebral arterioles and minimal disruption of the blood- brain barrier was observed (Mayhan et al. 1986).

Research studies on the pathogenesis of intestinal inflammation in general, and inflammatory bowel disease (IBD) in particular, have been performed and reported by Stenson(1988). Incubation of arachidonic acid

with IBD mucosa from surgical resections produced the synthesis of LTB_4 , 5-HETE, 12-HETE, PGE_2 and TxB_2. Similar experiments with rectal mucosal biopsies from IBD patient lead to the synthesis of LTC_4, LTD_4 and LTE_4 as well as LTB_4, indicating arachidonic acid metabolites as mediators that are involved in controlling the amplification of the inflammatory response in intestinal disease (Stenson 1988).

For many years SRS-A has been know as an important mediator of immediate hypersensitivity reactions, such as bronchoconstriction in allergic asthma(Dahlen et al. 1980). LTC_4 and LTD_4, as well as histamine, produce slowly developing contractions of human bronchial strips. The contraction that was induced by histamine disappeared after changing of the bath fluid, whereas LTC_4 or LTD_4 produced persistent contractile responses which were not readily reversed by washing(Dahlen et al. 1980). Antigen challenge of chopped parenchymal tissue obtained from asthmatic lung resulted in generation of LTC_4, LTD_4, and LTE_4. Likewise antigen challenge of isolated bronchial strips from the same lung caused contractions, which were slow in onset and of long duration (Feuerstein and Hallenbeck 1987)). In addition to the effects of leukotrienes on bronchial contraction, LTC_4 and

LTD_4 were demonstrated to stimulate mucous secretion from bronchial epithelial cells in airways that might be involved in asthma (Ford-Hutchinson 1985, Feuerstein and Hallenbeck 1987, Samuelsson et al. 1987).

Leukotrienes have been considered as powerful pulmonary vasoconstrictors (Dahlen et al. 1980, Samuelsson et al. 1980, Smedegard et al. 1982), however, there is evidence indicating that the pulmonary vasoconstrictions induced by SRS-A could be mediated by other vasoactive compounds of the cyclooxygenase pathway. Intravenous injection of LTC_4 or LTD_4 causes a bronchoconstriction that is reversible by indomethacin as an inhibitor of cyclooxygenase pathway, however, when administered by aerosol the leukotrienes induce bronchoconstriction, which is not reversed by indomethacin. This suggests that the mechanism of their action depends upon the route of administration(Piper 1983). Initially, the interaction between products of the lipoxygenase and cyclooxygenase pathway of arachidonic acid metabolism was observed by Engineer and co-workers(1978) when they found that prostaglandins and TxA_2 are released by guinea-pig lung in response to SRS-A. This was confirmed by observing in vivo generation of TxA_2 and PGI_2 from guinea-pig lung (Omini et al. 1981), as well as TxA_2 from isolated guinea pig-lung(Folco et al. 1981) following stimulation of LTC_4.

Furthermore, the effect of LTD4 as a pulmonary vasoconstrictor in sheep was markedly attenuated in the presence of sodium meclofenamate, a cyclooygenase inhibitor. Moreover, the inhibition of TxA_2 synthesis also caused decreasing of pressor response to LTD4, suggesting that a portion of the pressor response to LTD_4 is due to the action of TxA_2(Kadowitz and Hyman 1984).

Hypotensive shock, arrhythmias, myocardial depression, coronary constriction and ultimately profound heart failure are clinical manifestations of systemic anaphylaxis (Letts et al. 1986, Feuerstein and Hallenbeck 1987). The potential role of leukotrienes in cardiac anaphylaxis has been studied extensively in vivo and vitro preparations. Electrocardiographic changes characteristic of coronary artery constriction have been reported from humans during anaphylxis indicating that LTC_4, LTD_4 and LTE_4 are the major constituents of SRS-A that involve in constriction of coronary arteries (Michelassi et al. 1982; Hammarstrom 1983a).

In a study of guinea-pig isolated hearts both LTC_4 and LTD_4 produced dose-dependent decrease of coronary flow (Letts and Piper 1982). Although the two leukotrienes were shown to have similar actions on the heart, LTC_4 had the slower onset of action on guinea-pig isolated hearts with the greater efficacy in reducing coronary flow when compared with LTD_4. Moreover,

pre-treatment with indomethacin, the LTC_4-induced coronary flow reduction was halved, while the LTD_4-induced reduction in coronary flow was not significantly changed. These results suggested that the vascular effects of LTC_4 on guinea-pig isolated hearts are more dependent on the release of cyclooxygenase products (TxA_2, PGD_2, PGF_2, and PGI_2) when it was compared with LTD_4 (Letts and Piper 1982). In a recent study, in vivo injection of LTC_4 into the right heart of porcine caused a pronounced but transient increase in pulmonary arterial pressure and pulmonary arterial wedge pressure out of proportion to the increase in left ventricular end-diastalic pressure, suggesting that LTC_4 produces both pulmonary artery and pulmonary vein constriction (Ohtaka et al. 1987).

A number of other effects of leukotrienes on biological systems have been reported such as induction of lymphocytes supression, augmentation of natural cytotoxicity (Borgeat et al. 1985),as well as the contractility actions of LTC_4 and LTD_4 on isolated glomeruli and cultured mesangial cells in rat (Barnett et al. 1986). Leukotrienes as the modulators of the immune response were investigated by in vitro treatment of human peripheral blood mononuclear leukocytes with LTB_4 and LTD_4 stimulated with mitogens such as concanavalin A (conA) or phytohemagglutinin (PHA).

A significant inhibition of the lymphoproliferative response was observed (Rola-Pleszczynki et al 1982) indicating that T lymphocytes were affected by leukotrienes (Borgeat et al. 1985). The results were similar to the findings in mice when they were treated wiht LTD_4 and LTE_4 (Webb et al. 1982).

Moreover, it has been reported that LTB_4 added to human peripheral blood mononuclear leukocytes caused a significant increase of natural cytotoxicity suggesting that LTB_4 may be involved in the activation of natural cytotoxic cell activity (Borgeat et al. 1985). It has also been reported that the inhibition of natural cytotoxic activity by lipoxygenase inhibitor nordihydroguaiaretic acid(NDHGA) could be significantly reversed by LTB_4, indicating that the inhibition of endogenous lipoxygenase could be compensated by exogenously administered lipoxygenase products (Borgeat et al. 1985).

CHAPTER III

MATERIAL AND METHODS

A. Animals

For each experiment 3 to 4 male Sprague-Dawley derived rats, weighing 220-320g were used to obtain peritoneal macrophages. The animals were given Purina Rat Chow 5012 and water ad libitum. This species was selected because of its availability at any time of the year, and its relatively short life span. It is also advantageous to use a rat, because it is large enough to obtain a large number of cells from the peritoneal cavity.

B. Peritoneal Macrophage Cell Harvesting

The procedure utilized for isolation and culturing of macrophage cells was a modification of the techniques that were described previously (Stuart et al. 1973; Garvey et al. 1977). Each rat was killed by CO_2 asphyxiation by placing the rat in a closed container on a platform above dry ice. The fur over the entire abdominal wall was soaked thoroughly with 80% ethanol. By lifting the skin upward from the posterior abdominal wall with forceps, a small incision was made through the layers of skin and then carefully extended to the front legs and the skin was removed without damaging the abdominal

wall. Rapidly, after removal of abdominal skin, 20-24 mL modified Hanks balanced salt solution (mHBSS) was injected into the peritoneal cavity, using a 12-mL syringe and 21-gauge needle. The injected fluid was circulated by massaging or prodding the abdomen gently. It has been recommended that the peritoneal fluid should be aspirated within 5 min of injection into the peritoneal cavity, otherwise it is likely to become infected by organisms from the gut(Stuart et al. 1973). The cellular exudate was removed using a syringe inserted into the right or left flank and then the exudate was transferred into a sterile centrifuge tube placed in an ice bucket. The blockage of the needle by fat or intestine could be prevented by application of lateral traction that formed a pocket of fluid behind the spleen. The peritoneal cavity washing was repeated with the same volume of mHBSS and the washing was added to the first cellular exudate.

The centrifuge tubes containing the combined washings were centrifuged for 10 minutes at 300 xg (1200 rpm) at 0-2 ^{o}C. If there were red blood cells in the pellet, after discarding the supernatant, cells were resuspended in 4 mL of mHBSS and the suspension was layered on top 3 mL Ficol-Paque (Pharmacia Inc., Piscataway, NJ) gradient in a centrifuge tube and centrifugated at 400 xg (1400 rpm) for 30 min at room temperature. The resulting interface layer between mHBSS and the gradient containing the macrophage cells and possibly small lymphocytes was carefully removed with

a Pasteur pipet and washed 3 times with mHBSS. For each washing the cells were mixed in 6 mL of mHBSS and centrifuged for 10 min at 300 xg. If red blood cells were absent from the pellet, the centrifugation in Ficol-Paque gradient was omitted. The cells were then resuspended in 10 mL complete medium.

Prior to incubation, a small sample of the cell suspension in complete medium was removed into a white blood cell diluting pipet. The cells were diluted 1 : 20 with complete medium and then this solution was diluted 1:1 with Trypan Blue stain (Gibco Laboratories, Grand Island, NY) for determination of the cell concentration in a hemocytometer chamber. The total nucleated cell count per each rat was usually between 6 x 10^6 and 8 x 10^6 cells of which about 80 to 90 percent were macrophages. Consequently, the number of cells in complete medium was adjusted to 2 x 10^6 cells/mL, then 3 mL of this solution containing 6 X 10^6 cells was added in a 25 cm^2 plastic tissue culture flask (Gibco) and then the cultures were kept in a moist atmosphere in an incubator at 37 ^oC and 5% CO_2. After six hours pre-incubation the medium was removed using a sterile Pasteur pipet to remove non-adherent cells, then the cells were overlaid with fresh complete medium. The cell cultures then were incubated for 24 h prior to being labeled with ^{14}C- or ^3H-arachidonic acid.

C. Preparation of mHBSS and Complete Medium:

Hank's balanced salt solution was modified by mixing 0.0565 g of heparin (Gibco Laboratories, Grand Island, NY) in 100 mL of 10X HBSS to make 100 unit/mL of l0X HBSS. The HBSS was then sterilized by filtration using a 115 mL Nalgene disposable filter unit (Nalge Company,Rochester NY) with 0.2 µL filter pore size. Then the solution was diluted to IX concentration by adding 10 mL of this solution to 90 mL of sterile water (Abbott Laboratories). Prior to harvest of the macrophage cells from the peritoneal cavity, this solution was further modified by adding 1 mL of heat inactivated fetal bovine serum(FBS) (Gibco Laboratories, Grand Island NY) to 100 mL of diluted HBSS(1X) containing 10 unit/mL heparin to make mHBSS.

Complete medium was prepared for each experiment prior to culturing or feeding of the cells. Dulbecco's modified eagle medium (dMEM) with L-glutamine, D-glucose and 1000 mg/L sodium pyruvate (Gibco Laboratories, Grand Island, NY) was supplemented with antibiotic-antimycotic solution (Gibco) in 1 : 100 mL ratio (1%) and heat-inactivated FBS (Gibco) in 10% quantity.

D. Labeling of Cellular Phospholipids with Radioactive Arachidonic Acid:

Both ^{14}C- and ^{3}H-arachidonic acids were used for labeling of the membrane phospholipids of the macrophage cells. In some experiments

macrophage cells were incubated for 12 h with fresh complete medium containing [1-^{14}C] arachidonic acid(Research Products International Corp. , Mount Prospect, IL) at 0.27 uCi/mL(0.81 μCi/culturing flask). The specific activity of [14C]arachidonic acid was 58 mCi/mmol. In other experiments, cells were labeled with [5,6,9,11,12,14,15-^{3}H] arachidonic acid(New England Nuclear) in fresh complete dMEM at 1.0 μCi/mL for 12 h incubation. The specific activity of the [^{3}H]arachidonic acid was 230 Ci/mmol. At the end of 12 h labeling period, the medium containing the radioactivity was removed from each flask, and cultures were washed three times with pre-warmed dMEM to remove excess of radioactive material. Thereafter, the cultures were ready for stimulation. The radioassays of the washings showed that about 60% of the [^{14}C]- or [^{3}H]arachidonic acid was incorporated into the macrophage cells.

E. Calculation of Radiactivity to Millimol(mmol)

Tha radioactivity of [14C]- or [3H]arachidonic acid(AA) and its metabolites was calculated as:

1. Efficiency(%) of radioassay = Experimental CPM* / Expected CPM

2. Experimental DPM** = Experimental CPM / Efficiency

3. Activity(mCi) = Experimental DPM/ 2.22 x 10^{-9} DPM/mCi

4. Millimol(mmol) = Activity (mCi) / Specific activity of standard AA (mCi/mmol)

*= designates count per minute.

**= designates disintegration per minute.

F. Stimulation of Macrophage Cells with Calcium Ionophore

Calcium ionophore solution was prepared by adding of 1.0 mg Ca-ionophore A23183 (Sigma Chemical Company, Saint Louis, MO) in 0.2 mL dimethylsulfoxide (DMSO) and refrigerated as a stock solution. Prior to stimulate the macrophage cells, Ca-ionophore from the stock solution was added to complete dMEM(containing serum) to make 10^{-5}M in concentration. However, in some experiments dMEM was supplemented with Ca-ionophore (10^{-5}M) without serum to observe any quantitative differences in the arachidonic acid metabolites produced by stimulated cells.

Three millilitres of dMEM containing Ca-ionophore was added to each of the radiolabeled cultures and then the cultures were incubated for 1 h. After this incubation period, the cell cultures were ready for extraction of the leukotrienes as well as other related compounds.

G. Irradiation of Macrophage Cells with Cobalt-60 Gamma Radiation

The [^{14}C]- or [^{3}H]arachidonic acid labeled cells were overlaid with 3 mL pre-warmed dMEM (37 ^{o}C) with and without serum, then the cultures were incubated for about 30 min to reach pH and temperature equilibrium. At the end of 30 min, cultures were removed from the incubator and exposed to gamma irradiation with single dose of 2 Gy, 4 Gy, 8 Gy, 16 Gy and 32 Gy using a U.S. Nuclear Corporation GR-9 gamma irradiator. Following

exposure, the irradiated cultures were kept in the incubator for 1 h before extraction of arachidonic acid metabolites.

H. Control Cultures

The control cultures were simply [^{14}C]- or [^{3}H]arachidonic acid labeled cells that were not treated or stimulated in any way. Cultures in the presence or absence of serum were incubated for 1 h, and at the end of incubation were ready for extraction of any leukotriene or other related compounds that may have been produced by macrophage cells during the incubation time.

I. Inhibition of Cyclooxygenase Enzyme

Free arachidonic acid released from cellular phospholipids is metabolized via lipoxygenase and cyclooxygenase pathways(Davies et al. 1984). In some experiments, the cyclooxygenase pathway was inhibited by ibuprofen(F.W. 206) supplied as a gift from Dr T. Yorio(Texas College of Osteopathic Medicine, Department of Pharmacology). The stock solution of ibuprofen was prepared by dissolving 5 mg ibuprofen in 2 mL of DMSO. In some experimental treatments as well as untreated cultures(controls), ibuprofen solution was added to an appropriate volume of dMEM containing serum or without serum to make a 10^{-5} M concentration of ibuprofen.

Following the labeling period and washing of the cultures three times

with fresh medium(without serum), 3 mL of medium containing 10^{-5} M ibuprofen was added to each of the cultures for 30 min prior to treatment.

J. Detection of Damaged Macrophages in Treated and Untreated Cultures

Damage to the cells induced by various doses of gamma irradiation that might cause morphological changes in cellular membrane or death was detected via trypan blue staining (cell viability) and electron microscopy observation.

Prior to seeding the macrophage cells, two sterile cover glasses were put in each culture flask, then about 0.1 mL cell suspension (2×10^5 cells) in complete medium was transferred on the surface of each cover glass. After 2-3 min another 2.8 mL of complete medium containing macrophage cells was added to each flask, then the culture was incubated at 37 ^{o}C, 95% moist air and 5% CO_2 as was done in the other experiments.

At the end of the experiment and prior to stopping the cell growth with isopropanol for extraction of the medium, the cover glasses were removed from each culture. Cell viability for each treatment was performed by placing one cover glass in a small container with trypan blue for about 3-5 min followed by immediate observation with the light microscope. Dead cells on the cover glass appeared blue while living cells remained unstained.

K. Extraction of Leukotrienes from Tissue Culture Fluid:

After one hour treatment, the cultures were removed from the incubator and a small aliquot (50 µL) of the medium was taken and saved for radioactivity assay. The cell growth was stopped by adding 1.5 mL isopropanol to the remaining medium and then the pH was adjusted to 3 with 5 M formic acid. The ratio of the culture medium, isopropanol and formic acid was 1 : 0.5 : 0.03 respectively before the extraction. In order to minimize non specific binding of lipid, all glassware was siliconized for the entire extraction procedure. After 5 min the mixture was transferred into a separatory funnel and 1.5 volume of diethylether was added, resulting in the development of two phases. The upper organic layer, which consists of isopropanol and diethylether mixture was transfered to a glass tube. The aqueous phase was re-extracted with ether, the extracts were pooled together and then evaporated to dryness under a stream of nitrogen. The tube containing organic residue was capped, sealed with parafilm and stored at -80 °C until just prior to processing for high performance liquid chromatography (HPLC) analysis.

L. Separation and Identification of Leukotrienes and Other lipoxygenase Products:

The experimental samples for each treatment were dissolved in 200-240

µL HPLC grade methanol and transferred into a polypropylene microfuge tube. Separation of the arachidonic acid metabolites was achieved using a HPLC (Waters Associates, Milford, Massachusetts) equipped with a Model 6000A high-pressure pumping system, and a C_{18} (4.6 x 25 cm) reverse phase Partisil 5 ODS-3 column (Whatman Chemical Separation Inc., Clifton, NJ). The column had been pretreated with filtered MeOH (100 mL), H_2O (100 mL), 0.01 M HNO_3 (50 mL), 3% EDTA (100 mL), H_2O (200 mL) and again with MeOH (100 mL) before sample application. The mobile phase solvent was 75% acetonitrile and 25% H_2O mixture that was adjusted with acetic acid to pH 4.5. The mobile phase was filtered with 0.2 µ pore size filter and degassed prior to use.

The column was equilibrated for at least 25 min under solvent system condition at a flow rate of 1.0 mL/min. The experimental sample and appropriate standards were injected into a 100 µL HPLC loop and the column was eluted for about 50-60 min. The separation of radioactive compounds was detected using a Radioactive Flow Detector Model CT (Radiomatic Instrument and Chemical Co. Inc. , Tampa, Florida) and recorded by a dot matrix printer M8510 + (Soletic Corporation, Sun Valley, California). The solvent cocktail system for the radioactive detector was Flo-Scint ™ II (Radiomatic Instrument and Chemical Co. Inc., Tampa, Florida) at flow rate of

3 mL/min. The radioactivity of each eluted compound showing a peak with its retention time was recorded.

In some experiments separation of the metabolites was obtained using a weaker mobile phase[60% acetonitrile in water and acetic acid(pH 4.5)] for 30 min, and then followed by a stronger mobile phase[75% acetonitrile in water and acetic acid(pH 4.5)] to remove more nonpolar compounds more rapidly from the column.

Fractions were collected in siliconized tubes, then the aliquots of the column effluent for each detected compound were pooled together and evaporated to dryness under stream of nitrogen gas, sealed and stored at -80 °C for further analysis.

CHAPTER IV

Results

A. Isolation of Peritoneal Macrophage Cells, Appearance and Morphological Changes in Cell Culture:

The resident macrophage cells were isolated from adult rats immediately after sacrifice by washing the peritoneal cavity with mHBSS. Prior to culture of the macrophage cells, a small portion of cell suspension in complete medium was used to calculate the number of cells per ml. The results indicate that more than 8×10^6 living cells (99% of total) were collected from each rat. In addition to the macrophage cells, there are also a number of other cells including lymphocytes, eosionophils, and, in the case of internal injury, erythrocytes which are contained in the peritoneal exudate.

Peritoneal macrophages are phagocytic cells. Their phagocytic activity causes them to engulf materials in the culture medium and after a few hours of incubation they adhere to the surface of glassware as well the plastic culture flask. Therefore, the non-adherant cells accompanying macrophages from the peritoneal cavity can be removed easily after a few hours postincubation by replacing the medium with fresh medium. In general, macrophages exhibit two basic types of morphology: they are either round shape with abundent

cytoplasm or phagocytic with extended cytoplasm. In older cultures, it was found that the proportion of satellate cells increased with increasing of time in culture. Fig. 4(A) shows the normal appearance of a young macrophage culture(1 h) including other type of cells that are removed from peritoneal cavity. After a few hours of incubation following changing the culture medium and replacing with fresh complete medium all non adherent cells are removed from the culture. Fig. 4(B) shows the morphology of adhered macrophage cells excluding all other nonadherant cell from peritoneal cavity.

To increase the yield of macrophages, in some of the experiments mitotic stimulation was performed by intraperitoneal injection of paraffin oil or 10% proteose peptone broth 3-4 days prior to sacrificing the rats. Because of the presence of free oil in exudate and possible histochemical changes due to droplets of oil inside the cell, induction of macrophages by parafin oil was discontinued. In several experiments the induction of macrophages by 10% proteose peptone broth also caused stickiness of the cells and a high frequency of cells clumping together.

B. Determination of Arachidonic Acid Uptake and Release of Metabolites by Macrophage Cells:

At the end of the labeling period and prior to stimulating of the macrophage cells with Ca-ionophore or gamma irradiation, radioassays

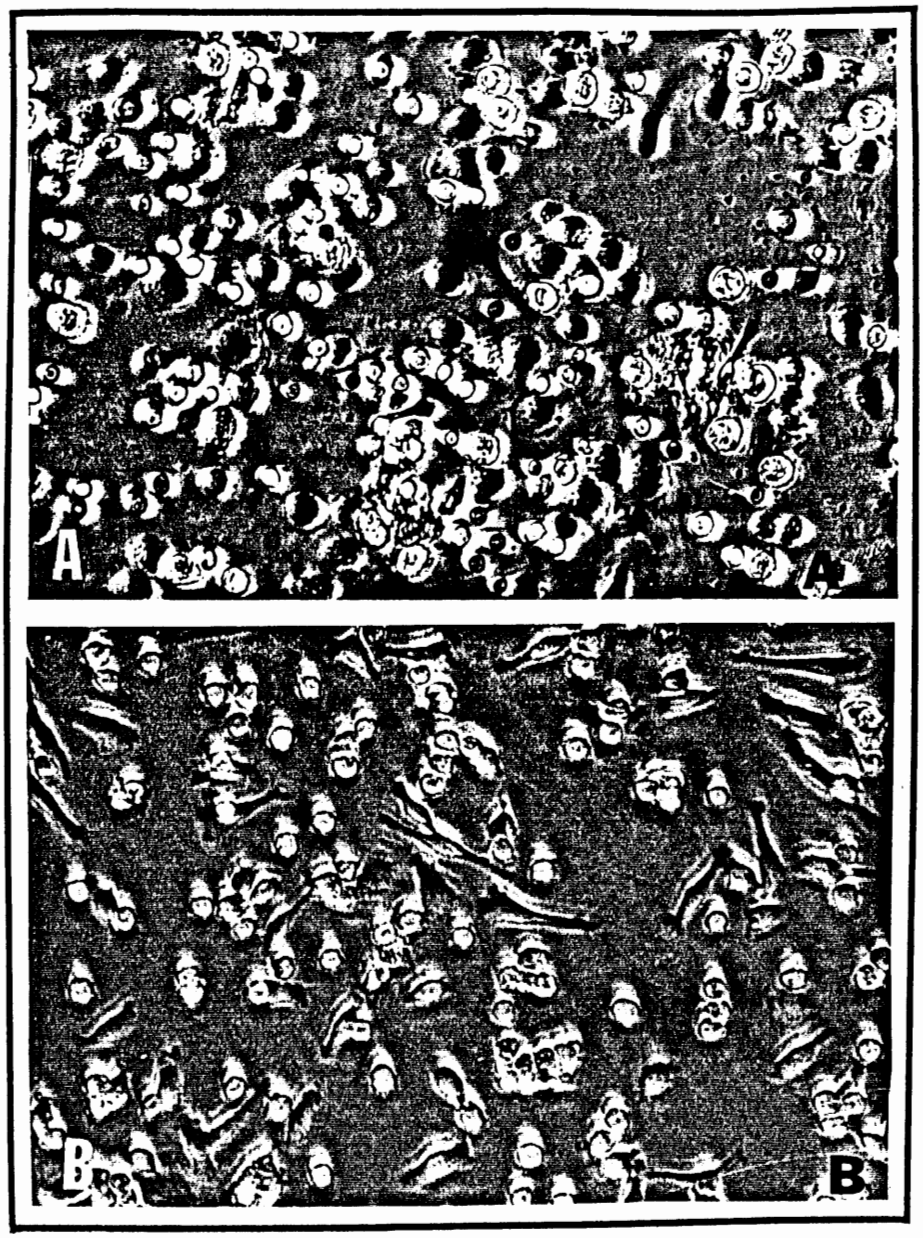


Fig. 4: (A) Morphology of macrophages and some other type of cells from the peritoneal cavity cultured for one hour incubation. Fig. 1A also shows some erythrocyte contamination of peritoneal cells. (B) shows the morphology of macrophage cells following exclusion of nonadherent cells after 6 h incubation.

showed that about 55% of the total amount of administered exogenous arachidonic acid was incorporated into the cells. The culture medium of all treated cells and control were used to separate leukotrienes and other related compounds released from the macrophage cells. Radioassays of the culture medium after stimulation but before extraction showed that 42-63% of the total incorporated arachidonic acid was released as leukotrienes, other related compounds and unmetabolized arachidonic acid. Table 1 and Fig. 5 show the mean and standard error of the mean of arachidonic acid administered, arachidonic acid incorporated and compounds released.

C. Lethality and Morphological Studies of Treated Macrophage Cells:

At the end of the treatment period prior to stopping the cell growth, some of the macrophage cells that had been grown on a glass cover slip inside of the plasic culture flask were removed from culture flask and immediately stained with trypan-blue for 5 min. The trypan-blue stained slides were examined with a light microscope. More than a thousand cells were counted in a zig zagging manner to calculate the percentage of viable cells following each treatment. Similarly, a direct counting of live and dead cells from each culture flask was performed to determine any changes in the cell viability of macrophages grown on the glass cover slip or plastic culture flask. No significant differences between the cell viability of macrophages grown on the

TABLE 1

Mean and standard error of the mean of total amount of [^{14}C]arachidonic acid administered to macrophage cells, incorporated in the cells, and metabolites including unmetabolized arachidonic acid released from the macrophage cells.

Treatment	Mean ± SEM mmoles Administered	Mean ± SEM mmoles Incorporated	Mean ± SEM Percent Incorporated	Mean ± SEM mmoles Released	Mean ± SEM Percent Released
Control (6)*	12.11 ± 0.77	6.70 ± 0.80	55.21 ± 5.74	3.86 ± 0.42	58.43 ± 4.02
2 Gy (3)*	11.45 ± 1.69	5.51 ± 0.89	48.04 ± 3.84	3.33 ± 0.09	63.34 ± 13.58
4 Gy (4)*	11.88 ± 1.60	6.27 ± 1.44	51.76 ± 5.97	2.35 ± 0.16	41.56 ± 9.27
8 Gy (5)*	12.77 ± 0.95	6.70 ± 0.90	52.25 ± 4.52	3.08 ± 0.4	46.64 ± 05.19
16 Gy (5)*	12.26 ± 1.27	6.25 ± 1.08	50.38 ± 4.73	3.02 ± 0.35	50.67 ± 6.19
32 Gy (6)*	12.03 ± 0.97	8.26 ± 1.01	67.60 ± 4.260	4.02 ± .61	48.85 ± 4.82
Ca-ionoph. (10^{-5}M) (6)*	13.30 ± 0.36	7.04 ± 0.59	53.60 ± 6.06	4.13 ± 0.20	60.97 ± 6.77

*= indicates number of experiments per treatment.

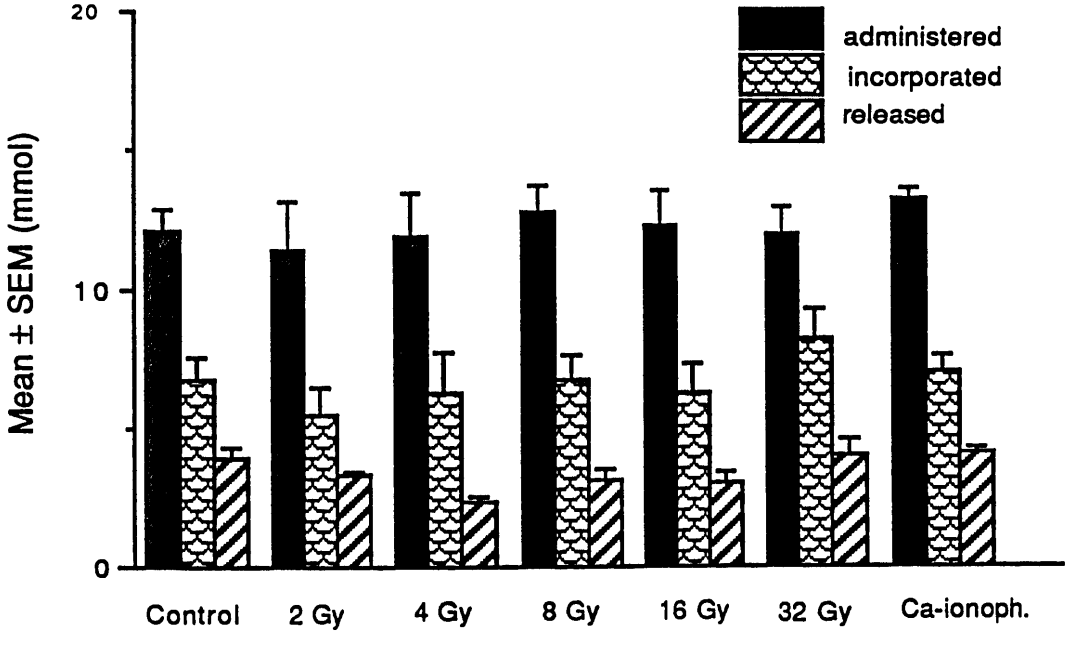


Fig. 5: Mean and standard error of the mean of total amount of [^{14}C]arachidonic acid administered to macrophage cells, incorporated in the cells, and released metabolites including unmetabolized arachidonic acid from macrophage cells in the culture medium.

glass cover slip and plastic flask was observed. The results also indicated that survival of macrophage cells was not significantly affected with various doses of gamma irradiation as well as Ca-ionophore(10^{-5}M) as a stimulating agent when compared to the control. However, the number of dead cells was slightly increased at the higher doses of gamma irradiation (Fig. 6).

Morphological studies of stimulated cells that had been treated with Ca-ionophore (10^{-5} M) or 32 Gy gamma irradiation were performed with light microscopy (Fig. 7). No significant changes in the morphology of the 32 Gy or Ca-ionophore-stimulated macrophage cells were observed when compared to the control. However, in Ca-ionophore treated cultures, macrophage cells appeared with more extended cytoplasm and phagocytic activity when compared to unstimulated cells.

D. Metabolism of Arachidonic Acid by Gamma Irradiated Macrophage Cells:

The [^{14}C]arachidonic acid labeled macrophage cells exposed to various doses of gamma irradiation (2 Gy, 4 Gy, 8 Gy, 16 Gy and 32 Gy) were incubated for one hour prior to the ether extraction of the arachidonic acid metabolites from the culture medium. HPLC analysis of the ether extract of each treatment indicated that the peaks eluting at 4.20 min and 10.00 min were as LTC_4 and arachidonic acid, respectively(Fig. 8 and 9). These

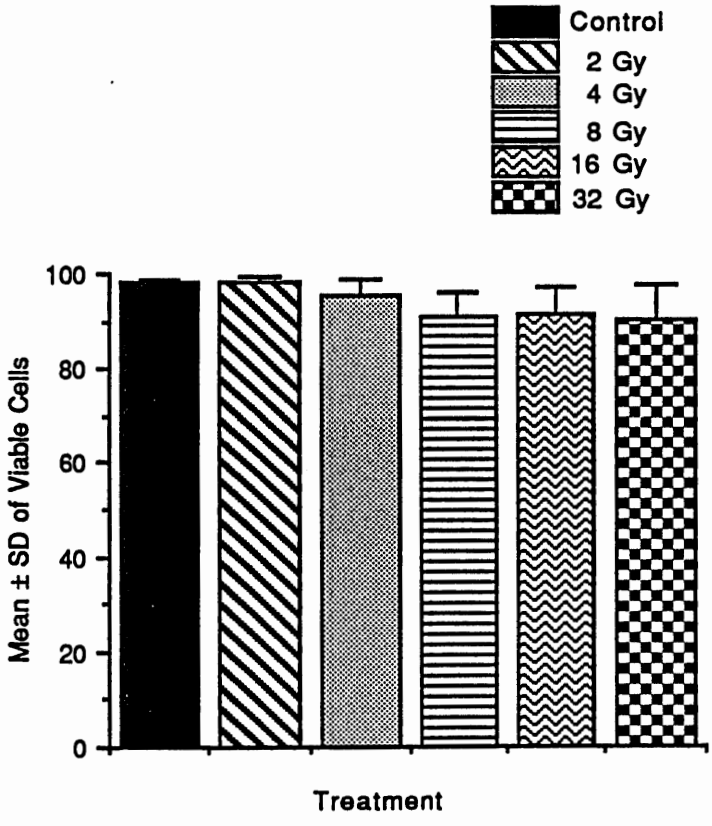
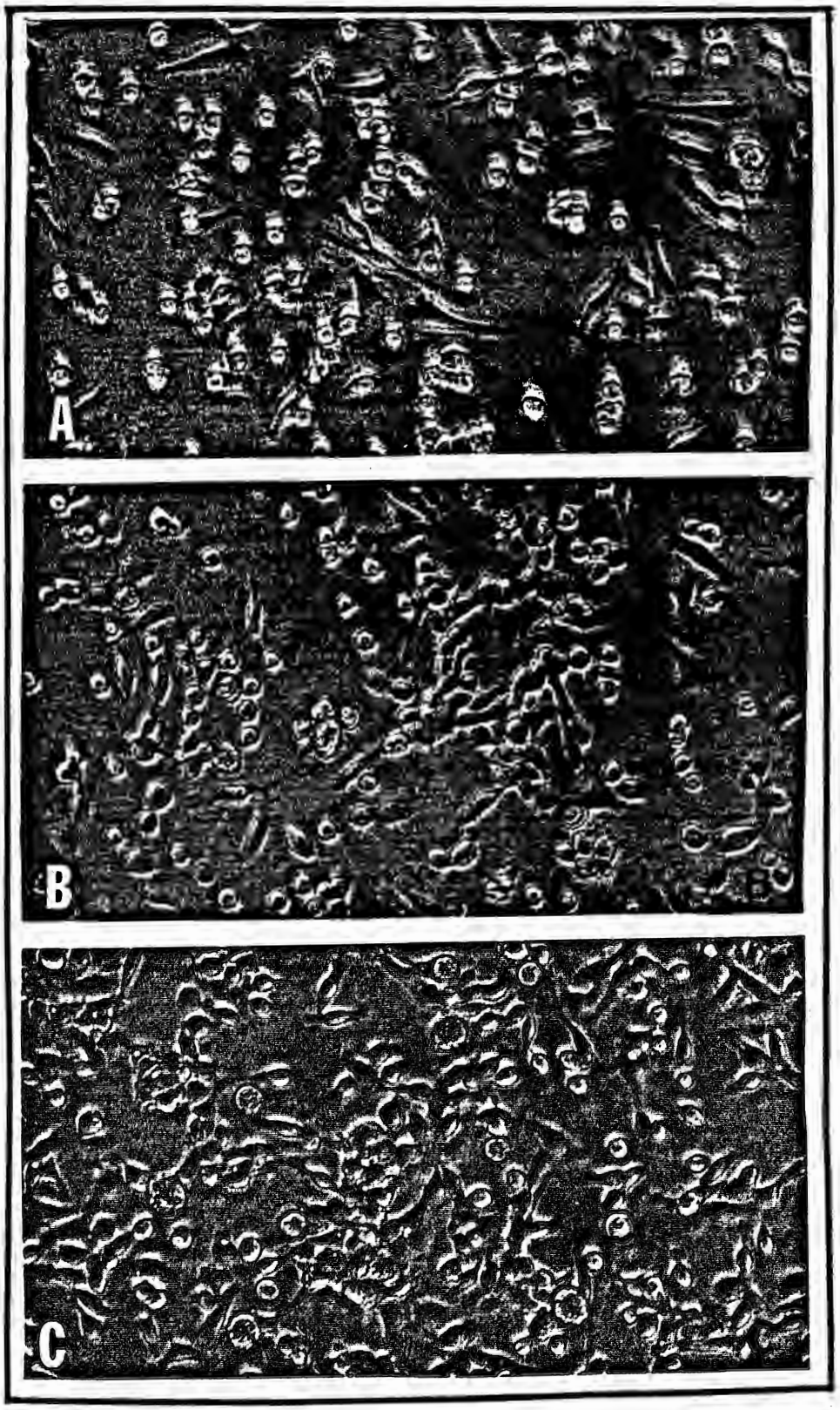


Fig. 6: Mean and standard deviation of the percentage of the total viable macrophage cells exposed to 0 Gy(control), 2 Gy, 4 Gy, 8 Gy,16 Gy and 32 Gy after treatment.

Fig. 7: Light microscopy of unstimulated macrophages(A) compared to 32 Gy gamma irradiated (B) and Ca- ionophore stimulated(C) macrophages after treatment period. No significant changes in the morphology is observed, however, Ca- ionophore(10^{-5}M) stimulated macrophage cells appeared to have more phagocytic activity.


A
B
C

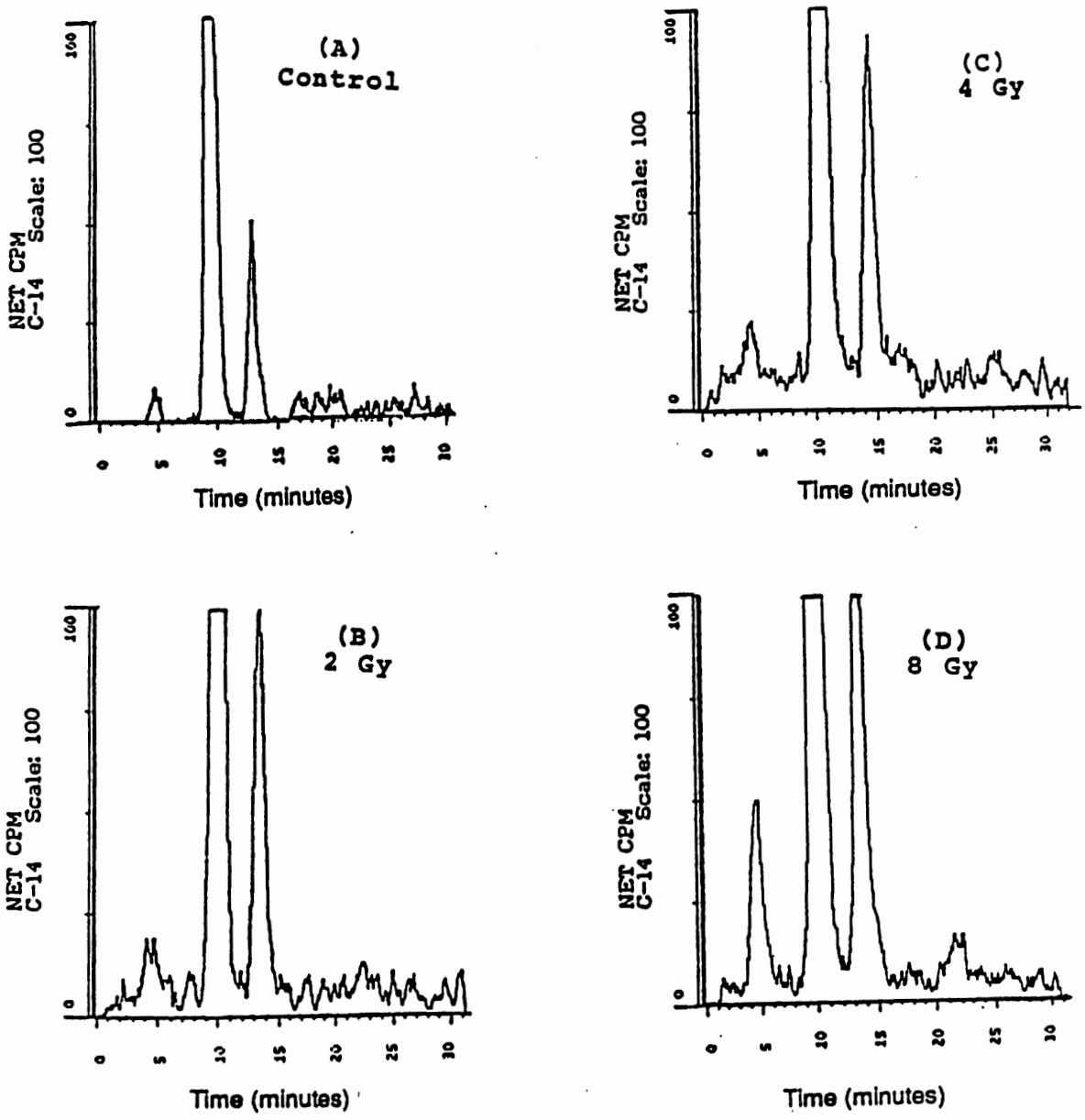


Fig. 8: Comparison of RP- HPLC analysis of ether extracts obtained from unstimulated (control), 2 Gy, 4 Gy and 8 Gy gamma irradiated macrophage cells respectively (A-D) using a C_{18} column and aceto-nitrile: water: acetic acid (75: 25: 0.02)pH 4.5 as a mobile phase. The peaks eluting at 4.20 min, 10.00min of retention time coelute with standard [^{3}H]LTC_4 and [^{14}C]arachidonic acid.

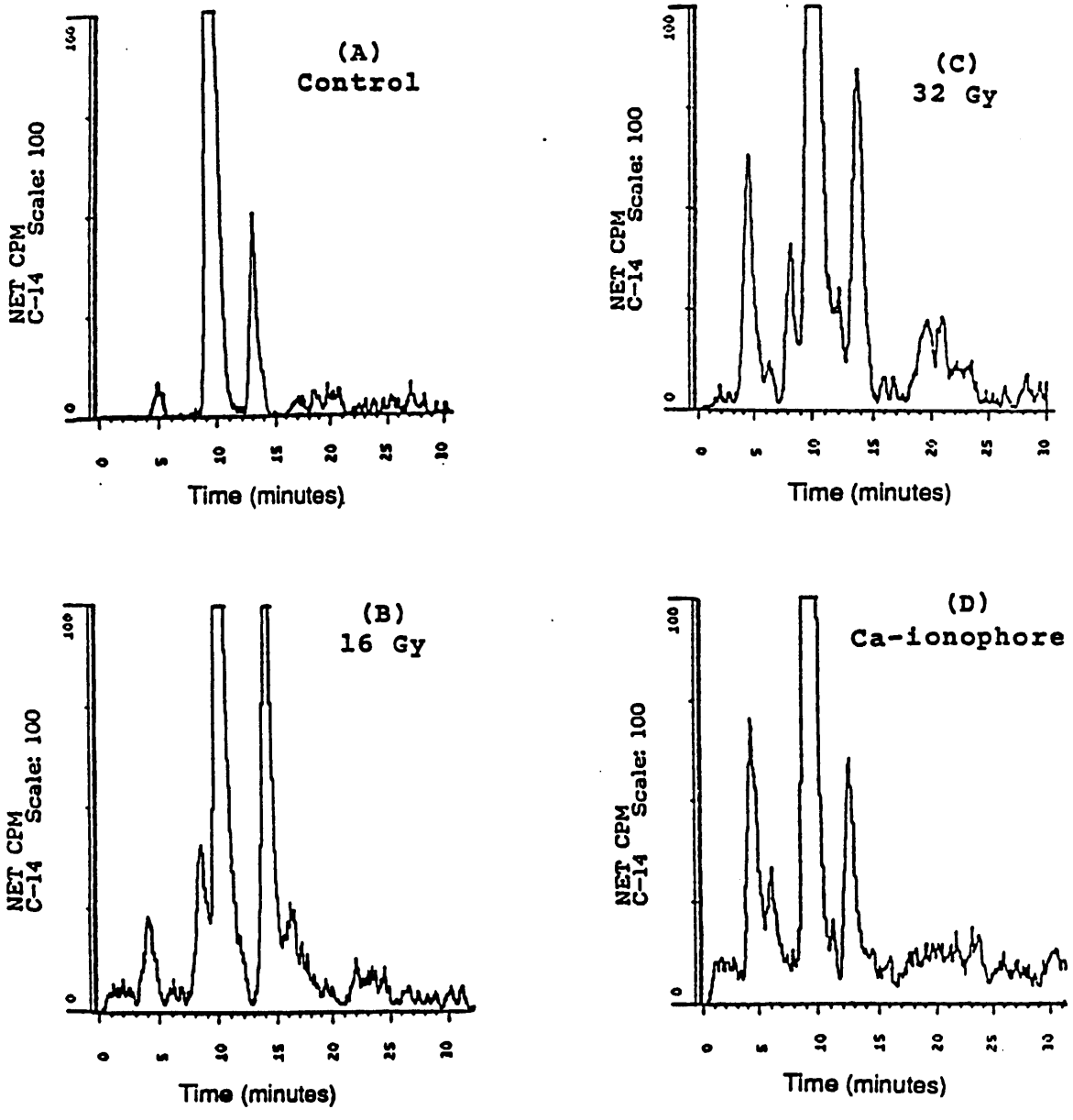


Fig. 9: Comparison of RP- HPLC analysis of ether extracts obtained from unstimulated (control), 16 Gy 32 Gy, and Ca-ionophore stimulation of macrophage cultures respectively (A-D) using a C_{18} column and acetonitrile: water: acetic acid (75: 25:0.02)pH 4.5 as mobile phase. The peaks eluting at 4.20 min and 10.00 min of retention time (A-D) co-elute standard [^{3}H]LTC_4 and [^{14}C]arachidonic acid.

materials co-eluted with standard [^{3}H]LTC_4 and [^{14}C] arachidonic acid(Fig. 10). The detected arachidonic acid was considered to be the endogenous unmetabolized arachidonic acid that was released from macrophage cells into the culture medium. The peak eluting at 6.20 min(peak 2) is probably LTB_4 or one of its rapidly produced isomers(6-trans-LTB_4 and 12-epi-6-trans-LTB_4), because the LTB_4 standards had similar but not identical retention times. The peak eluting at 8.50 min(peak 3) may contains mono-HETE compounds. This conclusion is based on results to be presented later(Fig. 11 and Fig. 20) and published results of others(Kouzan et al. 1985; Balter et al. 1988). A very non-polar arachidonate-derived compound eluting at 14.00 min was produced consistently from all irradiated as well as unstimulated macrophage cultures. This compound has not been identified but is thought to be a diacylglycerol.

HPLC experiments using a more polar mobile phase improved the separation of the more polar compounds. RP-HPLC analysis of a portion of the ether extract obtained from 32 Gy irradiated macropahges culture mixed with synthetic standard [^{3}H]LTC_4 and [^{3}H] 15- HETE as internal standards was performed using a C_{18} column and acetonitrile: water: acetic acid (60: 40: 0.02)pH 4.5. The peaks eluting at 4.50 min and 9.70 min (Fig. 11A)

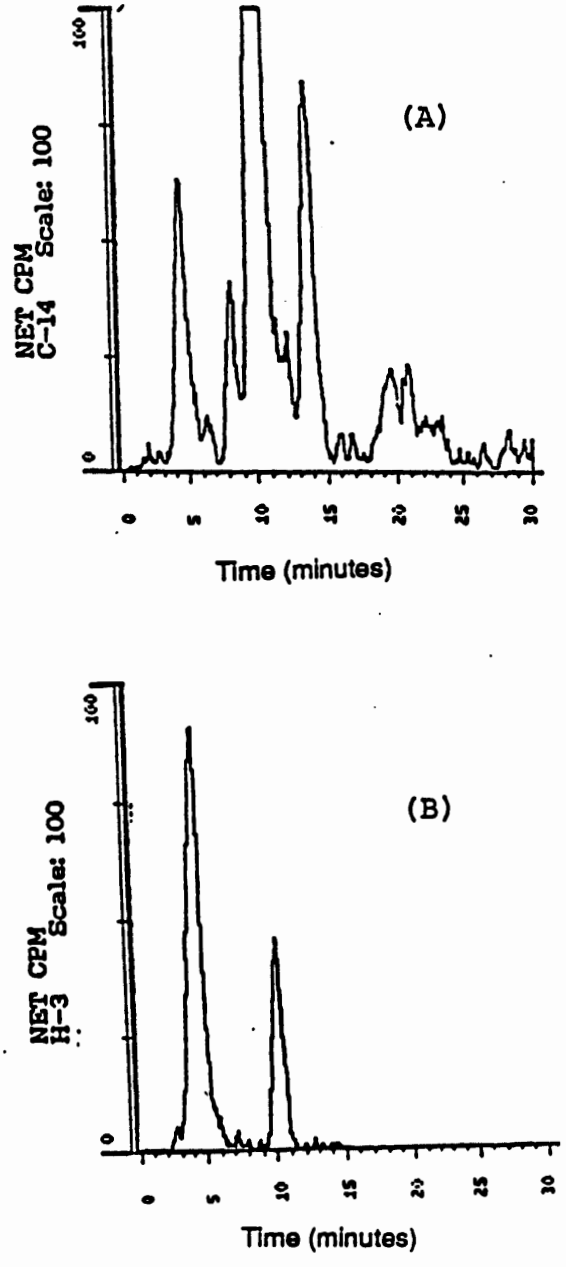


Fig. 10: RP- HPLC analysis of ether extract obtained from 32 Gy gamma irradiated macrophage culture(A). Treatment was conducted in the presence of FBS in the culture medium. The peaks eluting at 4.2 min and 10.00 min are LTC_4 and unmetabolized arachidonic acid respectively,(B) HPLC of standard [^{3}H]LTC_4 and [^{14}C]arachidonic acid.

represent LTC_4 and 15-HETE of biological origin as well as the internal standards mixed with the experimental sample. The elution of 15-HETE (Fig. 11A) was also coeluted with standard [^{3}H]15-HETE (Fig. 11B) under the same HPLC conditions. The more non-polar compounds, including arachidonic acid were not eluted until the mobile phase of the HPLC was changed to a stronger solvent [acetonitrile: H_2O: acetic acid (75: 25: 0.02)pH 4.5] at 21.00 min(Fig. 11A). The total amounts of LTC_4 and unmetabolized arachidonic acid as well as other compounds produced from macrophage cells were calculated according to the procedure described in the previous section (page 56). These data is shown in Table 2.

Statistical analysis of variance and multiple range tests were performed in order to compare any quantitative differences between the compounds from similar peaks eluting at the same retention time from samples obtained following various doses of gamma irradiation as well as other treated groups. It was shown that the amount of LTC_4 produced from all irradiated macrophage cells (2 Gy - 32 Gy) was significantly higher than the LTC_4 produced from macrophage cells that were not exposed to gamma irradiation (Table 2). Also, the LTC_4 produced by macrophage cells at different dose levels was not significantly different from each other. However, the highest

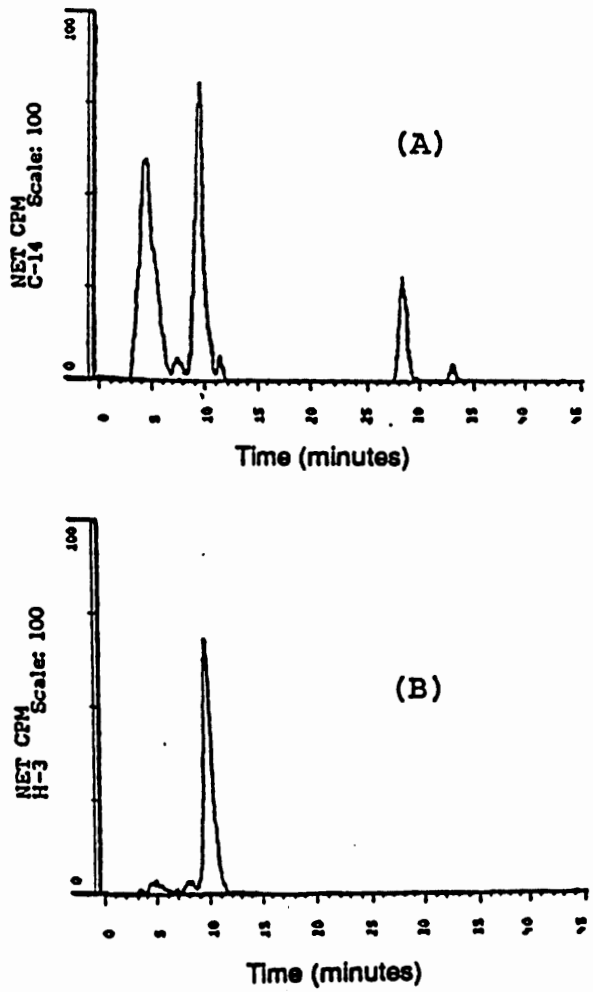


Fig. 11: RP- HPLC analysis of ether extract obtained from 32 Gy gamma irradiated macrophage culture and mixed with standard [^{3}H]LTC_4 and [^{3}H]15-HETE as internal standards prior to elution(A). Treatment was conducted in the presence of FBS in the culture medium during the treatment period. Analysis was performed using a C_{18} column and acetonitrile: water: acetic acid (60: 40: 0.02) pH 4.5 and then after 21.00 min the mobile phase was changed to the original solvent (75% acetonitrile). The peaks eluting at 4.50 min and 9.70 min(A) represent both standards and biologically produced LTC_4 and 15-HETE. (B) shows the elution of standard [^{3}H]15-HETE.

TABLE 2

Mean and standard error of the mean of total amounts of arachidonic acid metabolites produced from irradiated, Ca-ionophore stimulated and unstimulated macrophage cultures in the presence of FBS in the culture medium during the treatment period.

Treatment	Peak 1(LTC_4) Mean ± SEM pmole	Peak 2 Mean ± SEM pmole	Peak 3 Mean ± SEM pmole	Peak 4(AA) Mean ± SEM pmole	Peak 5 Mean ± SEM pmole
Control (5,6)*	8.33 ± 2.40[b] (6)**	—	22.04 ± 11.17[d] (5)**	157.92 ± 66.48d (6)**	68.76 ± 33.51[b] (2)**
2 Gy (2,2)*	16.88 ± 4.65[a] (2)**	—	24.06 ± 18.46[d] (2)**	433.17 ± 100.92[d] (2)**	238.51 ± 169.65[a] (2)**
4 Gy (3,3)*	14.24 ± 4.29[a] (3)**	—	22.06 ± 1.60[d] (2)**	266.98 ± 58.08[d] (3)**	1 55.0 ± 24.32a (2)**
8 Gy (4,6)*	35.92 ± 6.56[a] (6)**	16.14 ± 2.02[d] (2)**	—	390.67 ± 75.15[d] (6)**	126.19 ± 7.16[a] (6)**
16 Gy (5,6)*	22.65 ± 4.16[a] (6)**	12.97 ± 4.75[d] (3)**	48.81 ± 0.23[d] (2)**	231.73 ± 43.05[d] (6)**	120.30 ± 21.92[a] (5)**
32 Gy (5,7)*	48.58 ± 11.43[a] (7)**	24.66 ± 23.63[d] (2)**	34.06 ± 5.82[d] (5)**	334.33 ± 64.22[d] (7)**	97.90 ± 11.60[a] (5)**
Ca-ionoph. (10-5M) (5,6)*	86.59 ± 14.19 [c] (6)**	25.88 ± 8.13[d] (4)**	—	377.13 ± 53.94[d] (6)**	40.28 ± 9.37[b] (6)**

a= significantly different from b in the same column at the 0.05 level.
c= significantly different from a and b in the same column at 0.05 level.
d= not significantly different from each other in the same column.
*= first number in the parentheses indicates total experiments per treatment, and second number is equal to the number of HPLC runs
**= designates the number of repeated peaks detected by HPLC.

amount of LTC_4 was produced from 32 Gy irradiated macrophage cells compared to lower doses of gamma irradiation (Fig.12 and 13). For the different treatments the peaks eluting at 6.50 min, 8.50 min and 10.00 min(peaks 2,3 and 4 in Table 2) were not significantly different at 0.05 level from those of the control samples. However, the amount of arachidonic acid(peak 4) released from gamma irradiated or Ca-ionophore (10^{-5}M) stimulated macrophage cultures was higher than that of the control (Table 2, Fig. 10 and 11). The production of the most polar radioactive component in the RP- HPLC eluting after arachidonic acid was significantly higher in all gamma irradiated groups than the unstimulated macrophage culture (Table 2, Fig. 12 and 13).

E. Metabolism of Arachidonic Acid by Ca-ionophore Treated Macrophage Cells:

A typical trace from the radioactivity detection of the compounds produced from Ca-ionophore(10^{-5}M) treated macrophage cultures is shown in Fig. 9(D). The peaks eluting at 4.20 min, and 10.00 min co-elute with standard [^{3}H] LTC4 and [^{14}C] arachidonic acid. The peak eluting at 6.10 min which is less polar than LTC_4 is probably LTB4 or an LTB4 isomer, based on similar retention times presented earlier for data presented in Table 2 and

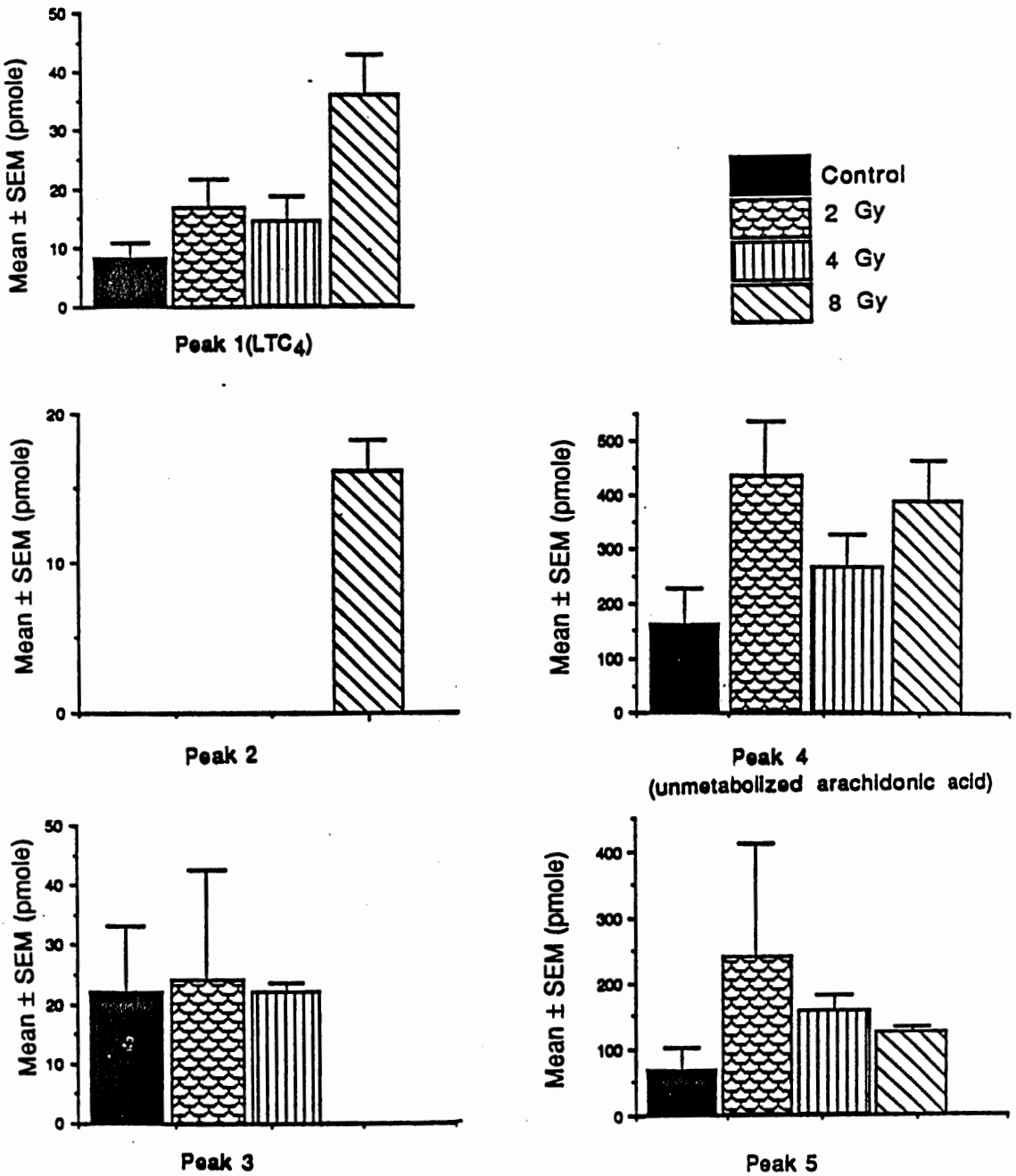


Fig. 12: Mean and standard error of the mean of total amounts of arachidonic acid metabolites produced from control, 2 Gy, 4 Gy and 8 Gy gamma irradiated macrophage cultures in the presence of FBS in the culture medium during the treatment period.

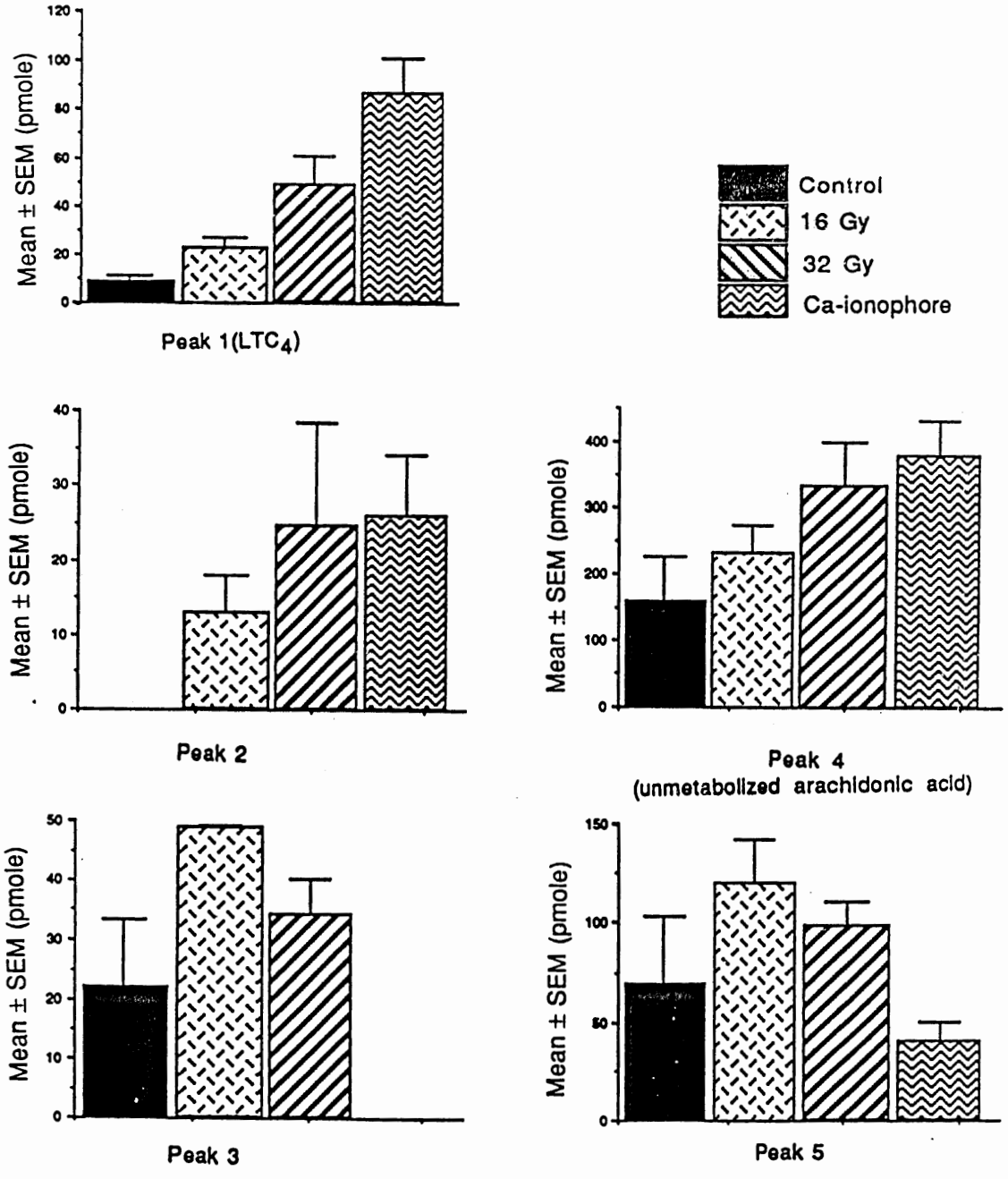


Fig. 13: Mean and standard error of the mean of total arachidonic acid metabolites produced from control, 16 Gy, 32 Gy and Ca-ionophore (10^{-5}M) stimulated macrophage cultures in the presence of FBS in the culture medium during treatment period.

previously reported results(Kouzan et al. 1985; Hardcastle and Minoui 1987). A peak that was eluted at 8.50 min of the HPLC trace from gamma irradiated(16 Gy and 32 Gy) macrophage cells was not detected in the Ca-ionophore trace(Fig. 9B-D). The most nonpolar peak eluted at 13.50 min was not identified, but it was considered as a diacyl glyceride compound.

Statistical analysis of variance and multiple range tests were performed on total amounts of substances produced by Ca-ionophore stimulated macrophage cultures. It was shown that the amount of LTC_4 production was significantly higher than in the other treated groups as well as in unstimulated macrophages (Table 2 and Fig. 13). However, there was no significant difference between the quantity of the other compounds released from Ca-ionophore(10^{-5}M) treatment and other treatments.

F. Metabolism of Arachidonic Acid by Unstimulated Macrophage Cells:

For each treatment, at least one [^{14}C]arachidonic acid labeled macrophage culture was used as a control for comparison of the results obtained from gamma irradiated or Ca-ionophore(10^{-5}M) treated macrophage cells. RP-HPLC analysis of ether extract obtained from culture medium was performed similar to other treatments. The peaks eluting at 4.20 min and 10.00 min co-eluted with standard [^{3}H] LTC_4 and [^{14}C] arachidonic acid.

A non-polar peak eluting at 14.00 min was not identified (Fig. 8 and 9). The total amount of LTC_4 produced from unstimulated macrophages was significantly lower than the LTC_4 production from gamma irradiated and Ca-ionophore (10^{-5}M) treated macrophage cells. However, the release of unmetabolized arachidonic acid and the non-polar compound produced with the peak eluting at 14.00 min were not significantly different from other treated group at 0.05 level, but in comparison with all other treatments the lowest amount of unmetabolized arachidonic acid was released from unstimulated control macrophage cultures (Table 2, Fig 8(A), 9(A), 12, and 13).

G. Metabolism of Arachidonic Acid by Macrophage Cells in the Absence of FBS:

In some of the experiments following radiolabelling of macrophage cells with [^{14}C]arachidonic acid, the culture medium had no FBS during the treatment period. HPLC analysis of the ether extract obtained from the culture medium under exactly the same analytical conditions as used previously showed that the release of unmetabolized arachidonic acid from macrophage cells was significantly decreased (Fig. 14 and 15) when compared to the macrophage cultures that had FBS in the medium during the treatment period (Table 2 and Fig. 9).

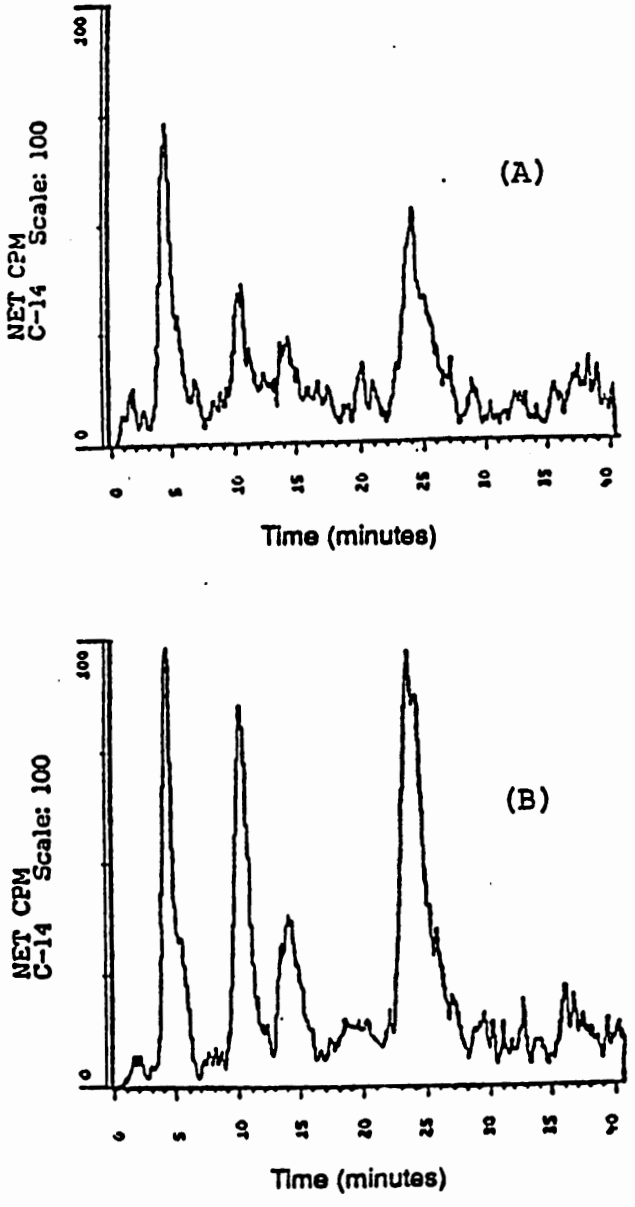


Fig. 14: Comparison of RP- HPLC analysis of ether extracts obtained from Ca-ionophore (A) and 32 Gy gamma irradiated macro- phage culture (B) in the absence of FBS during the treatment period. Analysis was performed using a C_{18} column and acetonitrile: water: acetic acid (75: 25: 0.02) pH 4.5 as a mobile phase. The peaks eluting at 4.20 min and 10.00 min of retention time co-elute with standard [^{3}H]LTC_4 and [^{14} C]arachidonic acid respectively.

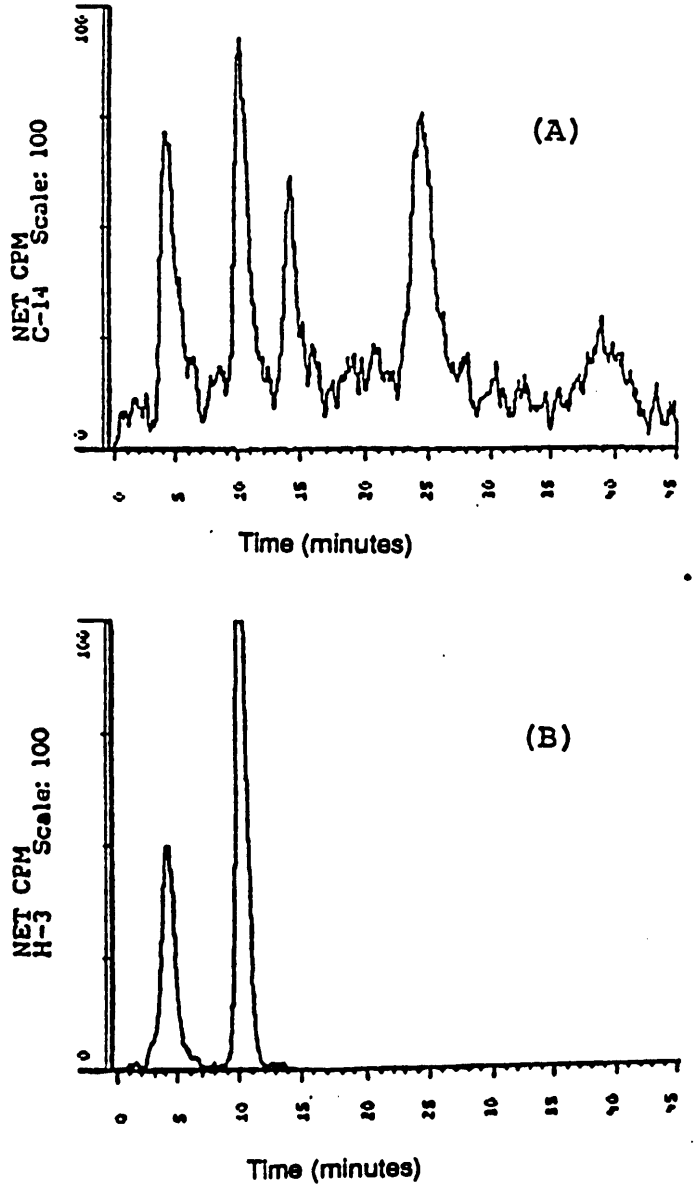


Fig. 15: RP- HPLC analysis of ether extract obtained from 32 Gy gamma irradiated macrophage culture in the absence of FBS during the treatment period. Analysis was performed using a C_{18} column and acetonitrile: water: acetic acid (75: 25: 0.02) pH 4.5 as a mobile phase. The peaks eluting at 4.20 min and 10.00 min of retention time (A) co-elute with standard [^{3}H]LTC_4 and [^{14} C] arachidonic acid respectively(B).

Statistical analysis of variance and multiple range test were performed and indicated that in the absence of FBS there was no significant difference at the 0.05 level in total amounts of LTC_4 and arachidonic acid as well as other radiolabeled compounds released from Ca-ionophore stimulated and irradiated 32 Gy macrophage cells. However, while the difference was not statistically significant, the irradiated macrophage cells produced higher amounts of LTC_4 and unmetabolized arachidonic acid(Table 3). The results also indicate that a peak eluting at 24.00 min from irradiated macrophage cells was significantly higher than in the Ca-ionophore (10^{-5}M) treated macrophage cells (Table 3 and Fig. 16). A quantitative comparison of unmetabolized arachidonic acid released from stimulated macrophages in the presence and absence of FBS is also shown in Table 4 and Fig.17).

H. Metabolism of Arachidonic Acid by Macrophage Cells in the Presence of Ibuprofen:

Free arachidonic acid released from cellular lipids is metabolized via two enzymatic pathways. Cyclooxygenase converts arachidonic acid into prostaglandin type substances and lipoxygenases metabolize the arachidonic acid into leukotrienes and other types of eicosanoids including hydroperoxyeicosatetraenoic acids(HPETEs) and

TABLE 3

Mean and standard error of the mean of total metabolites released from macrophage cells treated with Ca-ionophore and 32 Gy gamma irradiation in the absence of fetal bovine serum during the treatment period.

Treatment	Peak 1(LT_4) Mean ± SEM pmole	Peak 2 Mean ± SEM pmole	Peak3 Mean ± SEM pmole	Peak 4(AA) Mean ± SEM pmole	Peak 5 Mean ± SEM pmole	Peak 6 Mean ± SEM pmole
Ca-ionoph. (10^{-5}M) (4,6)*	69.92 ± 12.04[c] (6)**	25.52 ± 2.82[c] (4)**	36.11 ± 6.30[c] (5)**	51.13 ± 12.85[c] (6)**	35.04 ± 8.15[c] (5)**	76.06 ± 24.29[b] (4)**
32 Gy (3,4)*	94.00 ± 20.44[c] (4)**	25.58 ± 5.45[c] (3)**	39.85 ± 11.82[c] (2)**	104.04 ± 34.75[c] (4)**	54.37 ± 16.79[c] (4)**	219.82 ± 9.98[a] (2)**

a= significantly different from b at the 0.05 level.
c= not significantly different from each other at the 0.05 level.
*= first number in the parentheses indicates total experiments per treatment, and the second number is equal to number on HPLC runs.
**= designates number of repeated peaks detected by HPLC.

Fig. 16: Mean and standard error of the mean of total arachidonic acid metabolites released from macrophage cells exposed to Ca-ionophore and 32 Gy gamma irradiation in the absence of FBS during treatment period.

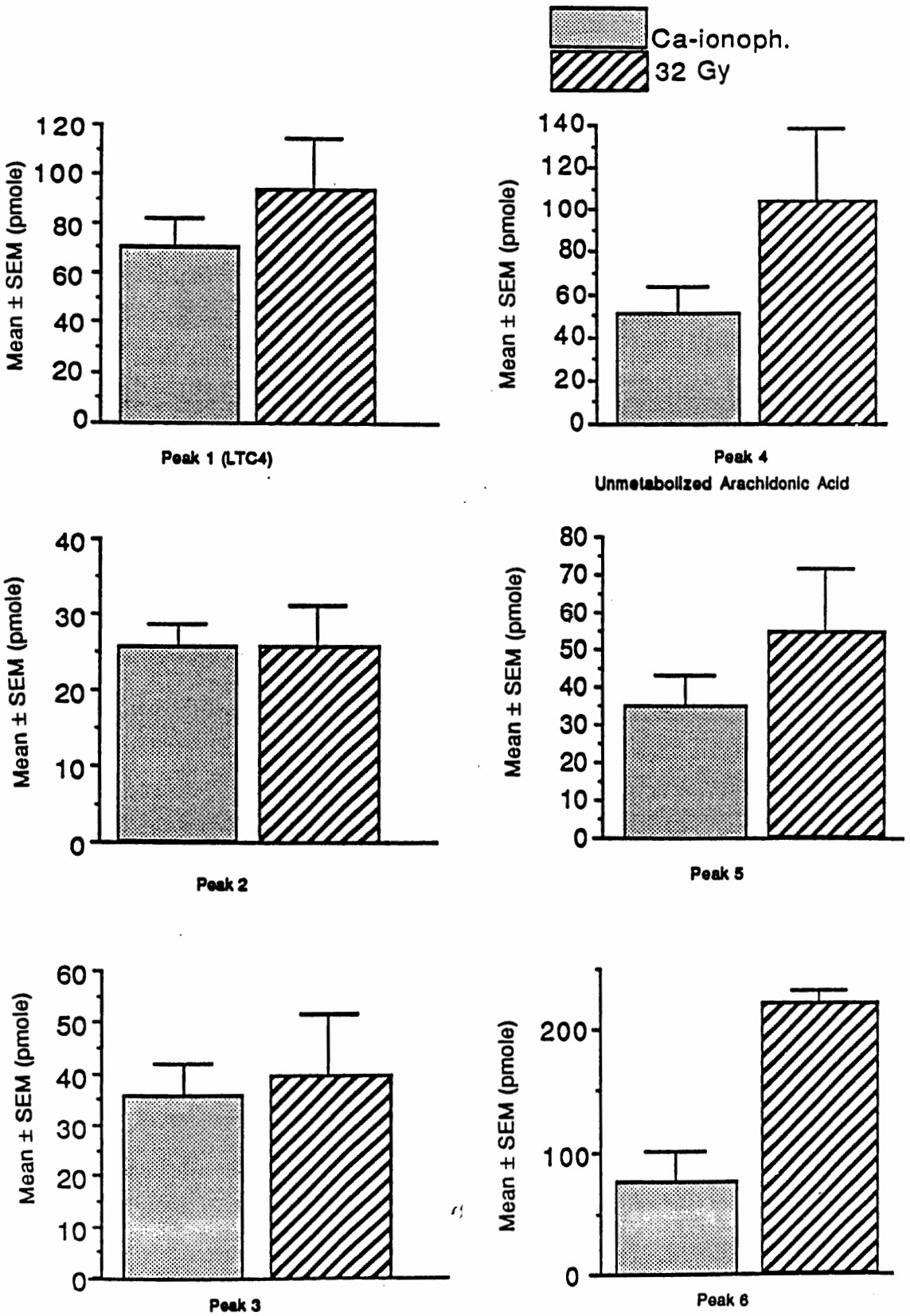

Ca-ionoph.
32 Gy
Mean ± SEM (pmole)
120
100
80
60
40
20
0
Peak 1 (LTC4)
140
120
100
80
60
40
20
0
Peak 4
Unmetabolized Arachidonic Acid
40
30
20
10
0
Peak 2
80
70
60
50
40
30
20
10
0
Peak 5
60
50
40
30
20
10
0
Peak 3
200
100
0
Peak 6

TABLE 4

Comparison of the total amount of unmetabolized arachidonic acid released from 32 Gy gamma irradiated and Ca-ionophore stimulated macrophage cells in the presence and absence of fetal bovine serum in the culture medium during the treatment period.

	with FBS	without FBS
Treatment	Mean ± SEM pmole	Mean ± SEM pmole
Ca-ionophore (10^{-5}M)	377.13 ± 53.94	51.13 ± 12.13
32 Gy	334.33 ± 64.22	104.04 ± 34.75

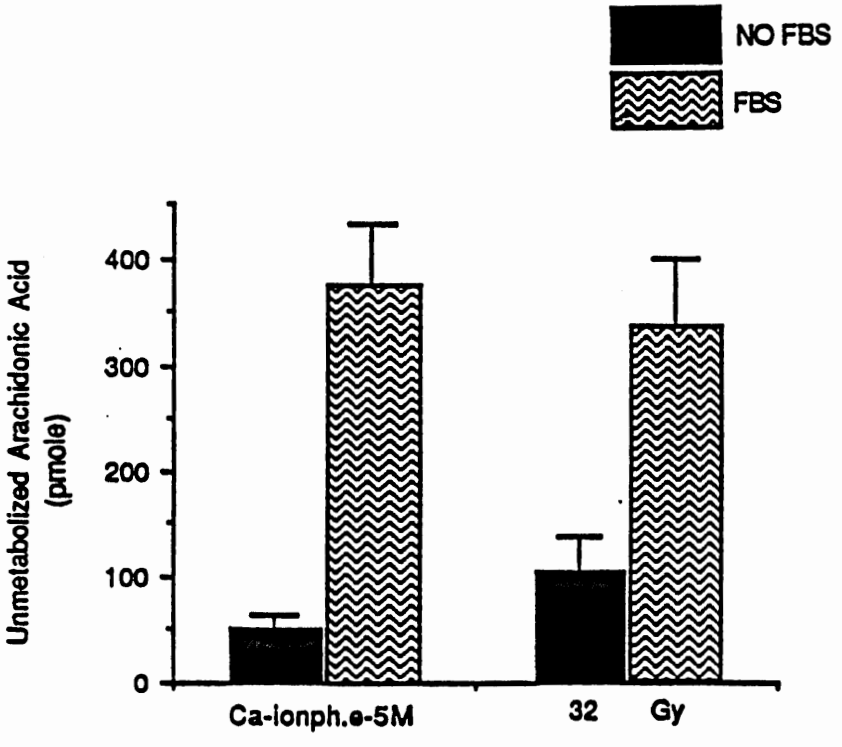


Fig. 17: Mean and standard error of the mean of unmetabolized arachidonic acid released from macrophage cells in the presence of FBS compared to the macrophge cultures that had no FBS in their medium during the treatment period.

monohydroxyeicosatetraenoic acids(HETEs)(Davies et al. 1984). In some of the experiments ibuprofen, an inhibitor of the cyclooxygenase pathway, was added (10^{-5}M in concentration) to the culture medium of [^{3}H]arachidonic acid labeled macrophage cell cultures 30 min prior to being stimulated with Ca-ionophore (10^{-5} M) or 32 Gy gamma irradiation. Similarly, the controls also received ibuprofene(10^{-5}M) for 1.5 h prior to ether extraction.

Qualitative detection of radioactive substances produced from macrophage cells exposed to 32 Gy, Ca-ionophore (10^{-5} M) and control in the presence of ibuprofen is shown in Fig. 18. In Ca-ionophore(10^{-5}M) stimulated macrophage cultures and in the absence of FBS, the peaks eluting at 4.50 min, 7.30 min and 14.30 min of retention time were identified as LTC_4, 12-HETE and unmetabolized arachidonic acid by co-eluting with the standards [^{3}H] LTC_4 , [^{3}H] 12- HETE and [^{3}H] arachidonic acid mixture (Fig. 18(D)) as well as single standard [^{3}H] 12- HETE shown in Fig.19. A non-polar post arachidonic acid peak eluting at 21.6 min was not identified with our standards.

Comparing data from Ca-ionophore treated macrophage cultures with those from irradiated cultures and control (Fig.18), the LTC_4 and unmetabolized arachidonic acid released from macrophage cells in both gamma irradiation and unstimulated control were co-eluted with standards

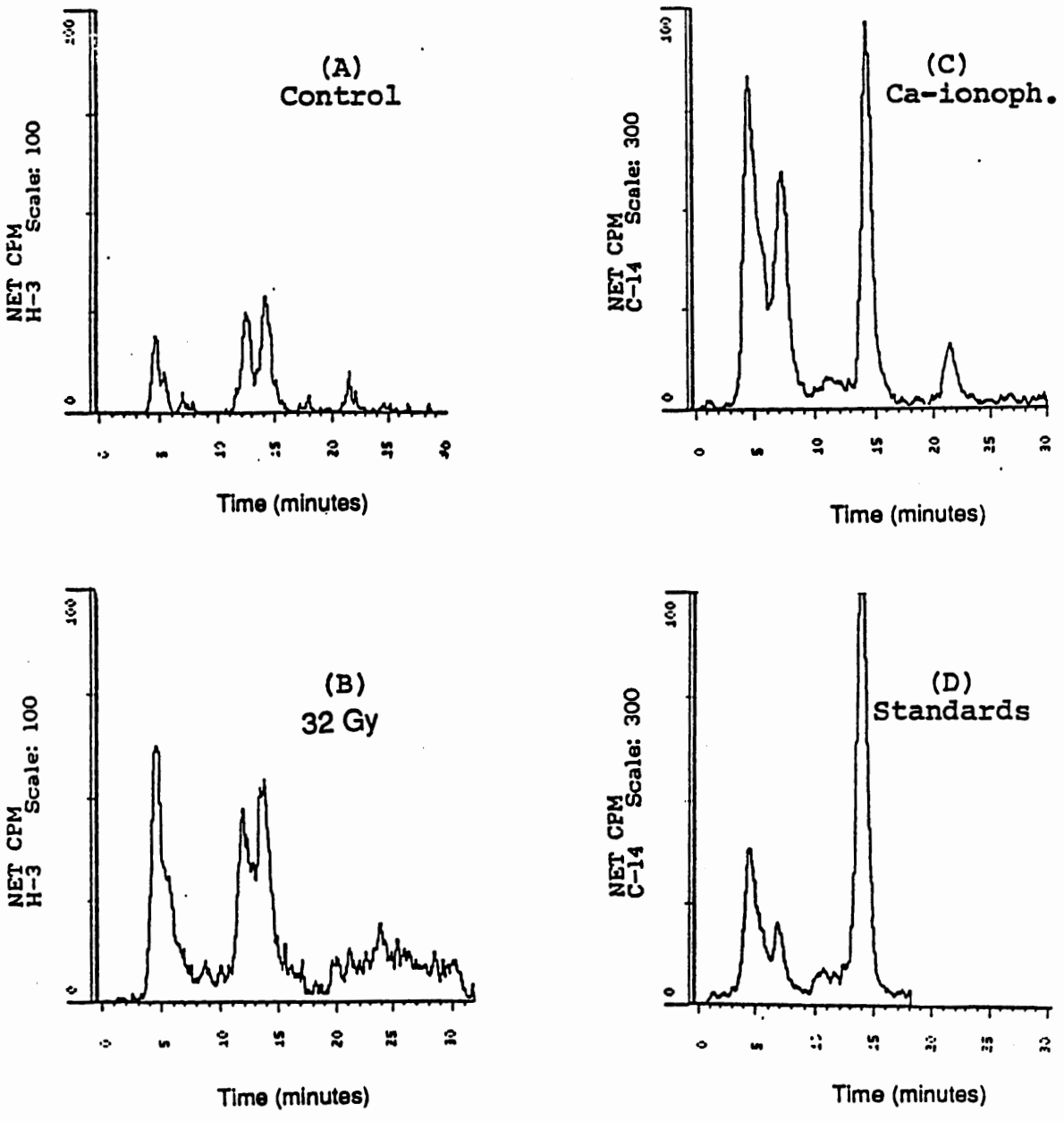


Fig. 18: RP- HPLC analysis of ether extracts obtained from (A) unstimulated (control), (B) 32 Gy and (C) Ca- ionophore stimulated macrophage cells in the presence of ibuprofen but without FBS in their culture medium during treatment period. The peaks eluting at 4.60 min, 7.30 min and 14.30 min co-eluted with standard [^{3}H]LTC$_4$, [^{3}H]12-HETE and [^{3}H]arachidonic acid (D).

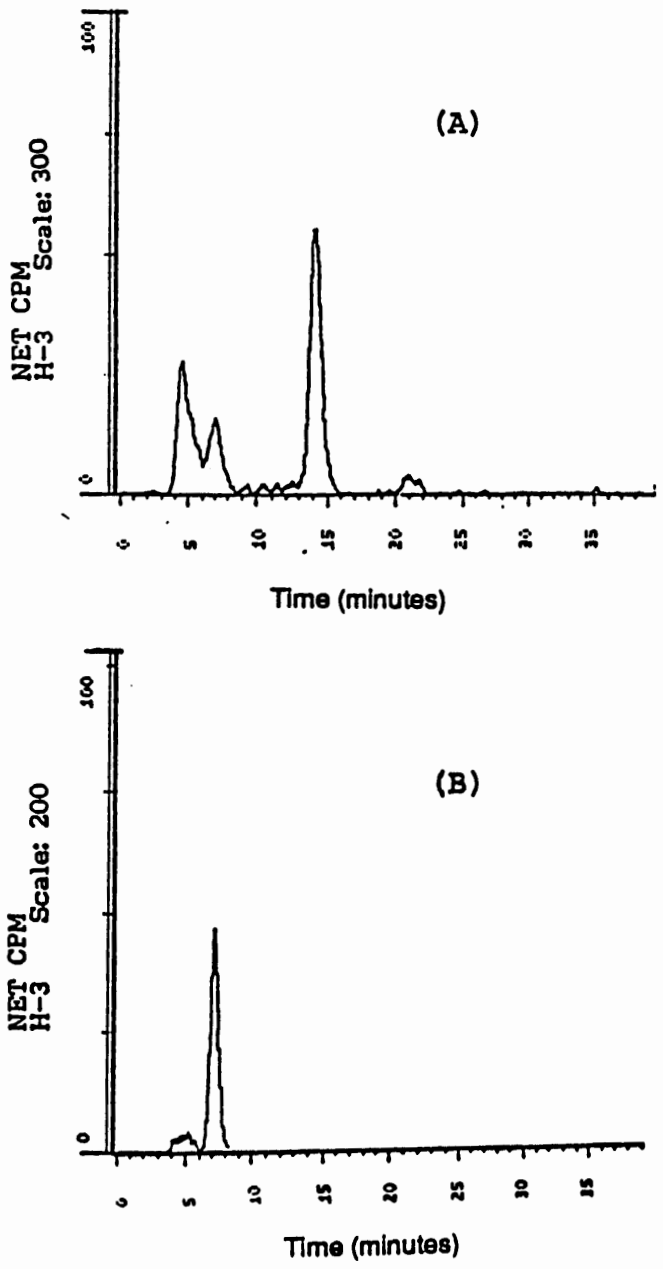


Fig. 19: RP- HPLC analysis of ether extract obtained from Ca-ionophore (10^{-5} M) treated macrophage cells in the presence of ibuprofen and without FBS in the culture medium during treatment period. The peak eluitng at 7.30 min of retention time (A) co-elute with standard [^{3}H]12-HETE (B).

[^{3}H]LTC_4 and [^{3}H] arachidonic acid but the other peaks eluting at 12.00 min and 22.00 min in both radiation and control were not identified (Fig. 18).

Similar results were observed when a weaker solvent (60% acetonitrile, 40% water and 0.02% acetic acid) pH 4.5 was used(Fig. 20). However, using a weaker solvent caused more separation of the eluting peaks(Fig. 20). The additional peaks eluting at 6.80 min and 8.30 min(Table 5, peak 2 and 3) from both Ca-ionophore and radiation treatments may be LTB_4 or LTB_4 isomers. This conclusion is based on similar retention times of standards in other experiments. The peak eluting at 12.90 min (Fig. 20B) was coeluted with standard 12-HETE. Other peaks eluting at 11.80 min(Fig. 20B) and 14.10 min(Fig. 20 A and B) are probably 15-HETE and 5-HETE metabolites.

Quantitative study of arachidonic acid metabolism by macrophage cells in the presence of ibuprofen indicates that macrophages exposed to Ca-ionophore(10^{-5} M) produced significantly higher amount of LTC_4 and a less polar substance(see Table 5 peak 2) as well as unmetabolized arachidonic acid (Table 5) when compared to other treated groups. The substances that were produced by unstimulated macrophage cells however, were lower in total amount when compared to all other treatments (Table 5 and Fig. 21).

Fig. 20: RP- HPLC analysis of ether extracts obtained from Ca-ionophore and 32 Gy gamma irradiated macrophage cell cultures in the presence of ibuprofen and without FBS in their culture medium. A weaker mobile phase solvent, acetonitrile: water: acetic acid (60: 40: 0.02) pH 4.5 was used to separate the more polar from non-polar substances. At 25.00 min the solvent was changed to the original mobile phase (75% acetonitrile) to elute most non-polar compounds. The peaks eluting at 4.70 min and 35.00 min from both treatments (A and B) co-eluted with standard [^{3}H]LTC_4 and [^{3}H] arachidonic acid (A). The peaks eluting at 12.90 min (B) from Ca-ionophore (10^{-5} M) treated co-elutes with standard [^{3}H]12- HETE (C).

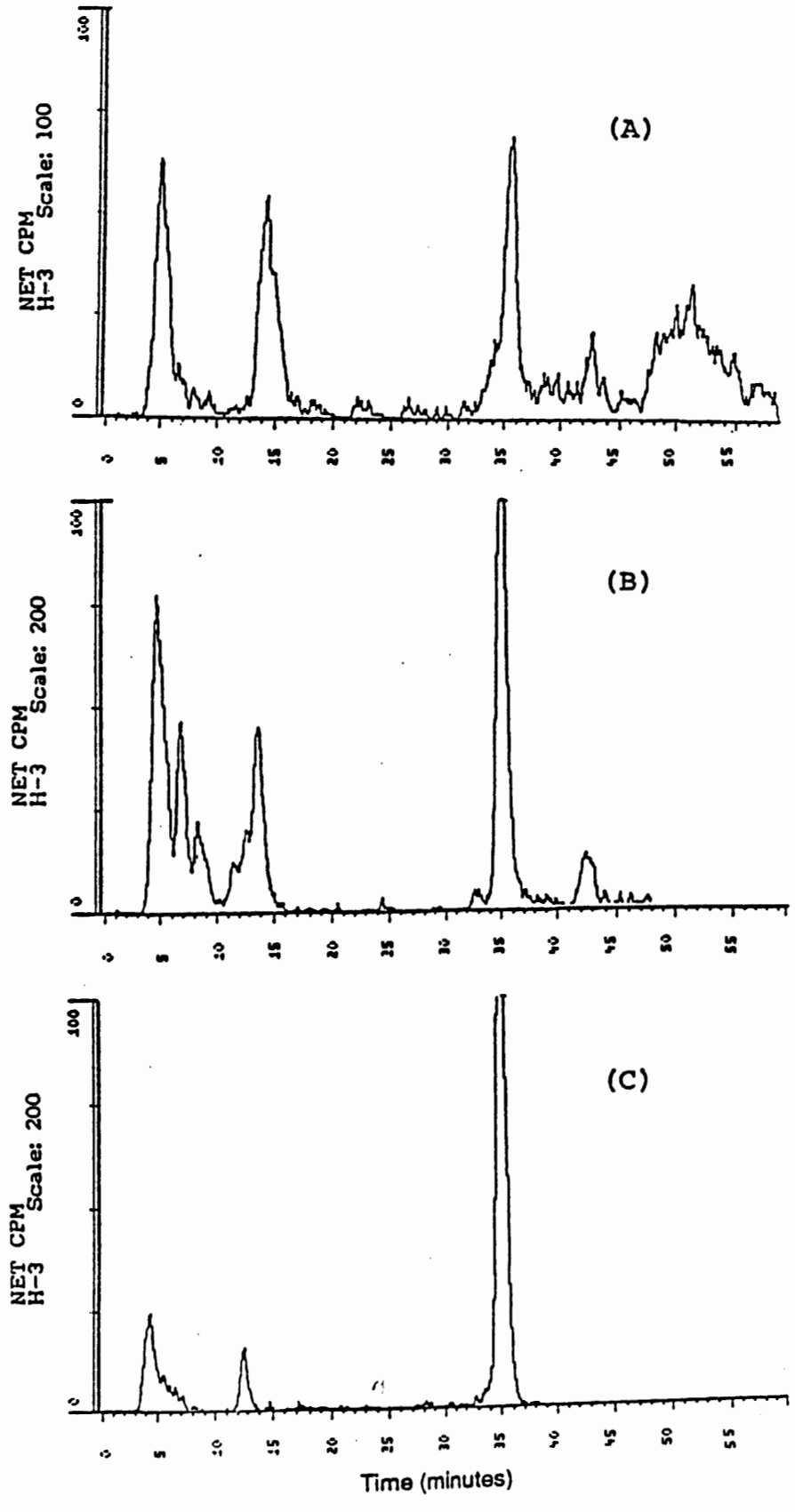

NET CPM
H-3
Scale: 100
(A)
NET CPM
H-3
Scale: 200
(B)
NET CPM
H-3
Scale: 200
(C)
Time (minutes)

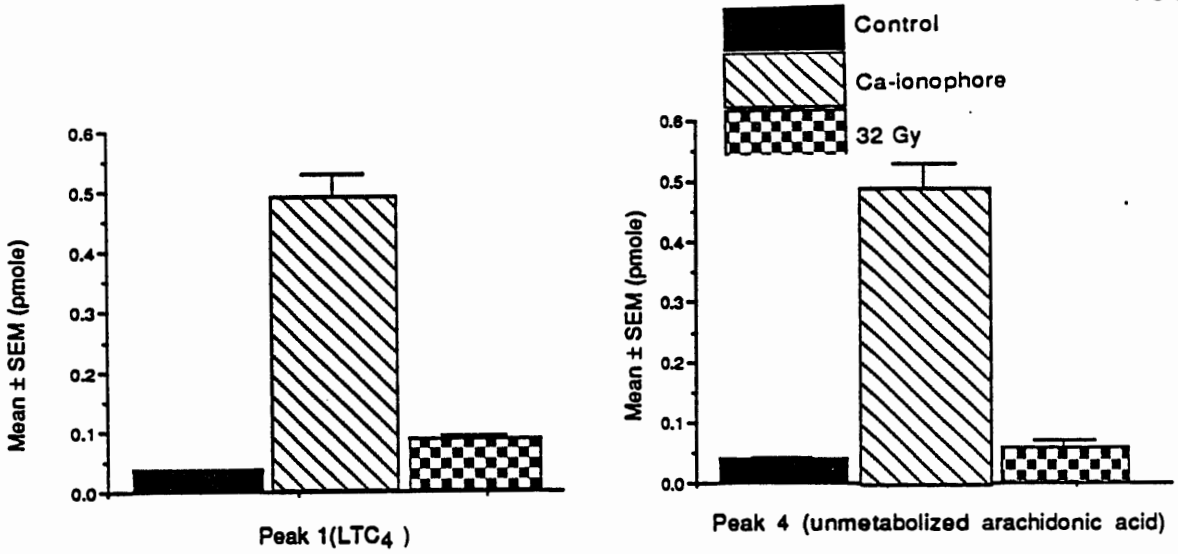
TABLE 5

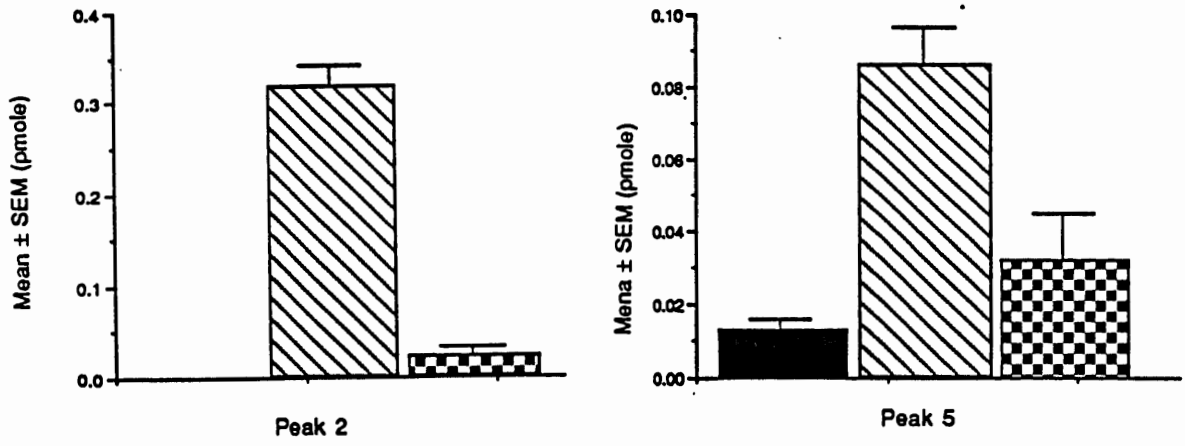
Mean and standard error of the mean of total metabolites released from macrophage cells treated with Ca-ionophore, 32 Gy gamma irradiation and unstimulated cells in the presence of ibuprofen but no fetal bovine serum for 30 min prior and during the treatment period.

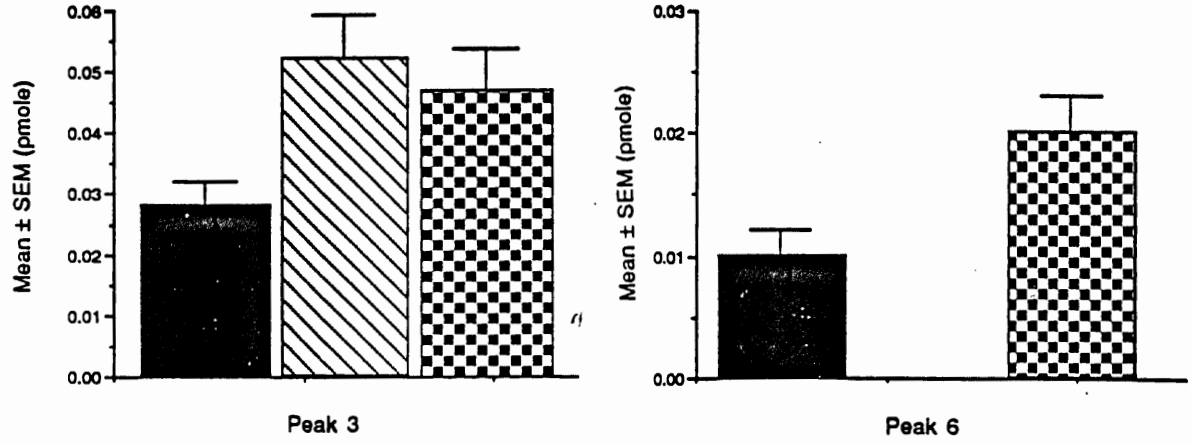
Treatment	Peak1(LTC_4) Mean ± SEM pmole	Peak 2 Mean ± SEM pmole	Peak 3 Mean ± SEM pmole	Peak 4(AA) Mean ± SEM pmole	Peak 5 Mean ± SEM pmole	Peak 6 Mean±SEM pmole
Control (2,2)*	0.034 ± 0.000[b] (2)**	—	0.028 ± 0.004[c] (2)**	0.037 ± 0.004[b] (2)**	0.013 ± 0.003[b] (2)**	0.010 ± 0.002[c] (2)**
Ca-ionoph. (10^{-5}M) (3,6)*	0.490 ± 0.037[a] (6)**	0.316 ± 0.024[a] (6)**	0.052 ± 0.007[c] (4)**	0.491 ± 0.043[a] (6)**	0.086 ± 0.010[a] (6)**	—
32 Gy (3,3)	0.086 ± 0.006[b] (3)**	0.022 ± 0.009[b] (3)**	0.047 ± 0.007[c] (3)**	0.059 ± 0.012[b] (3)**	0.032 ± 0.013[b] (2)**	0.020 ± 0.003[c] (3)**

a= significantly different from b at the 0.05 level.
c= not significantly different from each other at the 0.05 level.
*= first number in the parentheses indicates total experiments per treatment, and the second number is equal to the number of HPLC runs.
**= designates number of repeated peaks detected by HPLC.

Fig. 21: Mean and standard error of the mean of total amounts arachidonic acid metabolites produced from macrophage culutures in the presence of ibuprofen and without FBS in the culture medium when exposed to 32 Gy gamma irradiation and Ca-ionophore compared to unstimulated macrophage cells(control).


Control
Ca-ionophore
32 Gy
Mean ± SEM (pmole)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Peak 1(LTC4)
Peak 4 (unmetabolized arachidonic acid)


Mean ± SEM (pmole)
0.0
0.1
0.2
0.3
0.4
Peak 2
Mena ± SEM (pmole)
0.00
0.02
0.04
0.06
0.08
0.10
Peak 5


Mean ± SEM (pmole)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
Peak 3
Mean ± SEM (pmole)
0.00
0.01
0.02
0.03
Peak 6

I. FBS Effects on the Arachidonic Acid Metabolism by Macrophage Cells in the Presence of Ibuprofen:

A qualitative study was also performed in order to compare the effect of fetal bovine serum in the culture medium on arachidonic acid metabolism by stimulated macrophage cells in the presence of ibuprofen.

Data from pre-labled macrophage cells stimulated with Ca-ionophore (10^{-5}M) in the presence of ibuprofen and FBS in the culture medium were compared with those that had ibuprofen but no FBS in the culture medium. The HPLC analysis of the ether extracts obtained from the culture medium of the both treatments with a trace of radioactivity detection is shown in Fig. 22 and 23, and in Tables 6 and 7. Peaks eluting at 4.50min, 7.30 min and 14.30 min co-eluted with standard [^{3}H]LTC4, [^{3}H]12-HETE and [^{3}H]arachidonic acid respectively. A comparison of a typical trace of recorded radioactivity of the eluted compounds released from Ca-ionophore stimulated macrophage cells in the presence of ibuprofen and FBS with cultures that had no FBS in the culture medium during the treatment period is shown in Fig. 22 and Table 6. The release of exogenous arachidonic acid from macrophage cells that had ibuprofen and FBS was significantly higher than the release of arachidonic acid from macrophage cells that had ibuprofen without any FBS in the culture medium during the treatment period (Fig. 22 and Table 6). However, LTC_4

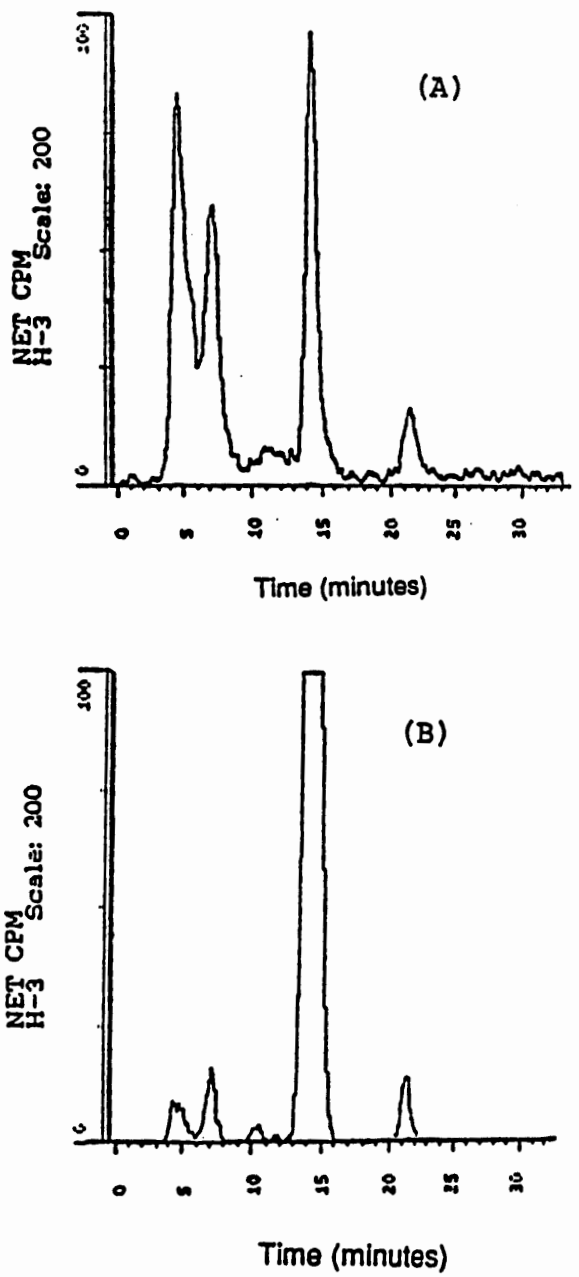


Fig. 22: Comparison of RP- HPLC analysis of ether extracts obtained from Ca-ionophore stimulated macrophage cells in the presence of ibuprofen without FBS (A) and with FBS (B) in their culture medium during the treatment period. Quantitatively the release of unmetabolized arachidonic acid from stimulated macrophage cells in the presence of FBS was significantly higher (B) while LTC_4 and 12- HETE production were lower when compared with macrophage cells exposed to Ca-ionophore (10^{-5} M) in the absence of FBS (A).

TABLE 6

Comparison of total radioactivity detected per each peak following HPLC analysis of the ether extract obtained from Ca-ionophore stimulated macrophage cultures with ibuprofen in the presence or absence of FBS during the treatment period.

	Ibuprofen + FBS		Ibuprofen, no FBS	
	CPM	%	CPM	%
Total	436000	100	443680	100
HPLC Peak 1(LTC4)	16640	3.82	148240	33.40
HPLC Peak 2(12-HETE)	13160	3.02	96000	21.64
HPLC Peak 3	7920	1.82	6440	1.45
HPLC Peak 4(AA)*	358000	82.17	121440	27.37
HPLC Peak 5	18080	4.15	25920	5.84

*= indicates the release of unmetabolized exogenous arachidonic acid from macrophage cells during the treatment period.

and 12-HETE released from Ca-ionophore stimulated macrophage cells in the absence of FBS was significantly higher than in the FBS containing macrophage cultures (Fig. 22 andTable 6). A similar observation was evident in cultures of gamma irradiated macrophage cells in the presence of ibuprofen and FBS when compared to the results obtained from macrophage cells exposed to 32 Gy in the presence of ibuprofen and without FBS in their culture medium during the treatment period (Fig. 23 and Table 7).

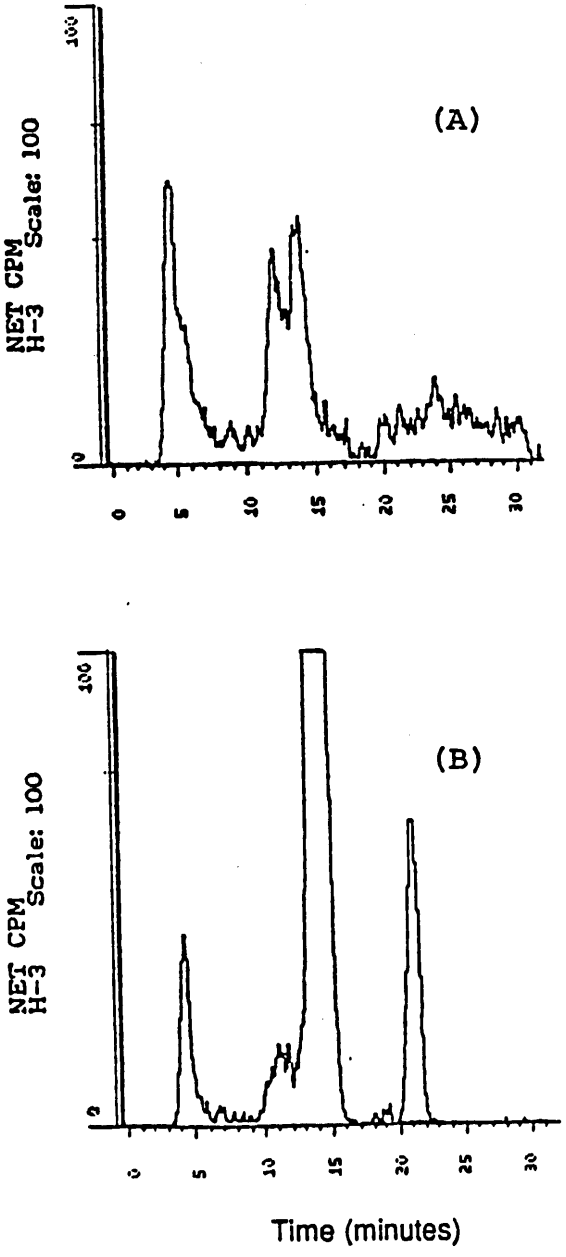


Fig. 23: Comparison of RP-HPLC analysis of ether extracts obtained from 32 Gy gamma irradiated macrophage cells in the presence of ibuprofen without FBS (A) and with FBS (B) in their culture medium during the treatment period. Quantitatively the release of unmetabolized archidonic acid from macrophage cells in the presence of FBS was significantly higher (B) while the release of LTC_4 and other compounds was lower when compared with irradiated macrophage cells in the absence of FBS (A).

TABLE 7

Comparison of total radioactivity detected per each peak following HPLC analysis of ether extract obtained from 32 Gy gamma irradiated macrophage cultures with ibuprofen in the presence or absence of FBS during the treatment period.

	Ibuprofen + FBS		Ibuprofen, no FBS	
	CPM	%	CPM	%
Total	111720	100	64968	100
HPLC Peak 1(LTC_4)	7008	6.27	12924	19.89
HPLC Peak 2	1236	1.11	1092	1.68
HPLC Peak 3	2448	2.19	6204	9.55
HPLC Peak 4(AA)*	83268	74.53	7776	11.93
HPLC Peak 5	10308	9.23	3540	5.45
HPLC Peak 6	_	_	2784	4.29

*= indicates the release of unmetabolized exogenous arachidonic acid from macrophage cells +during treatment time period.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Macrophages have been used extensively for both in vivo and vitro study of arachidonic acid metabolism, especially with regard to leukotriene synthesis. In the present study peritoneal macrophage cells labeled with [^{14}C]arachidonic acid were used to investigate leukotriene biosynthesis following exposure to ionizing gamma irradiation and Ca-ionophore stimulation. Using the total released radioactivity as an indicator of the activity of the entire arachidonic acid metabolic cascade, the amount of unmetabolized arachidonic acid released when macrophage cells were stimulated with ionizing gamma irradiation and Ca-ionophore was greater than that released from resting cells. This finding is in agreement with other reported results indicating that the UV irradiated human keratinocytes(De Leo et al. 1984) as well as zymosan stimulted mouse peritoneal macrophage cells (Scott et al. 1980; Humes et al. 1982) released relatively high amount of unmetabolized arachidonic acid into the culture medium. Recently the study of arachidonic acid metabolism in Ca-ionophore stimulated human alveolar macrophage cells has shown that the release of free arachidonic acid into the culture medium was significantly increased when compared to unstimulated

macrophages(Balter et al. 1988).

Stimulating agents such as Ca-ionophore which increase the intracellular calcium concentration trigger a signal for phospholipase A and C activation to promote the release of arachidonic acid from the phospholipid layer(Balter et al. 1988). Irradiation may cause release of arachidonic acid by a different mechanism (Hahn et al. 1983) perhaps peroxidation by a free radical mechanism(Ziboh et al. 1982).

The specific objective of the present study was to determine in vitro biosynthesis of leukotrienes by macrophage cells following exposure to Cobalt-60 gamma radiation. The various doses of gamma radiation(2 Gy-32 Gy) stimulated the metabolism of arachidonic acid to produce LTC_4 and some other arachidonate metabolites probably the isomers of LTB_4 and the HETEs(Table 2). The results of the recent investigations have shown that ionizing irradiation causes in vitro increase of cyclooxygenase products of arachidonic acid in the guinea-pig parenchymal lung tissues (Steel and Catravas 1982) and in calf endothelial cells (Hahn et al. 1983) and in vivo in domestic pig skin (Ziboh et al. 1982) and in mice (Steel et al. 1986). Other recent investigations have shown that the mouse peritoneal cells exposed to gamma irradiation(10 Gy) released a higher amount of cyclooxygenase (PGE_2) and lipoxygenase (LTC_4) products of arachidonic acid metabolism

when compared to unstimulated control cells(Steel et al. 1988).

The most nonpolar compound that was detected as a post-arachidonic acid peak shown in HPLC traces(Fig. 8 and 9) obtained from the culture medium of all irradiated macrophage cultures was considered to be a diglyceride compound, an intermediate compound that is released from cellular phospholipids prior to hydrolysis with diglyceride lipase to produce arachidonic acid. According to the reported studies on thrombin-stimulated platelets (Billah et al. 1980; Lapetina et al. 1981b) as well as other type of cells(Oliw et al. 1983), stimulation of the phospholipids led to the formation of 1,2-diacylglyceride and inositol phosphate through phospholipase C hydrolysis. The diacylglyceride is then hydrolyzed by diacylglyceride lipase with liberation of arachidonic acid(Oliw et al. 1983; Martin and Wysolmerski 1987).

The data presented in this study(Fig. 12 and 13, Table 2) demonstrate that increases in irradiation dose up to 32 Gy are associated with increasingly elevated LTC_4 production without any significant changes in the release of other metabolites. These observations are in agreement with the studies that have previously been reported, indicating that ionizing irradiation stimulates arachidonic acid metabolism leading to the production of leukotrienes(Steel et al. 1988) and prostaglandins(Ziboh et al. 1982; Das et al. 1985; Steel et al. 1986).

In comparison to ionizing irradiation used in the present study, it has been reported that ultraviolet irradiation causes the stimulation of the macrophage cells to release arachidonic acid from cellular phospholipids to produce leukotrienes LTC_4 and LTB_4 and some other metabolites(Hardcastle and Minoui 1987). Although it is not quite clear how irradiation stimulates the metabolism of arachidonic acid in macrophage cells, the results obtained in the present investigation may confirm the results of previous studies(Ziboh et al. 1982; Friedman et al. 1986) suggesting that radiation, possibly by generating free radicals, can activate phospholipase A_2 to promote the release of arachidonic acid from phospholipid stores.

Compared to irradiated macrophage cells, the resting cells(unirradiated macrophage cells) produced a very low amount of arachidonate metabolites, including LTC_4. The initiation of arachidonic acid metabolism in resting macrophage cells may be due to the contact between the adhered macrophage cell membrane and the plastic culture flask when the phagocytic cells recognize as a foreign particles (Kouzan et al. 1988).

In addition to ionizing gamma irradiation, in the present study Ca-ionophore was used as a non-immunological stimulating agent to investigate the production of leukotrienes by macrophage cells. Previous investigation of arachidonic acid metabolites has shown that pre-labeled

human peritoneal polymorphonuclear leukocytes exposed to Ca-ionophore produced 5-HETE and a more polar dihydroxy acid metabolite namely LTB_4 (Borgeat and Samuelsson 1979a; Samuelsson et al. 1980). Similar results have been reported following Ca-ionophore stimulation of alveolar macrophage cells(Hsueh and Sun 1982; Martin et al. 1984). Arachidonic acid metabolism in different types of cells of different species have shown that the Ca-ionophore stimulation of mouse peritoneal macrophage cells(Humes et al. 1982; Hardcastle and Minoui 1987), human monocytes(Williams et al. 1984) and human polymorphonuclear leukocytes (Lee et al. 1984) caused the production of both LTC_4 and LTB_4. The results obtained in the present study(Fig.13 and Table 2) are partially in agreement with the reported findings(Humes et al. 1982; Williams et al. 1984; Laviolette et al. 1988; Steel et al. 1988), that Ca-ionophore stimulated macrophage cells produced significantly higher amounts of LTC_4 when compared to the unstimulated cells. In this study LTD_4 and LTE_4, which are produced from LTC_4 through the sequential loss of glutamic acid residue and glycine residue(Piper 1983; Ford-Hutchinson 1985), apparently were not produced from any of the treated or untreated macrophage cultures. This observation is in agreement with the recent reported results suggesting that the peritoneal macrophage cells do not have LTD_4 and LTE_4 synthetases (Abe and Hugli 1988).

In the present study LTB_4 was detected in smaller quantities than LTC_4 in Ca-ionophore stimulated cells. This observation may support the statement that has been made previously suggesting that LTB_4 production from Ca-ionophore stimulated macrophage cells reached a plateau by 5 min, whereas the LTC_4 plateau was reached in 60 min, the longest incubation time studied(Laviolette et al. 1988). Other arachidonate metabolites that were less polar than LTC_4, are speculated to be LTB_4 isomers(Martin et al. 1984). The most nonpolar compound that was detected corresponded to the peak observed in irradiated cells and was thought to be a diacylglyceride; an intermediate compound that is hydrolyzed to form arachidonic acid.

In the present study, some of the macrophage cultures were administered FBS for the entire experiment, while in other experiments the addition of FBS to the culture medium was omitted after the incubation of macrophage cells with arachidonic acid and during the treatment period. A significant decrease in the release of free arachidonic acid with a larger amount of arachidonate metabolites production by all treated macrophage cells was observed in the absence of FBS compared to treated macrophage cultures that had FBS during the treatment period. Although the type of cells and the pattern of FBS administration to the culture medium are not similar to those used in this study, Kouzan et al. (1985) observed that the pattern of

arachidonic acid metabolism by alveolar macrophage cells after stimulation was dramatically changed following the omission of FBS from the culture medium during the period of cell adhesion and arachidonic acid incorporation. Furthermore, in a recent investigation it has been shown that in the presence of FBS during macrophage cell adhesion, the production of arachidonic acid metabolites was reduced by more than 50%. It was suggested that this reduction is possibly due to the inhibitory activity of FBS during cell adhesion. This inhibition may involves a modification of the nature of the adhesion between the plastic and the cell membrane by reducing the intensity of the signal triggering the initial arachidonic metabolism(Kouzan et al. 1988). The addition of FBS to the cells that had been adhered in FBS-free medium significantly increased the release of eicosanoids after stimulation with Ca-ionophore(Kouzan et al. 1988). The results obtained in this study and those reported above support the statement by Kouzan et al. (1988), suggesting that FBS is involved in the regulation of arachidonic acid metabolism.

Since the discovery of aspirin and indomethacin as nonsteroidal anti-inflammatory drugs(NSAID's), there have been numerous attempts to correlate the effects of these drugs with their ability to interfere with the activity of endogenous production of biological mediators, more specifically the cyclooxygenase products of arachidonic acid metabolism(Kuehl and Egan

1980; Corey and Park 1982). It has also been reported that the NSAID's inhibit prostaglandin production but simultanously enhance the synthesis of leukotrienes from Ca-ionophore stimulated mouse peritoneal macrophage cells(Brune et al. 1984). Aspirin, indomethacin and ibuprofen are among the anti-inflammatory drugs that have been used as an inhibitor of arachidonic acid metabolism in the cyclooxygenase pathway without affecting LTC_4 synthesis(Bonney and Humes 1984; Baud et al. 1987). However, it has been reported that using these drugs at higher concentrations blocks both cyclooxygenase and lipoxygenase pathways(Bonney and Humes 1984). The results obtained in the present investigation show clearly that Ca-ionophore stimulated macrophage cells in the presence of ibuprofen produced significantly higher amount of LTC4 and some other arachidonic acid metabolites. The results of this study support the findings indicating that anti-inflammatory agents including ibuprofen enhance leukotriene synthesis in Ca-ionophore stimulated macrophage cultures (Brune et al. 1984; Bonney and Humes 1984).

In the presence of ibuprofen, the total radioactivity released from gamma irradiated macrophage cells was increased but to a lesser extent than in Ca-ionophore stimulated cells. The relatively smaller effect of ionizing radiation as compared to Ca-ionophore may suggest that the action of the ibuprofen is attenuated by ionizing gamma irradiation. Perhaps the reduced

production of LTC_4 and other arachidonates from gamma irradiated macrophage cells may be due to a partial degradation of the enzymes involved in the metabolism of arachidonic acid.

In conclusion, the results obtained in the present study indicate that the ionizing gamma irradiation either sublethal(2 Gy) or lethal(32 Gy) caused the stimulation of arachidonic acid metabolism in peritoneal macrophage cells to produce a significantly higher amount of LTC_4 and some other arachidonates. Elevated LTC_4 production by irradiated macrophage cells was also closely associated with increases in irradiation dose up to 32 Gy, this may be related to the effect of ionizing radiation on phospholipase or lipoxygenase activities. Although the exact mechanism which is responsible for the enzymatic stimulation leading to the increased production of arachidonic acid metabolites is not quite clear, it is possible that the free radicals generated by ionizing irradiation may activate the arachidonic acid metabolic cascade.

In the absence of FBS during treatment, macrophage cells exposed to gamma irradiation produced a relatively higher amount of arachidonic acid metabolites with a significant decrease in the released free arachidonic acid when compared to macrophage cultures in FBS containing medium. It is concluded that FBS play a role in regulation of the release as well as the metabolism of arachidonic acid in macrophage cells and possibly in other

types of cell.

In the absence of FBS during treatment, Ca-ionophore stimulation of macrophage cultures in the presence of ibuprofen produced both qualitatively and quantitatively higher amounts of arachidonates including LTC_4.

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