ACTIONS OF ANTIOXIDANTS ON HUMAN T LYMPHOCYTES AND LEUKAEMIA CELLS

THESIS

presented for the degree of

Doctor of Philosophy

in the

Faculty of Medicine

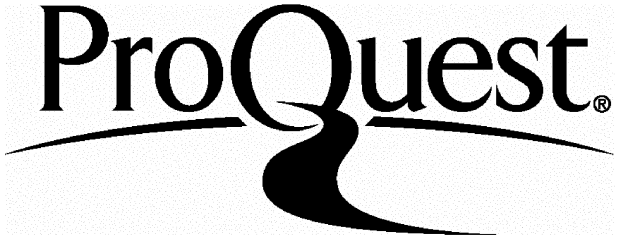
(Field of study-Biochemistry)

by

Dr.Mohammad Ayyub MBBS (Peshawar)

From: The Department of Haematology
The Royal Free Hospital School of Medicine
University of London

September 1992



I dedicate this work to my parents, my wife, Saba and my sons, Adil and Harris.

Acknowledgements

My sincere thanks are owed to Professor A.V. Hoffbrand, for introducing me to haematology and providing me with a friendly yet challenging atmosphere in which to carry out my work. His kind encouragement and efforts made my four years stay here pleasant and fruitful.

I am particularly indebted to my supervisor, Dr. Stan Wickremasinghe; without his nurturance, guidance, enthusiasm and efforts the work described here would have been impossible.

Special-thanks are due to Dr.S. Jabbar and A. Parsonson for their valuable counsel in computing. I also wish to extend my thanks to Mr. J. Jeremy, T. Hallinan and J. Tateson for their kind help and interesting discussions. I was fortunate indeed to work alongside such nice people as Guru, J. Yaxley, Emilio and too many other colleagues in this department to mention who were always kind, helpful and co-operative.

I am grateful to the Ministry of Science and Technology, Government of Pakistan for funding expenses during my PhD and the department of Haematology Royal Free Hospital for financial support for research. This project was co-sponsored by the Armed Forces Institute of Pathology Rawalpindi, Pakistan and Major General M. Saleem to whom gratitude is particularly expressed.

My special thanks go to my parents for their love, support and the many sacrifices that they have made for me to pursue my education. I also wish to thank my wife for her confidence, encouragements and continued support.

Last, but by no means the least, a very special vote of thanks goes to the Barnet Overseas Housing Association for providing excellent accommodation facilities.

Abstract

The roles of leukotrienes (LTs) in the mediation of specific cellular responses in immunity and inflammation are well established. Some authors have suggested that the endogenous generation of LTs may play a role in the regulation of the proliferation of both normal and malignant haemopoietic cells. I have therefore investigated effects of 5-lipoxygenase (LO) inhibitors on the proliferation of normal and malignant haemopoietic cells.

Piriprost, nordihydroguiaretic acid (NDGA), and BW755C, inhibitors of LO, inhibited DNA synthesis (as measured by the incorporation of [^{3}H] thymidine) in freshly isolated leukaemia blasts and in the leukaemia cell lines HL60, K562, and Jurkat. However, DNA synthesis and proliferation (as measured by counting trypan blue-excluding cells) of the above cell lines were not affected by the novel, highly specific LO inhibitors MK 886 and BWA4C in concentrations in excess of those required for substantial inhibition of leukotriene generation in whole blood. Furthermore, MK886 and BWA4C, did not impair DNA synthesis in PHA-stimulated T lymphocytes and normal haemopoietic cells.

The data suggest that endogenous leukotriene production does not play a role in regulating the proliferation of either normal haemopoietic cells or leukaemia cell lines.

I have also studied effects of LO inhibitors on mitogen-stimulated inositol lipid breakdown, one of the specific signal transduction pathways which regulate the proliferation of haemopoietic cells. Piriprost, NDGA, and BW755C inhibited

PHA- stimulated breakdown of inositol lipid in human T lymphocytes. However, inositol-lipid breakdown in PHA- and CD3 monoclonal antibody (McAb)-stimulated T lymphocytes and Jurkat leukaemia cells was not affected by the lipoxygenase inhibitors, BWA4C and MK886 in concentrations which were shown in parallel studies to substantially inhibit leukotriene generation in whole blood. Furthermore, leukotrienes generation (as measured by radioimunoassay) was not detected following PHA stimulation of T lymphocytes. These results imply that inhibition of mitogen-stimulated inositol lipid breakdown by NDGA, BW755C, and piriprost must be due to a mechanism other than inhibition of lipoxygenase. Because these LO inhibitors also have antioxidant properties, I studied the effects of several general antioxidants on growth regulatory signal transduction pathways.

Butylated hydroxytoluene (BHT), dithiothreitol, and N-acetylcysteine abrogated PHA- and CD3 McAb- stimulated inositol lipid breakdown in T lymphocytes. The effects of antioxidants on inositol-lipid breakdown were not attributable to irreversible damage to cellular structures or inhibition of PtdIns4,5-P_2-PLC. These observations imply that an antioxidant-sensitive step was obligatory in coupling cell-surface receptors to the inositol signalling system in T lymphocytes.

By contrast, antioxidants did not perturb PHA- or CD3 McAb- stimulated inositol lipid breakdown in Jurkat T acute lymphoblastic leukaemia cell line. Therefore, the biochemical mechanisms which couple cell-surface receptors to the inositol lipid signalling system are different in normal peripheral blood T lymphocytes and in Jurkat leukaemia cells.

Abbreviations

AA - arachidonic acid

AMP - adenosine 5'-monophosphate

APC - antigen presenting cell

BAB - bromophenacyl bromide

BHA - butylated hydroxyanisole

BHT - butylated hydroxytoluene

CD - cluster differentiation

cGMP - cyclic 3',5'-guanosine monophosphate

CO - cyclooxygenase

CSF-1 - colony-stimulating factor-1

DAG - diacylglycerol

DFO - desferrioxamine

DMSO - dimethyl sulphoxide

DTT - dithiothreitol

EGF - epidermal growth factor

FLAP - 5-lipoxygenase activating protein

G protein - guanine nucleotide-binding proteins.

GAP - GTPase activating protein

GM-CSF- granulocyte-macrophage colony-stimulating factor

GSH - glutathione

GST - glutathione-s-transferase

HBSS - Hanks balanced salts solution

HLA - human leukocyte antigen

HETE - hydroxyeicosanetraenoic acid

HPETE - hydroperoxyeicosatetraenoic acid

Ig - immunoglobulin

Ins 1,4,5-P_3 - inositol 1,4,5-triphosphate

IL-1 - interleukin-1

IL-2 - interleukin -2

IL-6 - interleukin-6

IP - inositol phosphate

IFN - interferon

kDa - kilodaltons

LFA - leukocyte function associated

LiCl - lithium chloride

LO - lipoxygenase

LT - leukotriene

LTA4 - leukotriene A4

LTB4 - leukotriene B4

LTC4 - leukotriene C4

LTD4 - leukotriene D4

LTE4 - leukotriene E4

McAb - monoclonal antibody

MHC - major histocompatibility complex

NADP - nicotinamide adenine dinucleotide phosphate

NDGA - nordihydroguiaretic acid

NO - nitric oxide

$O_2^{\cdot}$ - superoxide

PDGF - platelet-derived growth factor

PG - prostaglandins

PHA - phytohaemagglutinin

PKC - protein kinase C

PLC - phospholipase C

Ptd Ins 4,5-P_2 - phosphatidylinositol 4,5-biphosphate

PTK - protein tyrosine kinase

SOD - superoxide dismutase

TCA - trichloroacetic acid

TdT - terminal deoxynucleotide transferase

TCR - T cell antigen receptor

TLC - thin layer chromatography

TNF - tumour necrosis factor

Contents

CHAPTER 1

MECHANISMS OF MITOGENIC SIGNAL TRANSDUCTION IN T LYMPHOID CELLS.

The experiments described in this thesis were designed to evaluate the following: (a) The actions of lipoxygenase inhibitors and antioxidants on the proliferation of normal haemopoietic cells, malignant haemopoietic cell lines and freshly isolated leukaemia blasts; (b) Using T lymphocytes and the Jurkat T lymphoblastic leukaemia cell line as model systems, to investigate the mechanism of action of lipoxygenase inhibitors and antioxidants on a specific mitogenic signal transduction pathway viz. the inositol lipid signalling pathway. Therefore, the introduction to this thesis is divided into two sections. The first focuses on mitogenic signalling in T lymphocytes, and the second on oxidative processes and free radicals in biological systems, with an emphasis on the lipoxygenase system.

1.1 The Ontogeny of T lymphocytes

T lymphocytes are derived from the pluripotential stem cell (Wu et al 1968). Early T cell precursors do not express the membrane markers of mature T cells and their T- cell receptor genes are still in the unrearranged state (Shortman et al 1984).T cell progenitors migrate to the thymus where they undergo a series of differentiation steps under the influence of hormones produced in the

thymus including thymopoietin (Ezine et al 1984). The thymus is divided into cortical and medullary regions which each consist of epithelial cells and lymphocytes. In the cortex T lymphocytes undergo massive cell division and acquire both T cell functions and cell- surface markers characteristic of mature T cells.The cortical T lymphocytes contain terminal deoxynucleotide transferase and express CD4 (helper) and CD8 (cytotoxic T cell) antigens. The medulla contains mature T cells which exhibit the phenotypic characteristics of peripheral blood T lymphocytes.Here they also acquire CD3 surface antigen (Royer et al 1985).

1.2 Cell Surface Molecules Important in T Cell Activation

1.2.1 The T Cell Antigen Receptor (TCR) and CD3

The T cell antigen receptor (Fig.1) plays a central role in initiation of biochemical signals during T cell activation (Weiss et al 1987). It consists of two polypeptide chains, alpha (49 kDa) and beta (43kDa) linked to each other by disulphide bridges. Each chain has a transmembrane region and a cytoplasmic domain (Meuer et al 1983). The overall structure of these chains is very similar to that of the light chain of the immunoglobulin molecule, each having variable and constant regions.

The genes encoding the alpha and beta chains lie on chromosomes 14 and 7 respectively and are rearranged in T cells in a manner similar to the

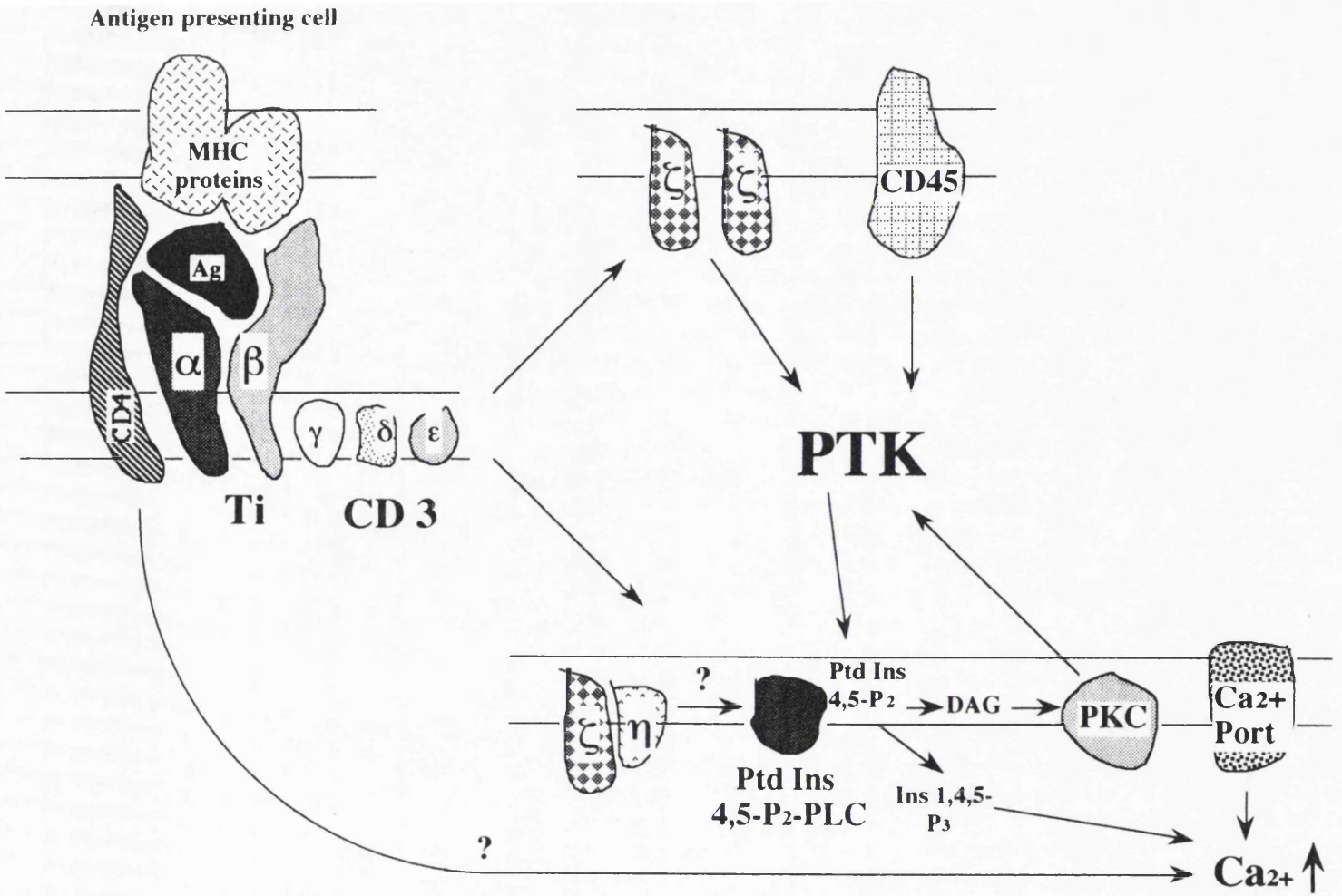


Figure 1 Model of T cell activation *via* the TCR
See text for details.

rearrangement of immunoglobulin genes in B cells, resulting in a wide diversity of specific antigen-recognizing structures (Barker et al 1984). In the germline configuration, the TCR beta gene comprises a set of alternative V (variable) regions,followed by a set of alternative D (diversity) regions and J (joining) regions and a C (constant) region. The beta gene additionally contains a second set of D,J, and C genes (Gascoigne et al 1984).

The overall organization of the alpha locus is similar but this gene contains only a single constant region ,multiple J segments, and no D segment (Caccia et al 1985). Clonotypic structures capable of specific recognition of antigen in association with products of the major histocompatibility complex (MHC), are generated by gene rearrangements which juxtapose individual V,D and J regions (Tonegawa 1983). Three hypervariable regions within the variable region of each of the two TCR chains form the antigen binding pocket (Wu et al 1970).

The non-clone specific CD3 complex is non- covalently associated with the TCR, and consists of gamma,delta, epsilon,zeta,and eta polypeptide chains (Clevers et al 1988). These chains are encoded by gene clusters located on chromosome 11 (except the zeta chain). These chains do not share structural homology with immunoglobulins. The CD3 polypeptides possess substantial intra-cytoplasmic domains which are probably important in transduction of signals derived from interaction of antigen-MHC with the TCR to the cell interior.

The zeta subunits have a unique structure,unrelated to the other polypeptides

of the CD3 complex and are encoded on a separate chromosome (Weissman et al 1988). The zeta subunits belong to a novel protein family that includes the gamma chain of the high affinity IgE receptor (FC epsilon RI) (Jin et al 1990). The probable function of the zeta dimers is to link the TCR to critical biochemical pathways involved in the activation of T cells (Irving et al 1991; Frank et al 1990) . However,TCR/CD3 complexes having intact gamma,delta, and epsilon chains but devoid of the zeta chain secrete IL-2 in response to antigenic stimulation (Wegener et al 1992), suggesting the existence of at least two parallel transducing units,one composed of gamma,delta,and epsilon subunits and the other a dimer of zeta chains.

A small percentage (3-10%) of peripheral blood T cells express a gamma-delta TCR cell complex instead of alpha-beta heterodimers (Koning et al 1987). The structure of the gamma chain is essentially similar to that of the other TCR chains in containing V,J, and C elements (Hayday et al 1985). The delta chain also contains an immunoglobulin like extra-cellular domain (Gold et al 1987). T cells expressing these chains exhibit MHC restriction (Matis et al 1987).

1.2.2. Proteins encoded by the major histocompatibility complex (MHC) and functions of CD4 and CD8 molecules:

Several other surface molecules also play important roles in efficient T cell antigen recognition and activation. The CD8 and CD4 glycoproteins bind to MHC class I and class II molecules on antigen presenting cells(APC)

respectively, and facilitate antigen recognition by T cells (Marrack et al 1986). The human major histocompatibility complex (MHC) is located on the short arm of chromosome six. It comprises five closely linked genes designated as HLA genes: HLA-A,B,C,D, and HLA-DR. The MHC- encoded proteins are glycoproteins of two sub-types. Class I molecules are encoded by HLA A,B, and C genes and are expressed by most somatic cells . The class I molecules consist of two chains; a 43 kDa polymorphic transmembrane glycoprotein and 12 kDa nonpolymorphic protein, beta2-microglobulin which is encoded outside the HLA region on chromosome 15 (Bjorkman et al 1987).

Class II antigens are also glycoproteins and are encoded by genes in the D region. There are at least three class II regions: HLA-DP,HLA-DQ,HLA-DR.They are expressed primarily on antigen presenting cells and B lymphocytes. The MHC encoded glycoproteins function in presenting antigen to specific T lymphocyte subsets(Engleman 1981). CD4 helper lymphocytes recognize antigen in the context of MHC class II molecules, whereas CD8 cytotoxic cells bind antigen associated with class I structures. The CD4 and CD8 molecules bind to non- variant domains of the class II and class I molecules respectively, and this binding initiates the generation of a co-signal which synergizes with that generated by the binding of the polymorphic domain of the MHC molecule, in association with the TCR complex (Marrack et al 1988).The capacity of T cells to recognize antigen only in the context of self MHC molecules is acquired in the thymus (MHC restriction). T cells reactive

with self antigens are destroyed in the thymus. The cytoplasmic domains of the CD4 and CD8 molecules are highly conserved and may be involved in transmembrane signal transduction. These molecules share homology with other receptor molecules encoded by genes within the immunoglobulin supergene family (Littman et al 1987). CD8 is a 32 kDa glycoprotein (Snow et al 1983), whose amino terminus closely resembles the variable region of the kappa light chain. CD8+ cells play a role in T cell- mediated cytotoxicity (Swain et al 1981), their main function being to recognize and kill virally infected cells.These cytotoxic T cells can recognize and lyse only those virus infected cells that display viral antigens in association with self MHC class I molecules.

MHC class II molecules are involved in specific antigen recognition by CD4+ helper T cells (Doyle et al 1987). The CD4 molecule is a 55 kDa cell surface glycoprotein (Terhorst et al 1980), whose extra-cellular domain binds to non-polymorphic regions of MHC class II molecules on antigen presenting cells(APC) independently of the TCR (Gay et al 1986). This probably promotes adhesion between CD4+ T cells and APC (Marrack et al 1983). It is possible to disrupt this adhesion using anti -CD4 or anti- MHC II antibodies (Gay et al 1987).

1.2.3 Leucocyte function-associated (LFA) molecules

The leucocyte function associated (LFA) molecules comprise a family of glycoproteins that play important roles in cell adhesion (Martz et al 1987).

Three such molecules identified as important in T cell binding to APC are designated LFA-1, LFA-2(CD2) and LFA-3.Interaction between LFA-1 and its ligand (CD54) facilitates antigen recognition and plays a critical role in mediating adhesion between T cells and antigen presenting cells.

Ligation of the LFA-2 (CD2) Ag has also been shown to be capable of stimulating T cells (Meuer et al 1984). The CD2 Ag is a receptor like structure (Sayre et al 1987).

Phytohaemagglutinin(PHA) probably stimulates T cells at least in part by interacting with CD2 (^{O}'Flyn et al 1986). CD2 facilitates cell-cell adhesion by binding to LFA-3, a surface glycoprotein expressed on connective tissue-cells, endothelial cells, leucocytes and red blood cells. Antibodies directed against LFA-3 inhibit cytotoxic T cell mediated killing by binding to LFA-3 on target cells, thus blocking binding of CD2 to LFA-3.

1.2.4 The CD28 molecule

CD28 is a homodimer expressed by T cells. Molecular cloning of this T cell surface molecule revealed that it too belongs to immunoglobulin gene super-family (Aruff et al 1987).

CD28 has been shown to mediate cell-cell adhesion by binding to its co-receptor the B7/BBI molecule (Springer et al 1987). B7/BBI transduces signals to T cells through the CD28 molecule (Linsley 1991; Bierer et al 1985).

1.3 Protein tyrosine kinases(PTKs) and the regulation of cell proliferation.

Protein phosphorylation is a well established mechanism for the rapid and reversible regulation of enzyme activity (Krebs 1986). Protein kinases and phosphatases respectively add phosphate residues to or remove them from serine/threonine residues of proteins (Edelman et al 1987) or tyrosine residues (Hunter et al 1985). Protein tyrosine kinases(PTKs), are classified into receptor and non-receptor kinases (Hanks et al 1985). The extra-cellular glycosylated domains of the receptor kinases bind specific ligands.Ligand binding by these receptors stimulates the intracellular kinase domain with the consequent activation of various second messenger systems (Yarden et al 1988).

Many growth factors receptors including the receptors for epidermal-growth factor(EGF),platelet-derived growth factor (PDGF), colony-stimulating factor-1 (CSF-1) and insulin possess an intracellular protein tyrosine kinase whose activity is stimulated following ligand binding (Yarden et al 1988). The c-kit proto-oncogene encodes a trans-membrane tyrosine-kinase, which shares close homology with the CSF-1 and PDGF receptor kinases (Yarden et al 1987; Ullirich 1986). The ligand for the c-kit receptor has been cloned. It is expressed and secreted by fibroblasts, and is believed to play an important role in the regulation of proliferation of early haemopoietic cells.(Nocka et al 1990).

Some important cellular targets regulated by PTKs include phospholipase C, the ras GTPase-activating protein (GAP), phosphatidylinositol 3-kinase and the serine/threonine protein kinase encoded by the c-raf protooncogene (Samelson et al 1986; Cockroft et al 1984; Baniyash et al 1988).

The non receptor PTKs are smaller intra-cellular enzymes associated with receptors which are themselves devoid of kinase activity(e.g; p56 lck linked to CD4 and CD8) or with kinase receptors e.g association of the PDGF receptor with a member of the src family tyrosine kinase (Veillette et al 1988; June et al 1990).

1.3.1 The role of PTKs in T cell proliferation

T cells express several PTKs including those encoded by the c-src, lck and c-fyn genes. Tyrosine phosphorylation of the CD3 zeta chain and serine phosphorylation of the CD3 polypeptide gamma and to a lesser extent epsilon are rapid consequences of binding of mitogenic anti TCR/CD3 monoclonal antibodies(McAbs) to the TCR complex (Patel et al 1987). TCR stimulation also results in serine phosphorylation of c-src (Ledbetter et al 1988). Tyrosine phosphorylation of the CD3 zeta chain and other proteins probably play a crucial role in T cell activation and proliferation as illustrated in Fig.1 (Samelson et al 1986; Baniyash et al 1988). Increased tyrosine phosphorylation of multiple proteins has been observed following TCR

stimulation (June et al 1990).

The 56 kDa PTK encoded by the lck proto oncogene ($p56^{lck}$) is the most likely candidate as enzyme responsible for the phosphorylation of the zeta chain (Marth et al 1985; Voronova et al 1986). Transfection experiments using point-mutated lck have suggested that the lck gene product probably plays a role in regulation of cell proliferation and differentiation. Veillette et al (1988) have shown that $p56^{lck}$ is non- covalently but tightly associated with the cytoplasmic domain of both CD4 and CD8 molecules. This association may underlie the mechanism by which ligation of the TCR is coupled to tyrosine phosphorylation events (Weissman et al 1989).Recently the $p^{59c\text{-}fyn}$ kinase, a member of the src family of PTKs, has been proposed as another candidate for the TCR coupled PTK (Volarevic et al 1990).

The mechanism by which $p56^{lck}$ or $p59^{c\text{-}fyn}$ are activated via the TCR remains elusive. Therefore, the existence of other molecules playing a role in TCR signal-transduction can not be ruled out.For example the cytoplasmic domain of the CD45 molecule present on T cells contains tyrosine phosphatase activity . Signalling through the TCR is impaired in Jurkat cells lacking the CD45 molecule. In addition, the CD45 molecule can associate with CD4 and the TCR complex (Volarevic et al 1990), forming a stable complex in mitogen- primed T cells.

Ligation of the CD4 molecule stimulates Ca++ mobilization (Rosoff et al 1987), and activation of tyrosine kinases including p^{56lck}. These PTKs induce in turn the tyrosine phosphorylation of specific cellular substrates (Veillette et al 1989).

The cytoplasmic domain of the CD4 molecule contains serine and threonines residues, one or more of which are phosphorylated upon exposure of T cells to specific antigen or treatment with phorbol ester.Neither CD4 nor CD8 possess PTK domains within their cytoplasmic tails (Maddon et al 1985). Stimulation of CD2 also stimulates a rise in intracellular Ca^{++} and induction of mitosis (Weiss et al 1984). CD2 cross-linking induces tyrosine phosphorylation in T cells(Samelson et al 1990) , but the mechanism is unclear.

The proto-oncogene vav encodes a protein with transcription regulatory functions. It is expressed in haemopoietic cells and its gene product p95vav contains multiple structural motifs, including the SH2 domain (for src-homologous domain 2). SH2 is a common non-catalytic domain possessed by several proteins implicated in the regulation of cellular responses to mitogenic stimuli. This domain mediates their interaction with activated tyrosine protein kinases. Stimulation of the T cell antigen receptor on normal human peripheral blood lymphocytes and leukaemic T cells results in phosphorylation of tyrosine residues of vav (Margolis et al 1989). All of these observations further consolidate the evidence that the tyrosine phosphorylation plays an important role in the regulation of T cell proliferation.

1.4 Guanine nucleotide-binding proteins(G Proteins)

Numerous cell-surface receptors are coupled to second messenger-generating enzymes (effector enzymes) via guanine nucleotide-binding proteins (G

proteins)(Birnbaumer et al 1990). Classical G proteins involved in signal transduction comprise a family of heterotrimers, consisting of alpha, beta, and gamma sub-units (Simon et al 1991; Neer et 1988). The beta and gamma sub-units are tightly associated with each other. The alpha sub-unit is specific to each G protein, and has characteristic properties of binding with guanine-nucleotide and interactions with specific receptors and effectors. More than 16 members of the G protein super-family have been identified. Three G proteins have been particularly well characterized: Gs, Gi(see below) and transducin, which controls cGMP phosphodiesterase activity in retinal-rods(Spiegel et al 1987). The best studied system involves the control of adenylate cyclase: the two G proteins, Gs(stimulatory) and Gi (inhibitory) couple receptors to adenylate-cyclase. Following receptor stimulation activation of Gs occurs when GDP bound to the alpha sub-unit of the heterotrimer is exchanged for GTP. The resulting Gs alpha-GDP dissociates from the beta - gamma complex and activates adenylate cyclase. The alpha sub-unit possesses an intrinsic GTPase activity which hydrolyzes GTP to GDP. Consequently the G alpha-GDP complex formed re-associates with beta-gamma sub-units to form the inactive heterotrimer(Bourne et al 1990).

Signalling via Gi involves an analogous mechanism, but the generation of free Gi alpha results in inhibition of adenylate cyclase.

Cholera and pertussis toxins have been used to elucidate the role of G proteins in signal transduction pathways. Cholera toxin modifies an arginine residue at position 201 of Gs.,thus reducing the intrinsic GTPase activity of Gs alpha,

resulting in continuous activation of adenylate-cyclase.

The functional activities of the ras-encoded protein bear some similarities to those of the classical G proteins (Hurley et al 1984). All the three members of the ras gene family i.e; H-ras, K-ras and N- ras encode 21 kDa proteins (p21), which are located on the inner side of the plasma-membrane, bind to guanine-nucleotides and possess weak intrinsic GTPase activity (Chang et al 1982).However, this GTPase activity is stimulated by a separate GTPase activating protein (GAP). GAP is a target for phosphorylation by PTKs, and this may provide a link between PTKs and ras proteins. In fact activation of the TCR leads to increases in the amount of ras in the GTP bound state (Downward et al 1990).The effector systems regulated by the p21ras protein have not been identified.

1.5 Phospholipase C(PLC)

Eukaryotic cells express a family of phospholipase C (PLC) enzymes highly specific for inositol lipids. Their activation results in the hydrolysis of a rare membrane lipid phosphatidyl inositol 4,5-biphosphate(PtdIns-4,5-P_2) resulting in the generation of two potent second messengers inositol 1,4,5- trisphosphate(Ins 1,4,5-P_3) and diacylglycerol (DAG) (Dougherty et al 1984). Multiple iso-enzymes of PLC have been described (Fain et al 1990). PLC beta is stimulated mainly via G proteins, while the activity of PLC gamma is regulated by tyrosine phosphorylation. Therefore, the stimulation of PTKs

following ligand binding is potentially able to regulate the crucial second messenger pathway triggered by PLC (Mustelin et al 1990).

In both neutrophils and platelets, G proteins are involved in receptor- mediated activation of PLC (Cockcroft et al 1984; Brass et al 1986).

Introduction of the ras oncogene which encodes a permanently activated form of p^{21ras}, into fibroblasts results in increased inositol lipid turnover (Fleischman et al 1987). This increased turnover can be blocked by injecting antibodies against Ptd Ins 4,5-P_2 (Fukami et al 1988). Although these observations suggest that GTP-bound ras can activate PLC, no direct link between the ras oncogene product and PLC has been demonstrated to date.

1.6 The biochemistry of the inositol-lipid signalling pathway

The binding of many growth factors to their receptors triggers the inositol lipid signalling pathway (Fig 2). Stimulation of PtdIns-4,5-P_2 specific phospholipase C results in the generation of diacylglycerol(DAG) and inositol 1,4,5-triphosphate(Ins 1,4,5-P_3) (Berridge et al 1985). Ins 1,4,5-P_3 is degraded via inositol bisphosphate (IP2), inositol monophosphate (IP) and inositol. The reaction of inositol with cytidine monophosphate-activated phosphatidic acid results in the resynthesis of phosphatidylinositol(PtdIns) , as shown in Fig 2.

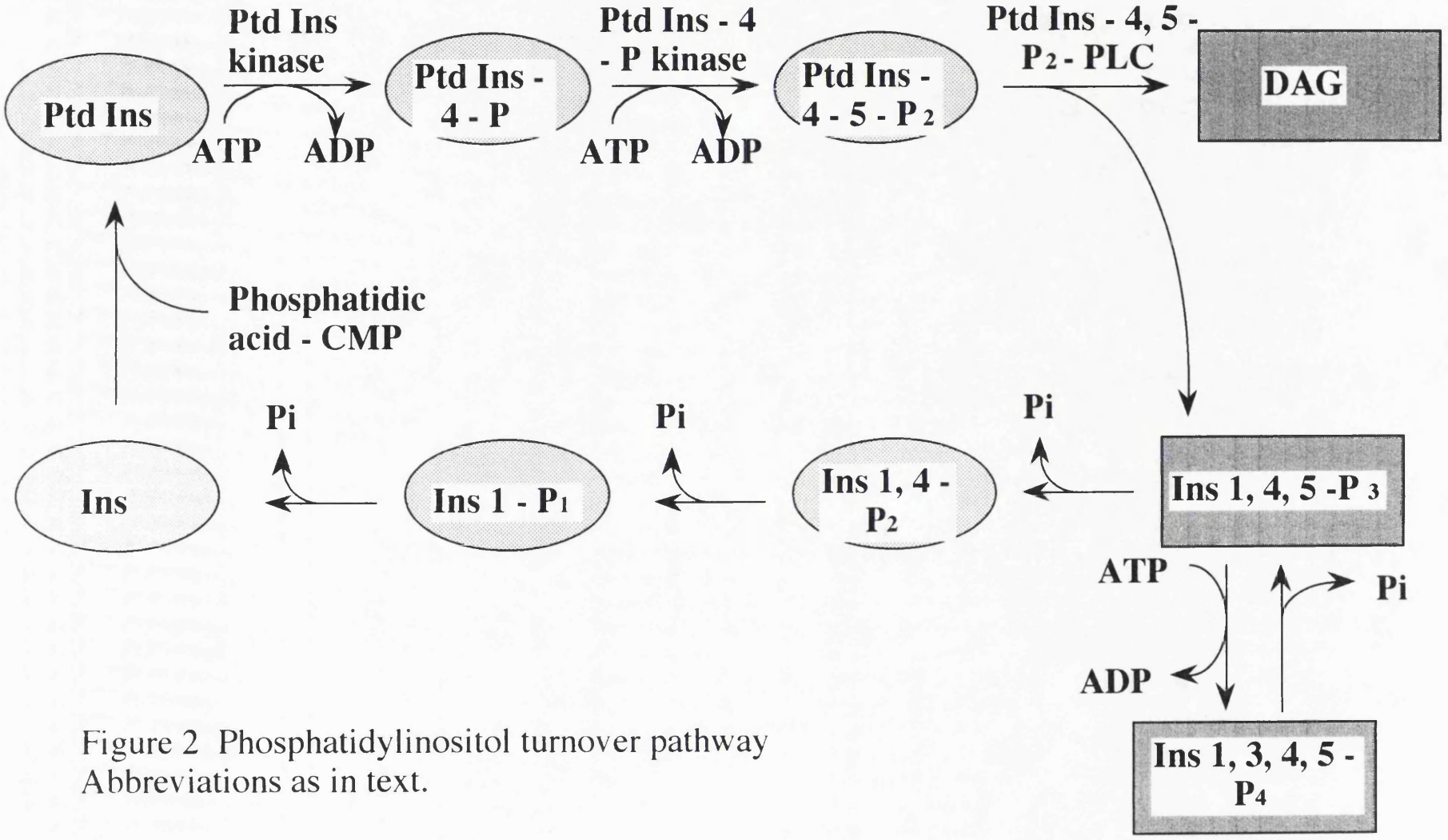


Figure 2 Phosphatidylinositol turnover pathway
Abbreviations as in text.

This pathway first described by Hokin in 1953, has been identified as a signalling mechanism in numerous cellular responses including secretion, contraction, metabolism and cellular proliferation (Majerus et al 1985). Most importantly it is believed that increased inositol lipid turnover is necessary for transformation by certain oncogenes and mitogenic stimulation by growth factors (Kaplan et al 1987; Coughlin et al 1989).

1.7 The second messenger roles of DAG,Ins 1,4,5-P_3 and inositol 1,3,4,5,tetrakisphosphate(Ins 1,3,4,5-P_4)

DAG and Ins 1,4,5-P_3 are potential proliferation-regulating second messengers in T cells (Imboden et al 1985). DAG is a neutral lipid which remains in the cell membrane and activates the serine/threonine kinase, protein kinase C (PKC) (Berridge et al 1987). Protein kinase C has been implicated in the regulation of diverse cellular functions. Following the binding of DAG, PKC shows an increased affinity for phospholipids and Ca^{++}, cofactors required for activation of the enzyme (Nishizuka 1984). In subsequent reactions, DAG can either be phosphorylated to phosphatidic acid or deacylated by a diglyceride-lipase, yielding monoglyceride and arachidonic-acid.

Ins 1,4,5-P_3 is a water soluble compound which binds to specific sites on the endoplasmic reticulum consequently triggering the release of stored Ca^{++} (Nishizuka,1986). Ins 1,4,5-P_3 can be phosphorylated by a specific kinase, giving rise to inositol 1,3,4,5-tetrakisphosphate(Ins 1,3,4,5-P_4). In concert with Ins

1,4,5-P_3, Ins 1,3,4,5-P_4 regulates the entry of extracellular Ca^{++}, thereby prolonging the Ca^{++} signal initiated by inositol lipid hydrolysis (Irvine et al 1986). The elevation of cytosolic Ca^{++} levels is an important component of mitogenic signalling pathways,and results in the activation of Ca^{++}-dependent enzyme systems (Irvine et al 1986).

1.8 The protein kinase C (PKC) family

PKC belongs to a family of protein kinases which phosphorylate proteins on serine and threonine residues, thus altering their functional activities (Nishizuka 1988). Certain mitogens, e.g phorbol esters can directly stimulate PKC, thereby short-circuiting ligand- induced pathways. The simultaneous elevation of Ca^{++} and activation of protein kinase C modulate diverse cellular functions including activation of inflammatory cells, secretion, smooth muscle contraction and cell-division. (Nishizuka 1988). Several iso-forms of PKC with different catalytic and/or regulatory properties have been identified including alpha,beta 1,beta 2, gamma, delta,epsilon,zeta and eta. T cells contain the alpha and beta iso-enzymes, the level of the beta iso-enzymes being slightly higher compared to that of the alpha iso-enzyme (Shearman et al 1988).

Genes encoding the different iso-enzymes of PKC have been cloned (Parker et al 1986) . The iso-enzymes alpha, beta and gamma are composed of four homologous domains (C1-4),C1 and C2 domains are unique for these iso-enzymes and have been implicated in the Ca^{++} dependent activation of PKC

(Schaap et al 1989), while the C1 domain have been shown to bind phorbol ester .The C2 domain of PKC shares structural homology with related regions in GAP (Vogel et al 1988), and phospholipaseA2(PLA-2) and may be involved in the translocation of PKC to the cell membrane (Clark et al 1991).

1.9 The inositol lipid signalling pathway in T cell activation

Several changes in membrane phospholipids have been observed in response to stimulation of the TCR by lectin (Wickremasinghe et al 1987; Hasegawa-Sasaki 1983), antigen (Patel et al 1987),or anti TCR/CD3 Mc Abs (Imboden 1985). Perturbation of the TCR/CD3 complex results in activation of PLC, with consequent hydrolysis of PtdIns-4,5-P_2 and generation of Ins 1,4,5-P_3 and DAG. DAG elevation results in the translocation of PKC from the cytoplasm to the plasma membrane (Farrar et al. 1986), where it catalyses phosphorylation of membrane protein substrates. The serine phosphorylation of CD3 gamma chain has been shown to be mediated by PKC (Cantrell et al 1985). The central role of the inositol lipid signalling pathway in T cell activation is emphasized by experiments in which direct stimulation of PKC by phorbol esters has been shown to synergise with elevation of Ca^{++} levels by ionophores in by-passing receptor stimulation in mitotic activation (Truneh et al 1983).

1.10 Mechanism of TCR-induced inositol lipid turnover in T lymphocytes

The precise mechanism by which ligation of the TCR/CD3 complex triggers inositol lipid turnover has not been elucidated. Early evidence suggested that stimulation of the TCR activated inositol lipid turnover via coupling to guanine-nucleotide binding protein (G protein) (Sassaki et al 1987, O,Shea et al 1987). Cholera toxin inhibited the TCR mediated increase in inositol lipid turnover and cytoplasmic Ca^{++} (Imboden et al 1986), also suggesting a role for a G protein in this coupling. However, no direct evidence implicating guanine nucleotide-binding proteins in T cell signalling pathways has been reported.It has been shown that tyrosine phosphorylation activated PLC gamma in T cells. Stimulation of the TCR failed to stimulate inositol lipid turn-over in the presence of inhibitors of tyrosine kinases (Mustelin et al 1990; June et al 1990). Transient activation of PLC was observed following activation of CD2, CD5, or CD28 with specific McAb(Ledbetter et al 1987).However, activation of PLC by these events depended upon the prior initiation of tyrosine phosphorylation. The CD4 and CD8 molecules are non-covalently associated with P^{56lck} and the association of CD4 or CD8 with the TCR also augments the activity of PLC (Ledbetter et al 1988). Increased tyrosine phosphorylation of multiple proteins was detected as early as 5 seconds after stimulation of the TCR, while stimulation of inositol lipid turnover was seen only after a lag period of 30-40 seconds (June et al 1990). All of these observations place PTK

activation as a proximal event to inositol lipid turnover, and suggest that the former may regulate in turn the latter process.

1.11 The regulation of gene expression during T cell activation

The early biochemical events described above are followed by increased transcription of the proto-oncogenes c-myc, c-fos,c-myb and the alpha subunit of the interleukin 2 (IL2) receptor (Linch et al 1987). The precise mechanisms involved in conveying signals to the nucleus are not fully understood. T cells normally require both the activation of antigen receptor and protein kinase C for IL2 gene expression (Rosenstreich et al 1979, Farrar et al 1986). IL2 gene expression and proliferation of the T cell can be initiated in the absence of phosphoinositide hydrolysis or increases in intracellular calcium (Susaman et al 1988; Truneh et al 1985). The induction of c-fos mRNA was seen by 10-15 minutes following mitogenic stimulation, and the elevated level persisted for 2-3 hours with a subsequent decline to the pre-stimulation level (Moore et al,1986). The protein encoded by the c-fos gene (P59c-fos) dimerizes with the product of the c-jun gene to form the transcription control factor AP-1 (Bohmann et al 1987). The IL2 gene contains a binding site for AP-1, suggesting that c-fos may play a role in the activation of the IL2 gene. The induction of c-myc mRNA follows mitogenic stimulation by about 30 minutes, with a maximal level at 2-10 hours with a subsequent decline. Inhibition of the activation of the c-myc

gene using antisense oligonucleotides results in delayed entry into S phase (Heikkilla et al 1987). The precise function of c-myc has yet to be delineated but these observations imply that it too may contribute to entry into S phase during T cell activation(Kelly et al 1983). In fact, the c-myc-encoded protein also plays a role in the control of transcription (Studzinski et al 1986). The association of c-myb transcription with DNA synthesis suggests that expression of this proto-oncogene is probably also required for entry into S phase.Control of the transcriptional activation of c-jun,c-fos and c-myc is complex, but it is clear that the activation of PKC and elevation of Ca^{++} can play important roles in their regulated expression.

In summary, the available data suggest that early biochemical signals generated following the ligation of cell-surface molecules results in the induced expression of a set of genes which encode transcription control factors (Bohmann et al 1987). and that these factors may induce in turn the expression of other genes involved in cell cycle progression.

1.12 Interleukin 1 (IL1) and T cell activation

At least two signals are required for the activation of the resting T cell. Signal 1 is provided by the activation of TCR/CD3 complex and associated cell-surface proteins. This primes the T cell to signal 2 which is provided by interleukin 1(IL1) released by antigen presenting cells (Shirakawa et al 1988). IL1 in turn triggers IL2 synthesis. Interaction of IL2 with its receptor then secures

commitment of the T cell to S phase entry (Cantrell et al 1984).

1.13 Structure of the IL-2 receptor

The cell surface receptor for IL2 consists of an alpha chain (55kDa) and beta chain (75kDa) which are not covalently linked (Dukovich et al 1987). The alpha chain has a low affinity for IL2, which binds and dissociates from it rapidly. However, this interaction between IL2 and the alpha chain facilitates the binding of IL2 with the beta chain from which it dissociates more slowly thus generating a high affinity receptor. The presence of adequate number of high affinity receptors is essential for the mitogenic action of IL2 (Sharon et al 1986; Tsudo et al 1986)

1.14 Mechanism of IL-2 signal transduction

The biochemical mechanism of IL-2 signal transduction is incompletely understood. There is no evidence to implicate inositol lipid breakdown in IL2 signal transduction (Gelfand et al,1987; Wickremasinghe et al 1987). IL2 stimulates generation of cAMP (Wickremasinghe et al 1987) suggesting a possible role for cAMP-dependent kinases in the transduction of IL2 signals. The IL2 receptor does not possess protein kinase activity. Nevertheless binding of IL2 to its receptor results in phosphorylation of proteins on serine and threonine residues (Mire-Sluis et al 1985, Ishi et al 1987). Recent evidence also

suggests that binding of IL2 to its receptor also triggers rapid tyrosine phosphorylation events catalysed by an unidentified PTK(Fung et al 1991)

1.15 The role of other cytokines in T cell activation:

In addition to IL2, number of cytokines play a role in the growth modulation of the T cell including IL4,IL6, tumour necrosis factor(TNF),and interferon (IFN) gamma.Interaction of IL4 and IL6 with cognate receptors on activated T cells augments the proliferative effects of IL2 (Houssiau et al 1988). Gamma interferon appears to be involved in the mediation of several immunological activities e.g induction or enhancement of expression of both class I and class II antigens of the major-histocompatibility-complex. It also appears to be of central importance in enhancing specific cytotoxic T cell activity (Johnson et al 1982; Torres et al 1982).

In summary, the available data suggest that mitogenic stimulation via ligation of cell-surface molecules on T lymphocytes may use some of the signal transduction pathways commonly used by other cell types, e.g; tyrosine phosphorylation, activation of the inositol lipid pathway, protein kinase C, Ca^{++} elevation and the activation of genes including c-fos,c-myc and c-myb. However, the precise mechanisms involved in coupling cell-surface stimulation to early biochemical events have remained elusive. This is due in part to the complexity and variety of cell-surface structures involved in mitogenesis, and because the

PTKs of T cells are not an integral part of the receptors. Some observations have suggested that anti-oxidants, free-radical scavengers and lipoxygenase inhibitors interfere with T lymphocyte activation (Chaudhri et al 1986; Sekkat et al 1988; Novogrodsky et al 1982). I have therefore focused on studying the actions of these agents on mitogen-stimulated activation of the inositol lipid signalling pathway of T lymphocytes.

CHAPTER 2

THE ROLE OF FREE RADICALS AND RADICAL-MEDIATED PROCESSES IN BIOLOGY.

2.1 Free-radical chemistry

A free radical is defined as a species having one or more unpaired electrons in an outer orbital and which is capable of independent existence. As electron pairs are more stable than unpaired electrons, the presence of unpaired electrons confers a high degree of reactivity on radical species i.e. by readily accepting electrons from or donating them to other species, thus initiating a chain reaction of free radical generation . Interaction between two radical species terminates the chain reactions giving rise to non-radical products.

2.1.1 Oxygen-derived radicals

Molecular oxygen is a biradical which normally undergoes a four electron reduction to water. However, the presence of intracellular oxygen also allows the inadvertent production of partially reduced toxic intermediate oxygen species. Addition of one electron gives superoxide ($O_2^{\cdot-}$),the dot denoting an unpaired electron.It is generated either directly during auto-oxidation in mitochondria, or enzymatically by cytoplasmic enzymes such as xanthine

oxidase, cytochrome P450, and other oxidases. Once produced,superoxide can be inactivated either spontaneously or, more rapidly, by the enzyme superoxide dismutase(SOD), forming H_2O_2.

2.1.2 Generation of hydroxyl-radicals($OH^{\cdot}$)

Superoxide possesses a high capacity to reduce transition metals such as iron and copper, resulting in the formation of more aggressive hydroxy-radicals, as described in the following reactions.

a. Generated in the reaction of ferrous iron with H_2O_2, the Fenton reaction:

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + {\cdot}OH + OH^-$$

b.Through the Haber-Weiss reaction

$$H_2O_2 + O2^{\cdot-} \longrightarrow {\cdot}OH + OH^- + O_2$$

These radicals show a high degree of reactivity towards many types of molecules found in living cells including phospholipids, nucleotides, and amino-acids (Black et al 1987). Hydroxyl-radicals react with unsaturated fatty acids of membrane phospholipids to generate organic acid free radicals, which in turn react quickly with oxygen to form peroxides. Peroxides thus generated then also act as free radicals, triggering auto-catalytic chain reactions which result in further membrane damage (Halliwell and Gutteridge. 1985a).

Interactions with DNA induces mutations , which, if not repaired result in DNA damage and inhibition of DNA replication(Imlay et al 1988).

2.2 Detoxification of free radicals

Several systems contribute to the termination of free radical reactions. These include:-

a. Endogenous or exogenous-antioxidants e.g., vitamin E, serum proteins, ceruloplasmin,transferrin, sulfhydryl- containing compounds such as cysteine and D-pencillamine.

b. Enzymes. Catalases and glutathione-peroxidases play important roles in controlling the level of superoxides produced during normal metabolism. Glutathione (GSH) is the main intracellular defence against oxidative stress.It is a cysteine-containing tripeptide(gamma-glutamyl-cysteinyl-glycine) which maintains protein sulphydryl redox status. Glutathione detoxifies peroxides which are reduced to the corresponding hydroxyl compound with concurrent oxidation of the glutathione co-factor. This is regenerated by the enzyme glutathione reductase, which utilises NADPH derived the from hexose monophosphate shunt as a co-factor.

$$2OH^{\cdot} + 2\ GSH \longrightarrow 2H_2O + GSSG$$

Glutathione-s-transferase (GST) detoxifies peroxides as well as such products as conjugated dienes and epoxides by forming a glutathione-S-conjugate which is further metabolised and excreted in urine. In this reaction the glutathione co-factor is irreversibly consumed.

2.3 Mechanisms of regulated free radical generation in biological systems

The regulated generation of free radicals play an important role in several cellular mechanisms. Some of these are summarized below.

2.3.1 The oxidative burst of leucocytes

Activated leucocytes produce large quantities of reactive oxygen metabolites, including H_2O_2, $O^{\cdot}$,$OH^{\cdot}$ and hypochlorous acid. These reactive oxygen metabolites are generated by NADPH-dependent oxidases. These oxidases are comprised of cytochrome (Segal et al 1987), flavoprotein and ubiquinone (Crawford et al 1982).

2.3.2 The generation of nitric oxide (NO)

Nitric-oxide is synthesized from L-arginine by a number of tissues including the vascular endothelium(Palmer et al 1988), platelets (Radomski et al 1990), neutrophils (Salvemini et al 1989), mast-cell (Salvemini et al 1990), nervous tissue (Bult et a 1990; Garthwaite et al 1988; Collier 1989) and T lymphoid cells (Kirk et al 1990). Nitric-oxide is generated by the oxidation and cleavage of the terminal guanido nitrogen-atom of L-arginine (Moncada et al 1989). Recent evidence suggests that nitric-oxide acts as a chemical messenger

between and within cells.It is a highly lipophilic compound (Ignarro et al 1987), having a half life of a few seconds. After release, it is rapidly oxidized to nitrite and nitrates. It acts by stimulating guanylate cyclase, with consequent elevation of cGMP (Feelisch et al 1987). Within the vascular endothelium, nitric oxide accounts for the biological activity of the vasodilator endothelium derived relaxing factor (Palmer et al 1987), and its main action seems to be the dilatation of blood vessels (Furchgott et al 1990). In platelets, nitric-oxide act as a second messenger regulating platelet function (Radomski et al 1990).It inhibits platelet aggregation (Furlong et al 1987) and adhesion (Radomski et al 1990) with the vessel wall. Nitric-oxide produced by macrophages in response to cytokine activation is involved in macrophage cytotoxicity (Hibbs et al 1988; Steuhor et al 1989).

2.3.3. The role of free radicals in the expression of nuclear transcription control factors NF-kB and AP-1.

The ubiquitous transcription control factor NFkB regulates the expression of a variety of genes, including those encoding several cytokines (Baeuerle et al 1988). Physiological activation of NFkB occurs following the binding of tumour necrosis factor or IL1 to their cell-surface receptors. In unstimulated cells, NFkB exists as an inactive complex bound to an inhibitor protein, IkB. Schreck et al (1991) have shown that the activation of NFkB, which is dependent on its dissociation from IkB, is mediated by a signalling process dependent on the

generation of reactive oxygen species. The molecular details involved in this intriguing mechanism have not been elucidated (Staal et al 1990).

The transcriptional activity mediated by the AP-1 binding factor is also regulated by redox mechanisms. Oxidation with diamide inhibited the DNA-binding activity of AP-1. It has been suggested that cysteine residues of the AP-1 polypeptides are important for DNA binding and that their reduction was required for association with DNA (Abate et al 1990)). Recent evidence suggest that IL2-induced signal involves the activation of nuclear NF-kB expression (Arima et al 1992).

2.3.4 Ribonucleotide reductase

Ribonucleotide reductase catalyzes the generation of deoxyribonucleotides required for DNA synthesis This enzyme requires non haem iron as a cofactor (Hoffbrand et al 1976), and also utilizes a tyrosine-based free radical intermediate that is essential for its activity (Reichard et al 1983).

2.4 Eicosanoid metabolism

Free radicals are generated as intermediates during the oxidation of arachidonic acid to prostaglandins and leukotrienes. The generation of these eicosanoids and

some of their biological roles are considered below.

2.4.1. Generation of arachidonic acid(AA)

Arachidonic-acid is a 20- carbon polyunsaturated fatty-acid containing four double bonds (5,8,11,14-eicosatetraenoic-acid). It is normally esterified in membrane phospholipids particularly at the Sn2 positions of phosphatidylcholine and phosphatidylinositol. Various stimuli trigger the release of arachidonic-acid from these phospholipids via the activation of phospholipase A2 (PLA2).PLA2 exists in cytosolic (cPLA2) and secreted(sPLA2) forms. There is substantial evidence that ligand- induced arachidonic acid release is mediated through cPLA2 (Jelsema et al 1987). The gene encoding cPLA2 has been cloned. Examination of its sequences shows that it does not share structural homology with the secreted form (Clark et al 1991). The secreted form($sPLA_2$) contains seven disulphide bridges (which are absent in cPLA2) and would therefore be unlikely to be functional in the reducing environment of the cytosol (Davidson et al 1990).

Structural analysis of cPLA2 has revealed that it possesses a Ca^{++} dependent phospholipid binding domain which shares close homology with similar domains present in PKC,P^{56}, and GAP. The activities of cPLA2 are Ca^{++} dependent,and the increase in Ca^{++} concentrations in response to ligand stimulation results in the association of cPLA2 with the plasma membrane (Channon et al, 1990; Yoshihara et al 1990) where it hydrolyses arachidonic

acid- containing phospholipids. The activation of PLA2 may be mediated via a G protein (Burch et al 1986). However, G proteins specifically coupled to PLA2 have not been characterized. Studies using cholera toxin and pertussis toxin have revealed that multiple G proteins may be linked to PLA2 (Jelsema, 1987, Burch et al 1987). Arachidonic acid can also be released from DAG and phosphatidic-acid by the action of specific lipases. Arachidonic acid is then metabolised by one of two major pathways with the formation of either prostaglandins or leukotrienes (eicosanoids). The pathways of eicosanoid metabolism are summarised in Fig.1. The actions of lipoxygenase and cycloxygenase enzymes respectively initiate the formation of leukotrienes and prostaglandins.

2.4.2 Lipoxygenases(LO)

Lipoxygenases catalyze the incorporation of one molecule of oxygen into unsaturated fatty-acids, resulting in the initial generation of hydroperoxyeicosatetraenoic acids (HPETEs). LO was first isolated from soya bean (Tappel 1963; Lewis and Austen 1984). A cDNA clone encoding human 5-LO was isolated from dimethyl sulfoxide differentiated HL60 cells. Substrates for lipoxygenases are long chain fatty-acids which have at least two double bonds interrupted by a methylene group. The target oxygenation sites in the substrate fatty acid molecule depend on the particular LO enzyme. Thus, 5-LO oxygenates C-5 of arachidonic-acid. Human neutrophil LO introduce oxygen

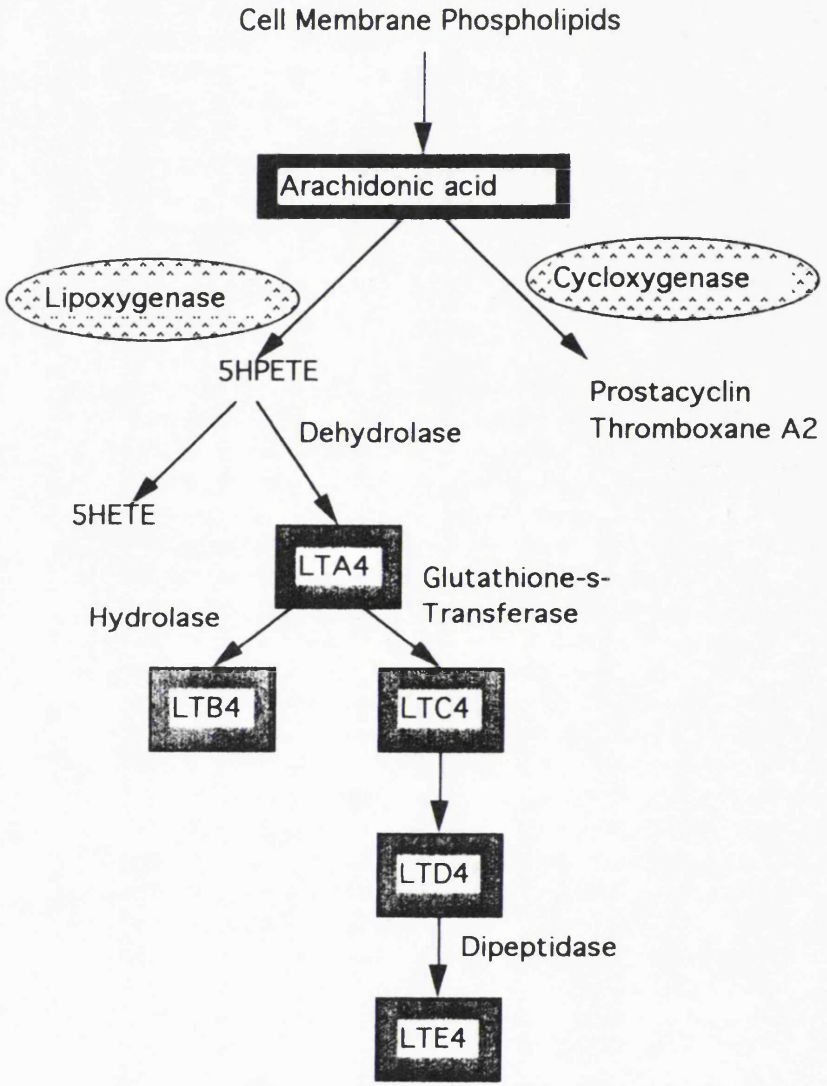


Fig. 1 Formation of leukotrienes from arachidonic acid

atoms at the 15 position(15-LO) or 5 position (5 LO) of arachidonic acid resulting in the formation of 15-HPETE and 5- HPETE. Platelets contain a 12-LO which introduces oxygen at the 12 position. The HPETEs are unstable, and are rapidly converted to the corresponding hydroxyeicosanetraenoic acids (HETEs). The 5-LO is a cytosolic-enzyme. Following cellular activation, it is translocated from the cytosol to the cell membrane where it binds to a specific high affinity 18 kDa target protein (Miller et al 1990). This protein the 5-LO activating protein (FLAP) , has been purified and its cDNA cloned (Dixon et al 1990). The presence of FLAP as well as the 5-lipoxygenase is essential for cellular activation of the latter enzyme. (Dixon et al 1990).

5 HPETE is the precursor of the leukotrienes (LTs), which have a triene structure (three double bonds separated from each other by single bond). LTA4 is an unstable 5,6-epoxide structure, that is enzymatically hydrolysed to LTB4 or converted to LTC4 by addition of a glutathione residue, LTC4 is then converted to LTD4 by the removal of a glutamic- acid residue by glutamyl-transferase (Rokach 1989). The metabolic relationships of HPETEs,HETEsand the LTs are shown in Fig 1.

2.4.3 Second messenger roles of arachidonic acid

Arachidonic acid can selectively activate the gamma sub-species of protein kinase C (Shearman et al 1989) and also it mobilizes cytosolic Ca^{++} independent of any other second messenger (Wolf et al 1986). One possible

mode of action of arachidonic acid is via inhibition of the GTPase activating protein(GAP) which negatively regulates the action of the c-ras protooncogene-encoded protein (Han et al 1991). The GAP protein is known to be inactivated by an unknown mechanism following mitogenic stimulation of T cells (Downward et al 1990). Arachidonic-acid and its metabolites can also augment other second messenger systems in the cell such as cAMP (Bitonti et al 1980) and have been implicated in transport of ions and amino-acids (Burgoyne,1987).

2.4.4. Biological functions of LTs

The classical roles of leukotrienes are in the mediation of specific cellular responses in immunity and inflammation (Samuelsson et al 1983; Rola-Pleszczynski 1982). In some instances, binding of LTs to cell surface receptors triggers the generation of the potent second messengers DAG and Ptd Ins 1,4,5-P_3 via the breakdown of inositol lipids (Mong et al 1988). Increased production of eicosanoids has been observed following the stimulation of cells by PDGF,EGF, phorbol-ester (Hassegawa-Sassaki et al 1985; Parker et al 1989; Margolis et al 1988; Goerig et al 1988) and GM-CSF (Claesson et al 1989; Estrove et al 1988).

Recent evidence has suggested that LO metabolites may function as second messengers in the nervous system (Piomelli et al 1987) and cardiac-muscle (Kim et al 1989).

2.4.5. Cyclooxygenases and prostaglandins

Arachidonic acid is also metabolised to prostaglandins via a pathway initiated by the cyclooxygenase (CO) enzyme. Prostaglandins also function as intercellular mediators (Samuelsson et al 1983). They have been shown to exert growth regulatory effects on some cells. PGE is known to elevate cAMP which has been shown to play a growth regulatory role in different cells systems.

PGE-stimulated cAMP elevation synergises with insulin in securing the commitment of 3T3 cells to mitosis (Rozengurt et al 1987). The insulin receptor also possesses an intrinsic tyrosine kinase activity which is augmented in response to ligand binding (Hunter et al 1985). Therefore the cAMP dependent kinase pathway may also synergise with events initiated by tyrosine phosphorylation to promote mitogenesis in some circumstances.

PGF2 alpha was found to be mitogenic for fibroblasts (Otto et al 1982). PGF2 alpha has also been shown to trigger the generation of Ptd Ins 4,5-P_2 derived second messengers (Weiland et al 1987).

2.4.5.1 The action of exogenous PGs and cAMP elevating agents on T-lymphocyte mitogenesis

PGs play subtle and complex roles in T-cell mitogensis.Mitogenic activation of T lymphocytes was inhibited by PGE2. PGF2alpha was neither inhibitory nor

did it reverse the action of PGE2 . PGE2 affects several aspects of T cell mitogenesis including secretion of IL2 and the expression of transferrin receptor (Chouaids et al 1985). In T lymphocytes also, PGE2 caused elevation of cAMP. Dibutyryl cAMP mimicked the action of PGE2, suggesting a role for cAMP dependent kinases in mediating PGE2 inhibition (Makoul et al 1985).

On the other hand , Mire-Sluis et al (1986) have shown that phosphorylation of a 42 kDa protein was an early event in T cell mitotic activation. It was activated indirectly via both the kinase C and kinase A pathways. These results suggest that kinase A pathway may also contribute to positive aspects of mitotic-regulation.

2.4.6 Inhibitors of eicosanoid synthesis

Several LO and CO inhibitors with various degrees of specificity have been synthesized. The properties of some of these antagonists are summarized in Table 1.

Piriprost is a selective inhibitor of 5-LO which does not inhibit 12-LO, 15- LO or CO. ETYA is a competitive inhibitor of lipoxygenase as well as cycloxygenase pathways of arachidonic-acid metabolism. Nordihydroguiaretic acid(NDGA) and pyralozones including BW755C inhibit the lipoxygenase enzyme and also have some cycloxygenase inhibitory effects. Most of these compounds have free radicals scavenging and antioxidant properties i.e they have the capability to inactivate 5-LO presumably by reducing the iron at the active site (Salari et al

TABLE I

Published IC50 values for the inhibition of arachidonic acid-metabolizing enzymes.

	5-LO	12-LO	15-LO	CO
NDGA	0.5	5	30	5
BW755C	10	50	100	10
Piriprost	2	>100	>200	>100
Indomethacin	50	80	>100	<0.1
BWA 4C	0.1	nd	nd	16.6
MK886	<0.01	nd	nd	nd

Data as published by Salari et al 1984 except piriprost (Sun et al 1983), BWA4C (Tateson et al 1988) and MK886 (Gillard et al 1989) " All values are μ M"

1984).

BWA 4C, a hydroxycinnamic acid, is an antioxidant which inhibits 5-LO directly (Tateson et al 1988). MK886 does not inhibit the 5-LO enzyme itself, but interferes with its interaction with the 5-LO activating protein (Gillard et al 1989), which is essential for the activation of the enzyme in intact cells. Both these compounds inhibited 5-LO of intact cells with IC_{50} values in the nanomolar range.

2.5 The role of eicosanoids in the regulation of cell proliferation

2.5.1 Non-haemopoietic cells

LTs have been shown to modulate the growth of several types of normal cells.Leukotrienes addition resulted in increases DNA synthesis in a number of tissues including epidermal keratinocytes (Kraqbulle et al 1985) skin fibroblasts (Baud et al 1987) and glomerular epithelial cells (Baud et al 1985). Mouse skin tumour promotion was inhibited by inhibitors of arachidonic acid metabolism (Fischer et al 1982).

2.5.2 Normal haemopoietic cells

Myeloid colony formation by normal human bone marrow cells in vitro was inhibited by LO antagonists including NDGA, piriprost and caffeic acid (Pasquale et al 1991).Inhibition of myeloid colony formation by 5-LO antagonists was reversed by the addition of leukotrienes C4 and D4 (Miller et al 1986), suggesting that exogenous LT generation by normal bone marrow cells plays a role in signalling the proliferation of colony-forming cells. Inhibition of myeloid colony formation by LO inhibitors may, however, be dependent at least in part on accessory cells; tumour necrosis factor or interleukin-1 stimulated generation of GM-CSF by two stromal cell lines is abrogated by the LO inhibitors NDGA and caffeic acid, and LTB4 apparently synergizes with interleukin-1 in inducing GM-CSF (Rizzo et al 1991).

2.5.3 Leukaemia cell lines

DNA synthesis in a variety of leukaemic cell lines was inhibited by inhibitors of 5-LO but not of cyclooxygenase (Snyder et al 1989; Ondrey et al 1989; Tsukada et al 1986), suggesting that the endogenous generation of leukotrienes may play some role in the proliferation of these cells.

2.5.4. The role of eicosanoids in T lymphocyte proliferation

The formation of eicosanoids by lymphoid- tissue is a controversial subject. Some authors believe that T lymphocytes are unable to produce leukotrienes (Poubelle et al 1987). On the other hand Farrar and Humes (1985) have demonstrated the apparent formation of both 5 and 15 HETE in murine T cells lines. Recently, it was shown that T cells contain no FLAP. The occurrence of FLAP has been investigated in a variety of cells and a positive relationship have been found between the expression of FLAP and the ability of cells to produce LTs. The absence of FLAP in T-lymphocytes suggest that they are unable to synthesize LT (Reid et al, 1990). LO antagonists inhibited the proliferation of lymphocytes. Inhibition of LO abrogated lectin- induced lymphocyte proliferation (Gualde et al 1985). The specific mechanism of these actions has not been elucidated.It has also been suggested that 5-LO metabolites play a mediator role in the signal transduction process triggered by IL1 and IL2 (Dinarello et al 1983; Farrar and Humes 1985). However, recently it has been shown that there is little evidence to suggest the involvement of LO metabolites in these immunoregulatory signalling mechanisms (Liu et al 1989).

2.5.5 Role of free radicals

The role of free radicals in the activation of T lymphocytes is incompletely understood. Various studies have indicated that free radicals are involved in

triggering T cell activation. Novogrodsky et al (1982) reported that free radical scavengers, dimethyl-sulfoxide,benzoate,thiourea and mannitol inhibited lymphocyte mitogenesis induced by phorbol ester. Expression of interleukin-2 receptor on stimulated T cells was found to be inhibited by butylated hydroxyanisole (BHA) which blocks lipid peroxidation and by the non-permeant electron acceptor ferricyanide which blocks the reduction of oxygen to superoxide. These agents also inhibited proliferation of T cells in a dose dependent manner (Chaudhri et al 1986). Irradiation(20 Gy) increased oxidative product formation from membrane phospholipids (Greenstock 1981) and also enhanced IL2 synthesis by T cells suggesting the involvement of oxidative processes in T cell activation. Enhanced IL2 synthesis observed in response to 20 Gy irradiation in T cells was inhibited by the hydroxy radical scavenger dimethyl sulphoxide (DMSO) and by the anti-oxidant nordihydroguaretic-acid (NDGA) (Sekkat et al 1988).

The cellular source of free radical generation which may play a role in T cell signal transduction pathways is obscure. PHA and phorbol ester treatment of T cells and Jurkat malignant T line lead to oxidative product formation as evaluated by flow cytoflurometric-studies (Sekkat et al 1988). In T cells, a possible role of cGMP in mediating the mitogenic-signals has been postulated (Hadden et al 1987), and in turn activities of cGMP are believed to be regulated by oxidative-reductive mechanisms. PHA and phorbol ester treatment of T cells has been reported to increase cGMP (Coffey et al 1981; Sekkat et al 1988). All

these observations tentatively suggest that free radical generation play some role in T cell signalling pathways.

I have therefore focused on studying the actions of antioxidants on ligands-stimulated activation of the inositol lipid signalling pathway of T lymphocytes and Jurkat cells, in order to elucidate the involvement and the mechanisms of action of putative free radical species in normal human T lymphocytes and their malignant counterpart, the Jurkat T lymphoblastic leukaemia cell line.

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of T lymphocytes

3.1.1 Isolation of peripheral blood mononuclear cells

Lymphocytes and monocytes were separated from granulocytes and erythrocytes by buoyant density centrifugation over a layer of Ficoll-hypaque (Lymphoprep, Nycomed, UK). Buffy coats from normal peripheral blood (about 20-25ml each), obtained from The Regional Blood Transfusion Centre, Edgware, were diluted with 30 ml Hanks' balanced salts solution (HBSS, Life Technologies, UK) and layered over 25 ml Ficoll-hypaque (Nycomed, U.K) in a sterile 50 ml Falcon centrifuge tube.The tubes were centrifuged for 25 min at 800 x g at room temperature.The layer of mononuclear cells was aseptically removed from the interface.The cells were washed twice in HBSS and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (Life Technologies, UK).

3.1.2 Preparation of sheep erythrocytes

Sheep erythrocytes in Alsevers medium (Tissue Culture Services , Claydan), were washed three times in HBSS. The washed erythrocytes (0.5 ml packed

cells) were incubated with 0.2 ml neuraminidase (1 unit ml^{-1} , Sigma) in 5 ml HBSS at 37^0 C for 30 min. The cells were washed three times in HBSS and resuspended at 5%(v/v) in RPMI 1640 medium.

3.1.3 Rosetting procedure

Materials

3.1.3.1.Ammonium chloride (NH_4Cl) lysis solution.

Stock solution x 10.

Ammonium chloride 8.290g.

Potassium hydrogen carbonate ($KHCO_3$) 1.0g.

Ethylenediamintetra-acetic acid disodium salt (EDTA) 0.02g.

Reagents were dissolved individually in a total volume of 100 ml distilled water pH 7.4, stored at 4^0 C. Before use diluted in 1:10 in distilled water.

3.1.3.2 Procedure

T lymphocytes were enriched by rosetting with neuraminidase-treated sheep erythrocytes (Minowada et al 1972).

One ml of the 5% sheep erythrocyte suspension (section 3.1.2) was added to 5 ml of mononuclear cell suspension (1x 10^6 $ml^{-1)}$ and the mixture incubated at 37^0 C for 15 min. The tube was centrifuged at 140 x g for 5 min at room temperature and then placed on ice for 60 min. The suspension was gently

mixed and layered on a Ficoll-hypaque gradient.The tubes were centrifuged at 800xg for 25 min at room temperature. T lymphocyte-erythrocyte rosettes were removed from the bottom of the tubes. Erythrocytes were lysed by suspension in a hypotonic solution (Section 3.1.3.1) at 4^0 C. HBSS (15 ml) was added as soon as lysis was observed, to restore tonicity.

T lymphocytes were recovered by centrifugation for 3 min at 800x g and washed twice in HBSS. The purified cells were cultured in RPMI 1640 medium containing 10% fetal calf serum,100 μg ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

3.1.4 Assessment of purity of T lymphocytes by immunophenotyping

Reactivity with CD3, CD19 ,CD33 McAbs was determined using the alkaline phosphatase anti alkaline phosphatase (APAAP) immunocytochemistry technique to assess purity of T lymphocytes.I am grateful to Faith Wright for carrying out these determinations.Cells prepared as described above were >96% $CD3^+$ (T lymphocytes) and contained <2% CD19 positive cells (B lymphocytes) and <2% CD33 positive cells (granulocytes and monocytes).

3.2 Preparation of leucocytes

Leucocytes were prepared by the Dextran sedimentation technique described by Baron and Ahmed (1969). Venous blood (10 -20 ml) was collected in

heparinised tubes (2 iu ml^{-1} final concentration). An equal amount of buffer (Solution I, pH 7.4), pre-warmed in a water bath at 37 C, was added. Dextran from Sigma,U.K. (Solution III, pH to 7.4) was warmed and added to the diluted blood in a ratio of 1:4. The mixture was shaken very gently and air bubbles(if any) at the top of the column were removed. The mixture was left to stand for 15 min. The blood cells sedimented to the bottom of the tube and the resulting leucocyte-rich supernatant was removed into sterile tubes and centrifuged for 4 minutes at 250 x g and the supernatant discarded. The pellet contained the leucocytes. Contaminating red blood cells were lysed hypotonically by the addition of 3 ml distilled water followed by whirlimixing for 8-10 seconds. One ml Solution II was rapidly added and mixed to restore isotonicity. The mixture was centrifuged at 250 x g for 3 min. The layer of haemoglobin and red cell ghosts was gently removed using a Pasteur pipette along the side of the conical tube without disrupting the tightly packed leucocytes. The pellet containing a mixed population of leucocytes was resuspended in RPMI 1640 medium.

3.3 Preparation of platelets

Venous blood (10-20 ml) was collected in heparinised tubes(2 iu ml^{-1} final concentration), transferred to 20 ml universal tubes and centrifuged immediately at 250 x g at room temperature for 30 min to sediment the red cells. The platelet-rich plasma was removed and centrifuged at 1400 x g at room

temperature for 5 min to sediment the platelets (Murphy et al 1970).

3.4 Preparation of mononuclear cells from blood or bone marrow of leukaemia patients

Bone marrow from leukaemia patients was first diluted 50:50 with HBSS. Diluted bone marrow or blood was layered over 10 ml of Ficoll-hypaque in a 20 ml universal tubes. The tubes were centrifuged for 25 min at 800 x g,at room temperature.

The layer of mononuclear cells was removed from the interface, washed twice in HBSS and resuspended in RPMI 1640 medium.

3.5 Culture of leukaemia cell lines

All tissue culture materials were from Life Technologies, UK.

Cell lines were maintained in RPMI 1640 medium supplemented with 100 µg ml^{-1} penicillin , 100 µg ml^{-1} streptomycin and 10% fetal calf serum.The following cell lines were used in this study: HL60 (acute promyelocytic leukaemia), K562 (Philadelphia-positive chronic granulocytic leukaemia in blast crisis), Jurkat (T-acute lymphoblastic leukaemia) and Daudi (Burkitt Lymphoma).

The cells were grown at 37°C in an 8% CO_2 atmosphere.

3.6 Determination of viability

Viable cell counts were determined by trypan blue-exclusion.

3.6 Drugs

NDGA, staurosporine and indomethacin were purchased from the Sigma Chemical Company, UK. BW755C and BW A4C were gifts from Dr. Lawrence Garland, Wellcome Research Laboratories, UK. Piriprost was donated by Upjohn, Kalamazoo, USA and MK 886 by Professor A.W. Ford-Hutchinson, Merck-Frosst, Canada. D609 was a gift from Merz and Co, Germany.
All inhibitors were freshly dissolved in ethanol and were added to cultures at suitable dilutions. The final ethanol concentration did not exceed 0.1%. Control incubations including 0.1% ethanol were therefore included in all studies.

3.7 The release of (^{3}H) arachidonic acid-labelled metabolites.

3.7.1 Materials

(^{3}H)arachidonic acid 222 Ci mmol^{-1} was from(Amersham International, UK)
Thin layer chromatography (TLC) was carried out using Kiselgel 60 pre-coated plastic-backed plates from Merck, Darmstadt, Germany.

3.7.2 Labelling of cells and cell lines with [^{3}H] AA.

Cells(10^6. ml^{-1}) were labelled with 0.1μCi. ml^{-1} [5,6,8,9,11,12,14,15 3H] AA overnight. Cells were then transferred into universal tubes and centrifuged at 700 x g for 3 min.The supernatant was removed and the pellet was washed twice with HBSS. Cells were resuspended in RPMI medium supplemented with 10% fetal calf serum and incubated at 37^0 C for two hours. At the end of this chase period, cells were washed twice in HBSS and resuspended in serum free RPMI 1640 medium at 5x 10^6. ml^{-1}.Mitogens(PHA or anti CD3 monoclonal antibody) were then added to cell cultures.

3.7.4 TLC procedures

Following 30 min incubation with mitogen, incubations were terminated by the addition of 2.5 vol ethyl acetate to 1 vol of culture. After vigorous vortexing, the tubes were placed at -70^0 C for 10 min. The ethyl acetate phase was separated from the frozen aqueous phase. The samples were dried in vacuo and dissolved in 50 μl ethyl acetate.Three μl unlabelled AA (10mg. ml^{-1} in CHCl3 and 0.1% BHT) were added and samples streaked on TLC plates. The plates were developed in hexane:diethyl ether:acetic acid (25:25:1) (Hurst et al 1987). This solvent system resolves arachidonic acid from HPETES and HETES.[^{3}H]12-HETE was prepared by incubating sonicated platelets with [^{3}H] arachidonic acid (Barradas et al 1989). This compound together with unlabelled arachidonic

acid, were used as chromatographic markers.

3.7.5 Detection of substances separated by TLC

The following procedures were used for the location of AA and its metabolites on TLC plates:

a. Auto-radiography:

Plates were sprayed with Amplify (Amersham, UK) and exposed to X-ray film at -70^0 C for 48 hours.

b. Iodine-Staining

Lipids were identified by exposure to iodine vapours.

c. Scintillation Counting

Plates were cut into 1-3 cm segments and counted following addition of 10 ml 299 Liquid Scintillator.

3.8 Detection of eicosanoid generation by radioimmunoassay

T lymphocytes were suspended in RPMI-1640 at $2x10^6$ ml^{-1} or whole blood were aliquotted into 1.5 ml Eppendorf tubes and drugs were added as appropriate. Following 20 min incubation at 37^0 C PHA/ionophore were added and incubation continued for 30 min.At the end of the incubation period cells were removed by centrifugation at 1500xg for 5 min. The supernatant were stored at-70^0 C. Radioimmunoassay for LTB4 was carried out as described (Tateson et al

1988). I am grateful to J.Tateson for performing radioimmunoassays for the estimation of the LTB4 and HETE.

3.9 Detection of lipid peroxides

T lymphocytes were stimulated with mitogens and lipids peroxides were determined by measuring reactivity with thiobarbituric acid as described (Hallinan et al 1991). I am grateful to Dr. T. Hallinan for carrying out these assays.

3.10 The metabolism of [3H] inositol pre-labelled compounds

3.10.1 Materials

myo- [2-^{3}H]inositol (19 Ci mmol^{-1}) was from Amersham International UK.

Preparation of Dowex-1(formate) resin

Fifty grams Dowex x A1-X8 resin (BDH,UK) were washed with 1 N NaOH. The resin was then washed with 1l N formic acid, and rinsed repeatedly with distilled water until the p^{H} approached that of the water.

3.10.2 Labelling of cells and cell lines with [^{3}H] inositol

Inositol-free RPMI-1640 (GIBCO) and dialysed fetal calf serum were used in inositol labelling experiments. The cells were grown at 37^{0} C in a 5% CO2

atmosphere.

Myo-[2-^{3}H] inositol (20 Ci/mmol, Amersham) at 5 2μCi/ml was added to cell cultures seeded at a density of $2x10^{6}$ cells.ml^{-1}. Cells were labelled for 24 h (Mire-Sluis et al 1989).

3.10.3 Stimulation of prelabelled cells

At the end of the 24 h labelling period, cells were washed three times in HBSS and resuspended in RPMI 1640 medium at $8x10^{6}$ ^{cells}.ml^{-1}. M LiCl was then added to give a final concentration of 10mM and the tubes equilibrated at 37^{0}C for 20 min. LiCl is an inhibitor of IP1 phosphatase.The presence of this compound therefore facilitates the detection of inositol phosphates generated by ligand-stimulated inositol lipid breakdown (Berridge et al 1983) . Cells were then aliquotted into 1.5 ml Eppendorf tubes and drugs were added as appropriate. Following a 20 min incubation at 37^{0} C cells were challenged with 2μl (1mg ml^{-1} PHA).In some experiments, 2μl 1/10 dilution of an antiCD3 monoclonal antibody was added as the activating agent. These concentrations of ligands gave optimal stimulation of inositol phosphates. Following incubation for 30 min, the cells were recovered by centrifugation at 1200xg for 20sec and supernatant discarded. One ml of 10% trichloroacetic acid (TCA) was added to each pellet, vortexed and incubated on ice for 30 min. The tubes were centrifuged again, and the acid-soluble supernatants processed for the determination of inositol phosphates. In some experiments, the acid-

insoluble material was dissolved in CHCl3:CH3OH:HCl (20:40:1), and the radiolabel in [^{3}H] inositol lipids determined by scintillation counting in order to ensure that equal numbers of cells were present in each aliquot.

3.10.4 Dowex column analysis of water-soluble [3H] inositol-labelled metabolites

Supernatants retained from TCA washes were extracted three times with 4 ml of diethyl ether to remove TCA and neutralized with 6-8 µl 3M Tris-HCl buffer, P^H 8.8as described (Porfiri et al 1989) . Aliquots were applied to columns containing 1 ml Dowex A1-X8(formate-form Section 3.10.1) as described (Berridge et al 1983) . [^{3}H] inositol was eluted with 10 ml water.[^{3}H] glycerophosphoinositol was eluted with 10 ml 5mM sodium tetraborate, 60mM sodium formate and [^{3}H] inositol phosphates with six successive 1 ml aliquots of 0.2M formic acid, 2 M ammonium formate.

3.10.5 Counting of fractions

Aliquots (1 ml) of each eluate were added to 10 ml scintillation fluid and counted for ^{3}H radiolabel.

3.10.6.Statistics

Data were analysed by paired t testing.

3.11. Assay for PtdIns-4,5-P_2 phospholipase C

Ptd Ins-4,5-P_2 phospholipase C was assayed by measurement of water soluble radiolabelled compounds from phosphotidyl[2-^{3}H Ins 4,5-P2) as described (Porfiri et al 1991).

3.11.1 Materials

Unlabelled PtdIns 4,5-P_2 was from Sigma. Ptd[^{3}H]Ins 4,5-P_2 was from Amersham International, U.K.

3.11.2 Cell fractionation

Lymphocytes or Jurkat cells were harvested by centrifugation and washed twice with ice cold HBSS. The cell pellet was then resuspended at 10^7 cells ml^{-1} in ice cold lysis buffer (5mM Tris pH 6.8, 1mM EGTA, 10mM benzamidine, 1 µg. ml^{-1} leupeptin, 500 µm phenylmethyl sulphonyl fluoride).

After 30 min on ice cells were disrupted by passing them couple of times through a 21 gauge syringe needle, followed by the addition of sucrose to 0.25 M. Nuclei and remaining intact cells were removed by centrifugation at 700 x g for 15 min. The resulting post-nuclear fractions were stored in aliquots at -20^0 C. Protein concentrations were determined by a standard procedure (Schleif and Wensink 1981).

3.11.3.Substrate preparation

Two hundred and fifty µmol of unlabelled Ptd Ins 4,5-P_2 in CHCl3: methanol were mixed with 1.5 µCi Ptd[^{3}H] Ins 4,5-P_2 (1µCi/100 µl) and dried under N2. The lipids were resuspended in 0.2% sodium cholate, 20 mM Tris-HCl pH 6.8. and the mixture was sonicated on ice (2x10 sec bursts at 18 µm peak to peak). The final specific activity was 2.5-3 counts.min^{-1}.pmol^{-1}.

3.11.4.Assay procedure

The assay contained in a final volume of 50µl: 20 mM Tris HCl, pH 6.8, 250 µM Pt[^{3}H]Ins4,5-P_2, CaCl2-EGTA buffer, 5 µg proteins. Tubes were incubated at 37^0 C for 20 min. Reactions were stopped by the addition of 150 µl chloroform:methanol: c HCl(20:40:1 vol/vol). Phases were split by the addition of 100 µl each of CHCl3 and H2O. Tubes were centrifuged at 13000 rev/min in Beckman microfuge. One hundred and fifty µl aliquots of the upper aqueous phase were added to 5 ml scintillation fluid and radioactivity determined in a scintillation counter.

3.12 Assay of [^{3}H] thymidine incorporation

DNA synthesis was assayed by [^{3}H]thymidine incorporation(Taheri et al 1981) in RPMI 1640 medium without serum supplementation.

[methyl ^{3}H] thymidine 49 Ci/mmol was from Amersham International , U.K. [^{3}H]thymidine incorporation was estimated by incubating 2x105 cells(in triplicate) in 200 µl RPMI 1640 medium(without fetal calf serum and in the presence or absence of drug/ligand being tested) in the presence of 0.2µCi of [^{3}H]thymidine. Following 4h incubation period at 37^{0}C, labelling was terminated by adding 50 µl 1N NaOH, 1mg ml^{-1} DNA into each aliquot.

Precipitation, hydrolysis and counting

Incorporation of (3H) thymidine into DNA was estimated following precipitation with 0. 5M perchloric acid.

Tubes were heated at 80^{0} C in a water-bath to hydrolyse DNA.

The contents were transferred into 10 ml scintillation fluid and radioactivity determined in a scintillation counter.

CHAPTER 4

THE ACTIONS OF ANTIOXIDANTS,LIPOXYGENASE INHIBITORS AND OTHER COMPOUNDS ON LIGAND STIMULATED INOSITOL LIPID BREAKDOWN IN T LYMPHOID CELLS.

A number of well-known ("classical") inhibitors of lipoxygenase impaired phytohaemagglutinin (PHA) stimulated breakdown of inositol lipids in human T lymphocytes. Piriprost, NDGA, and BW755C, inhibitors of the 5-LO enzyme, inhibited PHA stimulated inositol phosphate generation in a dose dependent manner, whereas the cyclooxygenase inhibitor indomethacin did not (Mire-Sluis et al 1989). These observations tentatively suggest that the generation of arachidonic acid and its LO metabolites may be involved in the activation and proliferation of T lymphocytes.However, the ability of T lymphocytes to generate LO product(s) is a controversial issue. Poubelle et al (1987) failed to detect leukotriene generation by T lymphocytes, whereas some other authors have demonstrated the apparent formation of 5-HETE in murine T cells line (Farrar and Humes 1985). I have therefore studied the generation of arachidonic acid and LO product(s) by T lymphocytes to establish whether or not these metabolites may play a role in T lymphocytes signalling pathways.

I have further investigated the mechanism(s) by which these lipoxygenase inhibitors impair PHA and CD3 McAb stimulated inositol lipid turnover in T lymphocytes using novel,highly specific 5-LO inhibitors. Classical inhibitors of

5-LO are also able to inactivate free radicals. Since free radicals have been shown to play an important role in early stages of T-cell proliferation (Chaudhri et al,1986), I investigated the possible involvement of these species in the activation of inositol lipid breakdown in T lymphoid cells using the following classes of agents that prevent the formation or effects of oxygen radicals: lipid soluble general antioxidants including butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA); sulfhydryl reagents: N-acetylcysteine and dithithreitol (DTT); PLA2 inhibitors; the non-permeant electron acceptor ferricyanide; and the iron chelator desferrioxamine(DFO). For these studies, I have used highly purified T-lymphocytes as a model system. OKT3 a specific T cell mitogen, was used as an activating ligand in addition to PHA.

Results.

4.2 Effects of lipoxygenase inhibitors on stimulation of inositol lipid breakdown by the T lymphocytes mitogens PHA-P and OKT3.

PHA-P and OKT3 were used as the activating ligands. These concentrations of ligands gave optimal stimulation of inositol phosphate generation (Fig.1,2).

BW755C,a dual inhibitor of both lipoxygenase and cycloxygenase, abrogated OKT3 stimulated breakdown of inositol lipids (Fig.3)

Piriprost and NDGA, two inhibitors of lipoxygenase also inhibited PHA-P-stimulated breakdown of inositol lipids (Fig.4,5).

In order to eliminate the possibility that the inhibitors may directly affect Ptd Ins 4,5- P_2-PLC activity, I studied their actions on this enzyme in an in vitro assay.NDGA and BW755C inhibited Ptd Ins 4,5-P_2-PLC activity while piriprost did not (Table 1).

These results tentatively suggested that the generation of arachidonic acid and its LO product(s) may be involved in coupling cell-surface receptors of T lymphocytes to its inositol lipid signalling pathway.

4.2.1 MK886 and BWA4C does not abrogate the stimulation of inositol lipid breakdown by T lymphocyte mitogens PHA-P and OKT3.

MK886 did not impair the stimulation of inositol lipid breakdown by the T lymphocyte mitogens PHA-P (Fig.6A,6B) or OKT3(Fig.6C). BWA4C(100nM) also failed to inhibit the stimulation of Ptd Ins breakdown by either PHA-P (Fig.7A,7B) or OKT3 (Fig.7C).

I have verified that in my hands both of these inhibitors, MK886 and BW A4C inhibited leukotriene generation at the concentration (100nM) used in the above experiments.Eighty percent inhibition of calcium ionophore-stimulated LTB4 generation was obtained using MK886, and >98% inhibition using BW A4C. The above data suggested that 5- LO product(s) do not play a role in coupling cell-surface receptors to the inositol lipid signalling pathway in T lymphocytes. In order to reinforce this conclusion I have attempted to detect lipoxygenase metabolites of arachidonic acid following

stimulation of lymphocytes pre-labelled with [^{3}H] arachidonate.

4.3 Assays for oxidative metabolites of arachidonic acid in T lymphocytes.

Both TLC and radioimmunoassay were used to detect the generation of arachidonic and its metabolites. As a positive control I initially used TLC technique to study generation of LTs by platelets (Carey et al 1982) and white blood cells (Wright et al 1979) which are known to produce these metabolites. Radiolabelling by the procedure described in Materials and Methods (Section 3.7) resulted in the incorporation of approximately 12,500 counts. min^{-1} into the cellular phospholipids of 10^6 cells. Chromatographic analysis showed that 280 $\pm$19 counts. min^{-1}(duplicate mean and range) co-migrated with arachidonic acid. Following 30 min stimulation with PHA-P or OKT3 antibody, the radiolabel co-migrating with arachidonic acid increased to 677($\pm$67) counts.min^{-1}. There was no significant increase in radiolabel in the HETE/HPETE peaks following mitogenic stimulation. Therefore, these data are consistent with the previous data suggesting that leukotrienes are not involved in the initial stages of T lymphocytes activation.

A sensitive radioimmunoassay was also carried out in an attempt to detect the generation of lipoxygenase products following mitogen stimulation (Table II). PHA stimulation of T lymphocytes did not result in the detectable generation of immunoreactive LTB4. By contrast, a small increase in secreted LT was

detected when the cells were challenged with the calcium inophore A23187. This was probably accounted for by the action of this non-specific activator on residual myeloid cells in the T cell preparation. In a control experiment, LTB4 generation was readily detected when human whole blood was stimulated with the ionophore. We also failed to detect the generation of 15-HETE following PHA and ionophore stimulation of T cells(Table 2). Therefore, the radioimmunoassay data taken together with the chromatographic analysis of [^{3}H] arachidonic acid metabolites suggests that the stimulation of T lymphocytes with levels of PHA or OKT3 that were optimal for triggering the inositol lipid pathway did not result in the generation of 5-,12- or 15-lipoxygenase products of arachidonic acid.

Since arachidonic acid generation was detected in response to mitogen treatment, I therefore tested the effects of PLA2 inhibitors on PHA stimulated generation of inositol phosphates, in an attempt to determine whether arachidonic acid generation per se may play a role in coupling mitogen receptors to the inositol lipid signalling system.

4.4 The PLA2 inhibitor bromophenacyl bromide (BAB) inhibited stimulation of inositol lipid breakdown by T lymphocyte mitogen PHA.

BAB inhibits PLA2 activity and it is also capable of inhibiting oxidases (Maridonneau-Parini et al 1986, Bromberg and Pick 1983). IC_{50} value for the inhibition of superoxide generation is similiar to that of PLA2 inhibition

(Henderson et al 1989).BAB inhibited PHA-P stimulated generation of Inositol phosphatase (Fig.13A, 13C).

The response to the drug is clearly dose-dependent (Fig. 13B).

Impairment of mitogen simulated inositol lipid breakdown by BPB could be due to either inhibition of arachidonate release and/ antioxidant action.

4.4.1 Two novel PLA2 inhibitors- Ro 31-4493 and Ro 31-4693 inhibited PHA-P stimulated inositol lipid breakdown in T lymphocytes (Fig.14,15).

Both of these compounds in addition to inhibiting PLA2 activity, abrogate $O^{\cdot}$ generation (Davis et al 1988, Henderson et al 1989) and also inhibited Ptd Ins 4,5-P2-PLC (Table I). Therefore, no conclusion regarding the role of arachidonic acid generation in coupling to the inositol lipid signalling pathway could be drawn on the basis of the experiments using these reagents.

4.5 Antioxidants impaired the coupling of mitogen receptors to the inositol lipid signalling system in T lymphocytes.

The 5-LO inhibitors NDGA, BW755C and piriprost and the PLA2 inhibitor BPB also posses antioxidant and free radical-scavenging properties. I therefore investigated the possible involvement of antioxidant sensitive processes in the activation of the inositol lipid signalling pathway in T lymphoid cells. Lipid soluble general antioxidants, sulfhydryl compounds and non-permeant electron

acceptors were used in these studies.

4.5.1 Action of lipid-soluble general antioxidants on T lymphocytes.

The lipid-soluble antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) inhibited PHA-stimulated generation of inositol phosphates. BHT(10μM) abrogated PHA-P- (Fig.8A,8B) and OKT3- (Fig.8D) stimulated- inositol lipid breakdown in T lymphocytes. The response was clearly dose-dependent (Fig 8C). The effects of the antioxidant on inositol-lipid breakdown were not attributable to irreversible damage to cellular structures, as the effect of 10 μM BHT was reversed by washing and resuspension of cells (Fig. 8E)

BHA (100μM) also inhibited PHA-P stimulated inositol lipid breakdown in T lymphocytes (Fig.9A,9B). BHA was a less potent inhibitor, and an approximately 100μM concentration of this compound was required for half-maximal inhibition. Therefore, the ability of these compounds to inhibit early events in T lymphocytes signal transduction correlated with their potency as antioxidants (Halliwell and Gutteridge 1989).

4.5.2.Action of sulfhydryl reagents on T lymphocytes.

Thiol-containing compounds have been used in studies designed to investigate the possible role of oxidative metabolites in signal transduction pathways

activated following ligand binding to cell surface receptors (Staal et al 1990). N-acetyl-cysteine (20mM) impaired PHA-P stimulated inositol lipid breakdown in T lymphocytes (Fig.10A,10C). The response was clearly dose dependent(Fig.10B).

Dithiothreitol DTT (5mM) also inhibited PHA-P stimulated Inositol lipid breakdown (Fig.11A,11B).

4.5.3 The non-permeant electron acceptor ferricyanide abrogated stimulation of inositol lipid breakdown by the T lymphocyte mitogen PHA-P.

Ferricyanide blocks the reduction of oxygen to superoxide (Chaudhri et al 1986), inhibited PHA-P stimulated inositol lipid breakdown in T lymphocytes (Fig.12A,12B).

4.5.4 Antioxidants did not inhibit PtdIns 4,5-P_2-PLC

The data here suggested that different classes of antioxidants abrogated the mitogen stimulated generation of inositol phosphates in T lymphoid cells.In order to eliminate the possibility that these antioxidants may directly affect Ptd Ins 4,5-P_2-PLC activity, I studied their action on this enzyme in an in vitro assay. None of these antioxidants perturbed the activity of this enzyme (Table I) suggesting that the target for these compounds is an antioxidant-sensitive regulatory step in the coupling mechanism of T lymphocytes. I have attempted to characterize the putative oxidative metabolite involved in signal

transduction.I was unable to detect, in three separate experiments, the generation of thiobarbituric acid reactive lipid peroxides using the procedures as described by Hallinan et al (1990). The generation of the free radical nitric oxide is involved in inter- and intra-cellular signalling mechanisms (Murad et al 1990). I therefore carried out experiments to study the role of this reactive compound in T lymphocyte signalling pathway.

4.6 An Inhibitor of NO Biosynthesis does not impair inositol lipid signalling in T lymphocytes.

Some authors have reported the production of NO like substances by T-lymphoid cell lines (Kirk et al 1990). I therefore investigated the possible involvement of this agent in the stimulation of the inositol lipid signalling pathway in T cells.Since it was possible that antioxidants may inactivate NO, I studied the action of N-monomethyl-L-arginine (L-NMMA), an inhibitor of NO biosynthesis (Gukovskaya et al 1989) , on the activation of the inositol lipid signalling system in T lymphocytes.

L-NMMA (200μM) did not impair PHA-P stimulated inositol lipid breakdown in T lymphocytes (Fig.18). The addition of arginine the precursor of NO also did not perturb this growth signalling pathway.These results suggest that NO production does not play a role in coupling mitogen receptors to the inositol lipid signalling system in T lymphocytes.

4.7 DFO does not impair PHA-P stimulated Inositol lipid breakdown in T lymphocytes.

DFO is an effective iron chelator which also inhibits proliferation of T lymphocytes (Chaudhri et al 1986).I therefore attempted to show whether this compound also perturbed inositol lipid signalling in T lymphocytes. DFO did not affect ligand-stimulated inositol phosphates generation (Fig.16A,16B).

4.8 Staurosporine inhibited PHA-P stimulated inositol lipid breakdown in T lymphocytes (Fig.20A, 20C).

I studied the actions of the protein kinase inhibitor staurosporine on PHA stimulated generation of inositol phosphates. This compound abrogated inositol lipid breakdown even when used at 100nM,and 500nM concentrations (Fig.20B).It is an antimicrobial alkaloid which inhibits PKC (Tamaoki et al 1986) and tyrosine protein kinases (Fallon et al 1990). Therefore, inhibition of inositol phosphates generation by staurosporine is likely due to its inhibitory effects on PTKs.

4.9 D609 impaired PHA-P stimulated inositol lipid breakdown in T lymphocytes (Fig.17A,17B).

These effects of D609 are attributable to its inhibitory effects on Ptd Ins 4,5-P2-PLC (Muller-Decker 1989)

4.10 Cycloheximide does not impair PHA-P stimulated inositol phosphates generation in T lymphocytes.

The activation of NFkB, which is dependent on its dissociation from an inhibitor protein IkB, is mediated by a signalling process dependent on the generation of reactive oxygen species (Schreck et al 1991) . It was therefore plausible that the actions of antioxidants may be accounted for by the requirement of new protein synthesis, mediated by NFkB or a similar transcription factor for maintenance of ligand-stimulated inositol lipid breakdown. However, PHA-stimulated inositol phosphate generation was not inhibited by the protein synthesis inhibitor cycloheximide(Fig 19A,19B), ruling out a requirement for protein synthesis in this process.

Fig 1

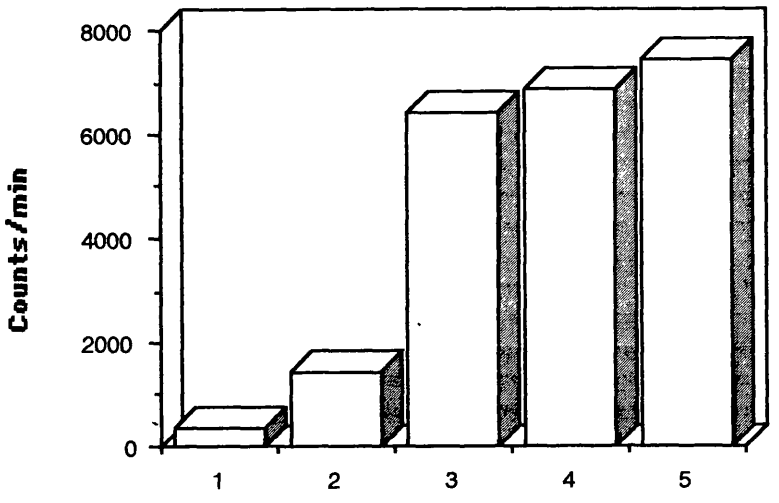

Counts/min
8000
6000
4000
2000
0
1
2
3
4
5

Fig 2

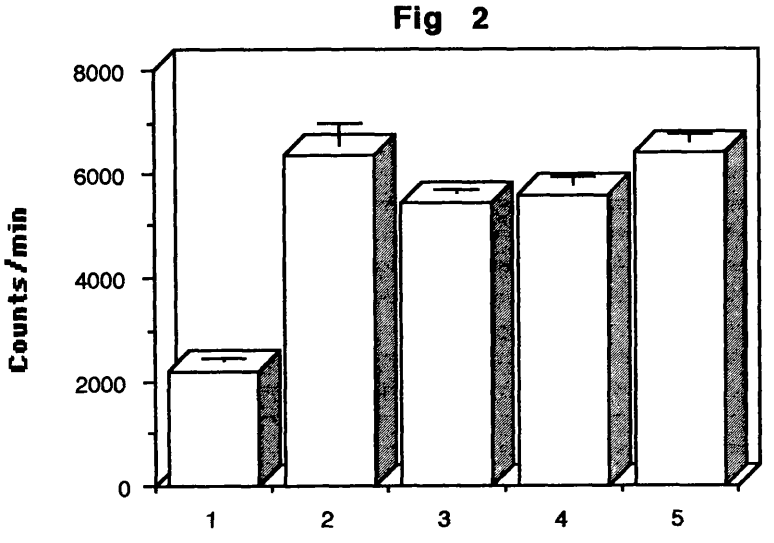

Counts/min
8000
6000
4000
2000
0
1
2
3
4
5

Fig.1. Effect of increasing concentrations of PHA-P on inositol lipid breakdown in T lymphocytes.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +.065 µg PHA-P $100\mu l^{-1}$ 10^6 cells.

3, +.13 µg PHA-P $100\mu l^{-1}$ 10^6 cells.

3, +.26 µg PHA-P $100\mu l^{-1}$ 10^6 cells.

4, +.50 µg PHA-P $100\mu l^{-1}$ 10^6 cells.

Fig.2. Effect of different concentrations of OKT3 on inositol lipid breakdown in T lymphocytes.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2,+5µl OKT3(1/10 diluted)

3,+10µl OKT3(1/10 diluted)

4,+20µl OKT3(1/10 diluted)

5,+40µl OKT3(1/10 diluted)

The bars indicate the range of duplicate measurements.

Fig.3

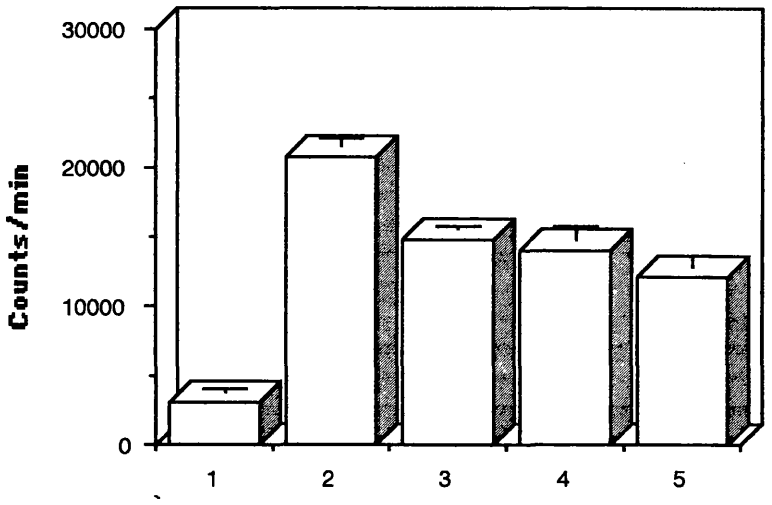


Fig.4

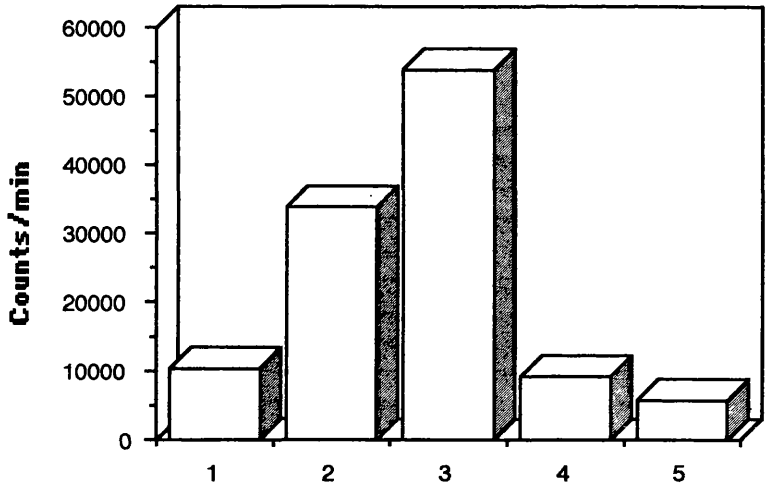


Fig.5

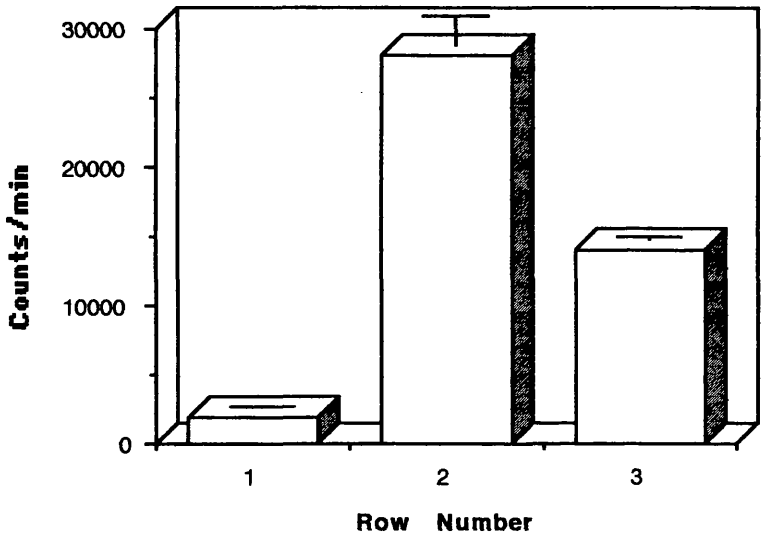


Fig 3. The action of BW755C on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, + 10 µl ml^{-1} OKT3.

3, + 10µl ml^{-1} OKT3 and 25µM BW755C.

4, + 10 µl ml^{-1} OKT3 and 50 µM BW755C.

The bars indicate the range of duplicate measurements.

Fig 4. The action of NDGA on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand. 2, +10 µl ml^{-1} PHA-P.

3 +10µl ml^{-1} PHA-P and 5 µM NDGA.

4, +10µl ml^{-1} PHA-P and 50 µM NDGA.

5, +10µl ml^{-1} PHA-P and 100 µM NDGA

Fig.5. The action of Piriprost on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P,

3, +10 µl PHA-P and 50µM piriprost.

The bars indicate the range of duplicate measurements.

Fig.6A

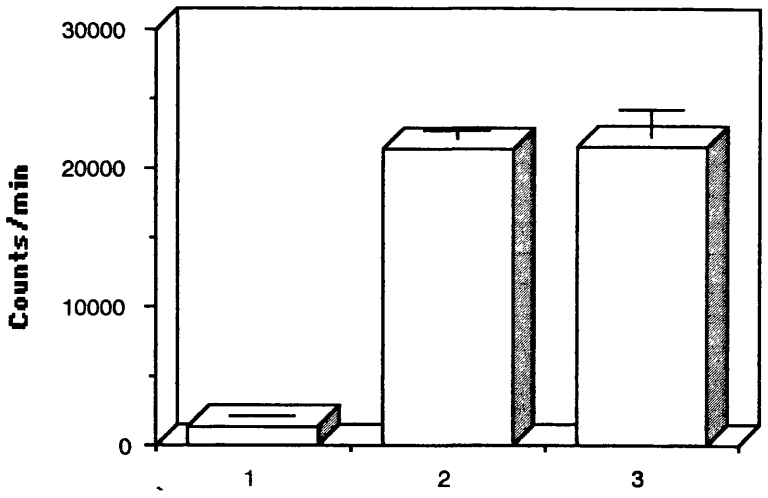


Fig.6B

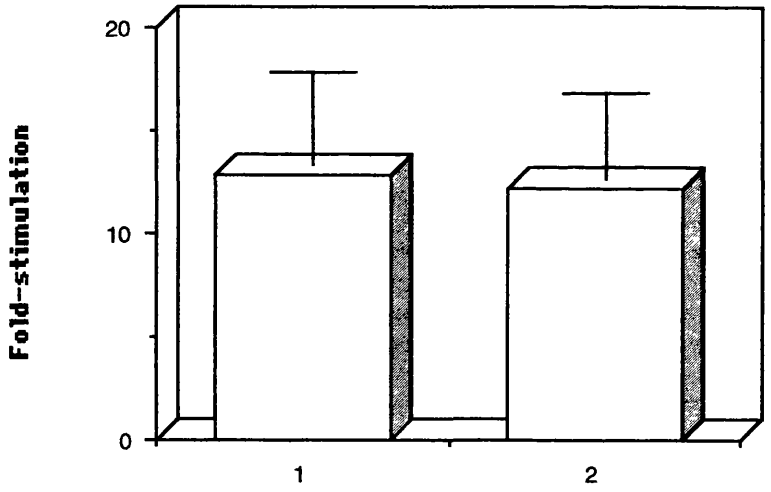


Fig.6C

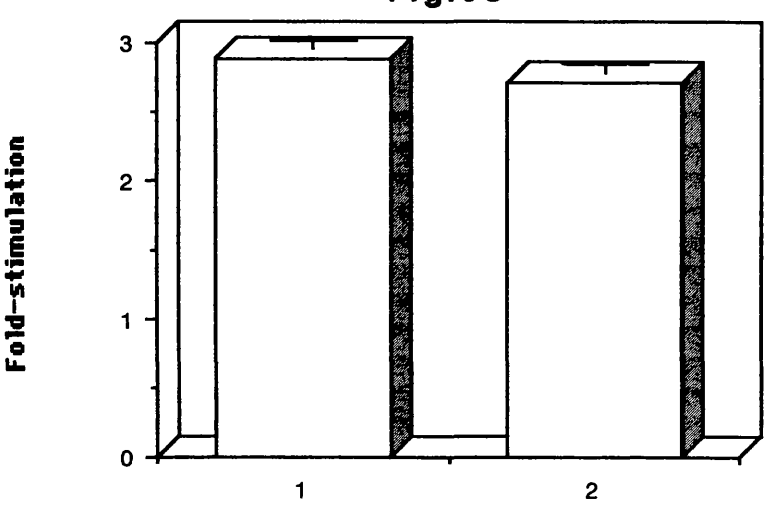


Fig. 6. The action of MK886 on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig. 6A. Results of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10 µl ml^{-1} PHA-P.

3, +10 µl ml^{-1} PHA-P and 100 nM MK886.

The bars indicate the range of duplicate measurements.

Fig. 6B. Pooled data from 8 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10µl ml^{-1} PHA-P compared to control.

1, PHA-P alone.

2, PHA-P 10 µl ml^{-1} + 100 nM MK886.

The bars indicate the SEM.

n=8, p=0.34

Fig. 6C. Pooled data from 4 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 12 µl ml^{-1} OKT3.

1, OKT3 alone.

2, OKT3 + 100 nM MK886.

The bars indicate the SEM.

n=4, p=0.18

Fig.7A

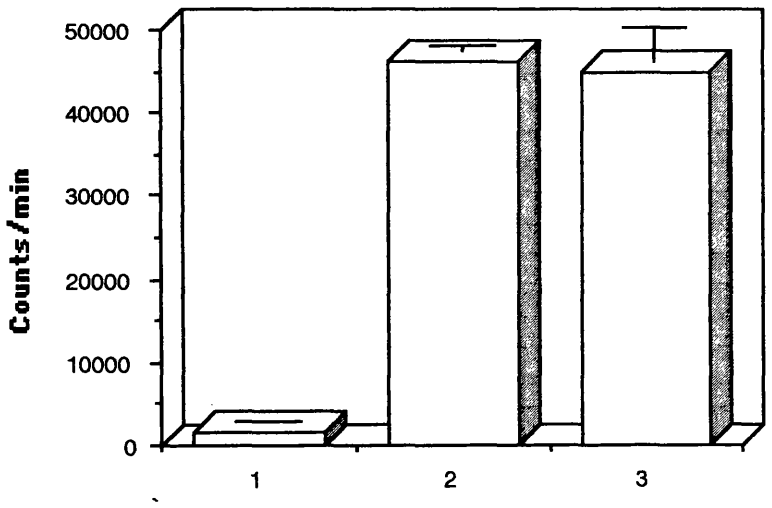


Fig.7B

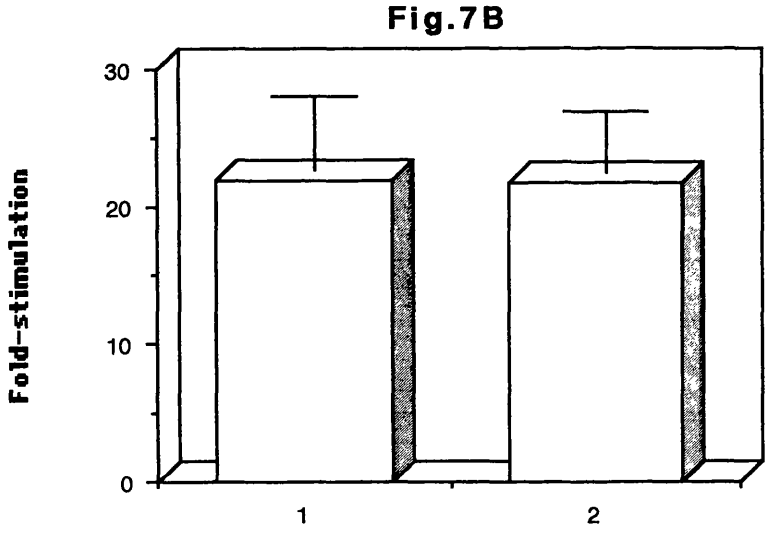


Fig.7C

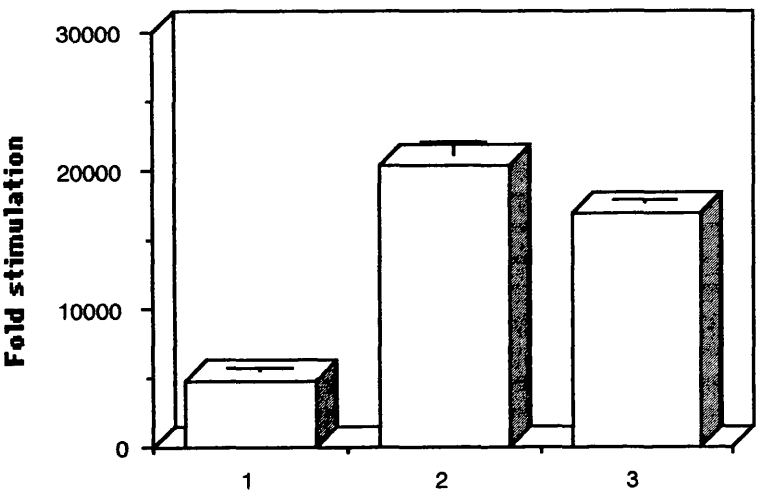


Fig. 7. The action of BWA 4C on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig. 7A. Results of a typical experiment.

The ordinate shows counts min^{-1} in inositol phosphate.

1, no ligand.

2, + 10µl ml^{-1} PHA-P.

3, + 10µl ml^{-1} PHA-P and 100 nM BWA 4C

The bars indicate the range of duplicate measurements.

Fig. 7B. Pooled data from 4 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10 µl ml^{-1} PHA-P compared to control.

1, PHA-P alone.

2, PHA-P 10µl ml^{-1} + 100nM BWA 4C.

The bars indicate the SEM.

n=4 , p=0.87

Fig. 7C. Pooled data from 4 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 12 µl ml^{-1} OKT3.

1, OKT3 alone.

2, OKT3 + 100 nM BWA 4C.

The bars indicate the SEM.

n=4 , p=0.19

Fig.8A

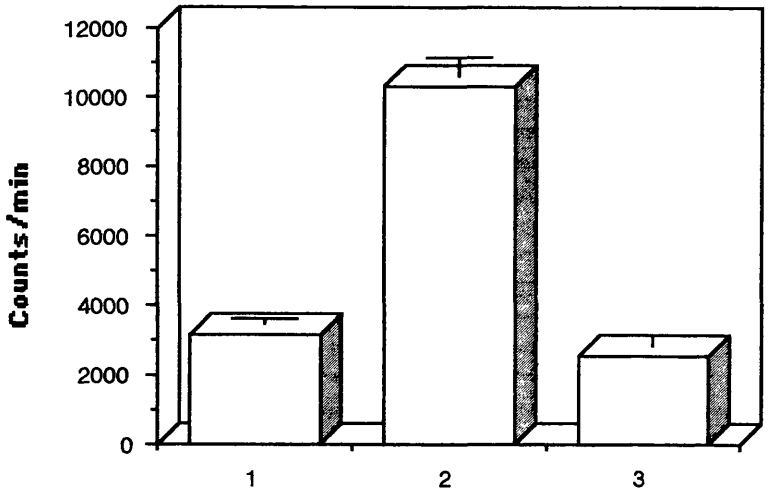


Fig.8B

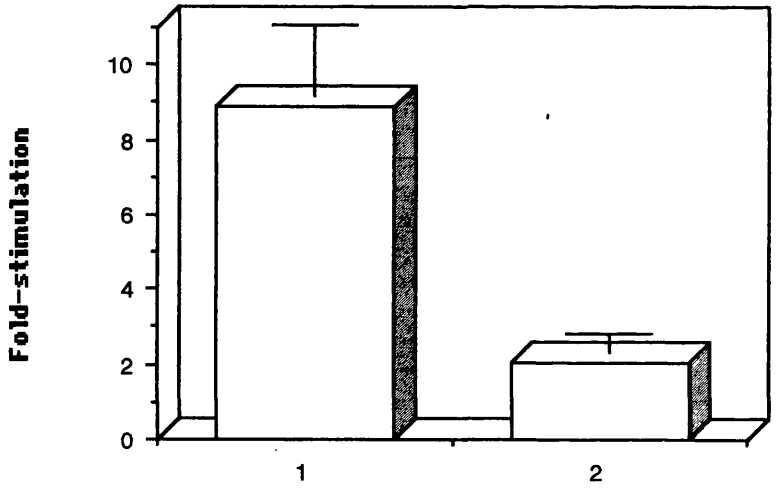


Fig.8C

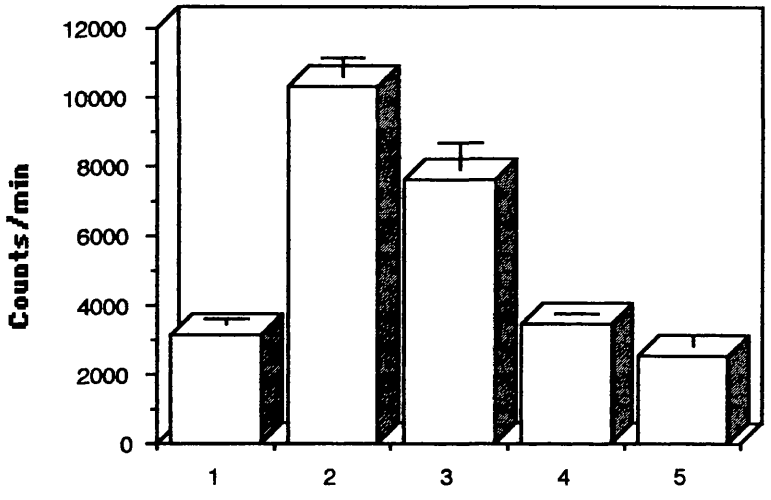


Fig.8. The action of BHT on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig. 8A. Result of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphate.

1, no ligand.

2, +10µl ml^{-1} PHA-P

3, +10µl ml^{-1} PHA-P and 10µM BHT.

The bars indicate the range of duplicate measurements.

Fig. 8B. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10 µl ml^{-1} PHA-P compared to control.

1, PHA-P alone.

2, +10µM BHT and PHA-P

The bars indicate the SEM.

n=6, p=0.054

Fig.8C. Dose-response curve.

The ordinate shows the counts min^{-1} in inositol phosphate.

1, no ligand. 2, +10µl PHA-P.

3, +2.5µM BHT+ PHA-P

4, +5µM BHT+ PHA-P

5, +10µM BHT+ PHA-P

The bars indicate the range of duplicate measurements.

Fig.8D

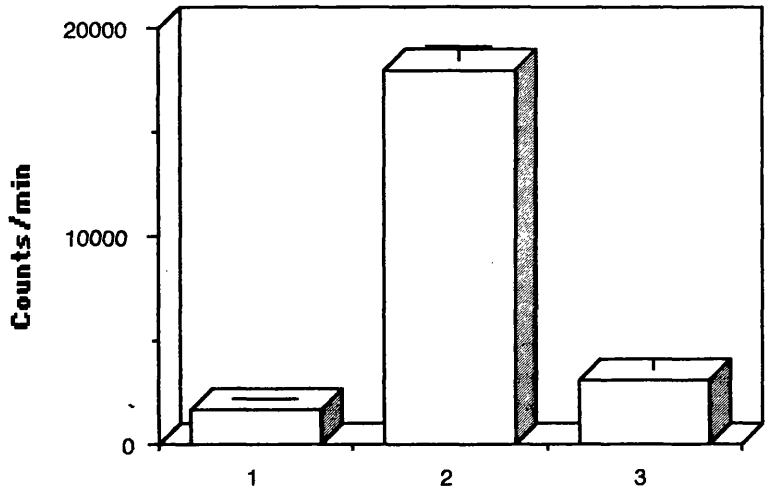

Counts/min
20000
10000
0
1
2
3

Fig.8E

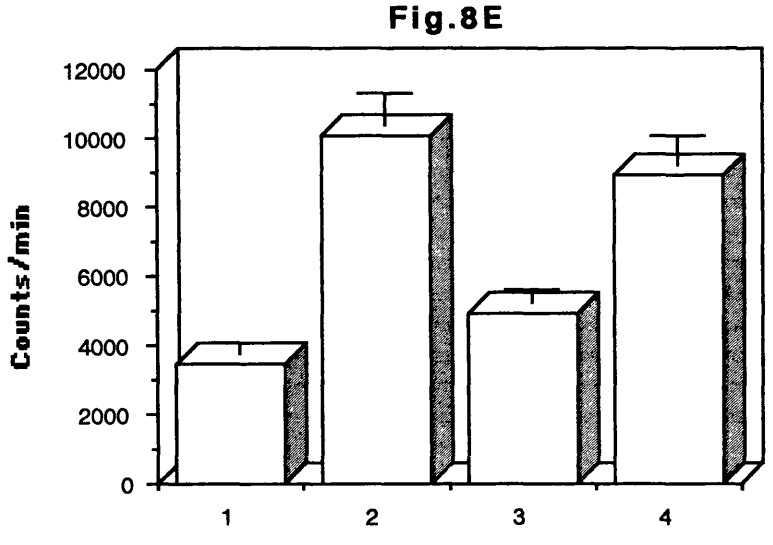

Counts/min
12000
10000
8000
6000
4000
2000
0
1
2
3
4

Fig.8D. Results of a typical experiment.

The ordinate shows counts min^{-1} in inositol phosphate.

1, no ligand.

2, OKT3 alone.

3. OKT3 + 10 µM BHT.

The bars indicate the range of duplicate measurements.

Fig.8E.[^{3}H]inositol pre-labelled T lymphocytes were incubated with and without 10 µM BHT for 30 min, and the PHA- stimulated generation of inositol phosphate was measured.One half of each culture was washed free of the drug immediately before the addition of PHA.

The ordinate shows counts min^{-1} in inositol phosphate.

1, no ligand.

2, + 10 µl ml^{-1} PHA-P.

3, + 10µM BHT+ PHA-P.

4, + 10 µl ml^{-1} PHA-P added immediately after washing culture free of drug.

The bars indicate the range of duplicate measurements.

Fig.9A

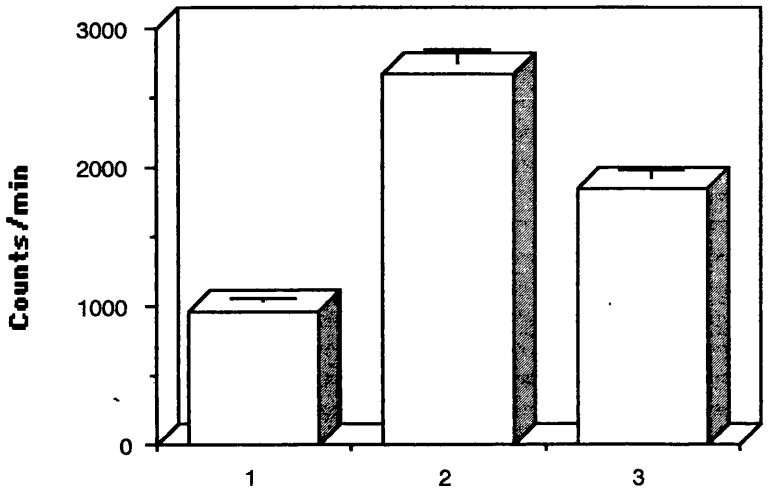

Counts/min
3000
2000
1000
0
1
2
3

Fig.9B

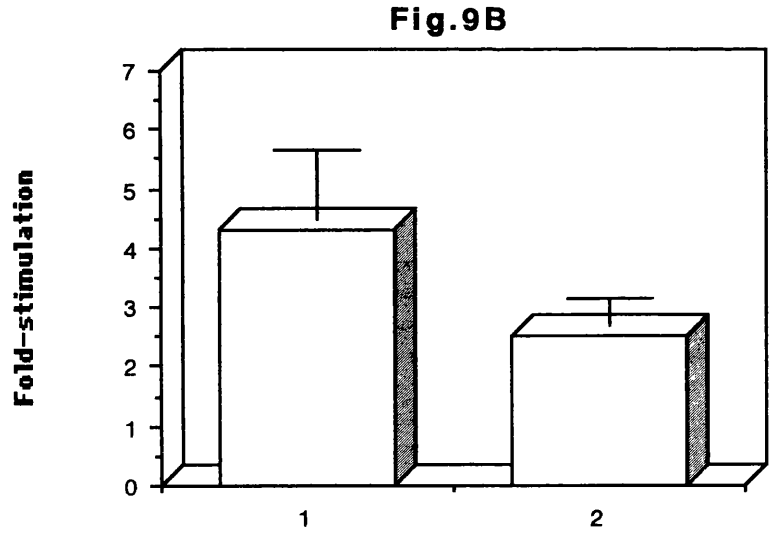

Fold-stimulation
7
6
5
4
3
2
1
0
1
2

Fig. 9. The action of BHA on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig.9A. Result of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphate.

1, no ligand.

2, +10µl PHA-P

3, +100µM BHA+ PHA-P

The bars indicate the range of duplicate measurements.

Fig. 9B. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10µl ml^{-1} PHA-P

1,PHA-P alone.

2,+100 µM BHA and PHA-P.

The bars indicate the SEM.

n=6 , p=0.06

Fig.10A

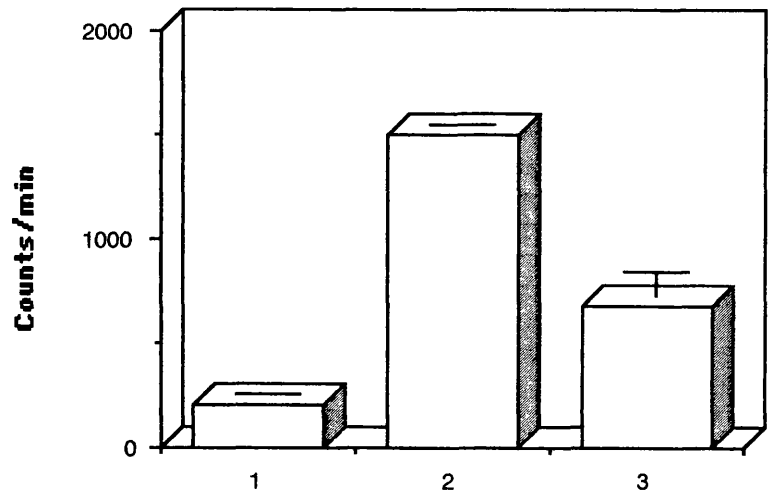


Fig.10B

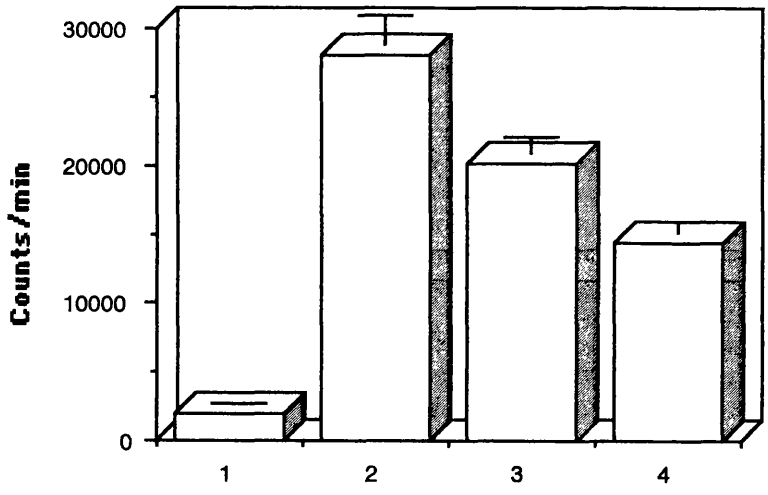


Fig.10C

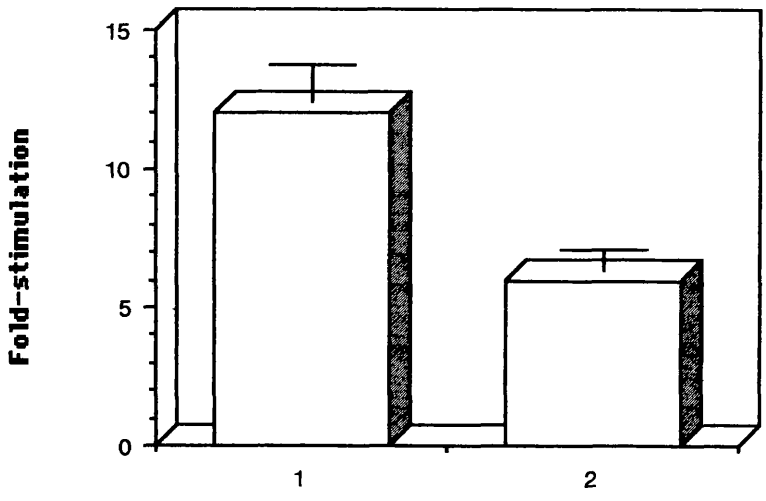


Fig. 10. The action of N-acetyl-cysteine on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig. 10A. Results of a typical experiments.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl PHA-P.

3,+20mM N-acetyl-cysteine+ PHA-P

The bars indicate the range of duplicate measurements.

Fig. 10B. Dose-response curve.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl PHA-P

3,+10mM N-acetyl-cysteine and PHA-P.

4,+20mM N-acetyl-cysteine and PHA-P

The bars indicate the range of duplicate measurements.

Fig. 10C. Pooled data from 8 experiments.

The ordinate indicates the fold-stimulation of inositol phosphates generation by 10µl ml^{-1} PHA-P

1, PHA-P alone.

2, +20mM N-acetyl-cysteine and PHA-P.

The bars indicate the SEM.

n=8, p=0.06

Fig.11A

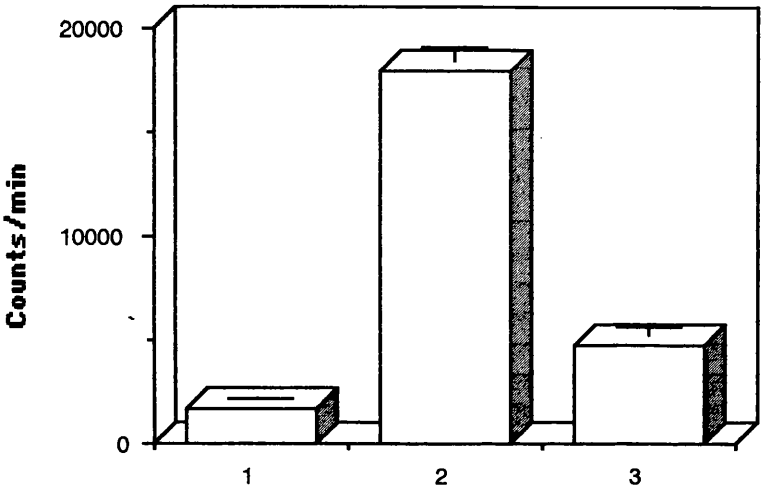


Fig.11B

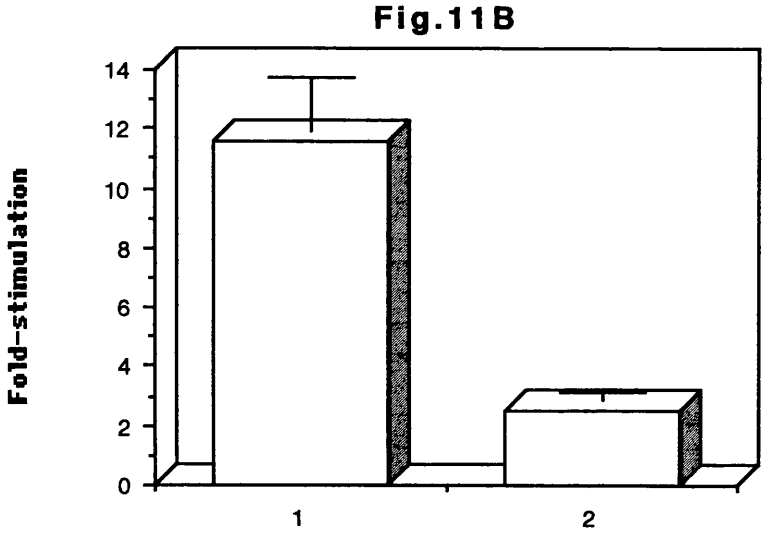


Fig. 11. The action of DTT on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig. 11A. Results of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P

3, +5mM DTT and PHA-P

The bars indicate the range of duplicates measurements.

Fig. 11B. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10µl ml^{-1} PHA-P compared to control.

1, PHA-P alone.

2, +5mM DTT and PHA-P

The bars indicate the SEM.

n=6 , p= 0.060

Fig. 12A

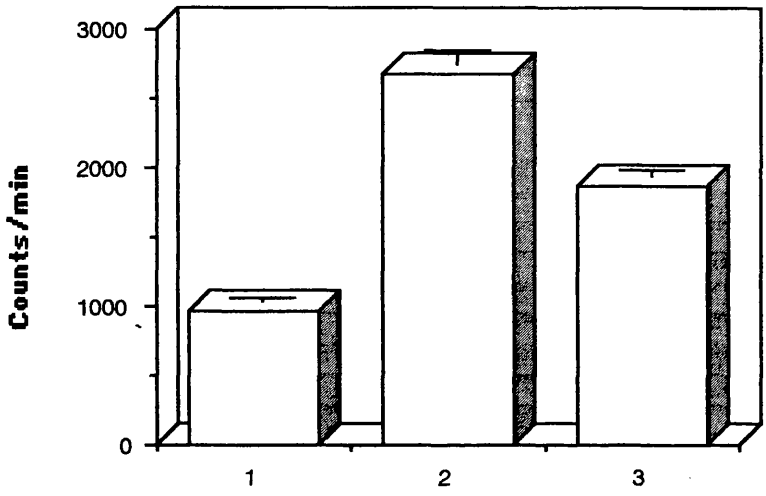


Fig.12B

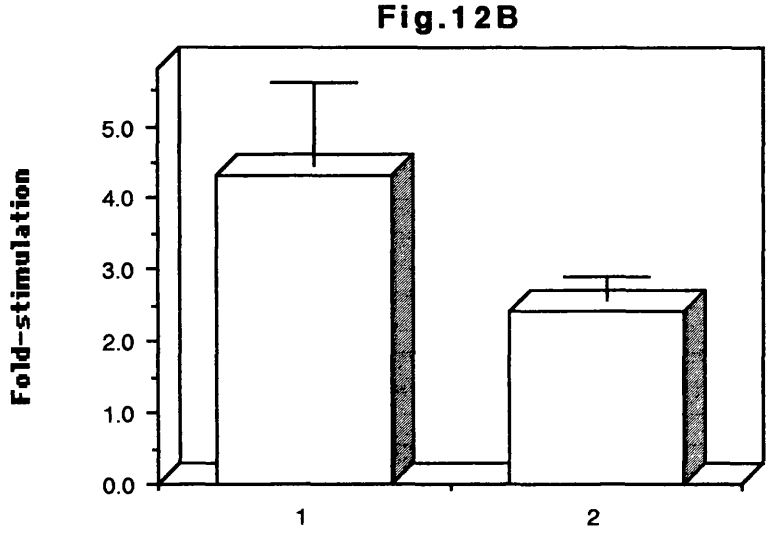


Fig.12. The action of ferricyanide on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig.12A. Results of a typical experiments.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P

3, +5mM ferricyanide and PHA-P

The bars indicate the range of duplicate measurements.

Fig. 12B. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphates generation by 10µl ml^{-1} PHA-P

1, PHA-P alone.

2, +5mM ferricyanide and PHA-P

The bars indicate the SEM.

n=6, p=.06

Fig.13A

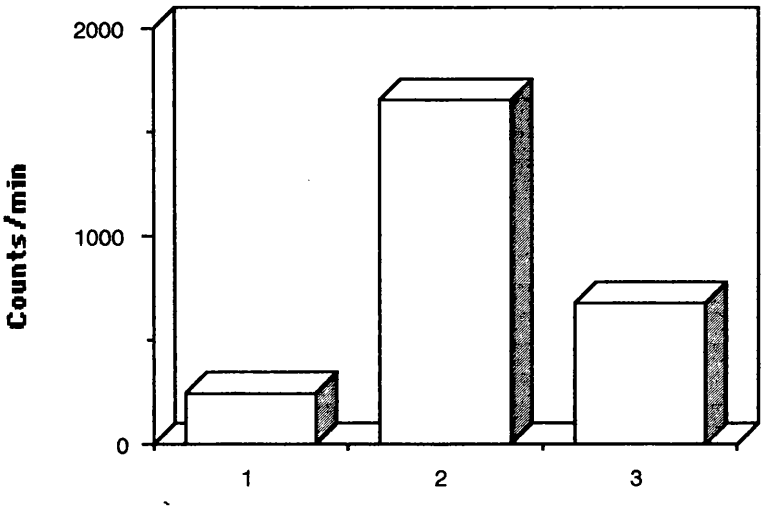


Fig.13B

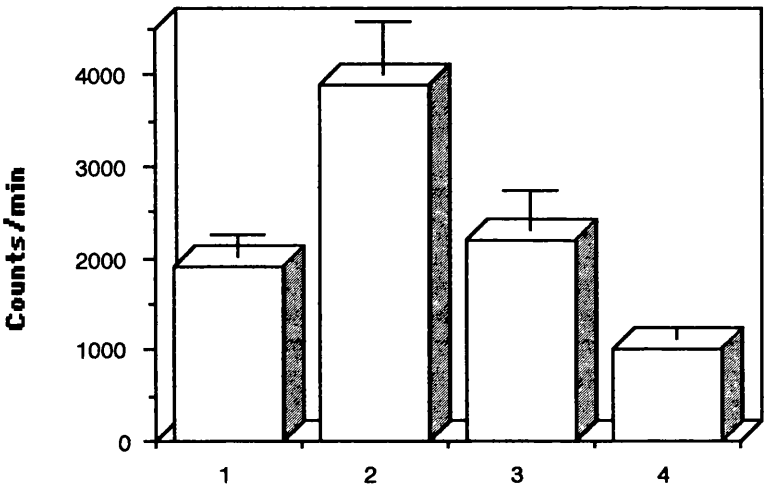


Fig.13C

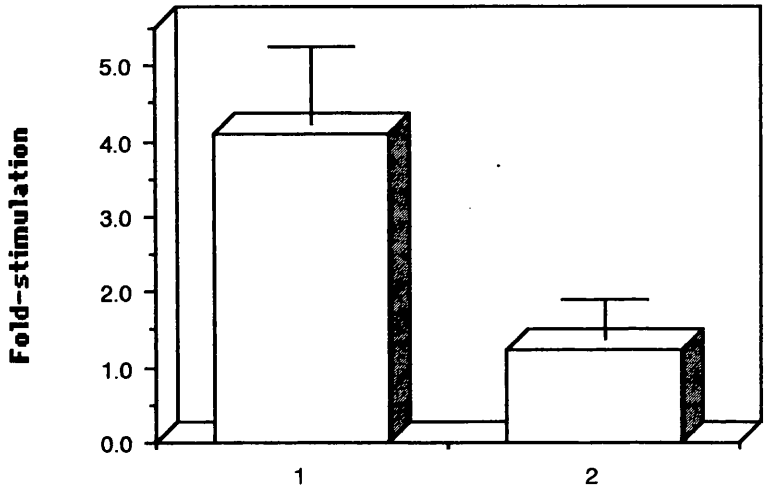


Fig.13. The action of BAB on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig. 13A. Results of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3, +10µM BAB and PHA-P

The bars indicate the range of duplicate measurements.

Fig. 13B. Dose-response curve.

1, no ligand.

2, +10µl ml^{-1} PHA-P

3, +5µM BAB and PHA-P

4, +10µM BAB and PHA-P

The bars indicate the range of duplicate measurements.

Fig. 13C. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10µl ml^{-1} PHA-P compared to control.

1, PHA-P alone

2, PHA-P + 10µM BAB

The bars indicate the SEM.

n=6, p= 0.05

Fig.14

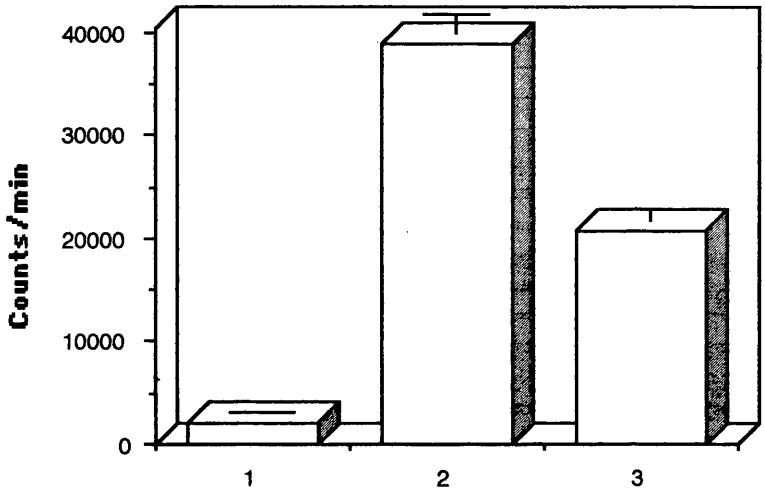

Counts/min
40000
30000
20000
10000
0
1
2
3

Fig.15

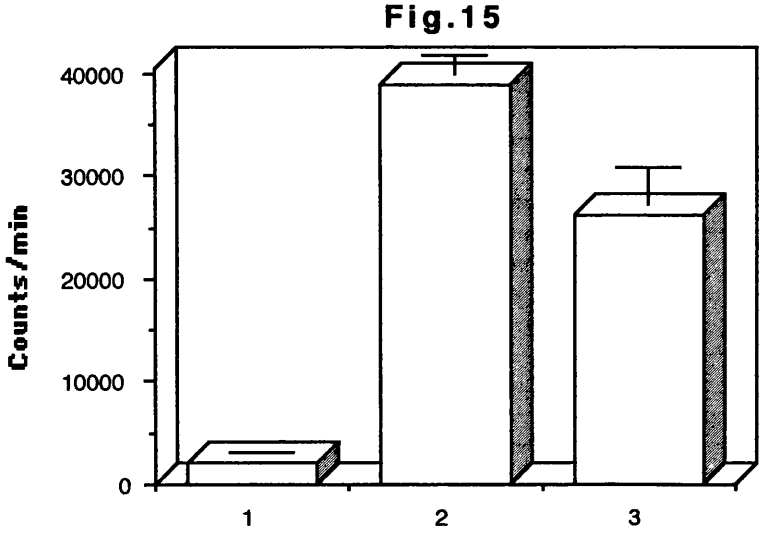

Counts/min
40000
30000
20000
10000
0
1
2
3

Fig.14. The action of Ro 31-4493 on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3, +25µM Ro 31-4493 and PHA-P

The bars indicate the range of duplicate measurements.

Fig.15.The action of Ro 31-4639 on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2,+10µl ml^{-1} PHA-P.

3, +25µM Ro 31-4693 and PHA-P

The bars indicate the range of duplicate measurements.

Fig.16A

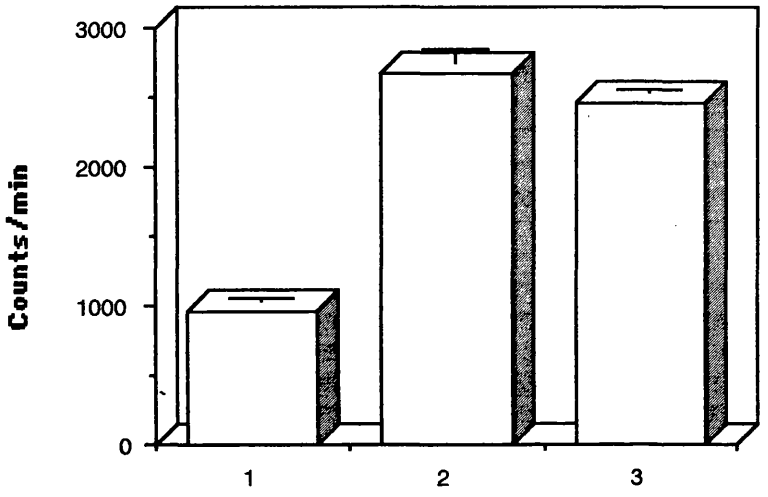


Fig.16B

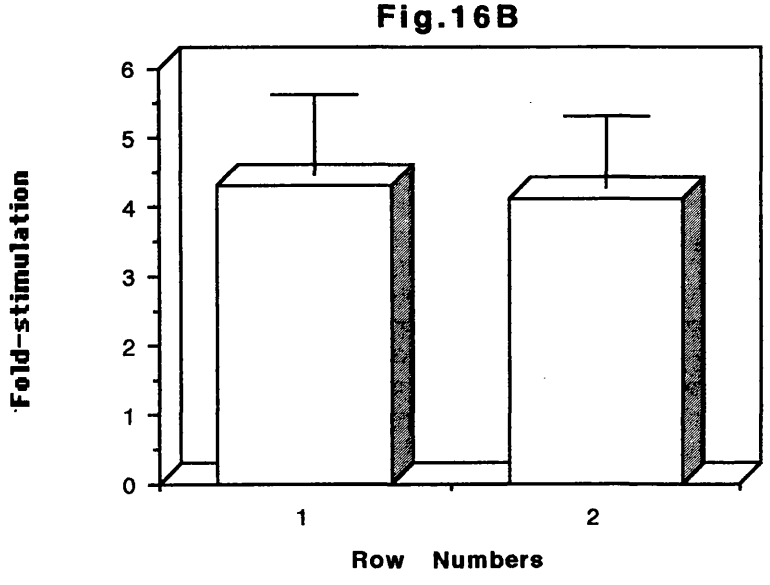


Fig.16. The action of DFO on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig.16A.Results of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3, +500µM DFO and PHA-P

The bars indicate the range of duplicate measurements.

Fig. 16B. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphates generation by 10 µl ml^{-1} PHA-P compared to control.

1, PHA-P alone.

2, +500 µM DFO and PHA-P

The bars indicate the SEM.

n=6 , p=0.368

Fig.17A

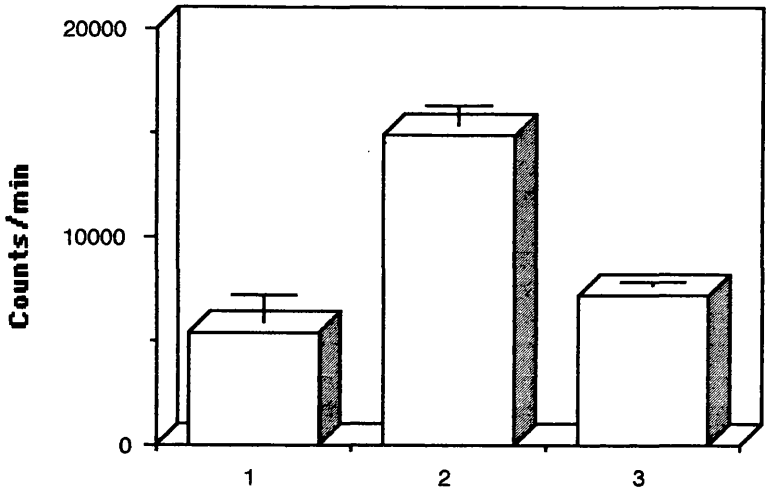


Fig.17B

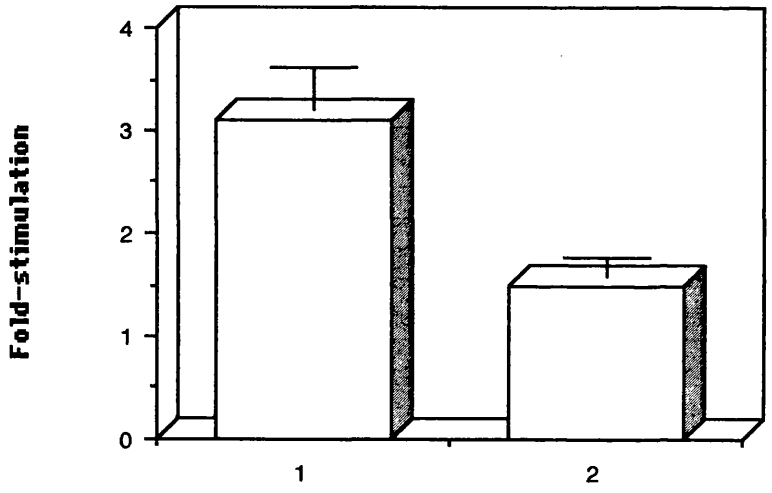


Fig.18

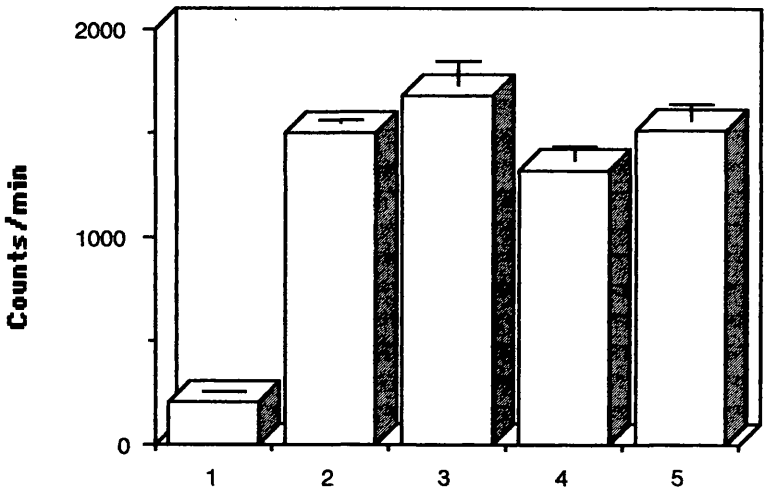


Fig. 17. The action of D609 on mitogen stimulated inositol lipid breakdown in T lymphocytes.

Fig.17A. Results of a typical experiment.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, + 10 µl ml^{-1} PHA-P.

3, +100µM D609 and PHA-P.

The bars indicate the range of duplicate measurements.

Fig. 17B.Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphates generation by 10µl ml^{-1} PHA-P compared to control.

1, PHA-P alone. 2, +100µM D609 and PHA-P

The bars indicate the SEM.

n=6, p=0.055

Fig. 18. The action of NMMA on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand. 2, + 10µl ml^{-1} PHA-P

3, +100µM NMMA and PHA-P.

4, +200µM NMMA and PHA-P

5, +100µM NMMA+ 1mM Arginine and PHA-P

The bars indicate the range of duplicate measurements

Fig.19A

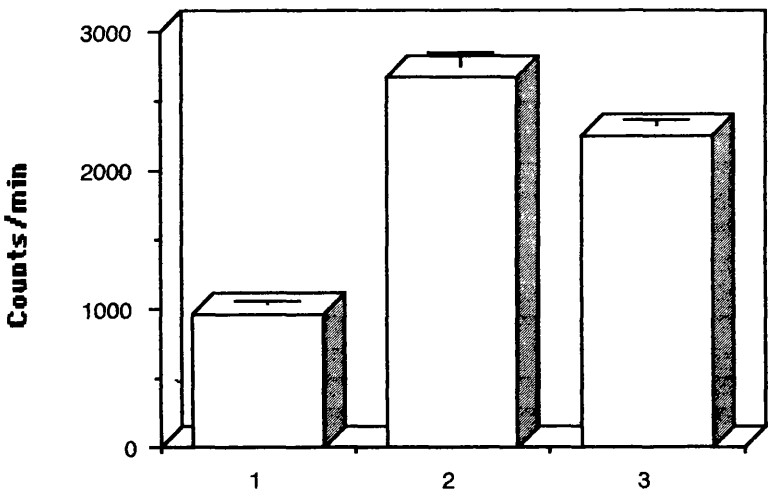


Fig.19B

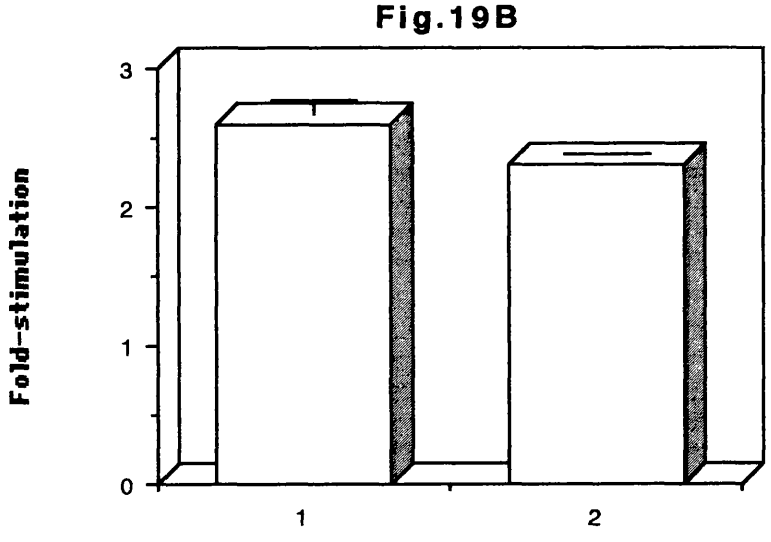


Fig.19. The action of cycloheximide on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig. 19A. Results of a typical experiment.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3,+ 1µg ml^{-1} cycloheximide and PHA-P

The bars indicate the range of duplicate measurements.

Fig.19B. Pooled data from 4 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10µl ml^{-1} PHA-P compared to control.

1, PHA-P alone.

2, + 1 µg ml^{-1} cycloheximide and PHA-P.

The bars indicate the SEM.

n=4, p=0.258

Fig.20A

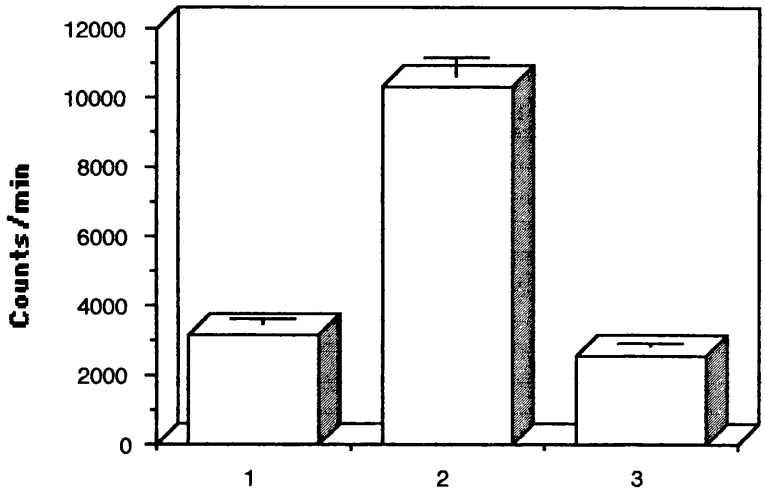


Fig.20B

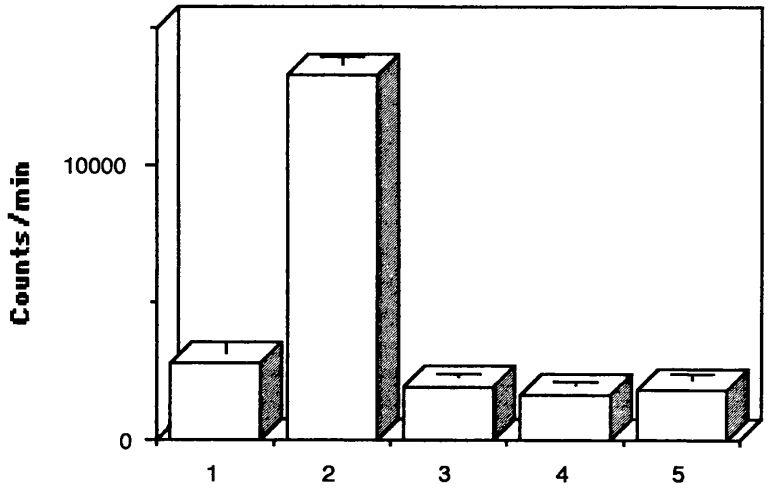


Fig.20C

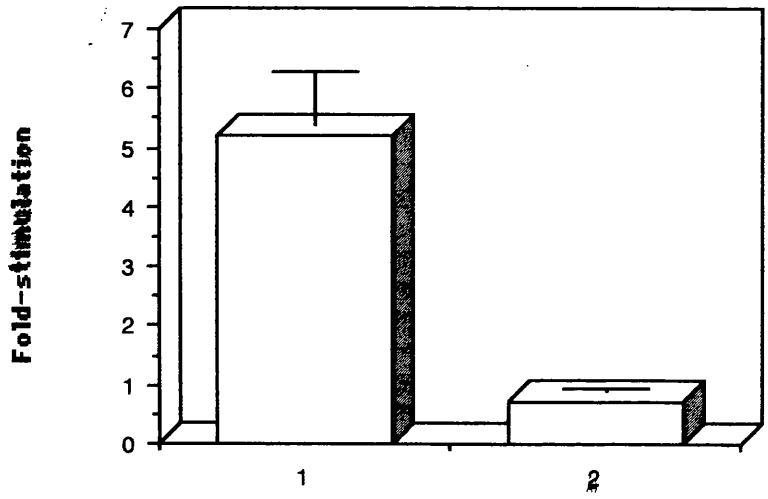


Fig.20. The action of staurosporine on mitogen-stimulated inositol lipid breakdown in T lymphocytes.

Fig. 20A. Result of a typical experiment.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3, +1µM staurosporine and PHA-P

The bars indicate the range of duplicate measurements.

Fig. 20B.Effects of increasing concentrations of staurosporine on PHA-stimulated inositol lipid breakdown in T lymphocytes.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand. 2, +10µl PHA-P.

3, +100nM staurosporine and PHA-P.

4, +500nM staurosporine and PHA-P.

5, +1µM staurosporine and PHA-P

The bars indicate the range of duplicate measurements.

Fig. 20C. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10µl ml^{-1} PHA-P compared to control.

1,PHA-P alone. 2, +1µM staurosporine and PHA-P

The bars indicate the SEM.

n=6, p=0.05.

[a] **Table 1 Actions of inhibitors on Ptd Ins 4,5-P2-PLC in vitro**

Drug	Concentration	PtdIns4,5-P2-PLC (pmol/30 min) [a]
NDGA	_	110(+10)
	20μM	80(+6)
	50μM	39(+5)
Ro 31-4493/001	_	110+14
	25 μM	47+8
	50 μM	47+8
	100μM	30+6
Ro 31-4639/001	_	110+14
	50μM	36+1
	100μM	25+1
BW755C	_	210(+20)
	25μM	150(+15)
	100μM	105(+11)
piriprost	_	155(+8)
	25μM	146(+11)
	50μM	158(+4)
MK886	_	56(+2)
	50nM	52(+17)
	100nM	47(+5)
BWA4C	_	76(+9)
	100nM	60(+19)
BHT	0μM	76(+9)
	10μM	125(+15)
N-acetylcysteine	_	76(+9)
	20mM	123(+11)

---

[a] **Ptd Ins-4,5-P2 -PLC activity as described in Materials and Methods**

TABLE 2

Radioimmunoassay of leukotriene B4 and 15-HETE in supernatants of T lymphocyte cultures and whole blood.

Cells	Additions	Leukotriene B_4 ($ng.ml^{-1}$)	15-HETE ($ng.ml^{-1}$)
T lymphocytes:	None	<0.5	<0.6
	1 μM A23187	25.6(±3)	31.7(±5.8)
	PHA	<0.5	<0.6
Whole blood:	No addition	<0.5	nd
	1 μM A23187	285(±110)	nd

T lymphocytes (10^6. ml^{-1} in RPMI medium) or human whole blood were incubated at 37°C for 30 min with the indicated additions. The supernatants of triplicate cultures were analyzed for leukotriene B_4 or 15-HETE by radioimmunoassay.

4.11 Discussion

I have studied the effects of LO inhibitors on mitogen-stimulated inositol lipid breakdown, one of the specific signal transduction pathways which regulates the proliferation of haemopoietic cells. Piriprost, NDGA, and BW755C inhibited PHA- stimulated breakdown of inositol lipid in human T lymphocytes. However, inositol lipid breakdown in PHA and CD3 monoclonal antibody(McAb) stimulated T lymphocytes was not affected by specific 5-LO inhibitors BWA4C and MK886. Furthermore, LT generation was not detected following PHA or anti CD3 stimulation of T lymphocytes. These results suggested that inhibition of mitogen-stimulated inositol lipid breakdown by NDGA, BW755C, and piriprost must be due to a mechanism other than the inhibition of lipoxygenase. Because the above inhibitors also have antioxidant properties, I studied the effects of several general antioxidants on growth regulatory signal transduction pathways. Butylated hydroxytoluene (BHT), DTT , and N-acetylcysteine abrogated PHA- and CD3 McAb- stimulated inositol lipid breakdown in T lymphocytes.

These antioxidants inhibitors did not have a direct action on the Ptd Ins 4,5-P2-PLC enzyme which catalyses inositol lipid breakdown, suggesting that the target for these compounds is an obligatory step in the coupling of TCR the with the inositol lipid pathway, which is regulated by a signalling process dependent on the generation of reactive free radical species. Others have shown that several aspects of T lymphocytes activation, including lymphokine secretion, DNA synthesis and Ca^{++} fluxes are also impaired by antioxidants (Dornand et

al 1989; Chaudhri et al 1986; Gukovskaya et al 1989).

Recent evidence suggests that the controlled generation of oxidative metabolites plays important roles in diverse cellular signalling mechanisms (Schreck et al 1991; Abate et al 1990).The data presented here are consistent with the interpretation that the generation of an oxidative metabolite may play a role in the activation of the inositol lipid signalling system following the ligation of cell-surface receptors of T lymphocytes. I have observed that the inhibition of ligand-stimulated inositol phosphate generation by T lymphocytes by the general antioxidants BHT is immediately reversed by washing the cells with serum-containing medium. This implies that BHT did not irreversibly damage the coupling mechanism in T lymphocyte.

I have carried out preliminary experiments in an attempt to characterize the putative oxidative metabolite involved in signal transduction. The generation of the free radical nitric oxide is involved in inter- and intra-cellular signalling mechanisms which depend on its ability to activate guanylate cyclase (Murad et al 1990). Although nitric oxide may be generated by ligand-stimulated T lymphoid cells (Kirk et al 1990), 100μM N-methyl L-arginine, an inhibitor of nitric oxide generation (Gukovskaya et al 1989), did not impair the PHA-stimulated generation of inositol phosphates by T lymphocytes. I was also unable to detect the generation of thiobarbituric acid-reactive lipid peroxides.Therefore, the chemical nature of a putative oxidative metabolite involved in coupling of ligand receptors to the inositol lipid signalling system remains to be determined.

CHAPTER 5

THE ACTIONS OF ANTIOXIDANTS,LIPOXYGENASE INHIBITORS AND OTHER COMPOUNDS ON LIGAND-STIMULATED INOSITOL LIPID BREAKDOWN IN JURKAT CELLS.

Stimulation of the Jurkat T lymphoblastic leukaemia cell line by PHA and monoclonal antibodies reactive with the T cell antigen receptor complex(CD3/Ti) lead to the activation of the inositol lipid signalling pathway.This pathway triggers the secretion of lymphokines in this cell line(Goldsmith and Weiss 1988; Imboden and Pattinson 1987).

In Chapter 4, I have shown that the activation of the inositol lipid signalling pathway by PHA and by OKT3 was abrogated by LO inhibitors and antioxidants suggesting that an oxidant-sensitive step is important in the coupling mechanism of normal T lymphocytes. Here I have studied the effects of 5-LO inhibitors and antioxidants on PHA- and OKT3- stimulated generation of inositol phosphate in Jurkat cells.

Results

5.1 Actions of lipoxygenase inhibitors s on ligand- stimulated inositol lipid breakdown in the Jurkat T lymphoblastic leukaemia

cell line.

PHA-stimulated inositol phosphate generation by Jurkat cells was not decreased by piriprost(Fig.1). In fact, piriprost enhanced inositol phosphate generation by ligand stimulated generation in these cells. These observations which are in marked contrast with those using T lymphocytes(Chapter.4,Fig.5), imply that the biochemical mechanisms which couple cell surface receptors to the inositol lipid signalling system are different in normal peripheral blood T lymphocytes and Jurkat leukaemia cells.

MK886 did not inhibit PHA and OKT3-stimulated inositol phosphate generation in Jurkat cells(Fig.2A,2B).

5.2 The action of antioxidants on ligand-stimulated inositol lipid breakdown in Jurkat cells.

The lipid soluble antioxidants BHT and BHA also failed to abrogate this early signalling event.BHT did not inhibit PHA- and OKT3-stimulated generation of inositol phosphate(Fig.3A,3B,3C), similarly BHA failed to decrease PHA- and OKT3-stimulated generation of inositol phosphate in these cells.(Fig.4A,4B,4C).

5.3 The action of sulphydryl reagents on ligand-stimulated inositol lipid breakdown in Jurkat cells.

The sulphydryl reagents N-acetylcysteine and dithiothreitol inhibited PHA-

stimulated inositol lipid breakdown in Jurkat cells(Fig.5,6A,6B)

5.4 Actions of staurosporine on ligand-stimulated inositol lipid breakdown in Jurkat cells.

Staurosporine, a protein kinase inhibitor, decreased ligand stimulated generation of inositol phosphate in Jurkat cells (Fig.7).

Fig.1

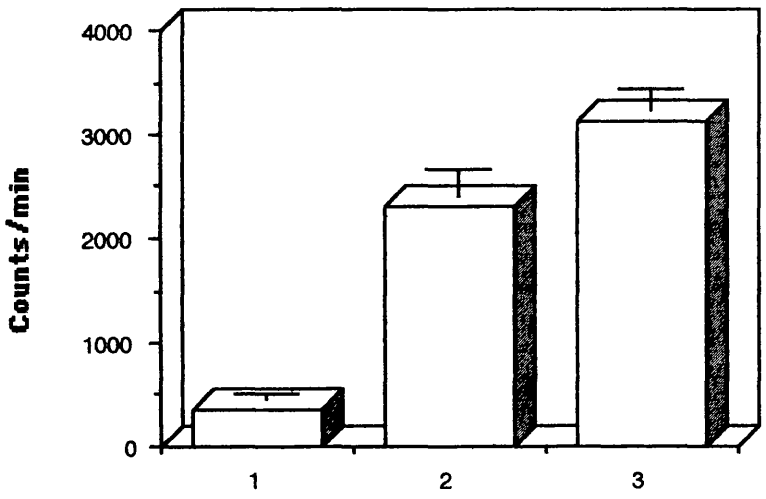


Fig. 2A

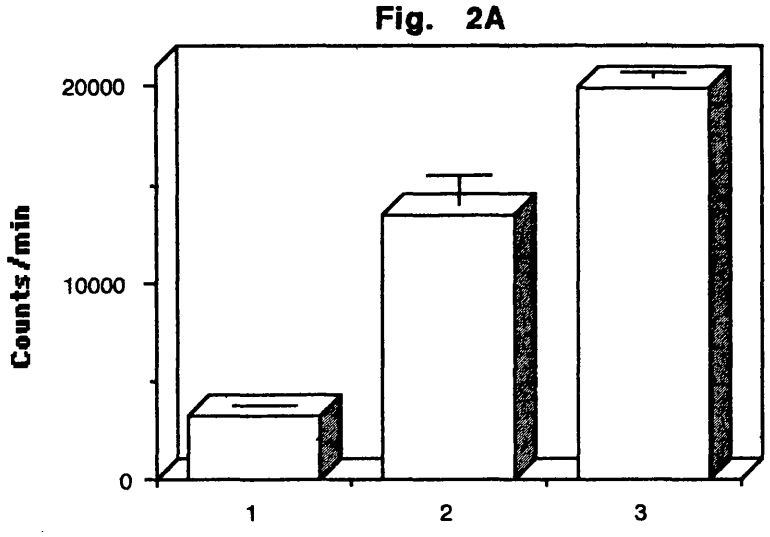


Fig.2B

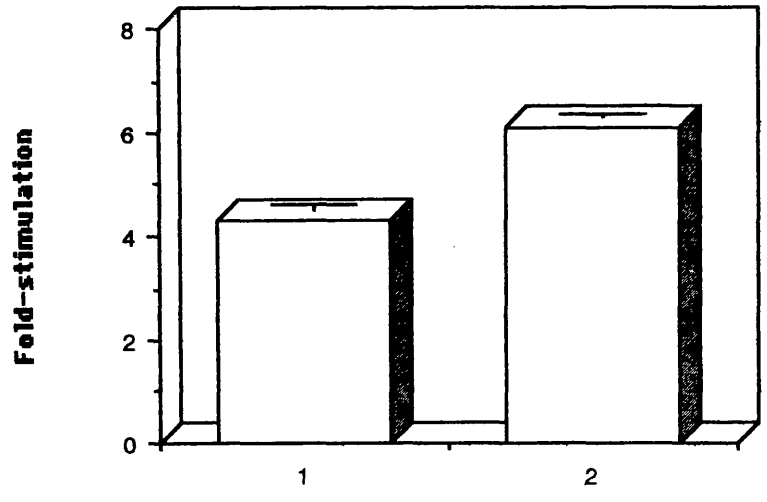


Fig.1. The action of piriprost on ligand-stimulated inositol lipid breakdown in the Jurkat leukaemia cell line.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3, +50µM Piriprost and PHA-P.

The bars indicate the range of duplicate measurements.

Fig.2. The action of MK886 on ligand-stimulated inositol lipid breakdown in Jurkat leukaemia cells line.

Fig. 2A. Results of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl PHA-P.

3, +100nM MK886 and PHA-P.

The bars indicate the range of duplicate measurements.

Fig.2B. Pooled data from 4 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 12µl ml^{-1} OKT3, compared to control.

1, OKT3 alone.

2, +100nM MK886 and OKT3.

The bars indicate the SEM.

n=4, p=0.086

Fig.3A

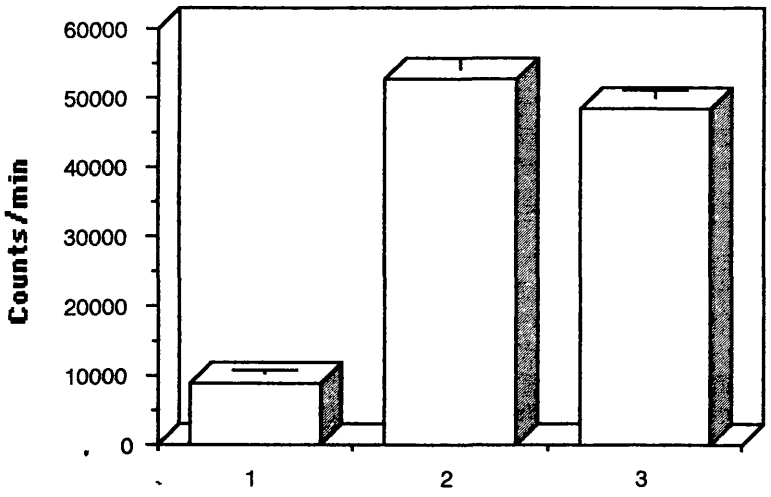


Fig.3B

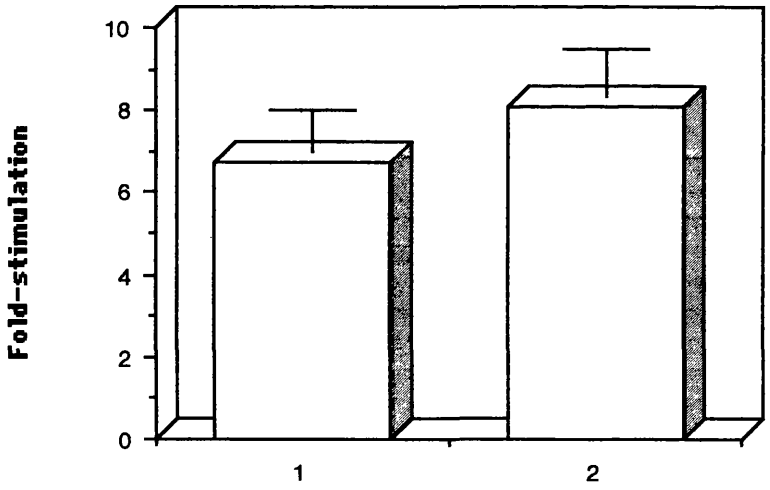


Fig.3C

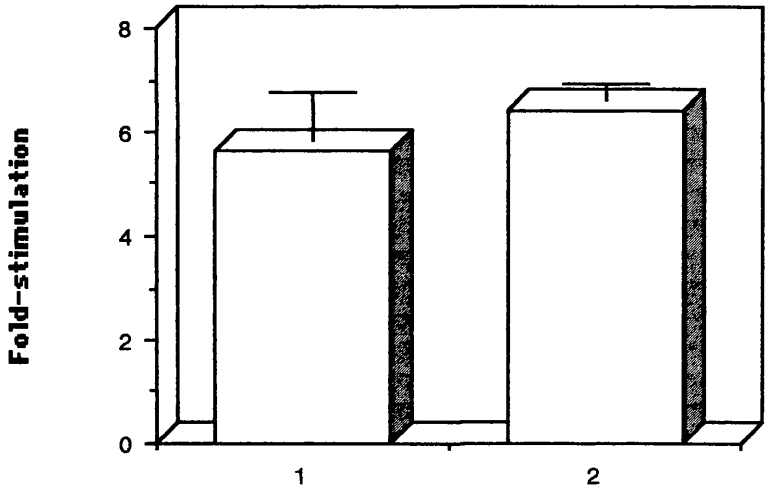


Fig.3. The action of BHT on ligand-stimulated inositol lipid breakdown in the Jurkat leukaemia cell line.

Fig.3A. Results of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3, 10µM BHT and PHA-P.

The bars indicate the range of duplicate measurements,

Fig.3B. Pooled data from 8 experiments.

The ordinate indicates the fold-stimulation of inositol phosphate generation by 10µl ml^{-1} PHA-P compared to control.

1, PHA-P alone.

2, +10µM BHT and PHA-P

The bars indicate the SEM.

n=8, p=0.081.

Fig.3C. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphates generation by 12 µl ml^{-1} OKT3, compared to control.

1,OKT3 alone.

2, +10µM BHT and OKT3.

The bars indicate the SEM.

n=6, p=0.478.

Fig.4A

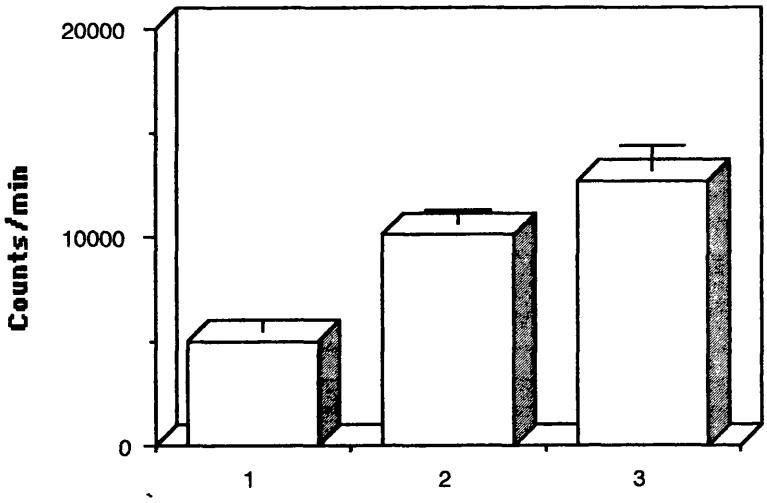


Fig.4B

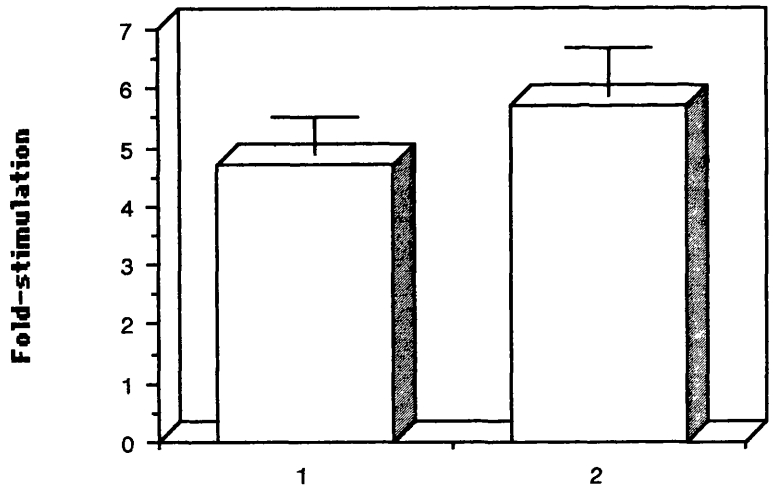


Fig.4C

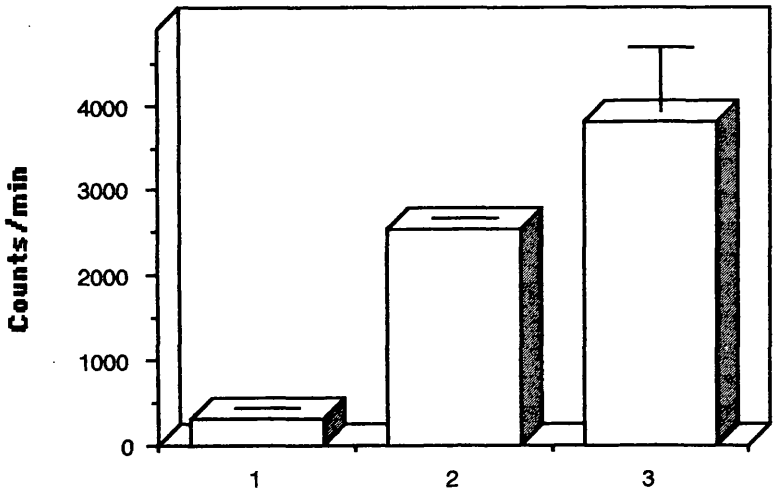


Fig.4. The action of BHA on ligand-stimulated inositol lipid breakdown in the Jurkat leukaemia cells line.

Fig.4A. Results of a typical experiment.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3, +100µM BHA and PHA-P.

The bars indicate the range of duplicate measurements.

Fig.4B. Pooled data from 4 experiments.

The ordinate indicates the fold stimulation of inositol phosphate generation by 10µl ml^{-1} PHA-P, compared to control.

1, PHA-P alone.

2,+100µM BHA and PHA-P

The bars indicate the SEM.

n=4, p=0.20

Fig.4C. The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +12µl ml^{-1} OKT3.

3, +100µM BHA and OKT3.

The bars indicate the range of duplicate measurements.

Fig.5

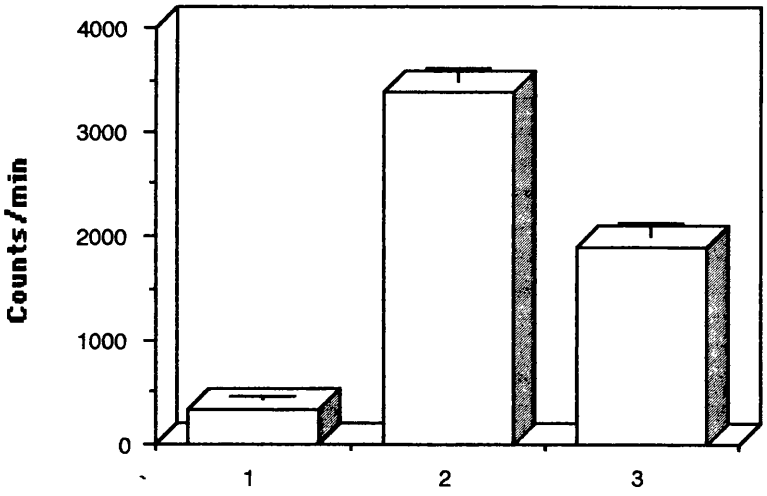


Fig.6A

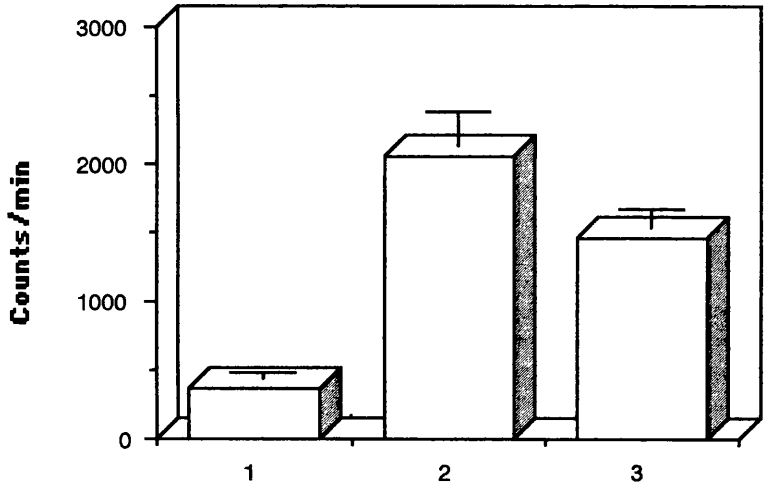


Fig.6B

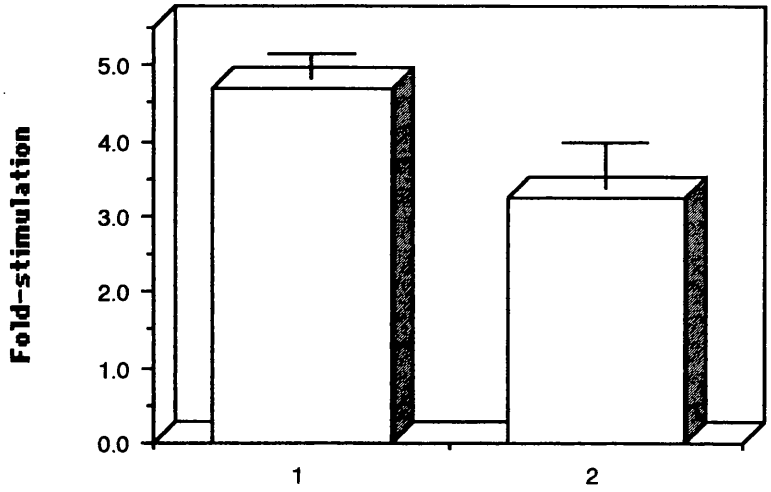


Fig.5.The action of N-acetylcysteine on ligand-stimulated inositol lipid breakdown in the Jurkat leukaemia cell line.

The ordinate shows the counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl PHA-P.

3, +20mM N-acetylcysteine and PHA-P

The bars indicate the range of duplicate measurements.

Fig.6. The action of DTT on ligand-stimulated inositol lipid breakdown in the Jurkat leukaemia cells line.

Fig.6A. Results of a typical experiment.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3,+1mM DTT and PHA-P.

The bars indicate range of duplicate measurements.

Fig.6B. Pooled data from 6 experiments.

The ordinate indicates the fold-stimulation of inositol phosphates generation by 10µl ml^{-1} PHA-P, compared to control.

1, PHA-P alone.

2, +1mM DTT and PHA-P

The bars indicate the SEM.

n=6, p=0.045.

Fig.7A

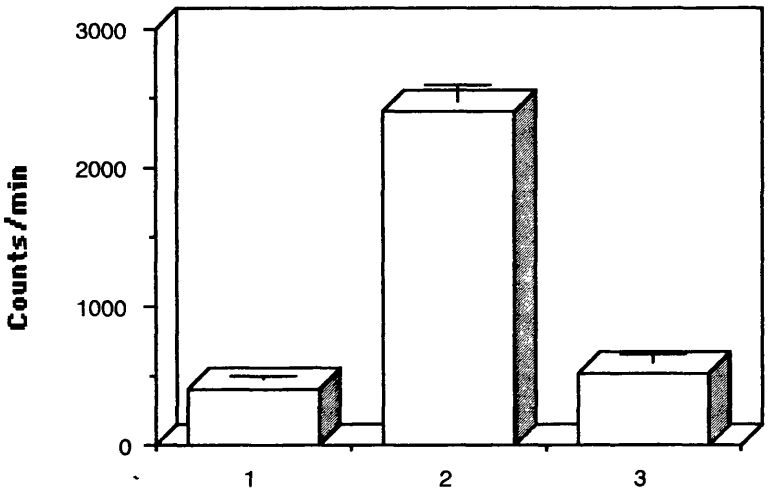


Fig.7B

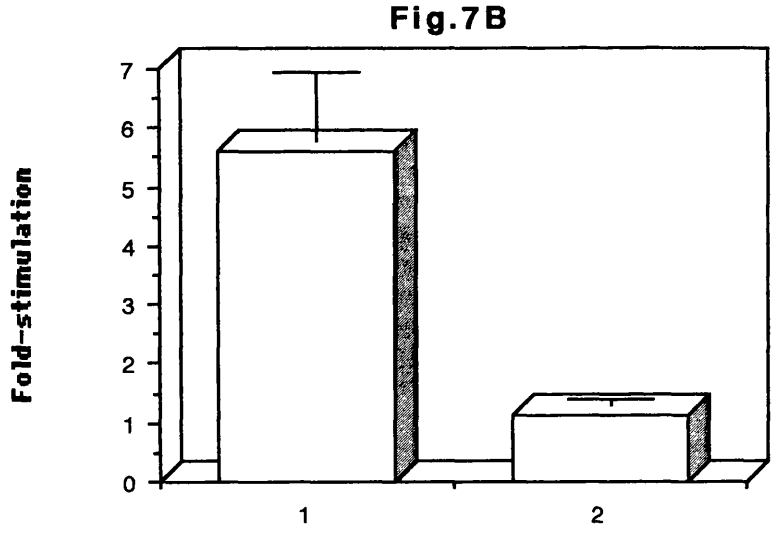


Fig.7C

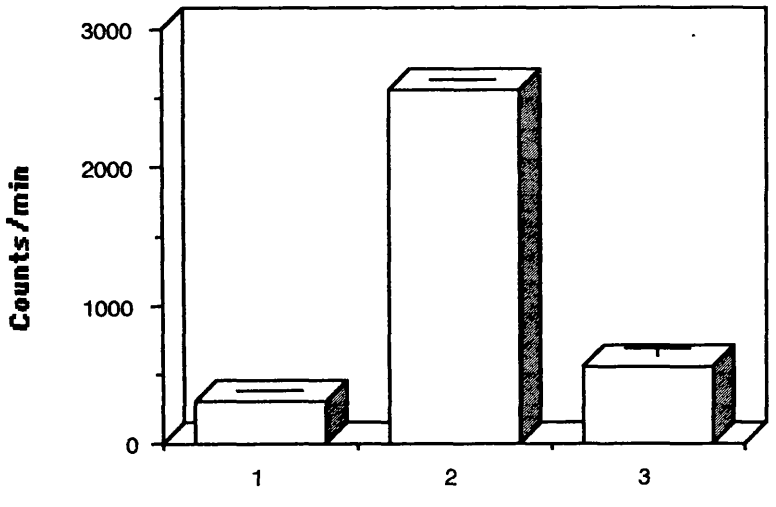


Fig.7. The action of staurosporine on ligand-stimulated inositol lipid breakdown in the Jurkat leukaemia cell line.

Fig.7A. Results of a typical experiment.

The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} PHA-P.

3, +1µM staurosporine and PHA-P.

The bars indicate the range of duplicate measurements.

Fig.7B. Pooled data from 8 experiments.

The ordinate indicates the fold-stimulation of inositol phosphates generation by 10µl ml^{-1} PHA-P, compared to control.

1, PHA-P alone.

2, +1 µM staurosporine and PHA-P

The bars indicate the SEM.

n=8, p=0.05

Fig.7C. The ordinate shows counts min^{-1} in inositol phosphates.

1, no ligand.

2, +10µl ml^{-1} OKT3.

3, +1 µM staurosporine and OKT3.

The bars indicate the range of duplicate measurements.

5.5 Discussion

Ligand-stimulated generation of inositol phosphates in Jurkat T lymphoblastic leukaemia cells line was not abrogated by the selective 5-LO inhibitors piriprost, MK886,or antioxidants including BHT and BHA.These findings are in marked contrast with those using T lymphocytes,in which piriprost, BHT and BHA markedly decreased ligand-stimulated inositol lipid turnover.

The studies presented here suggest that the early events in transmembrane signalling by which the TCR/CD3-complex activates inositol lipid signalling in Jurkat cells, do not involve an antioxidant-sensitive step. Therefore, the structural organization of signal transducing components linked to the TCR may be different in different T cell types.

Several lines of evidence have suggested heterogeneity of other aspects of the coupling mechanisms. Ward et al (1990) have shown that the ligand-stimulated generation of inositol phosphates was inhibited following activation of protein kinase C by phorbol esters in Jurkat cells but not in normal T lymphocytes.

The pattern of sensitivity of Jurkat cells to thiol reagents differed from their pattern of sensitivity to piriprost,BHT and BHA. It is therefore possible that thiol-containing compounds inhibit coupling to the inositol lipid signalling pathways at sites different to or in addition to those at which lipoxygenase inhibitors or lipid soluble antioxidants exert their actions.One possible explanation for the action of sulphydryl compounds is that they promote the dissociation of essential sulfhydryl-linked dimers of the signal transduction

pathway machinery eg the alpha-beta or zeta-zeta dimers of the T cell receptor.In fact, concentrations of thiol-containing compounds similar to those used here are known to dissociate sulphydryl bonds of immunoglobulin molecule (Dacie and Lewis 1991). Therefore, conclusions concerning the possible role of oxidative metabolites generation which are based on the use of sulfhydryl reducing agents should be interpreted with caution.

In order to investigate further the process of inositol lipid signalling in Jurkat cells, I studied the actions of the protein kinase inhibitor staurosporine on PHA-stimulated inositol phosphate generation. This compound effectively decreased inositol phosphate generation in Jurkat as well as T lymphocytes(Chapter 4.Fig 20).The inhibition of inositol phosphate generation, in turn, is probably attributable to inhibition of tyrosine protein kinases, which are known targets of staurosporine (Fallon et al 1990). Therefore, this aspect of the coupling mechanism is common to both Jurkat cells and to T lymphocytes.

CHAPTER 6

ACTIONS OF LIPOXYGENASE INHIBITORS ON THE PROLIFERATION OF NORMAL AND MALIGNANT HAEMOPOIETIC CELLS

The observations summarized in sections 2.5.2 and 2.5.3 suggest that LO metabolites may serve as autonomously generated signalling molecules which positively regulate the proliferation of normal and malignant haemopoietic cells. I have studied the effects of LO antagonists and other antioxidants on DNA synthesis and proliferation of normal bone marrow, freshly isolated leukaemia blasts, leukaemia cell lines and PHA-P stimulated T lymphocytes.The following lines were used in this study: HL60 (acute promyelocytic leukaemia), K562 (Philadelphia positive chronic granulocytic leukaemia in blast crisis), Jurkat (T-acute lymphoblastic-leukaemia) and Daudi (Burkitt lymphoma).

Results

6.1 Action of lipoxygenase inhibitors on DNA synthesis and proliferation of haemopoietic cell lines.

DNA replication in HL60 cells, as estimated by the incorporation of [^{3}H]thymidine into DNA, was inhibited by the selective 5-LO inhibitor

piriprost. Fifty percent inhibition (IC_{50}) was achieved at 40-60 μM drug concentration (Fig.1A, Table 1). [^{3}H]thymidine incorporation into K562 and Jurkat cells was also inhibited by piriprost with IC_{50} values in μM range (Table 1). NDGA inhibited DNA replication in HL60 and K562 cells (Table 1). The growth of HL60 cells was affected by piriprost(Fig IB,Table 2). The proliferation of K562 and Jurkat cells was also impaired in the presence of piriprost, with IC_{50} values in the μM range (Table 2).

In order to examine whether impairment of CO might be implicated in the inhibition of DNA synthesis, indomethacin which irreversibly inactivate CO enzyme was used in subsequent studies.This drug impaired DNA synthesis in HL60 and K562 cells (Table 1). However, the drug concentrations required for half-maximal inhibition were greatly in excess of the reported IC_{50} for CO inhibition (Chapter 2 Table 2).

DNA replication in Jurkat or K562 cells (Table 1) and HL60 (Fig.1A) was not inhibited by MK886 and BWA4C- two potent and specific inhibitors of 5-LO enzyme.

In addition, neither of these inhibitors significantly reduced proliferation of Jurkat or K562 cells (Table 2) and HL60 cells (Fig. 1B), even at 1 μM, a concentration significantly in excess of their IC_{50} value for the inhibition of 5-LO (Chapter 2, Table 1). I have verified that in my hands both of these inhibitors abrogated LT synthesis in a human whole blood system (Section 4.2.1)

6.2 Action of lipoxygenase inhibitors on DNA synthesis of freshly isolated leukaemia blasts.

[^{3}H]thymidine incorporation by freshly isolated acute myeloid leukaemia blasts as well as lymphoblasts was abrogated by NDGA in a dose dependent manner (Table 3). BW755C also impaired DNA synthesis in freshly isolated leukaemia blasts (Table 4). Haematological characteristics of the patients studied are summarized in Table 7, relevant details in case of AML patients 15,16,19,20,21 and 22 were not available.

All of these observations are in agreement with findings of Snyder et al (1989) and at first glance suggest that the endogenous generation of 5-LO metabolites of arachidonic acid may play a role in stimulating DNA replication and proliferation of leukaemic cell lines, and also in freshly isolated leukaemia blasts.

However, MK886 affected DNA synthesis in only four out of fifteen fresh acute leukaemia samples tested (Table 5). In one case this compound inhibited DNA replication at 1μM concentration significantly in excess of its IC_{50} value for the inhibition of 5-LO (Chapter 2. Table 1).

6.3 Action of lipoxygenase inhibitors on DNA synthesis of normal bone marrow and T lymphocytes.

I then studied the effects of 5-LO inhibitors on [^{3}H]thymidine incorporation by

PHA stimulated proliferation of T lymphocytes and normal bone marrow cells. Piriprost and BW755C impaired [^{3}H]thymidine incorporation into normal bone marrow cells (Fig.4). The same two agents inhibited DNA replication in PHA-P stimulated T lymphocytes (Fig 2).

These data are in agreement with previous observations of Gualde et al (1985), Pasquale et al (1991) and initially suggested that the endogenous generation of 5-LO metabolites of arachidonic acid may play a role in stimulating DNA replication and proliferation of T lymphocytes and normal bone marrow cells. However, MK886 and BWA4C did not inhibit DNA replication in T lymphocytes (Fig.3) and normal bone marrow cells (Fig.5).

TABLE 1

IC_{50} VALUES(μM) FOR THE INHIBITION OF [^{3}H]THYMIDINE INCORPORATION BY CELL LINES

	HL60	K562	JURKAT	DAUDI	NIH3T3
NDGA	3-5	3-6	nd	nd	7-10
Piriprost	40-60	90-110	16-20	nd	nd
Indomethacin	50-60	28-36	nd	nd	>200
MK886	>1[a]	>1[a]	>1[a]	>1[a]	nd
BWA4C	>1[a]	>1[a]	>1[a]	>1[a]	nd

a, no statistically significant inhibition (student's t test) was observed upto 1 μM of the drug.
nd, not determined.

TABLE 2

IC_{50} VALUES(μM) FOR THE INHIBITION OF PROLIFERATION OF CELL LINES

	HL60	K562	JURKAT
BW755C	30-38	nd	nd
Piriprost	80-100	75-85	36-42
MK886	>1[a]	>1[a]	>1[a]
BWA4C	>1[a]	>1[a]	>1[a]

---

a, no statistically significant (student's t test) inhibition was observed at concentrations of the drug upto 1 μM.
nd, not determined.

Table 3

Action of NDGA on [3H] thymidine incorporation by fresh leukaemic blasts. Figures in parentheses indicate the incorporation of radiolabel±SEM, into triplicate cultures of cells in the absence of added drug.

		Drug concentration(μM)					
No.		**1.6**	**3.3**	**6.6**	**16.6**	**33.3**	**66.6**
1	(6313 ±2600)	90± 20	100±6	69±50	28±15	16±5	3.5±1
`2	(2491 ±100)	90±15	80±15	57±5	34±10	12±4	8±1
3	(8102 ±2100)	96±11	95±10	35±10	20±5	1±10	1±1
4	(3217 ±106)	98±6	97±3	99±8	58±14	14±8	11±1
5	(1745 ±106)	90±4	71±18	34±9	21±5	21±4	17±3
6	(4905 ±360)	89 ±5	54±15	35±10	16±3	7±2	6±2
7	52156 ±1100)	80±5	62±13	55±15	14±3	6±2	2±2

Table 4

Action of BW755C on [3H] thymidine incorporation by fresh leukaemic blasts. Figures in parentheses indicate incorporation of radiolabel ±SEM, into triplicate cultures of cells in the absence of added drug.

	Drug concentration(μM)		
Patient No		**50**	**100**
8	(189944 ±7895)	---	4±3
9	(38679 ±7178)	---	8±2
15	(886285 ±19181)	64±8	---
10	(27260 ±842)	----	8±1
16	(2985 ±200)	35 ±5	---

Table 5

Action of MK886 on [3H] thymidine incorporation by fresh leukaemic blasts. Figures in parentheses indicate the incorporation of radiolabel ±SEM into triplicate cultures of cells in the absence of added drugs.

Patient No.		Drug concentration (nM) 2.5	5	10	25	50	100	1000	5000
8	(189944 ±7895)	---	---	---	---	---	---	8±5	---
9	(38679 ±7178)	---	---	---	---	---	3±2	---	---
10	(27260 ±842	---	---	---	---	---	15±10	---	---
11	(73871 ±18026)	99±14	90±13	---	80±23	40±20	22±15	---	---
12	(69489 ±1916)	---	---	---	---	---	99±13	---	---
13	(195470 ±80794)	---	---	80±10	100±15	90±11	99±5	---	---
14	(2872 ±200)	---	---	---	---	---	98±10	---	---
15	(866285 ±19180)	---	---	65±20	---	98±6	94±8	36±15	---
16	(2985 ±200)	---	---	---	99±5	---	98±6	---	---
17	(2370 ±62)	---	---	---	---	---	98±5	67±15	---
18	(62332 ±375)	---	---	---	---	---	81±12	---	30±5
19	(6347 ±665)	---	---	---	---	---	99±14	---	---
20	(36397 ±1464)	---	---	---	---	---	26±15	---	---
21	(356744 ±20620)	---	---	---	---	---	60±10	---	---
22	(138813 ±6908)	---	---	---	---	---	97±5	---	---

Table 6

Action Of BWA4C on [3H] thymidine incorporation by fresh leukaemic blasts. Figures in parentheses indicate the incorporation of radiolabel ±SEM into triplicate cultures of cells in the absence of added drug.

		Drug concentrations(nM)		
Patient No		**100**	**500**	**1000**
14	(2872 ±200)	98±5	---	---
17	(2370 ±62)	97±5	63±15	---
23	(243525 ±19558)	98±5	83±15	80±13
18	(62332 ±375)	89±10	---	---
21	(356744 ±20620)	99±5	---	80±10
22	(138813 ±6908)	91±10	3±25	---

Table 7

Haematological characteristics of patients studied. The figure in parenthesis is a percentage, ND-not determined

Patient No	Age/Sex	Diagnosis	WBC 10^9/L	immunophenot--yping details
1	55/F	AML	40	ND
2	50/M	AML	33	ND
3	14/F	AML	8	ND
4	41/M	AML	20	ND
5	23/M	AML	14	ND
6	12/M	ALL	12	TdT(89) CD19(96)
7	50/M	AML	50	ND
8	47/M	AML	34	CD34(80),HLA-II(82)
9	75/M	AML	12	CD33(70)CD13(73)
10	14/F	AML	30	CD34(24) CD33(81)
11	34/M	AML	5	CD13(79) CD33(70)
12	20/F	ALL	20.7	TdT(93) CD19(90)
13	33/M	AML	18	CD13(60) CD33(62)
14	29/M	AML	99	CD34(68)CD33(92) CD14(70)
17	28/M	AML	12	CD13(78) CD33(30)
18	35/F	AML	20.3	CD34(53) HLA-II(78)

Fig.1

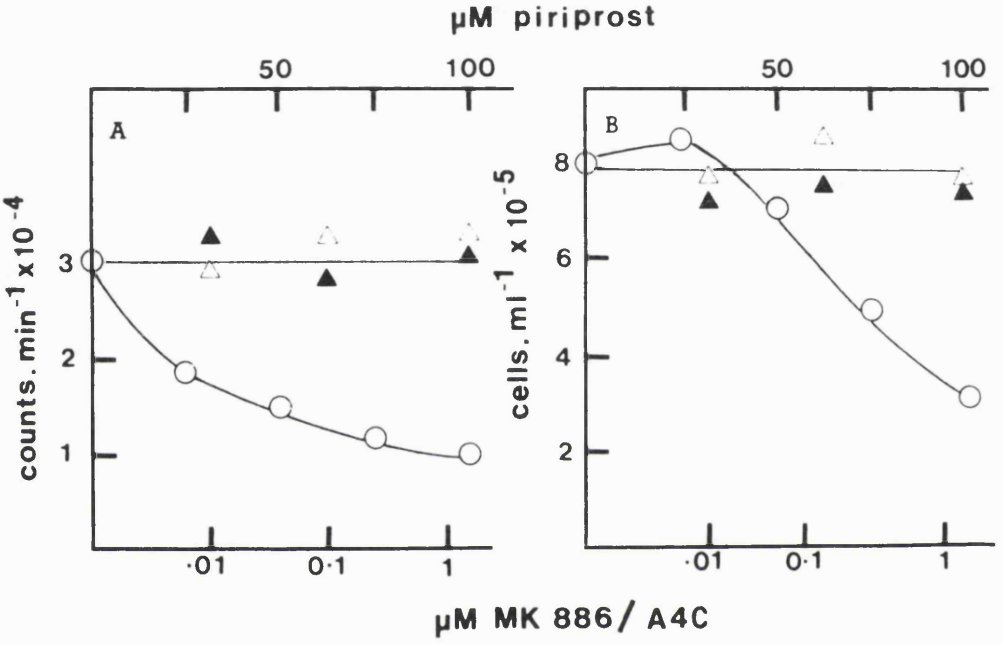

μM piriprost
50
100
A
B
counts.min⁻¹ x10⁻⁴
cells.ml⁻¹ x 10⁻⁵
3
2
1
8
6
4
2
·01
0·1
1
μM MK 886/ A4C

Fig. 1. A, actions of piriprost (o), BW A4C (Δ) and MK 886 (▲) on [3H] thymidine incorporation (A) and proliferation (B) of HL60 cells. Triplicate means are shown and standard errors are encompassed by the symbols in (A) . In (B), cells were seeded at $2.5x10^5$. ml^{-1}, and the cell counts at d3 (quadruplicate means)are shown. Standard errors were <10% of the values shown.

Fig.2

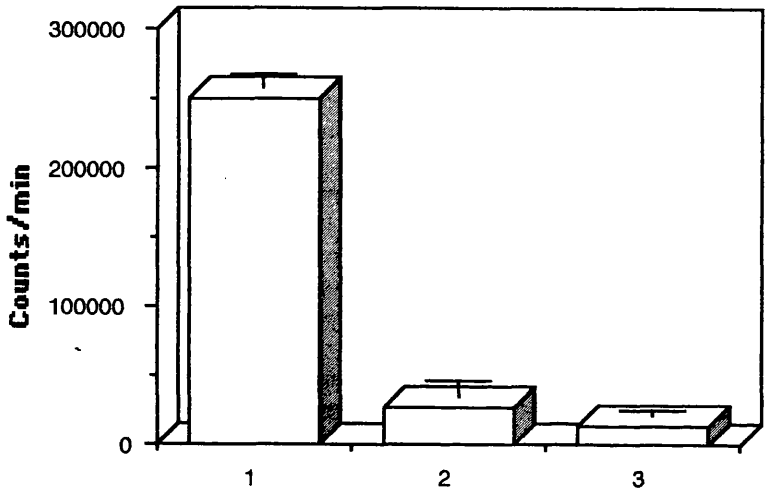

300000
200000
100000
0
Counts/min
1
2
3

Fig.3

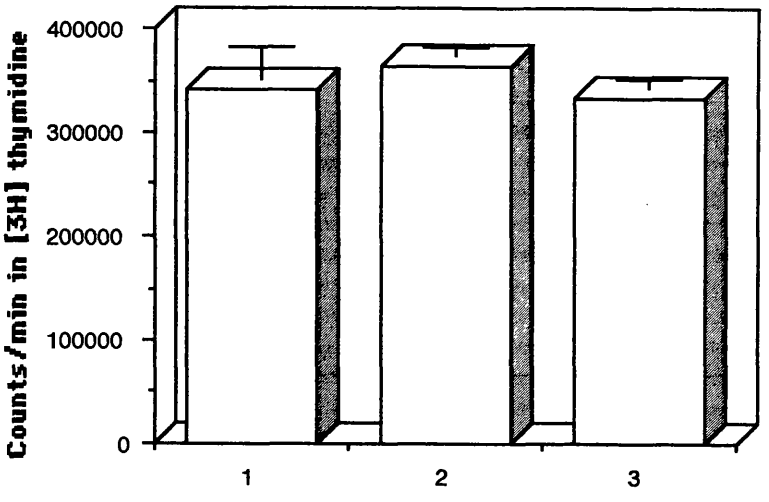

400000
300000
200000
100000
0
Counts/min in [3H] thymidine
1
2
3

Fig.2 Effects of piriprost and BW 755C on DNA replication in PHA-stimulated T lymphocytes.

The ordinate indicates counts min^{-1} in [^{3}H] thymidine incorporation into DNA.

1, no drug

2, 75 μM piriprost

3, 75 μm BW755C

The bars indicate the SEM.

Fig.3 Effects of BWA4C and MK886 on DNA replication in PHA-stimulated T lymphocytes.

The ordinate indicates counts min^{-1} in [^{3}H] thymidine incorporation into DNA.

1, no drug

2, 100 nM BWA4C

3, 100nM MK886

The bars indicate the SEM

Fig.4

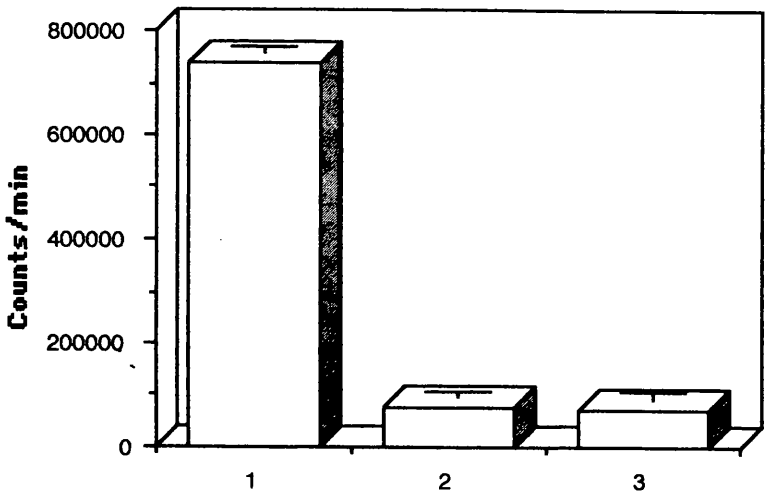

800000
600000
400000
200000
0
Counts/min
1
2
3

Fig.5

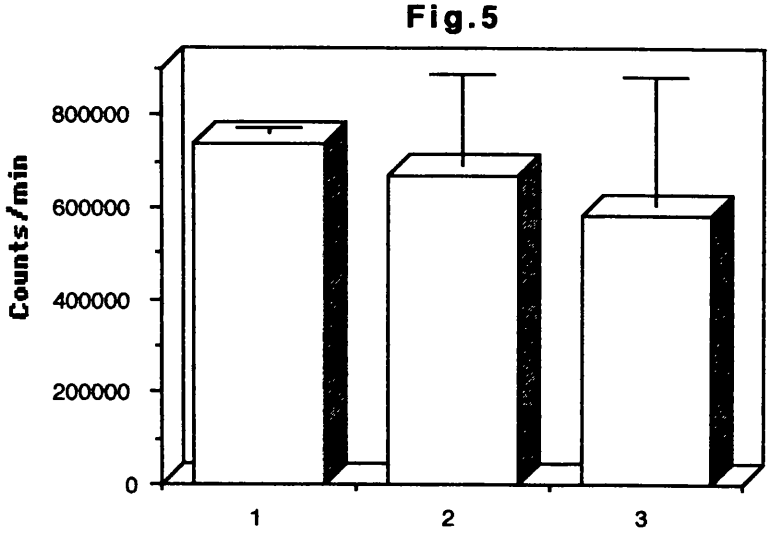

800000
600000
400000
200000
0
Counts/min
1
2
3

Fig.4 Effects of piriprost and BW 755C on DNA replication in normal human bone marrow.

The ordinate indicates counts min^{-1} in [^{3}H] thymidine incorporation into DNA.

1, no drug

2, 75 μM piriprost

3, 75 μm BW755C

The bars indicate the SEM.

Fig.5 Effects of BWA4C and MK886 on DNA replication in normal human bone marrow.

The ordinate indicates counts min^{-1} in [^{3}H] thymidine incorporation into DNA.

1, no drug

2, 100 nM BWA4C

3, 100nM MK886

The bars indicate the SEM

6.4 Discussion

Three 5-LO inhibitors NDGA, piriprost and BW755C suppressed DNA synthesis in leukaemic cell lines, freshly isolated acute myeloid leukaemia blasts, PHA- stimulated T lymphocytes and normal bone marrow cells. These agents also abrogated the proliferation of leukaemic cell lines. However, two novel, potent 5-LO antagonists, BWA4C and MK886, did not impair either function in normal haemopoietic cells or malignant haemopoietic cell lines.

I have repeatedly failed to detect any generation of LO metabolites by T lymphocytes (Chapter 4. Table 2). NDGA also inhibited [^{3}H]thymidine incorporation in NIH-3T3 fibroblasts (Table 1), which are unlikely to synthesize LTs. Most importantly, the IC_{50} values for the inhibition of DNA replication (Table 1) and proliferation (Table 2) of leukaemic cell lines, freshly isolated leukaemia blasts (Table 3) and T lymphocytes (Fig.3) by NDGA, BW755C and piriprosts were markedly in excess of the concentrations of these drugs required for the half-maximal inhibition of 5-LO (Chapter 2.Table I). Liu et al (1989) have also shown that spontaneous proliferation of T lymphoid cell lines was inhibited by BWA4C in the 10-40 μM dose range which is greatly in excess of the concentration of this compound required for the half-maximal inhibition of 5-LO (Chapter 2. Table 1). Taken together with the observations that HL60 and T lymphocytes produce virtually undetectable levels of LTs (Goerig et al 1988;

Reid et al 1990), the results reported here demonstrate that the actions of NDGA, BW755C and piriprost on these cells systems are attributable to a property other than inhibition of 5-LO, and that endogenous generation of LTs does not play a significant role in the regulation of their proliferation. Evaluation of the putative role of 5-LO in the regulation of normal and malignant cell proliferation is potentially of great importance. Recently, exogenous LTs have been shown to enhance DNA synthesis in a number of tissues including fibroblasts (Baud et al 1987) and glomerular epithelial cells (Baud et al 1985).Some of these conclusions were based in part on the use of relatively non-specific inhibitors of LTs generation.

The data here show that in case of normal and malignant haemopoietic cell lines, the use of highly specific inhibitors rule out a possible role for LTs in the regulation of DNA replication and proliferation. However, in a subset of AML DNA synthesis was inhibited by MK886. This raises the interesting possibility that LTs generation may play a modulatory role in the regulation of proliferation of some AML blasts. Studies on a larger number of cases of AML will indicate whether this drug may have therapeutic potential in this disease.

CHAPTER 7

FINAL DISCUSSION

7.1 The role of LTs in the modulation of transmembrane signalling processes.

The roles of LTs in the mediation of specific cellular responses in immunity and inflammation are well established (Rola-Pleszczynski 1983). Several actions of inhibitors of LT synthesis in transmembrane signalling processes have been documented in different cell types and have led to the conclusion that LTs may serve as second messengers in some signal transduction processes. These include the stimulation of T lymphocytes by PHA (Mire-Sluis et al 1989), the activation of specific transcription regulatory proteins by interferon alpha (Hannigan et al 1991), the regulation of gamma interferon production by interleukin-2 (Johnson and Torres 1984), and induction of tumour necrosis factor gene transcription by phorbol ester (Horiguchi et al 1989). Lipoxygenase inhibitors have been shown to suppress the synthesis of the lymphokine IL-2 by T leukaemia cells (Dornand et al 1987). Binding of LTs to cell surface receptors triggers the generation of potent second messengers DAG and PtdIns4,5-P_3 via the breakdown of inositol lipids (Mong et al 1988) and it is plausible that LTs play second messenger roles by stimulating the inositol lipid pathway.

Some of above conclusions were based in part on the use of relatively non-

specific inhibitors of LTs generation.

I have therefore focused on studying the actions of LO inhibitors on ligand-stimulated activation of the inositol lipid signalling pathway of T lymphocytes and their malignant counterpart, the Jurkat T lymphoblastic leukaemia cell line.

The T-cell antigen receptor regulates at least two signal-transduction pathways: the phosphatidylinositol and tyrosine kinase pathways (Weiss et al 1984; Imboden and Stobo 1985; Patel et al 1987). The TCR-initiated activation of PtdIns4,5-P_2 specific PLC is well documented. Ligand stimulation of the TCR induces the generation of inositol phosphates and DAG, responsible for mobilization of calcium and activation of protein kinase C (Berridge and Irvine 1984). These second messenger events contribute to later cellular responses, such as lymphokine gene expression (Farrar et al 1985).

However, many details of the mechanisms involved in the coupling of the transmembrane proteins and enzymes of these activation pathways are unclear. I have shown here that inositol lipid breakdown in PHA and CD3 monoclonal antibody stimulated T lymphocytes and Jurkat leukaemia cells was not affected by two highly selective 5-LO inhibitors MK886 and BWA4C. Furthermore, LTs generation was not detected following PHA and CD3 monoclonal antibody stimulation of T lymphocytes.These results imply that in case of the primary activation of T lymphocytes by mitogen, LTs are not involved in the coupling of cell-surface receptors to the inositol lipid signalling system.

The data here suggest that different classes of antioxidant compounds including some LO inhibitors abrogate the coupling of cell-surface receptors to the inositol

lipid signalling pathway in T lymphocytes. The effects of antioxidant compounds on inositol lipid breakdown were not attributable to irreversible damage to a component of the signal transduction machinery in T lymphocyte. Since these compounds did not have a direct action on the PtdIns 4,5-P_2 specific PLC which catalyses inositol lipid breakdown and because majority of them did not perturb activation of this pathway in Jurkat cells, I propose that the target for these compounds is an antioxidant-sensitive regulatory step in the coupling mechanism of T lymphocytes.

Others have shown that several aspects of T lymphocyte activation, including lymphokine secretion (Chaudhri et al 1988; Sekkat et al 1988), DNA synthesis (Novogrodsky et al 1982) and Ca^{++} fluxes(Dornand et al 1989) are also impaired by antioxidants .The experiments described here identify one possible site of action of these agents and the data here suggest that the generation of an oxidative metabolite may play a role in the activation of the inositol lipid signalling system following the ligation of cell-surface receptor of T lymphocyte. Recent evidence suggests that the controlled generation of oxidative metabolites play critical roles in diverse cellular signalling mechanisms. For example, the activation of the transcription factor NFkB by tumour necrosis factor is apparently mediated via the production of oxygen-centred free radicals (Shreck et al 1991). Redox reaction are also involved in the regulation of the AP-1 transcription factor (Abate et al 1990). The generation of the free radical nitric oxide is involved in inter- and intra- cellular signalling mechanisms in different cell types (Radomski et al 1990; Furlong et al 1987; Hibbs et al 1988; Moncada et al 1989). PHA and phorbol ester treatment of T lymphoid cells lead to

oxidative product generation as evaluated by flow cytoflurometric-studies (Sekkat et al 1988). Therefore, the coupling mechanism involved in T cell activation may represent a further example of the expanding role of regulated free radical generation in signalling processes. However, I was unable to characterize the putative oxidative metabolite involved in signal transduction in T lymphocyte.

7.2 Heterogeneity among different T cell types in coupling mechanisms with respect to antioxidant sensitivity.

The data here also suggest a heterogeneity among different T cell types in coupling mechanisms with respect to antioxidant sensitivity, since stimulation of inositol phosphate generation in Jurkat cells was not impaired by several of the antioxidants which are active on T lymphocytes. Heterogeneity in other aspects of the coupling mechanism have also been suggested by others. For example, activation of the inositol lipid signalling system was inhibited as the result of prior activation of PKC by phorbol ester in Jurkat cells but not in normal human T lymphocytes (Ward et al 1990). The different antioxidant sensitivity of OKT3-and PHA- mediated inositol phosphate generation in Jurkat cells and T lymphocytes may be a consequence of the different possible structural forms of the T cell receptor (Wegener et al 1992) or of different regulatory mechanisms acting on the isoenzymes of Ptd Ins4,5-P2 specific PLC (Rhee et al 1989).

Most importantly the resistance of ligand-activated inositol lipid breakdown to

antioxidants in Jurkat cells provides strong evidence that the action of these agents on the analogous process in T lymphocytes was not merely attributable to non-specific effects. It would be of interest to determine whether putative role of free radicals species suggested here is restricted to T lymphoid cells or is a general feature of other growth factor signalling pathways as well.

7.3 Modulation of normal and malignant haemopoietic cell proliferation by LTs.

LTs have been shown to modulate the growth of several types of normal and malignant cells (Fischer et al 1982). Exogenous LTs have been shown to enhance DNA synthesis in different types of tissues (Baud et al 1987).

Some authors have suggested that endogenous generation of LTs may play a role in the regulation of the proliferation of both normal and malignant haematopoietic cells (Snyder et al 1989; Ondrey et al 1989; Tsukada et al 1986; Pasquale et al 1991).

I have therefore studied the effects of 5-LO inhibitors on the proliferation of normal and malignant haematopoietic cells.The data here show that the three different LO inhibitors NDGA, piriprost, and BW755C suppressed DNA synthesis and proliferation of leukaemic cell lines, freshly isolated acute myeloid leukaemia blasts, ligand stimulated Tlymphocytes and normal bone marrow cells. However, two highly selective 5-LO antagonists, BWA4C and MK886, did not impair either function in normal and malignant haematopoietic cells. By contrast, MK886 inhibited a subset of freshly isolated acute myeloid leukaemia

blasts suggesting the intriguing possibility that some myeloid blasts may depend on endogenous LT generation for DNA synthesis.

Taken together with the findings that T lymphocytes and HL60 cells produced virtually undetectable levels of LTs (Goerig et al 1989), the data presented here suggest strongly that the actions of NDGA, piriprost and BW755C on normal and malignant haemopoietic cells are attributable to a property other than inhibition of 5-LO, and that the endogenous generation of LTs does not play a significant role in the regulation of their proliferation. Recently, exogenous LTs have been shown to enhance the proliferation of non malignant cells, including T lymphocytes (Atluru et al 1986),fibroblasts (Baud et al 1987) and glomerular epithelial cells (Baud et al 1985). Furthermore, myeloid colony formation by normal human bone marrow cells in vitro was also inhibited by LO antagonists (Pasquale et al) suggesting that endogenous LTs generation by normal bone marrow cells may play a role in initiating the proliferation of colony-forming cells. It is plausible that the suggested role of LTs in proliferation may, however, be dependant at least in part on accessary cells; tumour necrosis factor- or IL-1- stimulated generation of GM-CSF by two stromal lines is impaired by the LO inhibitors NDGA and caffeic acid, and LTB4 apparently synergizes IL-1 in inducing GM-CSF (Rizzo et al 1991).

However, the data here suggest that conclusions based on the actions of the 5-LO inhibitors NDGA, piriprost, and BW755C on proliferation and DNA synthesis in normal and malignant haematopoietic cells (Snyder et al 1989; Ondrey et al 1989; Tsukada et al 1986; Atluru et al 1986; Pasquale et al 1991;

Miller et al 1986) require re-assessment. Experiments using the highly selective LO inhibitors MK886 and BWA4C will be invaluable in determining more conclusively whether endogenous LT generation play a role in the regulation of proliferation of these cells.

Bibliography

1.Abate,C., Patel,L., Rauscher,FJ.,Curran,T. Redox regulation of Fos and Jun DNA-binding activity in vitro.Science.249:1157(1990).

2.Arima,N.,Kuziel,WA.,Grdina,TA., Greene,WC.IL2-induced signal transduction involves the activation of nuclear NF-kB expression. J.Immunol. 149:83-91(1992).

3. Aruff,A.,Seed,B. Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system. Proc.Natl.Acad.Sci. USA 84:8573-8577(1987)

4. Atluru, D., Goodwin,JS. LTB4 causes proliferation of IL-2- dependent T cells in the presence of sub-optimal levels of IL-2. Cell.Immunol. 99:444-452(1986).

5.Baeuerle,PA., Baltimore,D.I-kB: a specific inhibitor of the NF-kB transcription factor. Science. 242:538-546(1988)

6.Baniyash,M.,Morales,PG,Luong,E.,Samelson,LE.Klausner,RD. The T cell receptor zeta chain is tyrosine phosphorylated upon activation. J.Biol.Chem.263:18225-18230(1988).

7.Barker,PE.,Ruddle, FH.,Acuto,O., Reinherz,EL. Chromosomal location of human T-cell receptor gene Ti beta. Science 226:348-349(1984).

8.Barradas,MA., Jeremy,JY., Kontoghiorges,GJ., Mikhailidis,DP., Hoffbrand,AV., Dandona,P. Iron-chelators inhibit human platelet aggregation, thromboxane A2 synthesis and lipoxygenase activity. FEBS Lett.245:105-109(1989).

9.Baron,DN.,Ahmed,SA. Intracellular concentrations of water and of the principle electrolytes determined by cells of solid-tissues , erythrocytes, and leukocytes. Proc. Roy. Soc. Med. 62:945-953(1969).

10.Baud,L., Perez,J., Dennis,M., Ardaillou,R. Modulation of fibroblast proliferation by sulfido-peptide leukotrienes: effect of indomethacin. J.Immunol 138:1190-1195(1987).

11.Baud,L., Sraer,J., Perez,J., Nirez,MP., Ardaillon,R. LTC4 binds to glomerular epithelial cells and promote their proliferation in vitro. J.Clin.Invest.76:374-377(1985).

12.Berridge,MJ., Dawson,RMC., Downes,CP., Heslop,JP., Irvine,RF. Changes in the level off inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem.J.212:473-482(1983).

13. Berridge,MJ., Brown,KD., Irvine,RF., Heslop,JP. Phosphoinositides and cell proliferation. J.Cell.Sci. Suppl.3:187-198(1985).

14.Berridge,MJ. Inositol triphosphate and DAG: two interacting second messangers. Ann.Rev.Biochem. 56:159-193(1987).

15.Bierer, BE., Peterson,A., Gorga,JC., Harrmann,HS., Burakoff,SJ. Synergistic T cell activation via the physiological ligands for CD2 and the T cell receptor. J. Exp.Med. 168:1145-1153(1985).

16. Birnbaumer,L.,Abramowitz,J., Brown,M. Receptor-effector coupling by G proteins. Biochim.Biophy.Acta 1031:163-224(1990).

17. Bitonti,AJ., Moss,J., Tandom,NN., Vaughan,M.Prostagladins increase GTP hydrolysis by membranes from human mononuclear cells. J.Biol.Chem. 255:2026-2029(1980).

18. Bjorkman,PJ., Saper,MA., Samraoui,B. Structure of the human classl histocompatibility antigen, HLA-A2. Nature 329:506-512(1987)

19.Black,DR.,Allen,RE. Free radicals in biological system- a review oriented to inflammatory processes. Br.Med.Bull. 43:371-385(1987).

20. Bourne,HR.,Sanders,DA., McCormick,F. The GTPase superfamily: a conserved switch for diverse cell function. Nature 348:125-132(1990).

21.Bohmann,D.,Boss,TJ.,Adman,A., Vogt,PK., Tijan,K. Human protooncogene c-jun encodes a DNA binding protein with structural and functional property of transcription factor AP-1. Science 238:13486-1392(1992)

22. Brass,LF., Laposata,M., Banga,HS.Regulation of phosphoinositide hydrolysis pathway in thrombin-stimulated platelets by a pertussis toxin sensitive guanine nucleotide-binding protein.J.Biol.Chem. 261:16838-16847(1986).

23. Bult,H, Boeckxstaens,Ge., Jordaen,FG.,Herman,AG. Nitric oxide as an inhibitory non-adrenergic non-cholinergic transmitter. Nature 345:346-347(1990)

24.Burch,RM.,Luini,A.,Axelrod,J. PLA2 and phospholipase C are activated by distinct GTP-binding proteins in response to alpha 1-adrenergic stimulation in FTRL5 thyroid-cells. Proc.Natl.Acad.Sci.USA.83:7201-7205(1986)

25.Burgoyne,RD. G protein: Control of exocytosis. Nature. 328:112-113(1987).

26.Caccia,N., Bruns, GAP., Kirsch,IR., Hollis,GF.,Bertness,V., Mak,TW. T-cell receptor alpha chain genes are located on chromosome 14 at 14q11-14q12 in humans. J.Exp.Med. 161:1255-1260(1985).

27. Cantrell,DA.,Smith,KA.The interleukin -2 T-cell system: A new cell growth model. Science. 224: 1312-1316(1984)

28. Cantrell,DA., Davies,AA.,Crumpton,MJ.Activators of PKC downregulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. Proc.Natl.Acad.Sci. 82:8158-8162(1985)

29. Carey,F., Menashi,S.Localization of cycloxygenase and thromboxane synthetase in human platelets intracellular membrane. Biochem. J. 204:847-851(1982).

30.Chang,EH., Furth,ME., Scolnick,EM., Lowy,DR. Tumourogenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature.297:479-482(1982).

31.Channon,JY., Leslie,CC. A calcium dependent mechanism for associating a soluble PLA2 with membrane in the macrophage cell line RAW2647. J.Biol.Chem. 26:5409-5413(1990).

32.Chaudhri,G., Clark,IA., Hunt,NH., Cowden,WB., Ceredig,R. Effects of antioxidants on primary alloantigen-induced T cell activation and proliferation. J.Immuno. 137:2646-2652(1986).

33.Chaudhri,G., Hunt,NH., Clark,IA., Ceredig,R.Antioxidants inhibit proliferation and cell-surface expression of receptor for IL2 and transferrin in T lymphocytes stimulated with phorbol myristate acetate and ionomycin. Cell Immunol. 115:204-213(1988).

34.Chouaib,S. Welte,K., Dupont,B., Prostagladin E_2 acts at two distinct pathways of T cell activation: Inhibition of IL2 production and downregulation of transferrin receptor expression. J.Immunol. 135:1172-1176(1985).

35.Claesson,HE., Dahlberg,N., Gahrton,G. Stimulation of human myelopoiesis by leukotriene B4. Biochem. Biophys.Res. Comm. 131:579-585(1985).

36.Clark,JD., Lin,LL.,Kriz,RW., Sultzman,LA., Lin,AV., Knopf,JL
A novel arachidonic acid-selective cytosolic PLA2 contains a Ca++-dependent translocation domain with homology to PKC and GAP. Cell. 65:1043-1051(1991).

37.Clevers,H.,Alarcon,B.,Wileman., Terhorst,C. The T-cell receptor/CD3 complex; a dynamic protein assembly. Ann.Rev.Immunol 6:629-638(1988)

38.Cockcroft,S.,Baldwin,JM.,Allan,D. The Ca++ activated PtdIns phosphodiesterase of human and rabbit neutrophil membranes. Biochem.J. 221:477-481(1984).

39. Coffey,RG., Hadden,EM., Hadden,JW. Phytohaemagglutinin stimulation of guanylate cyclase in human lymphocytes. J.Biol.Chem. 256:4418-4424(1981)

40. Collier,J.,Vallance,P. Second messenger role of NO widens to nervous and immune system. Trends Pharmacol Sci 10:427-431(1989)

41.Couglin,SR.,Escobedo,JA.Role of phosphatidylinositol kinase in PDGF receptor signal transduction. Science. 243:1191-1194(1989).

42. Crawford,DR., Schneider,DL. Identification of ubiquinone-50 in human neutrophils and its role in microbicidal events. J.Biol.Chem. 257:6662-6667(1982)

43. Dacie,JV.and Lewis. Practical Haematology, 7th Edition. Churchill Livininingstone Publications. p493(1991).

44.Davidson,FF., Dennis,EA., Evolutionary relationship and implication for the regulation of PLA2 from snake venom to human secreted form. J.Mol-Evo 31:228-238(1990).

45. Davis,PD., Nixon,JS., Wilkinson,Se., Russel,MJN. Inhibition of PLA2 by some long chain alkylamines. Biochem.Soc.Trans. 16:816-817(1988).

46.Dinarello,CA., Marnoy,SO., Rosenwasser,LJ.Role of arachidonate metabolism in the immunoregulatory function of human leukocyte pyrogen/lymphocyte-activating factor/interleukin 1. J.Immunol. 130:890-895(1983)

47.Dixon,RAF.,Diehl,RE.,Opas,E.,Vickers,PJ.,Evans,JF., Miller,DK. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. Nature.343:282-284(1990).

48. Dornand,J., Sekkat,C., Manni,JC.,Gerber,M.Lipoxygenase inhibitors supress IL2 synthesis: Relationship with Ca++ rise and events dependent on PKC activation. Immunol.Lett. 16:101(1987b).

49. Downward,J., Graves,JD., Warne,PH.,Rayter,S., Cantrell,DA.Stimulation of p^{21ras} upon T-cell activation. Nature.346:719-723(1990)

50.Doyle,C., Strominger,JL. Interaction between CD4 and classII MHC molecules mediates cell adhesion. Nature 330:256-259(1987).

51.Dougherty,RW.,Godfrey,PP.,Hoyle,PC.,Putney,JW.,Freer,RJ. Secretagogue-induced phosphatidyl inositol metabolism in human leucocytes. Biochem.J.222:307-314(1984).

52.Dukovich,M., Wanoy,Y., Bich Thuy,L., Katz,P., Cullen,BR., Greene,WC. A second human IL2 binding protein that may be a component of high-affinity IL2 receptor. Nature 327:518-522(1987)

53. Edelman,AM., Blumenthal,DK., Krebs,EG. Protein serine-threonine kinases. Ann. Rev.Biochem. 56:567-613(1987).

54. Engleman,EG., Claudia,J., Benike,F., Grumet,C., Evans,RL.Activation of human T lymphocytes subsets: helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility antigen. J.Immunol. 127:2124-2129(1981).

55.Estrove,Z., Halperin,D.,Coceani,F., Freedman,MH. Modulation of human marrow haematopoeisis by leukotrienes in vitro. Brit.J. Haematol.69:321-327(1988).

56.Ezine,S., Irving, L., Weissman and Rouse RV. Bone marrow cells give rise to distinct cell clones within the thymus. Nature 309:629-631(1984).

57. Fain,JN. Biochim.Biophys.Acta. 1053:81-88(1990).

58.Fallon,RJ. Staurosporine inhibits tyrosine protein kinase in human hepatoma cell membrane. Biochem.Biophys.Res.Commun. 170:1191-1196(1990).

59.Farrar,WL.,Humes,JL. The role of arachidonic acid metabolism in the activation of interleukin 1 and 2. J.Immunol. 135:1153-1159(1985)

60. Farrar,WL., Ruscetti,FW. Association of PKC activation with IL2 receptor expression. J. Immunol 136:1266-1273(1986).

61. Feelisch,M., Noack,EA. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur.J.Pharmacol. 139:19-30(1987)

62. Fischer,AM.,Mills,GD.,Slaga,TJ.Inhibition of mouse skin tumour promotion by several inhibitors of arachidonic acid metabolism. Carcinogenesis. Vol 3,No11:1243-1245 (1982).

63.Fleischman,F., Chahwala,LF.,Cantley,L. Ras-transformed cells: altered levels of Ptd Ins 4,5-P_2 and catabolites. Science. 231:407-412(1987).

64. Frank,SJ., Niklinska,BB.,Orloff,DG., Mercep,M., Ashwell,JD.,Klausner,RD. Structural mutations of T cell receptor zeta chain and its role in T cell activation. Science. 249:174-177(1990).

65. Fukami,K., Mastsuoka,K., Nakanishe,O., Takenawa,T. Antibody to PtdIns 4,5-P_2 inhibit oncogene-induced mitogenesis. Proc.Natl.Acad.Sci. 85:9057-9064(1988).

Fung MR, Scearce RM, Hoffman JA, Reffer NJ, Hoskin JB, Kuziel WA, Mills GB, Greene WC. A tyrosine kinase physically associated with the β-subunit of human IL-2 receptor. J. Immunol 147:1253-1260 (1991).

66.Furchgott,RF., Zawadzki,JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373-376(1980)

67.Furlong,B., Henderson,AH., Lewis,MJ.Endothelium derived relaxing factor inhibits in vitro platelet aggregation. Br.J.Pharmacol. 90:687-692(1987)

68. Garthwaite,J., Charles,SL., Chess-William,R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intracellular messenger in brain. Nature 336:356-388(1988)

69.Gascoigne,NRJ.,Chien,Y., Becker, DM., Kavaler, J.,Davis,MM. Genomic organization and sequence of T-cell receptor Beta-chain constant- and joining- region gene.Nature 310:387-391(1984).

70.Gay,D., Coeshott, C., Golde,W., Kappler,J., Marrack,P.The MHC-restricted antigen receptor on T-cells. J.Immunol 136:2026-2032(1986).

71.Gay,D., Maddon,P., Sekaly,R.,Godfery,M.,Long,E., Goldstein,G., Chess,L., Axel,R., Kappler,J., Marrack,P. Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. Nature 328:626-629(1987).

72.Gelfand,EW., Mills,GB., Cheung,PK., Lee,JWW., Grinstein,S. Transmembrane ion fluxes during activation of human T lymphocytes. Role of Ca++,Na/H+ exchange and phospholipid turnover. Immunol Rev. 95:59-87(1987)

73. Gillard,J.,Ford-Hutchinson,AW., Cham,C et al. L-663,536(MK-886)(3-[1-(4-chlorobenzyl)-3-t-butyl-thiol-5-isopropylindol-2-yl]-2,2-diethyl propanoic acid), a novel,orally active leukotriene biosynthesis inhibitor. Can.J.Pharmacol. 67:456-464(1989).

74.Goerig,M., Habenicht,AJ., Zeh,W., Kommerrell,B.,Rothe,DE.,Glomset,JA. Evidence for co-ordinate selective regulation of eicosanoid synthesis by PDGF stimulated 3T3 fibroblasts and HL60 cells induced to differentiate into macrophages or neutrophils. J.Biol.Chem. 263:19384-19391(1988).

75.Gold,DP.,Clevers,H.,Alarcon,B.,Dunlap,S.,Williams,AF.,Terhorst,C.Evoiutionary relationship between the T3 chains of the T-cell receptor complex and the immunoglobulin supergene family. Proc.Nat.Acad.Sci. 84:7649-7653(1987).

76.Goldsmith,MA.,Weiss,A. Early signal transduction by antigen receptor without commitment to T celi activation. Science 240:1029-1031(1988).

77.Greenstock,CL.Redox processes in radiation biology and cancer. Radiat.Res.86:196-202(1981)

78. Gualde,N., Atluru,D., Goodwin,JS. Effect of lipoxygenase metabolites of arachidonic acid on proliferation of human T cells and T cell subsets. J.Immunol. 134:1125-1129(1985).

79. Gukovskaya,AS., Arias Pulido, Zinchenko,VP., Evtodienko,YV. Inhibitors of arachidonic acid metabolism eliminate the increase in cytosolic-free Calcium induced by the mitogen concanavalin A in rat thymocytes. FEBS.LETT. 244:461-464(1989)

80.Hadden,JW.,and Coffey,RG. in Mechanism of Lymphocyte Activation and Immunoregulation(Gupta,S., Paul,W.,and Fauci,A.,) pp 69-83, Plenum Press (1987).

81.Hallinan,T., Gor,J., Rice-Evans,CA.,Stanley,R.,O' Reilly,R., Brown,D. Lipid peroxidation in electroporated hepatocytes occurs much more readily than does hydroxyl radical formation. Biochem. J. 277:767-771(1991).

82.Halliwell,B. and Gutteridge,JMC. Free Radicals in Biology and Medicine(Clarendon Press,Oxford, 2nd Ed) p.188(1989).

83.Halliwell,B and Gutteridge,JMC., The importance of free radicals and catalytic metal ions in human disease. Mol.Aspects.Med.8:89-193(1985)

84. Han,JW., McCormick,F., Macara,IG. Regulation of Ras-GAP and neurofibromatosis-1 gene product by eicosanoids. Science. 252:576-579(1991)

85. Hannigan,GE., William,BRG. Signal transduction by alpha interferon through arachidonic acid metabolism. Science. 251:204-207(1991).

87. Hasegawa-Sasaki,H., Sasaki,T.PHA induces rapid degradation of PtdIns 4,5-P_2 and transient accumulation of phosphatidic acid and DAG in human T lymphoblastoid-cell line,CCRF-CEM Biochim Biophy Acta 754:305-314(1983)

88. Hayday,AC., Saito,H., Gillies,SD., Kranz,DM., Eisen,HN.,Tonegawa,S. Structure,organization,and somatic rearrangement of T-cell gamma genes. Cell 40:259-269(1985).

89. Henderson,LM., Chappel,JB., Jones,OTG.Superoxide generation is inhibited by phospholipase A2 inhibitors. Biochem.J. 264:249-255(1989

90.Heikkila,R., Schwab,G., Wickstrom,E.,Loke,SL., Watt,R., Neckers,LM. A c-myc antisense oligdeoxyonucleotide inhibit entry S phase but not progress from G_0 to G_1 Nature.328:445-449(1987)

91.Hibbs,JB., Taintor,RR., Vavrin,Z.,Rachlin,EM. Nitric-oxide: a cytotoxic activated macrophage effector molecule. Biochem.Biochem.Res.Commun. 157:87-94(1988)

92. Hoffbrand,AV.,Ganeshaguru,K.,Hooton,JWL., Tattersall,MHN.Effect of iron deficiency and desferioxamine on DNA synthesis in human cells. Br.J.Haematol. 33:517-526(1976)

93. Hokin,LE. Receptors and phosphoinositides generated second messangers. Ann.Rev.Biochem. 54:205-235(1985).

94.Horiguchi,J., Spriggs,D., Imamura,K., Stone,R., Luebbers,R., Kufe,D. Role of arachidonic acid metabolism in transcriptional induction of tumor necrosis factor gene expression by phorbol ester. Mol. Cell. Biol. 9:252-257(1989).

95.Houssiau,FA., Coulie,PG., Olive,D., Van Snick,J. Synergistic activation of human T cells by IL1 and IL6. Eur.J.Immunol. 18:653-656(1988)

96.Hurley,B.,Simon,MI., Teplon,DB., Robishaw,JD.,Gilman,AG.. Homologies between signal transducing G proteins and ras gene products. Science 226:860-862(1984).

97. Hunter,T., Cooper,JA. Protein tyrosine kinases. Ann. Rev. Biochem. 54:897-930(1985).

98.Hurst,JS.,Flatman,S., McDonald-Gibson,R. Thin layer chromatography(including radio thin-layer chromatography) of prostagladins and related compounds. In:Prostagladins and related substances; a practical approach.(Benedetto,C.McDonald-Gibson,RG., Nigam,S., Slater,TF. IRL Press,Oxford. p53

99. Ignarro,LJ., Buga,GM., Wood,KS., Chaudhuri,G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc.Natl.Acad.Sci 84:9265-9269(1987)

100. Imboden,JB.,Stobo,JD.Transmembrane signalling by the T cell antigen receptor. J.Exp.Med. 161:446-456(1985).

101. imboden,JB., Shoback,DM.,Pattison,G., Stobo,JD. Choiera toxin inhibits the T-cell antigen receptor-mediated increase in inositol triphosphate and cytoplasmic free calcium. Proc.Natl.Acad.Sci.83:5673-5677(1986).

102. Imboden,JB., Pattinson,G. Regulation of inositol 1,4,5-trisphosphate kinase activity after stimulation of human T cell antigen receptor. J.Clin.Ivest. 79:1538-1541(1987)

103. Imlay,JA., Linn,S.DNA damage and oxygen radical toxicity. Science 240:1302-1305(1988)

104.Irvine,RF., Moor,RM. Micro-injection of PtdIns1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca++. Biochem.J. 240-917-920(1986)

105. Irving,BA., Weiss,A. The cytoplasmic domain of the T-cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. Cell 64:891-901(1991)

106.Ishai,T.,Kohno,M., Nakamura,M.,Hinuma,Y.,Sugamura,K. Characterization of interleukin 2-stimulated phosphorylation of 67 and 63 kDa proteins in human T cells. Biochem. J. 242:211-219(1987)

107. Jelsema,CL., Axelrod,J. Stimulation of PLA2 activity in bovine rod outer segments by beta-gamma subunits of transducin and its inhibition by the alpha-subunit. Proc.Natl.Acad.Sci.USA. 84:3623-3627(1987).

108.Jin,Y., Clayton,LK., Howard,FD., Koyasu,S., Sieh,M., Tarr,GE., Reinherz,EL. Molecular cloning of the CD3eta subunit identifies a CD3 zeta-related product in thymus-derived cells. Proc.Natl.Acad.Sci. U.S.A. 87:3319-3323(1990).

109.Johnson,HMD., Archer,DL.,Torres,BA., cGMP as the second messenger in helper cell requirement for gamma interferon production. J.Immunol. 129:2570-2572(1982)

110. Johnson, HM., Torres, BA. Leukotrienes:Positive signal for regulation of gamma interferon production. J. Immunol. 132:413-416(1984).

111.June,CH., Fletcher,MC., Ledbetter,JA.,Samelson,LE. Increase in tyrosine phosphorylation are detectable before phospholipaseC activation after T cell receptor stimulation.J.Immunol.144:1591-1599(1990)

112.June,CH.,Fletcher,MC., Ledbetter,JA., Siegel,JN.,Philips,AF.,Samelson,LE. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. Proc.Natl.Acad.Sci. 87:7722-7726(1990)

113.Kaplin,Dr., Whitman,M.,Common elements in growth factors stimulation and oncogenic transformation: 85 kDa phosphoprotein and phosphatidylinositol kinase activity. Cell 50:1021-1029(1987).

114.Kelly,K.,Cochran,BH.,Stiles,CD.,Leder,P.Cell specific regulation of c-myc gene by lymphocyte mitogen and PDGF. Cell. 35:603-610(1983).

115. Kim,D., Lewis,DL., Neer,EJ., Clapham,DE.G protein beta-gamma subunits activate the cardiacv muscarinic K+ channel via PLA2. Nature. 337:557-560(1987).

116. Kirk,SJ.,Regan,MC., Barbul,A. Cloned murine T lymphocytes synthesize a molecule with the biological characteristic of nitric oxide. Biochem.Biophy.Res. Commun.173:660-665(1990)

117.Koning,F.,Stingl,G., Yokoyama,WM., Yamada,H., Maloy,WL.,Coligan,JE.Identification of T3-associated gamma-delta T-cell receptor on Thy-1+ dendritic-epidermal cell lines. Science 236:834-837(1987).

118. Kragballe,K., Desjarlais,L., Voorhees,JJ.Leukotrienes B4,C4,D4 stimulate DNA synthesis in cultured human epidermal keratinocytes. Br.J.Dermatol. 113:43-52(1985).

119.Krebs,EG. in The Enzymes(vol.17 1986). Protein kinases and phosphatases respectively add phosphate residues to or remove them from serine/threonine residues.

120.Ledbetter,JA.,June,CH.,Grosmaire,LS.,Rabinovitch,PS. Crosslinking of surface antigens cause mobalization of intracellular Ca++ in T lymphocytes. Proc.Natl.Acad.Sci.84:1384-1388(1987)

121.Ledbetter,JA., Tonk,NK., Fischer,EH., Clark,EA. CD45 regulates signal transdution and lymphocyte activation by specific association with receptor molecules on T or B cells. Pro.Natl.Acad.Sci. USA.85:8628-8632(1988).

122.Lewis,RA., Austen,KF. The biologically active leukotrienes. Biosynthesis,metabolism,receptors,functions and pharmacology. J.Cinc.Invest. 73:889-897(1984).

123.Linch,DC.,Wallace,DL.,°Flynn,K. Signal transduction in human T lymphocyte. Immunol.Rev.9:137-141(1987)

124. Linsely,PS., Clark,EA., Ledbetter,JA. T cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc.Natl.Acad.Sci. USA 87:5031-5035(1990).

125. Linsely,PS., Brady,W., Aruffo,A.,Damle,NK., Ledbetter,JA. Binding of the B cell activation antigen B7 to CD28 co-stimulates T cell proliferation and IL2 mRNA accumulation. J.Exp. Med. 173:721-730(1991).

126. Littman,DR. The structure of the CD4 and CD8 genes. Annu.Rev.Immunol 5:561-568(1987)

127. Liu,DS., Liew,FY., Rhodes,J. Immunoregulatory properties of novel specific inhibitors of 5-lipoxygenase. Immunopharmacology,Elsevier. 17:1-9(1989).

128. MacDonald,HR., Nabholz,M. T cell activation. Annu.Rev.Cell.Biol.2:231-239(1987).

129.Maddon,PJ., Littman,DR., Maddon,DE., Chess,L.Axel,R. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: A new member of immunogloulin gene family. Cell 42:93-104(1985).

130. Majerus,PW., Wilson,DB. Phosphoinositide turnover provides a link in stimulus-response coupling. Trends Biochem.Sci. 10:168-171(1985)

131.Makoul,GT.,Robinson,DR., Bhalla,AK. Prostagladin E_2 inhibits the activation of cloned T cell hybridomas. J. Immunol. 134:2645-2650(1985).

132.Margolis,B., Rhee,SG., Ullrich,A., Schlessinger,J., Lyall,R. EGF induces tyrosine phosphorylation of phospholipase: a potential mechanism for EGF receptor signalling. Cell 57:1101-1107(1989).

133.Margolis,BL.,Bonventre,JV.,Kremer,SG.,Kudlow,JE.,Skorechi,KL.Epidermal growth factor is synergistic with phorbol esters and vasopressin in stimulating arachidonate release and prostagladins production in renal glomerular messangial cells. Biochem.J.249:587-592(1988).

134. Maridonneau-Parini,I., Tauber,Al. Activation of NADPH-oxidase by arachidonic acid involves PLA2 in intact human neutrophils but not in a cell free system. Biochem. Biophys.Res.Comm. 138:1099-1105(1986).

135. Marrack,P., Endres,R., Zlotnik,A., Dialynas,D.,Fitch,F., Kappler,J. The MHC restricted antigen receptor on T-cells. J. Exp.Med. 158:1077-1091(1983).

136. Marrack,P., Kappler,J. The antigen-specific MHC-restricted receptor on T-cells. Adv.Immunol. 38:1-8(1986)

137. Marrack,P., Kappler,J. The T-cell repertoire for antigen and major histocompatibility complex. Immunol Today 9:308-315(1988).

138.Marth,JD.,Peet,R., Kreb,EG.,Perlmutter,RM. A lymphocyte-specific protein tyrosine kinase gene is rearranged and overexpressed in murine T cell lymphoma LSTRA. Cell 43:393-404(1985).

139.Matis,LA.,Cron,R., Bluestone,JA.MHC-linked specificity of gamma-delta receptor-bearing T lymphocytes. Nature 330:262-264(1987).

140. Martz,E. LFA-1 and other accessory molecules functioning in adhesions of T and B lymphocytes. Hum. Immunol 18(1):3-9 (1987).

141.Meuer,SC., Acuto, O., Husseuy, RE., Hodgdon, JC., Fitzgerald,KA., Schlossman, SF., Reinherz, EL. Evidence for the T3-associated 90K heterodimer as the T-cell antigen receptor. Nature 303:808-810(1983).

142. Meuer,SC., Hussey,RE., Fabbi,M., Fox,D., Acuto,O., Hodgdon,JC.Reinherz,E. An alternative pathway of T-cell activation: a functional role for the 50kDa T11 sheep erythrocyte receptor protein Cell 36:897-906(1984).

143.Miller,DK., Gillard,JN., Vickers,PJ., Sadowski,S., Mancini,JA,Dixon,RAF., Ford-Hutchinson,AW.et al. Identification and isolation of a membrane proteinnecessary for leukotriene production. Nature.343:278-281(1990).

144.Miller,AM., Weiner,RS.,Ziboh,VA.Evidence for the role of leukotrienes C4 and D4 as essential intermediates in CSF-stimulated human myeloid colony formation. Exp.Haematol. 14:76-81(1986).

145.Minowada,J., Ohnumo,T., Moor,GE. Rosette-forming human lymphoid cell line.I. Establishment and evidence for origin of thymus-derived lymphocytes. J.Natl.Canc.Inst. 49:891-896(1972).

146. Mire-Sluis,AR., Wickremasinghe,RG., Michalevicz, R,Hoffbrand,AV. Interleukin-2 induces rapid phosphorylation of an 85 kilodalton protein in permeabilized lymphocytes. Biochim.Biophys.Acta 847:159-163(1985)

147. Mire-Sluis,AR., Cox,CA., Hoffbrand,AV., Wickremasinghe,RG. Inhibitors of arachidonic acid lipoxygenase impair the stimulation of inositol phospholipid hydrolysis by the T lymphocyte mitogen PHA. FEBS. Lett. 258: 84-86(1989).

148. Mire-Sluis,AR., Hoffbrand,AV., Wickremasinghe,RG. Mitogen treatment of permeabilised human T lymphocytes stimulates rapid tyrosine and serine phosphorylation of a 42kDa protein. FEBS. Lett. 206:53-58(1986).

149.Moore,JP.,Todd,JA., Hesketh,TR., Metcalf,JC. c-fos and c-myc gene activation, ionic signals, and DNA synthesis in thymocytes. J. Biol.Chem. 261:8158-8162(1986)

150.Moncada,S.,Palmer,RMJ., Higgs,EA. Biosynthesis of nitric-oxide frm L-arginine: a pathway for the regulation of cell function and communication. Biochem. Pharmacol. 38:1709-1715(1989)

151. Mong, S., Wu, HL., Wong,A.,Sarau,HM., Crook,ST. LTD4 receptor-mediated phosphoinositol hydrolysis and calcium mobilization in rat basophilic-leukaemic cells. J.Pharmacol. Exp.Therapeu. 247:803-813(1988).

152. Muller-Decker,K. Interruption of TPA-induced signals by an antiviral and antitumoral xanthate compound: Inhibition of phospholipase C type reaction. Biochem.Biophys.Res.Comm. 162:198-205(1989)

153. Murad,F., Ishi,K.,Gorsky,L.,Kerwin,JF., Hellen,M. Endothelium derived relaxing factor is ubiquitous intracellular second messenger and extracellular paracrine substance for cGMP synthesis.In Nitric-oxide from L-arginine: a bioregulatory system. S.Moncada,E.A.Higgs,eds.Elsevier Science Publishers, Amsterdam.p301(1990).

154.Murphy,S., Sayer,S.N., Gardner,F.H. Storage of platelet concentrates at 22^{0}C. Blood. 35:549-553(1970).

155.Mustelin,T.,Coggeshall,KM.,Isakov,N., Altman,A. T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. Science.247:1584-1587(1990)

156. Neer,EG., Clapham,DE. Role of G protein subunits in transmembrane signalling. Nature. 333:129-134(1988).

157.Nishizuka,Y.The role of PKC in cell surface signal transduction and tumour promotion. Nature.308:693-698(1984).

158. Nishizuka,Y.Studies and perspectives of protein kinase C. Science. 233:305-312(1986).

159.Nishizuka,Y. The molecular heterogeneity of PKC and its implications for cellular regulation. Nature 334:661-665(1988)

160.Nocka,K., Tan,JC., Chiu,E., Ray,P.,Besmer,P. Molecular bases of dominant negative and loss of function mutations at murine c-kit/white spotting locus. EMBO.J. 9(6):1805-1813(1990)

161.Novogrodsky,A., Ravid,A.,Rubin,AL., Stenzel,KH. Hydroxyl-radical scanvengers inhibit lymphocytes mitogenesis. Proc.Natl.Acad.Sci.USA. 79:1171-1174(1982)

162.°Flyn,K., Knotts,LJ., Gaffar,A.,Morgan,G.,Linch,DC.CD2 and CD3 antigens mobalize Ca++ independently. Eur.J.Immunol. 16:580-583(1986)

163.°Shea,JJ.,Urdahl,KB., Luong,HT., Samelson,LE., Klausner,RD. Aluminum fluoride induces phosphatidylinositol turnover, elevation of cytoplasmic free Ca++, and phosphorylation of T cell antigen receptor in murine T cells. J.Immunol. 139:3463-3469(1987).

164. Ondrey,F., Harris,JE., Anderson,KM. Inhibition of U937 eicosanoid and DNA synthesis by 5,8,11,14-eicosatetrynoic acid, a competetive inhibitor of arachidonic acid metabolism. Cance Res. 49:1138-1142(1989)

165. Otto,AM., Hamilton,MN., Boss,BD., Ullrich.,Asua,LJ. Prostagladins E_1 and E_2 interact with prostagladin F_{a2} to regulate initiation of DNA replication and cell division in Swiss 3T3 cells. Proc.Natl.Acad.Sci. 79:4992-4996(1982).

166.Ozeki,T., Fujiki,H., Ohuchi,K. Dual-effects of staurosporine on arachidonic acid metabolism in rat peritoneal macrophages. Biochem. Biophys.Acta. 1047:141-147(1990).

167.Palmer,RMJ., Ferrige,AG., Moncada,S. Nitric-oxide release account for the biological activity of endothelium derived relaxing factor. Nature 327:524-526(1987)

168.Palmer,RMJ., Ashton,DS., Moncada,S.Vascular endothelium cells synthesize nitric-oxide from L-arginine. Nature 333:664-666(1988).

169.Parker,PJ., Totty,N., Rhee,L., Young,S., Chen,E.,Ullrich,A. The complete primary structure of protein kinase C- the major phorbol ester receptor. Science. 223:853-859(1986)

170.Parker,J.,Daniel,LW.,Waite,M. Evidence of PKC involvement in phorbol diester-stimulated arachidonic acid release and prostagladins synthesis. J.Biol. Chem. 262:5385-5393(1987).

171.Pasquale,D.,Monroy,R., Perrin,P.,Chikkappa,G.Lipoxygenase products regulate survival/proliferation of human CFU-GM. Blood 78,(Suppl.1),4219(1991)

172.Patel,MD.,Samelson,LE.,Klausner,RD.Multiple kinases and signal transduction:phosphorylation of T cell antigen complex. J.Biol.Chem. 262:5831-5838(1987).

173. Piomelli,D., Volterra,A., Dale,N., Kandel,Er., Schawartz,JH.,Belardetti,F. Lipoxygenase metabolites of arachidonic acid as second messengers for presynaptic inhibition of Aplysia secretory cells. Nature. 328:38(1987).

174. Poubelle,PE., Borgeat,P.,Rola-Pleszczynski,M.Assesment of leukotrienes B4 secretion in human lymphocytes by using high performance liquid chromatography and radioimmunoassay metods. J.Immunol. 139:1273-1277(1987).

175.Porfiri,E., Hoffbrand,AV., Wickremasinghe,RG. Granulocytic differentiation of the HL60 human promyelocytic leukaemia cell line is preceeded by a reduction in levels of inositol lipid-derived second messengers. Exp.Hematol. 16:641-646(1988).

176.Porfiri,E., Hoffbrand,AV., Wickremasinghe,RG. Retinoic acid induced granulocytic differentiation of HL60 human promyelocytic leukaemia cells is preceeded by downregulation of autonomous generation of inositol lipid-derived second messengers. Blood. 78:1069-1077(1991)

177.Radomski,MW.,Palmer,RMJ., Moncada,S. Endogenous nitric-oxide inhibits human platelet adhesion to vascular endothelium. Lancet ii:1057-1058(1987).

178. Radomski,MW., Palmer,RMJ., Moncada,S. An L-arginine nitric oxide pathway present in human platelet regulates aggregation.Proc.Natl.Acad.Scl. 87:5193-5197(1990)

179.Reichard,P.,Ehrenberg,A. Ribonucleotide reductase-a radical enzyme. Science. 221:514-519(1983)

180.Reid,GK.,Kargman,S.,Vickers,PJ., Mancini,JA. Leveille,C.,Miller,DK., Gillard,JW., Dixon,RAF., Evan,JF.Correlation between expression of 5-lipoxygenase-activating protein, 5-lipoxygenase, and cellular leukotriene synthesis. J.Biol.Chem. 265:19818-19823(1990)

181. Rhee, SG., Suh,PG., Ryu,SH.,Lee, SY. Studies of inositol phospholipids specifc PLC. Science. 244:546-550(1989).

182. Rizzo,MT.,Derigs,SG.,Boswell,HS. Arachidonate metabolites regulate c-jun expression in marrow stromal cells stimulated by IL-1 and TNFalpha: effect on cytokine gene expression. Blood. 78,(Suppl.1)),26a(1991).

183. Rokach,J. Leukotrienes and lipoxygenases.
(Elsevier,AMSTERDAM, 1989)

184.Rola-Pleszczynski,M. Immunoregulation by leukotrienes and other lipoxygenase metabolites. Immunol. Today 6:302-307(1982).

185.Rozengurt,E., Murray,M., Zachary,I., Collin,H. Protein kinase C activation enhances cAMP accumulation in Swiss 3T3 cells: inhibition by pertussis toxin. Proc.Natl.Acad.Sci. 84:2282-2286(1987).

186.Rosenstreich,DL., Mizel,SB. Signal requirements for T lymphocyte activation. J.Immunol. 123:1749-1754(1979)

187. Rosoff,PM.,Burakoff,SJ.,Greenstein,JL. The role of the L3T4 molecule in mitogen and antigen-activated signal transduction. Cell 49:845-853,(1987)

188.Royer, HD.,Ramarli,D., Acuto,O., Campen, TJ.,Reinherz. Genes encoding the T-cell receptor alpha and beta subunits are transcribed in an ordered manner during intrathymic ontogeny.
Proc.Nat.Acad.Sci USA 82:5510-5514(1985)

189. Salari,H., Braquet,P., Borgeat,P.Comparative effects of indomethacin,acetylenic acids, NDGA,and BW755C on the metabolism of arachidonic acid in human leukocytes and platelets. Prostagladins, Leukotriene Medicine 13:53-61(1984)

190. Salvemini,D., deNucci,G.,Vane,JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing nitric oxide-like factor. Proc.Natl.Acad.Sci. USA 86:6328-6332(1989)

191.Salvemini,D., Massioni,E.,Anggard,E,Vane,J. Synthesis of a nitric oxide-like factor from L-arginine by rat serosal mast cells: stimulation of guanylate cyclase and inhibition of platelet aggregation. Biochem. Biophy. Res. Commun. 169:596-
601(1990)

192.Samuelsson,B. Leukotrienes:Mediators of immediate hypersensitivity and inflammation. Science. 220:568-575(1983)

193.Samelson,LE.,Patel,MD.,Weissman,AM.,Harford,JB.,Klausner,RD.Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. Cell. 46:1083-1090(1986).

194. Samelson,LE., Fletcher,MC., Ledbetter,JA., June,CH. Activation of tyrosine phosphorylation in human T cells via the CD2 pathway. J. Immunol 145:2448-2454(1990).

195.Sassaki,T., Hasegawa-Sasaki,H. Activation of polyphosphoinositide phospholipase C by guanosine 5-O-(3-thio) triphosphate and fluoroaluminate in membrane prepared from human T cell leukaemia line Jurkat. FEBS.Lett. 218:87-92(1987)

196. Sayre,PH., Chang,HC., Hussey,RE., Brown, WR., Richardson, NE., Clayton,LK., Reinherz,EL. Molecular cloning and expression of T11 cDNA reveal a receptor like structure on human T lymphocytes. Proc.Natl.Acad.Sci. USA 84:2941-2945(1987).

197. Schaap,D., Parker,PJ., Bristol,A.,Kriz,R.,Knopf,J. Unique substrate specificity and regulatory properties of PKC-epsilon: a rationale for diversity. FEBS. Lettr. 243:351-357(1990).

198.Schleif RF and Wensink P. Chapter 4 page 74-75. Springer-Verlag publication. 1981.

199. Schreck,R.,Rieber,P.,Baeuerle,PA. Reactive oxygen intermediates as apparently widely used messenger in the activation of the NF-kB transcription factor and HIV. EMBO J.10:2247(1991)

200.Segal,AW. Absence of both cytochrome b_{-245} subunits from neutrophils in x-linked chronic granulomatous disease. Nature 326:88-91(1987).

201.Sekkat,C., Dornand,J., Gerber,M.Oxidative phenomena are implicated in human T cell stimulation. Immunology. 63:431-437(1988)

202.Sharon,M.,Klausner,RD.,Cullen,BR.,Leonard,WJ.Novel IL2 receptor subunit detected by cross-linking under high-affinity conditions.Science.234:859-863(1986)

203.Shearman,MS., Berry,M., Odo,T., Ase,K., Nishizuka,Y. Isolation of PKC subspecies from a preparation of human T lymphocytes. FEBS. Lett.234:387-391(1988).

204.Shearman,MS.,Naor,Z., Sekiguchi,K.,Kishimoto,A., Nishizuka,Y. Selective activation of the gamma-subspecies of PKC from bovine cerebellum by arachidonic acid and its lipoxygenase metabolites. FEBS.Lett. 243:177-182(1989).

205. Shirakawa,F,Yamashita,U., Mizel,SB. c-AMP-an intracellular second messenger for IL-1. Proc.Natl.Acad.Sci. 85:8201-8205(1988).

206. Shortman, K. T-cell development in the thymus. Nature. 309:583-584(1984).

207.Simon,MI.,Strathman,MP., Gautam,N. Diversity of G proteins in signal transduction. Science 252:802-808(1991)

209. Snyder,DS.,Castro,R.,Desforge,JF. Antiproliferative effects of lipoxygenase inhibitors on malignant human hemopoieticcell lines. Exp.Hematol.17:6-9(1989).

210. Spiegel,AM. Signal transduction by guanine nucleotide binding proteins. Mol. Cell. Endocrinology 49:1(1987)

211.Springer,TA., Dustin,ML., Kishimoto,TK., Marlin,SD. The lymphocyte function associated LFA-1,CD2 and LFA-3 molecule. Annu.Rev. Immunol. 5:223-229(1987).

212. Staal,FJT., Roederer,M.,Herzenberg,LA. Intracellular thiols regulate activation of nuclear factor kB and transcription of human immune deficiency virus. Proc.Natl.Acad.Sci.87:9943-9947(1990)

213.Steuhr,D., Gross,S., Levi,R., Nathan,C. Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxing factor and the chemical reactivity of nitric oxide. J.Exp.Med.269:1011-1020(1989)

214.Studzinski,GP., Brevli,ZS., Feldman,SC., Watt,RA. Participation of c-myc protein in DNA synthesis of human cells. Science. 234:467-470(1986).

215.Sussman,JJ.,Mercep,MS.,Germain,RN.,Ashwell,JD. Dissociation of phosphoinositide hydrolysis and Ca++ fluxes from the biological responses of a T-cell hybridoma. Nature.334:625-628(1988)

216. Swain, SL., Significance of Lyt phenotypes: Lyt2 antibodies block activities of T cells that recognize classI MHC antigen regardless of their functions. Proc.Natl.Acad.Sci. USA 78:7101-7105(1981).

217.Taheri,MR., Wickremasinghe,RG., Hoffbrand,AV. Alternative metabolic fates of thymine nucleotides in human cells. Biochem. J. 194:451-461(1981).

218.Tappel,AL. Lipoxidase. pp275-283 in The Enzymes. Second Edition,PD Boyer et al. Academic Press New York and London. 1963.

219.Tateson,JE., Randall,RW., Reynolds,CA.,Jackson,WP., Bhattacharjee,P.,Salmon,JA., Garland,LG.Selective inhibition of arachidonic 5-lipoxygenase by novel acetohydroxamic acid: biochemical assessment in vitro and ex vivo. Br.J.Pharmacol.
94:528-539(1988).

220. Terhorst,C.,Agthoven,AV., Reinherz,F., Schlossman,S., Biochemical analysis of human T lymphocyte differentiation antigen T4 and T5. Science 209:520-521(1980)

221. Tonegawa,S. Somatic generation of antibody diversity.Nature 302:575-581(1983).

222.Torres,BA.,Farrar,WL.,Johnson,HM. IL2 regulates IFN gamma production by normal and supressor cell culture. J.Immunol. 128:2217-2219(1982)

223. Truneh,A.,Albert,F.,Goldstein,P.,Verhulst,AM. Early steps of lymphocyte activation by passed by synergy between cacium ionophores and phorbol ester. Nature.313:318-320(1985)

224. Tsudo,M.,Kozak,RW.,Goldman,CK., Waldman,TA. Demonstration of a non-Tac peptide that binds IL2: a potential participant in a multichain IL2 receptor complex. Proc.Natl.Acad.Sci. 83:9694-9698(1986)

225.Tsukada,T., Nakashima,K.,Shirakawa,S. Arachidonate 5-lipoxygenase inhibitors show potent antiproliferative effects on human leukaemia cell lines. Biochem.Biophys.Res.Comm. 140(3):832-836(1986)

226.Ullrich,A., Gray,A., Tam,AW.,Collin,C., Henzel,W.,LeBon,T., Chen,E. Insulin-like growth factor 1 receptor primary-structure:comparison with insulin receptor suggests structural determinants that define functional specificity.EMBO.J. 5(10):2503-2512(1986).

227. Veillette,AM.,Bookman,MA., Horak,EM.,Bolen,JB. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p^{56lck}. Cell 55:301-308(1988).

228.Veillette,AM., Bookman,MA., Horak,EM., Samelson,LE., Bolen,JB. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine protein kinase $p^{56\ lck}$. Nature. 338:257-259(1989).

229.Vogel,US., Dixon,RAF., Diehl,RE., Marshall,MS., Sigal,IS., Gibb,JB. Cloning of bovine GAP and its interaction with oncogenic ras p^{21}. Nature. 335:90-93(1988).

230.Volarevic,S., Burns,CM.,Sussaman.,Ashwell,JD. Intimate association of Thy-1 and T-cell antigen receptor with the CD45 tyrosine phosphatase. Proc.Natl.Acad.Sci.USA. 87:7085-7089(1990).

231.Voronova,AF., Sefton,BM. Expression of a new tyrosine protein kinase is stimulated by a retrovirus promoter insertion. Nature. 319:682-685(1986).

232. Ward,SG and Cantrell,DA. Heterogeneity of the regulation of phospholipase C by phorbol esters in T lymphocytes.J. Immunol.144:3523-3528(1990).

233.Wegner,AK.,Letourneur,F., Hoevelor,A., Brocker,J., Luton,F., Malissen,B. The T-cell receptor/CD3 complex is composed of at least two autonomous transduction modules. Cell 68:83-95(1992).

234. Weiland,DA.,Farese,RV., West,LA. Prostagladin F_{2a} stimulate PtdIns 4,5-P2 hydrolysis and mobilizes intracellular Ca++ in bovine luteal cells. Proc.Natl.Acad.Sci. 84:3728-3732(1987).

235.Weiss,A., Imboden,D., Shoback,D.,Stobo,J.Role of T3 surface molecules in human T cell activation: T3 dependent activation result in an increase in cytoplasmic free calcium. Pro.Natl.Acad.Sci. 81:4169-4173(1984).

236.Weiss,A., Imboden, JB., Cell surface molecules and early events involved in human T lymphocytes activation. Adv. Immunol 41:1-6(1987)

237.Weissman,AM., Hou,D., Orloff,DG., Modi,WS.,O'Brien,SJ., Klausner,RD.Molecular cloning and chromosomal localization of the human T-cell receptor zeta chain:Distinction from the molecular CD3 complex. Pro.Nat.Acad.Sci. U.S.A. 85:9709-9713(1988).

238. Weissman,AM.,Bonifacino,JS.,Klausner,RD., Samelson,LE., O'Shea,JJ. T cell antigen receptor:structure,assembly and function. Year Immunol. 4:74-80(1989).

239. Wickremasinghe,RG., Mire-Sluis,AR., Hoffbrand,AV. Interleukin-2 binding to activated human T lymphocytes triggers generation of cyclic AMP but not inositol-phosphates. FEBS.Lett. 220:52-56(1987)

240.Wolf,BA.,Turk,J.,Shearman,WR., McDaniel,ML. Intracellular Ca++ mobilization by arachidonic acid. Comparison with myo-inositol 1,4,5-triphosphate in isolated pancreatic islets. J.Biol.Chem. 261:3501-3511(1986)

241.Wright,DG., Gallin,JI. Secretory response of human neutrophils:Exocytosis of specific-secondary grannules during adherence in vivo and during exudation in vivo. J.Immunol. 123:285-294(1979).

242.Wu,AM.,Till,JE.,Siminovitch,L., McCulloch, EA. Cytological evidence for a relationship between normal hematopoietic colony-forming cells and cells of the lymphoid-system.J.of Exp. Med. 127:445-464(1968)

243.Wu,TT., Kabat,EA. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. J.Exp.Med 132:211-250(1970).

244.Yarden,Y.,Escobedo,JA.,Daniel,TO.,Chen,EY.,Ando,ME.,Harkins,RN.,Francke,U. Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. Nature. 323(6085):226-232(1986).

244.Yarden, Y.,Kuang, WJ.,Coussens, L., Dull,TJ.,Francke,U.,Ullrich,A. Human proto-oncogene ckit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO.J. 6(11):3341-51(1987).

245.Yarden Y.,Ullrich,A. Molecular analysis of signal transduction by growth factors.Biochemistry.27(9):3113-3119(1988).

246.Yarden,Y.,Ullrich,A. Growth factor receptor tyrosine kinases. Ann.Rev.Biochem.57:443-478(1988).

247.Yoshihara,Y., Watanabe,Y. Translocation of PLA2 from cytosol to membranes in rat brain induced by calcium ions.Biochem.Biophy.Res.Comm. 170:489-490(1990).

PUBLICATIONS

Abstracts

1. Wickremasinghe, RG., Khan, MA., Tateson, JE., Hoffbrand, AV. The proliferation of malignant haemopoietic cell lines is not regulated by the endogenous generation of leukotrienes. Proceedings of the 24th Congress of the International Society of Haematology. p 118 (1992).

2. Wickremasinghe, RG., Khan, MA., Hoffbrand, AV., Tateson, JE. Antioxidants impair the coupling of mitogen receptors to the inositol lipid signalling system in T lymphocytes but not in Jurkat cells. Proceedings of the 24th Congress of the International Society of Haematology. p 170 (1992).

3. Wickremasinghe, RG., Mire-Sluis, AR., Khan, MA., Jeremy, JY., Cox, CA., Hoffbrand, AV. Lipoxygenase inhibitors impair mitogen-stimulated inositol lipid breakdown in human T lymphocytes. Brit. J. Haematol 74:35(1990).

Full papers

1. Khan, MA., Tateson, JE., Hoffbrand, AV., Wickremasinghe, RG. Evidence that endogenous generation of leukotrienes does not regulate proliferation of malignant haemopoietic cell lines (Leukaemia Research submitted, 1992)

2. Khan, MA., Jeremy, JY., Hallinan, T., Tateson, JE., Hoffbrand, AV., Wickremasinghe, RG. Antioxidants impair the coupling of cell-surface ligand receptors to the inositol lipid signalling pathway in human T lymphocytes but not in Jurkat T lymphoblastic leukemia cells (J. Immunol submitted, 1992)

3. Wickremasinghe, RG., Khan, MA., Hoffbrand, AV. Do leukotrienes play a role in the regulation of proliferation of normal and leukemic haemopoietic cells? (Prostaglandins, Leukotrienes and Essential Fatty acids, in press, 1993)