# CHARACTERISATION AND STUDIES OF T-LYMPHOBLASTOID PROCOAGULANT ACTIVITY

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**by**

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*This thesis is dedicated to the memory of my mother and grandparents.*

*Gladys Agnes Mary Pickering (1923 – 1998)*
*William George Henry Honébon (1892 – 1979)*
*Elizabeth Mary Honébon (1900 – 1985)*

## Abstract

Coagulation is an interaction between tissue factor (TF) and clotting factors, assembled on a negatively-charged phospholipid surface. Previously it was reported that malignant T-lymphoblastoid cells might have the ability to support procoagulant activity (PCA). In the present study human T-cell lines (CEM-CCRF, Jurkat, Molt-4, A3.01) representing different stages of differentiation were cultured and their basal PCA compared with that of a monocytoid cell line (THP-1). Various phospholipid or TF-dependent coagulation tests were used to investigate this PCA. The effect of TF on the phospholipid-dependent assays was also investigated.

The phospholipid dependent PCA was reported as phospholipid units/mL by comparison with a bovine brain phospholipid standard. There was variation in the procoagulant activity between the cell lines. Using calcium ionophore to stimulate this activity, and annexin A5 and an inhibitory phosphatidylserine antibody (3G4) to inhibit it, along with flow cytometry data, it was concluded that this activity was due to the exposure of anionic phospholipid.

Two pathophysiological processes, apoptosis and lipid peroxidation significantly enhanced the PCA of T-Lymphoblastoid cells. This enhanced PCA was higher than that observed following calcium ionophore treatment, suggesting the increase in activity was not solely due to anionic phospholipid exposure. Whilst annexin $A5^{FITC}$ and 3G4 bound to cells undergoing apoptosis only annexin $A5^{FITC}$ bound to cells exposed to oxidative stress. This implies that apoptosis increases PCA by causing the translocation of oxidised/native phosphatidylserine to the outer membrane, whilst lipid peroxidation appears to increase the PCA possibly due to malondialdehyde-adducts altering the net charge on the cell surface, which allows phospholipids other than phosphatidylserine to participate in thrombin generation. The possible pathophysiological significance of these observations with regard to thromboembolic complications of leukaemia, chemotherapy and atherosclerosis is discussed.

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# PUBLICATIONS

## (A). Papers related to this thesis that have been published.

1. Pickering W, Gray E, Goodall AH, Ran S, Thorpe PE, Barrowcliffe TW. Characterisation of the cell-surface procoagulant activity of T-lymphoblastoid cell lines. *Journal of Thrombosis and Haemostasis 2004*; **2**: 459 – 467.

## (B). Published reports related to this thesis that have been presented at national and international meetings in abstract form.

1. Pickering W, Gray E, Goodall AH, Barrowcliffe TW. Tissue factor independent PCA of T- lymphoblastoid cell lines: relationship to stage of differentiation. *Blood Coagul. Fibrinolysis 2001*, **12:**(7), A18.

2. Barrowcliffe TW, Fabregas P, Cancelas J, Rabaneda M, Pickering W, Gray E, Felez J. Promotion of Factor Xa and Thrombin Generation by T-lymphoblastoid cell lines. *Haemost 2001*, **31;** (Suppl. 1): p72.

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## Abbreviations

| | |
|---|---|
| $\alpha_1$AT | $\alpha_1$–Antitrypsin |
| $\alpha_2$-M | $\alpha_2$-macroglobulin |
| $\mu$g | Micrograms |
| $\mu$L | Microlitre |
| μM | Micromoles/litre |
| °C | Degrees centigrade |
| ACD-A | Acid citrate dextrose formula A anticoagulant solution |
| Ag | Antigen |
| Apaf-1 | Apoptosis protein activating factor-1 |
| aPC | Activated protein C |
| APTT | Activated Partial Thromboplastin Time |
| AT | Antithrombin |
| ATRA | all-*trans* retinoic acid |
| AUC | Area under the curve |
| BHT | Butylated hydroxytoluene |
| C1-inh | C1 inhibitor |
| $Ca^{2+}$ | Calcium ions |
| CD | Cluster of differentiation |
| CI | Confidence interval |
| cy | Cytoplasmic |
| Da | Daltons |
| DIABLO | Direct inhibition of apoptosis protein |
| DIC | Disseminated intravascular coagulation |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DVT | Deep vein thrombosis |
| EDTA | Ethylenediaminetetra-acetic acid |
| ER | Endoplasmic reticulum |
| ETP | Endogenous thrombin potential |
| EPCR | Endothelial cell protein C receptor |
| EPR-1 | Effector cell protease receptor-1 |
| $F(ab')_2$ | Bivalent antigen-binding fragment |
| Fab | Antigen-binding fragment |
| Fc | Crystalline fragment of antibody |
| Fgn | Fibrinogen |
| FII | Prothrombin; coagulation Factor II |
| FIIa | Thrombin, activated coagulation Factor II |
| FITC | Fluorescein isothiocyanate conjugated |
| FIX | Coagulation Factor IX |
| FIXa | Activated coagulation Factor IX |
| FnM | Fibrin monomer |
| FnPi | Fibrin-insoluble polymer |
| FnPs | Fibrin-soluble polymers |

| | |
|---|---|
| FV | Coagulation Factor V |
| FVII | Coagulation Factor VII |
| FVIIa | Activated coagulation Factor VII |
| FVIII | Coagulation Factor VIII |
| FVIIIa | Activated coagulation Factor VIII |
| FX | Coagulation Factor X |
| FXa | Activated coagulation Factor X |
| FXI | Coagulation Factor XI |
| FXIa | Activated coagulation Factor XI |
| FXII | Coagulation Factor XII |
| FXIIa | Activated coagulation Factor XII |
| FXIII | Coagulation Factor XIII |
| FXIIIa | Activated coagulation Factor XIII |
| g | Gravity |
| gla | γ-carboxyglutamic acid |
| Gly A | Glycophorin A |
| HcII | Heparin co-factor II |
| HDL | High density lipoprotein |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HMWK | High molecular weight kininogen |
| HSA | Human serum albumin |
| IAP | Inhibitor of apoptosis proteins |
| ICAM-1 | Intercellular adhesion molecule-1 |
| IL | Interleukin |
| IU | International Unit |
| IU.seconds/mL | The units in which the area under the curve is calculated |
| $K_{cat}$ | Catalytic constant |
| $K_d$ | Dissociation rate constant |
| $K_m$ | Michaelis constant (concentration of substrate giving half-maximal velocity) |
| L | Litre |
| LDL | Low density lipoprotein |
| LP | Lipid peroxidation |
| LPS | Lipopolysaccharide (endotoxin) |
| M | Moles/Litre |
| Mab | Monoclonal antibody |
| MDA | Malondialdehyde |
| Mg | Milligram |
| mL | Millilitre |
| mM | Millimoles/Litre |
| mRNA | Messenger ribonucleic acid |
| Mw | Molecular weight |
| NFκB | Nuclear factor kappa B |
| NIBSC | National Institute for Biological Standards and Control |
| nM | Nanomoles/Litre |
| ns | Not statistically significant |

| | |
|---|---|
| NO | Nitric oxide |
| NT | Not tested |
| *p* | Probability |
| PAR | Protease activated receptor |
| PARP | Poly (ADP-ribose) polymerase |
| PBS | Phosphate buffered saline |
| PCA | Procoagulant activity |
| pH | $-\log_{10}$ H |
| PI | Propidium iodide |
| PK | Prekallikrien |
| PL | Phospholipid |
| PLM | Phospholipid membrane surface |
| PLU | Phospholipid Units |
| pM | Picomoles/Litre |
| PA | Phosphatidic acid |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PC | Phosphatidylcholine |
| PE | Phosphatidylethanalomine |
| PK | Prekallikrien |
| PS | Phosphatidylserine |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| PUFA | Polyunsaturated fatty acids |
| $r^2$ | The square of the coefficient of correlation |
| ROS | Reactive oxygen species |
| rpm | Revolutions per minute |
| RPMI | Roswell Park memorial Institute |
| rTF | Recombinant tissue factor |
| RVVT | Russell's Viper Venom Time |
| s | Seconds |
| sc | Single chain |
| sIg | Surface immunoglobulin |
| SERPIN | Serine protease inhibitor |
| SMAC | Second mitochondrial activator of caspases |
| ST | Staurosporine |
| T-ALL | T-cell Acute Lymphocytic Leukaemia |
| TAT | Thrombin-antithrombin complex |
| TBARS | Thiobarbituric acid reacting substances |
| TBS | Tris-buffered saline |
| TCR | T-cell receptor $\alpha,\beta,\delta,\gamma$-chain |
| TdT | Terminal deoxyribonucleotidyl transferase |
| TE | Thromboembolism |
| TF | Tissue Factor |
| TFPI | Tissue factor pathway inhibitor |
| TM | Thrombomodulin |
| TNF | Tumour necrosis factor |
| t-PA | Tissue plasminogen activator |

| | |
|---|---|
| U | Unit |
| u-PA | Urokinase plasminogen activator |
| UV | Ultra Violet |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| vWF | von Willebrand Factor |
| WCC | White cell count |
| XIAP | X-linked inhibitor of apoptosis |

Chapter

1

# INTRODUCTION

## 1.1 Introduction

Haemostasis is a fundamental biological process that protects humans from bleeding. Thus, rapid fibrin deposition must occur as a consequence of tissue injury. On the other hand, the system of haemostasis is under tight control by counterbalancing antithrombotic substances in order to prevent continuous fibrin deposition and excessive thrombus formation. In addition, there has to be a powerful fibrinolytic system that can dissolve fibrin deposits or thrombotic material once they are formed. Although the fibrinolytic system is an important component of haemostasis it does not form part of this thesis and reviews can be found elsewhere (Leopold & Loscalzo, 2000; Hajjar & Nachman, 2001). However, in spite of all these checks and balances, thrombosis and thromboembolic complications represent a significant worldwide medical problem. From a pathophysiological point of view, three predisposing conditions that may result in thrombus formation (termed Virchow's triad) have been described. These factors are [i] changes in the vessel wall, [ii] changes in the constituents of blood, and [iii] changes in the pattern of blood flow. Recently, new concepts of cellular haemostasis and additional "risk" factors that may contribute to or counteract the thrombophilic state have been proposed (Blann & Lip, 2001). Virchow's triad occurs in pathological conditions such as malignancy and atherosclerosis. T-cells are a cellular component of the atherosclerotic plaque and little is known about their contribution to atherothrombosis.

Certain malignancies, especially solid tumours of breast and pancreas are associated with thrombosis however little is known about the thrombotic mechanisms associated with T-cell leukaemia and its treatment. Although it has been reported that monocytes and monocytoid cell lines can be procoagulant this is thought to be mainly due to the activity of tissue factor (TF). Recently it has been reported that T-lymphoblastoid cells can also support coagulation (Barrowcliffe *et al*, 1989; Barrowcliffe *et al*, 2002). This study specifically addresses the characteristics of the procoagulant activity associated with T-lymphoblastoid cells.

## 1.2 A brief historical review of the coagulation system

The earliest known references to coagulation were by Aristotle and Plato in the 4th Century BC who believed that the coagulation of blood was due to the cooling of blood as it left the body. Malphigi in 1686 described the threadlike fibres of the material forming the clot, and called them fibrin. This finding was extended by William Hewson in 1771 and B.G. Babbington in 1830 who demonstrated that these fibres were derived from a precursor in plasma that we now know as fibrinogen. This was contrary to the prevalent belief that fibrin was derived from the nuclei of red blood cells. It was Buchanan in 1845, who suggested the existence of a soluble precursor of fibrin, which was later purified and called fibrinogen. In 1892 Alexander Schmidt demonstrated that the transformation of fibrinogen into fibrin was a "fermentive" (enzymatic) process and named the fibrin ferment "thrombin"; he called its presumed plasma precursor "prothrombin". He also showed that tissue extracts had coagulant properties. In 1890, Arthus and Pages observed that removal of calcium ions ($Ca^{2+}$) from blood inhibited coagulation and Hammarsten in 1911 postulated that it was an essential component in reactions preceding thrombin formation. It was also suspected that platelets, first described convincingly by Giulio Bizzozero in 1882, were probably a necessary element in blood coagulation. In 1905, Paul Morawitz synthesized these observations into the first "Classical four-factor theory" of the biochemistry of blood coagulation: prothrombin, he hypothesized, was converted into the enzyme thrombin by "thrombokinase" (tissue factor) in the presence of calcium; thrombin, in turn, converted fibrinogen to fibrin (Morawitz, 1905). Owing to the influence of Schmidt and Morawitz, it was widely accepted in the first half of the 20th Century that the initiating event in blood coagulation was the exposure of plasma to damaged tissues and the action of tissue factor (TF). On the basis of the classical four-factor theory" Armand Quick in 1935 introduced the one-stage prothrombin test. Its use led to the discovery of other coagulation factors and along with mounting clinical evidence, in particular the observation that blood from haemophiliacs apparently clotted normally after the addition of TF, eventually forced a reassessment of the coagulation mechanism.

## 1.3 Models of coagulation

Since the classical "four factor" model of Morawitz, three other models of coagulation have been postulated. These are the cascade, physiological and cell-based models of coagulation. The purpose of these models is to evaluate and predict *in vivo* responses to alterations in the concentration of procoagulants and their inhibitors, occurring either naturally or as a consequence of pharmacological intervention.

### 1.3.1 The cascade model of coagulation

In 1964, two groups (Davie & Ratnoff, 1964; MacFarlane, 1964) independently proposed a model of coagulation that incorporated a series of sequential steps whereby activation of one clotting factor led to the activation of another, finally leading to a burst of thrombin generation (Figure 1.1).

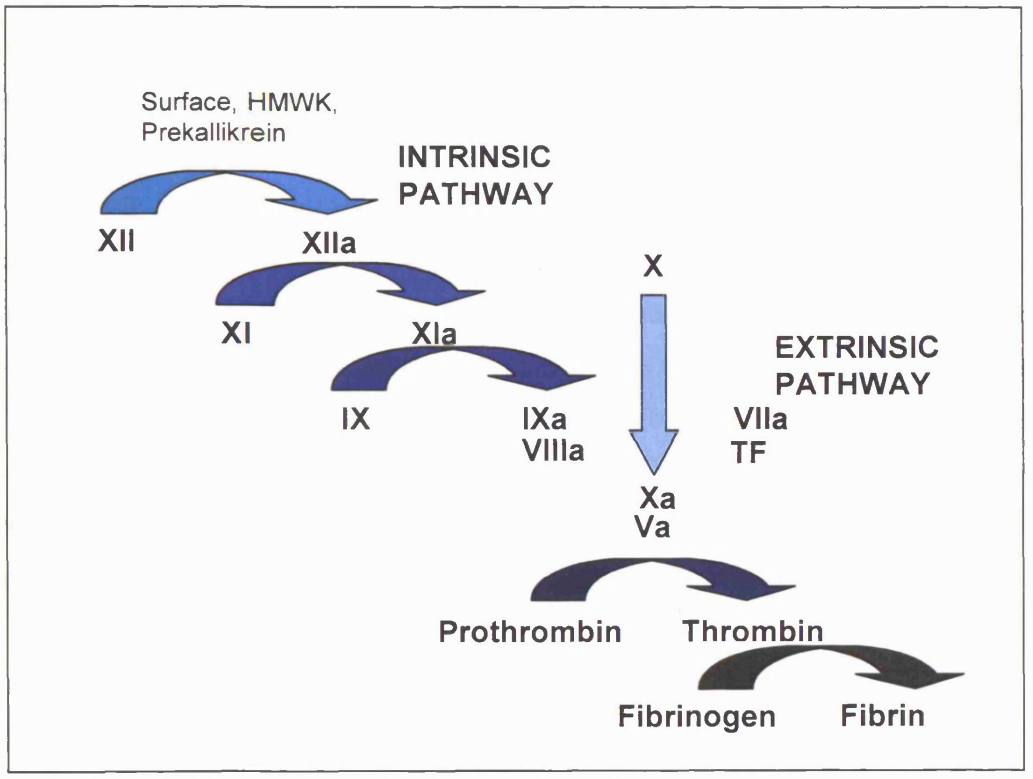



*Figure 1.1: The cascade or waterfall model of haemostasis.*

In this "cascade" model, the clotting pathways were divided into the "extrinsic" and "intrinsic" systems. Both pathways could activate factor X (FX) which in complex with its cofactor Va, then converted prothrombin to thrombin. This view of the coagulation cascade is a reasonably good model to explain the usefulness of the coagulation screening tests the prothrombin time (PT) and the activated partial thromboplastin time (APTT), which correspond to the extrinsic and intrinsic pathways, respectively.

### 1.3.2 The physiological plasma coagulation model

The cascade model is however inadequate to explain the pathways leading to haemostasis *in vivo* and is inconsistent with clinical observations in several key respects. If there are separate intrinsic and extrinsic coagulation pathways *in vivo*, why then, does the activation of FX by the extrinsic FVIIa/TF-initiated pathway not compensate for a lack of factor VIII (FVIII) or factor IX (FIX) in haemophiliacs? In fact, the activation of haemostasis by an intrinsic pathway *in vivo* is questionable, since deficiencies of FXII, high-molecular-weight kininogen (HMWK), or prekallekrein (PK) do not cause a tendency to clinical bleeding. Factor XI (FXI) deficiency is much less predictable in causing a bleeding diathesis and indeed has a less severe clinical picture than FVIII or FIX deficiencies. Thus it is clear that some components of the intrinsic pathway, such as FVIII and FIX, are essential for haemostasis, since their deficiency leads to a severe bleeding diathesis. It is also known that deficiencies of FX, factor V (FV) and factor VII (FVII) lead to serious clinical bleeding syndromes. Thus it was concluded that it was highly unlikely that separate extrinsic and intrinsic pathways operate under normal *in vivo* conditions. As a result, the need for a more physiological model of coagulation was recognised (Figure 1.2).

Key observations previously made by several groups led to the revision of the earlier models of coagulation. One major observation was that FVIIa/TF activated not only FX but also FIX (Osterud & Rapaport, 1977; Gailani & Broze, Jr., 1991). Other important observations by several groups led to the conclusion that the major initiating event in haemostasis *in vivo* was the formation of a FVIIa/TF complex at the site of injury

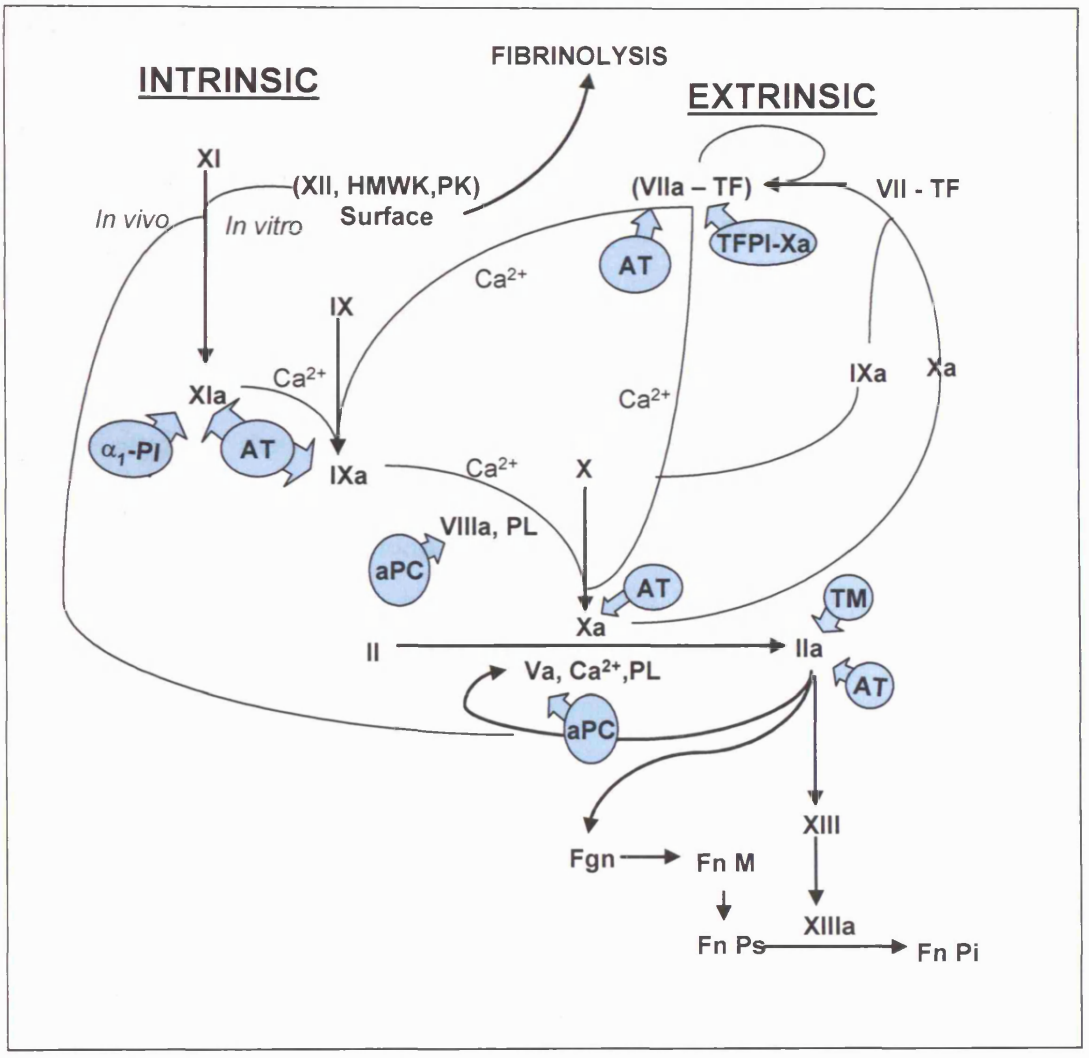



*Figure 1.2: The physiological model of blood coagulation and its regulation. Abbreviations used in this figure: AT, antithrombin; $\alpha_1$-PI, $\alpha_1$ - protease inhibitor, TFPI, tissue factor pathway inhibitor; aPC, activated protein C; Fgn, fibrinogen; Fn M, Fibrin monomer; Fn Ps, Fibrin soluble Polymer; Fn Pi, Fibrin-insoluble Polymer; TM, Thrombomodulin; PL, Phospholipid. Other abbreviations used are detailed on page 20.*

(Nemerson, 1992; Oliver *et al*, 1999). Also of importance was the finding that thrombin could directly activate FXI on a negatively charged surface (Gailani & Broze, Jr., 1991). The fact that activated platelets could provide surface for activation of FXI by thrombin under physiologic conditions (Baglia & Walsh, 1998; Baglia & Walsh, 2000; Baglia *et al*, 2002) explained why FXII, HMWK, and PK might not be required for haemostasis.

An emphasis on the role of the coagulation proteins in controlling coagulation in this model suggests that we should be able to understand the physiology and pathophysiology of haemostasis if we have a sufficiently good understanding of the enzymology and kinetics of individual reactions in the model. This has not been the case as evidenced by our inability to answer the question "why do haemophiliacs bleed?" Conversely, studies on the cellular control model of coagulation (see section 1.3.3) have helped us to explain the pathophysiology of FVIII deficiency (Haemophilia A) as a failure of platelet-surface FXa generation.

### 1.3.3 The cell-based model of coagulation

The newer cell based model of haemostasis (Figure 1.3) does not artificially separate the extrinsic and intrinsic pathways that together operate *in vivo* (Hoffman & Monroe, III, 2001). In a first step, small amounts of thrombin are generated after vessel injury, followed by a thrombin burst with clot formation (priming and propagation).

#### *1.3.3.1 Step 1: Initiation of Coagulation*

Low concentrations of activated factor VII (FVIIa) circulate under physiological conditions in the bloodstream (Morrissey, 1996). When trauma damages vascular integrity plasma FVIIa comes into contact with TF expressed on extravascular cells, mainly fibroblasts, and binds to it. While free FVIIa is a weak enzyme, binding to TF markedly increases its enzymatic activity. The membrane-bound complex of TF:FVIIa

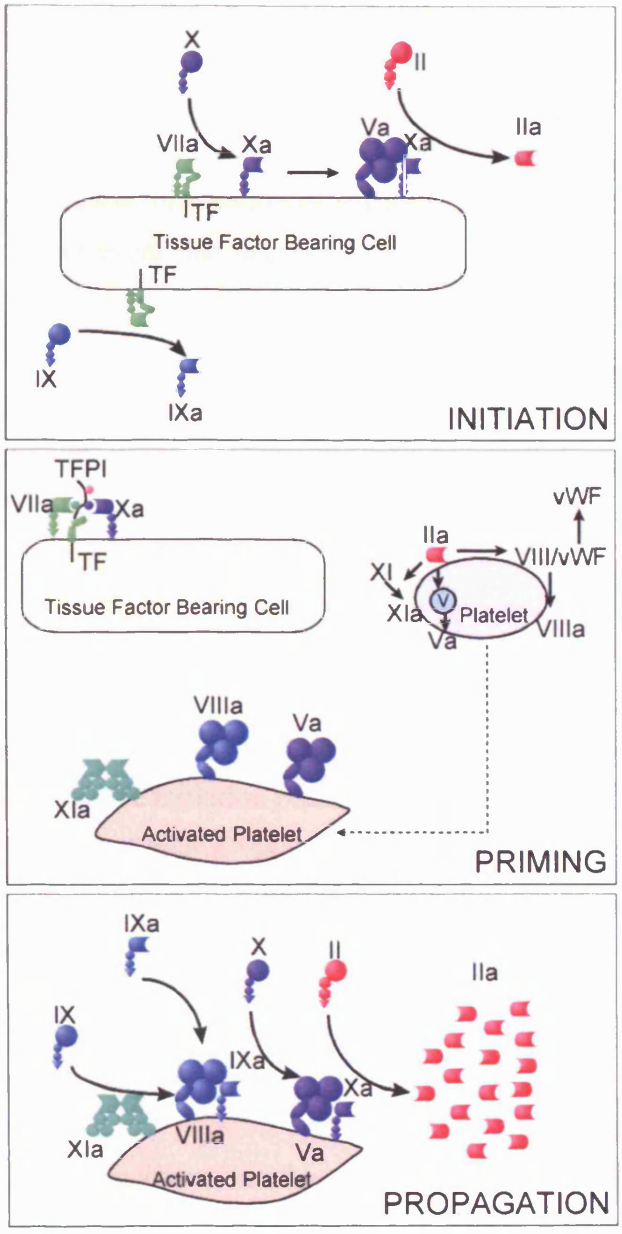



*Figure 1.3: The cell-based model of coagulation (Monroe et al, 2002). The abbreviations used are detailed on page 20.*

initiates coagulation. Factor X binds to this complex and becomes activated to FXa. The FXa that leaves the protected environment of the extravascular cell surface is rapidly inactivated by AT or TFPI. However, FXa that remains on the TF-bearing cell surface leads to limited proteolysis of inactive factor V, thus generating FVa. The FXa/Va complex on the TF-bearing cell results in prothrombin cleavage and the generation of small amounts of thrombin. The subsequent amplification and propagation steps are needed for creation of sufficient amounts of thrombin to lead to clot formation. The other event that occurs in the initiation phase is the activation of FIX to FIXa by the TF:FVIIa complex. However FIXa is unable to be enzymatically active to any significant degree during this phase: it cannot activate FX to FXa efficiently, since the necessary cofactor FVIIIa has not yet been formed. FVIIIa will only become available after the initial small amount of thrombin has been generated and has enzymatically cleaved FVIII to FVIIIa.

#### *1.3.3.2 Step 2: Priming*

The small amount of thrombin generated on the surface of the TF-bearing subendothelial cells during the initiation phase leads to activation of platelets that have adhered to the subendothelial matrix via vWF at the site of injury, partial proteolysis and thus activation of platelet-bound FVIII to FVIIIa, activation of FXI to FXIa, activation of FV to FVa, and further activation of FIX to FIXa.

#### *1.3.3.3 Step 3: Propagation*

During the propagation phase the activated coagulation factors that have been generated in the priming phase combine with their cofactors and form the "tenase" and "prothrombinase" complexes, resulting in large-scale thrombin generation. The so-called "thrombin burst" leads to fibrinogen cleavage and fibrin polymerisation. The main pathway for this explosive thrombin formation is via TF:VIIa complex-induced activation of FIX to FIXa. The direct activation of FX to FXa by the TF:VIIa complex plays a minor role in this propagation step.

## 1.4 The role of platelets in the haemostatic response

Platelets play an important role in thrombin generation, and form a pivotal part of the cell-based model of coagulation (Hoffman & Monroe, III, 2001; Monroe *et al*, 2002). The haemostatic response to endothelial damage is contingent upon the co-ordinated interaction between platelets and vessel wall constituents (primary haemostasis) and coagulation proteins (secondary haemostasis).

### 1.4.1 Platelets and primary haemostasis

Primary haemostasis is the process by which a breach in the vessel wall is sealed, preventing excessive blood loss. Platelet shape change, aggregation and secretion of granule components form essential steps in the cessation of bleeding (Leblond & Winocour, 1999). Platelet activation after vascular injury involves platelet adhesion to subendothelium structures and platelet aggregation to form a haemostatic plug at the wound site. The most important is probably collagen, for which platelets have a number of specific receptors but it is von Willebrand Factor (vWF), a multimeric glycoprotein that circulates in a complex with FVIII (Zimmerman, 1987), bound to exposed collagen that makes initial contact with the platelet via the GP 1b/IX/V complex. Once this contact has been made, the other types of collagen receptors may bind to the exposed collagen, and yet more receptors are able to bind specifically to ligands such as fibronectin, vitronectin and laminin. Platelet aggregation is also mediated via exposure of fibrinogen binding sites on the platelet receptor complexes, GP IIb-IIIa (Peerschke, 1985). Some of these receptors are also coupled to intracellular signalling systems that lead to the activation of the platelet.

Unactivated platelets appear to contain binding proteins for thrombin. The GP Ib/IX/V complex can also function as a high affinity receptor for thrombin (De Cristofaro *et al*, 2001) and a binding site for thrombin has been identified to be between residues 271 – 284, at the top of the macroglycopeptide 'stalk' region of GP 1bα (De Marco *et al*, 1994). An additional binding site has also been identified between residues 219 – 240

(McKeown *et al*, 1996) where inhibitory peptides were able to prevent platelet aggregation via GP Ibα or the main thrombin receptor on the platelet membrane, protease activated receptor-1 (PAR-1). A monoclonal antibody, SZ2 that binds to this region, has been shown to inhibit the development of platelet procoagulant activity in response to thrombin (Dormann *et al*, 2000). Thrombin cleaves GP V from the surface of the platelet possibly following its interaction with GP Ibα, and this may orientate thrombin in a optimal way to cleave PAR-1 (De Candia *et al*, 2001). Once PAR-1 has been cleaved a signalling cascade is triggered and the platelet can become activated with subsequent exposure of PS (Andersen *et al*, 1999). A review of platelet membrane glycoproteins and haemostasis can be found elsewhere (Thomas, 2002; Andrews & Berndt, 2004).

### 1.4.2 Platelets and secondary haemostasis

Secondary haemostasis is the formation of a fibrin clot. This clot is built on the scaffold of aggregated platelets that form the platelet plug and there are a number of ways in which platelets facilitate the formation of a clot. Activation of platelets can lead to procoagulant activity due to the surface exposure of phosphatidylserine (PS) via the "flip-flop" reaction (Bevers *et al*, 1983; Zwaal & Schroit, 1997) which serves as a catalytic membrane surface for both procoagulant and anticoagulant reactions thus localising thrombin formation at the site of injury. Platelets can facilitate these reactions further by carrying some of the relevant coagulation proteins (fibrinogen) and cofactors (FV) on or inside their membrane or on parts of their membrane that become 'pinched off' after activation to form microparticles.

Thus there is a sequence of platelet-mediated haemostatic effects that follow damage to the endothelium and lead to the formation of a fibrin clot. Platelet activation by interaction with sub-endothelium-bound vWF leads to platelet shape change, membrane "flip-flop" and the activation of GP IIb/IIIa. As this happens, the cofactor FVIIIa binds to the membrane with FIXa to form tenase that cleaves FX to create FXa that then combines with FV released from the platelet, activated by thrombin, and is bound to the

platelet membrane to create prothrombinase. Prothrombin that is carried on the membrane of resting platelets is displaced from the now activated GP IIb/IIIa, which can now bind the larger fibrinogen molecule for which it has a higher affinity, and the prothrombin is cleaved to create thrombin that in turn cleaves the fibrinogen to create fibrin that forms the clot.

## 1.5 Membrane phospholipids and blood coagulation

The primary and most important function of membrane phospholipids is to provide an efficient permeability barrier between two completely different aqueous compartments. However phospholipid-protein interactions are of mutual importance: particular proteins may govern the phospholipid organisation which controls the specific structural and functional requirements of a given membrane, whereas phospholipids in turn may play an essential role in the regulation of specific functions of the membrane such as haemostasis. It is phospholipid-protein interactions, and changes in conformation of the membrane cell-surface that are key to the ability of a cell to support coagulation.

### 1.5.1 Structure and properties of phospholipids

The primary lipids of biological membranes are phospholipids, a group of phosphate-containing molecules with structures related to the triglycerides. Glycerol forms the backbone of the molecule but only two of its binding sites (hydroxyl groups) link (acylated) to fatty acid residues. The third hydroxyl group links, instead, to a bridging phosphate group to which may be bonded one of a number of possible 'head-groups'. The other end of the phosphate bridge links to another organic subunit, most commonly a nitrogen-containing alcohol. Other organic subunits that may link at this position include the amino acids serine and threonine or a sugar, inositol. The phosphate plus head-group region of the molecule, usually bearing both positive and negative charges, is usually very polar and hydrophilic, while the fatty acid chains (which may be

saturated or partially unsaturated) confer a hydrophobic nature to the molecule. Phospholipids may therefore be termed amphiphilic, or both hydrophilic and hydrophobic.

In aqueous suspension, phospholipids tend to form structures in which their hydrophilic head-groups are in contact with polar water molecules, while the hydrophobic alkyl chains are orientated away from polar interactions: most commonly this is achieved by the spontaneous formation of micelles, or liposomes with a bilayer structure. Thus an interface is formed between the solution and the phospholipid surface, and dissolved molecules such as inorganic ions or proteins may interact either with only the polar head-group or via hydrophobic 'patches' with the interior of the bilayer as well.

The phosholipid bilayer forms the structural and functional basis for all eukaryote membranes and is arranged with the negative charge on the inside of the bilayer. A diagrammatic representation of a phospholipid molecule is shown in Figure 1.4. The main phospholipids found in mammalian cell membranes are: phosphatidylcholine (PC; 1,2-diacyl-sn-glycero-3-phosphocholine), phosphatidylethanolamine (PE; 1,2-diacyl-sn-glycero-3-phosphoethanolamine) and phosphatidylserine (PS; 1,2-diacyl-sn-glycero-3-phospho-L-serine). Other phospholipids which are found in lower or trace quantities in mammalian membranes include, phosphatidylinositol (PI; 1,2-diacyl-sn-glycero-3-phospho-1D-myoinositol) and phosphatidic acid (PA; 1,2-diacyl-sn-glycero-3-phosphate). Table 1.1 shows the phospholipid composition of the membrane of human platelets: the major components are PC and PE with PS and PI present in lower quantities (Bevers *et al*, 1982). Sphingomyelin, a phosphate-containing non-glyceride lipid, is included, as it is a major constituent of cell membranes. In addition to its being highly polar due to the charges present on the phosholipid head-groups, the surface of a liposome or vesicle may possess an overall net charge, dependent on the ambient pH and type(s) of head-group present. At neutral pH, PS and PA possess a high net negative charge, PI a smaller negative charge, while PC and PE are essentially uncharged, since in the zwitterionic state they possess one negative and one positive charge each.

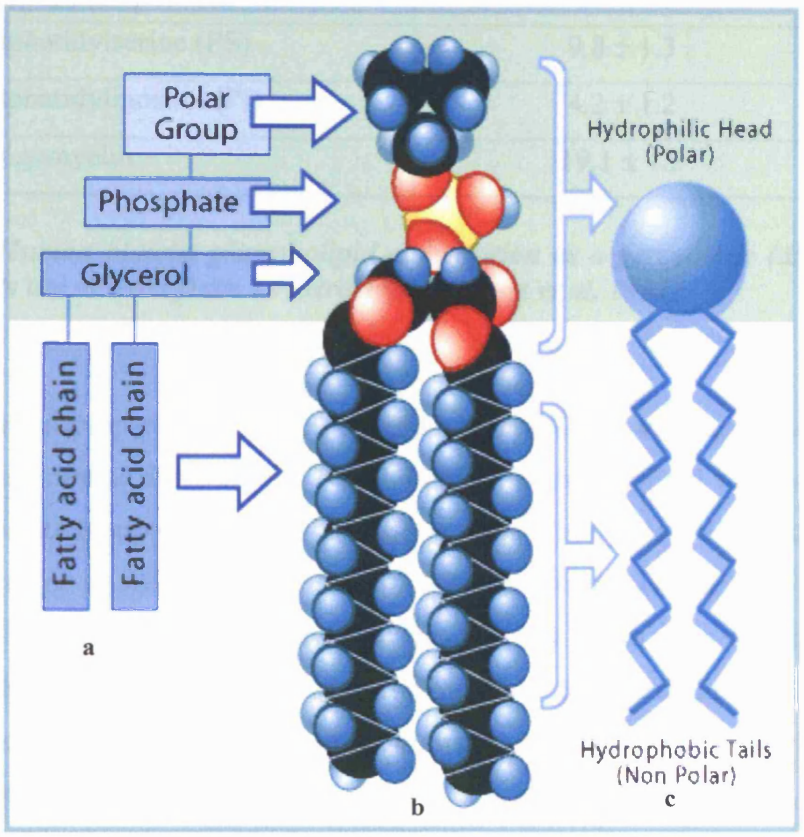



*Figure 1.4: A diagrammatic representation of a phospholipid. (a) Shows the arrangement of the subunits of a phospholipid molecule (b) The space-filling model of phosphatidylcholine (c) Is the diagram widely used to depict a phospholipid molecule. The circle represents the polar end of the molecule and the zig-zag lines the non-polar carbon chains of the fatty acid residues. Diagram courtesy of Professor A.H. Goodall.*

| Phospholipid Type | Membrane Composition (% of total) |
|---|---|
| Phosphatidylcholine (PC) | 36.9 ± 2.1 |
| Phosphatidylethanolamine (PE) | 27.6 ± 1.8 |
| Phosphatidylserine (PS) | 9.8 ± 1.3 |
| Phosphatidylinositol (PI) | 4.2 ± 1.2 |
| sphingomyelin | 19.1 ± 1.2 |

***Table 1.1: Human platelet phospholipid composition as a percentage (±S.D.) of the total. Results are derived from 12 extractions (Bevers et al, 1982).***

The biochemical properties of a phospholipid membrane may vary according to its net charge, in addition to its polarity. The fluidity of a phospholipid membrane is also dependent on its composition for two main reasons. Firstly, cell membranes contain large amounts (up to 50% in some cases) of cholesterol, which can markedly reduce the freedom of phospholipid molecules to diffuse in the plane of a liquid-crystalline membrane. Secondly the fatty acid composition of the phospholipid and the extent to which the acyl chains are unsaturated (how many carbon-carbon double bonds there are, and their conformation) affects membrane behaviour.

The fatty acid composition of phospholipids can vary with cell type, species, age and diet. Phospholipids with fewer double bonds have higher melting temperatures (the point at which a gel-phase membrane with restricted phospholipid motility 'melts' to become liquid crystalline), so the mobility of surface-bound and intrinsic proteins would be much reduced.

### 1.5.2 The role of membrane asymmetry in blood coagulation

The plasma membrane of healthy cells is characterised by an asymmetric distribution of the various phospholipid species over the two membrane leaflets (Figure 1.5).

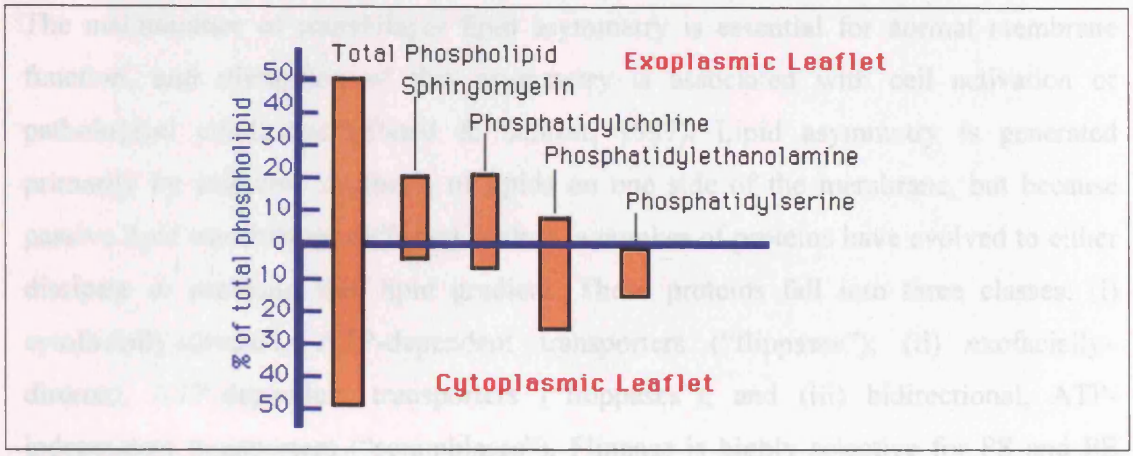



***Figure 1.5: Distribution of phospholipid species across the cell membrane***

The inner leaflet facing the cytosol contains PS, PI, PE and PC. The outer leaflet facing the environment, harbours PE (but to a lesser degree than the inner leaflet), PC and sphingomyelin. This asymmetry is generated and maintained by energy-dependent processes, which rely on the activity of an aminophospholipid translocase (APT). APT is defined by its ability to transport PE and PS from the outer to the inner leaflet, but has not been identified at the molecular level thus far. Reviews of membrane phospholipid asymmetry in health (Zachowski, 1993) and disease (Zwaal & Schroit, 1997) have been published.

The appearance of anionic phospholipids, particularly PS, on the cell surface participates and enhances the activity of the prothrombinase complex culminating in the formation of thrombin (Bevers *et al*, 1982; Connor *et al*, 1989). Changes in the

disposition of PE and sphingomyelin may interfere with ability of PS containing membranes to activate prothrombinase (Smeets *et al*, 1996).

### 1.5.3 Maintenance of phospholipid asymmetry

The maintenance of transbilayer lipid asymmetry is essential for normal membrane function, and disruption of this asymmetry is associated with cell activation or pathological conditions (Zwaal & Schroit, 1997). Lipid asymmetry is generated primarily by selective synthesis of lipids on one side of the membrane, but because passive lipid transbilayer diffusion is slow, a number of proteins have evolved to either dissipate or maintain this lipid gradient. These proteins fall into three classes: (i) cytofacially-directed, ATP-dependent transporters ("flippases"); (ii) exofacially–directed, ATP-dependent transporters ("floppases"); and (iii) bidirectional, ATP-independent transporters ("scramblases"). Flippase is highly selective for PS and PE and functions to keep these lipids sequestered from the cell surface. Floppase activity has been associated with the ABC class of transmembrane transporters. Although they are primarily non-specific, at least two members of this class display selectivity for their substrate lipid. Scramblases are inherently non-specific and function to randomise the distribution of newly synthesised lipids in the endoplasmic reticulum or plasma membrane lipids in activated cells. Inhibition of the ATP-dependent activities stops lipid movement, but does not result in loss of asymmetry for at least several days *in vitro* (Zwaal & Schroit, 1997). However, influx of $Ca^{2+}$ into the cytoplasm and subsequent activation of intacellular calpain, activates a scramblase activity that results in rapid transbilayer phospholipid mixing that leads to a nearly symmetric distribution of phospholipids across the membrane bilayer (Bevers *et al*, 1999). Loss of membrane lipid asymmetry is often accompanied by blebbing and subsequent shedding of lipid-symmetric microparticles from the cell surface. Thus it is the combined action of these proteins and the physical properties of the membrane bilayer that generate and maintain transbilayer plasma membrane phospholipid asymmetry (Figure 1.6).

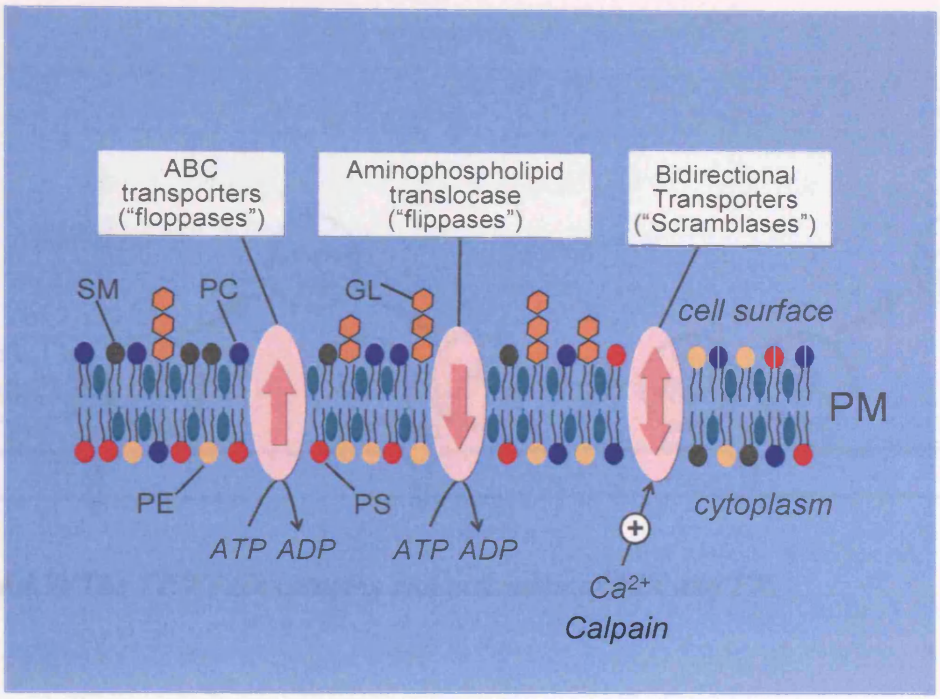



***Figure 1.6: Maintenance of membrane phospholipid asymmetry. The plasma membrane (PM) distribution is maintained by PS being inwardly directed by flippase, whilst PC is outwardly directed by floppase. A non-specific, $Ca^{2+}$ stimulated scramblase randomises phospholipid distribution in activated cells.***

In summary, at physiological (*ie*, low) cytoplasmic $Ca^{2+}$ levels, both "flippases" and "floppases" are active, and phospholipid asymmetry is maintained. Conversely, high cytoplasmic $Ca^{2+}$ concentrations activate lipid scramblase and block the co-operative action of "flippases" and "floppases", leading to randomisation of phospholipids across the membrane bilayer and consequent membrane collapse.

### 1.5.4 The Tissue Factor / FactorVII complex

Tissue factor is a 47-kD membrane glycoprotein which when tightly associated with phospholipids and FVIIa, is the *in vivo* trigger of blood coagulation. The TF molecule consists of three domains: an intracellular region, a transmembrane domain, and an extracellular domain which following binding of FVII and its active form FVIIa activates FIX and FX, leading to fibrin formation (Figure 1.7).

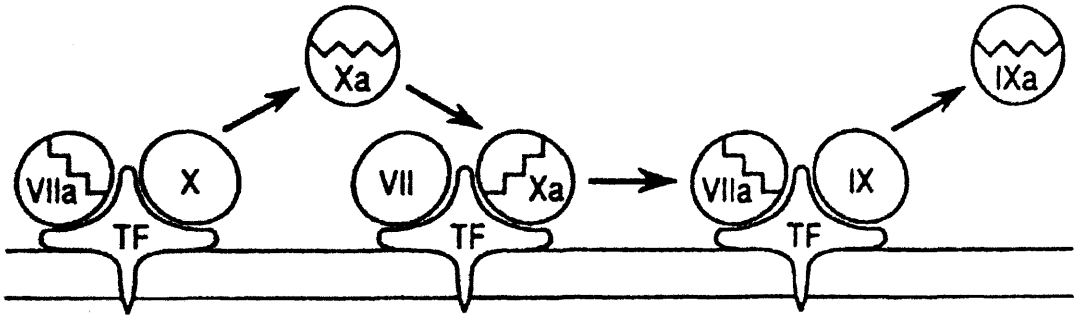



***Figure 1.7: The TF/FVIIa complex and activation of FIX and FX.***

TF:VIIa has an extremely restricted substrate specificity. Until the end of the 20th Century, only three protein substrates for this enzyme were known: factors VII, IX and X. Factor X is the favoured substrate in solution. However, activation reactions taking place on intact cells can show a preference for either FIX or FX, the reasons for this are not clear (Bom *et al*, 1991; Rao *et al*, 1992). When FIX is activated, two peptide bonds must be cleaved. TF:VIIa catalyses the cleavage of the peptide bond at Arg180 more efficiently than the other at Arg145. Furthermore, FXa and TF:FVIIa can synergize to activate FIX to FIXa more efficiently (Lawson & Mann, 1991). Factor VII also serves as a substrate for FVIIa in an autoactivation reaction enhanced by TF (Pedersen *et al*, 1989). This reaction requires that the TF:VIIa and TF:VII complexes encounter each other by lateral diffusion in the plane of the membrane. Factor VIIa can also catalyse the activation of FVII on sphingosine-containing phospholipids in the absence of TF (Iino & Kisiel, 1996). Therefore the composition and fluidity of the membrane are important in regulating TF:FVIIa activity.

Immunohistochemical studies have localised TF at tissue barriers between the body and the environment, in a pattern consistent with a 'haemostatic envelope' role of TF, whose function is to minimize blood loss following vessel damage. The procoagulant

activity of TF on the cell surface is largely dormant (TF encryption) until alterations of the plasma membrane occur. Thus, disrupted cells generate more procoagulant activity than intact cells. One explanation is that TF encryption is the result of sequestration of PS, which acts as a cofactor for TF procoagulant activity, and that the membrane alterations during cell injury enhance TF procoagulant activity due to TF decryption (Greeno *et al*, 1996).

Monocytes and endothelial cells, which under normal conditions do not express TF, upon appropriate stimulation, can be induced to synthesize and express it on their membranes. TF gene expression is regulated mainly at the level of transcription, when monocytes and endothelial cells are exposed to lipopolysaccharide (LPS) or inflammatory cytokines. Components of the NF-κB transcription factor family, c-Rel/p65 heterodimers, which are retained in the cytoplasm by the binding of the inhibitor IκBα, migrate to the nucleus where they bind to the κB site in the TF promoter, inducing TF gene transcriptional activation. At variance with endothelial cells and monocytes, tumour cells express intrinsic procoagulant properties without the need of exposure to any inducing agent.

Free FVIIa catalyses the proteolytic activation of its major protein substrates, FIX and X extremely slowly. When FVIIa binds to TF that has been incorporated into suitable phospholipid vesicles, this activity is enhanced (Nemerson & Gentry, 1986). Three main reasons for this rate of enhancement have been proposed. Firstly, TF enhances the rate of substrate hydrolysis by FVIIa, mainly by enhancing the affinity of the substrate for FVIIa rather than altering the rates of any of the subsequent catalytic steps – ie: an allosteric activator of FVIIa enzymatic activity (Payne *et al*, 1996). Secondly, TF appears to provide an extended binding site for the macromolecular substrates FX and FIX, for which there is evidence from site-directed mutagenesis studies (Huang *et al*, 1996). Thirdly, the reversible binding of FIX and FX to anionic phospholipid surfaces greatly raises the concentration of these substrates in the vicinity of the TF:VIIa complex. Thus phospholipids have the effect of reducing the apparent $k_m$.

### 1.5.5 Tenase and Prothrombinase complexes

Over the past three decades it has become clear that, with only two exceptions (activation of FIX by FXIa and cleavage of fibrinogen by thrombin), the activation steps of coagulation occur upon formation of macromolecular complexes involving at least three proteins and a phospholipid surface containing negatively-charged head groups. The three major activator complexes involved in thrombin generation are the TF/FVIIa (described in section 1.4.2), FVIIIa/FIXa (tenase) and FVa/FXa (prothrombinase) complexes that activate respectively FIXa and/or FXa, FXa and prothrombin (Figure 1.8).

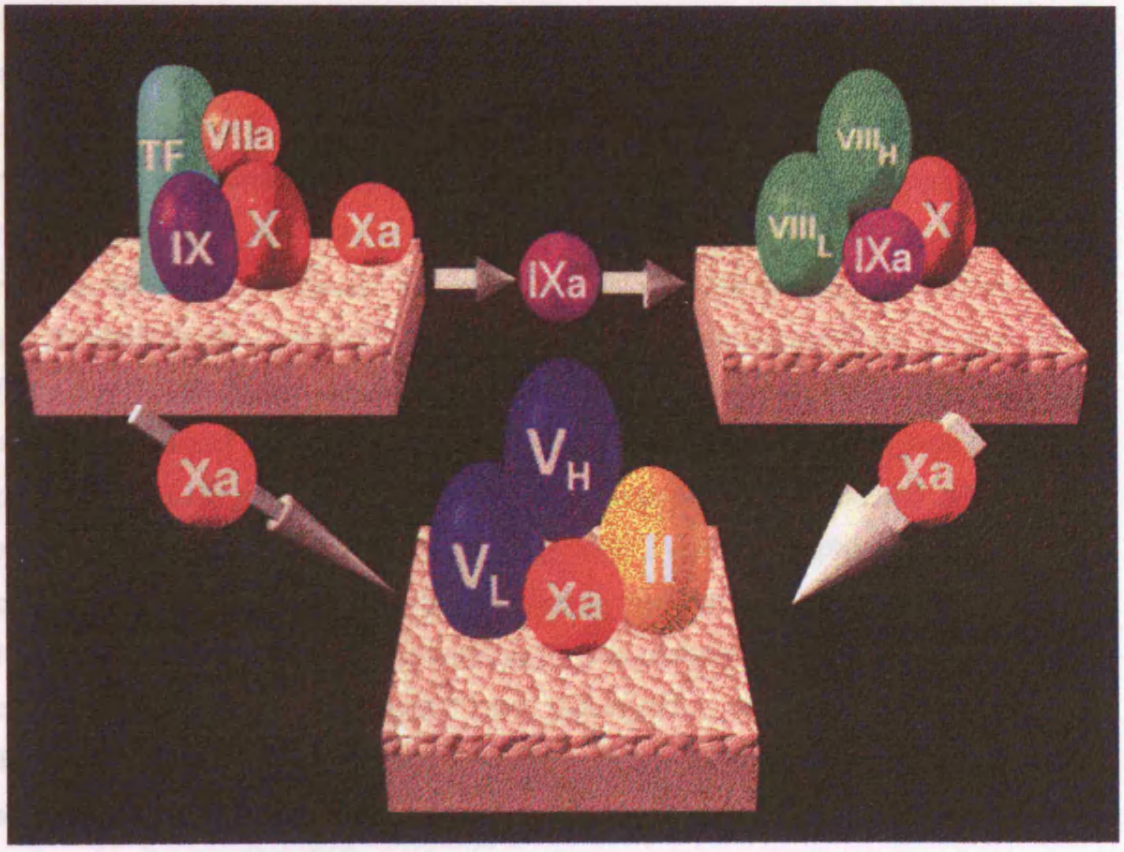



***Figure 1.8: Schematic representation of the vitamin K-dependent complexes required for normal clot formation; upper left complex - extrinsic tenase, upper right complex – intrinsic tenase, and bottom complex – prothrombinase (Kalafatis et al, 1997).***

### *1.5.5.1 The tenase complex*

Activation of the vitamin-K dependent FX is central to the blood coagulation cascade. Efficient generation of FXa is catalysed by enzymatic molecular complexes assembled on phospholipid membranes in the presence of calcium. Proteolytic activation of FX is accomplished by two distinct pathways, the intrinsic and extrinsic pathways. Activation of FX by the FVIII:FIXa complex is usually referred to as "intrinsic," whereas activation via TF:FVIIa is designated "extrinsic" (Figure 1.8). Both FIXa and FVIIa are serine proteases present in plasma in zymogen form as FIX and FVII. Factor VII is converted to FVIIa by traces of FXa, whereas FIXa can be generated by either FXIa or by the TF:FVIIa complex. Factor VIII is a phosphatidyl-L-serine binding cofactor for the vitamin K-dependent serine protease FIXa, which also binds to phosphatidyl-L-serine containing membranes (Gilbert & Drinkwater, 1993). The membrane bound FVIIIa:FIXa complex cleaves FX, to FXa which is then responsible for catalysing prothrombin activation. The importance of these two FX activating reactions *in vivo* has been deduced from the clinical observations that deficiencies in either FIX or FVII result in defective haemostasis. Factor X cleavage sites and resulting reaction products are identical for the "intrinsic" and "extrinsic" FX activating reactions. *In vitro*, FXa formation is efficiently catalysed by FVIII:FIXa complexes when assembled on negatively charged phospholipid vesicles, a catalytic surface which *in vivo* is usually provided by activated blood platelets. FIXa gains more than 100,000 fold greater efficiency in activating FX by assembling with FVIIIa on a phosphatidyl-L-serine containing membrane than when free in solution (van Dieijen *et al*, 1981). Membranes in which phosphatidyl-L-serine is replaced by phosphatidyl-D-serine, phosphatidic acid or phosphatidylglycerol are at least 10-fold less effective for enhancing the $k_{cat}$ (Gilbert & Arena, 1996). Thus it appears that although phosphatidyl-L-serine enhances the alignment of the enzyme with its cofactor and substrate, its biggest effect is activation of the assembled FVIIIa:FIXa enzyme complex.

#### *1.5.5.2 The prothrombinase complex*

Prothrombin is activated to α-thrombin in an anionic phospholipid-dependent process that includes the actions of the serine protease FXa (from the tenase complex) its cofactor FVa, and calcium ions assembled into a macromolecular complex on an anionic phospholipid membrane usually provided by stimulated platelets. The fully assembled complex is termed "prothrombinase" (Figure 1.8).

## 1.6 Regulation of the coagulation system

When thrombin is generated it leads to thrombus formation at the site of vascular injury or possibly in the environs of malignant cells. Natural anticoagulant systems prevent the extension of the thrombus outside the area of injury. There are several control mechanisms to ensure this happens, which include tissue factor pathway inhibitor (TFPI), the protein C pathway, and antithrombin (Figure 1.2).

### 1.6.1 Thrombin

Thrombin is a central bio-regulatory enzyme in haemostasis. Positive and negative feedback reactions, co-operation between platelets and soluble factors, blood flow and interaction with phospholipid surfaces together govern thrombin formation at the site of a wound or thrombus. Positive feedback reactions described in the cell-based model of coagulation leads thrombin to activate FXI, FV and FVIII, thus increasing its own formation. It also activates FXIII which cross-links fibrin, making it less susceptible to degradation by plasmin (Folk & Finlayson, 1977). Thrombin is also a potent activator of platelets (Vu *et al*, 1991) and can induce the "flip-flop" reaction which results in the exposure of procoagulant, negatively charged phospholipids. Alternatively thrombin can stimulate secretion of prostacyclin (Weksler *et al*, 1978) which prevents the adhesion and aggregation of platelets to endothelial cells, thus preventing thrombus formation on endothelial walls (Weiss & Turitto, 1979). In a negative feedback reaction, thrombin also plays a pivotal role in activating the protein C anticoagulant pathway.

### 1.6.2 Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is found primarily on the luminal surface of endothelial cells, and is synthesised by the microvascular endothelium and by megakaryocytes. It functions as a anticoagulant by blocking the extrinsic procoagulant pathway of thrombin generation by forming an inactive complex with FVIIa, TF and FXa (Mann, 1999). Tissue factor pathway inhibitor has three Kunitz type protease inhibitory domains, and the first stage of its inhibitory action is to bind to and inhibit FXa via the Kunitz 2 domain. This binary complex then binds to FVIIa in the TF-FVIIa complex on the cell surface, via the Kunitz 1 domain on TFPI, to form a quaternary complex. Continuation of coagulation is now dependent on the formation of additional thrombin via feedback activation by the small amounts of thrombin generated by the extrinsic pathway. Thus TFPI is fundamental component of the cell-based model of coagulation and participates in the priming phase of the model. This additional thrombin is essential to protect the clot from fibrinolysis (Bouma *et al*, 1998).

### 1.6.3 Thrombomodulin and the Protein C Pathway

The components of this pathway are thrombomodulin, thrombin, protein C, protein S and the endothelial cell protein C receptor (EPCR). Thrombomodulin is an integral membrane protein located on the surface of all vascular endothelia, lymph ducts, and placental vessels (Esmon & Owen, 1981; Maruyama *et al*, 1985). It acts as a cofactor for thrombin. Binding of thrombin to thrombomodulin switches the specificity of thrombin from the cleavage of fibrinogen and activation of factors V,VIII,XI,and XIII to the activation of protein C. In addition, thrombin loses its ability to activate platelets. The inhibition of the procoagulant activity of thrombin is probably caused by the binding of thrombomodulin to the anion binding exosite of thrombin. This site is also the binding site for a variety of substrates and inhibitors of thrombin, such as fibrinogen and hirudin (Esmon, 1993).

Activation of protein C by the thrombin-thrombomodulin complex on endothelial cells leads to an anticoagulant pathway by its ability to proteolytically degrade Va and VIIIa.

Protein S partly bound to C4b binding protein in plasma enhances the anticoagulant effect by serving as a co-factor to protein C. Protein S also potentiates the binding of activated protein C to the platelet and endothelial cell surface. As well as interacting with thrombomodulin protein C also binds to a specific receptor on the endothelial cell membrane, EPCR which alters the substrate specificity of activated protein C in a manner analogous to the specificity switch induced in thrombin by thrombomodulin (Esmon, 2000). This binding is mediated via the γ-carboxyglutamic acid domain of protein C and enhances the rate of its activation by thrombomodulin (Stearns-Kurosawa *et al*, 1996).

The protein C system is well established as a critical negative regulatory mechanism for controlling the haemostatic process. A polymorphism in factor V (factor $V_{Leiden}$) is the most frequent risk factor for familial thrombosis identified to date (Dahlback, 1994). The $FV_{Leiden}$ mutation affects the anticoagulant response to activated protein C at two distinct levels of the coagulation pathway, firstly, it impairs degradation of both FVa and FVIIIa, and secondly, FV containing the Leiden mutation is a poor cofactor for activated protein C (Nicolaes & Dahlback, 2003). In addition, homozygotes for the R2 haplotype of FV which is a series of linked polymorphisms, have a thrombotic tendency resulting from lower plasma concentrations of FV and lower co-factor activity in activated protein C catalysed inhibition of FVIIIa (Hoekema *et al*, 2001). Homozygous deficiency of protein C is associated with a lethal thrombosis in neonates (purpura fulminans) that can be corrected by protein C infusion. Protein S deficiency also can lead to major thrombotic problems. Heterozygous thrombomodulin deficiency in humans appears to be a potential risk factor for thrombosis, and gene deletion of thrombomodulin in mice results in embryonic lethality (Ohlin & Marlar, 1995; Healy *et al*, 1995).

### 1.6.4 Antithrombin and Heparin Co-factor II

Antithrombin (AT) is a vitamin K-independent plasma glycoprotein of 432 amino acids with three disulphide bonds and a molecular weight of 58,200 (Lane *et al*, 1994). It exists in two isoforms, a α-isoform with four carbohydrate side chains and a β-isoform with three side-chains and an increased affinity for heparin (Swedenborg, 1998). In plasma 90% of AT is present as the α-isoform (Peterson & Blackburn, 1985), whilst in the vessel wall the proportion of the two isoforms is equal (Bock *et al*, 1997). It has a major physiological role as an inhibitor of thrombin and other proteases, including factors Xa, IXa, XIa and XIIa.. This depends on the formation of a 1:1 stoichiometric complex between AT and the activated coagulant factor. On its own AT slowly inactivates thrombin and FXa, but in the presence of a therapeutic anticoagulant, heparin its activity increases 10,000 fold (Harper & Carrell, 1994). The effect of heparin is mediated through the pentasaccharide sequence, which has a high affinity to the positively charged heparin binding site of AT (van Boven & Lane, 1997). The binding of heparin to the heparin binding site of AT produces a conformational change in the AT molecule, and thus accelerates its inactivating ability. Inherited AT deficiency is a well-recognised cause of familial thrombophilia. *In vivo*, the major antithrombotic component of endothelial cells is heparan sulphate, a similar glycosaminoglycan to heparin, and the binding of AT to endothelial heparan sulphate potentiates AT inhibitory action.

Heparin cofactor II (HcII) is a 480-amino acid glycoprotein, with a molecular weight of 66,000, produced in the hepatocyte and circulates in plasma. HcII inhibits thrombin but not FXa or other coagulation factors. HcII has an advantage over AT in that it also inhibits surface bound thrombin. HcII probably participates in the *in vivo* inhibition of thrombin, as indicated by low levels of thrombin-HcII complexes in normal plasma samples, but its main action may be in the extravascular space as an inhibitor of thrombin, with dermatan sulphate proteoglycans on fibroblasts and smooth muscle cells serving as cofactors (McGuire & Tollefsen, 1987). Binding of heparin also potentiates HcII thrombin inhibitory activity approximately 1,000 fold (Tollefsen, 1997).

### 1.6.5 The role of the endothelium

The endothelium plays an active and central role in the haemostatic mechanism (Nawroth *et al*, 1986; Stern *et al*, 1986; Pearson, 1994). There is a balance between the clot inhibiting and clot promoting activities of the endothelium so that detrimental intravascular clotting is prevented. Damage to the endothelium results in exposure of procoagulant factors such as collagen and TF in underlying tissue, which can trigger coagulation; however, under normal conditions endothelial cells produce prostacyclin and nitric oxide which prevent platelet adherence and aggregation (Vane, 1994; Leblond & Winocour, 1999). Thrombomodulin molecules on the endothelial surface bind to thrombin and this complex activates protein C as already described (section 1.6.3). Endothelial cells also possess heparin like molecules on their surface which bind antithrombin as already described (section 1.6.4). The endothelium is also a major site of t-PA production and therefore promotes fibrinolysis at the surface and prevents local thrombus formation.

## 1.7 Leucocytes and tumour cell prothrombotic properties

The role of cellular procoagulants in the pathogenesis of fibrin deposition has been recognised since the studies of the "coagulative properties" of leucocytes in 1841 (Addison, 1841).

### 1.7.1 Leucocyte involvement in coagulation and thrombotic disease

The pathophysiology of ischaemic vascular disease is still unresolved despite many studies describing the gross and microscopic lesions within the blood vessels. The role of the platelet and red cell in thrombosis has become increasingly recognised, as has the importance of plasma factors such as fibrinogen. Less attention, however, has been paid to the contribution of the leucocyte to thrombosis at the lesion site.

The blood white cell count (WCC) is a powerful and consistent predictor of cardiovascular events in epidemiological studies. A number of studies have found that an increased WCC within the 'normal range' following myocardial infarction (MI) independent of other risk factors is predictive of further coronary events (Schlant *et al*, 1982; Haines *et al*, 1983; Lowe *et al*, 1985; Mueller *et al*, 2003). It is not known whether these results merely reflect the severity of the first infarct - the larger the initial area of tissue damage, the brisker the acute phase response following MI, with a concomitant increase in WCC - or whether leucocytes are having a direct effect with the procoagulant response being sustained at the site(s) of vascular injury by the recruitment of leucocytes to the growing thrombus. The risks associated with high WCC are not limited to coronary vessels. The total WCC also correlates with the risk of thrombotic stroke (Prentice *et al*, 1982) and with the risk of re-thrombosis following peripheral vascular grafting (Dormandy *et al*, 1988).

Recently a report using a porcine model system to investigate arterial thrombosis found that the thrombotic mass was more regulated by blood components than by the particular arterial segment used (Karnicki *et al*, 2002). Unexpectedly they also found that the thrombus mass and platelet deposition correlated with the lymphocyte count. Leucocytes might contribute to thrombosis in a number of ways: (1) by physical obstruction, (2) by release of noxious chemicals, and (3) by interaction with other blood constituents.

#### *1.7.1.1 Monocyte involvement in blood coagulation*

Monocytes appear to be unique among blood cells apart from activated platelets in that they can provide the appropriate membrane surface for the assembly and function of all the coagulation complexes involved in thrombin generation. The procoagulant activity of monocytes has been ascribed to TF. They can be induced to synthesise and express TF antigen at their cell surface by bacterial LPS and cytokines (Schwartz *et al*, 1982). *In vitro*, monocytes TF activity is up-regulated by a number physiological agonists such as C-reactive protein and interleukin–1 (Drake *et al*, 1989; Cermak *et al*, 1993).

Circulating monocytes and neutrophils interact with aggregated platelets by their constitutive expression of P-selectin glycoprotein ligand-1 (PSGL-1) (Yang, *et al* 1999), the ligand that binds P-selectin an α-granule membrane protein expressed subsequent to platelet activation. This recruitment allows the generation of additional thrombin because prolonged stimulation of monocytes with the cytokines released at the site of injury as well as P-selectin expressed by activated platelets can increase the synthesis and expression of functional TF (Celi *et al* 1994; Morrissey, 2001). After the formation of FIXa and FXa by a TF/FVIIa complex, the coagulant response can be propagated by the assembly and function of intrinsic tenase and prothrombinase on the monocyte surface, which express catalytic efficiencies identical to those expressed on activated platelets (Tracy *et al*, 1983; McGee *et al*, 1994). The ability of monocytes to generate FXa via intrinsic tenase is critical for sustained thrombin generation at the monocyte surface because functional TF activity can be inhibited by co-expression of TFPI (McGee *et al*, 1994).

The assembly and function of prothrombinase appears to be facilitated/amplified by several mechanisms unique to monocytes. Similar to observations made with platelets, FVa bound to monocyte surface is resistant to inactivation by activated Protein C, suggesting that the cofactor activity is sustained at the cell surface and that monocytes are unable to effectively down regulate prothrombinase (Colucci *et al*, 2001). Thus it is likely that a membrane component present on both activated platelets and monocytes protects FVa from inactivation by activated Protein C to promote sustained thrombin generation at sites of vascular injury (Colucci *et al*, 2001). In a proposed alternative pathway for the initiation of thrombin generation at the monocyte surface, agonist-induced binding of FX to Mac-1 (CD11b/CD18) triggers release of the enzyme cathepsin G from monocyte azurophil granules, which activates FX to FXa (Plescia *et al*, 1996). The cathepsin G-activated FXa remains associated with the monocyte membrane through its interaction with effector cell protease receptor-1 (EPR-1). FXa bound to monocytes in the absence of FVa can activate prothrombin to thrombin, but at a rate substantially less than that catalysed by monocyte-bound prothrombinase (Altieri *et al*, 1990). A factor Xa-EPR-1 complex can also generate FIX activation

intermediates. Because monocyte-associated cathepsin G can also activate FV to FVa, monocytes may be able to initiate blood coagulation via FX binding to Mac-1 followed by assembly of functional prothrombinase (Allen *et al*, 1995). Mac-1 also functions as an inducible, high-affinity receptor for fibrinogen in a reaction associated with increased fibrin deposition at the cell surface. Once formed, fibrin may function to modulate the coagulation response through its ability to down-regulate lipopolysaccharide-induced (LPS) TF expression by monocytes (Rossiello *et al*, 2000).

Thus blood coagulation pathways can operate efficiently to generate thrombin on the monocyte or macrophage membrane. Once generated thrombin can bind to the monocyte membrane (Goodnough & Saito, 1982) and cleave bound fibrinogen to fibrin monomers (Gonda & Shainoff, 1982; Altieri *et al*, 1988), which are subsequently cross-linked by FXIIIa-like molecule released from these cells (Weisberg *et al*, 1987).

### *1.7.1.2 Neutrophil and lymphocyte involvement in blood coagulation*

Neutrophils modulate activation of coagulation through their production and release of reactive oxygen species (Cadroy *et al*, 2000). Depending on the number of neutrophils present, the released neutrophil-reactive oxygen species can have either a positive or negative effect on the regulation of TF expression subsequent to monocyte stimulation by LPS. Controversially, it has also been reported that neutrophils can be induced to express TF, and they can also assemble a prothrombinase complex that is analogous and has a catalytic efficiency similar to that expressed by activated platelets and monocytes (Tracy *et al*, 1983). They can also form close cell/cell contacts with activated platelets (Bazzoni *et al*, 1991) and endothelial cells (Parent & Eichacker, 1999). However they do not support the assembly and function of intrinsic tenase (McGee & Li, 1991).

Even less is known about the assembly and function of procoagulant complexes on lymphocytes. Similar to neutrophils, normal lymphocytes do not support assembly and function of intrinsic tenase. (McGee & Li, 1991) but prothrombinase can assemble and

function on isolated lymphocytes (Tracy *et al*, 1983; Tracy *et al*, 1985). The kinetics of prothrombin activation shows that these cells participate in thrombin generation in a manner analogous to monocytes in that FVa and FXa form a 1:1 stoichiometric, calcium dependent complex on their membrane surfaces. Neutrophils and lymphocytes express approximately 8000 and 45000 prothrombinase binding sites per cell, respectively, governed by an apparent $K_d \approx 10^{-10}$ mol/L (Tracy *et al*, 1985). Lymphocytes express an apparent $K_m$ of 0.62 μmol/L that is similar to that expressed by monocytes and platelets. In contrast, the lymphocyte-bound complexes generate thrombin at a considerably slower rate (Table 1.2). Prothrombinase assembled on neutrophils express a catalytic efficiency that is nearly identical to that of monocytes. However, prothrombinase is assembled on lymphocytes with a catalytic efficiency that is considerably less than that of monocytes or platelets. Nonetheless normal lymphocytes do have the ability to support the assembly of the prothrombinase complex.

| | $K_d$app (mol/L x$10^{10}$) | Number of binding sites/cell | $K_{cat}$ (S) | $K_m$app (mol/L x$10^{10}$) |
|---|---|---|---|---|
| Monocytes | 0.4 | 16000 | 34 | 0.45 |
| Neutrophils | 0.2 | 8000 | 28 | Not Done |
| Lymphocytes | 1.3 | 45000 | 8 | 0.62 |
| Platelets | 1.2 | 2700 | 35 | 0.82 |

***Table 1.2: kinetic and binding parameters defining prothrombinase complex assembly and function on the surface of peripheral blood cells (Tracy et al, 1985).***

### 1.7.2 Classification of T-lineage acute lymphoblastic leukaemia (T-ALL)

The malignant T-cells used in this study were all immortalised cell lines from patients suffering from childhood acute lymphoblastic leukaemia of T-cell lineage (T-ALL). It is an aggressive haematopoietic malignancy that accounts for 10 – 15% of paediatric and 25% of adult ALL cases. Patients with T-ALL often show very high circulating blast cell counts, mediastinal masses, and infiltration of the central nervous system at

the time of diagnosis. In the early days soon after the development of combination chemotherapy for ALL, T-ALL patients had a dismal prognosis, with cure rates of 10% or less. However, after the introduction of modern intensified chemotherapeutic regimens, a significant improvement was observed in the prognosis of children and adolescents with T-ALL, so now there are relapse-free survival rates of 60-75% at five years post diagnosis. Similar advances have been made in the treatment of adults with T-ALL, also based on more aggressive combination chemotherapy, with cure rates now exceeding 50%. Further improvement is hampered by the lack of prognostic markers, and the increased risk of thrombosis associated with chemotherapy.

T-lineage ALL is derived from early, precursor T-cells found in the thymus, which is the 'central' T-cell organ (Zuniga-Pflucker & Lenardo, 1996). During the process of T-cell precursor differentiation, the cells rearrange their T-cell receptor (TCR) genes during which the enzyme terminal deoxyribonucleotidyl transferase (TdT) is expressed and active. Once a functional TCR molecule has been produced the process of positive and negative selection take place. During 'positive selection', the ability of the nascent TCR to bind to self class I or class II antigens of the major histocompatability complex (MHC) is tested, and cells that fail to do so are clonally deleted since they will be unable to perform their required function of recognising foreign peptide when associated with self MHC (Chan *et al*, 1994). Conversely, cells that bind with too great an avidity to self peptide presented by MHC are also deleted since they are likely to be autoreactive (negative selection)(Clayton *et al*, 1997). Prior to this period of positive and negative selection, T-cell precursors do not express either CD4 or CD8 ('double negative' cells). During the selection process, they express both CD4 and CD8 ('double positive') before becoming committed to being either single $CD4^+$ or $CD8^+$ T-cells (Zuniga-Pflucker & Lenardo, 1996). In terms of the immunological classification of T-lineage ALL, CD4 positivity is not sufficient to assign lineage specificity since a significant percentage of patients with AML (especially those with a monocytic component) are also $CD4^+$. Similarly, the T-cell antigens CD2 and CD7 are expressed by a proportion of those with AML (Bene *et al*, 1995). As T-cell precursors differentiate along these pathways, they move physically from the thymic cortex

towards the medulla. Cortical thymocytes express CD1a. The defining feature of T-lineage ALL is the presence of cytoplasmic or membrane CD3 (Bene *et al*, 1995). CD3 forms an integral part of the TCR antigen complex and once the TCR-CD3 complex is expressed on the cell membrane, then the cell is defined as a 'T-cell'. Prior to this stage, T-cell precursors express CD3 in the cytoplasm (cytCD3) in an analogous manner to pre-B-cells, which express cytoplasmic IgM (μ) heavy chains.

Patients with T-lineage ALL are classified according to their place within this sequence of differentiation. The early MIC classification simply divided T-lineage ALL into 'early precursor T-ALL' ($CD2^-$) and 'T-ALL' ($CD2^+$) (First MIC Cooperative Study Group, 1986). Both groups are predominantly $TdT^+$ and $CD7^+$. Subsequent classifications have tried to mirror thymic T-cell precursor differentiation in more detail and have highlighted the paramount importance of cytoplasmic $CD3^+$ or surface membrane $CD3^+$ ($smCD3^+$) in assigning T lineage (Table 1.3) (Bene *et al*, 1995).

In T-lineage ALL, HLA-DR and TdT positivity is the rule especially in the less mature cases. In contrast to B-lineage ALL, immunologically defined subtypes of T-lineage ALL are not specifically associated with certain cytogenetic abnormalities or prognostic categories. However, generally, the more immature subtypes have a worse prognosis (Thiel *et al*, 1989) while $CD10^+$ is a favourable prognostic indicator (Pui *et al*, 1993). A number of recurring chromosomal translocations are found in T-lineage ALL and many of these involve the TCR α/γ locus (14q11-13), the TCR β locus (7q32-36) or the TCR γ locus (7p13) (Thandla & Aplan, 1997).

| T-ALL type | Description | Immunophenotype |
|---|---|---|
| T-1 | Pro-T | $CD1a^{-}CD2^{-}cytCD3^{+}smCD3^{-}CD5^{-}CD7^{+}$ |
| T-II | Pre-T | $CD1a^{-}CD2^{+}cytCD3^{+}smCD3^{-}CD5^{+}CD7^{+}$ |
| T-III | Cortical T | $CD1a^{+}CD2^{+}cytCD3^{+}smCD3^{+/-}CD5^{+}CD7^{+}$ |
| T-IVa | Mature T (TCR $\alpha\beta^{+}$) | $CD1a^{-}CD2^{+}cytCD3^{+}smCD3^{+}CD5^{+}CD7^{+}$ and usually $CD4^{+}$ or $CD8^{+}$ |
| T-IVb | Mature T (TCR $\gamma\delta^{+}$) | $CD1a^{-}CD2^{+}cytCD3^{+}smCD3^{+}CD5^{+}CD7^{+}$ and usually $CD4^{-}$ or $CD8^{-}$ |

***Table 1.3: The EGIL classification of T lineage acute lymphoblastic leukaemia (T-ALL).***

EGIL, European Group for Immunological Classification of Leukaemias; TCR, T-cell receptor. All patients are postive for cytoplasmic (cyt) or surface membrane (sm) CD3 and terminal deoxribonucleotidyl transferase (TdT); some are $CD10^{+}$.

### 1.7.3 Pathogenesis of thrombosis associated with cancer

Thrombosis was identified as a complication of cancer by Trousseau in 1865, and the combination of the two conditions is still often called Trousseau's syndrome, although in an earlier report in 1823, Bouillard, another French physician described three patients with deep vein thrombosis and cancer. The first to report the observation of venous thromboembolism as a sign of truly occult malignancy were Illtyd James and Matheson, in 1935.

Arterial and, more commonly, venous thrombosis is a frequent complication of cancer, and sometimes a harbinger of occult cancer. Moreover, the use of new and aggressive therapy for cancer increases the risk of thrombosis. There are many causes of thrombosis in cancer. Cancer itself is often the underlying mechanism. Possible contributory mechanisms for blood clotting activation in cancer patients include general factors related to the patient's response to the tumour and more specific factors such as the clotting activities expressed by tumour cells and tumour-associated macrophages.

These mechanisms are summarised in Table 1.4, specific cancer cell procoagulant activities are described in the following sections.

| ***GENERAL*** |
|---|
| Inflammation<br>Local necrosis<br>Acute-phase reaction<br>Dysproteinemia<br>Hemodynamic disorders |
| ***SPECIFIC FACTORS*** |
| Cancer cell activities<br>Procoagulant<br>Fibrinolytic<br>Interactions with platelets<br>Interactions with mononuclear phagocytes<br>Interaction with endothelial cells<br>Neovascularisation<br>Chemotherapy<br>Hormone therapy. |

***Table 1.4: Possible mechanisms of thrombosis in malignancy.***

### *1.7.3.1 Role of tissue factor in coagulation and presence in tumour cells*

Tissue Factor has been found in many cancer tissues (Edwards *et al*, 1993) and its expression can be upregulated by tumour necrosis factor alpha (TNFα), interleukin-1β (IL-1β) and other cytokines (Carmeliet & Collen, 1998). The high expression of TF may be characteristic of the malignancy of a cell, as illustrated in the case of the acute promyelocytic leukaemia cell line HL-60. The upregulated messenger RNA (mRNA) of TF in the cell returns rapidly to normal levels upon the differentiation of the cell to normal neutrophils after treatment with all-*trans* retinoic acid (ATRA) (Rickles *et al*, 1995). The procoagulant functions of TF that lead to thrombin generation are critically

important to support metastasis, in part through the generation of fibrin, which allows prolonged arrest of tumour cells in organs. The importance of clotting activation in the invasive process is indicated by the inhibition of metastasis by anticoagulant treatments. In addition, the coagulation initiation complex, that is, TF:FVIIa, generates autocrine cell signalling through PAR-1 and 2 (Ruf & Mueller, 1999). Angiogenesis is a multi-factorial process of *de novo* vessel formation required for invasive tumour growth and metastasis. The finding that there was markedly defective vessel formation of yolk-sac vessels in TF knockout mice embryos followed by massive haemorrhaging suggests a role of TF in fetal angiogenesis (Carmeliet *et al*, 1996). Subsequently it was discovered that these TF-null mouse embryos could be rescued by human TF lacking the cytoplasmic domain, suggesting that embryogenesis requires the extracellular protease activity of the TF:VIIa complex (Parry & Mackman, 2000). A positive correlation between angiogenesis and levels of TF expression was found in human prostate carcinoma (Abdulkadir *et al*, 2000) and in non-small-cell lung carcinoma (Koomagi & Volm, 1998). Tissue factor and vascular endothelial growth factor have also been found to co-localise in human tumour cells (Contrino *et al*, 1996; Koomagi & Volm, 1998; Shoji *et al*, 1998). Thus co-operation of the TF cytoplasmic domain with protease signalling may explain the different contributions of TF to metastasis and angiogenesis.

### *1.7.3.2 Cancer procoagulant*

Cancer procoagulant (CP), E.C.3.4.22.26, is a cysteine proteinase produced by a broad spectrum of malignant or fetal tissue and amnion-chorion membranes but not in normally differentiated tissues (Gordon & Mielicki, 1997). Cancer procoagulant activates the coagulation cascade in the absence of FVII by a proteolytic cleavage of the heavy chain of FX. However, the site of FX hydrolysis by CP is slightly different from the site cleaved by FIXa/FVIIIa, FVIIa/TF or procoagulant from Russell's viper venom (RVV). CP recognises the peptidyl bond between $Tyr^{21}$ and $Asp^{22}$ while the other procoagulants activate FX at $Arg^{52}$ (Gordon & Mourad, 1991).

For a long time FX was considered as the only known physiological substrate for CP. However experiments on stimulation of platelet adhesion by CP suggest that PAR-1 may be another possible protein substrate for CP. Although the cellular activation events by CP may be initiated by a standard receptor (ligand – binding interactions), it seems that CP competes with thrombin for the PAR-1 receptor on the platelet surface (Olas *et al*, 2000).

Various types of natural and synthetic proteinase inhibitors and metal ions inhibit the enzymatic activity of CP. The effectiveness of most inhibitors increases in the presence of a reducing agent in the reaction environment. CP seems to be an enzyme regulated allosterically. Certain divalent metal ions may modulate the FX-activating activity of CP. It has been shown that the metals do not participate directly in the reaction within the active site, but metal-protein interactions takes place in other regions of the enzyme. It appears likely that the divalent metals are responsible for active, or inactive conformation of CP (Mielicki *et al*, 1994).

#### *1.7.3.3 Other cancer cell-derived procoagulant activities*

Procoagulants other than CP have been reported to activate FX. A less well-studied and characterised tumour cell procoagulant is CCA-1 (cancer cell-derived blood coagulating activity 1) that has been found in LK52 human squamous cell carcinoma cells. This procoagulant is enzymatically different from CP. A monoclonal antibody to CCA-1 inhibits the procoagulant activity and does not cross-react with either TF or CP (Inufusa *et al*, 1998). In addition to CCA-1, other procoagulant activities have been described. A substance has been partially purified from mucus-secreted adenocarcinomas that had factor X-activating activity (Pineo *et al*, 1973). Also a methylcholanthrene-induced rat fibrosarcoma has been reported to produce a serine protease FX activator which was inactivated by benzamidine and aprotinin (Pangasnan *et al*, 1992).

It has also been reported that one of the major histocompatibility antigens, HLA-DR, has procoagulant activity. HLA-DR does not require the presence of FVII to generate thrombin, but this PCA has not been observed in plasma deficient in factors V, VIII, IX, XI, or XII (Chelladurai *et al*, 1991). HLA-DR expression is observed not only in malignant tumours but in normal tissues as well; however, HLA-DR expressed on the intact surface of B cells does not have coagulation activity. Accordingly the mechanism whereby HLA-DR expresses procoagulant activity remains unclear. Also, high expression of glycosphingolipids has been reported to be involved in procoagulant activity of cancer (Suzuki *et al*, 1999).

Multiple myeloma, a B-cell malignancy, is associated with hypercoagulability and four basic mechanisms have been described: interference of immunoglobulins on fibrin structure, procoagulant autoantibody production (ie: lupus anticoagulant), effects of inflammatory cytokines on endothelium, and acquired activated protein C resistance.

Tumour cells can express all the proteins regulating the fibrinolytic system, including urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA), and the fibrinolysis inhibitors PAI-1 and PAI-2 (Falanga & Rickles, 1999). Among the activators, u-PA is the most widely expressed by tumour cells. Furthermore, cancer cells can carry the specific plasminogen activator receptor (uPAR) on their cell-surface. The presence of these receptors favours the assembly of all the fibrinolytic components on tumour cell membranes, facilitating the activation of the fibrinolytic cascade. Conversely, impaired plasma fibrinolytic activity in patients with solid tumours represents another tumour-associated prothrombotic mechanism (Falanga & Rickles, 1999). Importantly, fibrinolysis proteins can play a role in tumour cell proliferation, invasion and metastasis.

#### *1.7.3.4 Chemotherapy and Acute Tumour Lysis Syndrome (ATLS)*

Aggressive antitumour therapy with such commonly used agents as platinum compounds, high dose fluorouracil, mitomycin, tamoxifen, and growth factors

(granulocyte colony-stimulating factor, granulocyte-monocyte colony-stimulating factor, and erythropoietin) increases the risk of thrombosis (Delmas *et al*, 1998; Levine *et al*, 1998; Lee & Levine, 1999). The underlying mechanisms are poorly understood, but many of these therapeutic agents cause vascular damage as well as inducing apoptosis/necrosis in the tumour cells with subsequent exposure of procoagulant phospholipid.

Central venous catheters, commonly inserted for chemotherapy and hyperalimentation, are also associated with a risk of thrombosis and embolism. The thrombogenic surface of these catheters can activate platelets and serine proteases, such as Factors XIII and X. Moreover, gram-negative organisms that infect central venous catheters can release endotoxin, and gram-positive organisms can release bacterial mucopolysaccharides. These bacterial polysaccharides can activate Factor XIII, induce a platelet-release reaction, and cause sloughing of endothelial cells; each of these activities increases the risk of thrombosis. Endotoxin also induces the release of TF, tumour necrosis factor, and interleukin-1, which can incite thrombogenesis.

Acute tumour lysis syndrome (ATLS) was formally recognised in 1980, (Cohen *et al*, 1980) giving a name to a hyperuricemic syndrome in the setting of lymphoid malignancies, but now is recognised to be a specific oncological disorder that occurs when tumour cells are destroyed rapidly by natural means, chemotherapy, or radiation therapy. ATLS is an example of increased thrombogenicity of apoptotic and necrotising tumour cells. The laboratory findings include hyperuricemia, hyperkalemia, hyperphosphatemia, and hypocalcemia. The potassium and phosphate result from the intracellular contents of dying cells; the uric acid from the metabolism of purines in the degradation of nucleic acids; and because calcium is downregulated in the presence of high phosphate levels. Renal failure results from tubular precipitation of uric acid at acid pH and calcium at alkaline pH and classically shows evidence of calcification on renal ultrasound. The time of greatest risk of ATLS is during initiation of therapy, especially rapidly effective therapies such as induction therapy for acute lymphocytic leukaemia (ALL) in children. The syndrome is most commonly associated with

haematological malignancies, including lymphocytic leukaemia, chronic lymphocytic leukaemia, chronic myelogenous leukaemia, and classically with Burkitt's lymphoma (Jasek & Day, 1994). The severe degree of thrombogenicity often leads to microvascular coagulation and a disseminated intravascular coagulation picture.

#### *1.7.3.5 Phospholipid-dependent coagulation*

Recent studies have found that malignant T-lymphoblastoid cell lines are very active in promoting the generation of thrombin in plasma. This activity appears to be unrelated to TF, which is only present at very low levels in these cells, and it is postulated that this activity might be due to the exposure of negatively charged phospholipids which can promote the generation of FXa and thrombin (Barrowcliffe *et al*, 1989, 2002). The pathological processes that might lead to the exposure of procoagulant phospholipid are addressed in section 1.8.

## 1.8 Pathological changes in membranes that might support coagulation

The maintenance of an asymmetric distribution of phospholipids across the plasma membrane, with neutral phospholipids predominating on the external surface and anionic phospholipids confined to the cytoplasmic leaflet of the membrane, thus providing a non-thrombogenic surface, has now been well established. There are two main pathological processes that are capable of making the surface of a cell membrane thrombogenic. The first is apoptosis, where there is a loss of phospholipid asymmetry and PS is exposed on the outer leaflet. The second process is lipid peroxidation. Here the reasons why a membrane subjected to oxidative stress might become procoagulant are not so clear and are discussed later (see chapter 6). It must also be taken into account that oxidative stress, and lipid peroxidation in particular, have been implicated in the final pathway of apoptosis (Chandra *et al*, 2000), whilst oxidised lipids themselves can induce apoptosis (Wintergerst *et al*, 2000; Hsieh *et al*, 2001). Evidence for the synergy of these two pathological processes is evidenced by increased apoptosis

(Kockx *et al*, 1998; Mallat & Tedgui, 2000) as well as the presence of oxidised lipoproteins in atherosclerotic lesions (Okura *et al*, 2000; Rader & Dugi, 2000).

### 1.8.1 Apoptosis and cell death

Two alternative modes of cell death can be distinguished, 'apoptosis' that was first described by Kerr (Kerr *et al*, 1972) and accidental death usually defined as 'necrosis'. Necrosis due to ischaemia is especially referred to as 'oncosis'. Apoptosis is a death process that is controlled by intrinsic cellular mechanisms and is implicated in the steady state kinetics of healthy tissues. Although apoptotic cell death is widespread, dying cells are rarely seen *in vivo* because of their rapid clearance by phagocytes. When apoptotic cells are not phagocytosed they undergo secondary necrosis.

#### *1.8.1.1 Apoptosis*

Apoptosis is a highly conserved, biological process that allows the controlled removal of cells with genetic damage, those with improper developmental changes or those that are produced in excess. It was first described in 1842, by Vogt as the morphology of dying cells during the metamorphosis of amphibians, and the term "apoptosis" was first used in the early 1970's after the recognition that morphologically similar cell deaths were found in many pathological conditions as well as for normal tissue (Kerr *et al*, 1972).

Apoptosis is characterised by cell shrinkage, nuclear fragmentation and membrane blebbing. These morphological features appear to result from an energy-dependent process with a high degree of regulation at the biochemical level (Hengartner, 2000). Caspases, which constitute a family of cysteine-dependent-aspartate-directed proteases, play an important role in the biochemistry of apoptosis. They reside in the cytosol and intracellular organelles like mitochondria and endoplasmic reticulum as dormant zymogens. They are activated by proteolysis and then trigger a caspase cascade, which

generates a proteolytic burst within the cell. Caspase activation can start from receptor/ligand-associated signal transduction (extrinsic activation) or from the endoplasmic reticulum or mitochondrial-dependent apoptosome formation (intrinsic activation).

### *1.8.1.2 Necrosis and oncosis*

Necrosis is signalled by irreversible changes in the nucleus (karyolysis, pyknosis, and karyorhexis) and in the cytoplasm (condensation and intense eosinophilia, loss of structure and fragmentation). During pathological and physiological conditions such as severe hypoxia or ischaemia, major changes in environmental temperature, disruption of cell membrane by complement and exposure to toxins, the cell may instead directly undergo necrosis. This focal necrosis can often be seen in the centre of tumour nodules. Thus, necrosis is determined by dramatic environmental pertubations rather than intrinsic factors acting within the cell itself.

Cell death due to ischaemia is characterised by swelling and it was named in 1910 by von Recklinghausen as 'oncosis' (Majno & Joris, 1995). Although this pathology was first used primarily for bone cells, it can now be related to any ischaemic tissue. Oncosis involves a swelling of the cell and its intracellular organelles culminating in plasma-membrane rupture, blebbing and the release of intracellular proteins (see Table 1.5).

Therefore, necrosis can be diagnosed by tests of membrane permeability. Consequently, unlike apoptosis it induces a localised inflammatory response that can cause further tissue injury mainly due to leucocyte infiltration. However it is still unclear how necrotic cells trigger inflammation, this ability may be critical in linking tissue damage to the damage of immune responses.

### *1.8.1.3 Paraptosis*

A newly described type of programmed cell death (PCD) termed 'parapoptosis' has been described, it is driven by an alternative caspase-9 activity, and is distinct from apoptosis by criteria of morphology, biochemistry and response to apoptotic inhibitors (Sperandio *et al*, 2000). The main differences are that in parapoptosis there is a lack of nuclear fragmentation, DNA fragmentation, cytoplasm condensation or the formation of apoptotic bodies, together with an absence of caspase activity.

| | Apoptosis | Necrosis | Paraptosis |
|---|---|---|---|
| **Morphology** | | | |
| Nuclear fragmentation | + | - | - |
| Chromatin condensation | + | - | ± |
| Apoptotic bodies | + | - | - |
| Cytoplasmic vacuolation | - | + | + |
| Mitochondrial swelling | Sometimes | + | Late |
| **Genomic effect** | | | |
| Internucleosomal DNA fragmentation | + | - | - |
| **Caspase activity** | | | |
| DEVD-cleaving activity | + | - | - |
| Caspase-3 processing | + | - | - |
| PARP cleavage | + (85 kDa fragment) | + (50 to 62 kDa fragments) | - |
| **Inhibition by:** | | | |
| ZVAD.FMK | + | - | - |
| BAF | + | - | - |
| p35 | + | - | - |
| XIAP | + | - | - |
| Bcl-$x_L$ | + | Usually - | - |
| Actinomycin D | Sometimes | - | + |
| Cycloheximide | Sometimes | - | + |

***Table 1.5: Comparison of apoptosis, necrosis and paraptosis.***

### *1.8.1.4 Mechanism of cell death via apoptosis*

Apoptosis is an active cellular suicide either carried out following stimulation from outside the cell or intrinsically by the withdrawal of instruction to survive. Apoptosis is viewed as a two-stage process: **sentencing**, which has a variable duration and is carried out entirely at a biochemical level, and **execution**, which is the visible death of the cell and is universally of short duration. A chain of biochemical events leads to point where the cell is irreversibly committed to die. As in the cell cycle, cytoplasmic factors instruct the nucleus during this period. Proteins such as Bcl-2 retard entry to the execution phase, whilst p53 promotes it. This activates endonucleases which cleave internucleosomal regions causing a characteristic DNA-laddering effect. DNA cleavage patterns may however be less extensive in many cell types, being readily detected by end-labelling of single strand nicks in the chromatin. Other proteins diagnostic of apoptotic cells include proteases such as lamP, which attacks the nuclear envelope protein lamin at the time of nuclear dissolution, and type II transglutaminase which cross-links the apoptotic bodies. There are two key elements in the apoptotic cascade located in the cytoplasm. One is the mitochondrion and the other involves proteases termed caspases (Ormerod, 1998).

### *1.8.1.5 Involvement of proteases (caspases) in apoptosis*

Caspases are a family of cysteine proteases that have been identified as being key regulators and effectors of the apoptotic response. Following their activation by apoptotic signal, either through cell surface specific receptors or through intracellular signalling pathways, caspases act concertedly in a cascade cleaving a number of proteins, followed by cell disassembly and cell death. Caspases do not engage in indiscriminate digestion of proteins during apoptosis, but rather a select set of proteins are targeted and cleavage occurs at specific peptide sequences (Thornberry & Lazebnik, 1998). The destruction of these proteins is genetically programmed and is systematically carried out to ensure the proper demise of the cell and disposal of debris.

In humans, the caspase family consists of 14 proteolytic enzymes (caspases 1-14), which are expressed initially in the cell as zymogens or proenzymes. They contain a shared prodomain, and two "active domains", one small subunit and the other a large subunit resulting from intra-chain processing at cleavage sites similar among the caspases. Caspases can also be subdivided on the basis of their activity into "initiator" (cell death signaling) caspases and "effector" (cell disassembly) caspases, the latter of which may also target other caspases. The initiator caspases, such as caspases-8 and -9, appear to activate other caspases at the effector end of the cascade, such as caspases-3, -6, and -7. It is the effector caspases that are largely responsible for the morphological and biochemical changes that are the hallmark of apoptosis (Sun *et al*, 1999). Poly (ADP-ribose) polymerase (PARP) is one of the selected substrates of caspase-3. Once activated the activity of the caspase cascade proceeds quite rapidly and is manifested by the proteolytic cleavage of a wide range of substrates. The destruction of most of the key regulatory elements of the cell structure and replicative machinery is responsible for the ultimate death of the cell.

Caspases are largely found in the cytoplasm of the cell. There are a few exceptions. In particular, caspase-2 has been found in the nucleus as well as the Golgi apparatus. Caspase-12 is uniquely found in the outer endoplasmic reticulum (ER) membrane. Caspases-3 and -9, while predominantly expressed in the cytoplasm, have also been identified in small amounts in the mitochondria. Because of their catastrophic effects on the microenvironment, it is critical that the activation and activity of the caspase cascade be tightly regulated. This process is controlled by several mechanisms, such as sub-cellular localisation, and more likely through the activity of a specific family of caspase inhibitors, the inhibitor of apoptosis proteins (IAP) via interaction with the zinc binding baculovirus inhibitor of apoptosis repeat (BIR) domains, as well as directly and indirectly by members of the Bcl-2 family of proteins.

### *1.8.1.6 Apoptotic pathways*

Although the apoptotic process is highly complex and the cellular events involve the activation of many signalling cascades resulting from either external stress signals or internal organelle-specific initiation events, only three main pathways predominate: (i) the mitochondrial-mediated pathway; (ii) the endoplasmic reticulum (ER) stress-induced pathway and (iii) the death receptor or cell-surface mediated signal transduction pathway (Figure 1.9).

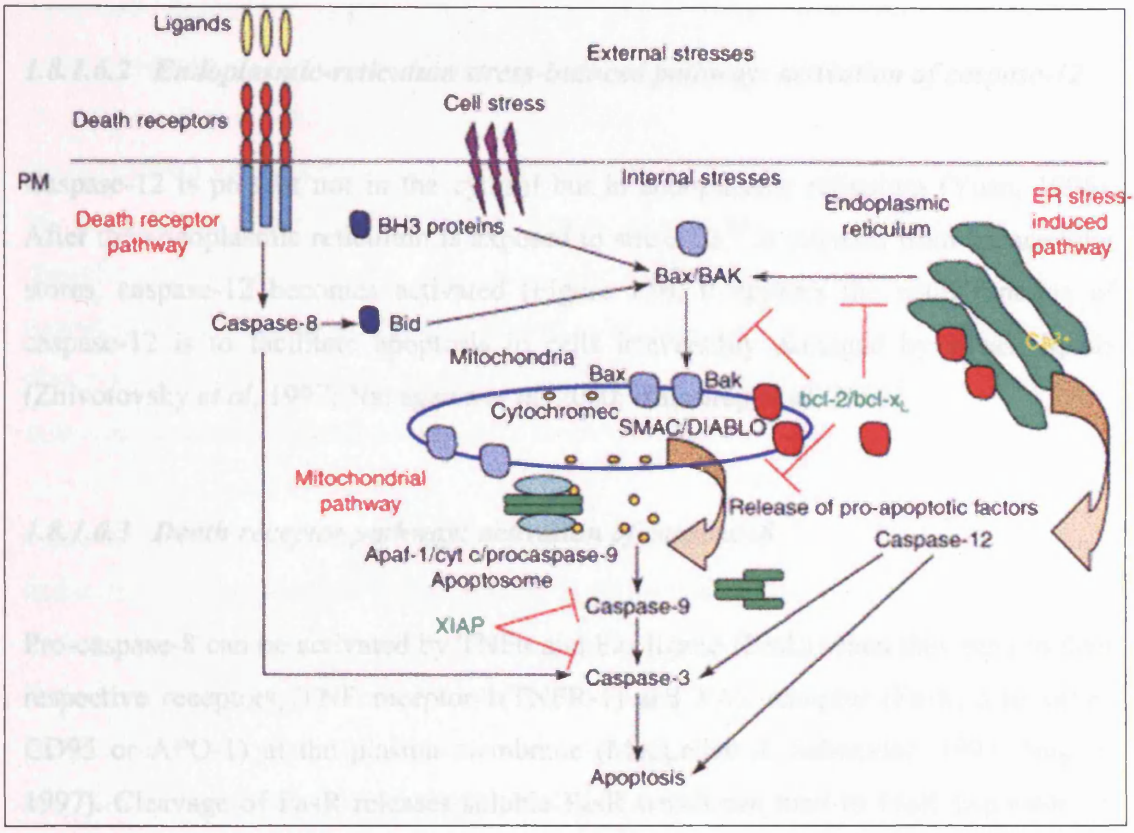



***Figure 1.9: Molecular pathways leading to apoptosis. This diagram shows the two main pathways that can be activated when cells encounter specific apoptotic stresses; [1] the mitochondrial-mediated pathway and [2] the endoplasmic reticulum (ER) pathway, and [3] the cell-surface mediated or death receptor pathway. Abbreviations used in diagram are: Apaf-1, apoptosis protein activating factor-1; cyt c, cytochrome c; DIABLO, direct inhibition of apoptosis protein (IAP) binding protein with low pI; SMAC, second mitochondrial activator of caspases; XIAP, X-linked inhibitor of apoptosis.***

#### *1.8.1.6.1 The mitochondrial pathway: activation of caspase-9*

Pro-caspase-9 is the major initiator caspase implicated in mitochondrial-mediated apoptosis (Hu *et al*, 1998). Pro-caspase-9 is activated in the presence of dATP, on binding apoptosis protein-activating factor-1 (Apaf-1) and cytochrome c (Zou *et al*, 1997), both of which are released from the mitochondria following stimulation or damage (Figure 1.9). Active caspase-9 then cleaves caspase-3, thus causing its activation (Kuida *et al*, 1998).

#### *1.8.1.6.2 Endoplasmic-reticulum stress-induced pathway: activation of caspase-12*

Caspase-12 is present not in the cytosol but in endoplasmic reticulum (Yuan, 1996). After the endoplasmic reticulum is exposed to stress $Ca^{2+}$ is released from intracellular stores, caspase-12 becomes activated (Figure 1.9). It appears the main function of caspase-12 is to facilitate apoptosis in cells irreversibly damaged by stress signals (Zhivotovsky *et al*, 1997; Nakagawa *et al*, 2000; Halestrap *et al*, 2000).

#### *1.8.1.6.3 Death receptor pathway: activation of caspase -8*

Pro-caspase-8 can be activated by TNFα and Fas ligand (FasL) when they bind to their respective receptors, TNF receptor-1(TNFR-1) and FAS receptor (FasR, also called CD95 or APO-1) at the plasma membrane (MacLellan & Schneider, 1997; Nagata, 1997). Cleavage of FasR releases soluble FasR which can bind to FasR expressed on other cells where, by blocking soluble FasL binding, it may have an anti-apoptotic effect. Following ligand binding, TNFR-1 or FasR oligomerise and associate with TNFR-1 associated death domain (TRADD) protein or Fas-associating protein with death domain (FADD), respectively. FADD contains an N-terminal death effector domain and can directly initiate apoptotic processes. Unlike FADD, TRADD does not contain an N-terminal death effector domain, but helps to recruit FADD. In this way, the FasR and the TNFR-1 use FADD as a common signal transducer. In addition,

TRADD can interact with receptor interacting protein which transduces death signals independent of kinase activity. Oligomerisation of both FasR and TNFR-1 ultimately results in recruitment and oligomerisation of caspase-8 which becomes activated by cleavage. Activated caspase-8 cleaves and activates caspase-3 (Figure 1.8), which in turn cleaves specific substrates (Nagata, 1997; Martin *et al*, 1998).

#### *1.8.1.7 Phosphatidylserine exposure during apoptosis*

Apoptosis generates signals that act on the plasma membrane by inhibiting aminophosholipid translocase ("flippase") and activating a phospholipid "scramblase", an enzyme that resides in or close to the bilayer. The combination of these actions results in the appearance of PS in the outer plasma membrane leaflet while the integrity of the plasma membrane remains largely uncompromised. The nature of the phospholipid scramblase is still not fully understood. It has been described unambiguously only by biological activity. Phospholipid "scramblase" transports bi-directionally all phospholipid species from the inner to the outer leaflet and *vice versa* in an aminophospholid translocase –independent manner.

It is still unclear how the apoptotic machinery inhibits aminophospholipd translocase and activates phospholipid "scramblase". It seems that active Caspase-3 does not act directly on these molecular complexes but likely via intermediates that may include the flux of calcium ions across the plasma membrane, activated protein kinase Cδ, and the ABC1-transporter. The Rho-1 kinase pathway previously thought to be involved, does not harbour candidates since inhibition of this pathway prevents membrane blebbing but not PS exposure.

The pathways responsible for PS exposure are not exclusively activated by caspases. For example, cathepsin B is capable of inducing PS exposure in a Caspase-independent fashion if it is translocated from the lysosome into the cytosol. Such translocation may arise from Caspase-independent processes in apoptosis-like programmed cell death.

The translocation of PS to the outer leaflet is one of the plasma membrane changes that occurs during programmed cell death. Other changes include looser packing of plasma membrane phospholipids. It is suggested that this is the consequence of scrambling the phospholipid species over the two leaflets of the plasma membrane. While restructuring of the plasma membrane is occurring its integrity and barrier function are kept intact. Gradually, however, as the apoptotic progresses to necrosis, the plasma membrane changes from a non-permeable barrier into a leaky one, which first allows small and later larger molecules to traverse across the plasma membrane. *In vivo*, the leakage of larger molecules across the plasmas membrane is unlikely to happen under normal circumstances since phagocytes recognise dying cells by changes in plasma membrane architecture and engulfment occurs before the plasma membrane integrity is compromised.

#### *1.8.1.8 Phagocyte recognition of apoptotic cells*

Removal of the majority of apoptotic cells is performed by phagocytes such as macrophages and dendritic cells, although other cell types including fibroblasts, epithelial cells and endothelial venule cells are also capable of phagocytosing apoptotic cells. Recognition and engulfment are mediated by interactions between phagocytic receptors and a variety of ligands on the surface of the dying cell. These ligands are either a part of the apoptotic plasma membrane (eg. PS for almost all apoptotic cells and ICAM-3 for apoptotic leucocytes) or appear at the surface from opsonisation as a consequence of altered plasma membrane architecture. The opsonins include members of the complement system C1q and iC3b, the glycoprotein β2-GPI, thrombospondin, pentraxin C-reactive protein (CRP) and serum amyloid protein. The latter two belong to family of phospholipid binding proteins with unknown function to date. The binding sites for the opsonins remain unidentified, although PS appears to function as a binding site for a number of them (C1q, iC3b, β2-GPI). The loose packing of the plasma membrane phospholipids may also expose binding sites to the opsonins.

### 1.8.2 Lipid peroxidation

All of the major classes of biomolecules may be attacked by free radicals but lipids are probably the most susceptible. Cell membranes are rich sources of polyunsaturated fatty acids (PUFAs), which are readily attacked by oxidising radicals. The oxidative destruction of PUFAs is known as lipid peroxidation, and is particularly damaging because it proceeds as a self-perpetuating chain-reaction. The chemical process of peroxidation can be divided into three stages: initiation, propagation and termination, and is shown in equation form below.

**INITIATION:** $L H + R^{\bullet} \rightarrow L^{\bullet} + R\text{-}H$

**PROPAGATION:** $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

$LOO^{\bullet} + L\text{-}H \rightarrow L\ OOH + L^{\bullet}$

$L\ OOH + Fe^{2+} \rightarrow L\ O^{\bullet} + OH^{-} + Fe^{3+}$

**Chain Branching:** $L\ O^{\bullet} + L\ H \rightarrow L\ OH + L^{\bullet}$

**TERMINATION:** $L^{\bullet} + L^{\bullet} \rightarrow$ **non-radical products**

$L^{\bullet} + L\text{-}OO^{\bullet} \rightarrow$ **non-radical products**

$LOO^{\bullet}$ **+ antioxidant-H** $\rightarrow$ **LOOH + antioxidant**$^{\bullet}$

($L^{\bullet}$, $LO^{\bullet}$, and $LOO^{\bullet}$ represent lipid alkyl, alkoxyl, and peroxyl radicals, respectively derived from lipid, LH, and lipid hydroperoxides, LOOH).

LH is the target PUFA and $R^{\bullet}$ the initiating, oxidising radical. Oxidation of the PUFA generates a fatty acid radical ($L^{\bullet}$) that rapidly adds oxygen to form a fatty acid peroxyl radical ($LOO^{\bullet}$). Hydroxyl radicals and singlet oxygen can react with the methylene groups of PUFA forming conjugated dienes, lipid peroxyl radicals, conjugated trienes and hydroperoxides. The formation of conjugated dienes occurs when free radicals attack the hydrogens of methylene groups separating double bonds and leads to rearrangement of the bonds. The peroxyl radicals are the carriers of chain-reaction, they can oxidise further PUFA molecules and initiate new chains, producing lipid

hydroperoxides (LOOH) that can break down to yet more radical species and to a wide range of compounds, notably aldehydes (Porter, 1990; Esterbauer *et al*, 1990).

The breakdown of lipid hydroperoxides often involves transition metal ion catalysis thus undergoing reductive cleavage by reduced metals, such as $Fe^{2+}$, yielding lipid peroxyl and lipid alkoxyl radicals. Aldehydes are always formed when lipid hydroperoxides break down as the hydroxyalkenals, whose best known member is 4-hydroxynonenal (Esterbauer *et al*, 1991). These compounds can diffuse from the original site of attack and spread the damage to other parts of the cell.

Lipid peroxidation is of particular significance as a damaging reaction consequent to free radical production in cells because: (i) it is a very likely occurrence, given the availability and susceptibility of PUFA in membranes; and (ii) it is a very destructive chain-reaction that can directly damage the structure of the membrane and indirectly damage cell components by the production of reactive aldehydes. Lipid peroxidation has been implicated in a wide range of tissue injuries and diseases (Halliwell & Gutteridge, 1990; Salomon *et al*, 2000; Tuma, 2002; Picklo *et al*, 2002).

Lipid peroxidation is regulated/terminated by the composition and organisation of lipids inside the bilayer, the degree of PUFA unsaturation, mobility of lipids within a bilayer, localisation of the peroxidative process in a particular membrane and the preventative antioxidant system, ROS scavenging and lipid peroxidation product detoxification.

#### *1.8.2.1 Sources of free radicals*

A free radical can be defined as a chemical species possessing an unpaired electron. It can also be considered as a fragment of a molecule. Free radicals can be formed in three ways: (i) by the homolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the paired electrons; (ii) by the loss of a single electron from a normal molecule; (iii) by the addition of a single electron to a normal molecule. The

latter, electron transfer, is a far more common process in biological systems than is homolytic fission, which generally requires high energy input from either high temperatures, UV light or ionising radiation. Heterolytic fission, in which the electrons of the covalent bond are retained by only one of the fragments of the parent molecule does not result in free radicals but in ions, which are charged. Free radicals can be positively charged, negatively charged or electrically neutral. The process by which free radicals and ions are formed is shown below (the unpaired electron and the radical nature of the species is denoted by superscript dot).

**Radical formation by electron transfer:** $A + e^- \rightarrow A^{-\bullet}$

**Radical formation by homolytic fission:** $X : Y \rightarrow X^\bullet + Y^\bullet$

**Ion formation by heterolytic fission:** $X : Y \rightarrow X:^- + Y^+$

### *1.8.2.2 Oxygen free radicals and reactive oxygen species*

The consensus is that the most important free radicals in biological systems are radical derivatives of oxygen. Reduction of oxygen by the transfer to it of a single electron will produce the superoxide free radical anion ('superoxide').

$$O_2 + e^- \rightarrow O_2^{-\bullet}$$

A two-electron reduction of oxygen would yield hydrogen peroxide:

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

Hydrogen peroxide is often generated in biological systems via the production of superoxide: two superoxide molecules can react together to form hydrogen peroxide and oxygen.

$$2O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$$

Because the free radical reactants produce non-radical products this is known as a dismutation reaction. It can take place spontaneously though at a slow rate, or can be catalysed by the enzyme superoxide dismutase. Hydrogen peroxide is not a free radical but is termed a 'reactive oxygen species' (ROS) that includes not only oxygen free radicals but also non-radical oxygen derivatives that are involved in oxygen radical production.

Hydrogen peroxide is an important compound in free radical biochemistry because it can rather easily break down, particularly in the presence of transition metal ions, to produce the most reactive and damaging of the oxygen free radicals, the hydroxyl radical (•OH):

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$$

The above reaction is referred to as the iron-catalysed Haber-Weiss reaction. The non-catalysed Haber-Weiss reaction is the reaction of superoxide directly with hydrogen peroxide:

$$O_2^{-\bullet} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + O_2$$

The spontaneous reaction is less likely in biological systems due to low steady-state concentrations of the reactants. The iron (or copper) catalysed reaction can still be considered to be dependent on superoxide as both the source of the hydrogen peroxide (via dismutation as described previously) and as the reductant of the transition metal ion:

$$O_2^{-\bullet} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$
$$O_2^{-\bullet} + Cu^{2+} \rightarrow Cu^{+} + O_2$$

Ferrous ($Fe^{2+}$) iron and cuprous ($Cu^{+}$) copper are much more reactive with hydrogen peroxide than their oxidised counterparts, ferric ($Fe^{3+}$) and cupric ($Cu^{2+}$), respectively.

The auto-oxidation of reduced transition metals can also generate superoxide:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet}$$
$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-\bullet}$$

Thus the reactions of the transition metal ions with oxygen can be considered reversible redox reactions and are extremely important in the promotion of free radical reactions.

Finally, singlet oxygen is another non-radical ROS often associated with oxygen free radicals; it can lead to and be generated by free radical reactions. Oxygen free radicals are not the only important free radicals in biochemistry, although they are often the initial species formed. Other free radicals of importance are the wide range of carbon-centred radicals ($\mathbf{R^{\bullet}}$) that arise from the attack of an oxidising radical (e.g. $\mathbf{OH^{\bullet}}$) on a biological molecule (**RH**) such as lipid, nucleic acid, carbohydrate or protein. These react very rapidly with oxygen to form the corresponding peroxyl radicals ($\mathbf{ROO^{\bullet}}$). In turn, these peroxyl radicals can participate in reactions that generate alkoyl radicals ($\mathbf{RO^{\bullet}}$). Sulphur atoms can also be the centre for free radicals (thiyl radicals, $\mathbf{RS^{\bullet}}$) formed for example, in the oxidation of glutathione. Finally, certain foreign toxic compounds such as carbon tetrachloride can be activated to free radical species.

### *1.8.2.3 Reactive nitrogen species*

Reactive nitrogen intermediates are now also recognised as important radicals. Nitric oxide (NO) is formed endogenously from the oxidation of L-arginine to L-citrulline by a family of NADPH-dependent enzymes, the NO synthases. NO exists in different chemical forms ($NO^{-}$, $NO^{\cdot}$ and $NO^{+}$) each with quite different chemical reactivities and functions in a variety of different biological roles (Stamler *et al*, 1992). These include regulation of the cardiovascular system, smooth muscle relaxation, neurotransmission, coagulation and immune regulation. Despite these beneficial functions, the molecule has a role as an oxidant. NO can combine with $O_2^{-}$ to form peroxynitrite ($ONOO^{-}$),

which shares some of NO's properties in that it too can diffuse freely intra- and intercellularly and also acts as a powerful oxidant.

### *1.8.2.4 Defences against free radicals (Antioxidant defences)*

Some free radical production in animal cells is inevitable, as they are essential for normal cell function. As free radicals can be very damaging, defences against the deleterious actions of free radicals have evolved. These antioxidant defences fall into two main categories: prevention of the generation of free radicals and removal or neutralisation of any that are generated. They exist in both the aqueous and membrane compartments of cells and can be enzymic or non-enzymic.

The preventive defences include decreasing the efficiency of electron transfer and sequestration of transition metal ions. An example is iron, which is held tightly bound to transferrin and ferritin. Some, iron, however, is postulated to exist in a more reactive low-molecular weight pool and, moreover, the generation of free radicals may actually release free transition metal ions (Halliwell *et al*, 1992). Another form of antioxidant defence is the removal of peroxides that react with transition metal ions to produce reactive free radicals. This includes both hydrogen peroxide and also the lipid hydroperoxides that are produced during lipid peroxidation. Catalase and glutathione peroxidase are enzymes whose role is to safely decompose peroxides. The former is mainly located in peroxisomes and acts upon hydrogen peroxide; the latter is found in the cytosol of most cells and is active towards both hydrogen peroxide and if first cleaved from membrane phospholipids by a phospholipase, fatty acid hydroperoxides.

$$\mathbf{2H_2O_2 \rightarrow catalase \rightarrow 2H_2O + O_2}$$

$$\mathbf{ROOH + 2GSH \rightarrow GSH\ peroxidase \rightarrow ROH + GSSG}$$

Other defences 'scavenge' free radicals. One of these is the only known enzyme whose substrate is a free radical, superoxide dismutase (SOD). However, most free radical scavengers are not enzymes. In cell membranes, the best characterised and possibly the

most important is α-tocopherol, the major member of the vitamin E family (Burton *et al*, 1983). This molecule is known as a 'chain-breaking antioxidant' because it functions to intercept lipid peroxyl radicals (LOO•) and so terminate lipid peroxidation chain reactions:

$$\mathbf{LOO^{\bullet} + \alpha\text{-tocopherol} - OH \rightarrow LOOH + \alpha\text{-tocopherol} - O^{\bullet}}$$

The resultant tocopheroxyl radical is relatively stable and, in normal circumstances, insufficiently reactive to initiate lipid peroxidation itself. Other lipid-soluble chain breaking antioxidants, such as ubiquinol, are as yet insufficiently characterised to establish their physiological importance.

In the aqueous phase other compounds act as free radical scavengers. Ascorbic acid (vitamin C) is an important antioxidant both within cells and in the plasma. It has been shown to regenerate α-tocopherol from the tocopheroxyl radical *in vitro* (Packer *et al*, 1979; Stocker & Frei, 1991). Uric acid in plasma and glutathione in cell cytosol also possess strong radical scavenging properties. It is apparent that cells have evolved an array of antioxidant defences designed to severely limit damage caused by free radicals wherever and when ever it might occur. This situation is possibly the most persuasive argument that exists for the significance of free radicals as a threat to the viability of cells and organisms.

A third category of natural antioxidant defences are repair processes, which remove damaged biomolecules before they can accumulate and before their presence results in altered cell metabolism or viability. Specific enzymes repair oxidatively damaged nucleic acids, oxidised proteins are removed by proteolytic systems. The regulated proteolysis of proteins is achieved by proteasome complexes which remove denatured or improperly translated proteins from the cell, whilst damaged, oxidised membrane lipids are acted upon by lipases, peroxidases and acyl transferases.

Finally there are antioxidant drugs that have been used for treatment or prevention of free radical-mediated tissue damage. Such compounds include metal-chelating agents and radical scavengers (eg: desferrioxamine). Very few have been proven to be of any value, with the possible exception of Probucol, which was used clinically as a lipid-lowering drug and was found to be an effective antioxidant that may help protect against atherosclerosis by preventing oxidation of low density lipoprotein (Parthasarathy *et al*, 1986).

## 1.9 Aims of the present study

The aims of the present study are described below and according to the current literature; the hypotheses proposed and technical approaches have not been investigated or previously employed.

### 1.9.1 Characterisation of the *in vitro* cell-surface procoagulant activity of T-lymphoblastoid cells

Previous studies had shown that T-lymphoblastoid cells were able to support thrombin generation despite having negligible or low levels of TF (Barrowcliffe *et al*, 1989; Barrowcliffe *et al*, 2002). The work presented in this thesis was instigated to further characterise the procoagulant activity of malignant T-lymphoblastoid cells. Childhood T-ALL cell lines of different stages of differentiation were chosen to investigate malignant T-cell procoagulant activity, and to add further information to the published data (Barrowcliffe *et al*, 1989; Barrowcliffe *et al*, 2002). The first aim of this thesis was to characterise the procoagulant activity of the cell lines in detail and in particular to compare and contrast the TF and phospholipid-dependence of malignant T-cell cell-surface phospholipid in various coagulation tests, and to determine whether cancer procoagulant played a significant role. As set out previously, the paradigm is that normal T-cells are not procoagulant, but there is little information as to whether malignant T-cells can be.

### 1.9.2 Investigation of the effect of anionic phospholipid exposure on T-lymphoblastoid procoagulant activity

Earlier studies had suggested that T-lymphoblastoid procoagulant activity was due to the exposure of anionic phospholipid (Barrowcliffe *et al*, 1989; Barrowcliffe *et al*, 2002). The second aim of the study was to confirm that this procoagulant activity was due to the exposure of procoagulant anionic phospholipid. This was determined by using annexin A5 and an inhibitory PS antibody 3G4 to inhibit T-lymphoblastoid procoagulant activity and to probe for anionic phospholipid exposure.

### 1.9.3 Effect of pathophysiological processes on T-lymphoblastoid procoagulant activity

There are two linked pathophysiological processes that are associated with the exposure of anionic phospholipid and the enhancement of procoagulant activity; apoptosis and lipid peroxidation. The possible contribution of these two processes was investigated by either inducing apoptosis (with or without inhibiting lipid peroxidation) or subjecting the cells to oxidative stress, and then measuring procoagulant activity in relation to the level of anionic phospholipid exposure and lipid peroxidation.

Chapter

2

# MATERIALS AND METHODS

## 2.1 Reagents

Common reagents are described below and reagents specific to a method will be described in detail in later chapters.

The following reagents and standards were from NIBSC, Potters Bar UK: Tissue Factor (relipidated human recombinant; X/95); bovine brain phospholipid (91/542); Factor VIII, (human recombinant; 96/598); Factor IXa (human recombinant; 97/562); Human Factor X (92/568).

Other coagulation reagents were from: Human Factors Xa, Va and Prothrombin were from Haematologic Technologies Inc (Essex Junction, VT, USA). Human placental annexin A5, russell's viper venom (RVV), papain, kaolin (Sigma, Poole, UK).

Other reagents were from: Chromogenic substrates S-2765 and S-2238 were from Chromogenix-Instrumentation Laboratory SpA (Milan, Italy). Human albumin 20% (ZENALB®20) was from Bio Products Laboratory, Elstree, Herts, UK.
2-meracaptoethanol was from Sigma (Poole, UK). Acetic acid was from (Merck, Poole, UK). The inhibitory IgG1 monoclonal anti-TF antibody, 4509, and the FITC conjugated IgG1 monoclonal anti-TF antibody, 4508CJ, were from American Diagnostica Inc. (Greenwich, CT, USA). Mouse IgG1 isotype control (FITC conjugated) was from Serotec Ltd (Oxford, UK). The inhibitory IgG3 Mouse anti-PS antibody, 3G4 was obtained from the Department of Pharmacology, University of Texas Southwestern Medical Center, (Dallas, USA). RNAguard™, RNase solution was obtained from Amersham Pharmacia Biotech Inc (NJ, USA). Annexin $A5^{FITC}$ propidium iodide and Z-VAD-FMK was from PharMingen (Becton Dickinson, San Jose, CA).

## 2.2 Buffers

Tris-buffered saline (TBS) was used as the dilution and washing buffer for all experiments unless otherwise stated. It comprised: 0.05M Tris (hydroxymethyl) aminomethane, 0.15M NaCl, pH 7.4 (BDH reagents from Merck, Poole, UK). Details of other buffers are described within the methods for specific tests.

## 2.3 Blood collection and handling

Blood was collected from healthy volunteers, who had not taken aspirin in the previous seven days, by venepuncture of the antecubital vein, 17 mL of blood was collected into 3 mL of acid-citrate-glucose formula A [ACD-A; 0.07 M sodium citrate, 0.04 M citric acid, 0.12 M glucose, pH 5.0].

### 2.3.1 Preparation of normal plasma pools

Blood was collected as above from 24 donors and centrifuged at 30000 g for 15 at 4°C minutes within 20 minutes of collection. The plasma was carefully aspirated from between the cell layer and any floating lipid. The plasma was pooled and centrifuged at 17000 g for a further 15 minutes before aliquots were snap frozen in liquid nitrogen and stored at -40°C.

### 2.3.2 Defibrination of normal plasma for use in the thrombin generation assay

Defibrinated normal plasma (DNP) was prepared immediately before use. Aliquots of pooled normal plasma were thawed at 37°C and defibrinated using ancrod (1$^{st}$ International Reference Preparation, 74/581) 0.5 IU per mL of plasma. The plasma was incubated for 30 minutes at 37°C with ancrod, the fibrin was removed by winding onto wooden sticks. Any remaining fibrin was removed by centrifugation at 1000 g for 5 minutes at room temperature.

## 2.4 Cell lines and cell culture

The procoagulant activity of four T-lymphoblastoid cell lines was investigated and one monocytoid cell line was also used for comparison.

### 2.4.1 Cell lines used in this study

The T-lymphoblastoid cell lines CEM-CCRF, Jurkat, Molt-4, and A3.01 as well as a monocytoid cell line, THP-1 were obtained from the Centralised Facility for AIDS Research Reagents, NIBSC, Potters Bar UK. The immunophenotype of each cell line is shown in Table 2.1.

#### *2.4.1.1 CEM-CCRF*

This cell line was derived from the peripheral blood buffy coat of a 3-year old Caucasian female (Foley *et al*, 1965) with T-cell acute lymphoblastic leukaemia (T-ALL) which had transformed from lymphosarcoma. The immunophenotype of this cell line is shown in Table 2.1. The optimum seeding density for this cell line is 1 x $10^5$ cells/mL and the doubling time was 24 hours.

#### *2.4.1.2 Jurkat*

This cell line was originally derived from a 14-year old male with T-ALL (Schneider *et al*, 1977). Morphologically, the cells are round to racquet shaped and express the T-cell antigen receptor. The immunophenotype of this cell lines is shown in Table 2.1. The optimum seeding density for this cell line is 1 x $10^5$ cells/mL and the doubling time is 25 – 35 hours.

| Marker | CD reactivity | Cell Lines | | | | |
|---|---|---|---|---|---|---|
| | | CEM-CCRF | Jurkat | Molt-4 | A3.01 | THP-1 |
| **T-/NK cell marker** | CD1a | - | + | + | - | - |
| | CD2 | - | + | + | - | - |
| | CD3 | + | + | + | + | - |
| | CyCD3 | + | + | + | + | - |
| | CD4 | + | + | + | + | + |
| | CD5 | + | + | + | + | NT |
| | CD6 | + | + | + | + | NT |
| | CD7 | + | + | + | + | + |
| | CD8 | - | - | - | - | NT |
| | CD27 | + | + | - | + | NT |
| | CD28 | + | + | + | + | - |
| | CD56 | - | NT | - | - | NT |
| | CD57 | - | (+) | - | - | - |
| | TCRαβ | + | + | - | + | - |
| | TCRγδ | - | - | - | - | NT |
| | cyTCRα | + | + | - | + | NT |
| | cyTCRβ | + | + | + | + | NT |
| | cyTCRγ | - | - | - | - | NT |
| **B-cell marker** | CD9 | - | + | + | - | NT |
| | CD10 | - | - | - | - | NT |
| | CD19 | - | - | - | - | - |
| | CD20 | - | - | - | - | - |
| | CD21 | - | - | - | - | NT |
| | CD22 | - | NT | - | - | NT |
| | CD23 | NT | NT | NT | NT | (+) |
| | CD24 | - | - | - | - | + |
| | CD80 | NT | NT | NT | NT | - |
| | CD86 | NT | NT | NT | NT | + |
| | cyIg | NT | NT | NT | NT | - |
| | sIg | NT | NT | NT | NT | - |
| **Myelomonocytic marker** | CD13 | - | - | - | - | + |
| | CD14 | - | - | - | + | (+) |
| | CD15 | + | - | - | - | + |
| | CD16 | - | - | - | - | NT |
| | CD33 | - | - | - | - | + |
| | CD68 | - | NT | NT | - | - |
| **Erythroid-megakaryocytic marker** | CD9 | NT | NT | NT | NT | - |
| | CD36 | - | NT | - | - | NT |
| | CD41 | - | NT | NT | - | NT |
| | CD41a | NT | - | - | NT | + |
| | CD61 | - | - | - | - | + |
| | GlyA- | - | NT | - | - | NT |
| **Progenitor/activation/ other marker** | CD34 | - | (+) | (+) | - | - |
| | CD38 | + | + | + | + | + |
| | CD71 | + | + | + | + | + |
| | CD90 | - | NT | NT | - | NT |
| | HLA-DR | - | - | - | NT | + |
| | TdT | + | + | + | + | NT |
| **Adhesion marker** | CD11b | - | - | - | - | + |
| | CD29 | + | + | + | + | NT |
| | CD54 | NT | NT | NT | NT | + |
| **Cytokine receptor** | CD25 | - | - | - | - | + |
| | CD117 | - | - | - | - | - |
| | CD122 | - | NT | - | - | NT |

***Table 2.1: The immunophenotypic characterisation of the cell lines.Abbreviations used in the table are: NT, not tested; +, positive; (+), weakly positive and -, negative.***

### *2.4.1.3 Molt-4*

This cell line was derived from a 19 year old male with acute T-ALL (Minowada *et al*, 1972). The morphology of the cells resembles that of relatively mature lymphocytes. The nuclear-cytoplasmic ratio is high in stained preparations. The immunophenotype of this cell line is shown in Table 2.1. The optimum seeding density for this cell line is 1 x $10^5$ cells/mL and the doubling time was 40 hours.

### *2.4.1.4 A3.01*

This cell line is a hypoxanthine / aminopterin / thymidine (HAT) sensitive derivative of the CEM cell line (Folks *et al*, 1985) described in section 2.4.1.1. Morphology is mature lymphocytic, though A3.01 contains a mutation in cholesterol biosynthesis which results in the complete replacement of membrane cholesterol with lanosterol and dihydrolanosterol (Buttke & Folks, 1992). The immunophenotype of A3.01 is the same as that of CEM-CCRF (Table 2.1) however the HLA phenotype could not be determined because of complement sensitivity. The optimum seeding density for this cell line is 1 x $10^5$ cells/mL and the doubling time of 24 hours.

### *2.4.1.5 THP-1*

THP-1 was derived from the blood of a 1-year old boy with acute monocytic leukaemia (Tsuchiya *et al*, 1980), the diagnosis was AML M5. Morphologically the cells are both round and somewhat polygonal. The cells had a moderate amount of basophilic cytoplasm which contained small azurophilic granules and a few vacuoles. The immunophenotypic characterisation is shown in Table 2.1. The optimum seeding density for this cell line is 1 x $10^5$ cells/mL and the doubling time was 35 – 40 hours.

### 2.4.2 Maintenance of Cultures

Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum (HIFCS) and 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in agreement with the guidelines for the characterisation and publication of human malignant hematopoeitic cell lines (Salvioli *et al*, 2000). For THP-1 cells, 50 mM 2-mercaptoethanol was also added to the medium. Cultures were incubated in 5% $CO_2$ and 95% air at 37°C, sub-cultured and fed every 3 to 4 days.

### 2.4.3 Monitoring Cell growth

Cultured cells were continuously assessed by regular observation under an inverted light microscope in order to monitor quantitative aspects of cell proliferation and cell viability. Accurate counting of the cells and monitoring of cell viability was achieved by a haemocytometer using 0.4% trypan blue solution. Trypan blue is a stain that is actively excluded from viable cells, but readily enters and stains dead cells. Only cell populations that were >90% viable by trypan blue exclusion were used in assays or before being subjected to apoptosis or oxidative stress.

### 2.4.4 Determination of Cell Growth

Each cell line was seeded at a cell number of 1 x $10^5$/mL and counted every 24 hours following subculture for 8 consecutive days in order to estimate a doubling time and to follow the dynamics of cell growth. Cell concentration was plotted against time of culture (Figure 2.1). Then the Lag, Log and Plateau phases were identified and were labelled for the culture of each cell line on the graph. In all cases the lag phase only lasted for a few hours. A period of time during the Log Phase was then selected and the doubling time was calculated. Doubling time was determined during the Log phase of cell growth, as the time required to double the number of cells per mL. It was calculated for each cell line, by using the linear part of the growth curve, in order to choose the optimal cell density depending on experimental requirements. The doubling

time for each cell line ranged from approximately 24 to 40 hours. For all experiments cells were tested after being cultured for between 72 and 96 hours.

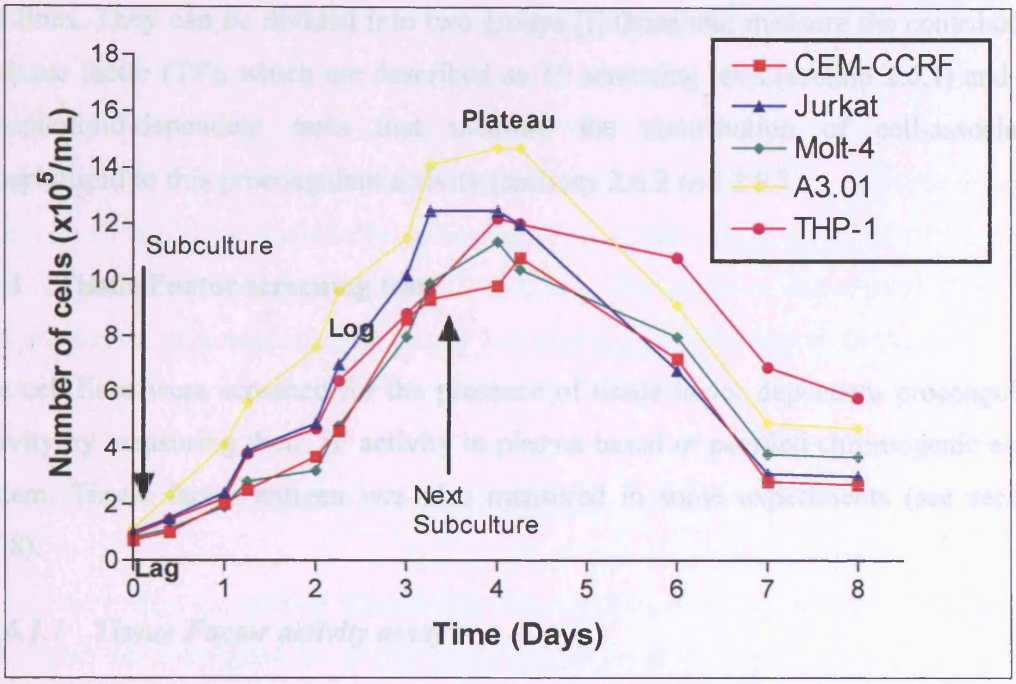



***Figure 2.1: Growth curves for CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1 cell lines. Cells were seeded at 1 x $10^5$ per mL and counted every 24 hours for 8 days on a haemocytometer using 0.4% trypan blue. Initially the cells are in a short lag phase and do not divide. This is followed by an exponential increase in cell number (log phase) up to about 1.2 to 1.6 x $10^6$/mL and then the cells start to die. Results shown are the mean of n = 3.***

## 2.5 Preparation of cells for use in coagulation studies

When analysing PCA, cells were washed three times in serum-free RPMI medium, and resuspended in 0.05 mol/L Tris HCl and 0.15 mol/L NaCl pH 7.4 buffer containing 1% human albumin. The cell concentration was adjusted to give a final concentration of 4 x $10^6$ cells per mL in the various tests for PCA unless stated otherwise. Cell viability was assessed by trypan blue dye exclusion with only preparations having >90% viability being used for analysis.

## 2.6 Coagulation assays used to measure T-lymphoblastoid procoagulant activity

A range of coagulation assays were selected to measure the procoagulant activity of the cell lines. They can be divided into two groups [i] those that measure the contribution of tissue factor (TF), which are described as TF screening tests (section 2.6.1) and [ii] phospholipid-dependent tests that measure the contribution of cell-associated phospholipid to this procoagulant activity (sections 2.6.2 and 2.6.3).

### 2.6.1 Tissue Factor screening tests

The cell lines were screened for the presence of tissue factor dependent procoagulant activity by measuring their TF activity in plasma based or purified chromogenic assay system. Tissue factor antigen was also measured in some experiments (see section 2.7.8).

#### *2.6.1.1 Tissue Factor activity assay*

Tissue factor activity was measured by a modification of the prothrombin time assay in human plasma, where TF is made the rate-limiting factor. To 100 µl of plasma (see section 2.3.1) were added 100 µL of cell suspension or a dilution of the TF standard. After 2 minutes incubation at 37°C 100 µL $CaCl_2$ (25 mM) was added and the clotting time recorded on a KC-4 coagulometer (Amelung GmbH, Lemgo, Germany). Clotting times were converted to tissue factor units (TFU) by comparison with a dilution curve of the TF standard, X/95. A 1/10 dilution of X/95 was assigned a value of 1000 TF units/mL, which gave a clotting time of 17.5 ±0.4 seconds (n=5), and a reagent blank time in the absence of TF or cells was 718 ±10.5 seconds (n=5).

#### *2.6.1.2 Two-Stage chromogenic assay for Tissue factor*

The amidolytic activity of TF was measured in a two-step FX-activation assay (Souter *et al*, 2001). The purified system consisted of FVIIa, purified human FX, $CaCl_2$ and a specific chromogenic substrate for FXa, S-2765. All reagents were kept on ice during

the assay. A combined reagent consisting of 0.35% albumin in TBS, FVIIa (final concentration 3 IU/mL, $CaCl_2$ (8 mM) and S-2765 (3 mM) was prepared. Immediately before use, purified FX at a final concentration of 0.063 IU/mL was added and 150 µL of the ice-cold mixture were transferred to the appropriate wells. Colour was allowed to develop for 60 minutes at 37°C. Absorbance at 405 nm was read using a Thermomax platereader (Molecular Devices, Menlo Park, CA, USA). The results obtained were compared with a recombinant human tissue factor (RecombiPlasTin, Ortho Diagnostic Systems, Hemoliance, Raritan, NJ) that was used as the standard and a 1/100 dilution was assigned an arbitrary potency of 1 TFU/mL. The batch of RecombiPlasTin used had previously calibrated against human recombinant TF X/95 from NIBSC.

### 2.6.2 Phospholipid-dependent screening tests

The phospholipid-dependent coagulation tests chosen to measure the contribution of cell-based phospholipids to T-cell procoagulant activity were the activated partial thromboplastin time (APTT) and the Russell's viper venom time (RVVT).

#### *2.6.2.1 Activated Partial Thromboplastin time (APTT)*

Intrinsic coagulation supported by the cells was measured by an APTT assay in human plasma (prepared as described in section 2.3.1). To 50 µL of cell suspension or a dilution of the phospholipid standard, was added 50 µL of plasma followed by 50 µL Kaolin (5mg/mL). After 10 minutes incubation at 37°C 50 µL $CaCl_2$ (25 mM) was added to start the reaction and the clotting time recorded on a KC-4 coagulometer. Clotting times were converted to phospholipid units (PLU) by comparison with a dilution curve of the phospholipid standard, 91/542. A 1/400 dilution of 91/542 was assigned a potency of 100 PLU/mL, and gave a clotting time of 35.7 ± 1.8 seconds (n=5), whilst the reagent blank gave a clotting time of 125.6 ± 2.4 (n=5).

#### *2.6.2.2 Russell's Viper Venom Time (RVVT)*

Russell's viper venom (RVV) can activate factor X in the presence of phospholipid, the co-factor FV and calcium ions, and can be used to investigate the activity of the prothrombinase complex. To 100 µL of plasma (prepared as described in section 8.5.1.2.) was added 100 µL of cell suspension or a dilution of the phospholipid standard. After 2 minutes incubation at 37°C 100 µL of diluted RVV (200 ng/mL) was added. The mixture was then incubated for a further 30 seconds after which 100 µL $CaCl_2$ (25 mM) was added and the clotting time recorded on a KC-4 coagulometer. Clotting times were converted to PL units (PLU) by comparison with a dilution curve of the phospholipid standard, 91/542. A 1/400 dilution of 91/542 was assigned a potency of 100 PLU/mL, and gave a clotting time of 38.3 ± 0.3 seconds (n=6), whilst the reagent blank with no phospholipid gave a clotting time of 104.4 ± 2.3 (n=6).

### 2.6.3 Measurement of the tenase and prothrombinase complex activities

More specific assays were used to investigate further the phospholipid dependent complexes assembled on the cell-surface of the cell lines. Both the tenase and prothrombinase complex activities were measured using a chromogenic sub-sampling method, with components of the complexes being provided by purified reagents.

#### *2.6.3.1 Intrinsic factor Xa generation*

FXa generation was based on a modification (by use of a stopping buffer) of a previously described chromogenic assay method (Barrowcliffe *et al*, 2002). One hundred and sixty microlitres of cells or phospholipid standard was mixed with 800 µL of combined reagent (FIXa, FVIII, and FX prepared to give a final reaction concentration of 1.0 IU/mL for each component) and warmed to 37°C. The recombinant FVIII product used in the assay has been shown to give rapid FXa generation in the absence of added thrombin (Kemball-Cook *et al*, 1993). FXa generation was initiated

by the addition of 80 μL of calcium chloride (25 mM). At various times 25 μL of the mixture was sampled and added to 50 μL of stopping buffer (Tris 0.05 mol/L, sodium chloride 0.1 mol/L, ethylenediaminetetra-acetic acid (EDTA) 6 mM, pH 8.4). FXa was then estimated by adding 100 μL of chromogenic substrate (3 mM, S-2765). The reaction in each well was stopped after 3 minutes at 37°C by the addition of 50 μL 50% acetic acid, and optical densities were read at 405 nm in a Thermomax platereader. Optical densities were converted to FXa U/mL from a dilution curve of a human FXa preparation ($M_r$ = 46 000 Da and a specific activity of 983 units/mg) and the activity of the cells, measured as initial rate of FX activation, was quantified by comparison with a phospholipid dilution curve. A 1/400 dilution of phospholipid standard (NIBSC 91/542) was assigned a potency of 100 PLU/mL.

#### *2.6.3.2 Prothrombinase assay*

Procoagulant phospholipid exposure by the cell lines was detected using a purified human prothrombinase assay in which PS promotes the activation of prothrombin by FXa in the presence of FVa. Thrombin generated by the assembled prothrombinase complex was measured using a specific thrombin chromogenic substrate. In the assay the clotting factor concentrations have been determined to ensure that phospholipid is the rate-limiting parameter of the reaction (Comfurius P *et al*, 1994). An aliquot (158 μL) of cell suspension or bovine phospholipid standard at varying concentrations were incubated for 3 minutes at 37°C with 5 μL of $CaCl_2$, 25 μL of FXa and 25 μL of FVa at final concentrations of 3 mM, 3 nM and 6 nM respectively. Twice the concentration of FVa compared to FXa was used to prevent FVa becoming a rate limiting factor as FV may be present to varying degrees in cell lines (Comfurius P *et al*, 1994). Prothrombin (60 μL) at a final concentration of 4 μM was then added to the reaction mixture. At various times 5 μL of the mixture was sampled and added to 200 μL of stopping buffer (Tris 0.05 mol/L, sodium chloride 0.1 mol/L, ethylenediaminetetra-acetic acid (EDTA) 6 mM, pH 8.4) in a 96-well microtitre plate. Thrombin generated was then estimated by adding 50 μL of chromogenic substrate (3 mM, S-2238). The reaction in each well was

stopped after 3 minutes at 37°C by the addition of 50 μL 50% acetic acid, and optical densities were read at 405 nm in a Thermomax platereader. Optical densities were converted to thrombin international units/mL from a dilution curve of a human α-thrombin standard (NIBSC 89/588) and the activity of the cells, measured as initial rate of thrombin generation, was quantified by comparison with a phospholipid dilution curve. A 1/400 dilution of phospholipid (NIBSC 91/542) was assigned a potency of 100 PLU/mL.

### 2.6.4 Measurement of direct activation of Factor X by the cell lines

Certain cells, especially malignant types have the ability to directly activate FX. The ability of the cell lines to directly activate FX was investigated by a modification of the intrinsic FXa generation method previously described in this chapter and by a cancer procoagulant screening assay.

#### *2.6.4.1 Direct activation of Factor X*

The method described in section 2.6.3.1 was modified to see if the cell lines could activate FX directly, and if this activation could be detected by the chromogenic substrate S-2765. The time course of FXa generation over 30 minutes was measured by omitting FVIII and FIXa from the reaction mixture. The experiment was controlled by the use of a sample blank where FX was replaced by buffer, and by using papain (1.0 mg/mL) a cysteine proteinase and RVV a complex venom mixture (0.3μg/mL), both known to be activators of FX, as positive controls.

#### *2.6.4.2 Cancer Procoagulant Screen*

Cancer procoagulant functional activity was determined using a three-stage chromogenic assay (Mielicki & Gordon, 1993). Fifty microlitres of cells at an initial

concentration of 2 x $10^6$/mL were mixed with 5 µL of human FX (1 µM) and 15 µL of 25 mM $CaCl_2$. After 30 minutes incubation at 25°C, 5 µL of human prothrombin (14 µM) and 15 µL of phospholipid/$Ca^{2+}$ mix were added to the cells [phospholipid/$Ca^{2+}$ mix = 1part of a 1:10 dilution phospholipid + 1 part of 50 mM $CaCl_2$ + 2 parts of 50 mM Tris buffer (pH 7.8)]. After a further 30 minute incubation at 25°C, 100µL 50 mM tris buffer pH 7.8, and 50 µl chromogenic substrate (1.2 mM, S2238) were added. The reaction rate was measured by monitoring the increase of the optical density of the sample at 405 nm in a Thermomax platereader over a 30 minute period. To calculate the level of direct factor X activator activity for each cell line, tris buffer was substituted for FX in the first stage of the assay and the absorbance at 405 nm was subtracted from the absorbance of the test sample. RVV and papain were used as positive controls in the assay.

### 2.6.5 Thrombin generation measurement

The main function of the clotting system is to generate thrombin in the amounts and at the site required for haemostasis. The time course of thrombin generation in a plasma sample can be monitored in different ways. It can be measured by a clot based method by sub-sampling into fibrinogen (Sas *et al*, 1975) or more recently by using chromogenic substrates that are specific to thrombin (Hemker *et al*, 1986). The chromogenic methods can be either sub-sampling or non-sub-sampling with thrombin generation being continuously monitored in a spectrophotometer (Hemker *et al*, 1993). In this study a chromogenic sub-sampling method was used. The total amount of thrombin estimated over time is represented by the area under the thrombin generation curve and is termed the endogenous thrombin potential (ETP) (Hemker *et al*, 1993). The test measures the combined action of the procoagulant and anticoagulant systems by measuring ETP curves. The curve is characterised by a lag phase, a rise to a peak value and a decline to zero in the time course of the reaction.

### *2.6.5.1 Determination of thrombin generation in plasma (chromogenic method)*

The coagulation system was activated by the addition of 80 μL of FIXa at an initial concentration of 1.1 IU/mL to 400 μL of defibrinated plasma and incubated at 37°C for 90 seconds. Then 100 μL of either cells, phospholipid or tris/HSA buffer at the desired concentration was added to the reaction mixture and incubated for a further 120 seconds. Thrombin formation was then initiated by the addition of 50 μL of $CaCl_2$ (90 mM) and the stopwatch started. Thrombin generated was assessed at 60 second intervals for 10 minutes and then at 120 second intervals for a further 20 minutes, by sub-sampling 10 μL of the reaction mixture into microtitre plate wells containing 140 μL of stopping buffer. Thrombin generation was visualised by adding 50 μL of S-2238 (1.2 mM) to each microtitre plate well. The reaction was carried out at 37°C. After exactly 180 seconds the reaction was stopped by adding 80 μL of 50% glacial acetic acid.

Optical density was read at 405 nm on a Thermomax platereader, and converted to thrombin units using standard curves made with the first International Standard for human α-thrombin (89/588) ranging from 0.1 - 50 IU/mL. The amount of thrombin generated was then plotted against incubation time to give a thrombin generation curve. The total amount of thrombin generated was obtained by computing the area under each curve using the trapezoidal rule. As the biologically inactive thrombin-$\alpha_2$ macroglobulin complex can still cleave S-2238, the data were then analysed using a program that calculates and subtracts the amidolytic activity of this complex (Hemker *et al*, 1986). Details of the calculations used for this correction are described in section 2.6.5.2. For each set of experiments, a control test was carried out with buffer instead of cells or phospholipid. Three to five sets of separate experiments were run for each cell line tested.

Thrombin generated was expressed as thrombin IU/mL and then converted to PLU/mL by comparison with a five point phospholipid standard curve, by using either the total thrombin generated from the area under the curve (AUC), or the peak of thrombin

generation (maximum thrombin concentration observed). When inhibitors of thrombin generation were used results were expressed as percentage of inhibition of thrombin generated versus untreated sample, using either AUC or the peak of thrombin generated and the time taken (lag phase) for this peak to appear.

### *2.6.5.2 Correction for thrombin/$\alpha_2$-macroglobulin complex*

A technical problem arises due to the fact that part of the thrombin in plasma binds to and is inactivated by $\alpha_2$-macroglobulin ($\alpha_2$-M). This complex has no procoagulant activity but can still cleave chromogenic substrates. The product formation observed is the result of the combined activities of free thrombin and $\alpha_2$-M-bound thrombin. A simple approximate method was used to calculate the progress curve for $\alpha_2$-M - thrombin complex formation which accounts for the final steady state activity using the chromogenic assay. In this way a final steady state level of activity due to the thrombin-$\alpha_2$-M complex is read from the progress curve and the attainment of this final level is extrapolated back to $t_0$. Thus a theoretical progress curve was calculated using the relationship:

1. thrombin-$\alpha_2$-M activity = final thrombin-$\alpha_2$-M [1-exp ($-k_\alpha t$)]

where $k_\alpha$ is the published pseudo-first order association rate constant for $\alpha_2$-M reaction with thrombin [$5.7 \times 10^{-3} s^{-1}$] (Hemker *et al*, 1986). The area under this curve was calculated from the integrated form of equation 1:

total thrombin-$\alpha_2$-M = final thrombin [$t_0 + 1/k_\alpha . \exp(-k_\alpha t)$] -$\alpha_2$-M activity.

For the purposes of these calculations $t_0$ was approximated to the time point showing the first significant rise in thrombin activity. This area was then subtracted from the total area to give a true measure of the total biologically free and active thrombin produced (Figure 2.2).

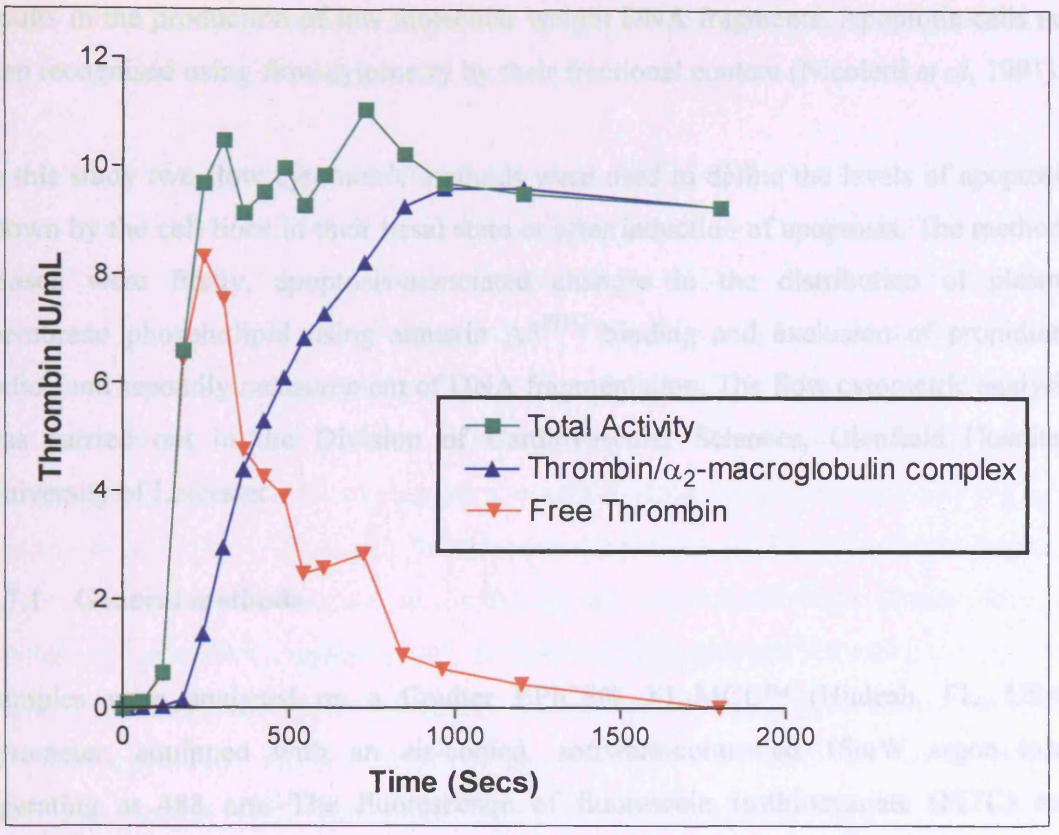



***Figure 2.2: An example of thrombin generation curve data, showing correction for the thrombin / $\alpha_2$-macroglobulin complex.***

## 2.7 Flow cytometry methods

Flow cytometry can be used for the identification and quantification of apoptotic or necrotic cells, and a variety of methods has been developed. The apoptosis associated changes in cell size and granularity can be detected by analysis of laser light scattered by the cell in forward and side directions (Ormerod, 1998). Some of the methods rely on the apoptosis associated changes in the distribution of plasma membrane phospholipids (Koopman *et al*, 1994) (Vermes et al 1995). Others measure the transport function of the plasma membrane, whilst other methods probe the mitochondrial transmembrane potential, which decreases during apoptosis (Petit *et al*, 1995; Zamzami *et al*, 1995). Another marker of apoptosis is the endonucleolytic DNA degradation that

results in the production of low molecular weight DNA fragments. Apoptotic cells are then recognised using flow cytometry by their fractional content (Nicoletti *et al*, 1991).

In this study two flow cytometric methods were used to define the levels of apoptosis shown by the cell lines in their basal state or after induction of apoptosis. The methods chosen were firstly, apoptosis-associated changes in the distribution of plasma membrane phospholipid using annexin $A5^{FITC}$ binding and exclusion of propidium iodide and secondly measurement of DNA fragmentation. The flow cytometric analysis was carried out in the Division of Cardiovascular Sciences, Glenfield Hospital, University of Leicester.

### 2.7.1 General methods

Samples were analysed on a Coulter EPICS® XL-MCL™ (Hialeah, FL, USA) cytometer, equipped with an air-cooled, software-controlled 15mW argon laser operating at 488 nm. The fluorescence of fluorescein isothiocyanate (FITC) and propidium Iodide (PI) was detected by using 525 ± 20 nm and 650 ± 20 nm bandpass filters respectively. The flow cytometer was calibrated daily using Flow-Check™ beads (Coulter, Hialeah, FL, USA). Light-scatter signals were detected at logarithmic gain. Where two colour analysis was performed, the signal was corrected for spectral overlap. Ten thousand events of interest were analysed. The optimum volume of all antibodies used was determined by titration. Buffers were filtered with a 0.2 μm filter prior to use.

### 2.7.2 Preparation of samples for flow cytometry

In all cases, cells were washed twice with RPMI and resuspended at an approximate density of 1 x $10^6$ cells/mL unless otherwise stated and aliquots of the cell suspension transferred to Falcon tubes before analysis.

### 2.7.3 Measurement of the exposure of negatively charged phospholipid by annexin $A5^{FITC}$ binding

The cells were harvested, washed twice in cold PBS, and then resuspended in either annexin A5 binding buffer (10 mM HEPES/NaOH (pH 7.4) 140 mM NaCl, 2.5 $CaCl_2$) or HEPES buffer devoid of $CaCl_2$ at a density of 2 x $10^6$/mL. One hundred microlitres of the cell solution were incubated with 5 µL of annexin $A5^{FITC}$ and 5 µL of PI in the dark for 15 minutes at room temperature. Four hundred microlitres of HEPES buffer with calcium was added to tubes to be analysed for anionic phospholipid exposure whilst 400 µL HEPES without calcium was added to the tubes being used as a negative control for annexin A5 binding. Samples were then kept on ice and analysed within 1 hour. A protocol was designed to distinguish the cells on the basis of size (forward scatter) and granularity (side scatter). A gate was manually positioned around the cell cloud on the 'log FS' versus 'log SS' histogram, and the gating settings were saved on the flow cytometer and not repositioned. The negative control for the conjugated antibody (annexin $A5^{FITC}$ minus $Ca^{2+}$ in HEPES buffer) was set at approximately 2% positive. Spectral overlap of the emission from annexin $A5^{FITC}$ and PI was corrected during signal processing.

### 2.7.4 The use of annexin $A5^{FITC}$ and propidium iodide to distinguish apoptotic cells using FACS analysis

In live cells there is membrane asymmetry, where PS is present at higher concentrations on the inner compartment with the outer leaflet of the cell membrane due to an energy dependent translocation of PS to the inner membrane. One of the first changes associated with cell death is the loss of this asymmetry and the consequent rapid exposure of PS on the outer membrane. This phenomenon has been used develop a method of discriminating dying cells from live cells by flow cytometric analysis. Annexin A5 is a 35 – 36kD calcium dependent, phospholipid binding protein that has a particular affinity for anionic phospholipids, especially PS, and binds to cell membranes following exposure of PS (Raynal & Pollard, 1994). Annexin A5 may be

conjugated to biotin or fluorochromes such as FITC or PE and still retain a high affinity for PS and therefore can be used as a sensitive probe for flow cytometric analysis of apoptotic and dying cells. Live cells do not bind annexin A5. However, labelling cells with annexin A5 alone does not allow discrimination between apoptotic and necrotic cells, as both will stain positive for annexin A5. During the transition from apoptosis to necrosis, the cell membrane becomes ruptured and cationic dyes such as PI, 7-amino-actino-actinomycin D (7-ADD) or To-PRO-3 iodide are no longer excluded from the cells (Nicoletti *et al*, 1991) (Philpott *et al*, 1996). Thus by simultaneous co-labelling of a cell population with annexin A5 and a nucleic acid dye it becomes possible to discriminate apoptotic cells from necrotic or live ones. An example of the typical data obtained is shown in Figure 2.3. Jurkat cells before treatment with staurosporine (Figure 2.3a) were mainly annexin $A5^{FITC}$ and PI negative, indicating that they were viable and not undergoing apoptosis. After 6 hrs of treatment with staurosporine (an apoptotic agent), three populations of cells can be identified, as shown in Figure 2.3b; (1) cells that were viable and not undergoing apoptosis (Annexin $A5^{FITC}$ and PI negative); (2) cells undergoing apoptosis (Annexin $A5^{FITC}$ positive, PI negative); (3) cells that that are necrotic or are in the late stage of apoptosis (annexin $A5^{FITC}$ and PI positive).

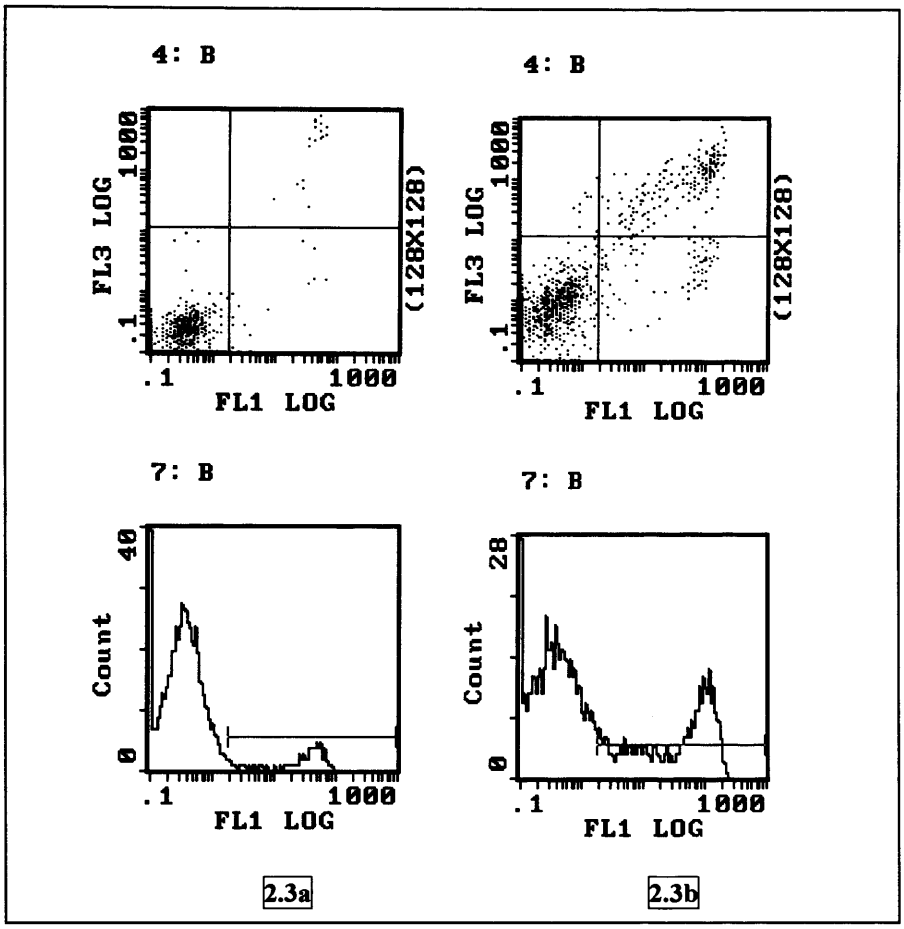



***Figure 2.3: An example of the dot plots and histograms of Jurkat cells before (2.3a) and after 6 hours of treatment with (1 μM) staurosporine (2.3b). For the dot plot FL1 axis shows annexin $A5^{FITC}$ fluorescence and propidium iodide fluoresence is shown on the FL3 axis. When annexin $A5^{FITC}$ fluorescence (FL1) is plotted against cell count a histogram is obtained that shows two populations, one healthy that does not bind annexin $A5^{FITC}$, and one undergoing cell death that binds to annexin $A5^{FITC}$.***

### 2.7.5 Generation of 3G4 mouse monoclonal antibody reactive with anionic phospholipids

The antibody 3G4 was generated by Dr S. Ran and Professor P.E. Thorpe, at the Simmons Comprehensive Cancer Center (University of Texas Southwestern Medical Center, Dallas, Texas USA). To generate a monoclonal antibody reactive with anionic phospholipids, female mice had been immunised with bEnd.3 endothelial cells that had been treated with 200 μM of hydrogen peroxide for 2 hours. The treatment caused

translocation of anionic phospholipids to the external surface in 70-90% of cells as detected by $^{125}$I-labelled annexin A5. Treated cells were washed, detached and counted. Two million cells were suspended in sterile PBS and injected five times intra-peritoneally with the interval of 3 weeks between injections. The titre of polyclonal antibodies to anionic phospholipids was determined 2 days after each immunisation. Hybridomas were obtained by fusing splenocytes from immunised mice with myeloma partner P3 X 63AG8.653 cells (American Type Culture Collection). The reactivity of the selected antibody, mouse $IgG_3$ 3G4, with PS and CL was established by screening hybridoma supernatants on PS, cardiolipin (CL), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) immobilised on plastic. Further studies showed that 3G4 bound strongly to PS, phosphatidic acid (PA), and CL but weakly to phosphatidylinositol (PI) and phosphatidylglycerol (PG). Recognition of anionic phospholipids by 3G4 was identical in the presence and absence of serum indicating that binding does not require serum cofactors. Cross-blocking experiments on PS coated plates showed that 3G4 and annexin A5 do not block each other's binding to PS, indicating that these two reagents recognise different epitopes on the PS molecule, or differently packed forms of PS (personal communication with Professor P.E. Thorpe).

### 2.7.6 Measurement of the exposure of phosphatidyserine using the anti-PS antibody 3G4

The anti-PS antibody 3G4, together with a secondary conjugated antibody (FITC-conjugated rabbit anti-mouse $F(ab')_2$, Dako Ltd, Ely, Cambs, UK) was used to determine the exposure of PS on the cell surface. The cell solution (100 μL) was incubated with 3 μL of 3G4 for 15 minutes. The secondary antibody (5 μL) was then added and incubated for a further 15 minutes. Both these incubations took place on ice. Four hundred microlitres of HEPES buffer without $Ca^{2+}$ was then added to each test and negative control tube and analysed within 1 hour. Samples incubated with secondary antibody alone served as the negative control, and were set at approximately 2%.

### 2.7.7 Monitoring apoptosis by DNA fragmentation using propidium iodide

DNA fragmentation was measured in a flow cytometric assay (Nicoletti *et al*, 1991). Briefly, cells were washed and resuspended in PBS and the count adjusted to 2.5 x $10^6$/mL. One mL of the cell suspension was then fixed and the cell membrane made permeable in 4 mL cold 70% ethanol at 4°C for 60 minutes, and stored at 4°C until analysed. The cells were then washed in PBS and resuspended to a count of 2.5 x $10^6$/mL. To a 400 µL cell sample, 100 µL of RNAse (1 mg/mL) was added followed, after gentle mixing, by 100 µL of PI (50 µg/mL). The cells were incubated in the dark for 30 minutes at 37°C and then measured within 1 hour.

### 2.7.8 Apoptosis, the cell cycle and DNA fragmentation

Flow cytometry can be used for studying DNA degradation in cells undergoing apoptosis (Johnson *et al*, 1995). During apoptosis the activation of endonuclease leads to DNA fragmentation and small molecular weight fragments are left free in the nucleus. The intercalcating DNA dye, PI can be used to measure this. Cells are permeabilised by fixation with 70% ethanol, which allows PI to enter the cell and bind to DNA and RNA. Binding of PI to RNA can be elimimated by pre-treating the cells with RNAse. Propidium iodide bound to DNA fluoresces, and the intensity of PI fluorescence is directly proportional to the amount of DNA present, and thus once in the cell gives us information about the DNA profile and the percentage of cells in each phase of the cell cycle.

The cell cycle is the sequence of growth, DNA replication, growth and cell division (Figure 2.4). The cell cycle is divided into the mitotic phase (M) and DNA-synthesis phase (S) separated by intervals, occupied by transcriptional processes, called the gap phases ($G_1$ and $G_2$). Cells that are not dividing are deemed to be in the $G_0$ phase. During division the cell first enters the $G_1$ phase where cells synthesise the components required for division, including lipids, carbohydrates and proteins and the amount of

RNA increases and replication of DNA starts to occur. At this point PI bound to DNA is observed as the $G_1$ peak.

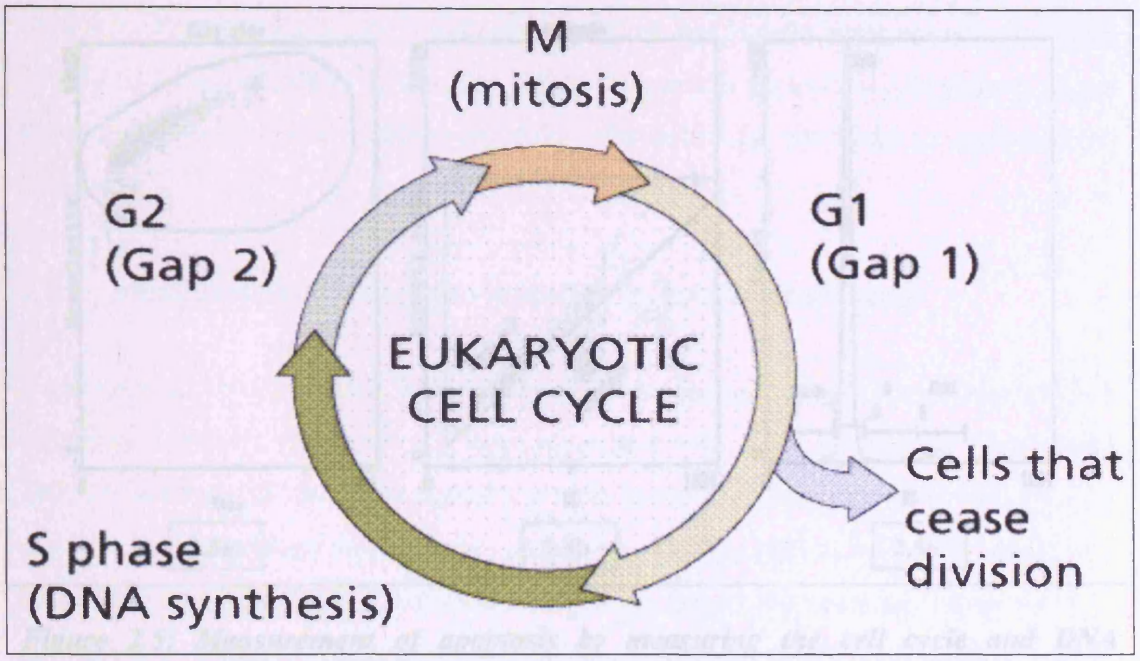



***Figure 2.4: The stages of the cell cycle: The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. Non-dividing cells not considered to be in the cell cycle. The stages, pictured to the left, are G1-S-G2-M. The G1 stage stands for "GAP 1". The S stage stands for "Synthesis". This is the stage when DNA replication occurs. The G2 stage stands for "GAP 2". The M stage stands for "mitosis", and is when nuclear (chromosomes separate) and cytoplasmic (cytokinesis) division occur.***

The daughter cells, which are small after cytokinesis, synthesise ATP and increase in size during the $G_1$ phase of interphase. Because interphase is the longest part of the cell cycle, most 'healthy' cells are observed in this phase. After acquiring sufficient size and ATP, the cells then enter the S phase where they replicate DNA and some proteins until the DNA content of the cell is double those in $G_0$ and $G_1$ phases. This phase is followed by a second growth phase ($G_2$), after which cells then undergo mitosis (the M phase) and divide into two identical daughter cells. Therefore cells at different stages of the cell cycle have different quantities of DNA. Cells in $G_0/G_1$ contain the standard compliment of DNA; cells in S-phase have increasing amounts of DNA, as during this

phase DNA is being synthesised, whilst cells in $G_2/M$ have double the quantity of DNA in cells in $G_0/G_1$.

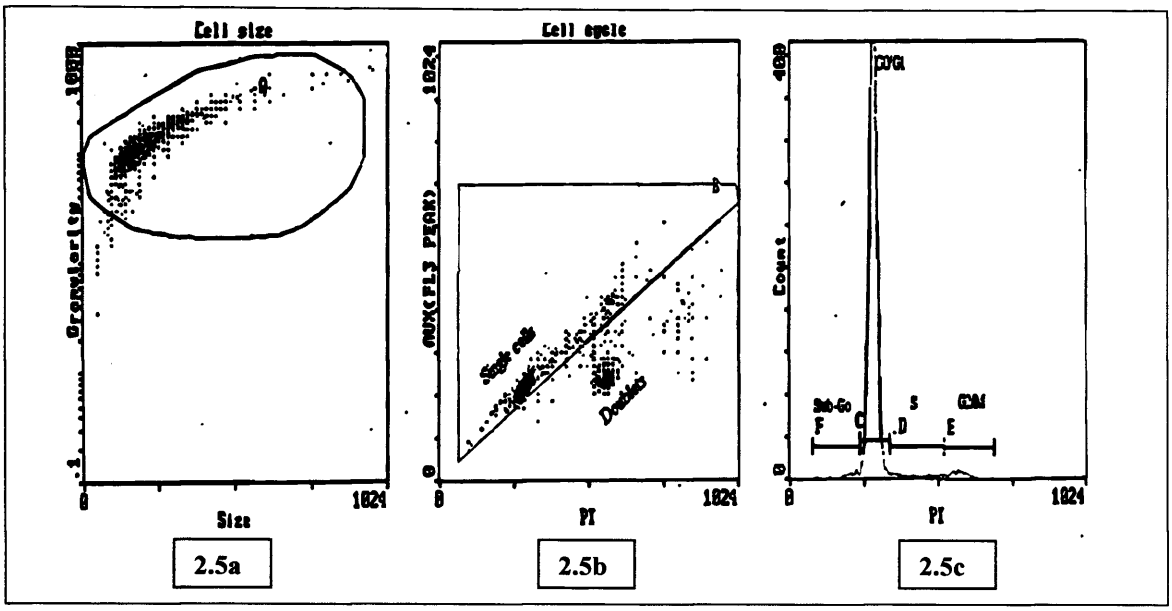



***Figure 2.5: Measurement of apoptosis by measuring the cell cycle and DNA fragmentation. The forward scatter (size) and side scatter (granularity) of the individual cells are displayed in 2.5a. Only cells gated in 2.5a are shown in 2.5b. Single cells are distinguished from doublets in 2.5b by comparing the fluorescent area and fluorescent peak for propidium iodide staining. Single cells are captured in the triangular gate shown in 2.5b. Only the DNA content of the single cells, gated in 2.5b are displayed in the frequency histogram 2.5c. Data in this histogram (2.5c) is gated into stages of the cell cycle; C = $G_0/G_1$ phases, D = S phase, E = $G_2/M$ phases, and F = Sub-$G_0$.***

There is also a proportion of the cell population that have entered apoptosis, resulting in DNA fragmentation with these cells having less DNA than cells in the $G_0/G_1$ phase of the cell cycle. This population of cells are said to be in sub-$G_0$. Due to the same mechanism, the DNA content of necrotic cells is even lower and these cells are found on the DNA histogram between the Y axis and the sub-$G_0/G_1$ peak. Thus, use of PI as well as giving us information about the cell cycle allows us to discriminate between live cells and those undergoing apoptosis. However, with this technique it is impossible to separate primary from secondary necrosis. To assess accurately the percentage of a population in each phase of the cell cycle, aggregated cells or doublets as well as cell

debris have to be removed from the analysis. If doublets are not eliminated $G_0/G_1$ cells would be incorrectly identified as single cells with $G_2/M$ nuclei, as the fluorescent signals from $G_0/G_1$ doublets labelled with PI have the same area as single $G_2/M$ cells. However, these doublets can be distinguished from single cells because the peak height is only half that of single $G_2/M$ cells. When this data is plotted on a histogram as red fluorescence against cell number, apoptotic cells fall to the left of the $G_o$ plus $G_1$ peak (Figure 2.5).

### 2.7.9 Identification of Tissue factor antigen by immunofluoresence

Expression of TF on the cell lines was analysed by immunofluorescence. One hundred microlitres of cells (4 x $10^6$/mL) were incubated with FITC conjugated monoclonal $IgG_1$ TF antibody (4508CJ, 50 μg/mL) or with isotype control $IgG_1$ (50 μg/mL) for 30 minutes at 4°C and kept in the dark. Cells were resuspended in 400 μL of phosphate buffered saline (PBS) before analysis. The mean channel fluorescence intensity (MFI) of 10,000 events was determined for each sample. The isotype negative control antibody for the conjugated antibody was set at approximately 2%.

## 2.8 Measurement of caspase-3/7 like activity

To confirm that apoptosis was taking place in the cells caspase-3/7 like activity was measured in cell extracts from the cell lines using colorimetric assay (Sigma, St Louis, USA). Cells were washed once in Dulbecco's PBS (pH 7.4) and resuspended at a concentration of 2 x $10^6$/mL. The cells were pelleted by gentle centrifugation at 800g for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 100 μL of ice-cold lysis buffer consisting of 50 mM HEPES (pH 7.5), 5 mM 3-[(3-cholamidopropyl)dimethyl-ammomio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), and allowed to stand at 4°C for 15 minutes to allow complete lysis to occur. The lysed cells were then centrifuged at 17000g in a microfuge at 4°C for 10 minutes and the supernatant removed and stored at -80°C until analysis.

For analysis, 5 μL extract was combined with 85 μL of assay buffer consisting of 20 mM HEPES (pH 7.5), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 10 μL colourimetric substrate (2 mM Ac-DEVD-*p*NA in dimethyl sulfoxide). The samples were incubated at 37°C, and the absorbance at 405 nm was measured, using a Thermomax platereader after 90 minutes. Caspase-3/7 like activity was also evaluated in extracts treated with 10 μL of 200 μM Ac-DEVD-CHO a peptide inhibitor of caspase-3. Activity was defined as μmol Ac-DEVD-*p*NA cleaved per $10^6$ cells/mL per minute by comparison with a *p*NA calibration curve.

## 2.9 Detection of products of Lipid Peroxidation

The appearance of the products of lipid peroxidation were detected using methods to measure conjugated dienes, conjugated trienes and thiobarbituric acid reactive substances (TBARS). In order to estimate the degree of lipid peroxidation, lipids have to be extracted from the cell membrane, and the amount of phospholipid in the extract has to be quantified.

### 2.9.1 Extraction of Lipids from cell membranes

Lipids were extracted by the classical method of (Folch *et al*, 1957) with the modification used by Cartwright, 1993. Membrane lipids from the cell lines (1 mL of cells in aqueous suspension) were solubilised using 20 mL of organic solvents (chloroform:methanol ratio of 2:1 (v/v)) to give a monophasic system after centrifuging the mixture at 1000 g for 10 minutes. The supernatant was removed, and contaminants (water and methanol soluble compounds) were removed by vigorously mixing the supernatant with 4 mL of 50 mM $CaCl_2$ for 2 minutes. This mixture was then centrifuged at 2000 g for 5 minutes and a biphasic system was formed, where the lower organic phase contained the lipids, and the upper aqueous phase contained soluble non-lipid contaminants. The upper water-methanol phase was aspirated and the lipid extract

was evaporated under a stream of oxygen-free nitrogen, and either stored at –80°C or redissolved immediately in cyclohexane and used for conjugated diene or triene determination.

### 2.9.2 Quantitation of total phospholipid

A fluorometric assay that had been designed to assay phospholipids in vesicles (London & Feligenson, 1978) and serum (Jouanel *et al*, 1980) was used to measure cell line phospholipid in Folch extracts. The method is based upon the fluorescence of a probe (1-6-diphenyl-1-3-5 hexatriene, DPH) in the presence of phospholipids and the results obtained have been shown to be comparable with those found by the phosphorous (Fiske & Subarrow, 1925) and enzymatic assays (Takayama *et al*, 1977).

Twenty microlitres of a fresh dilution (1/10, v/v) of lysed phospholipid extract in isotonic saline was added to 3 mL of aqueous solution of DPH (200 µL of 3 mM DPH, diluted in tetrahydrofuran, added to 60 mL of distilled water) , mixed and incubated at 40°C for 40 minutes in the dark. The tubes were then allowed to equilibrate to room temperature. The fluorescence of the mixture was read in a cell (1 cm light path) thermostatically held at 25°C. The fluorescence was measured using a Spectra MAX GEMINI XS spectrofluorometer (Molecular Devices Corporation, Sunnyvale CA, USA), at 365 nm for excitation and 460 nm for emission with 10 nm band pass slits. A standard curve was established for the range 50 – 600 mg/dL, using a commercial standard solution (Wako Chemicals USA Inc, Richmond, Va, USA).

### 2.9.3 Measurement of lipid peroxidation

The oxidative status of the cell line phospholipids pre and post treatment was determined by measuring conjugated dienes, conjugated trienes and TBARS.

### *2.9.3.1 Detection of lipid-conjugated dienes and trienes*

Spectrophotometric detection of conjugated dienes and trienes reflects the presence of fatty acid hydroperoxides in lipid extracts. The procedure was carried out according to an established method (Recknagel & Glende, Jr., 1984). An aliquot of cell line lipid extract was evaporated under a stream of nitrogen and redissolved in cyclohexane (spectrophotometric grade). Ultra-violet (UV) spectra of lipids were monitored using an Ultraspec 2400 spectrophotometer (Amersham Biosciences, Amersham, UK). The characteristic absorption maxima at 234 nm (conjugated dienes) and 274 nm (conjugated trienes) were measured. Intensity of conjugated diene and conjugated triene formation was quantified per μg of phospholipid determined by the method reported in section 2.9.2 and expressed as relative units (RU).

$$\mathbf{RU = Abs_{234nm\ or\ 274nm} / \mu g\ Phospholipid}$$

### *2.9.3.2 Thiobarbituric acid reactive substances (TBARS) test*

The levels of TBARS were determined as a marker of lipid peroxidation (Janero, 1990). Cells were washed and resuspended in 500 μL PBS. To avoid lipid peroxidation during the assay, 10 μL of the antioxidant butylated hydroxytoluene (100 mM) and 10 μL EDTA (1 mM) was added to the sample prior to assay. Then in sequence 100 μL of cells were added 100 μL of 8% sodium dodecylsulphate, followed by 2.5 mL of thiobarbituric acid (TBA) reagent (0.5% TBA [w/v] in 20% [w/v] trichloroacetic acid [TCA]). Subsequently the mixture was heated to 95°C for 60 minutes. After cooling and centrifugation (15 minutes x 10 000g) absorbance of the supernatant was measured at 532 nm using an Ultraspec 2400 spectrophotometer (Amersham Biosciences, Amersham, UK). The concentration of TBARS was expressed as equivalents of malondialdehyde Bis (dimethyl acetal) (Alexis Biochemicals, SanDiego, USA), which was used as a standard. The absorbance was then converted to *p*moles Malondialdehyde per 1 x $10^6$ cells.

## 2.10 Data analysis of the haemostasis tests

All the results reported in this study represent the arithmetic mean value of three or five observations for procoagulant activity, Cancer Procoagulant and apoptosis or lipid peroxidation assays ± 95% confidence interval (95% CI). Orthogonal regression was used to determine the coefficient of correlation ($r^2$) between different procoagulant activities and parameters. To compare results, differences in mean values were analysed using the t-test; $p$-values < 0.05 were considered to be statistically significant.

Chapter

3

# CHARACTERISATION OF THE *IN VITRO* CELL-SURFACE PROCOAGULANT ACTIVITY OF T-LYMPHOBLASTOID CELLS

## 3.1 Introduction

Leucocytes play an important role not only in the physiological processes of inflammation and wound healing but also in pathophysiological haemostatic processes such as malignancy and atherosclerosis. Integral to their role in these processes is the ability of leucocytes to participate in and regulate the molecular events leading to thrombin formation, which is supported by several observations. Firstly, there is an active fibroproliferative response (Ross, 1993) as well as fibrin deposition (Colvin & Dvorak, 1975; Bini *et al*, 1989) accompanying these processes. Thrombin promotes numerous cellular effects including chemotaxis, proliferation, cytokine release, and extracellular matrix turnover. Thus, it is likely that thrombin production at the leucocyte surface provides this important bioregulatory molecule at precise locations.

The majority of studies defining leucocyte involvement in coagulation reactions have been with monocytes; the contribution of lymphocytes remains virtually unknown. In contrast to monocytes, which express the thrombin receptor PAR-1, neither normal lymphocytes nor neutrophils appear to respond to thrombin through PAR receptors (Hoffman & Church, 1993). In addition neither support the assembly and function of intrinsic tenase (McGee & Li, 1991) but prothrombinase assembles and functions on both isolated cell populations (Tracy *et al*, 1983; Tracy *et al*, 1985). The initial objective of the present study was to characterise the ability of malignant T-cells to support thrombin generation via TF/FVIIa, intrinsic tenase, and prothrombinase catalysed blood coagulation reactions. Figure 3.1 shows the assays used in this work to characterise malignant T-cell procoagulant activity. They are sub-divided according to whether they are TF or phospholipid dependent or are looking for the direct activation of Factor X by the cell-surface of the cell line. Eight different assay systems were used: the prothrombin time (PT) and a chromogenic assay both dependent on the presence of TF, FVIIa and anionic phospholipid; the activated partial thromboplastin time (APTT), russell's viper venom time (RVVT) and purified intrinsic Xa and prothrombinase generating systems all requiring anionic phospholipid; finally a cancer procoagulant (CP) assay to investigate the direct activation of FX.

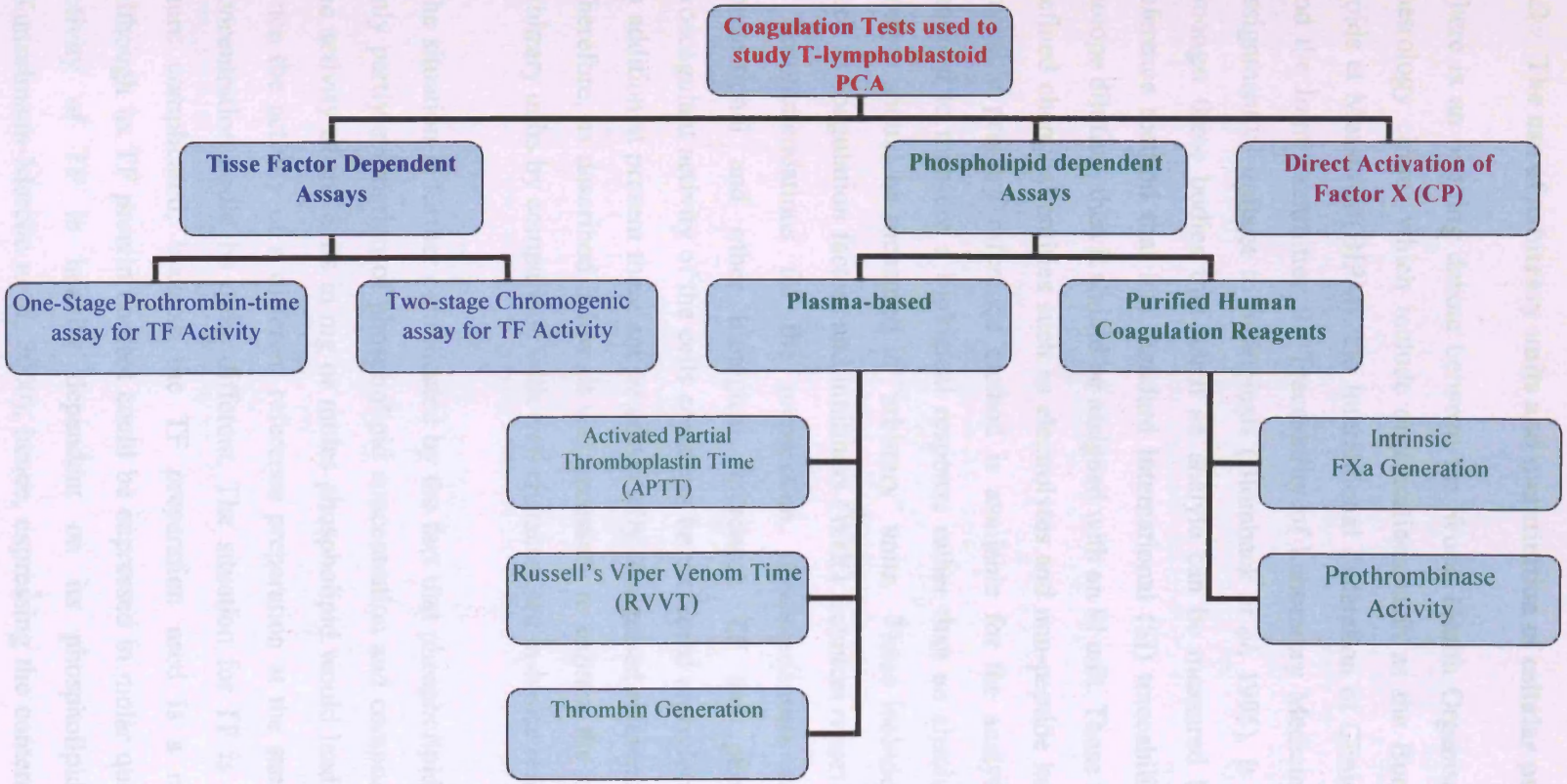



*Figure 3.1: Coagulation assays used in the study of T-lymphoblastpiod cell procoagulant activity (PCA). The type of assay used is classified according to whether it is tissue factor (TF) or phospholipid dependent and if it can detect the effect of Cancer procoagulant (CP). The phospholipid dependent coagulation assays are further subdivided as to whether they are plasma-based or use purified human coagulation factors.*

## 3.2 The use of arbitrary units and quantitation of cellular procoagulant activity

There is an ongoing debate between the World Health Organisation (WHO) and the metrology circles which include organisations such as the Bureau International des Poids et Measures (BIPM), the International Federation of Clinical Chemistry (IFCC) and the Joint Committee for Traceability of Laboratory Medicine (JCTLM), over the assignment of unitage to biologicals (Blomback *et al*, 1995). It has now been agreed amongst these bodies that when an analyte can be measured by a defined primary reference method that has Standard International (SI) traceability such as weight or isotope dilution, then it should be assigned with an SI unit. These materials include well defined chemical entities such as electrolytes and non-peptide hormones. When no SI traceable primary reference method is available for the analyte, and bioassays are required to measure a biological response rather than an absolute quantity, then the analyte should be measured in "arbitrary" units. These include complex biologicals such as coagulation factors and inhibitors (WHO technical report in preparation (2004) - Recommendations for the preparation, characterisation and establishment of international and other biological reference). TF and phospholipid dependent procoagulant activity of the cells could not be measured reproducibly in absolute units, in addition at present there are no universally recognised reference materials available. Therefore, as described below, it was necessary to express the results for the cells as arbitrary units by comparison with well characterised in-house reference preparations.

The situation is further complicated by the fact that phospholipid biological activity is only partly a function of phospholipid concentration and composition, and expressing the activity of the cells in mg or moles phospholipid would lead to its own problems, since the activity of a different reference preparation at the same phospholipid mass concentration could be quite different. The situation for TF is slightly different and more complicated, because the TF preparation used is a recombinant material. Although its TF protein content could be expressed in molar quantities the biological activity of TF is heavily dependent on its phospholipid micro-environment (Kunzelmann-Marche *et al*, 2000), hence, expressing the content of the TF reference

reagent in SI units without reference to its phospholipid content might not relate to its biological activity.

The choice of reference preparations and the use of arbitrary units was also influenced by previous studies, not least because it is important to demonstrate continuity with these studies, which had been carried out elsewhere (Barrowcliffe *et al*, 2002). The fact that the activities of the cells in this study, when expressed as arbitrary units were found to be similar to those reported in the previous study (Barrowcliffe *et al*, 2002) could be considered as a justification of the standardisation system adopted.

Tissue factor activity was measured as TF units (TFU/mL) by calibration with a TF preparation (X/95), where a 1/100 dilution was assigned a value of 100 TFU/mL. This dilution was equivalent to a tissue factor concentration of 1.96 μg/mL. This estimate is based on a value assigned to a preparation of lipidated recombinant TF (Product 4500L; Greenwich, CT, USA) by American Diagnostica, however there is no evidence of SI traceability.

To enable a direct comparison of the activities of the cell lines in each of the phospholipid dependent assays the results were reported as phospholipid equivalence (PLU/mL) by comparison with a bovine brain phospholipid standard (NIBSC 91/542). A 1/400 dilution of the PL standard (91/542) equivalent to a phospholipid concentration of 25 μg/mL (measured by phosphorous determination personal communication with Dr E Gray, NIBSC) was arbitrarily assigned a potency of 100 PLU/mL. APTT-like and RVVT activity was measured as phospholipid units (PLU/mL) by calibration with the NIBSC bovine brain PL standard (91/542). The FXa and thrombin generating activities supported by the cell surfaces were firstly quantified by comparison with purified human FXa and thrombin standards. These values were then converted to PLU/mL by comparison with a PL standard curve.

## 3.3 Tissue factor (TF) expression by the cell lines

Tissue factor is expressed on the surface of various cells including fibroblasts, monocytes and monocytes-like cell lines (Nemerson & Bach, 1982). Several tumours express TF constitutively (Rao, 1992; Sato N *et al*, 1995), frequently shedding microparticles carrying TF. The tendency to thrombotic complications in various cancers is possibly dependent on this cellular PCA. There is also evidence that TF is involved in metastasis and angiogenesis in tumours.

### 3.3.1 Expression of TF activity and antigen on the cell-surface of the cell lines

The pattern of TF cell surface expression of the various cell lines was assessed by measuring TF activity using two methods, as well as TF antigen. TF activity was measured by a clotting assay based upon a PT assay in human PPP in the presence of either cells or relipidated TF, and in a two-step amidolytic FX-activation assay. Both activities were compared with measurement of TF antigen ($TF_{Ag}$) on cell surfaces by flow cytometry (Figure 3.2). The monocytoid cell line (THP-1) showed high clotting activity (53.7 ± 8.7 TFU/ml), whilst the T-lymphoblastoid cell lines CEM-CCRF, Molt-4 and A3.01 had very low levels of activity (6.6 ± 1.1, 1.5 ± 0.2 and 1.1 ± 0.2 TFU/ml respectively). Jurkat cells showed an intermediate level of activity (15.5 ± 2.4 TFU/ml). A similar pattern was observed for amidolytic activity with THP-1 cells showing the highest activity (70.2 ± 0.58 TFU/mL), whilst CEM-CCRF, Molt-4 and A3.01 cells had low activity levels (10.4 ± 0.58, 17.7 ± 0.46 and 11.4 ± 1.46 TFU/ml respectively). Jurkat cells again showed an intermediate level of activity (24.5 ± 0.94 TFU/ml). Amidolytic activity was higher than that of clotting activity and is due to the extra promotion of FVIIa activation of FX by phospholipid, independent of TF. $TF_{Ag}$ expression showed a similar pattern and good correlation ($r^2$ = 0.98 v 0.99, respectively) with TF clotting and amidolytic activity. There was good correlation and no significant difference ($r^2$ = 0.98; p = 0.485) between the clotting and amidolytic TF activity levels. The amidolytic assay reported higher TF activity results than the clotting activity. This could be due to there being TF protein on the cell-surface of CEM-CCRF,

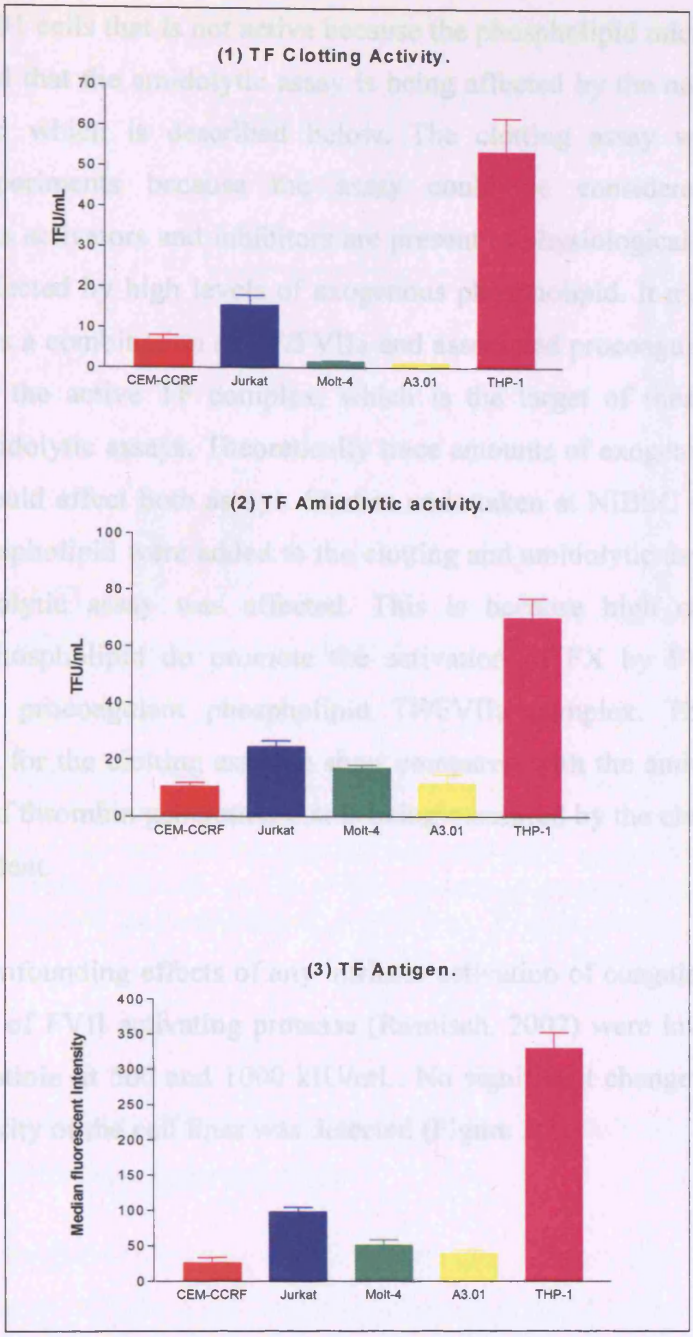



***Figure 3.2: Summary of TF procoagulant activities of the five cell lines using different assay systems. (1) Activities in clotting TF assay (2) Amidolytic activity, were compared with a TF standard (X/95) and (3) TF Ag expression measured as median fluorescent intensity by flow cytometry. All activities are measured at a final cell concentration of 4 x $10^6$/mL. Results are the mean ± 95% CI; n = 5.***

Molt-4 and A3.01 cells that is not active because the phospholipid microenvironment is not suitable, and that the amidolytic assay is being affected by the non-specific action of phospholipid which is described below. The clotting assay was used for all subsequent experiments because the assay could be considered to be more physiological, as activators and inhibitors are present in physiological amounts as well as not being affected by high levels of exogenous phospholipid. It must be taken into account that it is a combination of TF/FVIIa and associated procoagulant phospholipid that constitutes the active TF complex, which is the target of measurement of the clotting and amidolytic assays. Theoretically trace amounts of exogenous procoagulant phospholipid could affect both assays. Studies undertaken at NIBSC where increasing amounts of phospholipid were added to the clotting and amidolytic assays showed that only the amidolytic assay was affected. This is because high concentrations of procoagulant phospholipid do promote the activation of FX by FVIIa, but not as quickly as the procoagulant phospholipid TF/FVIIa complex. Thus because the incubation time for the clotting assay is short compared with the amidolytic assay the main pathway of thrombin generation that is being measured by the clotting assay is TF complex dependent.

The possible confounding effects of any intrinsic activation of coagulation by the cells or the presence of FVII activating protease (Romisch, 2002) were investigated by the addition of aprotinin at 500 and 1000 kIU/mL. No significant change in the PT-based clotting TF activity of the cell lines was detected (Figure 3.3)

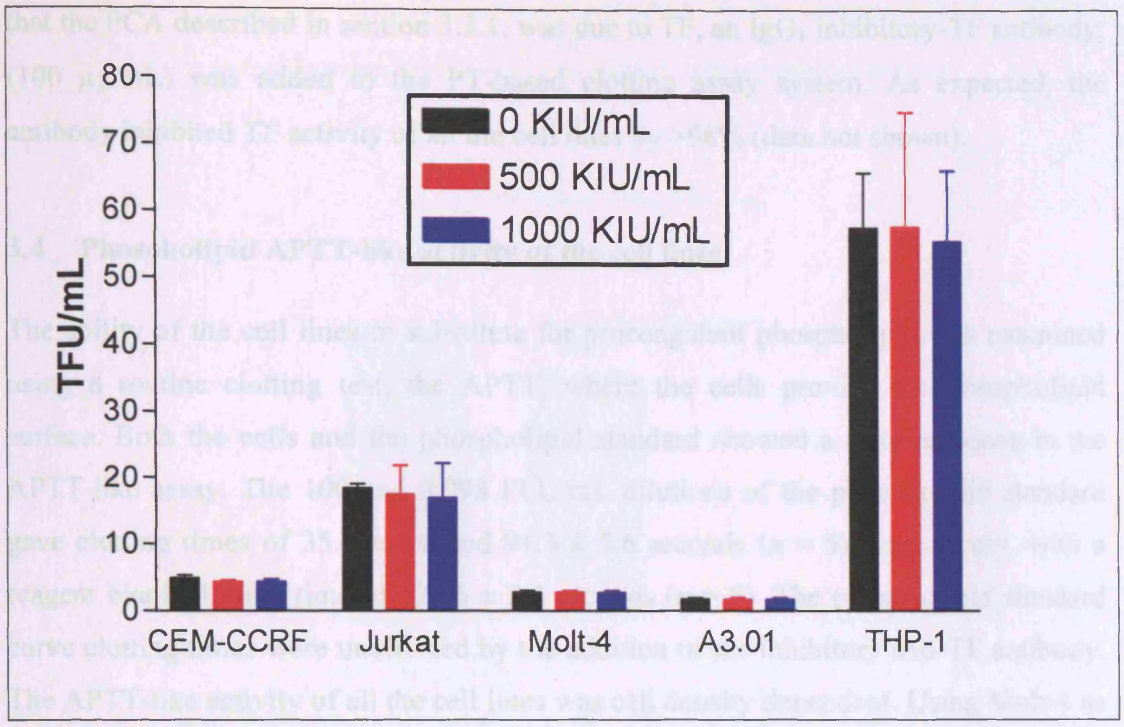



***Figure 3.3: The effect of Aprotinin at 0, 500 and 1000 KIU/mL on the PT-based TF clotting activity. The clotting time was converted to TFU/mL by comparison with a TF standard. Aprotinin was used to inhibit any activation of the clotting system by the intrinsic clotting pathway or by FVII activating protease. All activities are measured at a final cell concentration of 4 x $10^6$/mL. Results are the mean ± 95% CI; n = 3.***

### 3.3.2 Effect of inhibitory TF antibody on TF-FVII complex formation on the cell surface of the cell lines

It is now well established that monocytes/macrophages produce PCA in response to a variety of stimulants (Rivers *et al*, 1975; Schwartz *et al*, 1982) and that this is mainly due to expression of TF promoting the extrinsic coagulation system (Semeraro *et al*, 1983). TF is known as the principal initiator of blood coagulation. Furthermore it has been shown that TF is a true receptor capable of signalling mechanisms involved in angiogenesis, tumour growth and metastasis. The ability of TF to influence cellular behaviour may arise either directly from this protein, or via the activation of protease activated receptors, through TF-FVIIa or FXa activities (Chen *et al*, 2001). To confirm

that the PCA described in section 3.3.1, was due to TF, an $IgG_1$ inhibitory-TF antibody, (100 μg/mL) was added to the PT-based clotting assay system. As expected, the antibody inhibited TF activity of all the cell lines by >98% (data not shown).

### 3.4 Phospholipid APTT-like activity of the cell lines

The ability of the cell lines to substitute for procoagulant phospholipid was examined using a routine clotting test, the APTT, where the cells provide the phospholipid surface. Both the cells and the phospholipid standard showed a dose response in the APTT-like assay. The 100 and 0.098 PLU/mL dilutions of the phospholipid standard gave clotting times of 35.7 ± 1.8 and 91.3 ± 5.6 seconds ($n$ = 5) respectively, with a reagent blank clotting time of 125.6 ± 2.4 seconds ($n$ = 5). The phospholipid standard curve clotting times were unaffected by the addition of the inhibitory anti-TF antibody. The APTT-like activity of all the cell lines was cell density dependent. Using Molt-4 as an example, final cell counts of 4, 2 and $1x10^6$/mL gave clotting times of 58.1 ± 1.8, 62.2 ± 2.29 and 70.0 ± 2.16 seconds (n = 5), respectively.

The intrinsic APTT-like PCA of the cell lines is shown in Figure 3.4. The monocytoid cell line (THP-1) supported the highest APTT-like activity (4.5 ± 0.29 PLU/mL), whilst CEM-CCRF showed the lowest activity (1.14 ± 0.06 PLU/mL), closely followed by A3.01 (1.57 ± 0.36 PLU/mL). Jurkat and Molt-4 supported an intermediate level of activity (2.79 ± 0.36 and 2.29 ± 0.22 PLU/mL respectively).

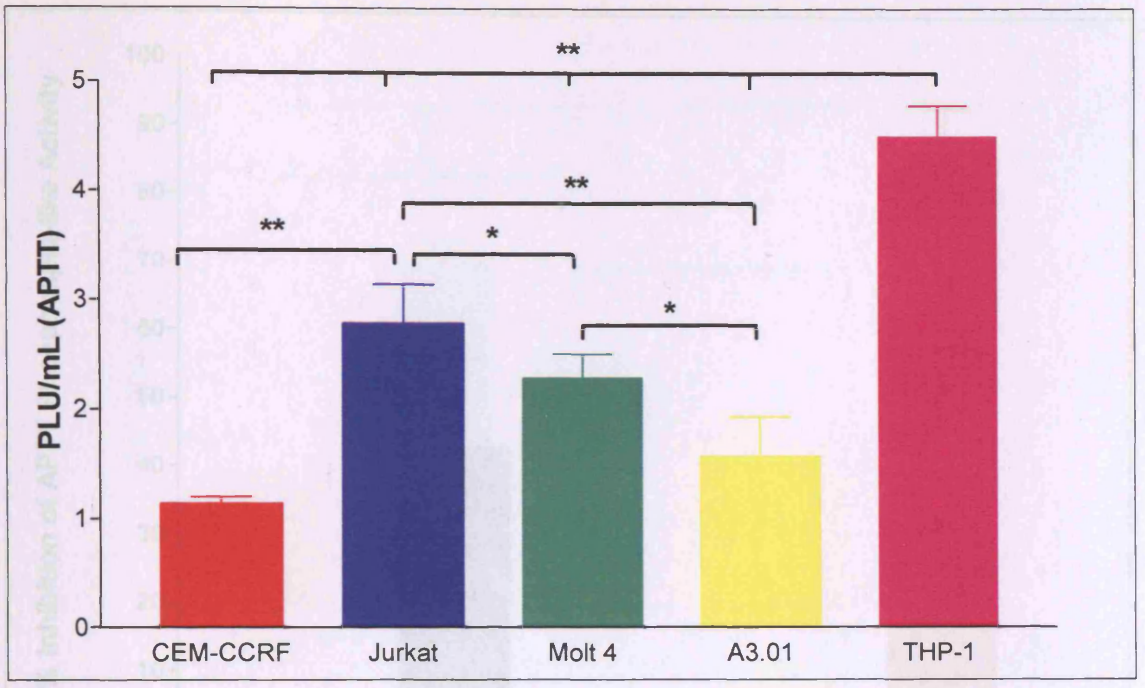



***Figure 3.4: APTT-like procoagulant activity of the five cell lines. The activity was measured at a final cell concentration of 4 x $10^6$/mL. Results are the mean ± 95% CI of n = 5; *p<0.05 **p<0.005.***

### 3.4.1 Effect of inhibitory TF antibody on the APTT-like activity of the cell lines

The contribution to this APTT-like PCA of TF was investigated by using an inhibitory antibody to block its action. The anti-TF antibody (100 µg/mL) inhibited the APTT-like activity of Jurkat and THP-1 cells by 41.2 ± 5.22 and 86.9 ± 2.55 %, respectively, but had no effect on the other cell lines (Figure 3.5). This suggests that cell associated TF influences this APTT system as the pattern of inhibition reflects the pattern of TF expression/activity of the cells. This was substantiated by the observation that the addition of recombinant TF (>0.46 nM) to a 1:400 dilution of phospholipid was able to shorten the APTT.

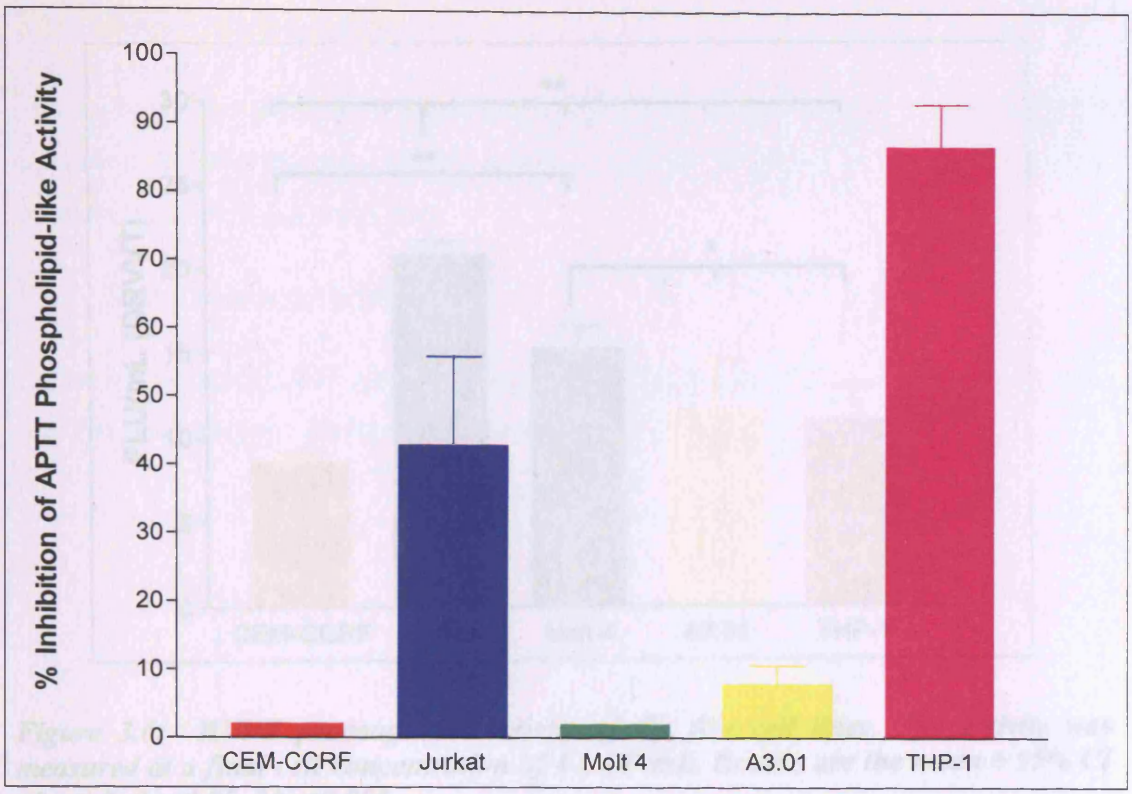



***Figure 3.5: Inhibition of APTT-like procoagulant activity using an inhibitory antibody to TF (100 µg/mL). Mean ± 95%; n = 3.***

## 3.5 Phospholipid-RVVT-like activity of the cell lines

The ability of the cell lines to substitute for procoagulant phospholipid was also examined using a routine clotting test, the russell's viper venom time (RVVT). Both the cells and the phospholipid standard showed a dose response in the RVVT assay. Using Molt-4 as an example to show the dependence on cell number, final cell counts of 4, 2 and 1 x $10^6$/mL gave clotting times of 54.2 ± 1.6, 57.9 ± 0.5 and 64.5 ± 1.0 seconds (n = 5) respectively. The 100 and 0.098 PLU/mL dilutions of the phospholipid standard curve gave clotting times of 38.6 ± 0.6 and 97.5 ± 1.5 seconds ($n$ = 5) respectively, with a reagent blank clotting time of 104.9 ± 1.9 seconds ($n$ = 5).

The cell surface of all the cell lines studied supported the activation of FX by RVV, and there were differences between their activities (Figure 3.6).

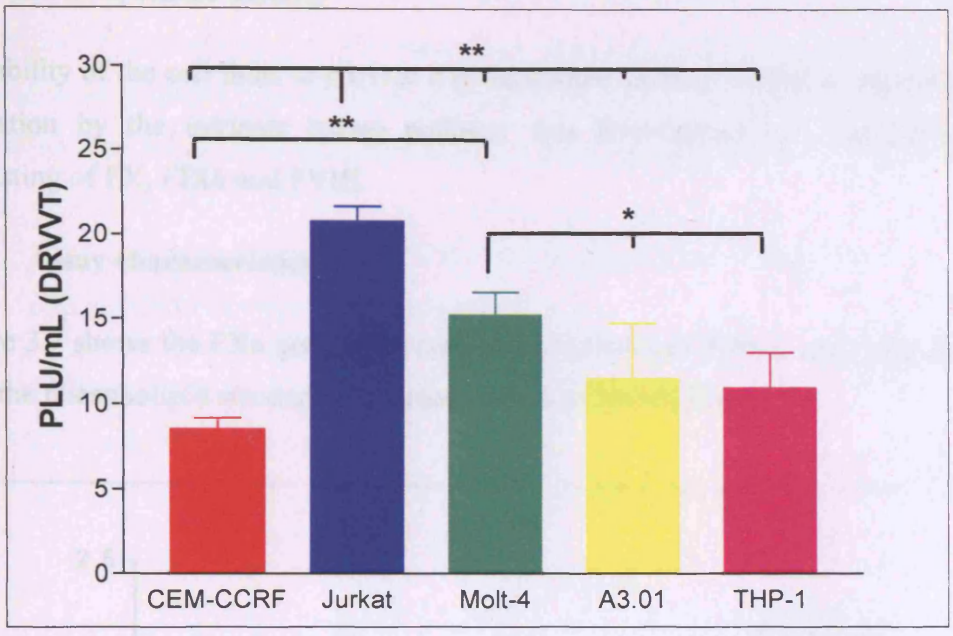



***Figure 3.6: RVVT procoagulant activity of the five cell lines. The activity was measured at a final cell concentration of 4 x $10^6$/mL. Results are the mean ± 95% CI of n = 5; *p<0.05 **p<0.005.***

The T-lymphoblastoid cell lines Jurkat and Molt-4 supported the highest RVVT-like activity (20.8 ± 0.4 and 15.3 ± 0.6 PLU/mL respectively), whilst CEM-CCRF showed the lowest activity (8.5 ± 0.3 PLU/mL). The monocytoid cell line (THP-1) and the T-cell A3.01 supported an intermediate level of activity (10.9 ± 0.9 and 11.5 ± 1.5 PLU/mL respectively).

### 3.5.1 Effect of inhibitory TF antibody on the RVVT-like activity of the cell lines

The contribution to this PCA of TF was investigated by using an inhibitory antibody to block its action. The anti-TF antibody (100 µg/mL) had no effect on the RVVT-like activity of the cell lines, or the clotting times of the phospholipid standard curve. The insensitivity of this assay to the presence of cell-surface associated TF is probably due to the high concentration of RVV (200 ng/mL) activating all the FX in the substrate plasma, thereby leaving little residual FX to be activated by the TF/FVII pathway.

### 3.6 Intrinsic tenase activity

The ability of the cell lines to provide a phospholipid surface capable of supporting FX activation by the intrinsic tenase pathway was investigated in a purified system consisting of FX, FIXa and FVIII.

#### 3.6.1 Assay characteristics

Figure 3.7 shows the FXa generation curves for Molt-4 and THP-1, and contrasts them with the phospholipid standard at a concentration of 100 PLU/mL.

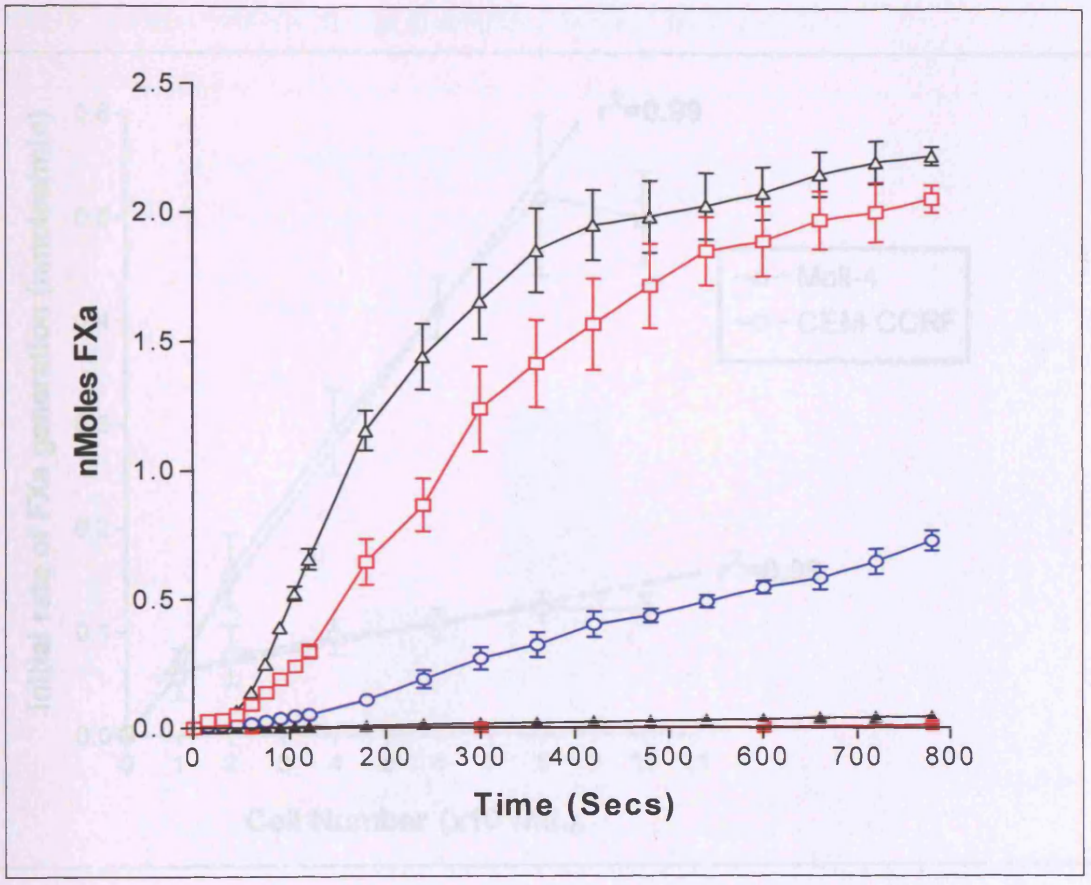



***Figure 3.7: Representative FXa generation curves with (open symbols) or without FIXa and FVIII (closed symbols) for Molt-4 (Squares), THP-1 (Circles) cells (final cell concentration is 4 x $10^6$/mL). Cell activity was compared with the phospholipid standard at a concentration of 100 PLU/mL (open triangle) and a reagent blank (closed triangle).***

The rate of FXa generation was calculated by comparison with a human FXa preparation (Haematologic Technologies Inc, Essex Junction, VT, USA). The assay was unable to generate FXa in the absence of FIXa, FVIII or phospholipid, giving FXa generation curves that were no different from that of the sample blanks (Figure 3.7).

### 3.6.2 Dose-response curve for intrinsic tenase generation, using cells and purified bovine phospholipid

Intrinsic FXa generated was dependent on the cell count, as shown in Figure 3.8, using CEM-CCRF and Molt-4 as examples.

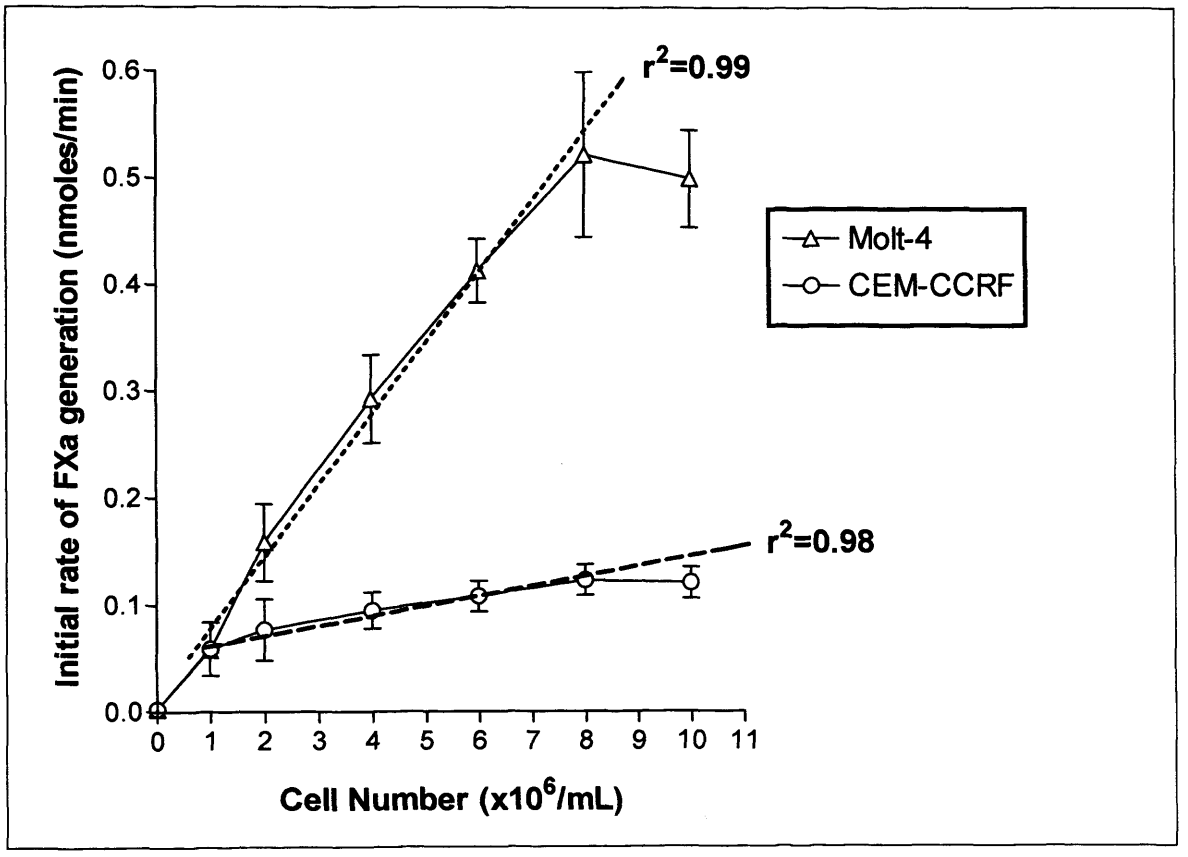



*Figure 3.8: FXa generation dose-response curves for CEM-CCRF and Molt-4 cell lines. The data shown is the initial rate of FXa generation at each cell concentration over the range of 1 – 8 x $10^6$/mL. Results are the mean ± 95% CI of n = 5.*

The rate of FXa generation showed a linear increase with increasing cell number and reached a maximum at a final cell concentration of 8 x $10^6$/mL. Similar results were obtained with the other cell lines. At a final cell concentration of 4 x $10^6$/mL, the slope of the cell lines were 0.0017, 0.0029, 0.005, 0.0058 and 0.0010 nmoles/min for CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1 cells, respectively.

### 3.6.3 Intrinsic Tenase activity of the cell lines

All the cell lines studied promoted the intrinsic pathway generation of FXa, but there were substantial differences between their activities (Figure 3.9). Activity showed a trend to increase with increasing maturation status of the T-cell lines.

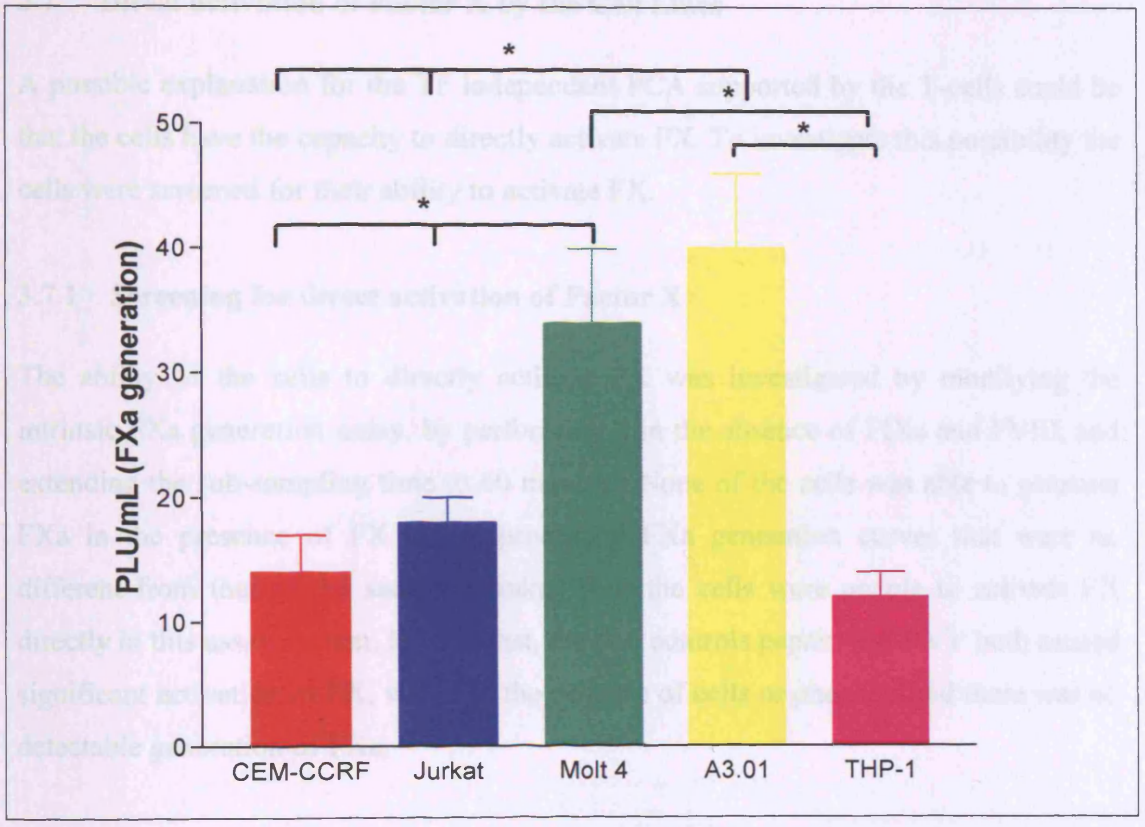



***Figure 3.9: Procoagulant activities of five cell lines in the intrinsic FXa generation assay system. All activities are measured at a final concentration of 4 x $10^6$/mL. Results are the mean ± 95% CI of n = 5; *p<0.05.***

A3.01 had the highest activity (40 ± 5.4 PLU/mL), and the least mature, CEM-CCRF showed the least (15 ± 3.6 PLU/mL). Jurkat and Molt-4 supported intermediate activities equivalent to 20 ± 3.9 and 34 ± 5.7 PLU/mL respectively. The monocytoid cell line THP-1 showed the lowest activity (12 ± 2.0 PLU/mL).

### 3.6.4 Effect of inhibitory TF on the "intrinsic tenase" activity of the cell lines

The inhibitory TF antibody (100 μg/mL) gave no inhibition with any of the cell lines when included in the FXa generation assay, indicating that this system is independent of TF.

## 3.7 Direct activation of Factor X by the Cell Lines

A possible explanation for the TF independent PCA supported by the T-cells could be that the cells have the capacity to directly activate FX. To investigate this possibility the cells were screened for their ability to activate FX.

### 3.7.1 Screening for direct activation of Factor X

The ability of the cells to directly activate FX was investigated by modifying the intrinsic FXa generation assay, by performing it in the absence of FIXa and FVIII, and extending the sub-sampling time to 60 minutes. None of the cells was able to generate FXa in the presence of FX alone, producing FXa generation curves that were no different from that of the sample blanks. Thus the cells were unable to activate FX directly in this assay system. By contrast, the two controls papain and RVV both caused significant activation of FX, whilst in the absence of cells or phospholipid there was no detectable generation of FXa.

### 3.7.2 Cancer Procoagulant

Activation of FX by Cancer Procoagulant (CP) has been documented for many years but efforts to use chromogenic substrates to measure this activity has been frustrated by poor sensitivity. It has been shown that this is because CP activates FX at a different

site than other serine proteinase activators (Gordon & Mourad, 1991). Furthermore, it has been demonstrated that the FXa generated by the proteolytic action of CP has a greater affinity for natural substrates (eg: prothrombin) than synthetic substrates because natural substrates have several amino acids within the cleaved region of the protein that provide specificity (Mielicki *et al*, 1997). This has led to the development of a more specific three-stage assay for CP using a thrombin specific chromogenic substrate (Mielicki & Gordon, 1993).

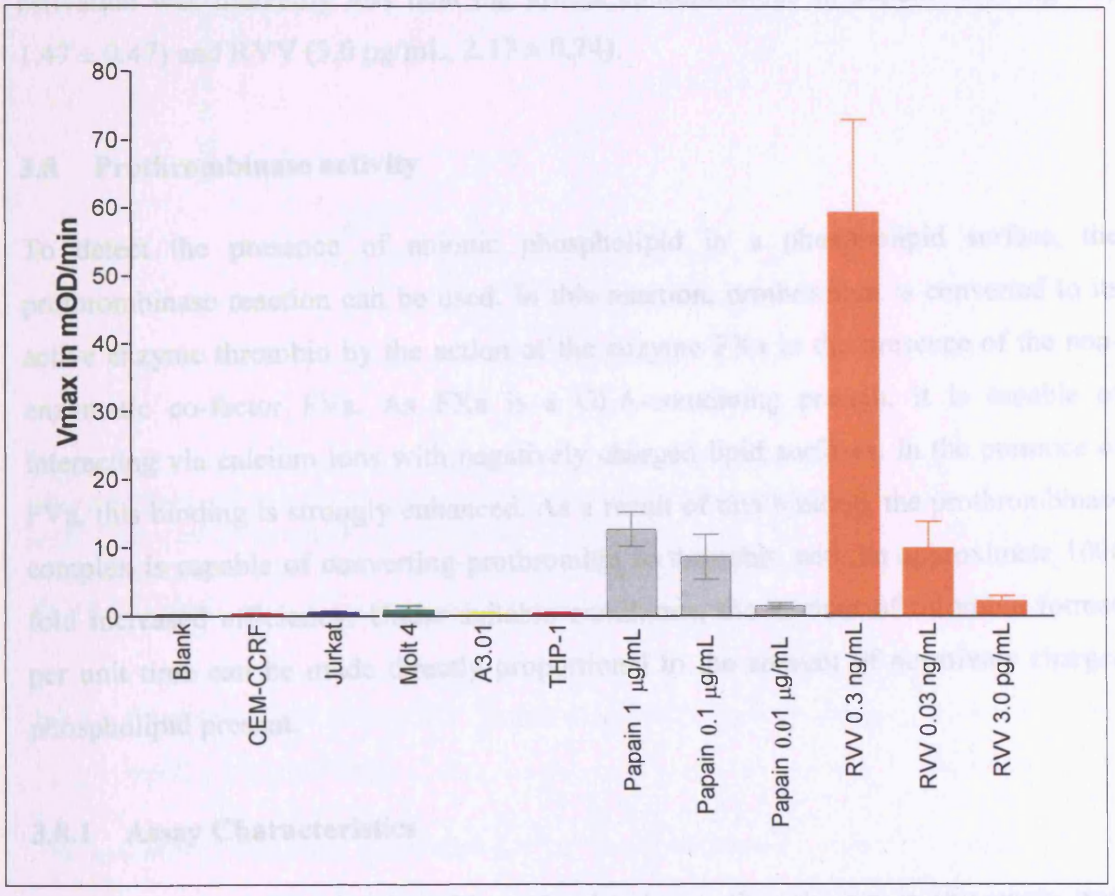



***Figure 3.10: Direct activation of FX in the three-stage cancer procoagulant assay using the chromogenic substrate S-2238). Papain and RVV were used as positive controls. Results are the mean of n =3 and 95% CI.***

The CP activity of the cell lines was determined by a three-stage chromogenic assay (Mielicki & Gordon, 1993) using the thrombin substrate S2238. Papain and RVV were used a positive controls and a reagent blank containing all the assay reagents but without cells was used as a negative control. The results were expressed as mOD/min, and both papain and RVV directly activated FX in a dose-dependent manner (Figure 3.10). CEM-CCRF, A3.01 and THP-1 cells were unable to activate FX directly. However the mOD/min for Jurkat cells (0.566 ± 0.15) and Molt-4 cells (0.902 ± 0.6) were significantly different ($p$ = 0.007 and 0.04 respectively) from the reagent blank (0.183 ± 0.018), indicating that there was slight activation of FX. This level of FX activation was markedly less than the lowest concentrations of papain (0.01 μg/mL, 1.47 ± 0.47) and RVV (3.0 pg/mL, 2.17 ± 0.74).

## 3.8 Prothrombinase activity

To detect the presence of anionic phospholipid in a phospholipid surface, the prothrombinase reaction can be used. In this reaction, prothrombin is converted to its active enzyme thrombin by the action of the enzyme FXa in the presence of the non-enzymatic co-factor FVa. As FXa is a GLA-containing protein, it is capable of interacting via calcium ions with negatively charged lipid surfaces. In the presence of FVa, this binding is strongly enhanced. As a result of this binding, the prothrombinase complex is capable of converting prothrombin to thrombin with an approximate 1000 fold increased efficiency. Under suitable conditions, the amount of thrombin formed per unit time can be made directly proportional to the amount of negatively charged phospholipid present.

### 3.8.1 Assay Characteristics

Initial experiments on prothrombin activation by prothrombinase in this assay were performed to demonstrate the sensitivity of this system to phospholipid and other components of the "prothrombinase" (Figure 3.11).

It was found that if any component of the "prothrombinase" complex was omitted no signal was observed after 10 minutes. The assay system was sensitive to the amount of phospholipid available to FXa and FVa. Phospholipid at 100 and 10 PLU/mL gave thrombin generation rates of 0.571 ± 0.004 and 0.262 ± 0.001 IU/mL/min respectively.

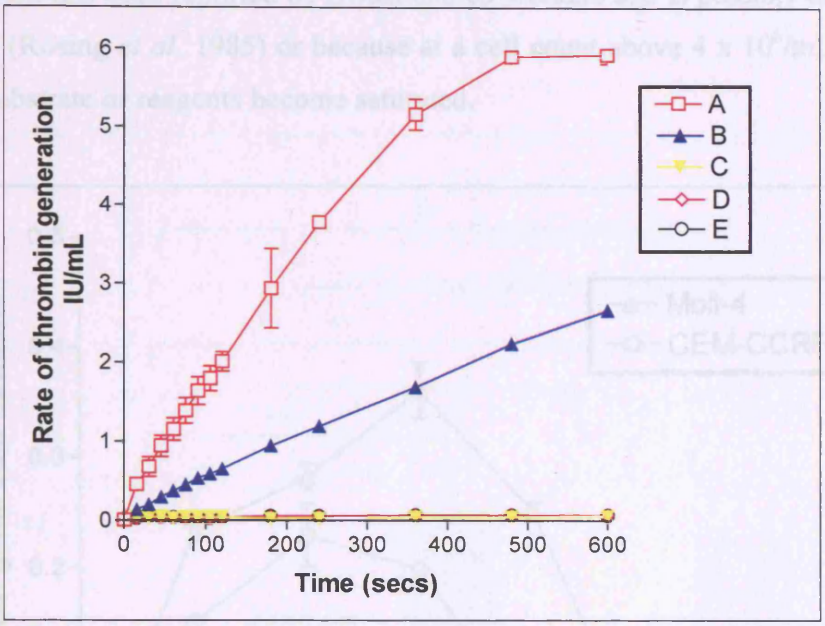



***Figure 3.11: Representative prothrombin (4 μM) activation curves with or without phospholipid (PL), FXa (3 nM) or FVa (6 nM). Curve (A) shows PL (100 PLU/mL) with FXa and FVa. (B) PL (10 PLU/mL) with FXa and FVa. (C) PL (100 PLU/mL) without FXa and with FVa. (D) PL (100 PLU/mL) with FXa and without FVa. (E) PL (0 PLU/mL) with FXa and without FVa. Results are the mean ± 95% CI of n = 3.***

### 3.8.2 Dose-response curves for "prothrombinase activity, using cells and purified bovine phospholipid

The amount of thrombin generated by the "prothrombinase" complex was affected by the cell count, as shown in Figure 3.12, using CEM-CCRF and Molt-4 as examples. "Prothrombinase" activity showed a linear increase until peak activity was reached at a final cell concentration of between 4 and 6 x $10^6$/mL. Similar results were obtained with other cell lines. After peak activity was reached, thrombin activation by "prothrombinase" rapidly diminished becoming almost undetectable at a cell

concentration of 10 x $10^6$/mL. The activity supported by the phospholipid standard increased in a linear fashion until a concentration of 100 PLU/mL. However, phospholipid at concentrations above 200 PLU/mL became inhibitory behaving in a similar fashion to the cell lines. A similar inhibitory effect of high anionic phospholipid concentrations has been reported by Zwaal and co-workers and is possibly due to steric hinderance (Rosing *et al*, 1985) or because at a cell count above 4 x $10^6$/mL either the available substrate or reagents become saturated.

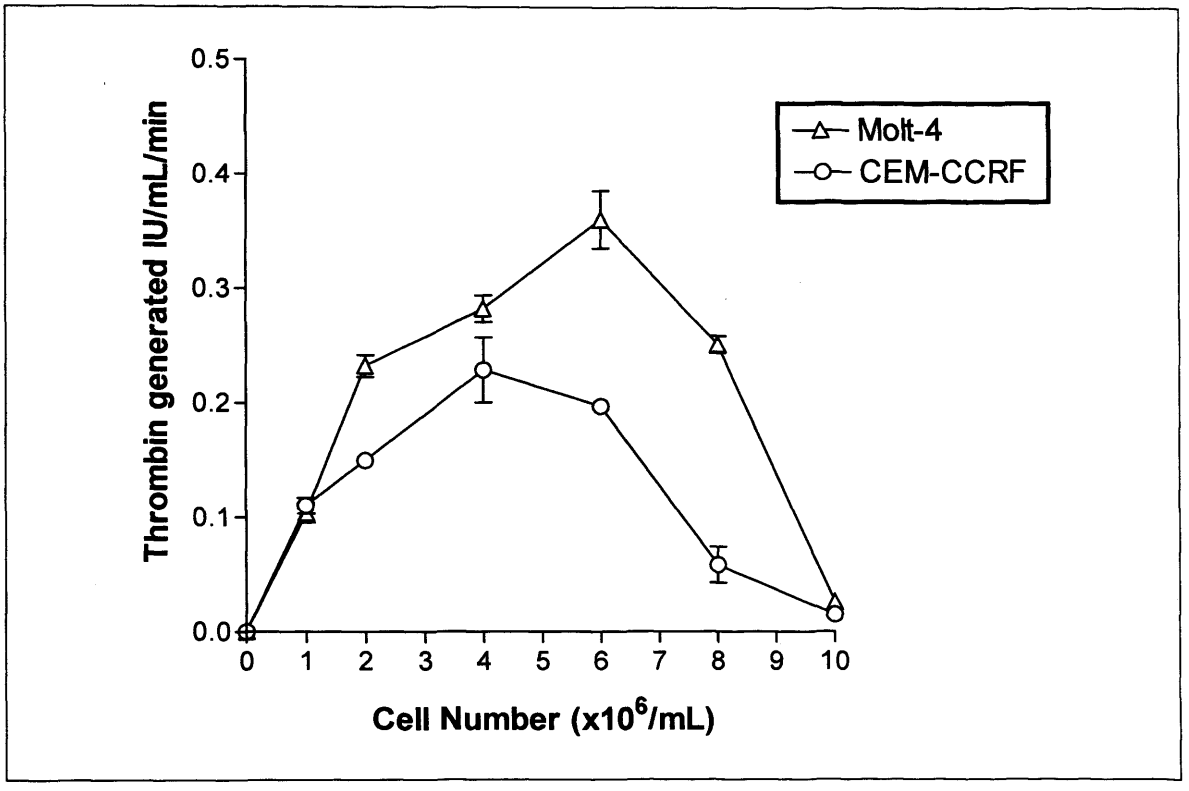



***Figure 3.12: Prothrombinase activity dose-response curves for CEM-CCRF and Molt-4 cell lines for cell concentration over the range of 1 - 10 x $10^6$/mL. Results are the mean ± 95% CI of n = 5.***

### 3.8.3 Prothrombinase activity of the cell lines

When tested at a cell count of 4 x $10^6$/mL all the cell lines studied supported the formation of "prothrombinase" and the subsequent generation of thrombin. Although there were differences between their activities (Figure 3.13), they were not as large as

those for the FXa generation assay. Molt-4 had the highest activity (21.4 ± 1.7 PLU/mL), and, CEM-CCRF showed the least (9.0 ± 1.0 PLU/mL). Jurkat and A3.01 supported intermediate activities equivalent to 11.6 ± 1.7 and 18.6 ± 0.7 PLU/mL respectively. The monocytoid cell line THP-1 also showed an intermediate activity (18.7 ± 1.9 PLU/mL).

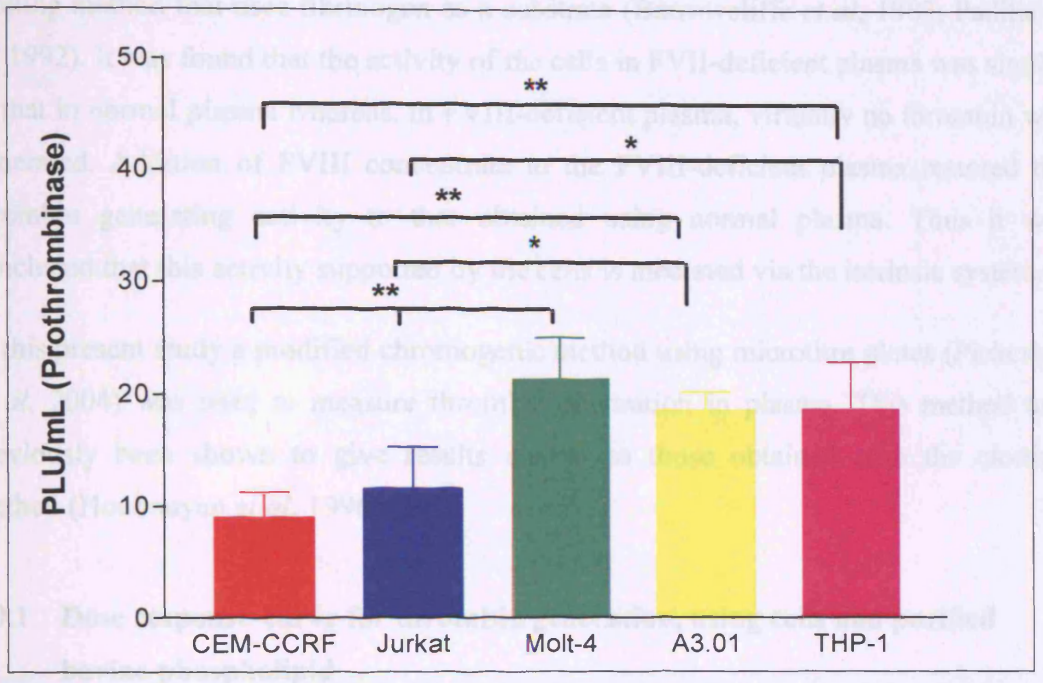



***Figure 3.13: Procoagulant activities of five cell lines using the prothrombinase assay system. All activities are measured at a final concentration of 4 x $10^6$/mL. Results are the mean ± 95% CI of n = 5; *P<0.05 **P<0.05.***

### 3.8.4 Effect of inhibitory TF antibody on the "prothrombinase" activity of the cell lines

When included in the prothrombinase assay the inhibitory TF antibody (100 µg/mL) gave no inhibition with any of the cell lines, indicating that this system is not dependent on TF.

## 3.9 Thrombin generation activity in plasma

The ability of the cell lines to promote the generation of thrombin in defibrinated plasma activated by FIXa was measured. The role of extrinsic and intrinsic systems in the thrombin generating activity of the Jurkat and Molt-4 cell lines had been investigated previously (Barrowcliffe *et al*, 1989; Barrowcliffe *et al*, 2002) using a clotting method that uses fibrinogen as a substrate (Barrowcliffe *et al*, 1983; Padilla *et al*, 1992). It was found that the activity of the cells in FVII-deficient plasma was similar to that in normal plasma whereas, in FVIII-deficient plasma, virtually no thrombin was generated. Addition of FVIII concentrate to the FVIII-deficient plasma restored the thrombin generating activity to that obtained using normal plasma. Thus it was concluded that this activity supported by the cells is mediated via the intrinsic system.

In this present study a modified chromogenic method using microtitre plates (Pickering *et al*, 2004) was used to measure thrombin generation in plasma. This method had previously been shown to give results similar to those obtained with the clotting method (Houbouyan *et al*, 1996).

### 3.9.1 Dose response-curve for thrombin generation, using cells and purified bovine phospholipid

Thrombin generation was measured by peak thrombin levels, time to achieve peak thrombin activity and total thrombin generation, estimated by calculating the area under the curve (AUC) utilising the trapezoidal method. The AUC is analogous to the endogenous thrombin potential (ETP) (Hemker & Beguin, 1995). Peak thrombin and ETP were found to increase with increasing cell number or phospholipid standard concentration. Results for the cell lines are tabulated in Table 3.1 and the thrombin generation curves, including those obtained with the phospholipid standard are shown in Figure 3.14. The maximum thrombin generating activity of the cell lines appears to be reached at a cell density of 8 x $10^6$/mL or above. Thus the increase in thrombin generation was dependent upon the cell density.

| | | $1x10^6$/mL | $2x10^6$/mL | $4x10^6$/mL | $6x10^6$/mL | $8x10^6$/mL | $10x10^6$/mL |
|---|---|---|---|---|---|---|---|
| **CEM-CCRF** | A | 2.2 ± 0.02 | 2.5 ± 0.1 | 3.6 ± 0.16 | 4.9 ± 0.17 | 5.6 ± 0.36 | 5.6 ± 0.35 |
| | B | 560 ± 39 | 600 ± 118 | 480 | 360 | 340 ± 39 | 260 ± 39 |
| | C | 1728 ± 75 | 1923 ± 54 | 1916 ± 19 | 2704 ± 131 | 2931 ± 128 | 2863 ± 90 |
| **Jurkat** | A | 4.1 ± 0.09 | 5.0 ± 0.14 | 5.9 ± 0.03 | 6.9 ± 0.10 | 7.0 ± 0.33 | 7.0 ± 0.32 |
| | B | 320 ± 39 | 360 | 300 | 200 ± 112 | 200 ± 39 | 240 |
| | C | 2226 ± 73 | 2458 ± 12 | 2565 ± 117 | 2646 ± 75 | 2864 ± 151 | 2746 ± 59 |
| **Molt-4** | A | 4.1 ± 0.12 | 5.8 ± 0.12 | 7.2 ± 0.16 | 7.6 ± 0.16 | 7.8 ± 0.07 | 8.3 ± 0.10 |
| | B | 440 ± 39 | 380 ± 39 | 240 | 180 | 180 | 180 |
| | C | 2667 ± 20 | 3146 ± 99 | 3279 ± 57 | 3246 ± 69 | 3639 ± 97 | 3627 ± 98 |
| **A3.01** | A | 4.5 ± 0.48 | 4.6 ± 0.10 | 5.3 ± 0.16 | 6.7 ± 0.31 | 6.3 ± 0.19 | 6.0 ± 0.34 |
| | B | 500 ± 39 | 480 | 360 | 240 | 220 ± 39 | 180 |
| | C | 2049 ± 78 | 2264 ± 29 | 2326 ± 59 | 2812 ± 64 | 2516 ± 59 | 2390 ± 84 |
| **THP-1** | A | 3.5 ± 0.09 | 3.4 ± 0.03 | 5.4 ± 0.10 | 6.9 ± 0.14 | 7.5 ± 0.21 | 8.1.1 ± 0.08 |
| | B | 400 ± 39 | 340 ± 39 | 320 ± 39 | 300 | 180 | 180 |
| | C | 1898 ± 73 | 1898 ± 52 | 2701 ± 43 | 3110 ± 91 | 3232 ± 204 | 3468 ± 169 |

***Table 3.1: Effect of cell number on thrombin generation. The indices measured are (A) peak thrombin activity (IU/mL), (B) time taken to reach peak activity (seconds), and (C) Endogenous thrombin potential (IU.seconds/mL). Results are the mean ± 95% CI of n = 5.***

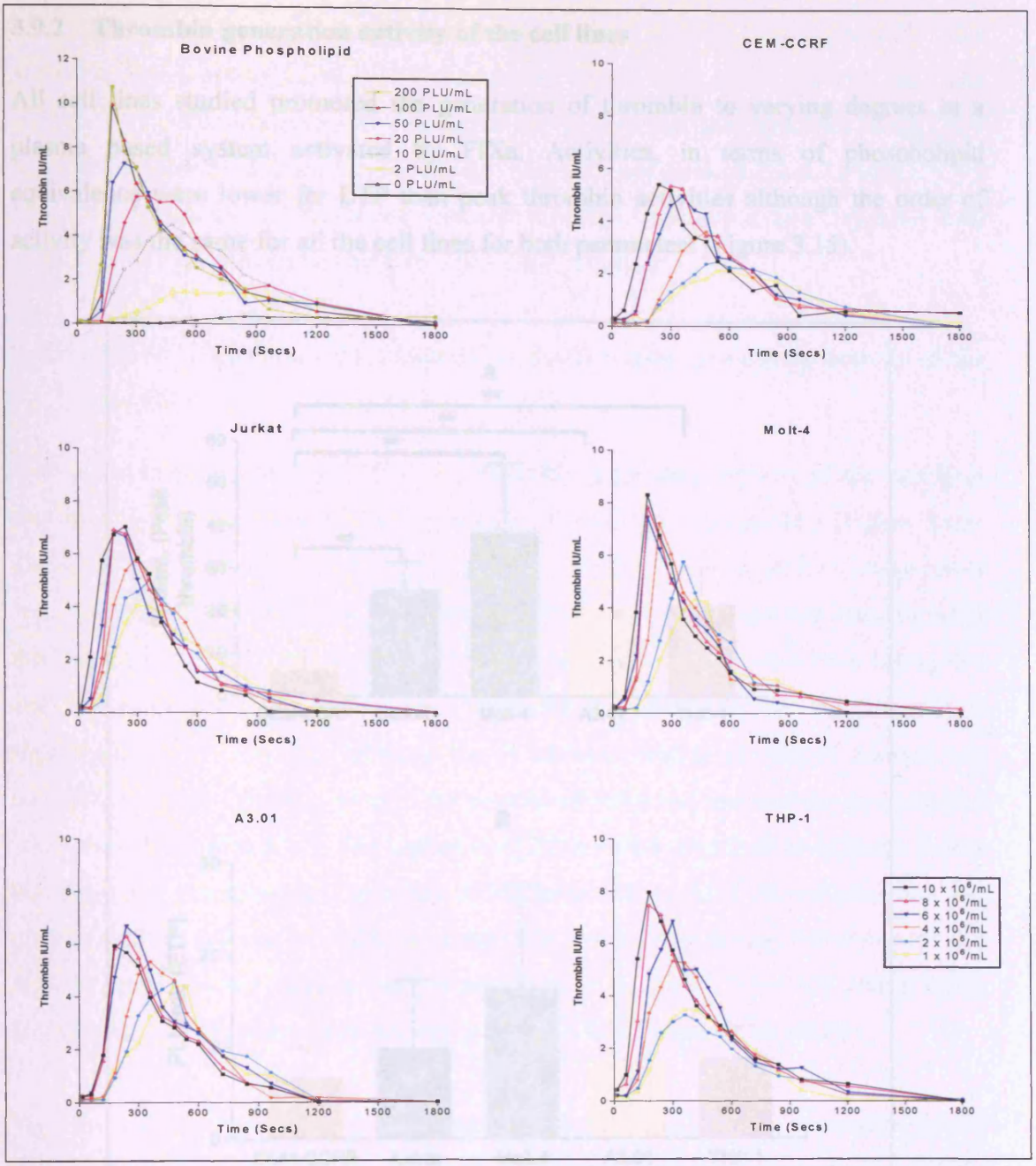



***Figure 3.14: Thrombin generation curves corrected for the effect of $\alpha_2$-macroglobulin showing the dose response effect of increasing concentrations of cells and bovine phospholipid standard. The concentrations are indicated in the legend. Results are the mean of n = 5.***

### 3.9.2 Thrombin generation activity of the cell lines

All cell lines studied promoted the generation of thrombin to varying degrees in a plasma based system activated by FIXa. Activities, in terms of phospholipid equivalence were lower for ETP than peak thrombin activities although the order of activity was the same for all the cell lines for both parameters (Figure 3.15).

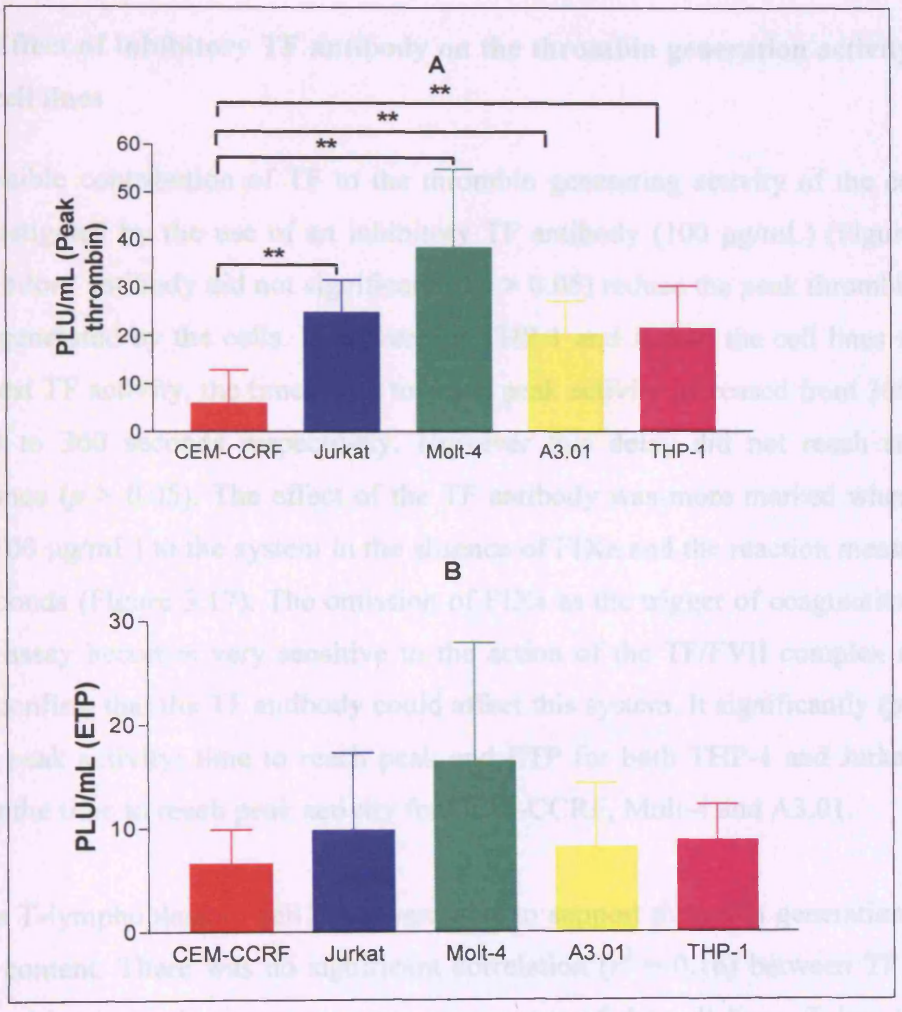



***Figure 3.15: Thrombin generation test activities of the cell lines. Activity expressed as PLU/mL was calculated by comparison with a phospholipid standard using (A) peak thrombin generated and (B) Endogenous thrombin potential (ETP). All activities are measured at a final cell concentration of 4 x $10^6$/mL. Results are the mean ± 95% CI of n = 5; **p<0.005.***

Molt-4 supported the highest peak thrombin level (38.5 ± 7.5 PLU/mL) and ETP (16.5 ± 8.8 PLU/mL), whilst CEM-CCRF showed the lowest level (5.9 ± 3.2 PLU/mL) and ETP (6.6 ± 2.5 PLU/mL). A3.01 and Jurkat showed similar peak thrombin levels (20.4 ± 3.2 and 24.9 ± 3.1 PLU/mL respectively) and ETP activities (8.2 ± 4.7 and 9.8 ± 5.7 PLU/mL respectively). The monocytoid cell line (THP-1) gave a peak thrombin level of 21.6 ± 4.0 PLU/mL and an ETP of 8.8 ± 2.6 PLU/mL.

### 3.9.3 Effect of inhibitory TF antibody on the thrombin generation activity of the cell lines

The possible contribution of TF to the thrombin generating activity of the cell lines was investigated by the use of an inhibitory TF antibody (100 μg/mL) (Figure 3.16). The inhibitory antibody did not significantly ($p > 0.05$) reduce the peak thrombin levels or ETP generated by the cells. However, for THP-1 and Jurkat, the cell lines showing the highest TF activity, the time taken to reach peak activity increased from 360 to 480 and 300 to 360 seconds respectively. However this delay did not reach statistical significance ($p > 0.05$). The effect of the TF antibody was more marked when it was added (100 μg/mL) to the system in the absence of FIXa and the reaction measured for 3600 seconds (Figure 3.17). The omission of FIXa as the trigger of coagulation means that the assay becomes very sensitive to the action of the TF/FVII complex and was used to confirm that the TF antibody could affect this system. It significantly ($p < 0.05$) reduced peak activity; time to reach peak and ETP for both THP-1 and Jurkat whilst delaying the time to reach peak activity for CEM-CCRF, Molt-4 and A3.01.

Thus the T-lymphoblastoid cell lines were able to support thrombin generation despite low TF content. There was no significant correlation ($r^2 = 0.16$) between TF activity and thrombin generation measurements among any of the cell lines. Taken together, this data suggests that the T-cell PCA measured by this assay is not significantly affected by the presence of TF.

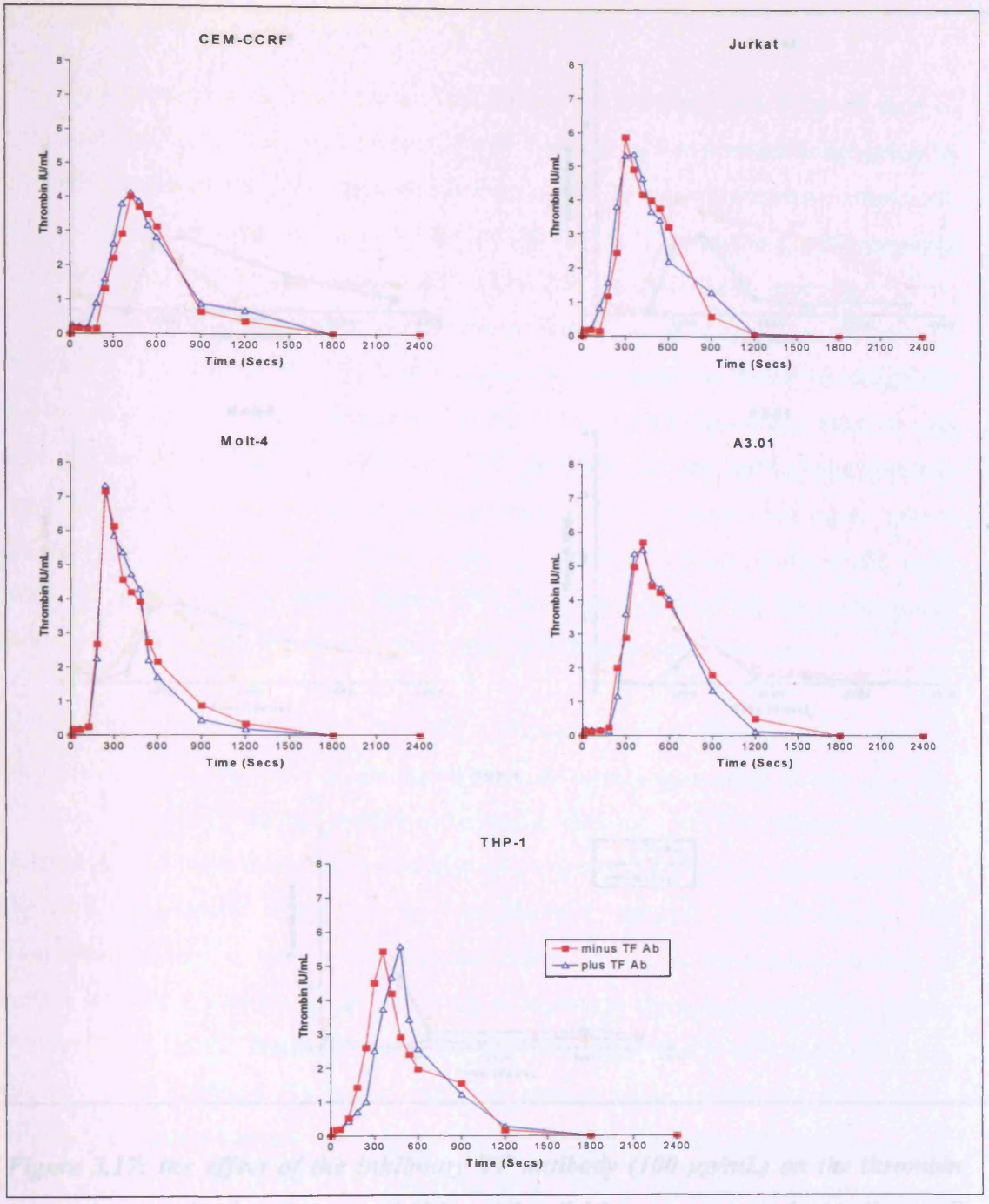



*Figure 3.16: The effect of the inhibitory TF antibody (100 µg/mL) on the thrombin generation test activated by FIXa (0.14 IU/mL). All activities are measured at a final cell concentration of 4 x $10^6$/mL. Results are the mean of n = 3.*

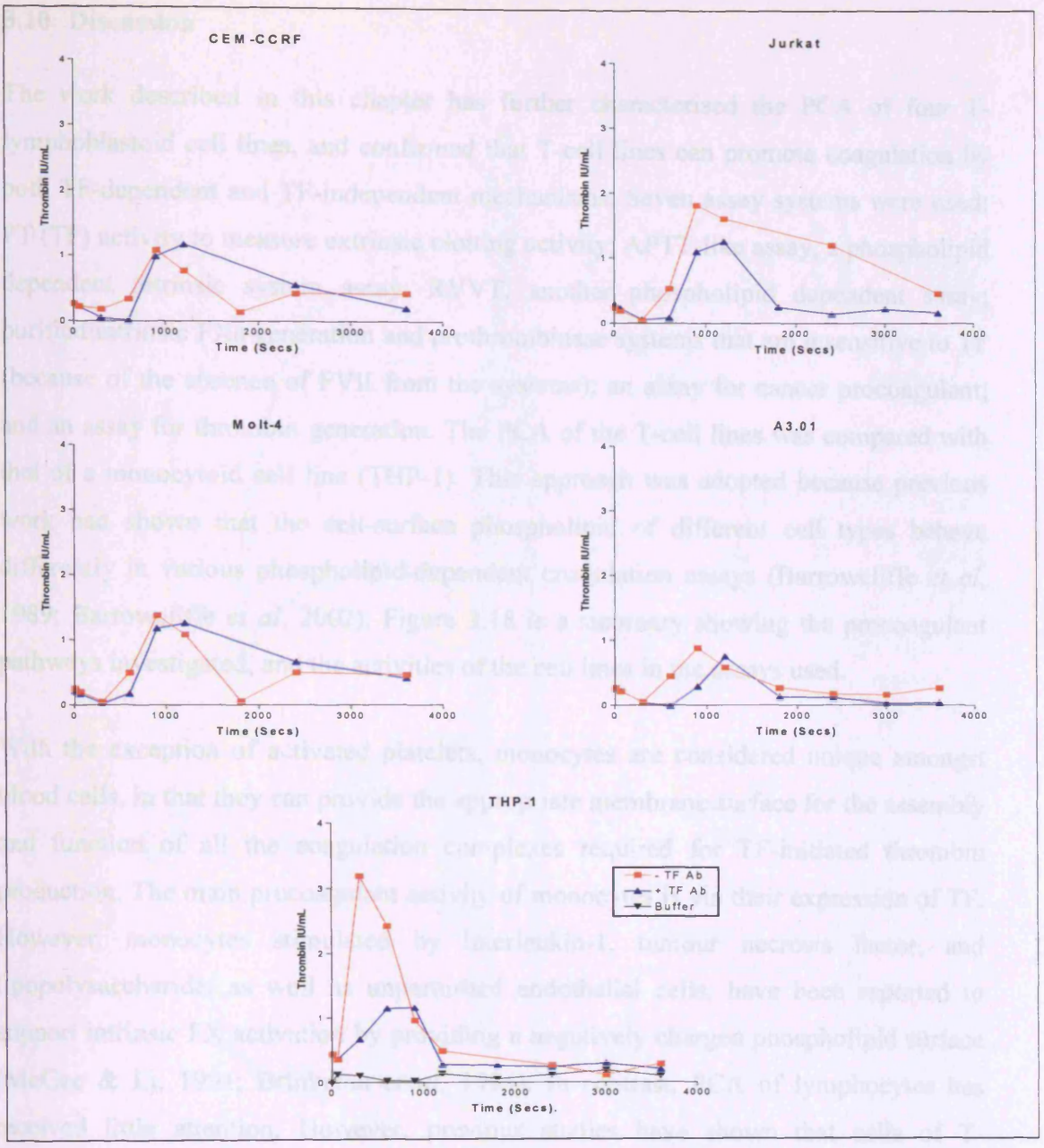


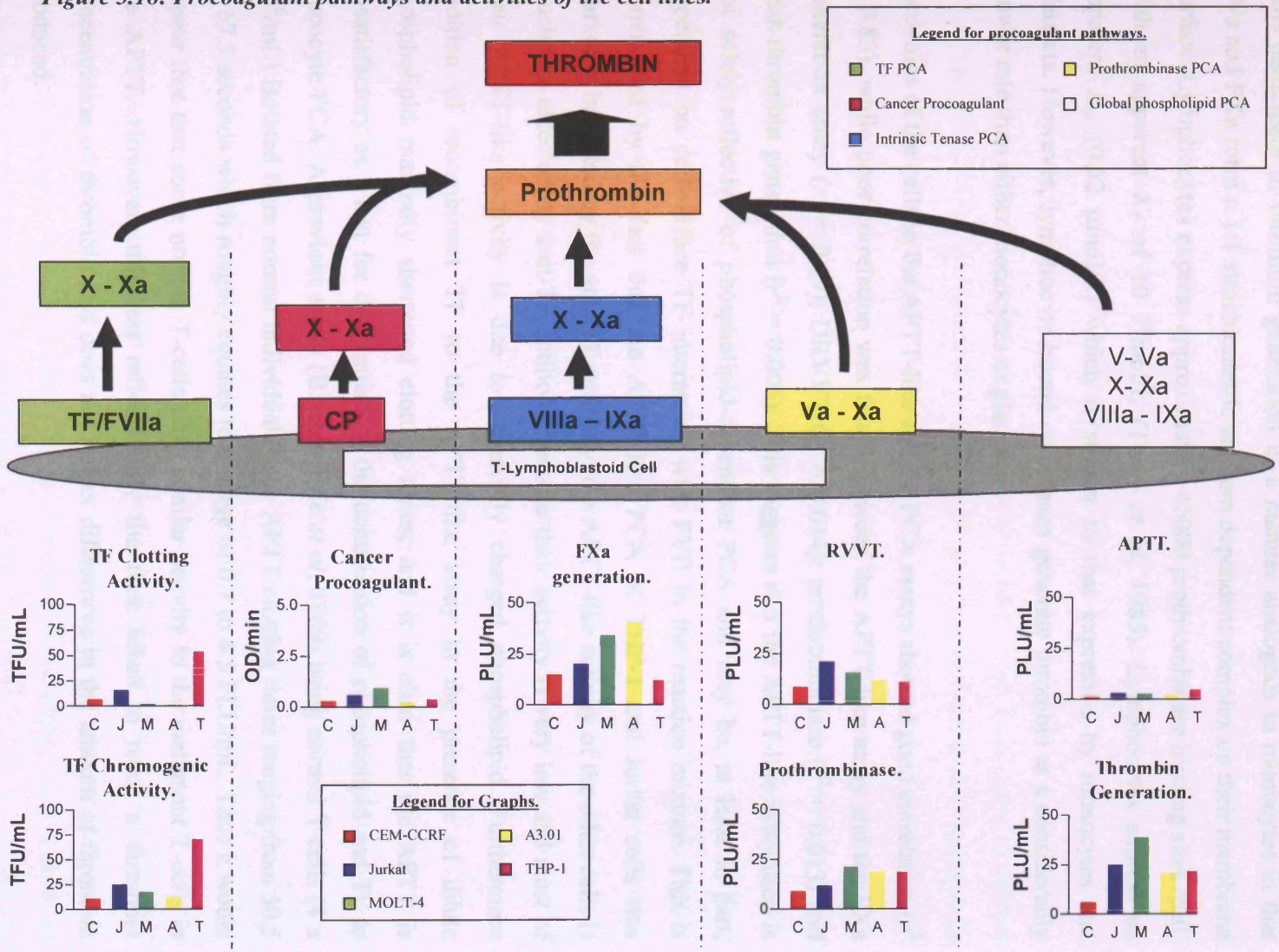

***Figure 3.17: the effect of the inhibitory TF antibody (100 µg/mL) on the thrombin generation test in the absence of FIXa. All activities are measured at a final cell concentration of 4 x $10^6$/mL. Results are the mean of n = 3.***

## 3.10 Discussion

The work described in this chapter has further characterised the PCA of four T-lymphoblastoid cell lines, and confirmed that T-cell lines can promote coagulation by both TF-dependent and TF-independent mechanisms. Seven assay systems were used: PT (TF) activity to measure extrinsic clotting activity; APTT-like assay, a phospholipid dependent intrinsic system assay; RVVT, another phospholipid dependent assay; purified intrinsic FXa generation and prothrombinase systems that are insensitive to TF (because of the absence of FVII from the systems); an assay for cancer procoagulant; and an assay for thrombin generation. The PCA of the T-cell lines was compared with that of a monocytoid cell line (THP-1). This approach was adopted because previous work had shown that the cell-surface phospholipid of different cell types behave differently in various phospholipid-dependent coagulation assays (Barrowcliffe *et al*, 1989; Barrowcliffe *et al*, 2002). Figure 3.18 is a summary showing the procoagulant pathways investigated, and the activities of the cell lines in the assays used.

With the exception of activated platelets, monocytes are considered unique amongst blood cells, in that they can provide the appropriate membrane surface for the assembly and function of all the coagulation complexes required for TF-initiated thrombin production. The main procoagulant activity of monocytes is via their expression of TF. However, monocytes stimulated by interleukin-1, tumour necrosis factor, and lipopolysaccharide, as well as unperturbed endothelial cells, have been reported to support intrinsic FX activation by providing a negatively charged phospholipid surface (McGee & Li, 1991; Brinkman *et al*, 1994). In contrast, PCA of lymphocytes has received little attention. However, previous studies have shown that cells of T-lymphocyte lineage can promote the generation of large amounts of thrombin, despite having little or no TF activity (Barrowcliffe *et al*, 1989). This group also investigated the ability of normal T-lymphocytes, isolated from peripheral blood, to generate thrombin. Normal T-cells gave a maximum peak of 6.4 IU/mL and were much less active than Jurkat cells (23.5 IU/mL). Similar results were obtained with normal T-cells isolated from three individuals. It has been reported that normal lymphocytes do not support the assembly and function of intrinsic tenase (McGee & Li, 1991). However

*Figure 3.18: Procoagulant pathways and activities of the cell lines.*



prothrombinase can assemble and function on normal lymphocytes (Tracy *et al*, 1983; Tracy *et al*, 1985). The observed kinetics of prothrombin activation indicate that these cells participate in thrombin generation in a manner analogous to monocytes in that FVa and FXa form a 1:1 stoichiometric calcium dependent complex on their membrane surface. Lymphocytes express approximately 45000 prothrombinase binding sites /cell, with an apparent $K_d$ of $10^{-10}$mol/L (Tracy *et al*, 1985). Lymphocytes express an apparent $K_m$ (0.62 μmol/L) which is similar to that expressed by monocytes and platelets. However, lymphocyte bound complexes generate thrombin at a considerably slower rate than either monocytes or platelets.

Activities of the cells in the APTT-like and TF PCA assays showed good correlation ($r^2$ = 0.83), while poor correlation was found between the APTT-like assay and the FXa generation assay ($r^2$ = 0.19); DRVVT ($r^2$ = 0.014); prothrombinase ($r^2$ = 0.013) and peak thrombin generation ($r^2$ = 0.006). This suggests that the APTT-like assay used is not solely reflective of phospholipid-dependent PCA and may be, at least in part, dependent on cell-surface TF interacting with FVII in the reaction mixture. This is emphasised by the fact that the APTT-like PCA of THP-1 and Jurkat cells was markedly inhibited by the anti-TF antibody; the APTT-like activity of the other cells is much less affected by anti-TF antibody because their activity is very low and most of their APTT-like activity is due to negatively charged phospholipid. Furthermore addition of recombinant TF to the APTT-like assay in the presence of dilute phospholipid markedly shortened clotting times, and it is clear that the APTT is unsatisfactory as a test for distinguishing the contribution of phospholipid and TF to leucocyte PCA. A previous study (Barrowcliffe *et al*, 1989) using normal T-cells (4 x $10^6$/mL) isolated from normal individuals gave APTT clotting times ranging from 50.5 to 67.5 seconds which roughly equates to a range of 0.7 to 4.5 PLU/mL. Thus it would appear that that some normal T-cells have similar activity to the malignant T-cells in the APTT. However, this test reflects only the time taken to reach a threshold concentration of thrombin and does not reflect differences in the amount of thrombin produced.

The purified FXa generation system appeared to be the most specific and sensitive assay system for studying phospholipid-dependent PCA. The amount of FXa generation supported by the T-cells increased with their degree of differentiation. This could be due to individual variation, or may be associated with membrane changes resulting from rearrangement of T-cell receptor (TCR) genes or the high expression of glycosphingolipids that have been reported to be involved in cancer cell PCA (Suzuki *et al*, 1999), though other explanations are possible.

There was wide variation in the ability of the cell-surface of the T-cell lines to support FXa generation, whilst the monocytoid cell line THP-1 had the least activity, despite its high TF content. The poor correlation between FXa generation and TF activity ($r^2$ = 0.45) and the lack of effect of the inhibitory TF antibody on FXa generation demonstrate that this assay is completely independent of TF. The activity seen in the FXa assay was also not due to direct activation of FX by the cells, as might occur with CP or other substances that activate FX such as cancer cell derived blood clotting activity-1 (CCA-1) (Gordon & Cross, 1981; Gordon & Mielicki, 1997; Inufusa *et al*, 1998). There was no activity when FVIII and FIXa were omitted from the incubation mixture, and when a more specific 3-stage assay for CP was employed (Mielicki & Gordon, 1993) three of the five cell lines showed no activity, with minimal levels for the other two.

It would appear from this data that the observed PCA of T-lymphoblastoid cells especially in the purified FXa generation and prothrombinase assay systems is not due to TF but is associated with the cell surface as evidenced by the dose-response data from the assay systems. The activity is likely to be due to exposed phospholipid, though the reason for the differential expression of anionic phospholipid by the cell lines is not clear. It does not appear to be an artefact of cell culture, since all the cells were cultured under the same conditions but there were substantial differences in their activities. Also PCA activity for each cell line did not increase during subculture (data not shown). In accord with previous studies Jurkat and Molt-4 were more active in phospholipid dependent tests than the monocytoid cell lines (THP-1 and U-937) and the B-cell line

Nalm-6 (Barrowcliffe *et al*, 2002). The possibility that some of the PCA might be due to absorption of clotting factors from the serum in the culture medium was discounted by a previous report for two reasons (Barrowcliffe *et al*, 1989). Firstly, when cells were grown in an artificial serum (CLEX), or on serum absorbed with $Al(OH)_3$ to remove prothrombin complex factors, their PCA was similar to those grown in normal serum. The second being that, in the thrombin generation system, the activity of the cells was similar when glass contact activation was replaced by plastic tubes and FIXa, indicating that this activity is not due to adsorption of contact factors.

In conclusion the studies described in this chapter have shown that T-cell lines can promote coagulation by both TF-dependent and TF-independent mechanisms. This phenomenon may be due to specific properties of cell surfaces, or to the amount and/or biological state of anionic phospholipid on the cell surface, and this is further explored in chapter 4.

### 3.11 Summary

The behaviour of four T-lymphoblastoid cell lines and one monocytoid cell line (THP-1) in TF and phospholipid dependent coagulation reactions was characterised. High levels of TF activity were seen only with THP-1 cells, though Jurkat did show some TF activity. The T-lymphoblastoid cells Molt-4 and A3.01 were more active than THP-1 in supporting FXa generation. In the RVVT and thrombin generation test, Jurkat and Molt-4 were more active in providing a suitable surface for prothrombin activation than THP-1. This pattern was not repeated for the APTT-like assay, which was related to cell surface TF activity since it was partially inhibited by an anti-TF antibody, which had no significant effect on the other assay systems, used. The cell lines were unable to directly activate FX, and CP like activity was not detected. Molt-4 and A3.01 were the most active cell lines in the prothrombinase assay, supporting an activity that was similar to that of THP-1. It was shown that T-lymphoblastoid procoagulant activity is cell concentration dependent with exposed negatively charged phospholipids serving as templates for the assembly of coagulation complexes on the outer membrane of the cell lines.

Chapter

4

# PHOSPHATIDYLSERINE EXPOSURE AND T-LYMPHOBLASTOID PROCOAGULANT ACTIVITY

## 4.1 Introduction

Procoagulant reactions occur at a physiologically significant rate only when the respective enzymes form multi-component complexes on lipid membrane surfaces (Zwaal *et al*, 1998). This chapter explores the role of inhibition of anionic phospholipids particularly the procoagulant activity of PS using annexin A5 and 3G4, an anionic phospholipid inhibitory antibody. The relative levels of anionic phospholipid making up the cell membrane of the cell lines, and their ability to expose anionic phospholipid is also investigated.

The molecular details of the mechanism or mechanisms by which various lipids affect coagulation are not fully understood. Many groups have observed that lipid vesicles containing negatively charged phospholipids, especially PS (Zwaal *et al*, 1998), bind and markedly enhance the rate of activation of procoagulant enzymes (Rosing *et al*, 1988). It has been proposed that the high rate of activation of procoagulant enzymes in the presence of vesicles containing PS is due in large part to enhancement of substrate binding to the negatively charged surface of the phospholipid bilayer. The resulting high concentration of substrate on the membrane surface can enhance diffusion of substrate to the activation complex in two dimensions rather than in three dimensions (Mann *et al*, 1990). Although the significance of this proposed mechanism has been controversial and alternative models have been proposed to explain many kinetic data (Lu & Nelsestuen, 1996a; Lu & Nelsestuen, 1996b), current paradigms most often assume that membranes only provide a surface template for the assembly and function of the various procoagulant complexes and that the procoagulant phospholipids do not play a more active role (such as enhancing the rate of activation of prothrombin) in the blood coagulation reaction.

Recently it has been reported that a short variant of PS, dicaproylphosphatidylserine (C6PS), can enhance the rate of activation of both prothrombin by the prothrombinase complex and factor X by the tenase complex (Koppaka *et al*, 1996; Gilbert & Arena, 1997). This data suggests the existence of a functionally significant binding site or sites

for C6PS on one or more procoagulant plasma proteins and that the occupancy of these sites by the phospholipid molecules activates the clotting factors.

Although both procoagulant and anticoagulant reactions are markedly enhanced by the presence of negatively charged surfaces *in vitro*, certain lipids and lipoproteins selectively enhance anticoagulant reactions in plasma (Barrowcliffe *et al*, 1984; Smirnov & Esmon, 1994; Griffin *et al*, 1999; Fernandez *et al*, 2000). The ability of cells to support intrinsic coagulation requires the presence of negatively charged phospholipids on their outer surface (Zwaal & Schroit, 1997; Bevers *et al*, 1998). Normal circulating blood cells have their negatively charged phospholipids on the interior of the membrane bilayer and hence are only weak procoagulants. Upon activation by thrombin and/or collagen, platelets transfer their negatively charged lipids from the interior to the exterior of the membrane. However it appears that leucocytes do not expose PS in response to thrombin or collagen (Hoffman & Church, 1993).

## 4.2 Surface area and phospholipid composition of the cell lines

Two possible explanations that could explain the differences in phospholipid procoagulant activity of the cell lines reported in chapter 3 are cell size / surface area, and the phospholipid composition – in particular anionic phospholipids – of the cell membranes.

### 4.2.1 Determination of surface area of the cell lines

Cell surface areas were calculated from the measurement of the cells diameter. Cells were stained with May-Grünwald-Giemsa (Sigma, Poole, UK) and the diameter of 100 cells of each cell line was measured by light microscopy, at a magnification of x 100. The surface area of the entire cell was calculated from the formula:

$$\text{Surface area of a sphere} = 4\pi r^2$$

Where $\pi$ is a constant value and is the ratio of the circumference to the diameter of a circle, and $r^2$ is the square of the radius of a circle.

For this method surface area was calculated under the assumption that the cells were perfect spheres. The cell lines were not significantly different ($p > 0.05$) in size from each other, with THP-1 having the largest surface area (446 ± 43$\mu m^2$) and A3.01 the smallest (395 ± 26 $\mu m^2$). Jurkat, Molt-4 and CEM-CCRF had similar surface areas (421 ± 33, 411 ± 35 and 414 ± 37 $\mu m^2$ respectively). Thus the surface area available as a template for procoagulant activity is similar for each cell line, though this does not preclude differences in membrane perturbations and ruffles, or the effect of shed microparticles after sample preparation.

### 4.2.2 Phospholipid composition of the cell lines

Lipid extraction was performed as previously described in section 2.9.1. The extracts were evaporated to dryness and stored at –80°C in glass vials prior to analysis by HPTLC. The HPTLC measurements were undertaken by Dr Ian Cartwright (Department of Molecular Biology and Biotechnology, University of Sheffield).

#### *4.2.2.1 Phospholipid analysis of cell lines*

Lipid extracts were re-dissolved in chloroform/methanol (1:1, v/v) and 10 μL aliquots applied to nanosil 20 x 10 cm HPTLC plates using a Camlab Desaga AS30 Autospotter. Phospholipid standards (0.5 – 20 μg) were also applied to the first five lanes on each plate. The following lipid standards (Sigma, Poole, UK) were used: L-α-phosphatidyl choline-dioleoyl; L-α-phosphatidyl ethanolamine-dioleoyl; L-α-phosphatidyl inositol (ammonium salt); DL-α-phosphatidyl-L-serine dipalmitoyl (sodium salt); and sphingomyelin.

Phospholipids were separated using the developing solvent system: ethyl acetate / propan-1-ol / ethanol / chloroform / methanol / 0.25% KCL (35/5/20/22/15/9 by volume). The plates were air-dried and dipped in cupric acetate (3%, w/v) dissolved in phosphoric acid (8%, w/v) for 30 seconds, air dried again and heated to 140°C on a hot plate for 30 minutes. After cooling, the individual lipid components were determined using a Camlab Desaga CD60 densitometer in reflectance mode at 340 nm. The following phospholipid components were quantified: phosphatidylcholine (PC); phosphatidylethanolamine (PE); phosphatidylinositol (PI); phosphatidylserine (PS) and sphingomyelin (SM).

#### *4.2.2.2 Lipid composition of the cell lines*

The total phospholipid content of the cell line and platelet Folch extracts was estimated using an fluorometric method (Jouanel *et al*, 1980). The cells were not significantly different in total phospholipid content from each other ($p > 0.05$), with CEM-CCRF having the most phospholipid (1081 ± 175 $\mu g/10^6$ cells) whilst Jurkat and THP-1 had the least (963 ±73 and 940 ± 40 $\mu g/10^6$ cells respectively). Molt-4 and A3.01 had a total phospholipid content of 1019 ± 190 and 1039 ± 95 $\mu g/10^6$ cells, respectively. Fresh platelet phospholipid composition was also measured as a control. Platelets were found to have a total phospholipid content of 74 ± 4 $\mu g/10^9$ platelets.

An example of HPTLC separation of phospholipids in the cell lines is shown in Figure 4.1. The proportion of each component phospholipid was calculated as a % of the total phospholipid obtained in the Folch extract (Table 4.1). Measurement of the relative phospholipid composition of the platelets showed that they had similar levels of PS but higher levels of SM than the cell lines. The micellar preparation of bovine brain phospholipid used as reference standard in all assays was found to have at least twice the amount of PS than the cells or platelets. The phospholipid composition showed little variation between the cell lines, and was therefore unlikely to explain the differences in PCA between the cell lines. However as the phospholipid distribution across the inner and outer membrane of the cells could not be investigated this explanation for the differences in the procoagulant activity of the cells cannot be ruled out completely. The situation is further compounded by the fact that the measurement of lipid composition used in this study does not distinguish between cellular phospholipid derived from the cell membrane and the proportion derived from cellular organelles such as mitochondria, Golgi apparatus and lysosomes. The question of phospholipid distribution across the cell membrane would have to be addressed using various independent techniques , such as non-lytic treatments with phospholipases $A_2$ and C, sphingomyelinase C and chemical labelling with fluorescent dyes such as fluorescamine (Perret *et al*, 1979; Rawyler *et al*, 1983; Rawyler *et al*, 1984; Rawyler *et al*, 1985).

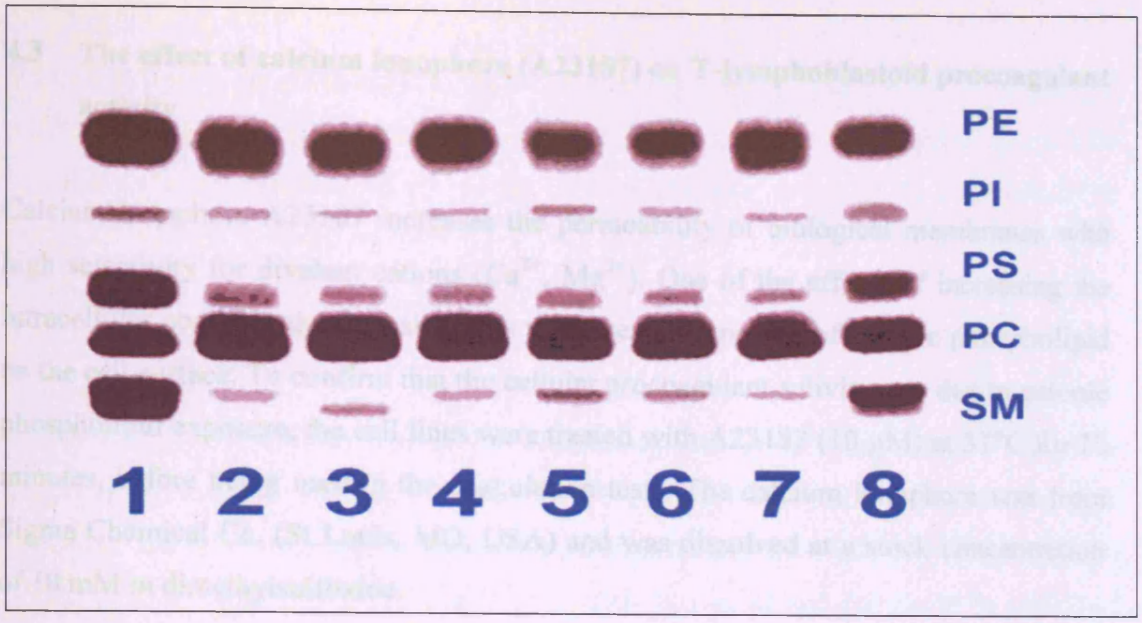



*Figure 4.1: A typical example of HPTLC separation of phospholipids found in the cell lines. Platelets B-cells were included for comparison. Lane 1 contains standard phospholipids: SM/PC/PS/PE (4 μg each) and PI (1 μg). Lanes 2 – 8 show the profiles of respectively: CEM-CCRF, Jurkat, Molt-4, A3.01, THP-1, normal immortalised B-cell and platelets. Phospholipid abbreviations are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM).*

| | PC | PE | PS | PI | SM |
|---|---|---|---|---|---|
| **CEM-CCRF** | 40.5 | 38.1 | 11.3 | 4.4 | 5.7 |
| **Jurkat** | 48.0 | 31.6 | 10.7 | 3.2 | 6.5 |
| **Molt-4** | 47.3 | 33.0 | 10.9 | 3.3 | 5.5 |
| **A3.01** | 37.1 | 37.2 | 11.9 | 5.3 | 8.5 |
| **THP-1** | 41.9 | 36.9 | 10.4 | 4.5 | 6.3 |
| **91/542** | 24.7 | 38.9 | 28.5 | 5.7 | 2.2 |
| **Platelets** | 36.6 | 28.1 | 12.0 | 6.2 | 17.1 |

*Table 4.1: Relative percentage phospholipid composition of the different cell lines, bovine brain phospholipid (91/542) and platelets. Phospholipid abbreviations are: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM).*

## 4.3 The effect of calcium ionophore (A23187) on T-lymphoblastoid procoagulant activity

Calcium ionophore A23187 increases the permeability of biological membranes with high selectivity for divalent cations ($Ca^{2+}$, $Mg^{2+}$). One of the effects of increasing the intracellular concentration of calcium is to cause the exposure of anionic phospholipid on the cell surface. To confirm that the cellular procoagulant activity was due to anionic phospholipid exposure, the cell lines were treated with A23187 (10 μM) at 37°C for 20 minutes, before being used in the coagulation tests. The calcium ionophore was from Sigma Chemical Co. (St Louis, MO, USA) and was dissolved at a stock concentration of 10 mM in dimethylsulfoxide.

### 4.3.1 Anionic phospholipid and phosphatidylserine exposure

Annexin $A5^{FITC}$, due to its strong affinity for anionic phosphoipid was used to probe for the exposure of PS and to confirm that A23187 at 10 μM was sufficient to cause maximal exposure of anionic phospholipid as measured by flow cytometry.

| Cell Line | Annexin $A5^{FITC}$ binding (%) –A23187 | Annexin $A5^{FITC}$ binding (%) +A23187 |
|---|---|---|
| **CEM-CCRF** | 10.92 ± 0.51 | 98.43 ± 0.13 |
| **Jurkat** | 12.96 ± 1.26 | 98.35 ± 0.36 |
| **Molt-4** | 12.55 ± 0.44 | 98.93 ± 0.82 |
| **A3.01** | 9.92 ± 0.60 | 95.77 ± 1.16 |
| **THP-1** | 12.34 ± 1.75 | 96.63 ± 0.64 |

***Table 4.2: Increase in anionic phospholipid exposure, detected by annexin $A5^{FITC}$ binding on the cell-surface of cell lines caused by incubating them with 10 μM calcium ionophore (A23187) for 20 minutes at 37°C. The results are the mean of n = 3; 95% CI.***

Table 4.2 shows the percentage of each cell line that bound annexin $A5^{FITC}$ to the cell surface with and without treatment by A23187 (10 μM). In all cases A23187 caused the translocation and exposure of PS on the outer membrane leaflet of the cell lines with >95% of the cells binding annexin $A5^{FITC}$.

### 4.3.2 Tissue Factor activity

The effect of calcium ionophore on the TF clotting activity of the cell lines is shown in Figure 4.2A. Calcium ionophore caused the TF activity of all the cell lines to increase. However this increase only reached statistical significance ($p < 0.05$) for the T-cell line Jurkat and the monocytoid cell line THP-1. This increased activity could be inhibited by > 98% (data not shown) using the inhibitory anti-TF antibody (100 μg/mL).

Expression of TF activity has been associated with changes in intracellular levels of calcium (Nawroth *et al*, 1985; Camerer *et al*, 1996). It has also been shown that the increase in intracellular calcium occurs in parallel with the increase in TF activity in a variety of ionophore treated cell types (Fibach *et al*, 1985; Bach & Rifkin, 1990). Previous studies have reported that calcium ionophore increases TF activity without increasing TF mRNA or protein (Fibach *et al*, 1985; Bouchard *et al*, 1997). It is thought that calcium ionophore has its effect both by increasing PS (and possibly PE) exposure and by a PS-independent component of TF activity regulation (de-encryption) that is currently unknown (Wolberg *et al*, 1999), and not release from intra-cellular stores (Drake *et al*, 1989).

### 4.3.3 APTT-phospholipid-like activity

The effect of calcium ionophore on the APTT-like activity of the cell lines is shown in Figure 4.2B. The APTT-like activity of all the cell lines was significantly ($p < 0.05$) increased by the action of calcium ionophore. The TF bearing cell lines (Jurkat and

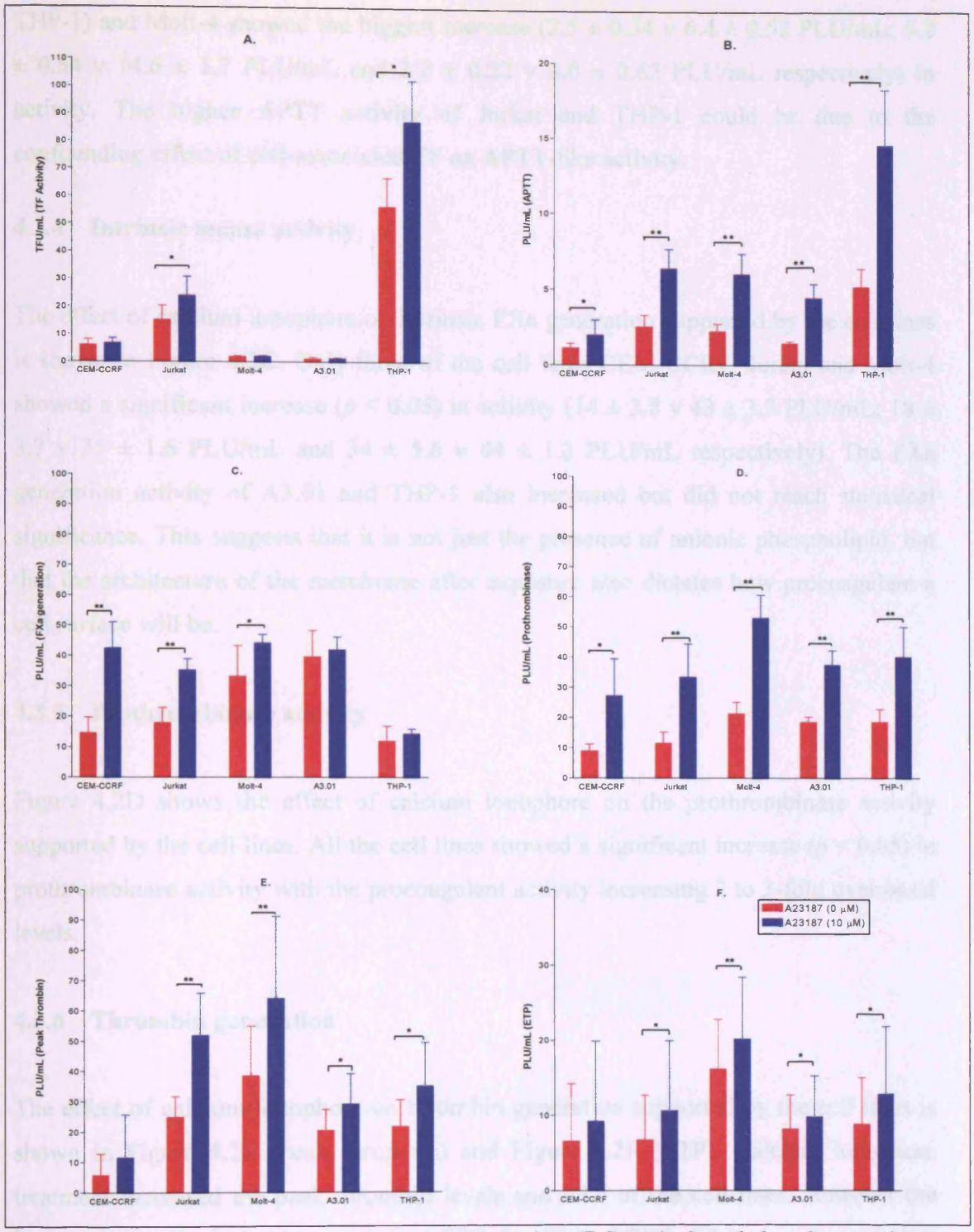



***Figure 4.2:*** ***Increase in procoagulant activity (PCA) induced by the calcium ionophore A23187 (10 µM) on (A) TF PCA (B) activated partial Thromboplastin time (APTT)-like PCA (C) intrinsic FXa generation (D) Prothrombinase activity (E) Peak thrombin activity (F) Endogenous thrombin potential (ETP). Mean ± 95%CI; n = 3; *p<0.05 **p<0.005.***

THP-1) and Molt-4 showed the biggest increase (2.5 ± 0.34 v 6.4 ± 0.52 PLU/mL; 5.2 ± 0.54 v 14.6 ± 1.7 PLU/mL and 2.2 ± 0.22 v 6.0 ± 0.62 PLU/mL respectively) in activity. The higher APTT activity of Jurkat and THP-1 could be due to the confounding effect of cell-associated TF on APTT-like activity.

### 4.3.4 Intrinsic tenase activity

The effect of calcium ionophore on intrinsic FXa generation supported by the cell lines is shown in Figure 4.2C. Only three of the cell lines CEM-CCRF, Jurkat and Molt-4 showed a significant increase ($p < 0.05$) in activity (14 ± 3.8 v 43 ± 3.9 PLU/mL; 18 ± 3.7 v 35 ± 1.6 PLU/mL and 34 ± 5.6 v 44 ± 1.2 PLU/mL respectively). The FXa generation activity of A3.01 and THP-1 also increased but did not reach statistical significance. This suggests that it is not just the presence of anionic phospholipid, but that the architecture of the membrane after exposure also dictates how procoagulant a cell surface will be.

### 4.3.5 Prothrombinase activity

Figure 4.2D shows the effect of calcium ionophore on the prothrombinase activity supported by the cell lines. All the cell lines showed a significant increase ($p < 0.05$) in prothrombinase activity with the procoagulant activity increasing 2 to 3-fold over basal levels.

### 4.3.6 Thrombin generation

The effect of calcium ionophore on thrombin generation supported by the cell lines is shown in Figure 4.2E (peak thrombin) and Figure 4.2F (ETP). Calcium ionophore treatment increased the peak thrombin levels and ETP of the cell lines. However the increase in peak thrombin activity or ETP for CEM-CCRF did not reach statistical significance ($p = 0.06$). After treatment with calcium ionophore, both peak thrombin and ETP measurements (the results expressed as phospholipid equivalence) showed

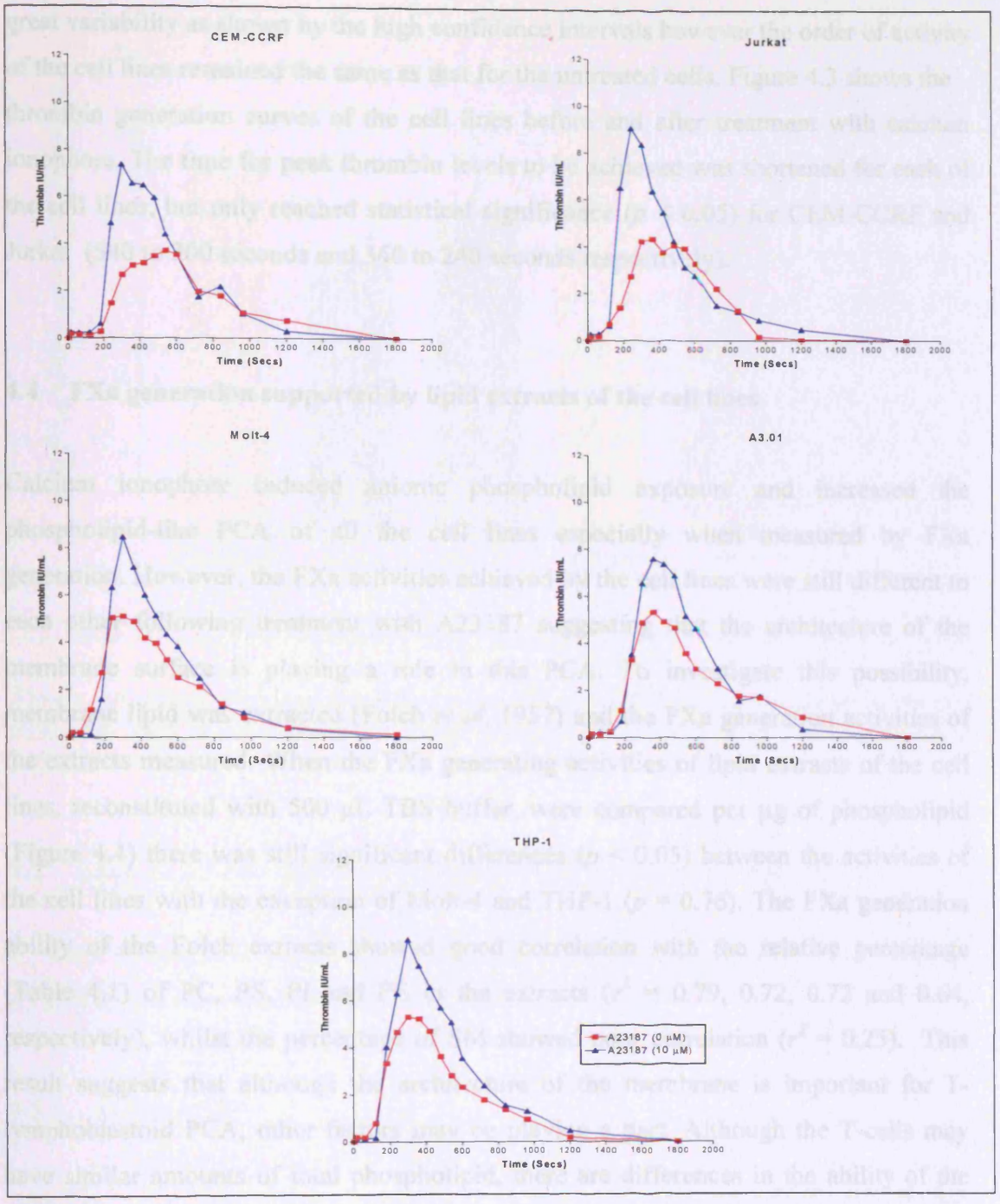



***Figure 4.3: The effect of calcium ionophore A23187 (10 μM) on thrombin generation curves supported by the cell lines. Mean of n = 5.***

great variability as shown by the high confidence intervals however the order of activity of the cell lines remained the same as that for the untreated cells. Figure 4.3 shows the thrombin generation curves of the cell lines before and after treatment with calcium ionophore. The time for peak thrombin levels to be achieved was shortened for each of the cell lines, but only reached statistical significance ($p < 0.05$) for CEM-CCRF and Jurkat (540 to 300 seconds and 360 to 240 seconds respectively).

## 4.4 FXa generation supported by lipid extracts of the cell lines

Calcium ionophore induced anionic phospholipid exposure and increased the phospholipid-like PCA of all the cell lines especially when measured by FXa generation. However, the FXa activities achieved by the cell lines were still different to each other following treatment with A23187 suggesting that the architecture of the membrane surface is playing a role in this PCA. To investigate this possibility, membrane lipid was extracted (Folch *et al*, 1957) and the FXa generation activities of the extracts measured. When the FXa generating activities of lipid extracts of the cell lines, reconstituted with 500 μL TBS buffer, were compared per μg of phospholipid (Figure 4.4) there was still significant differences ($p < 0.05$) between the activities of the cell lines with the exception of Molt-4 and THP-1 ($p = 0.76$). The FXa generation ability of the Folch extracts showed good correlation with the relative percentage (Table 4.1) of PC, PS, PI and PE in the extracts ($r^2 = 0.79$, 0.72, 0.72 and 0.64, respectively), whilst the percentage of SM showed poor correlation ($r^2 = 0.25$). This result suggests that although the architecture of the membrane is important for T-lymphoblastoid PCA, other factors may be playing a part. Although the T-cells may have similar amounts of total phospholipid, there are differences in the ability of the phospholipid extracts from the cell lines to support PCA. These differences may be due to the variation in sub-types, composition and oxidation state of the phospholipids present in the cell membranes.

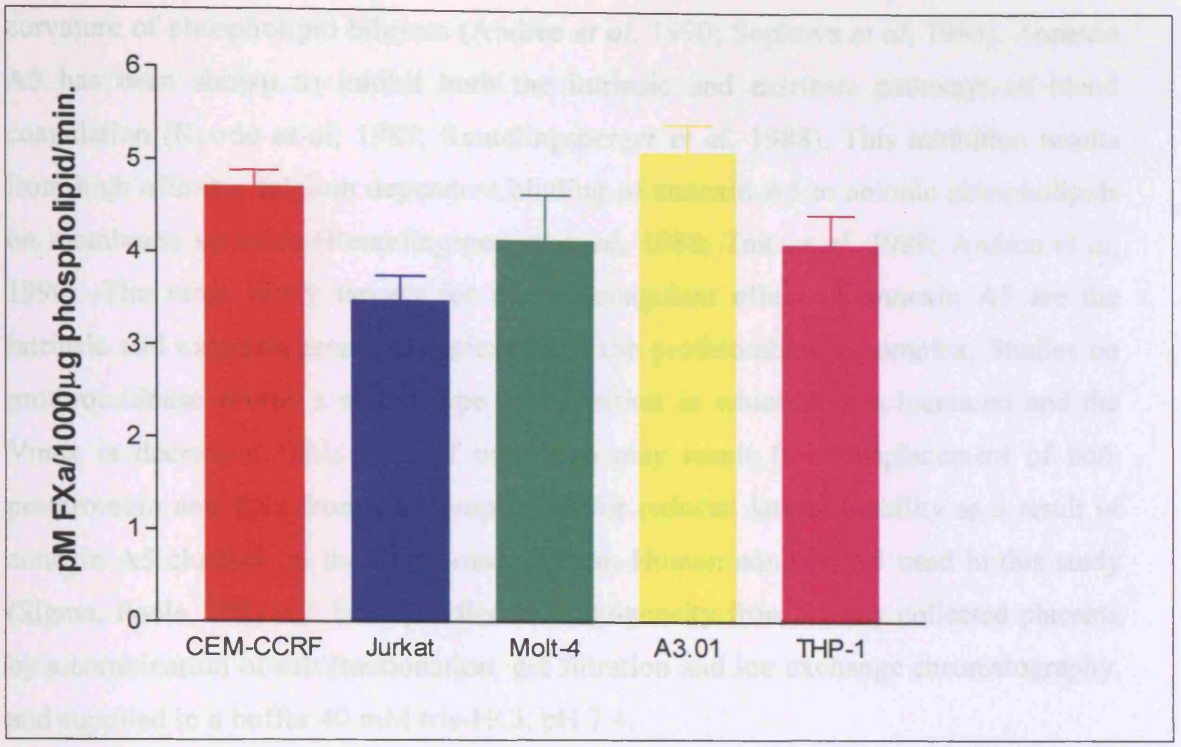



***Figure 4.4: Comparison of the FXa generating activities of Folch extracts of the cell lines. The data is normalised for a phospholipid concentration of 1000 µg (mean ± 95% CI; n = 3).***

## 4.5 Annexin A5 and inhibitory antibody (3G4) to anionic phospholipids

Annexin A5 and 3G4 an anionic phospholipid inhibitory antibody with a more specific affinity for PS were used to study T-lymphoblastoid PCA.

### 4.5.1 Annexin A5

Annexin A5 is a vascular anticoagulant protein normally present on the cytosolic side of the plasma membrane (Reutelingsperger *et al*, 1985; Reutelingsperger *et al*, 1988). Annexin A5 is a member of the calcium dependent, phospholipid binding annexin/lipocortin family. Electron micrographs show that annexin A5 forms trimers upon membrane binding, and that clusters of these trimers form large sheets on membrane surfaces causing local disorder and perturbation of permeability and

curvature of phospholipid bilayers (Andree *et al*, 1990; Sopkova *et al*, 1993). Annexin A5 has been shown to inhibit both the intrinsic and extrinsic pathways of blood coagulation (Kondo *et al*, 1987; Reutelingsperger *et al*, 1988). This inhibition results from high affinity, calcium dependent binding of annexin A5 to anionic phospholipids on membrane surfaces (Reutelingsperger *et al*, 1988; Tait *et al*, 1989; Andree *et al*, 1990). The most likely targets for the anticoagulant effect of annexin A5 are the intrinsic and extrinsic tenase complexes and the prothrombinase complex. Studies on prothrombinase reveal a mixed type of inhibition in which *K*m is increased and the Vmax is decreased. This type of inhibition may result from displacement of both prothrombin and FXa from the complex and/or reduced lateral mobility as a result of annexin A5 clusters on the membrane surface. Human annexin A5 used in this study (Sigma, Poole, UK) had been purified to homogeneity from freshly collected placenta by a combination of salt fractionation, gel filtration and ion exchange chromatography, and supplied in a buffer 40 mM tris-HCl, pH 7.4.

### 4.5.2 Mouse monoclonal antibody 3G4 reactive with anionic phospholipids

The generation of the mouse monoclonal antibody 3G4 is described in detail in section 2.7.5. The reactivity of the selected antibody, mouse $IgG_3$ 3G4, with PS and CL was established by Dr S. Ran and Professor P.E. Thorpe by screening hybridoma supernatants on PS, cardiolipin (CL), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) immobilised on plastic. Further studies showed that 3G4 bound strongly to PS, phosphatidic acid (PA), and CL but weakly to phosphatidylinositol (PI) and phosphatidylglycerol (PG). Recognition of anionic phospholipids by 3G4 was identical in the presence and absence of serum indicating that binding does not require serum cofactors though a requirement for β2 glycoprotein-1 involvement has not been ruled out. Cross-blocking experiments on PS-coated plates showed that 3G4 and annexin A5 do not block each other's binding to PS, indicating that these two reagents recognise different epitopes on the PS molecule, or differently packed forms of PS (personal communication with Professor P.E. Thorpe).

### 4.5.3 Use of inhibitory antibody (3G4) and annexin A5 in coagulation studies

The anti-PS antibody 3G4 and annexin A5 were added to each of the assays at the highest concentrations of 400 μg/mL and 33 μM, respectively. These concentrations had been selected as being able to completely inhibit the bovine brain phospholipid standard (91/542) at a concentration of 100 PLU/mL in the various assay systems used in this thesis (data not shown). An inconsequential isotype control antibody BBG3 used at the same concentration as 3G4 was employed as a negative control in all clotting studies. Antibody or annexin A5 was added in equal volume to cell suspension. The anti-PS antibody was immediate acting (data not shown), and was not preincubated with the cells. Annexin A5 was preincubated for 15 minutes at 37°C with the cell suspension before the assays were performed.

## 4.6 Effect of annexin A5 or the inhibitory phosphatidylserine antibody 3G4 on TF activity on the cell-surface of the cell lines

To investigate the requirement for anionic phospholipid of TF activity, annexin A5 and an inhibitory anti-PS antibody 3G4 were added to the TF PT clotting assay. Annexin A5 at concentrations ranging from 3.3 – 33 mM was used to see if it would inhibit TF activity (Figure 4.5). At the high concentration of 33 mM, annexin A5 was able to totally inhibit clotting TF activity. Even at the lowest concentration of 3.2 μM it was able to inhibit the complex by 18 - 22%. The inhibitory anti-PS antibody 3G4 was also a potent inhibitor of TF-FVII activity (Figure 3.3) and did so in a dose dependent manner. At 400 μg/mL complete inhibition was observed, and at 50 μg/mL there was 65 – 85% inhibition. 3G4 was also found to able to inhibit lipidated recombinant TF (Recombiplastin™). The activity of Recombiplastin™ at a concentration of 1.96 μg/mL (a 1/100 dilution of stock solution) was inhibited by 100, 92 and 87% by 3G4 at initial concentrations of 400, 100 and 50 μg/mL respectively. This inhibition cannot be attributed to the effect of annexin A5 and 3G4 on functional TF protein, but to their effect on procoagulant phospholipid associated with TF and the prevention of the

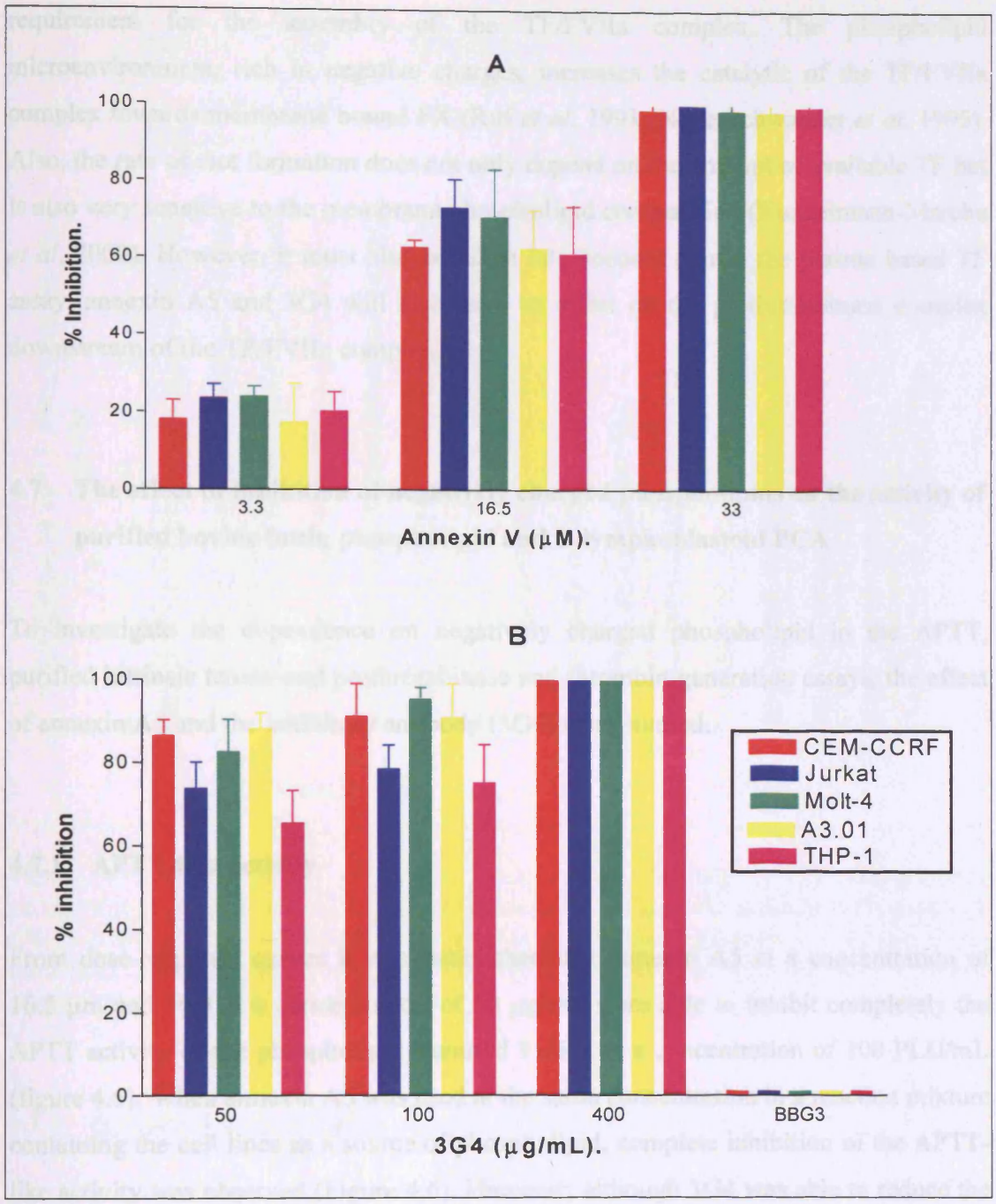



***Figure 4.5: The effect (% inhibition) of varying concentrations of (A) annexin A5 and (B) 3G4 on the cell line activity of the TF-FVIIa complex using the PT TF clotting assay. BBG3 (100 μg/mL) was the isotype control for 3G4. The cell concentration used was $4x10^6$/mL. Results are the mean of n=3 and 95% CI.***

assembly of the TF/FVIIa complex. This is because phospholipid is an essential requirement for the assembly of the TF/FVIIa complex. The phospholipid microenvironment, rich in negative charges, increases the catalytic of the TF/FVIIa complex towards membrane bound FX (Ruf *et al*, 1991; Neuenschwander *et al*, 1995). Also, the rate of clot formation does not only depend on the amount of available TF but is also very sensitive to the membrane phospholipid composition (Kunzelmann-Marche *et al*, 2000). However, it must also be taken into account that in the plasma based TF assay, annexin A5 and 3G4 will also have an effect on the prothrombinase complex downstream of the TF/FVIIa complex.

## 4.7 The effect of inhibition of negatively charged phospholipids on the activity of purified bovine brain phospholipid and T-lymphoblastoid PCA

To investigate the dependence on negatively charged phospholipid in the APTT, purified intrinsic tenase and prothrombinase and thrombin generation assays, the effect of annexin A5 and the inhibitory antibody (3G4) were studied.

### 4.7.1 APTT-like activity

From dose-response curves it was established that annexin A5 at a concentration of 16.5 μm and 3G4 at a concentration of 50 μg/mL were able to inhibit completely the APTT activity of the phospholipid standard 91/542 at a concentration of 100 PLU/mL (figure 4.6). When annexin A5 was used at the same concentration in a reaction mixture containing the cell lines as a source of phospholipid, complete inhibition of the APTT-like activity was observed (Figure 4.6). However, although 3G4 was able to reduce the APTT-like activity by 52.6, 48.1, 59.4, 58.0 and 69.1% respectively, for CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1, it was unable to inhibit it completely even when higher concentrations (400 μg/mL) were used (Figure 4.6).

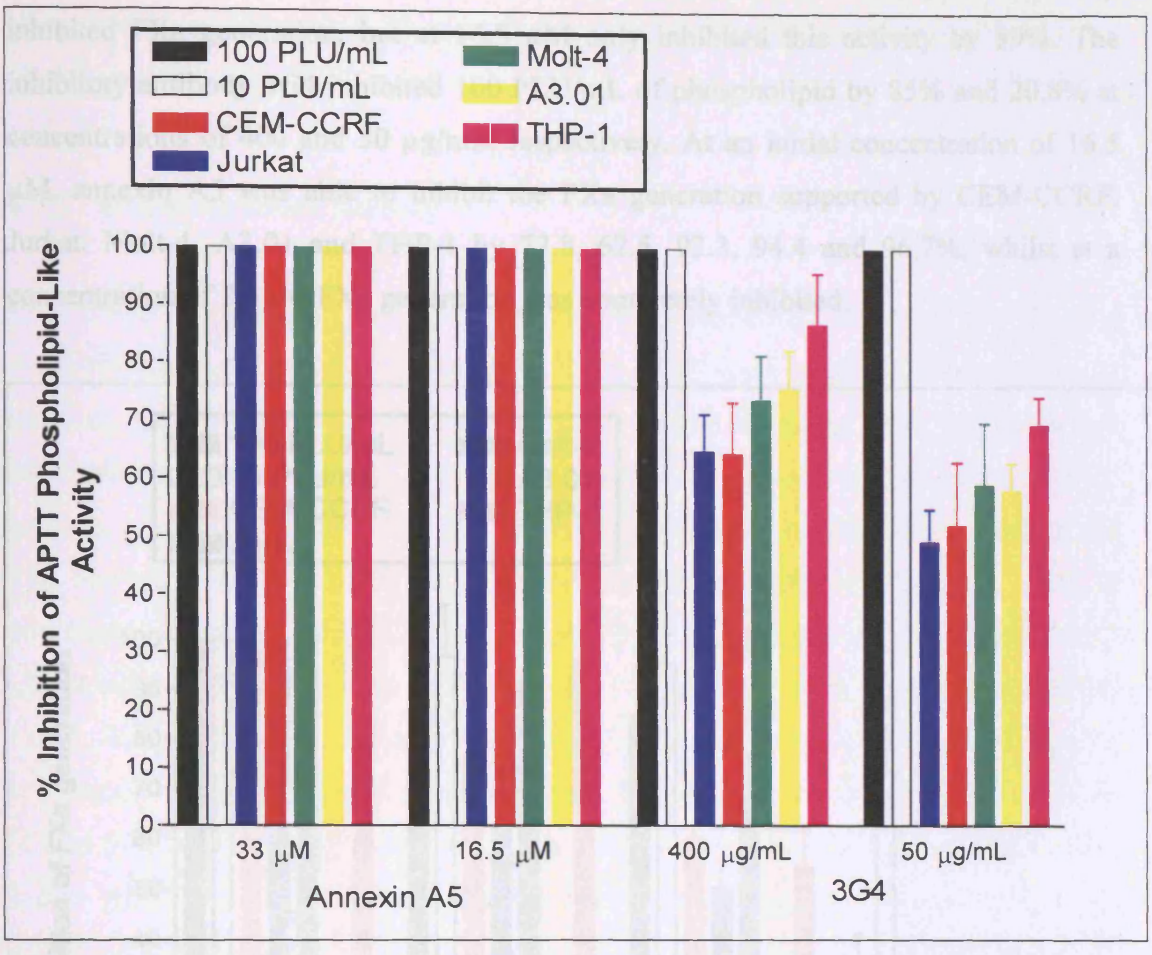



***Figure 4.6: Inhibition of APTT-like procoagulant activity of the phospholipid standard and cell lines (4 x $10^6$/mL) using annexin A5 and the inhibitory PS antibody (3G4). Activity of the inhibitors is reported as percentage inhibition of the control (no inhibitor). The concentration of each inhibitor and phospholipid standard is shown in the legend (mean ± 95% CI; n = 3).***

### 4.7.2 Intrinsic FXa generation

Figure 4.7 depicts the effect of annexin A5 and 3G4 on the purified FXa generation system. The phospholipid dependence of the assay had been shown by the absence of FX generation when tris buffer was substituted for phospholipid. Higher concentrations of both annexin A5 and 3G4 were required to inhibit the same concentration of phospholipid activity in the FXa generation assay than in the APTT system. For

phospholipid at a concentration of 100 PLU/mL, annexin A5 at 33 μM completely inhibited FXa generation, but at 16.5 μM only inhibited this activity by 89%. The inhibitory antibody 3G4 inhibited 100 PLU/mL of phospholipid by 85% and 20.8% at concentrations of 400 and 50 μg/mL, respectively. At an initial concentration of 16.5 μM, annexin A5 was able to inhibit the FXa generation supported by CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1 by 77.8, 62.5, 92.3, 94.4 and 96.7%, whilst at a concentration of 33 μM FXa generation was completely inhibited.

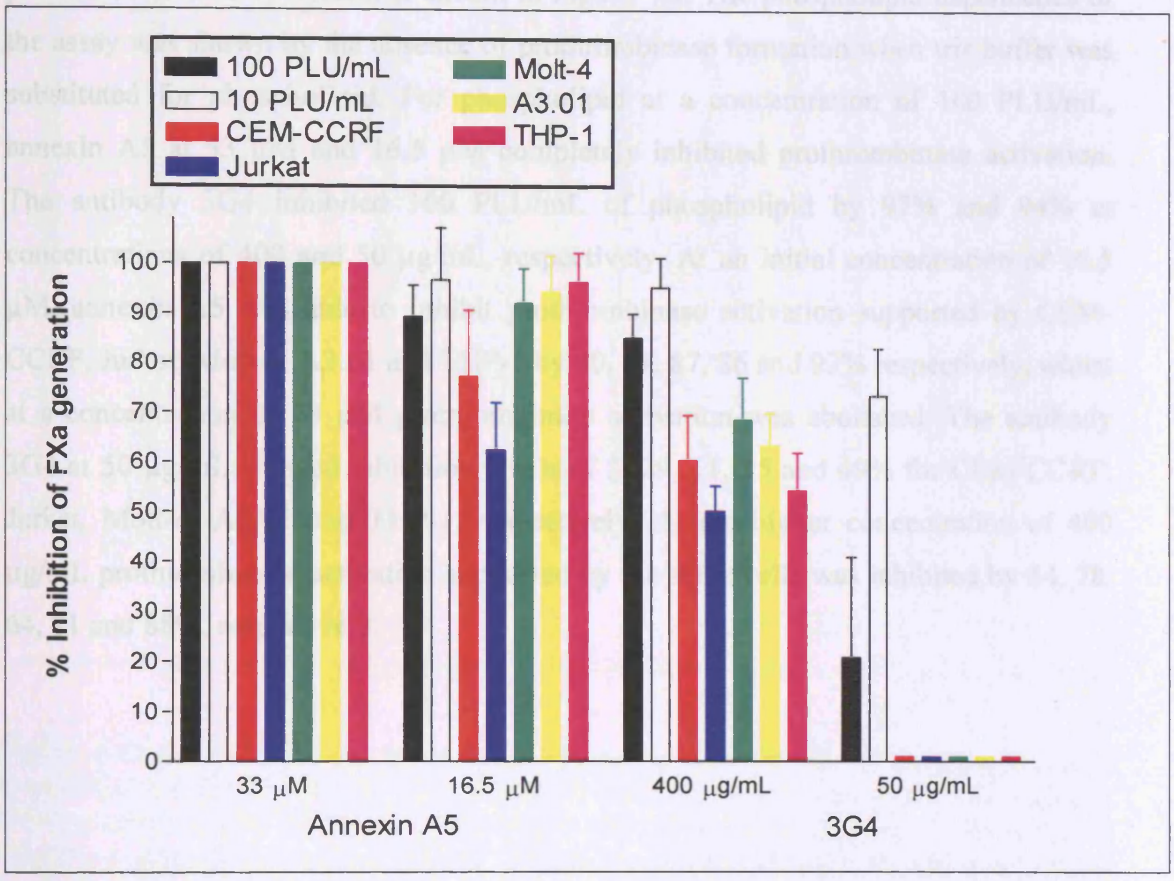



***Figure 4.7: Inhibition of FXa generation procoagulant activity of the phospholipid standard and cell lines (4 x $10^6$/mL) using annexin A5 and the inhibitory PS antibody (3G4). Activity of the inhibitors is reported as percentage inhibition of the control (no inhibitor). The concentration of each inhibitor and phospholipid standard is shown in the legend (mean ± 95% CI; n = 3).***

The inhibitory antibody 3G4 showed no inhibition at 50 μg/mL, but at an initial concentration of 400 μg/mL FXa generation of the same cells was inhibited by 60, 50, 68, 63 and 55% respectively.

### 4.7.3 Prothrombinase Activity

The effect of annexin A5 and the inhibitory antibody 3G4 on the purified prothrombinase assay system is shown in Figure 4.8. The phospholipid dependence of the assay was shown by the absence of prothrombinase formation when tris buffer was substituted for phospholipid. For phospholipid at a concentration of 100 PLU/mL, annexin A5 at 33 μM and 16.5 μM completely inhibited prothrombinase activation. The antibody 3G4 inhibited 100 PLU/mL of phospholipid by 97% and 94% at concentrations of 400 and 50 μg/mL, respectively. At an initial concentration of 16.5 μM, annexin A5 was able to inhibit prothrombinase activation supported by CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1 by 80, 91, 87, 86 and 93% respectively, whilst at a concentration of 33 μM prothrombinase activation was abolished. The antibody 3G4 at 50 μg/mL showed inhibition levels of 3, 29, 11, 35 and 49% for CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1, respectively. At the higher concentration of 400 μg/mL prothrombinase activation supported by the same cells was inhibited by 64, 78, 64, 81 and 88%, respectively.

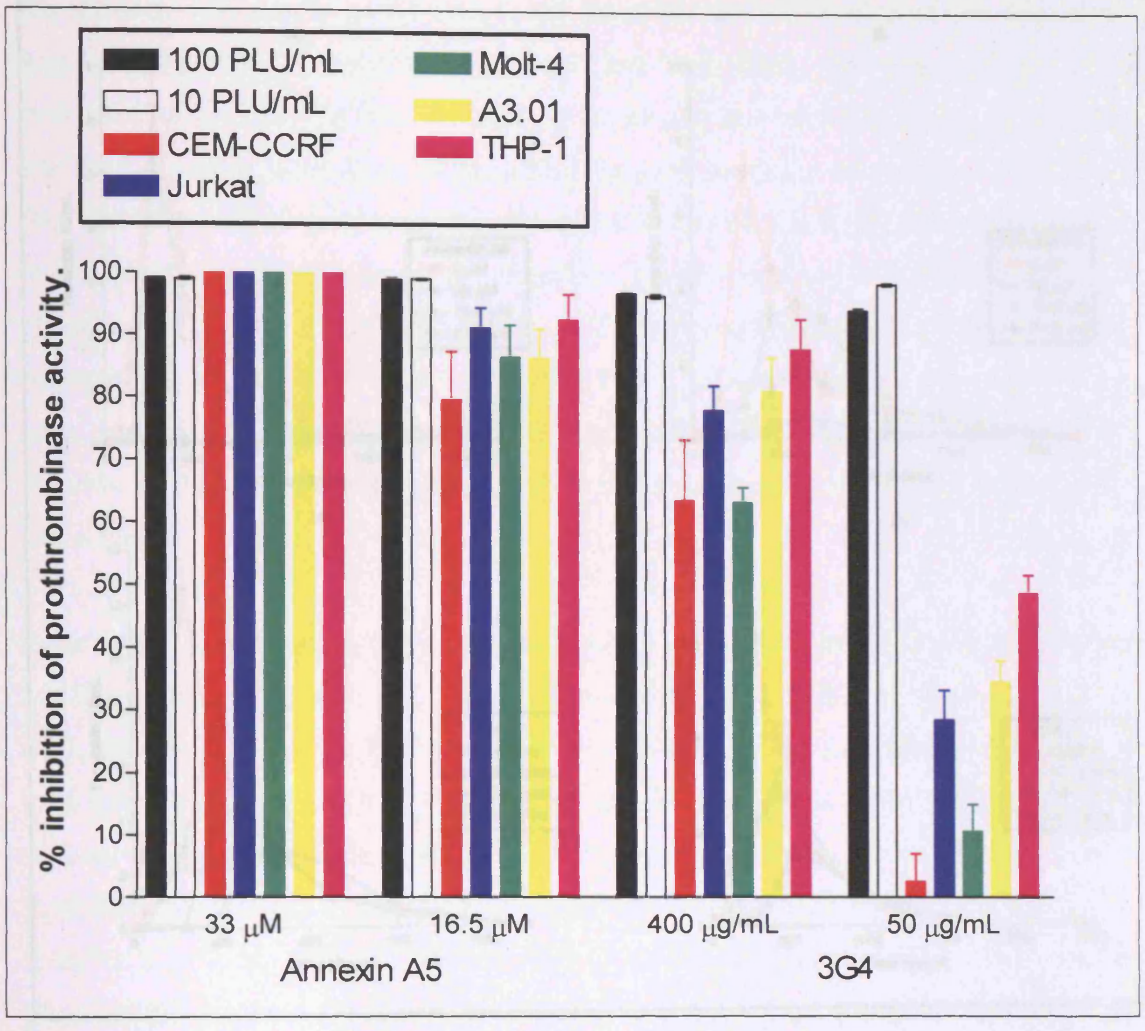



***Figure 4.8: Inhibition of prothrombinase procoagulant activity of the phospholipid standard and cell lines (4 x $10^6$/mL) using annexin A5 and the inhibitory PS antibody (3G4). Activity of the inhibitors is reported as percentage inhibition of the control (no inhibitor). The concentration of each inhibitor and phospholipid standard is shown in the legend (mean ± 95% CI; n = 3).***

### 4.7.4 Thrombin generation in plasma

Figure 4.9 shows representative thrombin generation curves for the effect of annexin A5 and the inhibitory antibody 3G4 on the phospholipid standard at 100 PLU/mL and

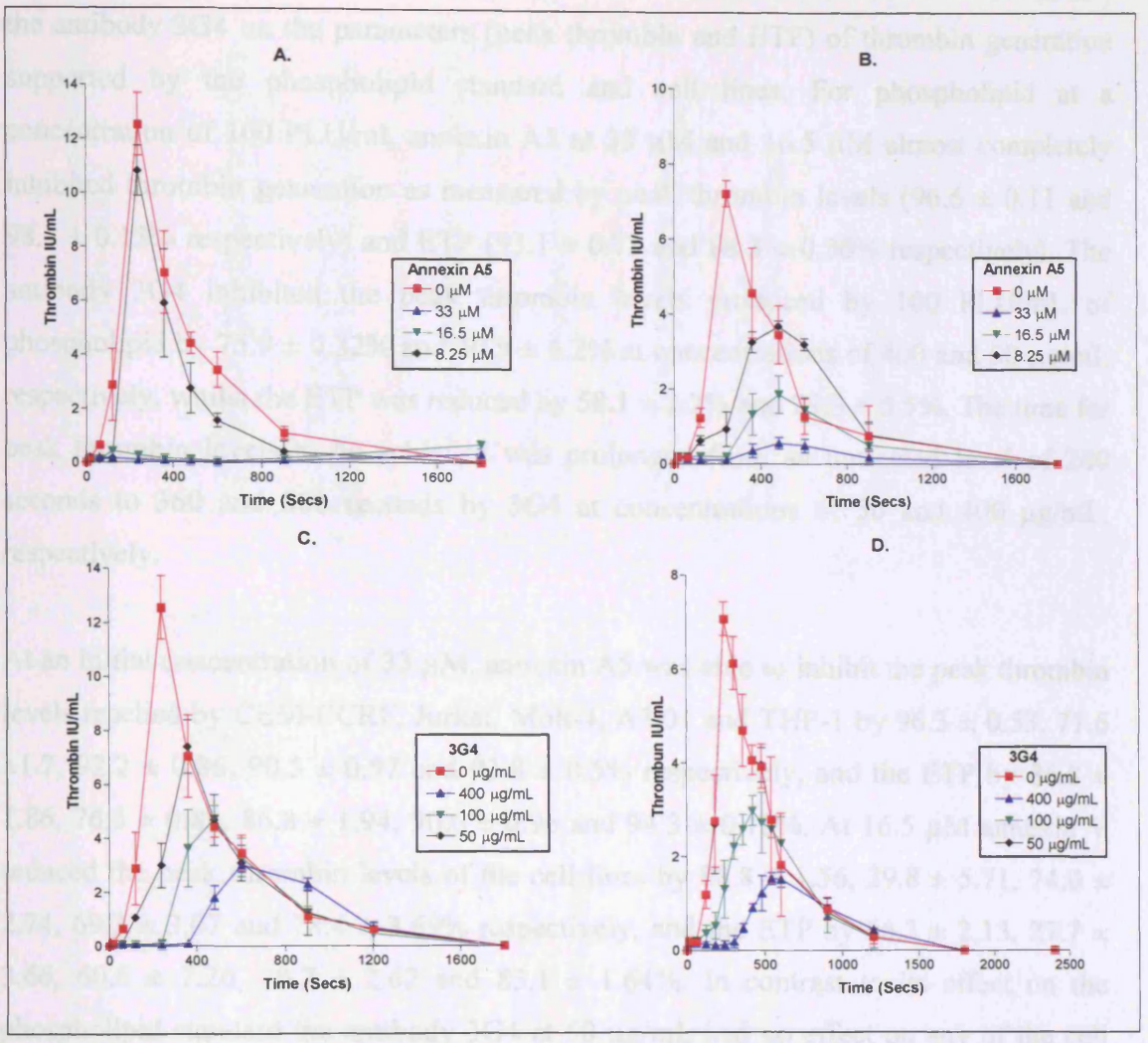



***Figure 4.9: Representative curves showing the effect of annexin A5 and the inhibitory PS antibody (3G4) on thrombin generation for the phospholipid standard (100 PLU/mL) and Molt-4 cell line (4 x $10^6$/mL). (A) Phospholipid standard and annexin A5 (B) Molt-4 and annexin A5 (C) Phospholipid standard and 3G4 (D) Molt-4 and 3G4. The concentration of each inhibitor is shown in the legend (mean ± 95% CI; n = 3).***

Molt-4 at a cell density of 4 x $10^6$/mL. Figure 4.10 depicts the effect of annexin A5 and the antibody 3G4 on the parameters (peak thrombin and ETP) of thrombin generation supported by the phospholipid standard and cell lines. For phospholipid at a concentration of 100 PLU/ml, annexin A5 at 33 μM and 16.5 μM almost completely inhibited thrombin generation as measured by peak thrombin levels (96.6 ± 0.11 and 98.4 ± 0.13% respectively) and ETP (93.1 ± 0.73 and 88.3 ± 0.36% respectively). The antibody 3G4 inhibited the peak thrombin levels produced by 100 PLU/mL of phospholipid by 75.9 ± 0.32% and 40.9 ± 6.2% at concentrations of 400 and 50 μg/mL respectively, whilst the ETP was reduced by 58.1 ± 2.2% and 29.3 ± 5.5%. The time for peak thrombin levels to be achieved was prolonged from an untreated level of 240 seconds to 360 and 580 seconds by 3G4 at concentrations of 50 and 400 μg/mL, respectively.

At an initial concentration of 33 μM, annexin A5 was able to inhibit the peak thrombin levels reached by CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1 by 96.3 ± 0.53, 77.6 ±1.7, 92.2 ± 0.86, 90.5 ± 0.97 and 93.8 ± 0.5% respectively, and the ETP by 86.8 ± 1.86, 76.5 ± 0.85, 86.8 ± 1.94, 90.6 ± 0.96 and 94.3 ± 0.12%. At 16.5 μM annexin V reduced the peak thrombin levels of the cell lines by 88.8 ± 1.56, 29.8 ± 5.71, 74.0 ± 2.74, 69.3 ± 3.07 and 78.4 ± 3.69% respectively, and the ETP by 66.3 ± 2.13, 27.7 ± 3.66, 60.6 ± 7.26, 70.7 ± 2.62 and 83.1 ± 1.64%. In contrast to its effect on the phospholipid standard the antibody 3G4 at 50 μg/mL had no effect on any of the cell lines. However at the higher concentration of 400 μg/mL it delayed the time taken to achieve peak thrombin levels to a maximum of 560 seconds, and inhibited the peak thrombin levels supported by CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1 by 67.7 ± 1.65, 50.4 ± 0.36, 77.6 ± 0.82, 72.8 ± 1.42% respectively. The ETP was inhibited by 58.7 ± 1.87, 57.4 ± 3.84, 60.8 ± 3.24, 68.5 ± 0.73 and 49.8 ± 4.05% for the same cell lines.

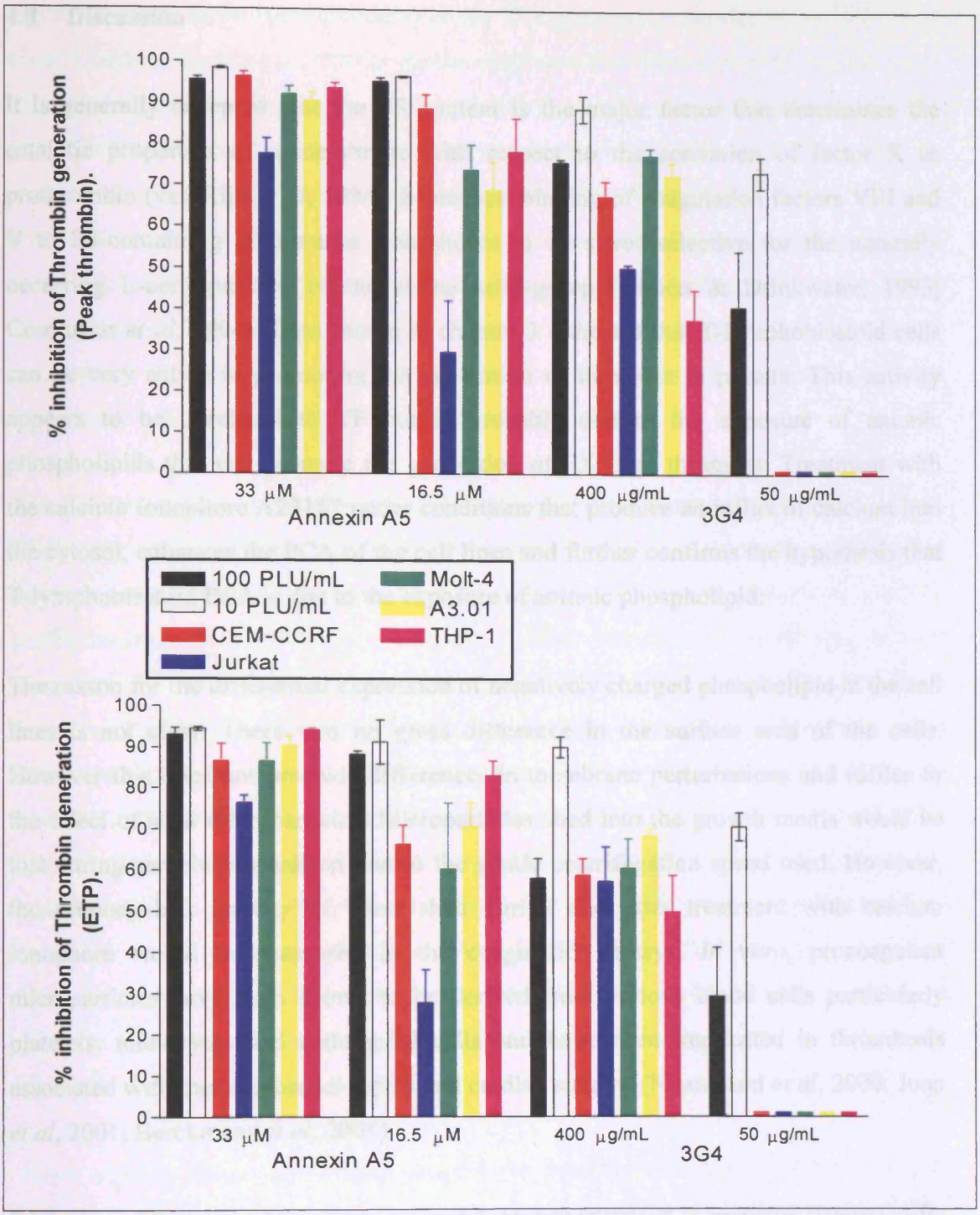



***Figure 4.10: Inhibition of thrombin generation (peak thrombin and ETP) supported by the phospholipid standard and cell lines (4 x $10^6$/mL) using annexin A5 and the inhibitory PS antibody (3G4). Activity of the inhibitors is reported as percentage inhibition of the control (no inhibitor). The concentration of each inhibitor and phospholipid standard is shown in the legend (mean ± 95% CI; n=3).***

## 4.8 Discussion

It is generally accepted that the PS content is the major factor that determines the catalytic properties of a membrane with respect to the activation of factor X or prothrombin (van Rijn *et al*, 1984). Moreover, binding of coagulation factors VIII and V to PS-containing membranes was shown to be stereo-selective for the naturally occurring L-configuration of the serine head-group (Gilbert & Drinkwater, 1993; Comfurius *et al*, 1994). Data shown in chapter 3 indicates that T-lymphoblastoid cells can be very active in promoting the generation of thrombin in plasma. This activity appears to be unrelated to TF but is probably due to the exposure of anionic phospholipids that can promote the generation of FXa and thrombin. Treatment with the calcium ionophore A23187 under conditions that produce an influx of calcium into the cytosol, enhances the PCA of the cell lines and further confirms the hypothesis that T-lymphoblastoid PCA is due to the exposure of anionic phospholipid.

The reason for the differential expression of negatively charged phospholipid in the cell lines is not clear. There was no gross difference in the surface area of the cells. However this does not preclude differences in membrane perturbations and ruffles or the effect of shed microparticles. Microparticles shed into the growth media would be lost during sample preparation due to the gentle centrifugation speed used. However, the procoagulant activity of those shed during and after treatment with calcium ionophore would be measured in the coagulation assays. *In vitro*, procoagulant microparticles have been shown to be derived from various blood cells particularly platelets, monocytes and endothelial cells and have been implicated in thrombosis associated with meningococcal sepsis and cardiac surgery (Nieuwland *et al*, 2000; Joop *et al*, 2001; Berckmans *et al*, 2001).

Inhibition of FXa generation by annexin A5, as demonstrated in previous (Barrowcliffe *et al*, 2002) and present studies, indicates that it is due to the expression of negatively charged phospholipids on the cell surfaces, and the antibody 3G4 with a high specificity for PS was used to attempt to confirm this hypothesis. However, as shown in

Figure 4.5, 3G4 only partially inhibited the FXa generating activity of the cell lines, even at high concentration. Similar partial inhibition was seen in the APTT-like assay (Figure 4.4), but interestingly, 3G4 was able to inhibit almost completely the TF activity of the TF-bearing cell lines (data not shown). This further confirms that the importance of PS in haemostasis is not restricted to its catalytic properties in tenase and prothrombinase complexes, and that it is necessary for the assembly of the TF enzymatic complex and its optimal activity (Kunzelmann-Marche *et al*, 2000). Furthermore, 3G4 also completely inhibited the activity of the phospholipid standard preparation in both the APTT and FXa generation systems, although a higher concentration was required for the FXa generation assay.

The prothrombinase complex showed similar inhibition patterns with annexin A5 and 3G4 to that observed in the FXa generation assay system. The inhibitory antibody 3G4 at high concentrations, unlike annexin A5 was unable to completely inhibit prothrombinase activity of the malignant T-cells, but able to inhibit the activity supported by bovine phospholipid at 100 PLU/mL. When thrombin generation supported by bovine phospholipid was measured, an interesting difference between inhibition by annexin A5 and 3G4 was observed (Figure 4.8). Annexin A5 at 8.25 μM inhibited peak thrombin levels supported by bovine phospholipid, but did not affect the time to reach this peak activity, whilst for 3G4 the time to reach peak thrombin activity was delayed and peak thrombin levels were decreased. When annexin A5 and 3G4 were used to inhibit the thrombin generation activity of the cell lines it was found that inhibition was similar to that to that seen with 3G4 action on bovine phospholipid. As observed in the other phospholipid-dependent assays, annexin A5 was better able to inhibit thrombin generation than 3G4.

Taken together, these observations suggest two possible hypotheses: (i) the activity of the cells is all due to PS, but its stereo-chemical environment on the cell surface is less accessible to the antibody than when in liposome form (phospholipid standard preparation). In support of this, the cell lines, which had the highest procoagulant activity, also gave the highest percentage inhibition by the inhibitory antibody 3G4;

perhaps the cells that show most accessibility for binding to the coagulation factors also allow most accessibility to the antibody; (ii) the activity of the cells is only partially due to PS and the remainder is due to other negatively charged phospholipid, or to oxidised phospholipid. In support of this hypothesis, annexin A5 does not have unique specificity for PS and can bind to other phospholipids such as PE, PI or SM (Andree *et al*, 1990; Blackwood & Ernst, 1990); also previous studies have shown that lipid peroxidation can enhance procoagulant activity (Barrowcliffe *et al*, 1984).

## 4.9 Summary

The inhibitory effect of annexin A5 and 3G4 on the TF and phospholipid dependent coagulation reactions supported by the cell lines and bovine phospholipid was studied. Annexin A5 totally inhibited the activity observed in all the phospholipid dependent assay systems studied, and together with the increase in PCA induced by the calcium ionophore A2387 indicates that the PCA of T-lymphoblastoid cells is primarily due to the expression of negatively charged phospholipids. However, the anti-PS antibody 3G4 even at high concentration gave only partial inhibition of the activity observed in the APTT, FXa generation, prothrombinase and thrombin generation systems for the cells compared with almost total inhibition for the bovine brain phospholipid standard, suggesting either that cellular PS is less accessible to the antibody, or that PS is not the sole negatively charged phospholipid responsible for this activity.

Chapter

5

# APOPTOSIS AND T-LYMPHOBLASTOID PROCOAGULANT ACTIVITY

## 5.1 Introduction

The association between thrombosis and malignancy has been confirmed by many clinical observations (Piccioli & Prandoni, 2001). The risk for thromboembolism has been related to the stage of the disease and the intensity of chemotherapy and hormonal therapy (Levine *et al*, 1998). Cancer cells as well as in some cases being rich in procoagulants can also have an increased population of apoptotic cells. In all the conditions associated with a high cancer associated thrombotic risk, such as high tumour burden, chemotherapy, radiation or hormonal therapy, apoptosis of the tumour cells is also increased (Lee & Levine, 1999). The thrombogenic changes in tumour cells undergoing apoptosis has been investigated by Wang *et al*, 2001. They found a correlation between TF activity, thrombin generation and the degree of apoptosis. Addition of an excess of anti-TF antibody or TFPI partially inhibited thrombin generation and they suggested that TF activation was responsible for the increase in thrombin generation.

Apoptosis, or cellular suicide, is important for normal development and tissue homeostasis, but too much or too little apoptosis can also cause disease. One of the first events of apoptosis is the loss of phospholipid asymmetry and the consequent exposure of PS on the outer membrane (Williamson *et al*, 2001a; Williamson *et al*, 2001b). Thus one of the consequences of apoptosis could be the exposure of a potent procoagulant surface for coagulation to take place. This chapter addresses the question of whether the PCA supported by the cell lines is solely due to apoptosis and explores the effect of staurosporine induced apoptosis on T-lymphoblastoid PCA.

## 5.2 Measurement of the basal levels of apoptosis in the cell lines and correlation with unstimulated T-lymphoblastoid procoagulant activity

To explore whether phospholipid dependent PCA supported by the cell-surface of T-lymphoblastoid cell lines was due to loss of PS asymmetry caused by apoptosis, the basal apoptotic state of the cells was measured using two assays, DNA fragmentation

and annexin A5 binding, in parallel with the measurement of intrinsic FXa generation. Factor Xa generation was chosen because it was the assay where the majority of the cell lines were the most active (see Chapter 3). DNA fragmentation of the cells was measured by determining the percentage of cells in sub-$G_0$ using propidium iodide (PI) staining of permeabilised cells. Apoptosis was also measured by determining the percentage of cells binding annexin $A5^{FITC}$ in the absence of membrane permeability (determined by leakage of PI). Full details of these methods are described in Chapter 2.

| Cell Line | Apoptosis (% of Cells in sub-$G_0$) | Apoptosis (% Cells binding Annexin $A5^{FITC}$) | FXa generation (PLU/mL) |
|---|---|---|---|
| **CEM-CCRF** | 2.1 ±1.9 | 10.9 ± 0.5 | 15 ± 3.6 |
| **Jurkat** | 2.7 ± 1.6 | 13.0 ± 1.3 | 20 ± 3.9 |
| **Molt-4** | 4.3 ± 1.4 | 12.6 ± 0.4 | 34 ± 5.7 |
| **A3.01** | 2.1 ± 0.5 | 9.9 ± 0.6 | 40 ± 5.4 |
| **THP-1** | 1.6 ± 0.5 | 12.3 ± 1.8 | 12 ± 2.0 |

***Table 5.1: Basal apoptotic level of the cell lines on day 3 after sub-culture: comparison of the sub-$G_0$ population, % of cells binding annexin $A5^{FITC}$ and intrinsic FXa generation levels (mean of n = 3 and 95% CI).***

As shown in Table 5.1, on day 3 after subculture - the time when cell lines were harvested for use in experiments - the percentage of cells in sub-$G_0$ was low in all cell lines (<5%). The percentage of cells binding annexin $A5^{FITC}$ was found to range from 9.9 to 13.0 %. The T-lymphoblastoid cells that supported the highest level of FXa generation did not correlate with the highest levels of spontaneous apoptosis as measured by DNA fragmentation ($r^2$ = 0.17) or annexin $A5^{FITC}$ ($r^2$ = 0.16). It can therefore be concluded that apoptosis alone does not account for the PCA seen in previous experiments.

## 5.3 Induction of apoptosis/cell death with Staurosporine

Apoptosis was induced by the antibiotic staurosporine, an alkaloid that inhibits topoisomerase II (Lassota *et al*, 1996) and is a potent inhibitor of protein kinase activity. Staurosporine induces apoptosis and caspase activation through a mitochondrion-mediated pathway (Sun *et al*, 1999). It has been reported to induce apoptosis *in vitro* in human umbilical vein endothelial cells (Bombeli *et al*, 1999), HL-60 (Shimizu *et al*, 1998; Salvioli *et al*, 2000) and other cell lines (Nakagawa *et al*, 2000). The cell lines were cultured in the absence or presence of staurosporine at a final concentration of 1 μM for up to 24 hours. The amount of PS exposure on the cell surface was measured by staining with PI-annexin $A5^{FITC}$, and an example showing data obtained using Jurkat cells is shown in Figure 5.1.

## 5.4 Changes in phosphatidylserine exposure and caspase measurement after staurosporine induced apoptosis

As shown in section 6.2 the basal degree of apoptosis of the cell lines in culture, measured as annexin $A5^{FITC}$ binding ranged from 9.9 ± 0.6 to 13.0 ± 1.3 % and compares well with levels of 6.1 ± 1.0 % reported in breast carcinoma cells and 12.4 ± 1.8 % in acute promyelocytic leukaemia cells (Wang *et al*, 2001).

The addition of staurosporine at a final concentration of 1 μM to the culture medium for a period of 24 hours resulted in an increase of apoptosis in the cell lines, as shown by the percentage of cells that entered into sub-$G_0$ (Table 6.2). The percentage of cells binding annexin $A5^{FITC}$ and the anti-PS antibody 3G4 also increased over the treatment period. This increase in anionic phospholipid exposure correlated with sub-$G_0$ measurements ($r^2$ = 0.475 for annexin $A5^{FITC}$ and $r^2$ = 0.601 for 3G4), and was therefore considered to be a consequence of apoptosis.

During Staurosporine induced apoptosis, cytochrome *c* is released from the mitochondria and activates caspase-9, which in turn activates caspase-3 (Sun *et al*, 1999). Caspase-3 plays a key effector role in apoptosis by cleaving specific substrates

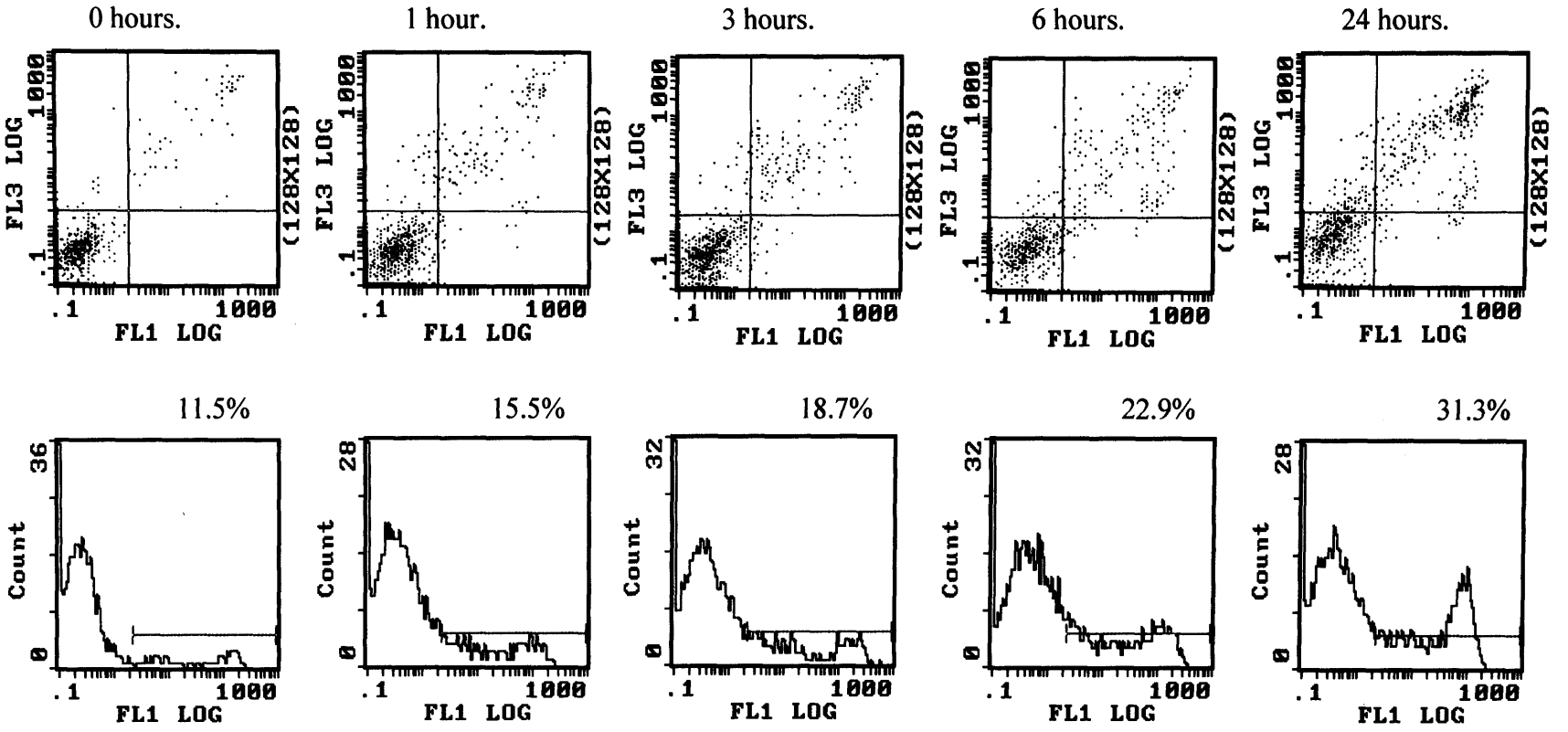



*Figure 5.1: Representative dot plot and histogram data showing the increase (%), over 24 hours in binding of annexin A5$^{FITC}$ to Jurkat cells after treatment with staurosporine (1 μM) to induce apoptosis.*

| Cell Line | Detection Method | Time (hrs) 0 | 1 | 3 | 6 | 24 |
|---|---|---|---|---|---|---|
| CEM-CCRF | $A5^{FITC}$ | 10.4 ± 0.4 | 13.0 ± 0.4 | 14.8 ±0.8 | 18.1 ± 1.0 | 20.8 ± 0.5 |
| | $p$ = | | 0.012 | 0.016 | 0.004 | 0.0008 |
| | 3G4 | 5.0 ± 0.5 | 5.9 ± 0.5 | 7.7 ± 0.9 | 11.8 ± 1.1 | 21.9 ± 2.9 |
| | $p$ = | | 0.054 | 0.060 | 0.011 | 0.005 |
| | Sub-$G_0$ | 2.9 ± 0.9 | 15.1 ± 2.1 | 18.1 ± 2.4 | 25.2 ± 5.0 | 61.2 ± 5.8 |
| | $p$ = | | 0.162 | 0.099 | 0.055 | 0.005 |
| Jurkat | $A5^{FITC}$ | 11.5 ± 0.9 | 15.5 ± 1.0 | 18.7 ± 0.3 | 22.9 ± 0.5 | 31.3 ± 0.9 |
| | $p$ = | | 0.018 | 0.001 | 0.005 | 0.001 |
| | 3G4 | 5.8 ± 1.8 | 10.8 ± 1.7 | 11.7 ± 0.5 | 15.9 ± 0.8 | 23.2 ± 1.1 |
| | $p$ = | | 0.131 | 0.024 | 0.014 | 0.003 |
| | Sub-$G_0$ | 3.8 ± 0.4 | 24.9 ± 4.0 | 26.9 ± 2.3 | 39.6 ± 2.7 | 51.7 ± 3.8 |
| | $p$ = | | 0.029 | 0.002 | 0.005 | 0.001 |
| Molt-4 | $A5^{FITC}$ | 12.2 ± 0.4 | 26.4 ± 2.0 | 39.6 ± 3.1 | 40.0 ± 0.4 | 45.0 ± 1.9 |
| | $p$ = | | 0.004 | 0.003 | 0.0002 | 0.001 |
| | 3G4 | 6.9 ± 0.6 | 10.9 ± 0.3 | 11.5 ± 1.2 | 13.2 ± 0.8 | 29.1 ± 2.1 |
| | $p$ = | | 0.002 | 0.033 | 0.009 | 0.002 |
| | Sub-$G_0$ | 5.4 ± 0.3 | 10.2 ± 1.3 | 23.3 ± 0.4 | 34.9 ± 0.45 | 53.3 ± 4.7 |
| | $p$ = | | 0.012 | 0.002 | 0.0002 | 0.002 |
| A3.01 | $A5^{FITC}$ | 9.5 ± 0.5 | 12.6 ± 0.9 | 21.6 ± 0.1 | 25.1 ± 3.5 | 29.7 ± 1.5 |
| | $p$ = | | 0.047 | 0.0004 | 0.011 | 0.002 |
| | 3G4 | 6.3 ± 0.6 | 6.9 ± 0.3 | 10.3 ± 0.3 | 12.9 ± 0.5 | 23.1 ± 1.8 |
| | $p$ = | | 0.150 | 0.012 | 0.0003 | 0.004 |
| | Sub-$G_0$ | 2.7 ± 0.5 | 3.1 ± 0.8 | 22.3 ± 3.9 | 31.9 ± 4.9 | 42.8 ± 3.6 |
| | $p$ = | | 0.325 | 0.012 | 0.008 | 0.003 |
| THP-1 | $A5^{FITC}$ | 12.6 ± 1.6 | 15.6 ± 1.0 | 18.5 ± 1.4 | 25.3 ± 0.3 | 28.7 ± 1.2 |
| | $p$ = | | 0.011 | 0.054 | 0.005 | 0.007 |
| | 3G4 | 6.4 ± 2.5 | 14.3 ± 0.3 | 16.9 ± 0.4 | 24.9 ± 1.7 | 26.0 ± 1.5 |
| | $p$ = | | 0.030 | 0.009 | 0.004 | 0.010 |
| | Sub-$G_0$ | 4.6 ± 0.7 | 6.7 ± 0.6 | 12.4 ± 3.3 | 22.9 ± 0.9 | 37.9 ± 0.7 |
| | $p$ = | | 0.001 | 0.051 | 0.001 | 0.0004 |

***Table 5.2: Phosphatidylserine (PS) exposure and staurosporine induced apoptosis. The cell lines were treated 1 μM for up to 24 hours to induce apoptosis. PS exposure was investigated by flow cytometry measuring the binding (%) of annexin $A5^{FITC}$ and the anti-PS antibody 3G4 to treated cells and compared with untreated ones. The degree of apoptosis was determined by the percentage of cells in sub-$G_0$. The data is the mean of n = 3 and 95% CI. p values were obtained by comparison with time 0 (hrs).***

important for downstream apoptosis signalling. Thus to further confirm that the cell lines were undergoing apoptosis, caspase-3/7 activity was measured using a colorimetric assay based on the hydrolysis of the peptide substrate Ac-DEVD-*p*NA (see section 2.8 for details of method). The results are shown in Figure 5.2. Interestingly, Jurkat and Molt-4 showed the highest basal caspase-3/7 activity (72.7 ± 20.6 and 86.1 ± 14.4 pmol/min/$10^6$ cells, respectively) when compared with CEM-CCRF, A3.01 and THP-1 (19.2 ± 0.2 and 26.7 ± 7.5 and 22.7 ± 6.5 pmol/min/$10^6$ cells, respectively).

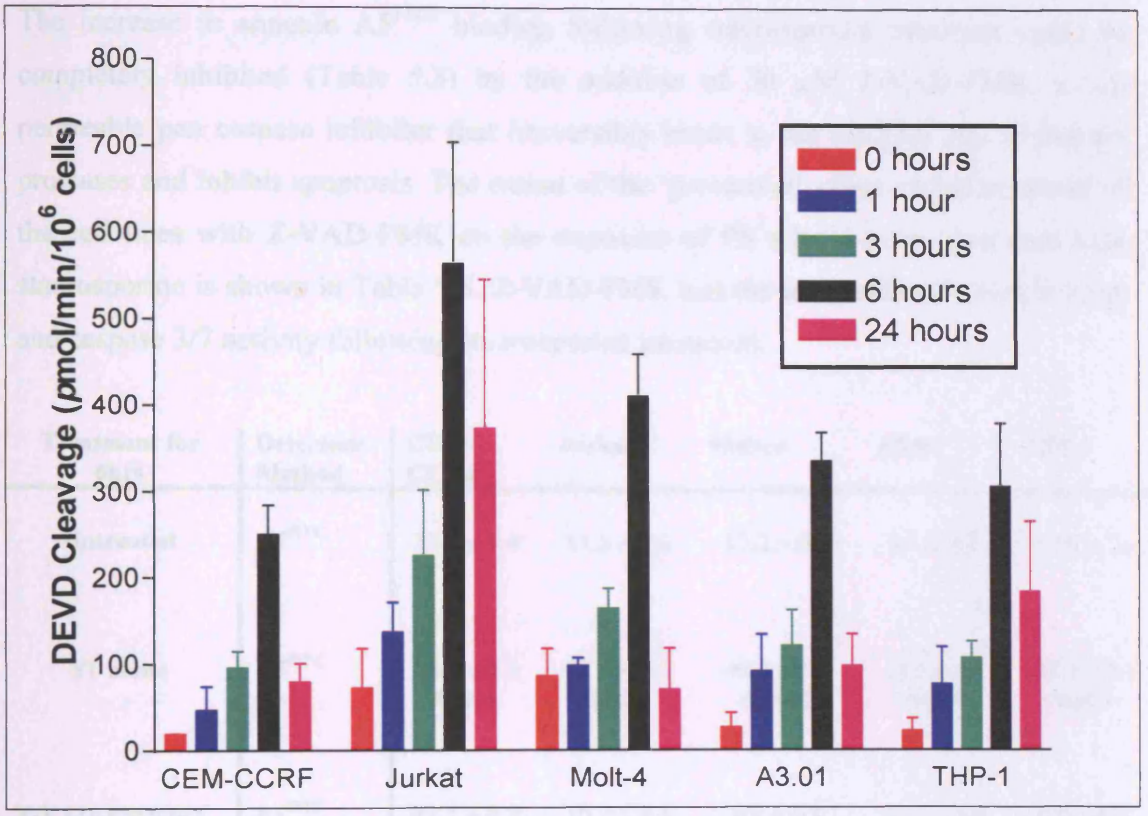



***Figure 5.2: Caspase-3/7 activity of the cell lines following staurosporine (1 μM) treatment. DEVD cleavage of cell lysates was measured at 0, 1, 3, 6 and 24 hours. The activity of the lysates were converted to pmol of DEVD by comparison with a standard curve generated with free pNA.The data shown are the mean of n = 3 and 95% CI.***

For all of the cell lines, maximum activity was reached 6 hours following assay initiation after which the activity decayed. Jurkat and Molt-4 showed the highest

caspase-3/7 activity after 6 hours (564.3 ± 64.2 and 411.1 ± 22.3 pmol/min/$10^6$ cells, respectively), whilst CEM-CCCF, A3.01 and THP-1 showed lower activities (250.5 ± 15.4, 336.0 ± 14.3 and 306.3 ± 33.7 pmol/min/$10^6$ cells, respectively). In contrast to the caspase-3/7 activation (Figure 5.2) and PS exposure, DNA fragmentation (sub-Go) occurs in the later stages of apoptosis (data reported in table 5.2). Taken together these results are consistent with the dogma, that apoptosis is a process that occurs over time and that not all cells in a population undergo apoptosis at once.

The increase in annexin $A5^{FITC}$ binding following staurosporine treatment could be completely inhibited (Table 5.3) by the addition of 50 μM Z-VAD-FMK, a cell permeable pan caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases and inhibit apoptosis. The extent of the 'protective' effect of pre-treatment of the cell lines with Z-VAD-FMK on the exposure of PS after 6 hours treatment with staurosporine is shown in Table 5.3. Z-VAD-FMK had the same effect on 3G4 binding and caspase 3/7 activity following staurosporine treatment.

| Treatment for 6hrs | Detection Method | CEM-CCRF | Jurkat | Molt-4 | A3.01 | THP-1 |
|---|---|---|---|---|---|---|
| **Untreated** | $A5^{FITC}$ | 10.4 ± 0.4 | 11.5 ± 0.9 | 12.2 ± 0.4 | 9.5 ± 0.5 | 12.6 ± 1.6 |
| **ST alone** | $A5^{FITC}$<br>$p$ = | 18.1 ± 1.0<br>0.004 | 22.9 ± 0.5<br>0.005 | 40.0 ± 0.4<br>0.0002 | 25.1 ± 3.5<br>0.011 | 25.3 ± 0.3<br>0.005 |
| **Z-VAD-FMK/ST** | $A5^{FITC}$<br>$p$ = | 11.1 ± 0.3<br>0.021 | 10.1 ± 0.4<br>0.033 | 9.9 ± 0.4<br>0.003 | 12.1 ± 0.8<br>0.053 | 9.0 ± 0.7<br>0.157 |

***Table 5.3: Inhibition of phosphatidylserine (PS) exposure due to treatment of the cell lines for 6 hrs with 1 μM staurosporine by the caspase inhibitor Z-VAD-FMK (50 μM). PS exposure was by investigated by flow-cytometry measuring the binding (%) of annexin $A5^{FITC}$ to treated cells compared with untreated ones. The data is the mean of n = 3 and 95% CI. The p-values indicate differences between ST or Z-VAD-FMK/ST treated cells and untreated ones.***

## 5.5 The effect of staurosporine induced apoptosis on T-lymphoblastoid procoagulant activity

When apoptosis was induced by staurosporine in the various cell lines, their respective procoagulant activities were studied for both baseline and apoptotic conditions.

### 5.5.1 Effect of apoptosis on tissue factor activity

Tissue factor clotting activity (method described in section 2.6.1.1) of the suspension of intact cells was assayed to explore TF activation on the cell membrane during apoptosis (Figure 5.3).

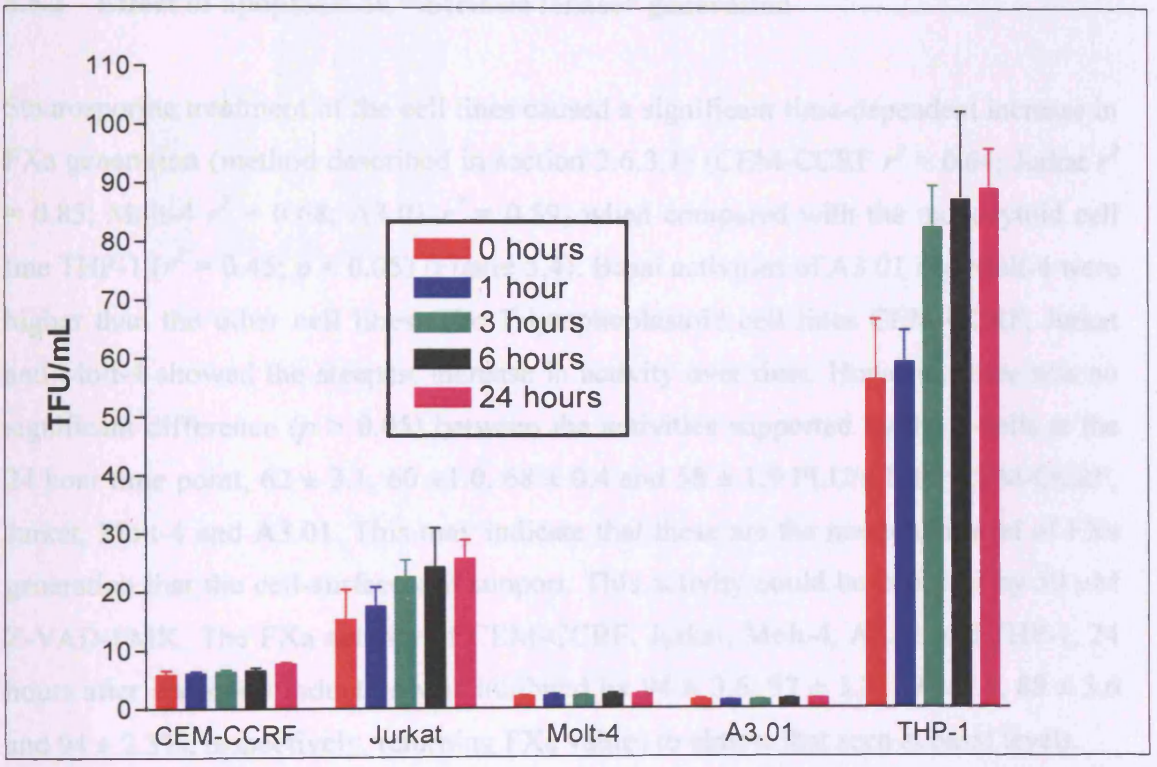



***Figure 5.3: Effect of staurosporine (1 μM) induced apoptosis over 24 hours on the tissue factor clotting activity of the cell lines (4 x $10^6$/mL). The data shown are the mean of n = 3 and 95% CI, and is expressed as TFU/mL.***

Not surprisingly only the TF bearing cell lines Jurkat and THP-1 showed a significant increase in TF clotting activity following staurosporine treatment ($p < 0.05$). Tissue factor clotting activity of these two cell lines was compared with the degree of apoptosis. Tissue factor activity of Jurkat and THP-1 cells correlated well with the number of cells in sub-$G_0$ ($r^2 = 0.75$ and 0.67, respectively) and also with caspase-3/7 activity ($r^2 = 0.55$ and 0.57, respectively). It was also found that the amount of cells binding annexin $A5^{FITC}$ also correlated with TF activity for Jurkat ($r^2 = 0.74$) and THP-1 ($r^2 = 0.79$) cells. This finding is in agreement with other workers (Hutter *et al*, 2004) who showed similar findings in macrophages undergoing apoptosis in conditions found in the lipid-rich plaque.

### 5.5.2 Effect of apoptosis on "intrinsic tenase" generation

Staurosporine treatment of the cell lines caused a significant time-dependent increase in FXa generation (method described in section 2.6.3.1) (CEM-CCRF $r^2 = 0.64$; Jurkat $r^2 = 0.85$; Molt-4 $r^2 = 0.68$; A3.01 $r^2 = 0.59$) when compared with the monocytoid cell line THP-1 ($r^2 = 0.45$; $p < 0.05$) (Figure 5.4). Basal activities of A3.01 and Molt-4 were higher than the other cell lines. The T-lymphoblastoid cell lines CEM-CCRF, Jurkat and Molt-4 showed the steepest increase in activity over time. However, there was no significant difference ($p > 0.05$) between the activities supported by the T-cells at the 24 hour time point, 62 ± 3.1, 60 ±1.0, 68 ± 0.4 and 58 ± 1.9 PLU/mL for CEM-CCRF, Jurkat, Molt-4 and A3.01. This may indicate that these are the maximum level of FXa generation that the cell-surface can support. This activity could be inhibited by 50 μM Z-VAD-FMK. The FXa activity of CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1, 24 hours after apoptosis induction was inhibited by 94 ± 3.6, 92 ± 3.3, 89 ± 7.5, 88 ± 3.6 and 94 ± 2.3%, respectively, returning FXa values to almost that seen at basal levels.

The increase in FXa PCA was found to relate to the degree of staurosporine induced apoptosis. When the FXa generating activity of the cell lines was compared at each time point with the number of cells in sub-$G_0$, $r^2$ values of 0.76, 0.84, 0.95, 0.87 and

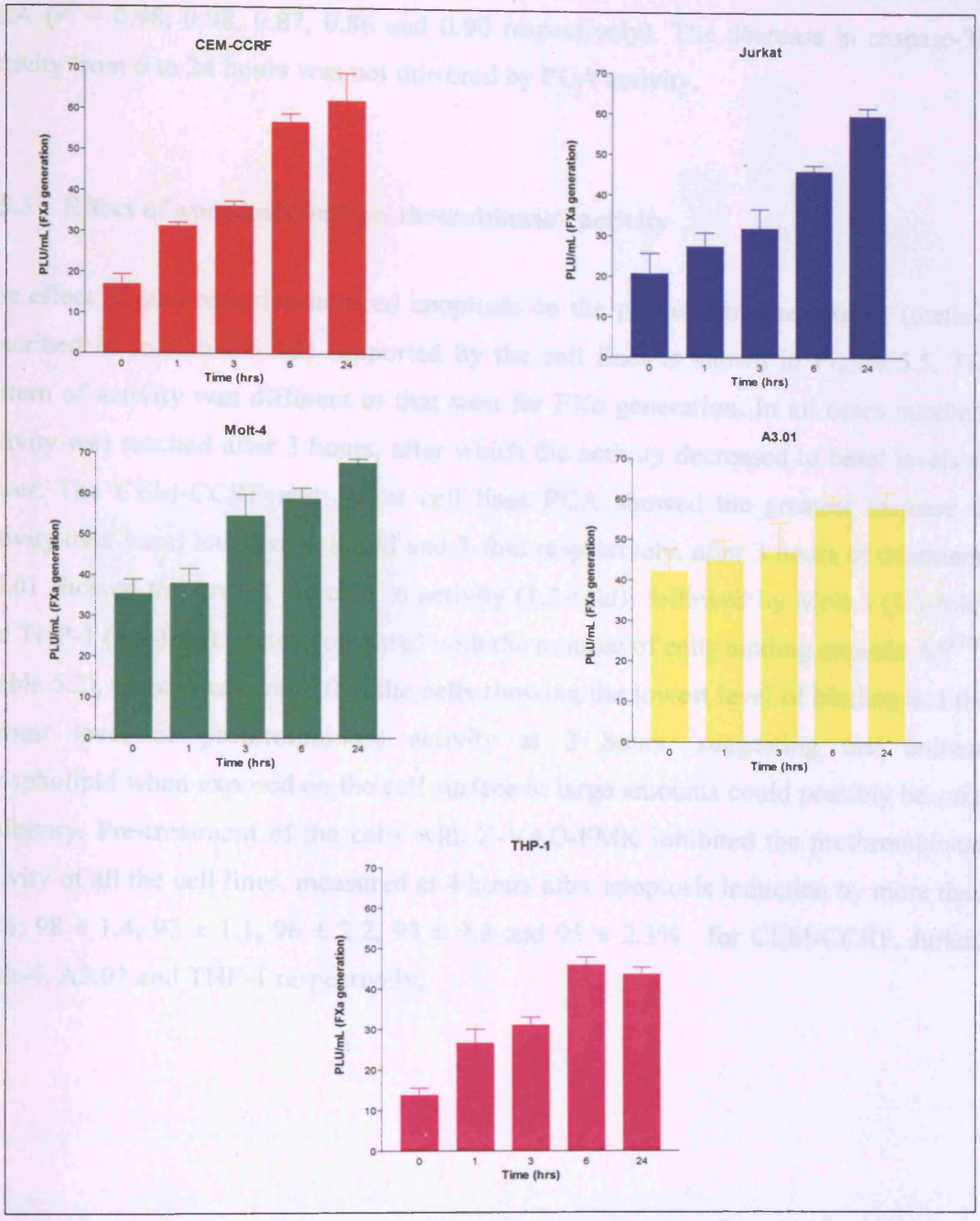



***Figure 5.4: Effect of staurosporine (1 μM) induced apoptosis over 24 hours on the FXa generation procoagulant activity of the cell lines (4 x $10^6$/mL). The data shown are the mean of n = 3 and 95% CI, and is expressed as PLU/mL.***

0.72 for CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1 were obtained. It was also found that the percentage of cells binding annexin $A5^{FITC}$ also correlated with the FXa PCA ($r^2$ = 0.98, 0.98, 0.87, 0.86 and 0.90 respectively). The decrease in caspase-3/7 activity from 6 to 24 hours was not mirrored by PCA activity.

### 5.5.3 Effect of apoptosis on "prothrombinase" activity

The effect of staurosporine-induced apoptosis on the prothrombinase activity (method described in section 2.6.3.2) supported by the cell lines is shown in Figure 5.5. The pattern of activity was different to that seen for FXa generation. In all cases maximal activity was reached after 3 hours, after which the activity decreased to basal levels or lower. The CEM-CCRF and Jurkat cell lines PCA showed the greatest increase in activity over basal levels of 4.1-fold and 3-fold respectively, after 3 hours of treatment. A3.01 showed the lowest increase in activity (1.2-fold), followed by Molt-4 (1.3-fold) and THP-1 (1.4-fold). When compared with the number of cells binding annexin $A5^{FITC}$ (Table 5.2), there was a trend that the cells showing the lowest level of binding had the highest level of prothrombinase activity at 3 hours, suggesting that anionic phospholipid when exposed on the cell surface in large amounts could possibly become inhibitory. Pre-treatment of the cells with Z-VAD-FMK inhibited the prothrombinase activity of all the cell lines, measured at 4 hours after apoptosis induction by more than 90%; 98 ± 1.4, 97 ± 1.1, 96 ± 2.2, 93 ± 9.3 and 95 ± 2.3%, for CEM-CCRF, Jurkat, Molt-4, A3.01 and THP-1 respectively.

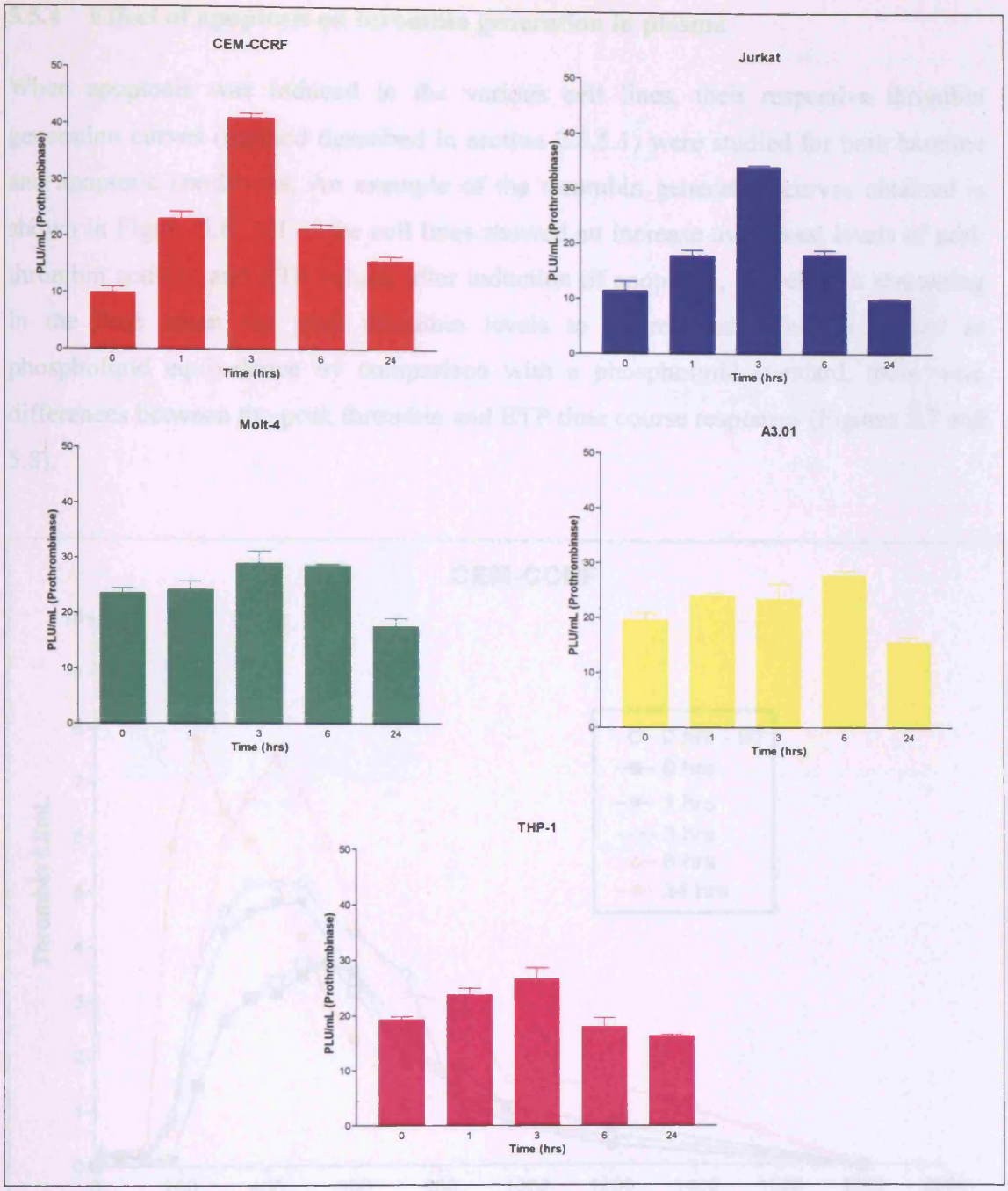



***Figure 5.5: Effect of staurosporine (1 μM) induced apoptosis over 24 hours on the prothrombinase procoagulant activity of the cell lines (4 x $10^6$/mL). The data shown are the mean of n = 3 and 95% CI, and is expressed as PLU/ml.***

### 5.5.4 Effect of apoptosis on thrombin generation in plasma

When apoptosis was induced in the various cell lines, their respective thrombin generation curves (method described in section 2.6.5.1) were studied for both baseline and apoptotic conditions. An example of the thrombin generation curves obtained is shown in Figure 5.6. All of the cell lines showed an increase over basal levels of peak thrombin activity and ETP values, after induction of apoptosis, as well as a shortening in the time taken for peak thrombin levels to be reached. When expressed as phospholipid equivalence by comparison with a phospholipid standard, there were differences between the peak thrombin and ETP time course responses (Figures 5.7 and 5.8).

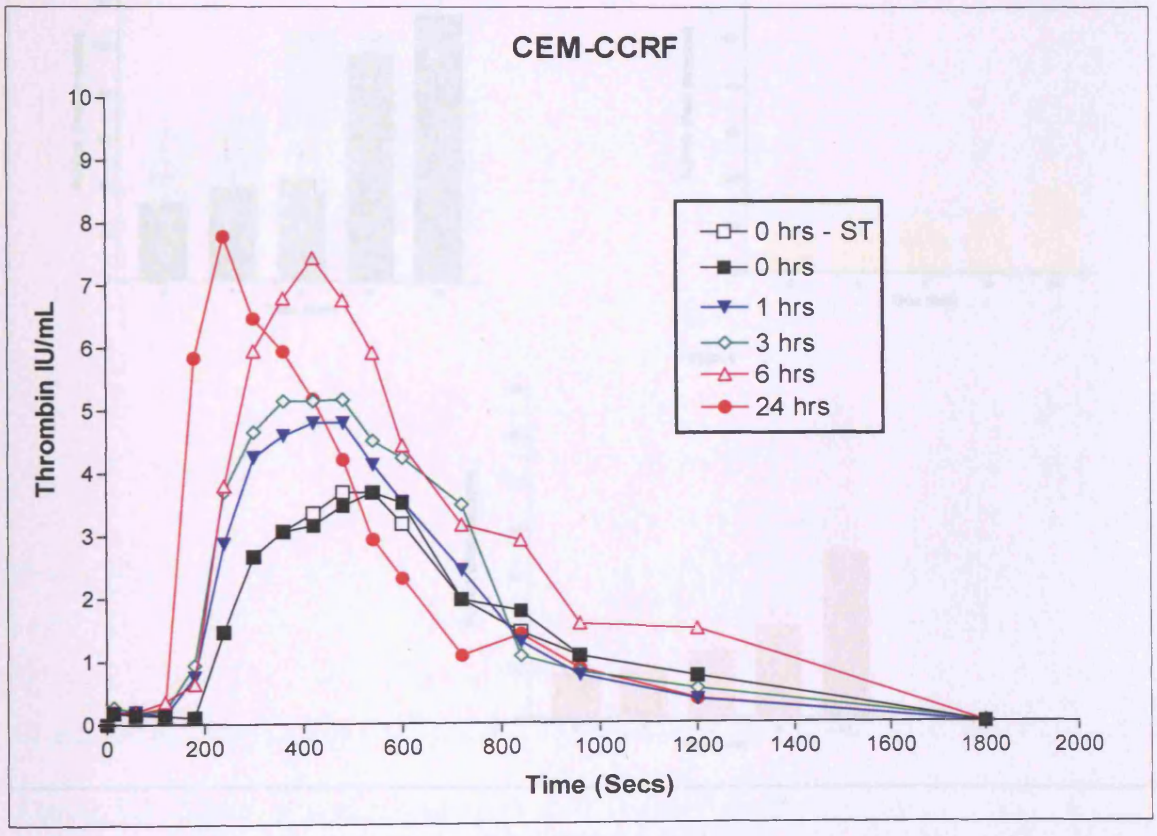



***Figure 5.6: The effect of 1 μM staurosporine (ST) induced apoptosis over 24 hours on the thrombin generation curves supported by the cell line CEM-CCRF (4 x $10^6$/mL). The data shown is the mean of n = 3.***

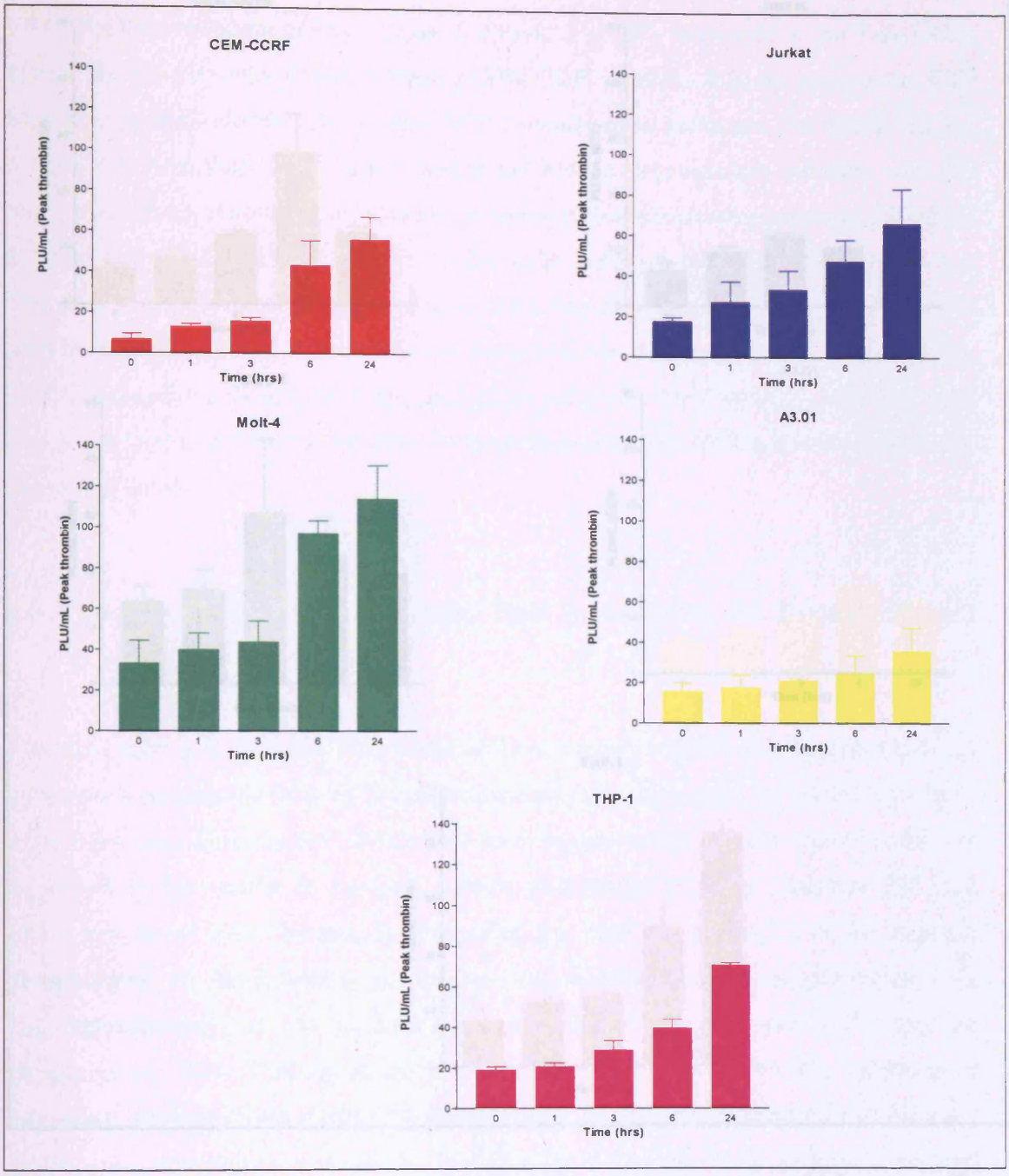



***Figure 5.7: Effect of staurosporine (1 μM) induced apoptosis over 24 hours on the thrombin generation (peak thrombin) activity of the cell lines (4 x $10^6$/mL) converted to PLU/mL, by comparison with a phospholipid standard. The data shown are the mean of n = 3 and 95% CI.***

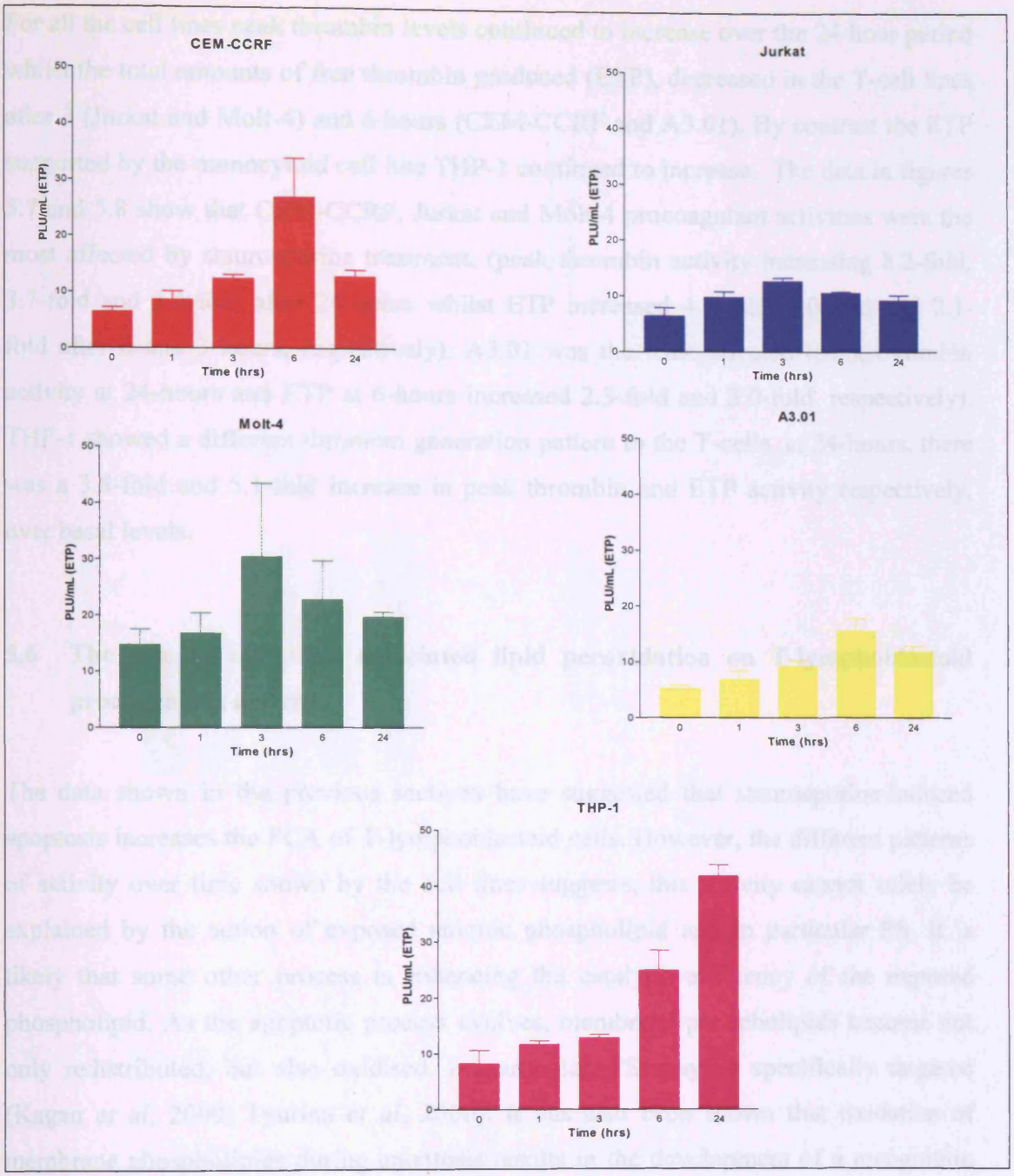



***Figure 5.8: Effect of staurosporine (1 μM) induced apoptosis over 24 hours on the endogenous thrombin potential (ETP) of the cell lines (4 x $10^6$/mL) converted to PLU/mL, by comparison with a phospholipid standard. The data shown are the mean of n = 3 and 95% CI.***

For all the cell lines peak thrombin levels continued to increase over the 24-hour period whilst the total amounts of free thrombin produced (ETP), decreased in the T-cell lines after 3 (Jurkat and Molt-4) and 6 hours (CEM-CCRF and A3.01). By contrast the ETP supported by the monocytoid cell line THP-1 continued to increase. The data in figures 5.7 and 5.8 show that CEM-CCRF, Jurkat and Molt-4 procoagulant activities were the most affected by staurosporine treatment, (peak thrombin activity increasing 8.2-fold, 3.7-fold and 3.4-fold after 24-hours whilst ETP increased 4.2-fold, 2.0-fold and 2.1-fold after 6 and 3-hours, respectively). A3.01 was the least affected (peak thrombin activity at 24-hours and ETP at 6-hours increased 2.3-fold and 3.0-fold, respectively). THP-1 showed a different thrombin generation pattern to the T-cells, at 24-hours, there was a 3.8-fold and 5.1-fold increase in peak thrombin and ETP activity respectively, over basal levels.

## 5.6 The role of apoptosis associated lipid peroxidation on T-lymphoblastoid procoagulant activity

The data shown in the previous sections have suggested that staurosporine-induced apoptosis increases the PCA of T-lymphoblastoid cells. However, the different patterns of activity over time shown by the cell lines suggests, this activity cannot solely be explained by the action of exposed anionic phospholipid and in particular PS. It is likely that some other process is enhancing the catalytic efficiency of the exposed phospholipid. As the apoptotic process evolves, membrane phospholipids become not only redistributed, but also oxidised. In particular, PS may be specifically targeted (Kagan *et al*, 2000; Tyurina *et al*, 2000). It has also been shown that oxidation of membrane phospholipids during apoptosis results in the development of a recognition ligand for macrophages that can be inhibited by mAbs that only recognise oxidised forms of choline-containing phospholipids of apoptotic but not viable cells (Chang *et al*, 1999). A model for the lipid oxidation signalling pathway in apoptosis has been proposed (Kagan *et al*, 2000). In this model apoptotic stimuli cause mitochondrial

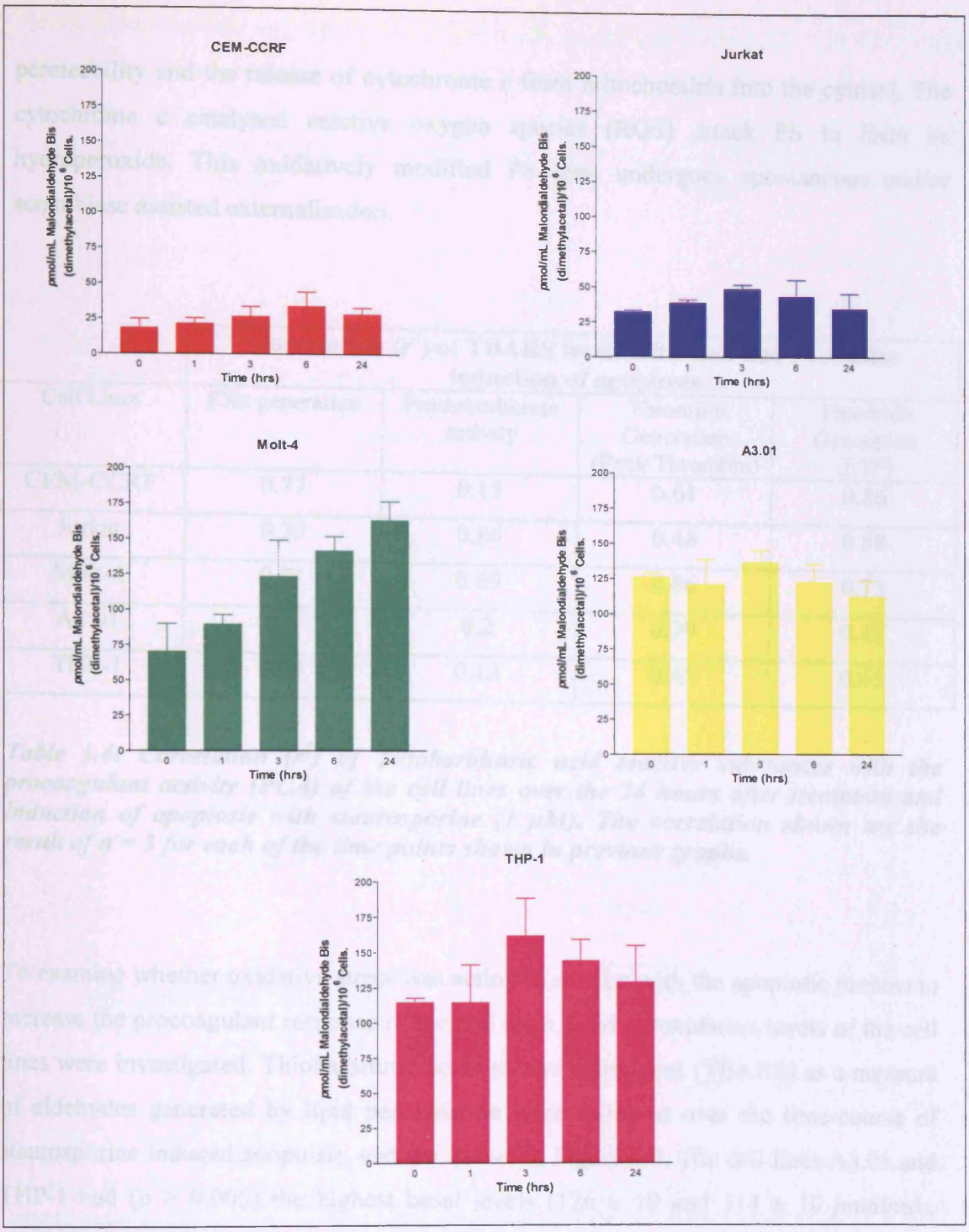



***Figure 5.9: Measurement of the level of oxidative stress during staurosporine (1 μM) induced apoptosis. Thiobarbituric acid reactive substances (TBARS) were measured at 0, 1, 3, 6 and 24 hours after treatment. The data shown are the mean of n = 3 and 95% CI.***

permeability and the release of cytochrome *c* from mitochondria into the cytosol. The cytochrome *c* catalysed reactive oxygen species (ROS) attack PS to form its hydroperoxide. This oxidatively modified PS then undergoes spontaneous and/or scramblase assisted externalisation.

| | **Correlation ($r^2$) of TBARS levels with Cell line PCA after induction of apoptosis.** | | | |
|---|---|---|---|---|
| Cell Lines | FXa generation | Prothrombinase activity | Thrombin Generation (Peak Thrombin) | Thrombin Generation (ETP) |
| CEM-CCRF | 0.77 | 0.15 | 0.61 | 0.86 |
| Jurkat | 0.39 | 0.86 | 0.48 | 0.88 |
| Molt-4 | 0.81 | 0.69 | 0.86 | 0.75 |
| A3.01 | 0.3 | 0.2 | 0.39 | 0.43 |
| THP-1 | 0.24 | 0.13 | 0.45 | 0.45 |

***Table 5.4: Correlation ($r^2$) of Thiobarbituric acid reactive substances with the procoagulant activity (PCA) of the cell lines over the 24 hours after treatment and induction of apoptosis with staurosporine (1 μM). The correlation shown are the result of n = 3 for each of the time points shown in previous graphs.***

To examine whether oxidative stress was acting in concert with the apoptotic process to increase the procoagulant response of the cell lines, lipid peroxidation levels of the cell lines were investigated. Thiobarbituric acid reactive substances (TBARS) as a measure of aldehydes generated by lipid peroxidation were followed over the time-course of staurosporine induced apoptosis, and are shown in Figure 5.9. The cell lines A3.01 and THP-1 had ($p > 0.005$) the highest basal levels (126 ± 10 and 114 ± 10 *p*mol/mL, respectively), whilst CEM-CCRF had the lowest basal levels (18 ± 3 *p*mol/mL). Jurkat and Molt-4 had basal levels of 33 ± 10 and 71 ± 9 *p*mol/mL, respectively. Apart from the cell line A3.01, TBARS increased after induction of apoptosis with the highest

levels being reached at 3 hours for CEM-CCRF, Jurkat and THP-1 (27 ± 3; 49 ± 1 and 162 ± 12 *p*mol/mL, respectively) and at 24 hours for Molt-4 (63 ± 6 *p*mol/mL).

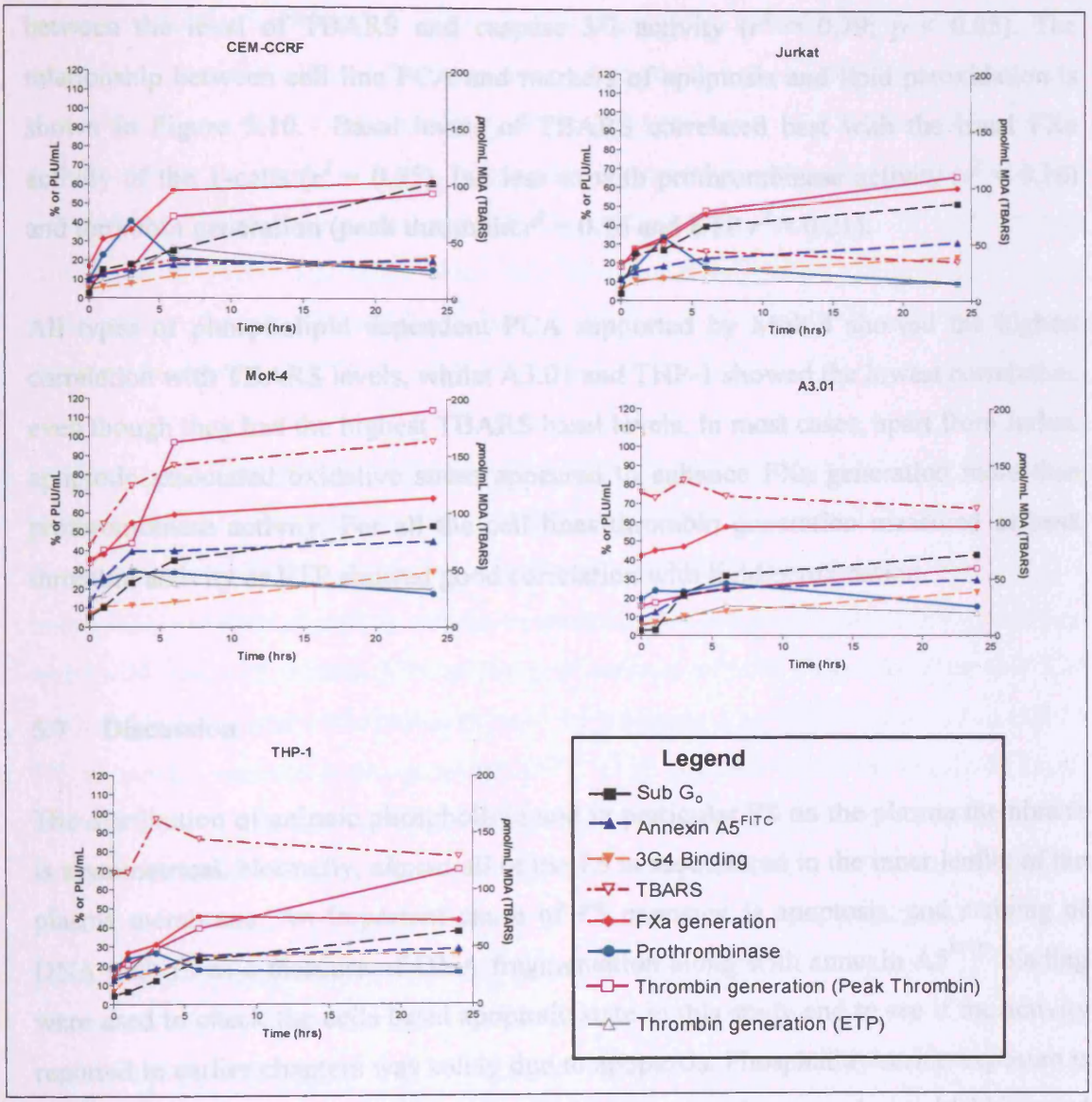



***Figure 5.10: The time course of the procoagulant activities (PLU/mL) of the cell lines (4 x $10^6$/mL), compared with a markers of apoptosis (% of cells in sub $G_0$; % of cells binding annexin $A5^{FITC}$ or 3G4) and lipid peroxidation (TBARS) after treatment with staurosporine (1 μM). The data shown are the mean of n = 3.***

Over the 24-hour time course of apoptosis induction there was significant correlation between the level of TBARS and caspase 3/7 activity ($r^2$ = 0.79; $p < 0.05$). The relationship between cell line PCA and markers of apoptosis and lipid peroxidation is shown in Figure 5.10. Basal levels of TBARS correlated best with the basal FXa activity of the T-cells ($r^2$ = 0.55), but less so with prothrombinase activity ($r^2$ = 0.16) and thrombin generation (peak thrombin $r^2$ = 0.16 and ETP $r^2$ = 0.21).

All types of phospholipid dependent PCA supported by Molt-4 showed the highest correlation with TBARS levels, whilst A3.01 and THP-1 showed the lowest correlation, even though they had the highest TBARS basal levels. In most cases, apart from Jurkat, apoptotic associated oxidative stress appeared to enhance FXa generation more than prothrombinase activity. For all the cell lines thrombin generation measured as peak thrombin activity or ETP showed good correlation with lipid peroxidation.

## 5.7 Discussion

The distribution of anionic phospholipid and in particular PS on the plasma membrane is asymmetrical. Normally, almost all of the PS is sequestered in the inner leaflet of the plasma membrane. An important cause of PS exposure is apoptosis, and staining of DNA with PI as a measure of DNA fragmentation along with annexin $A5^{FITC}$ binding were used to check the cells basal apoptotic state in this study and to see if the activity reported in earlier chapters was solely due to apoptosis. Phosphatidylserine exposure is an early event in apoptosis, preceding DNA fragmentation, membrane blebbing and loss of membrane integrity (Martin *et al*, 1995). Exposed PS on the outer-leaflet of the plasma membrane of apoptotic cells is thought to serve as a trigger for macrophage recognition and phagocytosis (Fadok *et al*, 1992; Bennett *et al*, 1995).

Cells undergoing apoptosis have been reported to be thrombogenic, with a significant correlation between thrombin generation supported by tumour cells having TF activity and the degree of apoptosis (Wang *et al*, 2001), but to date little data is available for T-lymphoblastoid cells. Also few studies have looked at the thrombogenic mechanisms in any detail. Less than 5% of the population of the cell lines studied were undergoing spontaneous apoptosis as measured by DNA fragmentation, and the level of annexin $A5^{FITC}$ binding was below 13.0%. Since the level of FXa generation supported by the T-cell lines did not correlate ($r^2$ = 0.17) with the highest levels of spontaneous apoptosis, it was concluded that the TF-independent PCA supported by the unstimulated T-lymphoblastoid cell lines studied was not solely due to PS exposure resulting from apoptosis.

In this study, staurosporine a topoisomerase II inhibitor was used to stimulate apoptosis through the mitochondrial pathway and activation of caspase-3. Staurosporine treatment caused an increase in caspase activity with the highest levels being seen at 6 hours. The numbers of cells in sub-$G_0$ increased over the time course of the experiment and by 24 hours more than 37% of the cells were in sub-$G_0$. Taken together this data confirms that apoptosis was being induced by staurosporine. The hallmark of apoptosis PS exposure, measured as annexin $A5^{FITC}$ and anti-PS antibody (3G4) binding, increased over time. The trend was for the percentage of cells binding annexin $A5^{FITC}$ to be higher than for 3G4. This is probably due to the specificity of 3G4 for PS and the fact that annexin A5 can bind to other anionic phospholipids such as phosphatidylethanolamine, phosphatidylinositol, or sphingomyelin (Andree *et al*, 1990; Blackwood & Ernst, 1990). Also malondialdehyde adducts a major product of cell surface lipid peroxidation, bind annexin A5 in a calcium-dependent manner similar to PS (Balasubramanian *et al*, 2001), and this is a theme that will be returned to later in this discussion and in the next chapter.

The effect of staurosporine on the PCA of the cell lines was investigated over a 24 hour period. The procoagulant activity of TF on the cell surface of Jurkat and THP-1 increased over the 24 hours of experiment (172 ± 32 and 158 ± 16 %, respectively). It is

noteworthy that this increase in TF activity was not significantly different ($p > 0.05$) to that observed after Jurkat and THP-1 were treated with the calcium ionophore, A23187 (10 μM) as described in chapter 4. This is different to the results found when ionophore and staurosporine treated cells were compared using TF independent assays, and this is discussed later in this section. The procoagulant activity of TF on the cell surface is mostly dormant due to TF encryption (Bach *et al*, 1986). When alterations of the plasma membrane occur, TF is able to become procoagulant (de-encrypted). One explanation of these results is that TF encryption is a result of sequestration of PS, which acts as a co-factor of TF PCA, and the externalisation of PS during apoptosis places PS in close proximity to TF and enables its activation. This explanation is also supported by experiments showing that treatment of HL-60 cells by cytotoxic drugs induces the generation of potent TF PCA without the *de novo* synthesis of TF mRNA and protein (Fibach *et al*, 1985). It has also been reported that addition of annexin A5 can block 70 – 80% of apoptosis-associated TF activity, further supporting the hypothesis that PS is enhancing TF activity (Wang *et al*, 2001). When further TF inhibition experiments, using a thrombin generation assay were carried out in the presence of an anti-TF antibody or TFPI only 50 – 60% of thrombin generated during apoptosis was neutralised, leaving the pathway responsible for the remaining 40 – 50% of thrombin generation unaccounted for (Wang *et al*, 2001). The most likely explanation is that alterations of the cell membrane caused by apoptosis are contributing to this thrombin generation.

When the effect of apoptosis on TF-independent FXa generation and thrombin activation/generation supported by the cell lines was investigated, in all cases there was a significant increase ($p < 0.05$) in phospholipid activity over basal levels. This increase in activity could be prevented by the addition of Z-VAD-FMK, a general inhibitor of caspases. There was also good correlation ($r^2 > 0.7$) between "intrinsic tenase" generation and the percentage of cells binding annexin $A5^{FITC}$ and the number of cells in sub-$G_0$. The effect of staurosporine on PCA was greater for FXa generation than for prothrombinase activation. However, for both assays CEM-CCRF and Jurkat were the most affected, showing the highest increase in activity. When thrombin generation in

plasma was measured a similar pattern was observed. Staurosporine caused the biggest increase in peak thrombin activity in Molt-4 cells, followed by Jurkat, CEM-CCRF and THP-1 cell lines. The cell line A3.01 was the most resistant to staurosporine, with peak thrombin and ETP levels showing the lowest increases. The PCA of the cell line CEM-CCRF was the most susceptible to staurosporine treatment and showed the highest increases in activity. CEM-CCRF showed the lowest increase in the percentage of cells binding annexin $A5^{FITC}$ over the 24 hour period (10.4 ± 0.4 % increasing to 20.8 ± 0.5 %), whist Molt-4 having higher basal PCA showed an increase in the binding of annexin $A5^{FITC}$ from 12.2 ± 0.4 % to 45.0 ± 1.9 %. This suggests that the early stages of apoptosis are procoagulant due to PS exposure, but as the process continues the amount of PS exposed and its biochemical state becomes inhibitory.

The discrepancy between the maximal phospholipid dependent procoagulant activities of the cell lines after treatment with calcium ionophore or staurosporine is an interesting observation. It appears that calcium ionophore treatment allows PS to be externalised, however, the higher levels of PCA observed after staurosporine treatment suggests that another biological process associated with apoptosis is either enhancing PS catalytic efficiency, and/or allowing other phospholipids to provide a potent coagulation surface. The most likely candidate was thought to be lipid peroxidation. Data presented in table 5.4 shows that there is a relationship between oxidative stress levels measured as TBARS and the phospholipid PCA supported by T-lymphoblastoid cells, especially Molt-4 and Jurkat. There is evidence in the literature that apoptosis can be associated with selective oxidation of specific phospholipid classes, most notably PS (Tyurina *et al*, 2000), and in some cases this can amplify the "eat me" signal recognised by macrophages. Triggering of the death receptor Fas on Jurkat cells results in the generation of reactive oxygen species with oxidation and externalisation of PS but not of the other major aminophospholipid, phosphatidylethanolamine. These cells were readily ingested by macrophages, whilst Raji cells, which are defective for Fas-induced PS exposure, were not engulfed (Kagan *et al*, 2002). It has also been shown using paraquat treated 32D cells that PS oxidation precedes PS externalisation as measured by annexin A5 binding and DNA fragmentation (Fabisiak *et al*, 1997). This data led to

the conclusion that although apoptosis is a procoagulant process it is greatly enhanced by the synergistic effect of lipid peroxidation.

T-lymphoblastoid PCA therefore appears to be due in part to the externalisation of PS via the apoptotic pathway, but this PCA is greatly enhanced by lipid peroxidation.

## 5.8 Summary

Apoptosis is involved in many biological processes and under certain circumstances such as chemotherapy can be thrombogenic. However, flow cytometry studies using propidium iodide and annexin $A5^{FITC}$ showed that the basal PCA of the cell lines, although linked to PS exposure, was not the result of apoptosis. Staurosporine induced apoptosis enhanced the procoagulant activities of all the cell lines and was found to be associated with membrane PS exposure. There was a direct correlation between thrombin generation and the degree of apoptosis, measured by the percentage of cells in sub-$G_0$. The TF clotting activity of the TF bearing cells Jurkat and THP-1 increased over the treatment period due to 'de-encryption' of TF caused by the exposure of PS. Previous reports had shown that PS-activated TF accounts for only 50 - 60% of thrombin generated (Wang *et al*, 2001). The remainder of the activity in TF-bearing cells and all the activity of the other cell lines (CEM-CCRF, Molt-4 and A3.01) was via the intrinsic pathway. The increase in FXa generation, prothrombin activation and thrombin generation correlated with the degree of apoptosis. Bell shaped activity curves of the cell lines were observed for prothrombinase and thrombin generation (ETP) PCA. This could be due to the amount of PS exposed hindering the assembly of the prothrombinase complex, or that the PS being exposed over the treatment period is "damaged" by the apoptotic process. The higher procoagulant activities observed after staurosporine treatment than seen after calcium ionophore treatment (at a concentration that caused maximal PS exposure) was found to be due to the additive effect of lipid peroxidation. This was shown by the correlation of TBARS levels with cell line PCA, and is probably due to the selective oxidation of anionic phospholipid in particular PS.

# SPECIAL NOTE

## ITEM SCANNED AS SUPPLIED
## PAGINATION IS AS SEEN

**Sanj Raut**

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**From:** Sanj Raut

**Sent:** 01 June 2005 17:40

**To:** Carl Dolman

**Subject:** Internal Audit Non Compliance - help!

Hi Carl

I came to see you earlier but unfotunately just missed you.

In a recent Internal Practical Audit, we had a non compliance in which the auditors were able to print three different version (nos) of the same SOP of the workbench.

Please can you help. I wonder if we could get together sometime Thursday (tomorrow) if you are available, to sort this out.

Thanks very much

Cheers
Sanj

Chapter

6

# INVESTIGATION OF THE EFFECT OF LIPID PEROXIDATION ON T-LYMPHOBLASTOID PROCOAGULANT ACTIVITY

## 6.1 Introduction

Conditions that can initiate and potentiate lipid peroxidation include hyperoxia, hypoxia and ischaemic insults, copper or iron toxicity and antioxidant deficiencies. Imbalance between pro-oxidant and antioxidant forces in which the former dominate are broadly termed as "oxidative stress", of which lipid peroxidation is a very important manifestation. The plasma membranes of most cells contain both polyunsaturated and monounsaturated lipids, which are susceptible to oxidative damage by free radical processes or electrophilic addition reactions. Such oxidative modification of the lipids is thought to lead to alterations in structure and fluidity of the membrane, affecting cell function, and can ultimately lead to loss of integrity (Halliwell & Gutteridge, 1990). Taken together with the susceptibility of lipoproteins to oxidative damage and the production of potent oxidising agents by platelets and other cells *in vivo* it could be possible that degradation of membrane phospholipid by reactive oxygen species (ROS) might contribute to or affect the cell line procoagulant activity.

Lymphocytes are one of the cell types that can be exposed to large amounts of oxidants because they migrate and function in inflammatory areas or in the region of tumour growth. Neutrophils and other phagocytic cells in these areas can generate oxidants in the process accompanying phagocytosis. Therefore the redox status in the lymphocytes has to be strictly regulated by the cells, and any breakdown in this process would lead to the peroxidation becoming self-perpetuating and could lead to procoagulant changes in the membrane surface. This chapter describes experiments investigating the effect of ROS induced by hydrogen peroxide ($H_2O_2$) and copper sulphate ($CuSO_4$) on T-lymphoblastoid procoagulant activity.

## 6.2 Measurement of the basal levels of lipid peroxidation in the cell lines and correlation with unstimulated T-lymphoblastoid procoagulant activity

Preliminary studies reported in chapter 5 showed that thiobarbituric acid reactive substances (TBARS) increased in apoptosis. To further explore whether the basal level of phospholipid-dependent PCA supported by the cell-surface of the cell lines was due to oxidative stress, the baseline levels of lipid peroxidation were measured. The assays chosen to measure lipid peroxidation were conjugated diene/triene measurement on Folch extracts of the cell lines, and TBARS levels released by the cells after being heated with thiobarbituric acid under acidic conditions. Full details of these methods are described in Chapter 2. The total phospholipid content of the cell line Folch extracts were estimated using the fluorometric assay described in section 2.9.2, so that conjugated diene and triene measurements could be quantified per μg of phospholipid. The results of these assays were compared with basal levels of intrinsic FXa generation (Table 6.1).

| Cell Line | CD ($A_{234}$)/pg phospholipid | CT ($A_{274}$)/pg phospholipid | TBARS (pmol MDA/$10^6$ cells) | FXa generation (PLU/mL) |
|---|---|---|---|---|
| **CEM-CCRF** | 9.7 ± 1.0 | 7.5 ± 1.0 | 18.0 ± 3.0 | 15 ± 3.6 |
| **Jurkat** | 18.5 ± 2.0 | 8.4 ± 1.0 | 28.0 ± 8.0 | 20 ± 3.9 |
| **Molt-4** | 20.9 ± 2.0 | 9.4 ± 1.0 | 76.0 ± 8.0 | 34 ± 5.7 |
| **A3.01** | 13.5 ± 1.0 | 4.3 ± 1.0 | 186.0 ± 20 | 40 ± 5.4 |
| **THP-1** | 8.6 ± 1.0 | 1.2 ± 1.0 | 118.0 ± 17 | 12 ± 2.0 |

***Table 6.1: Basal lipid peroxidation levels of the cell lines: comparison of conjugated diene/triene, TBARS and intrinsic FXa generation levels (mean of n = 3 and 95% CI). Abbreviations used in the table: CD, conjugated dienes; CT, conjugated trienes; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.***

Factor Xa generation was chosen because it was the assay where the majority of the cell lines were the most active. The basal FXa activity of the cell lines correlated well with basal TBARS levels ($r^2$ = 0.58), but less so with unstimulated conjugated diene ($r^2$ = 0.31) and conjugated triene ($r^2$ = 0.06) levels. When only the data for T-cells was

analysed the correlation with FXa generation increased for TBARS ($r^2$ = 0.83) and conjugated triene ($r^2$ = 0.20) levels, but decreased for conjugated dienes ($r^2$ = 0.12).

## 6.3 Induction of lipid peroxidation by $H_2O_2/CuSO_4$

The cell lines (all >90% viable by trypan blue exclusion) were subjected to oxidative stress and ROS degradation of cell membrane phospholipid by incubating intact cells (50 x $10^6$/mL in RPMI) with $H_2O_2$ (4 mM) and $CuSO_4$ (40 μM). As a negative control 50 mM PBS (pH 7.4) was added to the cells without $H_2O_2$ and $CuSO_4$. The presence of $CuSO_4$ acts as a catalyst, increasing the rate of breakdown of $H_2O_2$ (a reactive oxygen species) to form the phospholipid damaging hydroxyl radical. Cell lines were exposed to this oxidative treatment for 0.5, 1, 2, and 4 hours at 37°C, after which time, lipid peroxidation was stopped by the addition of 20 nM butylated hydroxytoluene (BHT). The cells were then washed three times in serum-free RPMI medium to remove traces of oxidants, and resuspended in 50 mM PBS (pH 7.4) containing 1% (w/v) human albumin before being used in assays. The cell concentration was adjusted to give a final concentration of 4 x $10^6$/mL in the various tests for PCA unless stated otherwise. For analysis of conjugated dienes/trienes, after exposure to oxidative treatment, lipids were extracted by the Folch method (Folch *et al*, 1957).

## 6.4 The peroxidation profiles of the cell lines: Conjugated dienes/trienes, and thiobarbituric acid reactive substances (TBARS) and changes in phosphatidylserine exposure, after $H_2O_2/CuSO_4$ induced oxidative stress

To confirm that treatment of the cell lines with 4 mM $H_2O_2$ and 40 μM $CuSO_4$ induced oxidative stress, markers of lipid peroxidation were measured over the time-course of treatment. Methods for the analysis of oxidative damage to lipids differ considerably in the sensitivity and information offered, and can be divided into two groups: those that measure initial products of oxidative attack, such as conjugated dienes and lipid

hydroperoxides, and those that measure lipid-oxidation breakdown products such as aldehydes (TBARS).

The term conjugated diene refers to two double bonds separated by a single bond, which are not present in unoxidised unsaturated fatty acids. The formation of conjugated dienes is generally accepted as evidence for lipid peroxidation, and is due to the conjugated diene structure, which in the presence of oxygen can form hydroperoxides. Conjugated dienes can be detected spectrophotometrically since they have a characteristic absorption at 234 nm. The application of measurement of conjugated diene formation by UV absorbance in this study was to monitor changes in absorbance of isolated membrane fractions in dynamic experiments rather than absolute quantification. The TBARS test is probably the most widely used single assay for measuring lipid peroxidation. The lipid material is heated with thiobarbituric acid (TBA) at low pH, and the formation of a pink chromogen is measured at 532 nm. The chromogen is formed by reaction of one molecule of malondialdehyde (MDA) with two molecules of TBA. Some of the MDA detected in the TBA test is formed during the peroxidation process itself, but most is generated by decomposition of lipid peroxides during the acid-heating stage of the test. In effect the peroxidation process beginning in the reaction mixture is amplified in the assay, thus making it very sensitive. The measurement of MDA by the TBARS test was used in this study to indirectly assess lipid peroxidation or susceptibility to peroxidation of the cell line membrane phospholipid. Detailed reviews of the advantages and disadvantages of the techniques available to measure lipid peroxidation have been reported elsewhere (Gutteridge & Halliwell, 1990; Moore & Roberts, 1998).

Finally the possible procoagulant effect of lipid peroxidation due to its effect on phospholipid and/or exposure of PS was investigated by flow-cytometry using annexin $A5^{FITC}$ and the PS antibody 3G4 to probe for PS exposure.

### 6.4.1 Conjugated dienes and trienes production by T-lymphoblastoid cells

The peroxidation profile of the cell lines is shown in Figure 6.1. The band of conjugated dienes at 234 nm is displayed as a shoulder over the higher band at about 220 nm due to the end absorption of non-peroxidised lipid. Lipid peroxidation of the cell lines resulted in an absorption peak at 234 nm at time 0 hours, which first increased with time (up to 1 hour) and then gradually decreased, representing the formation and decomposition of conjugated dienes. The absorption peak at 234 nm of the cell lines shifted with time toward 215 nm. This shift may be explained by formation of alkenals and hydroxyalkenals, typical products of lipid peroxidation (Kim & LaBella, 1987). Two other absorption peaks at 270 and 280 nm were observed, which probably represents the formation of conjugated trienes (Kim & LaBella, 1987). Conjugated triene formation started later and was lower than conjugated diene formation (Figure 6.2). Molt-4 and the monocytoid cell line THP-1 showed the highest lipid peroxidation profile, whilst A3.01 showed the lowest. Jurkat and CEM-CCRF showed intermediate peroxidation profiles (Figure 6.2). Figure 6.1 also shows the UV difference spectrum of tris buffer alone and $H_2O_2/CuSO_4$ with tris buffer as a control. The reasoning for this is that both lipids and $H_2O_2/CuSO_4$ are UV absorbing.

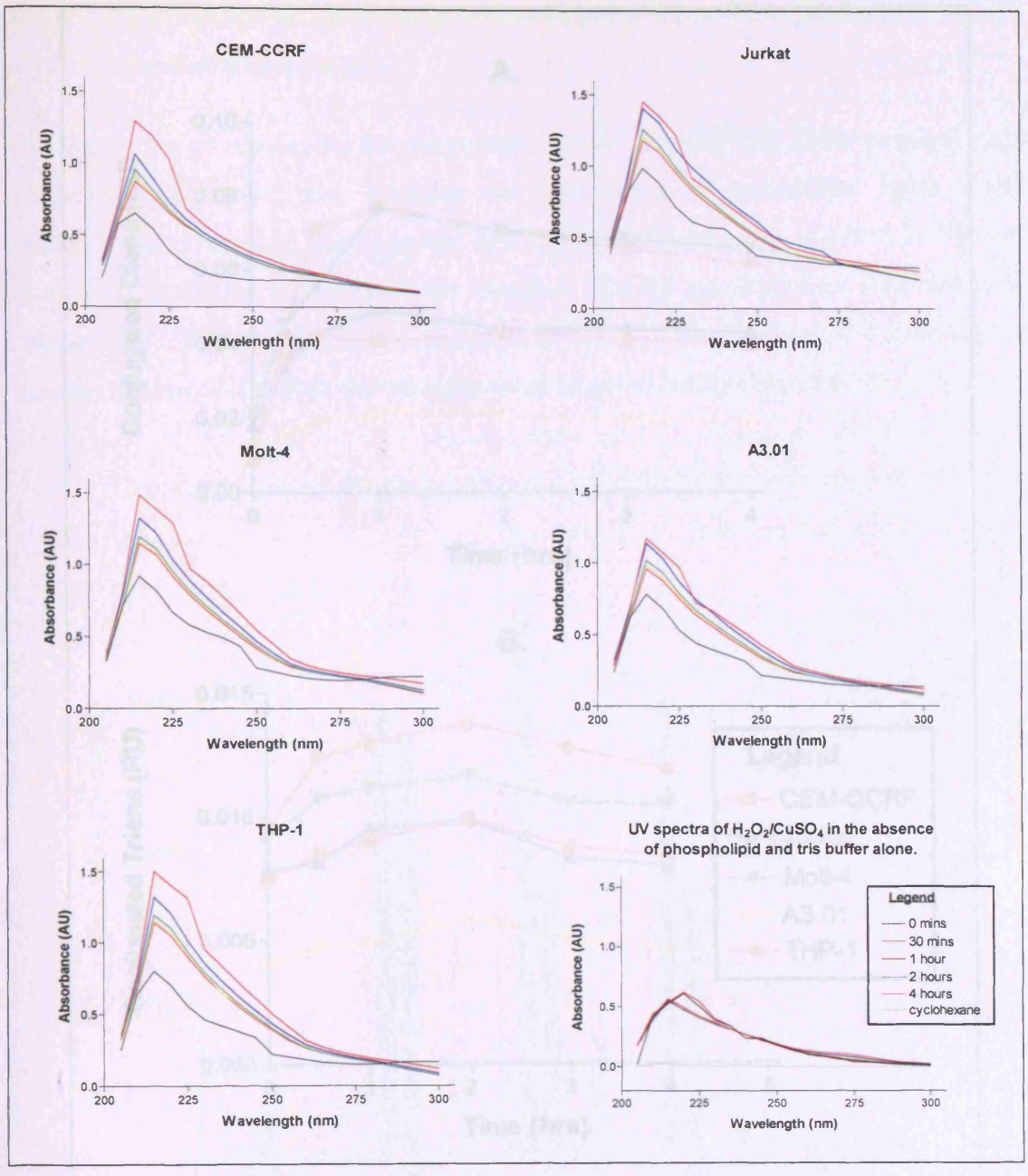



***Figure 6.1: Change of the ultraviolet (UV) difference spectrum of Folch extracts of cell line phospholipid. Conjugated diene/triene formation was monitored over range 205 – 300 nm. Peroxidation profiles are shown as UV absorption units (AU) against wavelength (nm) for the time points indicated.***

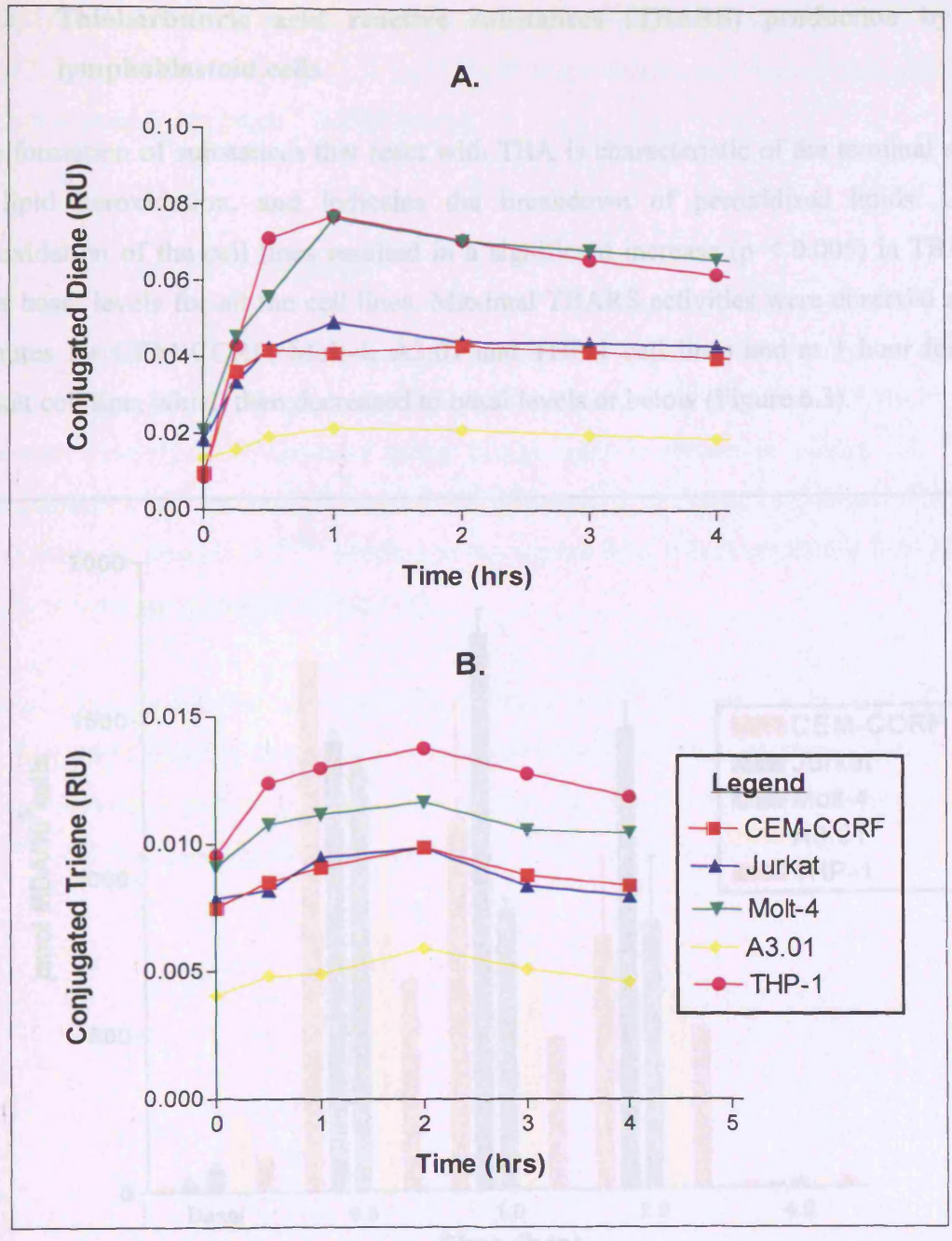



***Figure 6.2: The time scale of (A) conjugated diene (CD) and (B) conjugated triene (CT) formation in the cell line Folch extracts. Lipid peroxidation was induced by 4 mM $H_2O_2$ and 40 µM $CuSO_4$. Ultraviolet absorption at 234 nm (CD formation) and 274 nm (CT formation) is plotted against peroxidation time. The data shown is the mean of n = 3.***

### 6.4.2 Thiobarbituric acid reactive substances (TBARS) production by T-lymphoblastoid cells

The formation of substances that react with TBA is characteristic of the terminal stage of lipid peroxidation, and indicates the breakdown of peroxidised lipids. Lipid peroxidation of the cell lines resulted in a significant increase ($p < 0.005$) in TBARS over basal levels for all the cell lines. Maximal TBARS activities were observed at 30 minutes for CEM-CCRF, Molt-4, A3.01 and THP-1 cell lines and at 1 hour for the Jurkat cell line, which then decreased to basal levels or below (Figure 6.3).

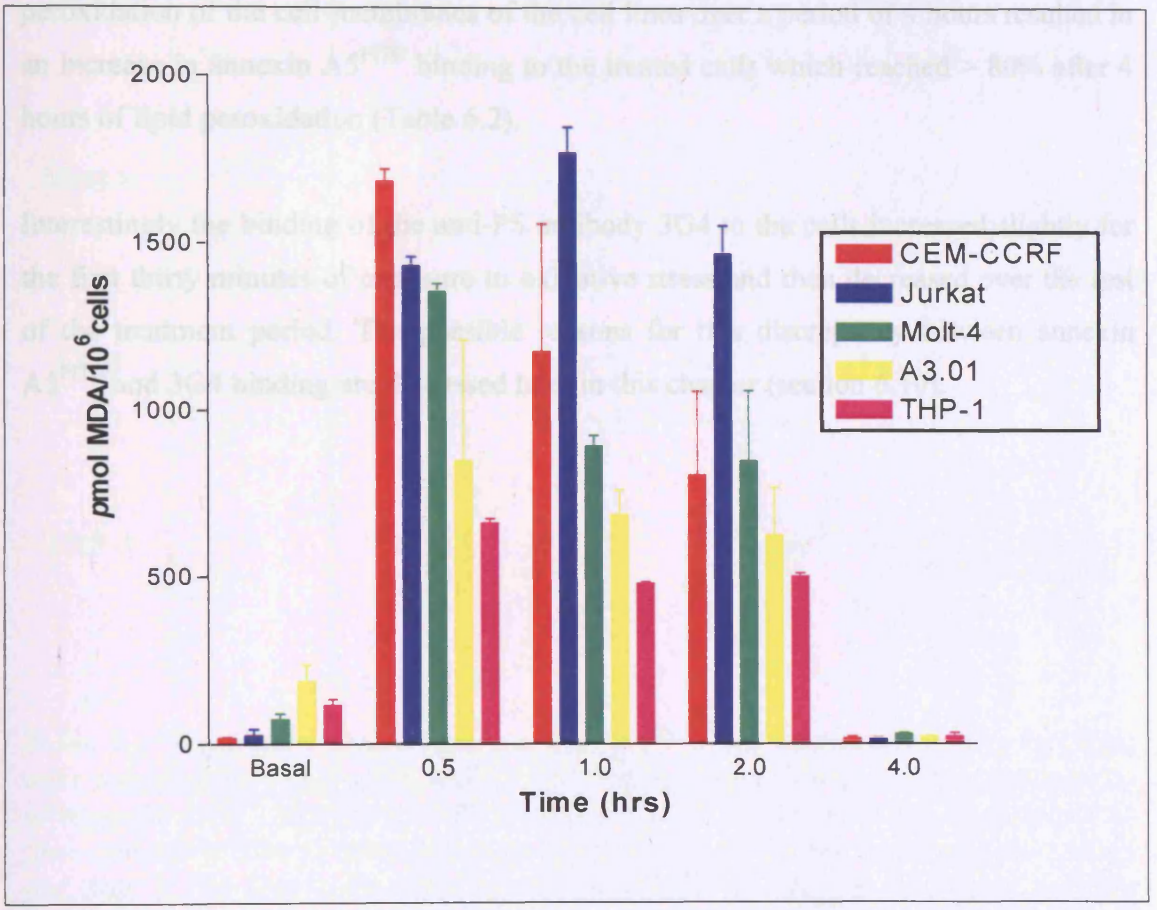



***Figure 6.3: The levels of thiobarbituric acid reactive substances (TBARS) produced by exposure of the cell lines to lipid peroxidation (4 mM $H_2O_2$ and 40 µM $CuSO_4$) after 4 hours. TBARS levels were quantified by comparison against a malondialdehyde (MDA) standard. The results are the mean of n = 3; ± 95% CI.***

The increase in TBARS activity over basal levels at 30 minutes for CEM-CCRF, Molt-4, A3.01 and THP-1 was 94, 18, 5 and 6-fold, respectively. At 1 hour Jurkat showed a 64-fold increase over basal TBARS levels.

### 6.4.3 Annexin $A5^{FITC}$ binding

The possible effect of lipid peroxidation on anionic phospholipid exposure and activity was investigated by flow-cytometry using annexin A5 and the anti-PS antibody 3G4, an example showing data obtained using Jurkat cells is shown in Figure 6.4. Lipid peroxidation of the cell-membranes of the cell lines over a period of 4 hours resulted in an increase in annexin $A5^{FITC}$ binding to the treated cells which reached > 80% after 4 hours of lipid peroxidation (Table 6.2).

Interestingly the binding of the anti-PS antibody 3G4 to the cells increased slightly for the first thirty minutes of exposure to oxidative stress and then decreased over the rest of the treatment period. The possible reasons for this discrepancy between annexin $A5^{FITC}$ and 3G4 binding are discussed later in this chapter (section 6.10).

| Cell Line | Detection Method | Time (hrs) 0 | 0.5 | 1 | 2 | 4 |
|---|---|---|---|---|---|---|
| **CEM-CCRF** | $A5^{FITC}$ | 8.9 ± 0.4 | 12.5 ± 1.0 | 21.8 ± 1.8 | 27.2 ± 2.4 | 94.9 ± 0.5 |
| | *p* = | | 0.005 | 0.0087 | 0.0079 | <0.0005 |
| | 3G4 | 5.2 ± 0.4 | 5.4 ± 0.5 | 1.8 ± 0.2 | 0.8 ± 0.2 | 0.0 |
| | *p* = | | 0.737 | NS | NS | NS |
| **Jurkat** | $A5^{FITC}$ | 11.7 ± 1.3 | 28.4 ± 1.7 | 48.5 ± 1.2 | 68.4 ± 3.3 | 98.9 ± 0.2 |
| | *p* = | | 0.002 | 0.0002 | 0.0010 | < 0.0005 |
| | 3G4 | 5.9 ± 0.9 | 10.1 ± 1.7 | 2.6 ± 0.5 | 1.0 ± 0.2 | 0.0 |
| | *p* = | | 0.055 | NS | NS | NS |
| **Molt-4** | $A5^{FITC}$ | 12.8 ± 0.7 | 13.1 ± 0.5 | 19.3 ± 1.6 | 28.2 ± 3.2 | 98.3 ± 0.7 |
| | *p* = | | 0.042 | 0.0060 | 0.0105 | < 0.0005 |
| | 3G4 | 6.5 ± 0.8 | 11.5 ± 1.2 | 2.6 ± 0.3 | 0.6 ± 0.3 | 0.0 |
| | *p* = | | 0.0017 | NS | NS | NS |
| **A3.01** | $A5^{FITC}$ | 9.0 ± 0.8 | 16.0 ± 0.6 | 30.1 ± 2.4 | 41.4 ± 2.8 | 86.7 ± 0.6 |
| | *p* = | | 0.001 | 0.0026 | 0.0028 | < 0.0005 |
| | 3G4 | 7.0 ± 1.1 | 8.3 ± 1.5 | 2.9 ± 0.9 | 1.2 ± 0.3 | 0.0 |
| | *p* = | | 0.030 | NS | NS | NS |
| **THP-1** | $A5^{FITC}$ | 10.7 ± 0.9 | 12.4 ± 0.3 | 26.1 ± 1.1 | 38.0 ± 1.6 | 89.7 ± 0.7 |
| | *p* = | | 0.079 | 0.0001 | 0.0007 | < 0.0005 |
| | 3G4 | 6.3 ± 0.5 | 11.8 ± 0.9 | 3.7 ± 1.0 | 2.1 ± 0.9 | 0.0 |
| | *p* = | | 0.0018 | NS | NS | NS |

***Table 6.2: Lipid peroxidation and annexin $A5^{FITC}$ and 3G4 binding: The cell lines were treated with 4 mM $H_2O_2$ and 40 μM $CuSO_4$ for up to 4 hours. The binding (%) of annexin $A5^{FITC}$ and the anti-PS antibody 3G4 to treated cells was investigated by flow cytometry. The p-value indicates differences between the treatment time-points and time 0. The data is the mean of n = 3 and 95% CI. NS = no significant increase in binding.***

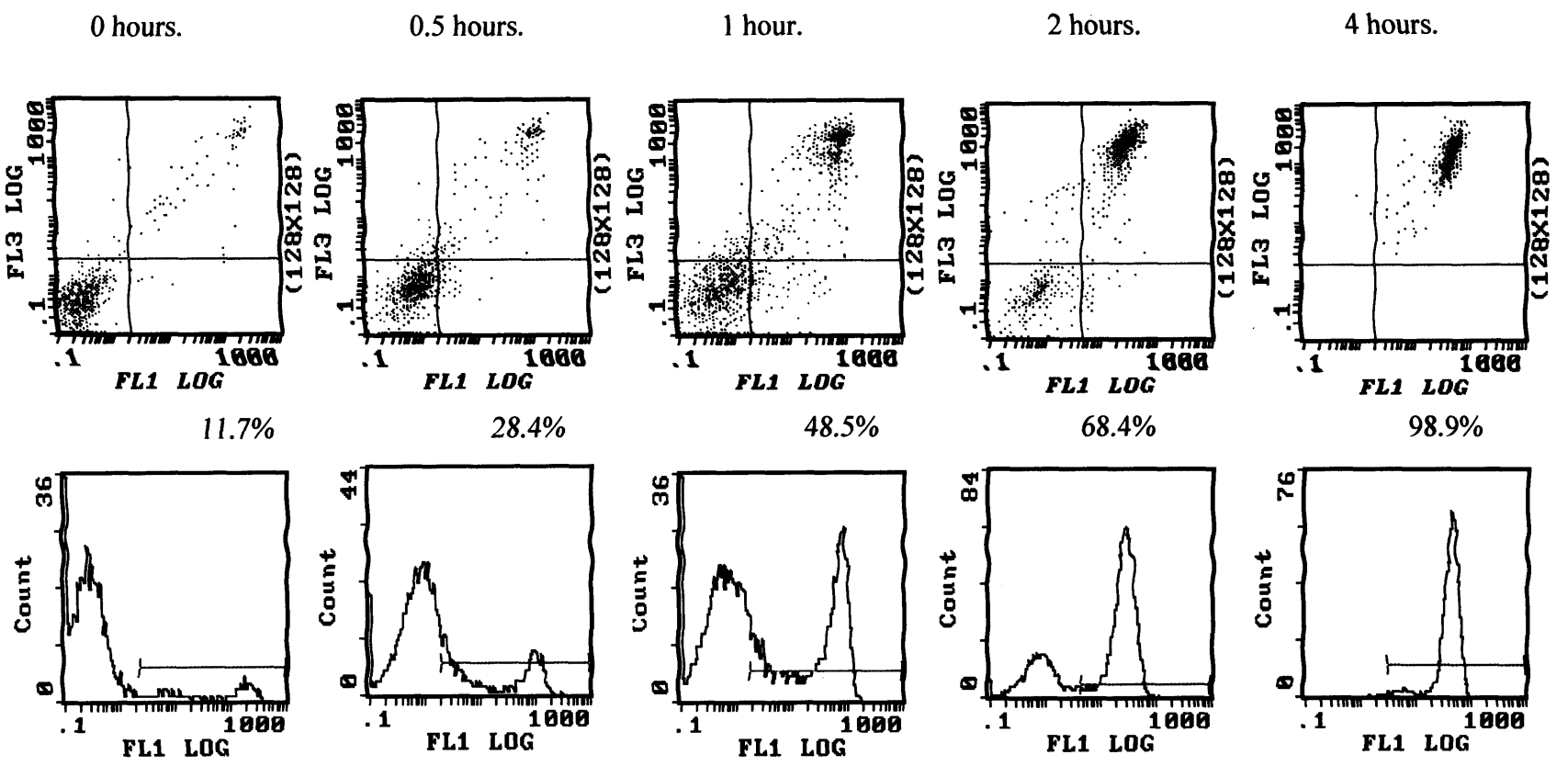



*Figure 6.4: Representative dot plot and histogram data showing the increase (%) in binding of annexin A5$^{FITC}$ to Jurkat cells after exposure to lipid peroxidation (4 mM $H_2O_2$ and 40 µM $CuSO_4$) for 4 hours.*

## 6.5 The effect of lipid peroxidation induced by $H_2O_2/CuSO_4$ on T-lymphoblastoid procoagulant activity

The cell lines were subjected to oxidative stress induced for 4 hours by $H_2O_2/CuSO_4$, and their respective procoagulant activities were studied over time.

### 6.5.1 Effect of lipid peroxidation on TF activity

Tissue factor clotting activity of a suspension of intact cells was assayed to explore whether there was TF activation on the cell membrane after exposure to oxidative stress. This would be of importance for the thrombin generation assay where high levels of TF might affect the assay. Only Jurkat and THP-1 cell lines showed a significant increase in TF clotting activity following $H_2O_2/CuSO_4$ treatment (Figure 6.5). Over the 4-hour treatment period there was a 4.1 and 3.3-fold increase in the TF clotting activity over basal levels supported by Jurkat and THP-1 cells respectively.

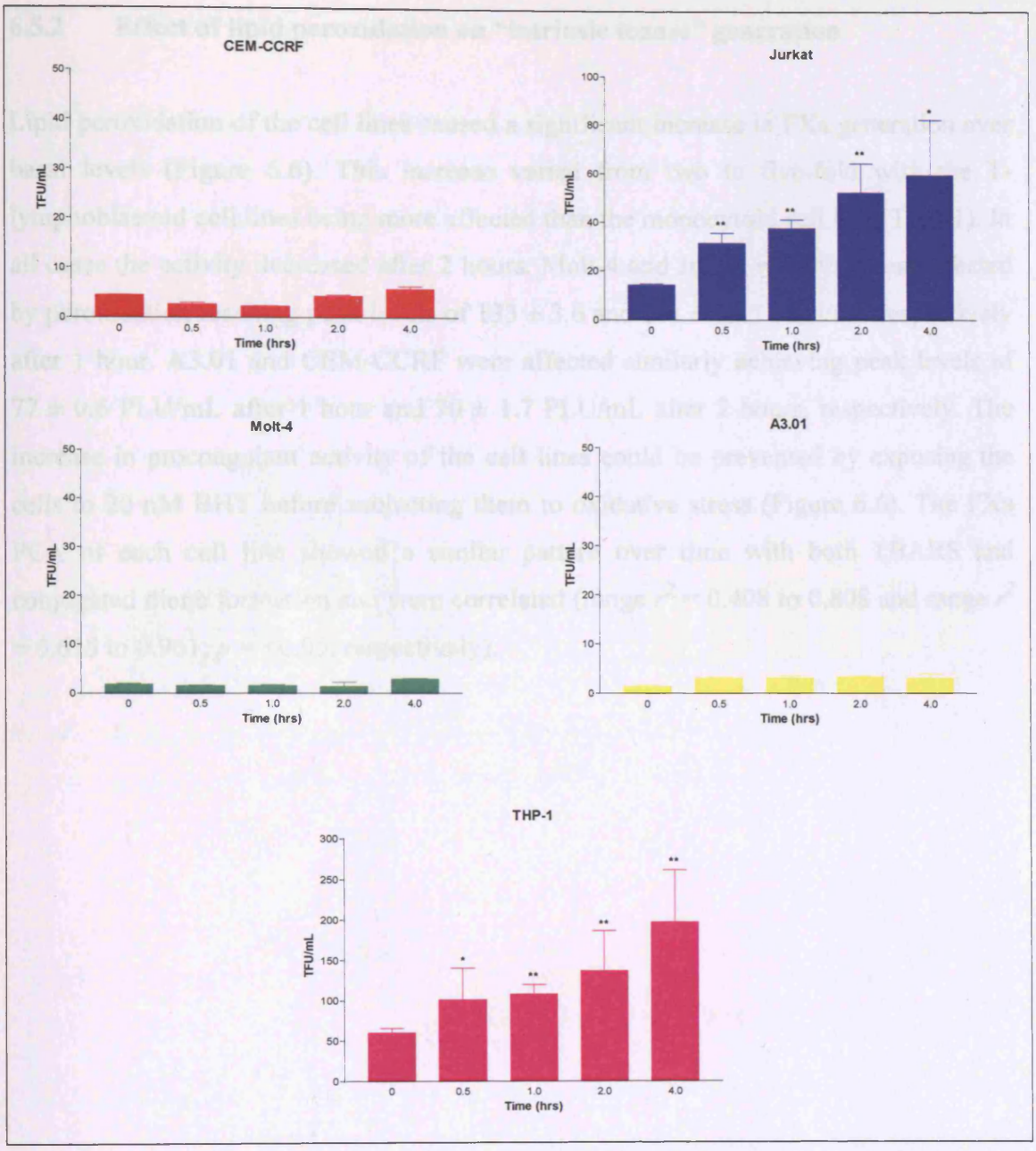



***Figure 6.5: Effect of $H_2O_2$ (4 mM) and $CuSO_4$ (40 μM) induced oxidative stress on clotting tissue factor activity of the cell lines (4 x $10^6$/mL). Factor Xa generation was measured at 0, 0.5, 1, 2 and 4 hours after treatment. At each time point the reaction was stopped by the addition of BHT (20 nM). The data shown is expressed as TFU/mL by comparison with a tissue factor standard curve, and is the mean of n = 3 and 95% CI, *p < 0.05, **p < 0.005 compared with time 0 (hrs).***

### 6.5.2 Effect of lipid peroxidation on "intrinsic tenase" generation

Lipid peroxidation of the cell lines caused a significant increase in FXa generation over basal levels (Figure 6.6). This increase varied from two to five-fold with the T-lymphoblastoid cell lines being more affected than the monocytoid cell line (THP-1). In all cases the activity decreased after 2 hours. Molt-4 and Jurkat were the most affected by peroxidation reaching peak levels of 133 ± 3.6 and 101 ± 13.5 PLU/mL respectively after 1 hour. A3.01 and CEM-CCRF were affected similarly achieving peak levels of 77 ± 0.6 PLU/mL after 1 hour and 70 ± 1.7 PLU/mL after 2 hours, respectively. The increase in procoagulant activity of the cell lines could be prevented by exposing the cells to 20 nM BHT before subjecting them to oxidative stress (Figure 6.6). The FXa PCA of each cell line showed a similar pattern over time with both TBARS and conjugated diene formation and were correlated (range $r^2$ = 0.408 to 0.808 and range $r^2$ = 0.615 to 0.961; $p$ = <0.05, respectively).

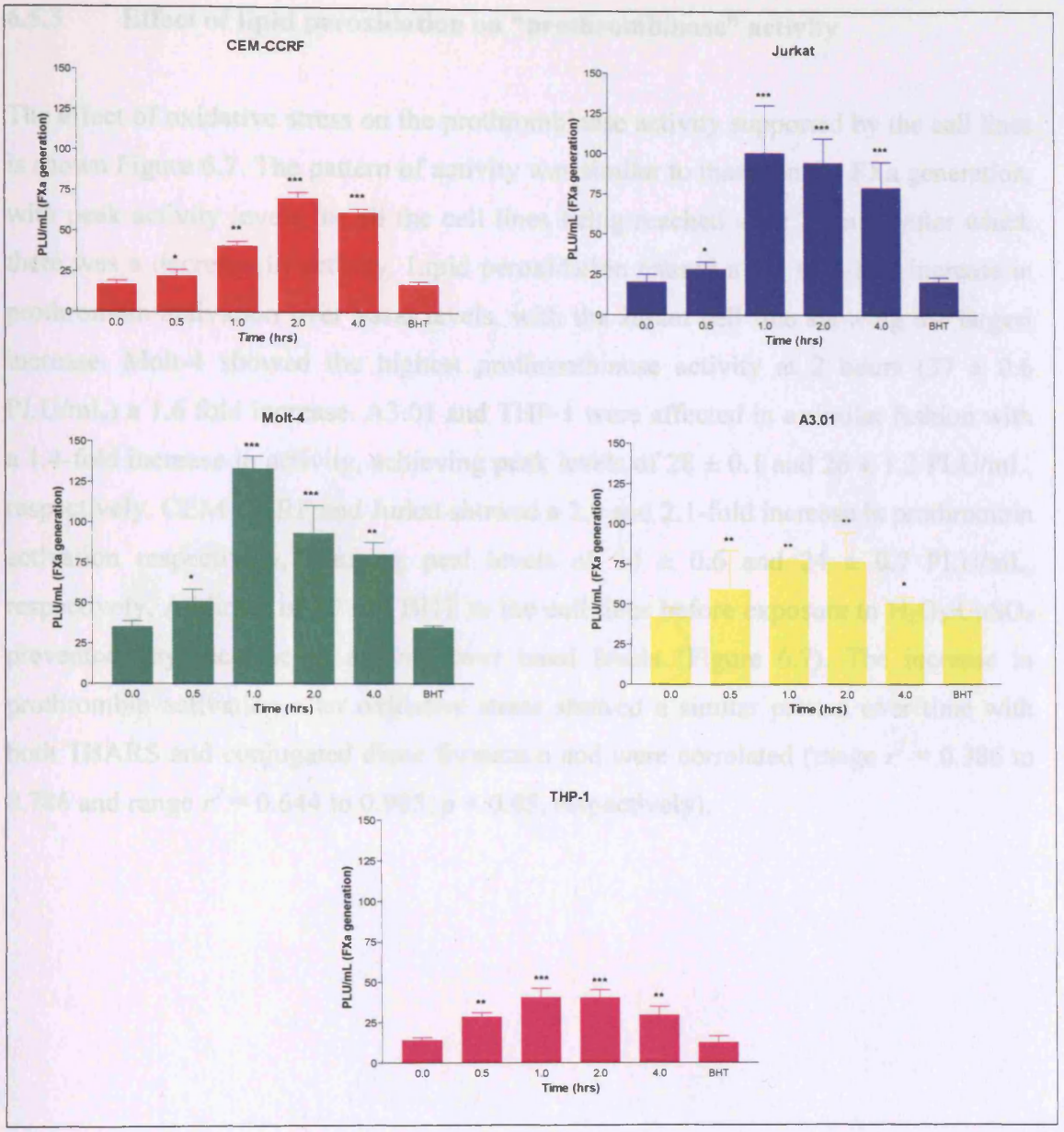



***Figure 6.6: Effect of $H_2O_2$ (4 mM) and $CuSO_4$ (40 µM) induced oxidative stress on the FXa generation procoagulant activity of the cell lines (4 x $10^6$/mL) over 4 hours. Control data is shown (BHT) where 20 nM BHT was added to the cell lines before being subjected to 4 hours of oxidative stress. At each time point the reaction was stopped by the addition of BHT (20 nM). The data shown is expressed as PLU/mL and is the mean of n = 3 and 95% CI, *p < 0.05, **p < 0.005, ***p < 0.0005 compared with time 0 (hrs).***

### 6.5.3 Effect of lipid peroxidation on "prothrombinase" activity

The effect of oxidative stress on the prothrombinase activity supported by the cell lines is shown Figure 6.7. The pattern of activity was similar to that seen for FXa generation, with peak activity levels for all the cell lines being reached after 2 hours, after which there was a decrease in activity. Lipid peroxidation caused a 1.4 to 2-fold increase in prothrombin activation over basal levels, with the Jurkat cell line showing the largest increase. Molt-4 showed the highest prothrombinase activity at 2 hours (37 ± 0.6 PLU/mL) a 1.6 fold increase. A3.01 and THP-1 were affected in a similar fashion with a 1.4-fold increase in activity, achieving peak levels of 28 ± 0.1 and 26 ± 1.2 PLU/mL, respectively. CEM-CCRF and Jurkat showed a 2.0 and 2.1-fold increase in prothrombin activation respectively, reaching peal levels of 20 ± 0.6 and 24 ± 0.7 PLU/mL, respectively. Addition of 20 nM BHT to the cell lines before exposure to $H_2O_2/CuSO_4$ prevented any increase in activity over basal levels (Figure 6.7). The increase in prothrombin activation after oxidative stress showed a similar pattern over time with both TBARS and conjugated diene formation and were correlated (range $r^2 = 0.386$ to 0.786 and range $r^2 = 0.644$ to 0.905; $p < 0.05$, respectively).

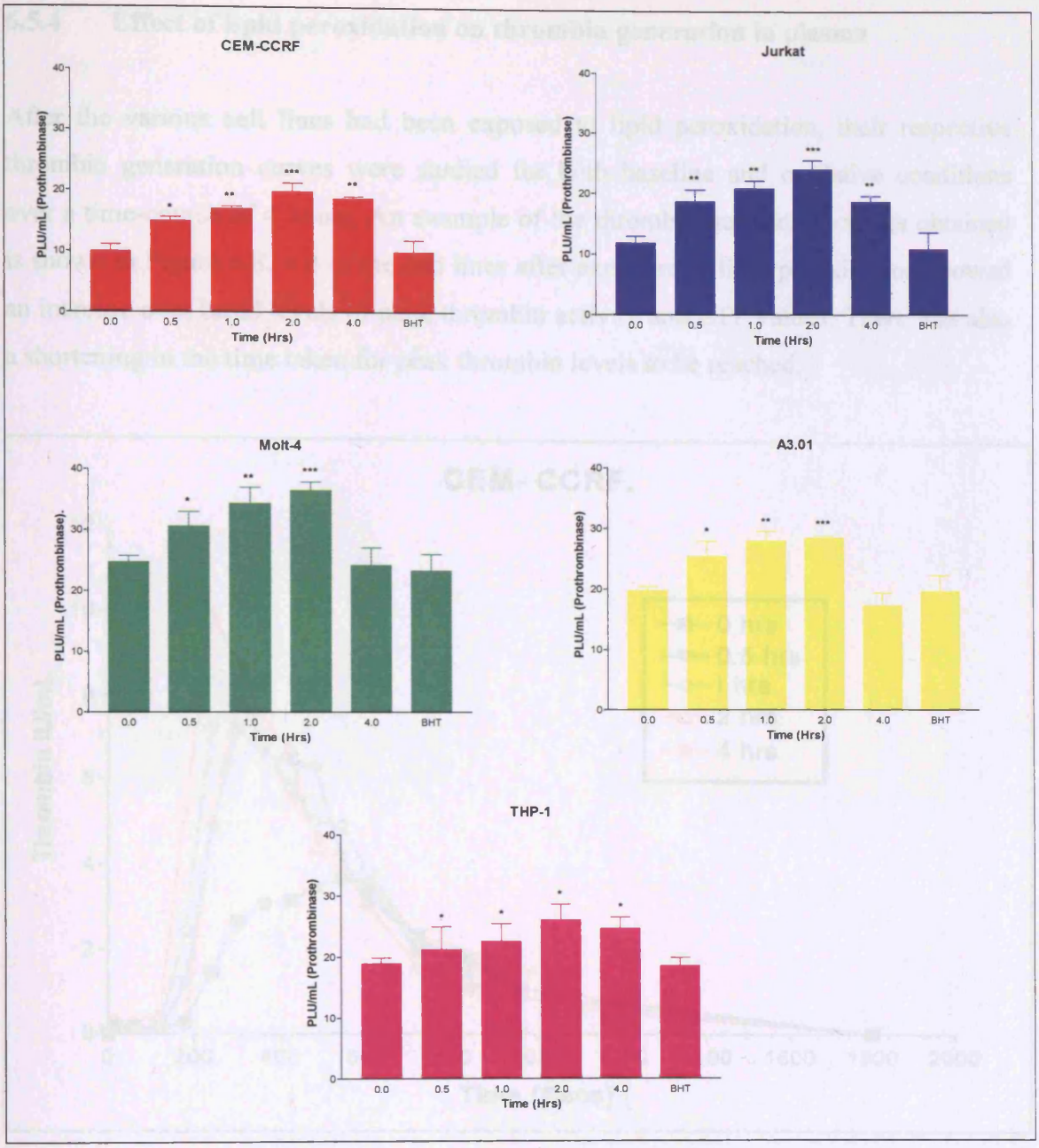



***Figure 6.7: Effect of $H_2O_2$ (4 mM) and $CuSO_4$ (40 µM) induced oxidative stress on the prothrombinase procoagulant activity of the cell lines (4 x $10^6$/mL) over 4 hours. Control data is shown (BHT) where 20 nM BHT was added to the cell lines before being subjected to 2 hours of oxidative stress. At each time point the reaction was stopped by the addition of BHT (20 nM). The data shown is expressed as PLU/mL and is the mean of n = 3 and 95% CI, *p < 0.05, **p < 0.005, ***p < 0.0005 compared with time 0 (hrs).***

### 6.5.4 Effect of lipid peroxidation on thrombin generation in plasma

After the various cell lines had been exposed to lipid peroxidation, their respective thrombin generation curves were studied for both baseline and oxidative conditions over a time-course of 4 hours. An example of the thrombin generation curves obtained is shown in Figure 6.8. All of the cell lines after exposure to lipid peroxidation showed an increase over basal levels of peak thrombin activity and ETP values. There was also a shortening in the time taken for peak thrombin levels to be reached.

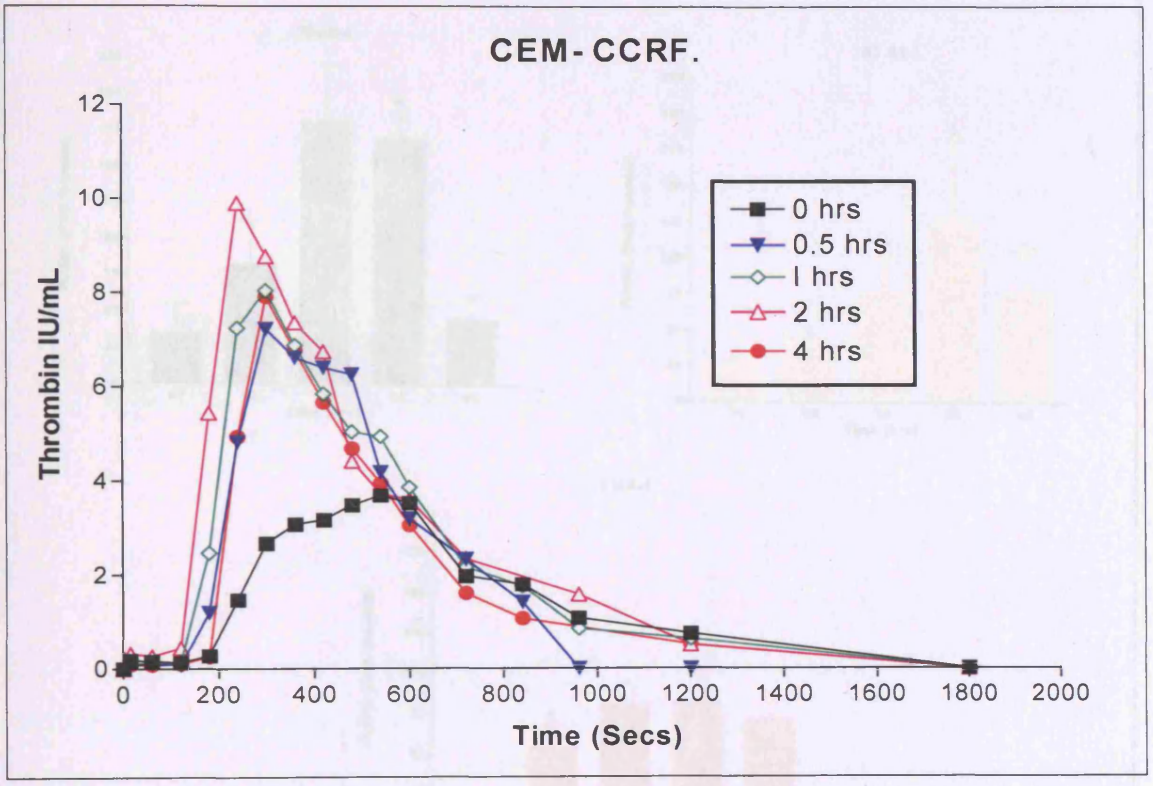



***Figure 6.8: A representative thrombin generation curve showing the effect of $H_2O_2$ (4 mM) and $CuSO_4$ (40 µM) induced oxidative stress on the procoagulant activity supported by the cell line CEM-CCRF (4 x $10^6$/mL), measured at 0, 0.5, 1, 2 and 4 hours after treatment. The data shown is the mean of n = 3.***

Figures 6.9 and 6.10 show thrombin generation responses (peak thrombin and ETP values) of the cell lines expressed as phospholipid equivalence after exposure to oxidative stress.

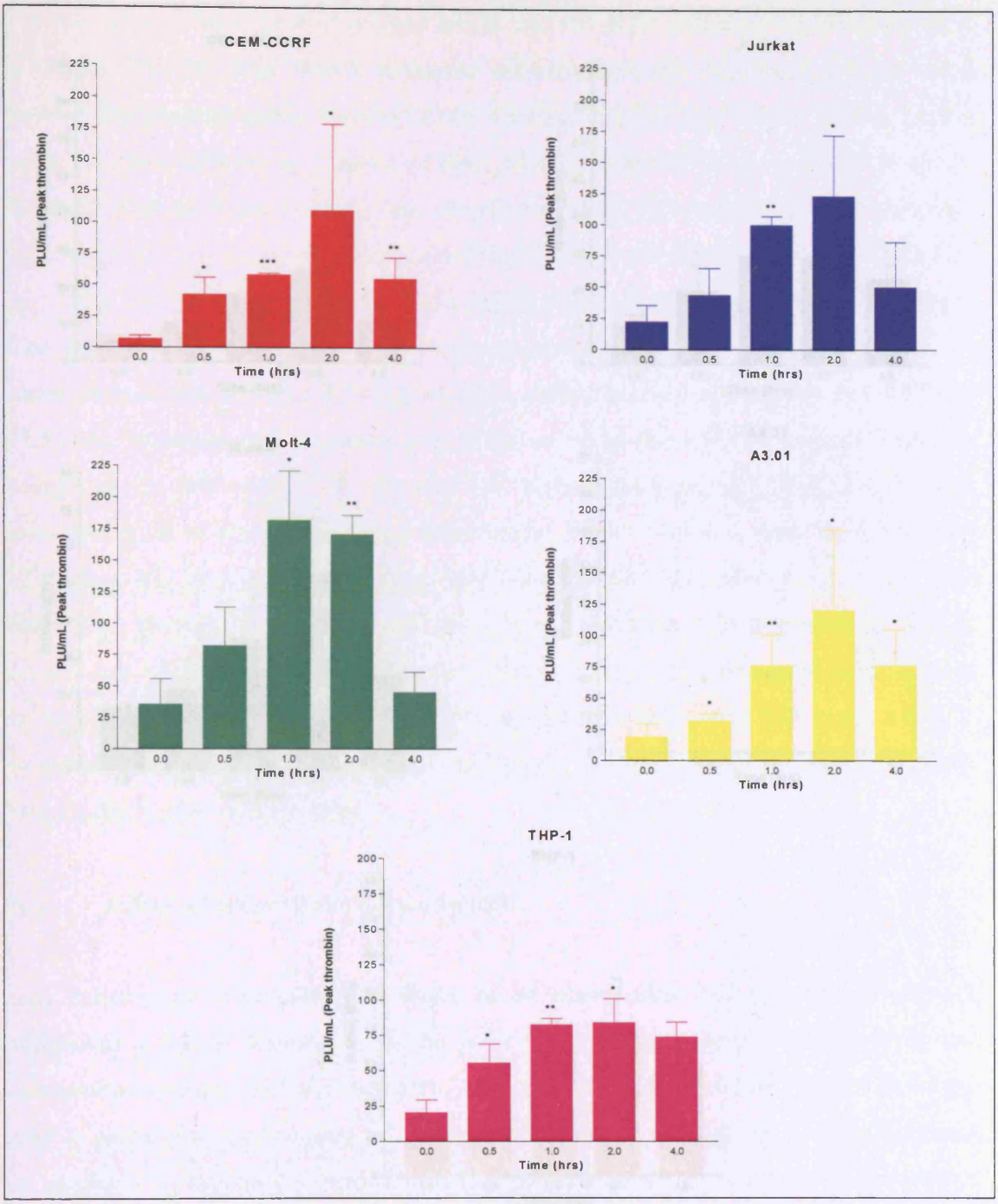



*Figure 6.9: Effect of oxidative stress induced by $H_2O_2$ (4 mM) and $CuSO_4$ (40 μM) on the thrombin generation (peak thrombin) activity of the cell lines (4 x $10^6$/mL) converted to PLU/mL by comparison with a phospholipid standard. Peak thrombin levels were measured at 0, 0.5, 1, 2 and 4 hours after treatment. The data shown is the mean of n = 3 and 95% CI, *p < 0.05, **p < 0.005, ***p < 0.0005 compared with time 0 (hrs).*

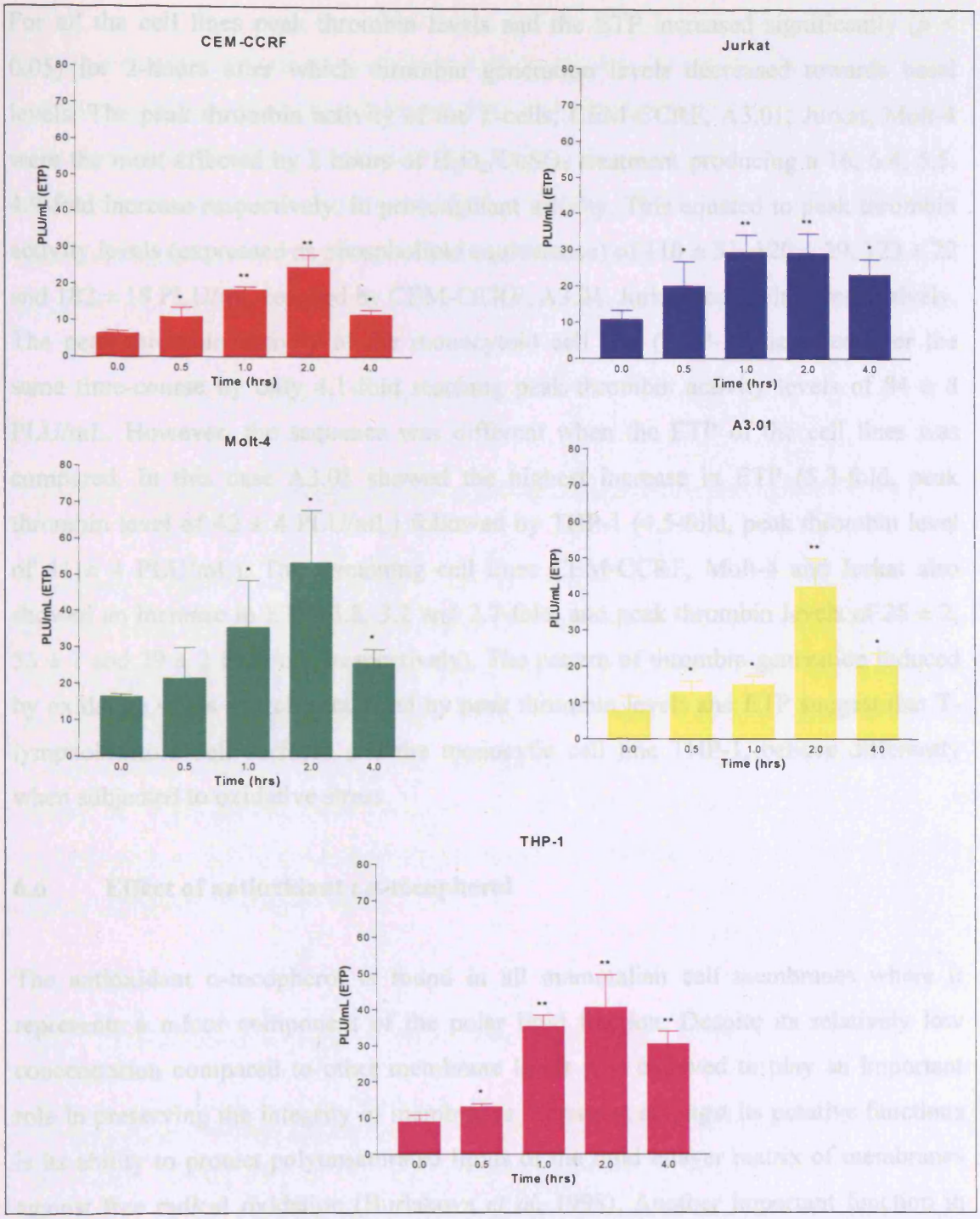



***Figure 6.10: Effect of oxidative stress induced by $H_2O_2$ (4 mM) and $CuSO_4$ (40 µM) on the endogeneous thrombin potential (ETP) of the cell lines (4 x $10^6$/mL) converted to PLU/mL by comparison with a phospholipid standard. ETP was measured at 0, 0.5, 1, 2 and 4 hours after treatment. The data shown is the mean of n = 3 and 95% CI, *p < 0.05, **p < 0.005, ***p < 0.0005 compared with time 0 (hrs).***

For all the cell lines peak thrombin levels and the ETP increased significantly ($p < 0.05$) for 2-hours after which thrombin generation levels decreased towards basal levels. The peak thrombin activity of the T-cells; CEM-CCRF, A3.01, Jurkat, Molt-4 were the most affected by 2 hours of $H_2O_2/CuSO_4$ treatment producing a 16, 6.4, 5.5, 4.9-fold increase respectively, in procoagulant activity. This equated to peak thrombin activity levels (expressed as phospholipid equivalence) of 110 ± 31, 120 ± 29, 123 ± 22 and 182 ± 18 PLU/mL reached by CEM-CCRF, A3.01, Jurkat and Molt-4, respectively. The peak thrombin activity of the monocytoid cell line (THP-1) increased over the same time-course by only 4.1-fold reaching peak thrombin activity levels of 84 ± 8 PLU/mL. However, the sequence was different when the ETP of the cell lines was compared. In this case A3.01 showed the highest increase in ETP (5.3-fold, peak thrombin level of 42 ± 4 PLU/mL) followed by THP-1 (4.5-fold, peak thrombin level of 41 ± 4 PLU/mL). The remaining cell lines CEM-CCRF, Molt-4 and Jurkat also showed an increase in ETP (3.8, 3.2 and 2.7-fold, and peak thrombin levels of 25 ± 2, 53 ± 7 and 29 ± 2 PLU/mL, respectively). The pattern of thrombin generation induced by oxidative stress and characterised by peak thrombin levels and ETP suggest that T-lymphoblastoid cell surfaces and the monocytic cell line THP-1, behave differently when subjected to oxidative stress.

## 6.6 Effect of antioxidant : α-tocopherol

The antioxidant α-tocopherol is found in all mammalian cell membranes where it represents a minor component of the polar lipid fraction. Despite its relatively low concentration compared to other membrane lipids it is believed to play an important role in preserving the integrity of membranes. Foremost amongst its putative functions is its ability to protect polyunsaturated lipids of the lipid bilayer matrix of membranes against free radical oxidation (Burlakova *et al*, 1998). Another important function in membranes is the formation of complexes between α-tocopherol and products of membrane lipid hydrolysis such as lysophospholipids and free fatty acids (Kagan, 1989). These complexes tend to stabilise membranes and prevent the detergent-like actions of lipid hydrolytic products on the membrane.

Tocopherols inhibit lipid peroxidation largely because they scavenge lipid peroxyl radicals much faster than these radicals can react with adjacent fatty acid side-chains or with membrane proteins. In addition they both quench and react with singlet $O_2$ and might protect against this species. α-tocopherol reacts slowly with $\mathbf{O_2^{\bullet-}}$ and, like most other biological molecules, at an almost diffusion- controlled rate with $\mathbf{OH^{\bullet}}$. The $\alpha Toc^{\bullet}$ radical might also react with further peroxyl radical to give non-radical products.

$$LO_2^{\bullet} + \alpha Toc\bullet \rightarrow \alpha TocOOL$$

Thus one molecule of α-tocopherol can probably terminate two peroxidation chains.

Tocopherols can reduce $Fe^{3+}$ to $Fe^{2+}$ and $Cu^{2+}$ to $Cu^{+}$ and thus at high concentrations they can exert pro-oxidant effects in some *in-vitro* systems. Blood tocopherol levels in humans varies from 18 μM in term infants to 20 μM in the elderly, achieving peak levels of 28 μM in children. From this data and a dose-response curve (data not shown) a α-tocopherol concentration of 25 μM was chosen to investigate inhibition of oxidative stress on FXa procoagulant activity. At this concentration in the absence of $H_2O_2/CuSO_4$ α-tocopherol had no significant effect ($p > 0.05$) on basal FXa generation levels supported by the cell lines.

## 6.7 The effect of α-tocopherol on T-lymphoblastoid procoagulant activity

To investigate whether the increase in procoagulant activity of the cell lines described previously in this chapter was due to lipid peroxidation, α-tocopherol was used to inhibit the effect of $H_2O_2/CuSO_4$. α-Tocopherol (25 μM) was added to the cell cultures 72 hours before the cells were used in coagulation studies. This period of 72 hours was chosen, as it would give time for α–tocopherol to be incorporated into the cell membrane and form complexes with lipid constituents.

Figure 6.11 shows that α-tocopherol ameliorated the increase in procoagulant activity of the cell lines by 37 – 79% in all the phospholipid-dependent coagulation assays used in this study.

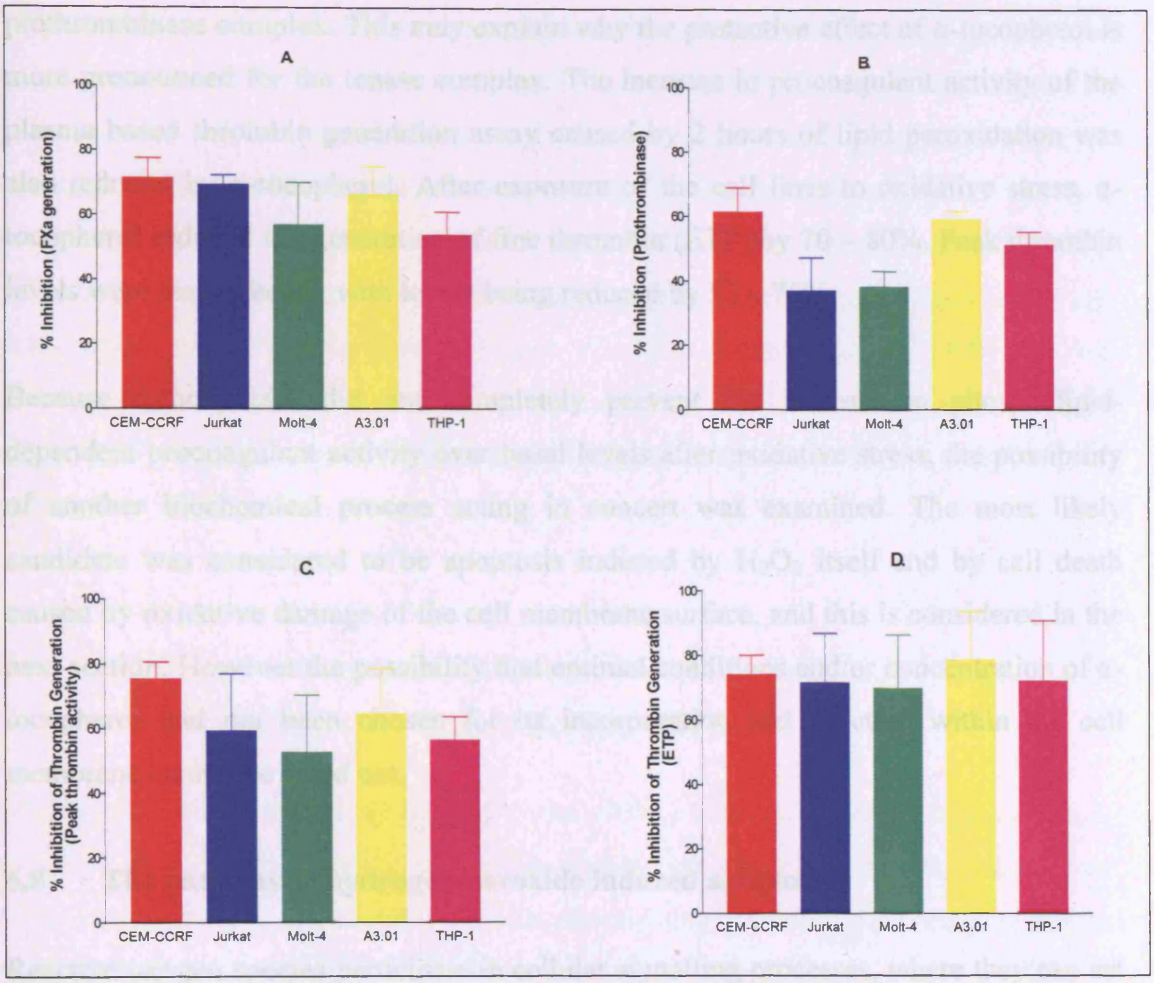



***Figure 6.11: Inhibition of procoagulant activity (PCA); (A) FXa generation (B) Prothrombinase activity and thrombin generation measured as (C) Peak thrombin or (D) endogeneous thrombin potential (ETP) by α-tocopherol (25 μM) after exposing the cell lines (4 x $10^6$/mL) to oxidative stress (4 mM $H_2O_2$ and 40 μM $CuSO_4$). The inhibition by α-tocopherol is reported as the % inhibition of the peak PCA of cells subjected to oxidative stress for 1 (A) or 2 hours (B, C, D) above basal levels. The data shown is the mean ± 95% CI; n = 3.***

The protective effect of α-tocopherol was greater for FXa generation than for prothrombinase activity. However, the increase in procoagulant activity following lipid peroxidation was much less for prothrombinase activity than for FXa generation (Figures 6.7 and 6.6), implying that lipid peroxidation products have less impact on the prothrombinase complex. This may explain why the protective effect of α-tocopherol is more pronounced for the tenase complex. The increase in procoagulant activity of the plasma based thrombin generation assay caused by 2 hours of lipid peroxidation was also reduced by α-tocopherol. After exposure of the cell lines to oxidative stress, α-tocopherol reduced the generation of free thrombin (ETP) by 70 – 80%. Peak thrombin levels were less affected, with levels being reduced by 52 – 75%.

Because α-tocopherol did not completely prevent the increase in phospholipid-dependent procoagulant activity over basal levels after oxidative stress, the possibility of another biochemical process acting in concert was examined. The most likely candidate was considered to be apoptosis induced by $H_2O_2$ itself and by cell death caused by oxidative damage of the cell membrane surface, and this is considered in the next section. However the possibility that optimal conditions and/or concentration of α-tocopherol had not been chosen for its incorporation and function within the cell membrane cannot be ruled out.

## 6.8 The pathway of hydrogen-peroxide induced apoptosis

Reactive oxygen species participate in cellular signalling processes, where they can act as second messengers of signal transduction (Nakamura *et al*, 1997). There are several species of ROS such as the short-lived superoxide ion ($O_2^-$) and the more stable hydrogen peroxide ($H_2O_2$). These molecules can form the highly reactive hydroxyl radicals ($OH^\cdot$) in the presence of transition metal ions (Sies, 1993). Moderately elevated concentrations of ROS have been found to induce apoptosis (Slater *et al*, 1995). Low micromolar concentrations of $H_2O_2$ do not affect the viability of the cells measured by PI exclusion, but concentrations around 100 μM kills 50% of the cells within 24 hours, almost exclusively by apoptosis ((Dumont *et al*, 1999). Hydrogen peroxide treatment of

T-cells leads to the generation of mitochondrial permeability transition pores, causing a rapid decrease of the mitochondrial transmembrane potential $\Delta\Psi_M$, followed by the release of cytochrome c and subsequent cleavage and activation of caspase-3. Inhibition of the mitochondrial permeability transition by bongkrekic acid (BA), or interference with the mitochondrial electron transport system by rotenone or medadione prevents the apoptotic effect of $H_2O_2$, whilst Antimycin A, an agent that increases the release of mitochondrial ROS, enhances apoptosis. Pharmacological and genetic inhibition of transcription factor NF-κB protects cells from $H_2O_2$-triggered cell apoptosis/death. The detrimental effect of NF-κB in mediating $H_2O_2$-induced apoptosis/cell death probably relies on the induced expression of death effector genes such as p53, which is NF-κB-dependently up-regulated in the presence of $H_2O_2$ (Dumont *et al*, 1999). To test whether apoptosis was contributing to the observed PCA after the cell lines were exposed to oxidative stress an anti-apoptotic agent was added to the cells prior to exposure to $H_2O_2$/CuSO4.

## 6.9 The effect on procoagulant activity of inhibition of apoptosis by bongkrekic acid after $H_2O_2/CuSO_4$ induced lipid peroxidation

Bongkrekic acid, an anti-apoptotic agent was added to the cells 1 hour before induction of oxidative stress. Bongkrekic acid is an inhibitor of adenine nucleotide translocase, which is a component of the mitochondrial permeability transition pore complex (Gross *et al*, 1999). Thus, bongkrekic acid prevents mitochondrial depolarisation, swelling, rupture of the mitochondria outer membrane, and the release of apoptogenic proteins such as cytochrome c and subsequent activation of poly (ADP – ribose) polymerase (PARP) and caspases. Bongkrekic acid was used at a final concentration of 50 μM. This concentration was chosen because it had been shown to prevent mitochondria membrane depolarisation in CEM-7 and Jurkat cells (Dumont *et al*, 1999; Scarlett *et al*, 2000) as well as being able to prevent staurosporine induced apoptosis in Jurkat cells (Scarlett *et al*, 2000).

To confirm that apoptosis was being inhibited, caspase 3/7 levels were measured in cell lysates that had been subjected to $H_2O_2/CuSO_4$ treatment for 2 hours with or without pre-treatment with BA. The lipid peroxidation associated increase in caspase 3/7 activity was inhibited by > 90% by the presence of 50 μM BA (Table 6.3). However, BA did not greatly reduce the % binding of annexin $A5^{FITC}$ to cells subjected to oxidative stress with only a maximum of 25% inhibition being observed (Table 6.3) implying that annexin $A5^{FITC}$ was not binding solely to anionic phospholipid and in particular PS exposed due to apoptosis.

| Treatment | Detection Method | CEM-CCRF | Jurkat | Molt-4 | A3.01 | THP-1 |
|---|---|---|---|---|---|---|
| **Untreated** | $A5^{FITC}$ | 8.9 ± 0.4 | 11.7 ± 0.3 | 12.8 ± 0.7 | 9.0 ± 0.8 | 10.7 ± 0.9 |
| | Caspase 3/7 | 17.2 ± 3.7 | 78.8 ± 15.7 | 70.0 ± 11.3 | 24.4 ± 8.7 | 22.7 ± 6.5 |
| **LP** | $A5^{FITC}$ | 27.2 ± 2.4 | 68.4 ± 3.3 | 28.2 ± 3.2 | 41.4 ± 2.8 | 38.0 ± 1.6 |
| | Caspase 3/7 | 84.2 ± 6.0 | 167.4 ± 7.7 | 168.3 ± 8.6 | 121.7 ± 17.3 | 102.2 ± 11.5 |
| **LP/BA** | $A5^{FITC}$ | 27.7 ± 1.5 | 51.2 ± 1.6 | 23.5 ± 2.6 | 35.8 ± 2.5 | 30.6 ± 3.2 |
| | % Inhibition* | 0 | 25.0 ± 2.7 | 16.2 ± 11.9 | 13.2 ± 11.5 | 19.6 ± 6.3 |
| | Caspase 3/7 | 18.5 ± 1.3 | 84.7 ± 6.5 | 78.7 ± 7.0 | 35.3 ± 4.6 | 30.6 ± 3.9 |
| | % Inhibition* | 98.1 ± 5.9 | 93.3 ± 8.6 | 91.1 ± 4.9 | 93.9 ± 7.5 | 90.1 ± 6.2 |

***Table 6.3: The inhibitory effect of 50 μM bongkrekic acid (BA) on caspase 3/7 activity (pmol DEVD cleavage/min/$10^6$ cells) and annexin $A5^{FITC}$ binding to cells exposed to lipid peroxidation (LP). The cell lines were treated with 4 mM $H_2O_2$ and 40 μM $CuSO_4$ for 2 hours. The binding (%) of annexin $A5^{FITC}$ was investigated by flow cytometry. The binding of annexin $A5^{FITC}$ to untreated cells as well as their caspase 3/7 activity is shown for comparison. *The effect of BA is reported as the % inhibition of annexin $A5^{FITC}$ binding and inhibition of caspase 3/7 activity. The data is the mean of n=3 and 95% CI.***

A typical example of the effect of BA on its own and in association with α-tocopherol on FXa generation, prothrombinase activation and thrombin generation is illustrated in Figure 6.12 using data obtained using the Jurkat cell line. For all the cell lines, the combined effect of BA and α-tocopherol in ameliorating the effect of lipid peroxidation

was additive, although the procoagulant activity was not reduced back to pre-treatment levels. This combined effect was the most pronounced for the plasma based thrombin generation assay, where BA increased inhibition levels attributed to α-tocopherol by an additional 12 – 32%. The least affected was prothrombinase activation where inhibition levels were only increased by an extra 2 – 9%.

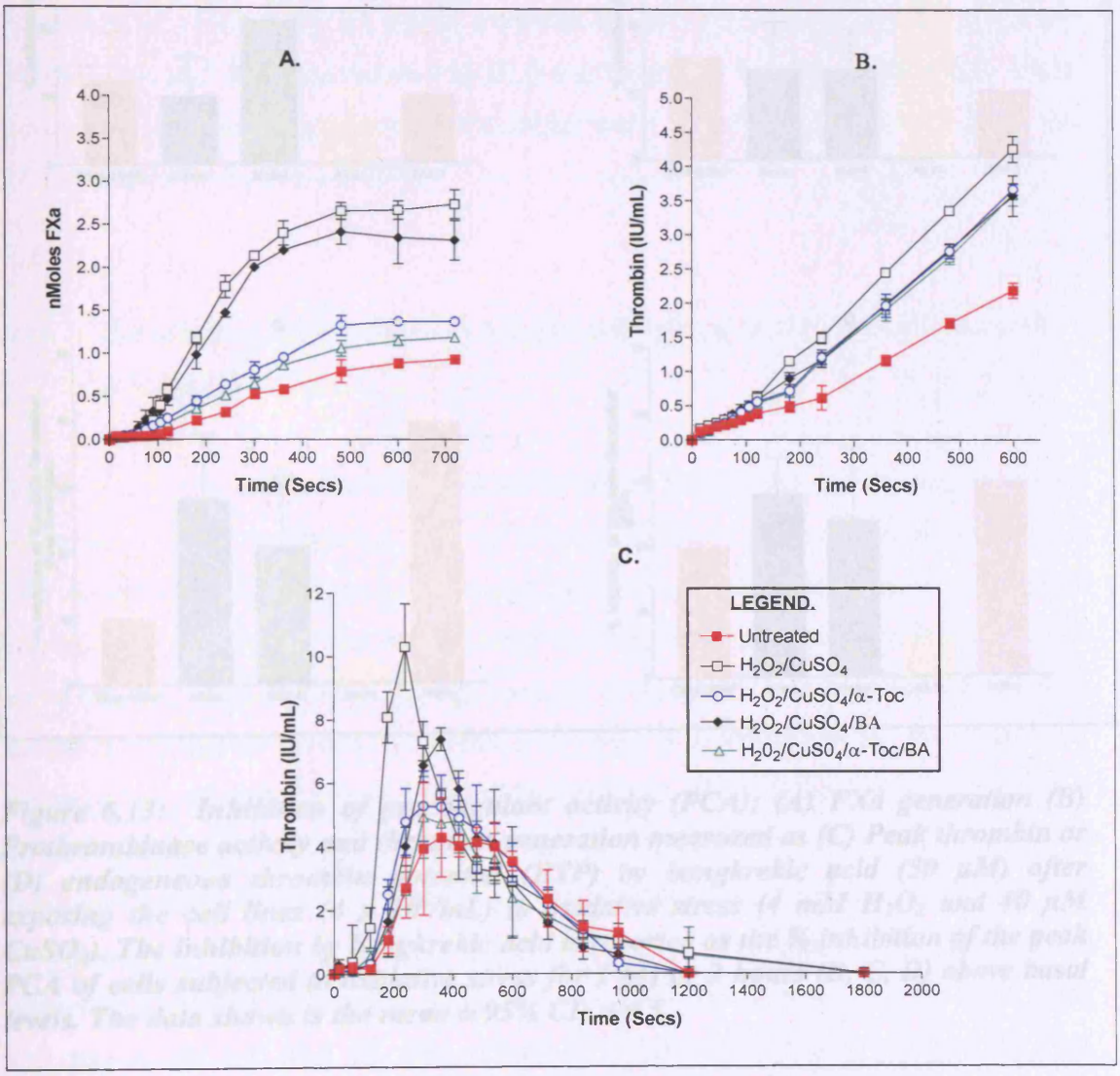



***Figure 6.12: A typical example of the effect of Bongkrekic acid (BA) and α-tocopherol (α-Toc) on (A) FXa generation (B) Prothrombinase activation and (C) thrombin generation of the Jurkat cell line (4 x $10^6$/mL) after exposure to oxidative stress (4 mM $H_2O_2$ and 40 μM $CuSO_4$) for 1 hour (A) or 2 hours (B, C). The data shown is the mean ± 95% CI; n = 3.***

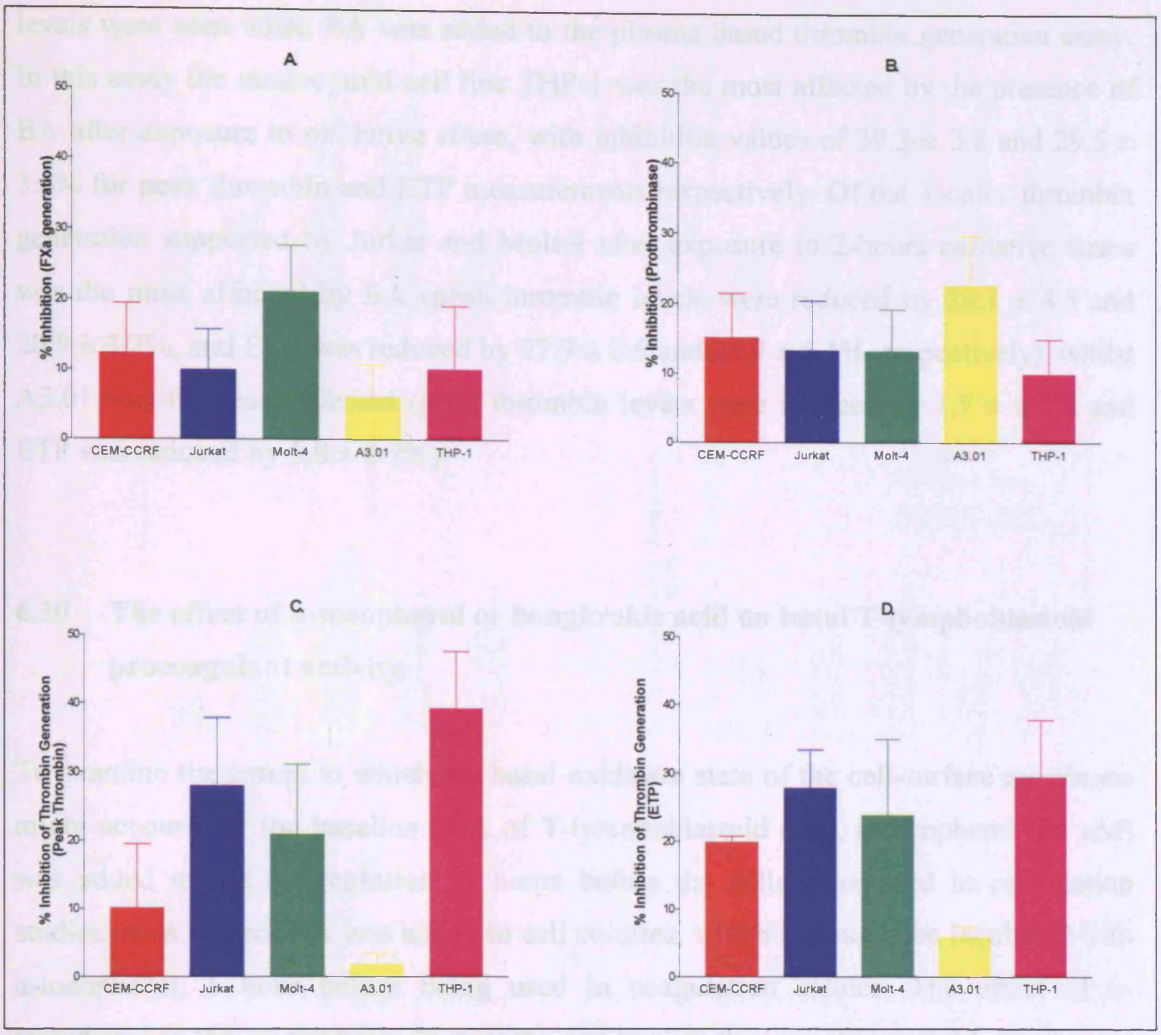



***Figure 6.13: Inhibition of procoagulant activity (PCA); (A) FXa generation (B) Prothrombinase activity and thrombin generation measured as (C) Peak thrombin or (D) endogeneous thrombin potential (ETP) by bongkrekic acid (50 μM) after exposing the cell lines (4 x $10^6$/mL) to oxidative stress (4 mM $H_2O_2$ and 40 μM $CuSO_4$). The inhibition by bongkrekic acid is reported as the % inhibition of the peak PCA of cells subjected to oxidative stress for 1 (A) or 2 hours (B, C, D) above basal levels. The data shown is the mean ± 95% CI; n = 3.***

Bongkrekic acid alone inhibited intrinsic tenase and prothrombinase activities by 4 – 21% and by 12 – 24 % after 1 and 2 hours of lipid peroxidation respectively (Figure 6.13). There were differences between the level of inhibition for FXa generation and prothrombinase activation by BA, with Molt-4 and A3.01 cells showing the highest

inhibition levels (21.1 ± 3.0 and 22.7 ± 3.2%, respectively). Much higher inhibition levels were seen when BA was added to the plasma based thrombin generation assay. In this assay the monocytoid cell line THP-1 was the most affected by the presence of BA after exposure to oxidative stress, with inhibition values of 39.3 ± 3.8 and 29.5 ± 3.8% for peak thrombin and ETP measurements respectively. Of the T-cells, thrombin generation supported by Jurkat and Molt-4 after exposure to 2-hours oxidative stress was the most affected by BA (peak thrombin levels were reduced by 28.1 ± 4.5 and 20.9 ± 4.7%, and ETP was reduced by 27.9 ± 2.6 and 23.9 ± 5.1%, respectively), whilst A3.01 was the least affected (peak thrombin levels were reduced by 1.7 ± 0.7% and ETP was reduced by 5.8 ± 0.7%).

## 6.10 The effect of α-tocopherol or bongkrekic acid on basal T-lymphoblastoid procoagulant activity

To examine the extent to which the basal oxidative state of the cell-surface membrane might account for the baseline PCA of T-lymphoblastoid cells, α-tocopherol (25 μM) was added to the cell cultures 72 hours before the cells were used in coagulation studies. As a control BA was added to cell cultures, which had not been incubated with α-tocopherol, 1 hour before being used in coagulation studies. The effect of α-tocopherol or BA on the basal PCA of the cell lines is shown in Figure 6.14. In all cases α-tocopherol reduced the basal PCA of the cell lines, whilst BA either had no effect or only slightly decreased this activity. α-Tocopherol inhibited basal; FXa generation, prothrombinase activity and thrombin generation measured as peak thrombin and ETP by between 11 – 17%, 5 – 18%, 10 – 29% and 17 – 29% respectively. For the same assays BA inhibited the basal PCA of the cells by 0 – 10%. Interestingly the PCA of the cell line A3.01 that had the highest basal TBARS level (Table 6.1) was the most affected by the addition of α-tocopherol, whilst the cell line, CEM-CCRF that had the lowest basal TBARS level was the least affected.

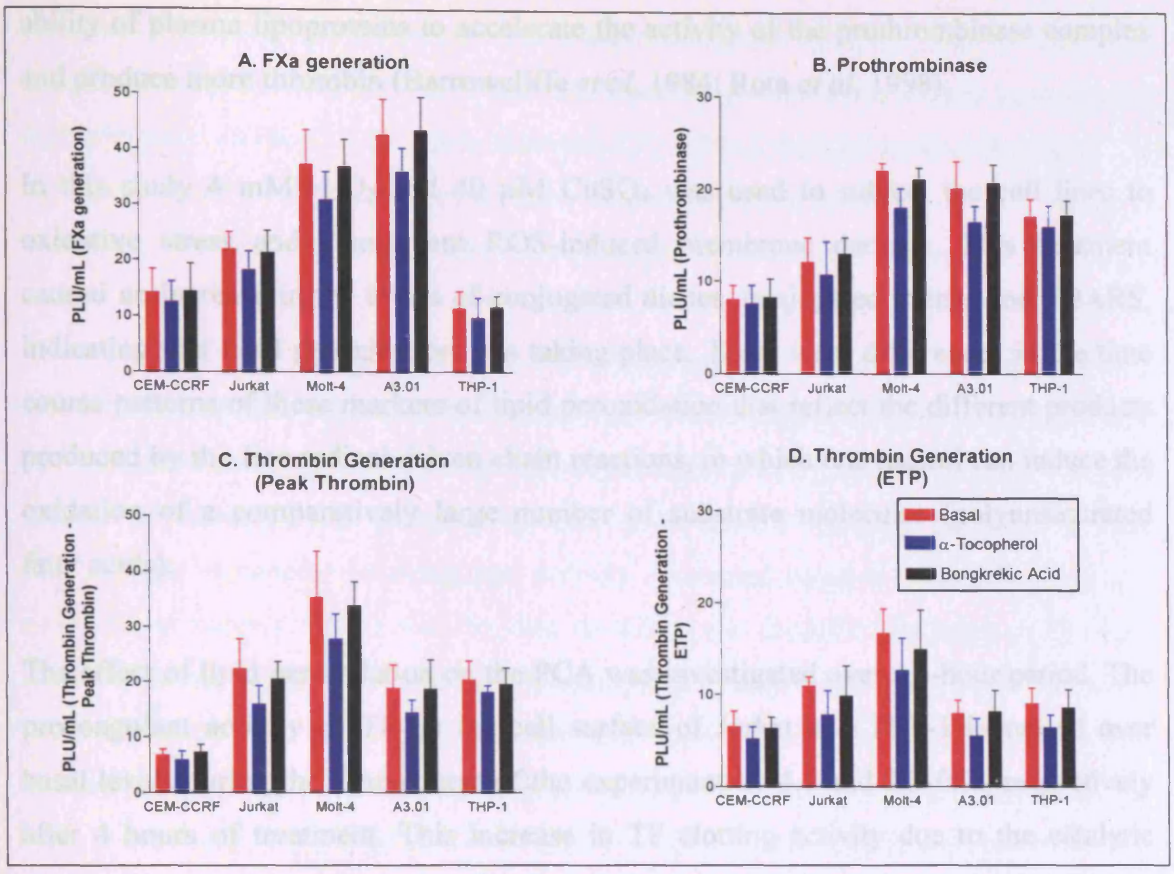



***Figure 6.14: Inhibition of basal procoagulant activity (PCA) of the cell lines (4 x $10^6$/mL) by α-tocopherol (25 μM) or bongkrekic acid (50 μM). The PCA was measured as; (A) FXa generation (B) Prothrombinase activity and thrombin generation as either (C) Peak thrombin or (D) endogeneous thrombin potential (ETP). The results are expressed as PLU/mL by comparison with a phospholipid standard. The data shown is the mean ± 95% CI; n = 3.***

## 6.11 Discussion

There are compelling reasons to consider the possible effects of oxidative phospholipid damage on coagulation and in particular thrombin production. For instance, oxidation profoundly alters the architecture and chemical properties of phospholipid bilayers (Lamba *et al*, 1991; Lamba *et al*, 1994), and the function of the prothrombinase complex in particular is sensitive to the physical state of the membrane (Govers-Riemslag *et al*, 1992). Many common cellular processes such as platelet activation release ROS that can oxidise lipoproteins. This oxidation dramatically increases the

ability of plasma lipoproteins to accelerate the activity of the prothrombinase complex and produce more thrombin (Barrowcliffe *et al*, 1984; Rota *et al*, 1998).

In this study 4 mM $H_2O_2$ and 40 μM $CuSO_4$ was used to subject the cell lines to oxidative stress and consequent ROS-induced membrane damage. This treatment caused an increase in the levels of conjugated dienes, conjugated trienes and TBARS, indicating that lipid peroxidation was taking place. There were differences in the time course patterns of these markers of lipid peroxidation that reflect the different products produced by the free radical-driven chain reactions, in which one radical can induce the oxidation of a comparatively large number of substrate molecules (polyunsaturated fatty acids).

The effect of lipid peroxidation on the PCA was investigated over a 4-hour period. The procoagulant activity of TF on the cell surface of Jurkat and THP-1 increased over basal levels during the time-course of the experiment by 4.1 and 3.3-fold, respectively after 4 hours of treatment. This increase in TF clotting activity due to the catalytic effect of copper in oxidation has also been reported by other researchers using THP-1 cells (Crutchley & Que, 1995) but appeared much later in their study (30 minutes versus 6 hours). This is probably due to the higher concentration of $CuSO_4$ used in this study (40 μM v 5 – 10 μM) and possibly due to TF de-encryption due to apoptosis induced rearrangement of the cell membrane. It is also possible that lipid peroxidation may be enhancing the efficiency of the TF/FVII complex in activating the extrinsic pathway. However, this increase in TF activity was significantly higher ($p < 0.05$) than that observed after treatment with staurosporine (1 μM) or with the calcium ionophore, A23187 (10 μM) as described in chapters 5 and 4 respectively. Thus it is possible that lipid peroxidation facilitates TF gene expression, especially as it is known that reactive oxygen intermediates can function as important and widespread signalling molecules, facilitating gene expression by activation of the nuclear transcription factor NF-κB. Evidence that this is the case comes from experiments, using THP-1 cells, showing that activation of NF-κB and the subsequent increase in TF activity can be prevented by pre-treatment of THP-1 cells with antioxidants (Crutchley & Que, 1995). It is also

possible that lipid peroxidation has post-translational effects, due to oxidative modification of TF that leads to a gain of function. Recently there have been reports that although oxidised LDL can increase TF mRNA levels, it also increases TF pathway activity, suggesting that lipid hydroperoxides some of which exist in atherosclerotic lesions, can activate this pathway (Penn *et al*, 2000; Cabre *et al*, 2004).

When the effect of lipid peroxidation on TF-independent FXa generation and thrombin activation/generation supported by the cell lines was investigated, in all cases there was a significant increase ($p < 0.05$) in phospholipid activity over basal levels. However, the pattern of activity was different to that seen for TF clotting activity, in that phospholipid dependent procoagulant activity decreased towards basal levels after 2 hours of treatment. This could be due to either the oxidative damage of the cell membranes becoming so severe that the complexes cannot assemble, or that the cell-surface phospholipid is so procoagulant that steric hindrance was preventing the assembly of macromolecular complexes. In this study the effect of lipid peroxidation on PCA was greater for FXa generation than for prothrombinase activation. However, for both assays Molt-4 and Jurkat were the most affected, showing the highest increase in activity. When thrombin generation in plasma was measured a similar pattern was observed for both peak activity and ETP values. Interestingly this increase in PCA did not continue for the whole time of the treatment period. After 2 hours of lipid peroxidation PCA values decreased towards basal levels.

The phenolic antioxidant (20 nM) butylated hydroxytoluene, which acts by terminating chain reactions involving free radicals, was able to completely inhibit lipid peroxidation induced PCA when added to the cells immediately before treatment with $H_2O_2/CuSO_4$. This increase in procoagulant activity could also be significantly inhibited ($p < 0.05$), though not completely by the addition of 25 μM α-tocopherol, suggesting that this antioxidant was having little or no effect on procoagulant phospholipids exposed due to apoptosis. When bongkrekic acid (50 μM), an inhibitor of the mitochondrial pathway of apoptosis, was used on its own it did not have much effect on the observed PCA induced by $H_2O_2/CuSO_4$. If bongkrekic acid, was used in addition to α-tocopherol there

was a small additional inhibitory effect on the cell line PCA, though this activity did not return to pre-treatment levels. Thus most of the increase in PCA could be inhibited by the action of α-tocopherol and is likely to be from a direct effect of lipid peroxidation, with only a smaller addition effect being due to apoptosis. The basal PCA of the cell lines was also inhibited by the addition of α-tocopherol, but only slightly affected by bongkrekic acid. It was also noteworthy that the cell line having the highest basal TBARS level was the most affected by α-tocopherol, whilst the cell line having the lowest basal TBARS levels was the least affected. Taken together with the lack of inhibition due to bongkrekic acid indicates that the basal PCA is mainly due to oxidised phospholipids.

In a similar fashion to that reported in chapter 5, for the effect of apoptosis on T-lymphoblastoid PCA there was a discrepancy between the maximal phospholipid dependent procoagulant activities caused by treatment with calcium ionophore or $H_20_2$/ $CuSO_4$. This can be partially explained by the synergistic effect of apoptosis caused by $H_2O_2$. Addition of 50 μM bongkrekic acid reduced the cell line PCA by up 40%, and the percentage of cells binding annexin A5 by up to 25%. Interestingly the flow cytometry data shown in Table 7.2, regarding the binding of annexin $A5^{FITC}$ and 3G4 to cells subjected to lipid peroxidation shows that after 30 minutes 3G4 no longer bound. However, over the treatment period annexin $A5^{FITC}$ binding increased to > 85%. The anti-PS antibody 3G4 was produced by treating bEND.3 endothelial cells with 200 μM $H_2O_2$. Hence it could be expected that this antibody would recognise oxidised PS caused by lipid peroxidation, but it appears that 3G4 only recognises non-oxidised PS or, that only low levels of PS are being exposed. Although annexin $A5^{FITC}$ bound strongly to cells subjected to oxidative stress this might not be solely due to the exposure of PS caused by apoptosis associated with lipid peroxidation. If bongkrekic acid is inhibiting apoptosis and its associated PS exposure, the small inhibitory effect on annexin $A5^{FITC}$ binding (Table 6.3) indicates that most of the increase in binding is due to increased negative charge on the cell-surface, not PS exposure. This, taken together with the lack of effect of bongkrekic acid on cell line PCA, suggests that either the catalytic efficiency of the cell-surface PS is being enhanced by lipid peroxidation or

that other phospholipid types such as PE are being modified and become able to participate in this PCA. There is evidence that endogeneously generated aldehydes formed as a result of lipid peroxidation are involved in the pathophysiological effects associated with oxidative stress in cells. Malondialdehyde (MDA), a major product of lipid production has been reported (Balasubramanian *et al*, 2001) to modify amines present on the cell surface and thus introduce alterations in the net charge on the cell surface. This is also reflected by the observation that TBARS measurements correlated better than conjugated diene or triene measurements with the cell line PCA. It appears that calcium dependent binding of annexin A5 to MDA-lipid adducts is comparable to its binding to PS, especially MDA-PE. In a similar fashion to oxidised LDL (Rota *et al*, 1998) and arachidonate-containing PC (Weinstein *et al*, 2000), MDA-lipid adducts also promote prothrombinase activation in a "PS-dependent" assay (Balasubramanian *et al*, 2001). These results indicate that annexin A5 binds to both PS and aldehyde-modified lipids, and that they can greatly enhance the rate of tenase, prothrombinase activation and subsequent thrombin generation. It is also important to take into account that unlike free-radicals, aldehydes are relatively long lived and are able to diffuse from the lipid bilayer and react with multiple intra and extracellular targets a distance from the initial free radical event (Esterbauer *et al*, 1991).

## 6.12 Summary

Lipid peroxidation, the non-enzymatic autocatalytic interaction of poly-unsaturated fatty acids with molecular oxygen, is a process common to all biological systems and can be involved in many pathological processes. Oxidative stress induced by $H_2O_2$ and $CuSO_4$ treatment enhanced the phospholipid-dependent procoagulant activities of all the cell lines and this increase was found to correlate with TBARS levels. This increase in procoagulant activity was greatest for the FXa generation assay. Lipid peroxidation also increased the TF procoagulant activity of the TF-bearing cell lines. This was possibly due to TF de-encryption, or less likely because of the time frame increased gene expression, or possibly oxidative modification of the protein that increased its

function or alterations in the net negative charge on the cell surface caused by lipid peroxidation. In all cases the effect of lipid peroxidation on phospholipid-dependent procoagulant activity could be ameliorated though not completely by addition of the antioxidant α-tocopherol. The remaining activity was thought to be due to the apoptotic effect of oxidative stress and the exposure of PS. Bongkrekic acid, an inhibitor of the mitochondrial pathway of apoptosis, was also able to reduce phospholipid-dependent procoagulant activity by preventing the translocation of PS to the outer membrane. There was only a slight additive effect when bongkrekic acid was used in combination with α-tocopherol. Bongkrekic acid also had only a small inhibitory effect on the binding of annexin $A5^{FITC}$ to cells subjected oxidative stress, suggesting that annexin $A5^{FITC}$ was not solely binding to PS. This taken with the difference between the binding of annexin $A5^{FITC}$ and that of the anti-PS antibody to the cell-surface of cells undergoing exposure to lipid peroxidation is probably due to the fact that the anti-PS antibody does not bind to MDA-adducts. It would appear that the increase in phospholipid dependent procoagulant activity is possibly caused by the MDA-adducts altering the net charge on the cell surface, which allows other phospholipids other than PS to participate in the propagation of thrombin generation.

Chapter

7

# GENERAL DISCUSSION AND CONCLUSIONS

## 7.1 Discussion

The work described in this thesis was instigated to further characterise phospholipid dependent procoagulant activity reported in earlier studies using various cell lines, especially T-lymphoblastoid cells (Barrowcliffe *et al*, 1989; Barrowcliffe *et al*, 2002), and to compare this activity with that shown by a monocytoid cell line (THP-1). THP-1 cells were used for comparison as they are cells that can express TF. Furthermore it was hoped to elucidate which pathophysiological processes might be responsible for the procoagulant response supported by the cell-surface of these cells. The following cell lines were investigated and tested in detail: CEM-CCRF, Jurkat, Molt-4, A3.01 (human leukaemic T lymphocytes - type II and III cortical – derived from childhood T-ALL) and THP-1 (human monocytic leukaemia – French, American and British (FAB) classification type M5).

### 7.1.1 T-lymphoblastoid procoagulant activity: Behaviour of the cell lines in different assay systems

Previous studies had shown that malignant T-cells (Jurkat and Molt-4) were able to support thrombin generation despite having negligible or low levels of TF (Barrowcliffe *et al*, 2002). The activity observed was presumed to be due to cell surface phospholipid. The approach adopted in the present study was to screen the cells for procoagulant activity by using various phospholipid dependent coagulation assays. To enable a direct comparison of the activities of the cell lines in each of the phospholipid dependent assays the results were reported as phospholipid equivalence (PLU/mL) by comparison with a bovine brain phospholipid standard (NIBSC 91/542). It was also necessary to investigate the confounding effect of TF on these assays. This was especially important as two of the cell lines, THP-1 and Jurkat were found to express TF. All the cell lines were able to support the assembly of phospholipid dependent coagulation reactions; however there were differences in the activities of the cell lines themselves, as well as between each cell line in each assay. This probably reflects, for

each assay, its sensitivity to the tenase and prothrombinase complexes and/or the influence of TF on the assay. As the prothrombinase and tenase complexes have different anionic phospholipid requirements, the difference in procoagulant activity can be partially explained by differences in membrane configuration between the different cell lines and the bovine brain phospholipid. The differences between cell line procoagulant activities in the different assays indicate that the observed activity is not just an artefact of cell culture.

The procoagulant activity supported by the cell lines was always lower in the plasma based assays than in the purified assay systems when the results are expressed as PLU/mL. The APTT-like assay was found to be significantly affected by the presence of cell associated TF, and this was the one phospholipid dependent assay where the monocytoid cell line THP-1 had the most procoagulant activity. When an inhibitory anti-TF antibody was added the procoagulant activity measured in the APTT-like assay was greatly reduced and correlated with data from the intrinsic FXa generation assay where THP-1 was the least procoagulant. Thus use of the anti-TF antibody in the APTT-like assay could make it specific for the measurement of cell surface phospholipid dependent procoagulant activity. The APTT is generally regarded as a screening test for intrinsic pathway defects, but data in this thesis have shown that it can be affected by extrinsic components such as TF.

### 7.1.2 T-lymphoblastoid procoagulant activity: Differences between cell lines, and the phospholipid reference preparation

The least differentiated T-cell line CEM-CCRF (cortical type II) was the least able to support coagulation reactions in all the assay systems studied. The more differentiated (cortical type III) Molt-4 and Jurkat cell lines in most cases were much more potent in supporting phospholipid dependent procoagulant activity. Thus, it might be that the more differentiated a malignant T-lymphoblastoid cell line is, the more procoagulant it is. However, this could solely be due to individual variation, and warrants further investigation (see section 8.4.3). This could also be explained by the basal levels of

apoptosis in the cell lines and/or the basal level of oxidative stress to which they have been subjected.

The procoagulant activity of the cell lines was further characterised using calcium ionophore (A23187) to cause anionic phospholipid exposure, and annexin A5 and the anti-PS antibody 3G4 to inhibit its activity. The ionophore A23187 was able to increase the phospholipid dependent procoagulant activity of all the cell lines by causing the translocation of anionic phospholipid to the cell surface. The activity of the cells in their basal state could be reduced by the action of annexin A5 and slightly less so by 3G4. The patterns of inhibition caused by annexin A5 and 3G4 were similar for the micellar bovine phospholipid and T-lymphoblastoid cell lines. Taken together these observations confirm and extend previous findings (Barrowcliffe *et al*, 2002) and lead to the conclusion that the observed procoagulant activity is due to the presence of negatively-charged phospholipid, but is not totally due to PS on the cell surface. It is also apparent from the data obtained when annexin A5 and 3G4 were used in assays that measured TF activity, that for TF to function effectively it has an absolute requirement for anionic phospholipid.

T-lymphoblastoid cells were better able to support intrinsic FXa generation than prothrombin activation. It is likely that the cell lines provide a "passive pseudo-platelet surface", and that the cell signalling seen in platelets stimulated by activation of thrombin and other receptors is not observed in T-lymphoblastoid cells. The question of how procoagulant malignant T-cells are still remains unresolved. However acute leukaemia patients at initial presentation can have high white cell counts sometimes greater than 100 x $10^6$/mL and certainly higher than 4 x $10^6$/mL, suggesting that the procoagulant activity supported by T-cells could be an additional risk factor for cancer associated thromboembolism.

### 7.1.3 T-lymphoblastoid procoagulant activity: Possible pathophysiological causes of increased activity

One of the possible explanations for the basal differences in the procoagulant activity of the T-cells could have been the degree of apoptosis in the cell populations. However it was found that basal FXa generation levels did not correlate with measurements of apoptosis (either the number of cells in Sub-$G_0$ or the percentage of annexin $A5^{FITC}$ / PI positive cells). To investigate whether apoptosis could increase malignant T-cell procoagulant activity, staurosporine, a topoisomerase II inhibitor, was used to stimulate apoptosis through the mitochondrial pathway and activation of caspase-3. That staurosporine treatment was inducing apoptosis was confirmed by the observation of increase in caspase activity with the highest levels being seen at 6 hours. Over the 24 hour treatment period there was a time dependent increase in the number of cells in sub$G_0$, as well as the number of cells binding annexin $A5^{FITC}$ and the antibody 3G4.

When the effect of apoptosis on TF-independent FXa generation and thrombin activation/generation supported by the cell lines was investigated, there was a significant increase in phospholipid activity over basal levels in all cases. The effect of apoptosis on malignant T-cell procoagulant activity was greater for FXa generation than for prothrombinase activation, and the increase in activity could be prevented by the addition of Z-VAD-FMK, an inhibitor of caspases. This increase in activity could not solely be explained by the action of exposed anionic phospholipid and in particular PS, as the observed activity was far higher than that induced by the calcium ionophore, A23187 at a concentration that caused maximal PS exposure. The increase in FXa generation, prothrombin activation and thrombin generation correlated with the degree of apoptosis.

When the cell lines were treated with staurosporine for 24 hours, and prothrombinase and thrombin generation (ETP) procoagulant activity measured at various time points, bell-shaped activity curves were obtained. This could be due to several factors. Firstly, that as apoptosis progresses the amount of PS exposed is so high that it hinders the

assembly of the prothrombinase complex due to steric-hinderance. Secondly, it could be that the binding sites for coagulation factors have become saturated, though the observed activity might be expected to plateau rather than produce a bell-shaped curve. Finally, that the PS being exposed over the treatment period is "damaged" or in some way affected by the apoptotic process. As the apoptotic process evolves, membrane phospholipids become not only redistributed, but also oxidised. In particular, PS may be specifically targeted (Kagan *et al*, 2000; Tyurina *et al*, 2000). The higher procoagulant activities observed after staurosporine treatment than seen after calcium ionophore treatment is probably due to the additive effect of lipid peroxidation. This was shown by the correlation of TBARS levels with cell line procoagulant activity, and is probably due to the selective oxidation of anionic phospholipid in particular PS.

Therefore a possible explanation for the differences in the basal procoagulant activity of the malignant T-cells could be the degree to which their membranes have been subjected to oxidative stress during cell culture. This hypothesis is supported by the fact that A3.01 which supported high FXa and prothrombinase activity had the highest basal TBARS level. The principal sites of both enzymatic and non-enzymatic oxidation in the membrane are the olefinic groups of unsaturated fatty acyl chains. The fatty acyl chains of membrane lipids have long been regarded as having little or no effect on the catalytic activity of coagulation factor complexes (Jones *et al*, 1985). However more recent studies have concentrated on the prothrombinase complex and have reported that unsaturated acyl chains increase the intrinsic $k_{CAT}$, compared with saturated acyl chains (Kung *et al*, 1994), even after accounting for changes in substrate transport due to altered lipid "fluidity", and this was confirmed using the soluble substrate, pre-thrombin-1. This implies that the phospholipid bilayer serves not merely as a surface for condensing the proteins but also as a functional element of the prothrombinase complex. Thus, the evidence suggests that the acyl chain composition of membrane lipids can influence prothrombinase activity, at least under some circumstances, though less is known about the effect of lipid peroxidation on other membrane complexes.

The effect of lipid peroxidation on malignant T-cell procoagulant activity was investigated by subjecting them to oxidative stress for 4 hours. Oxidative stress caused by $H_2O_2$ and $CuSO_4$ treatment enhanced the phospholipid-dependent procoagulant activities of all the cell lines and this increase correlated with the level of TBARS. Once again this increase of activity could not solely be explained by the action of exposed PS, as it was far higher than that induced by the calcium ionophore, A23187. Like staurosporine treatment of the cells, this increase in procoagulant activity was greatest for the FXa generation assay. Lipid peroxidation also increased the TF procoagulant activity of the TF bearing cell lines. This was most likely due to TF de-encryption; other explanations could be possible oxidative modification of the protein that increases its function or alterations in the net negative charge on the cell surface caused by lipid peroxidation or less likely increased gene expression/transcription.

In all cases the effect of lipid peroxidation on phospholipid dependent procoagulant activity was ameliorated, although not completely, by the addition of the antioxidant α-tocopherol. This remaining activity was attributed to apoptosis induced by oxidative stress and the consequent exposure of PS. Bongkrekic acid, an inhibitor of the mitochondrial pathway of apoptosis induced by $H_2O_2$, reduced the phospholipid dependent procoagulant activity caused by lipid peroxidation by preventing the translocation of PS to the outer membrane. There was only a slight additive effect when bongkrekic acid was used in combination with α-tocopherol. Also bongkrekic acid only had a small inhibitory effect on the binding of annexin $A5^{FITC}$ to cells subjected to oxidative stress, suggesting that annexin $A5^{FITC}$ was not solely binding to PS. This, taken together with the difference between the binding of annexin $A5^{FITC}$ and that of the anti-PS antibody 3G4 to the cell-surface of cells undergoing lipid peroxidation, suggests that annexin $A5^{FITC}$ is probably binding to something else on the cell surface. Malondialdehyde, a major but not the only product of lipid production, can modify amines present on the cell surface and thus introduce alterations in the net charge on the cell surface (Balasubramanian *et al*, 2001). This is also reflected by the observation that levels of TBARS correlated better with the cell line procoagulant activity than the level of conjugated dienes or trienes. It appears that calcium dependent binding of annexin

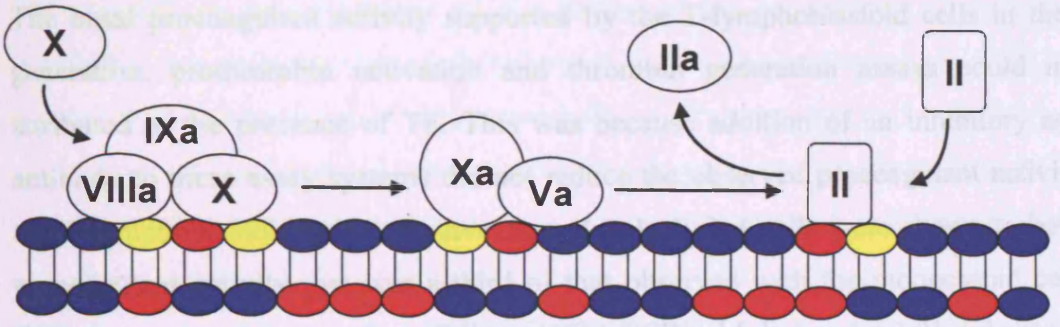
A5 to MDA-lipid adducts is comparable to its binding to PS, especially MDA-PE (Balasubramanian *et al*, 2001). In a similar fashion to oxidised LDL (Rota *et al*, 1998) and arachidonate-containing PC (Weinstein *et al*, 2000), MDA-lipid adducts also promote prothrombinase activity in a "PS-dependent" assay (Balasubramanian *et al*, 2001). Therefore an explanation is that annexin $A5^{FITC}$, but not the anti-PS antibody 3G4, is binding to MDA-adducts. Putting these observations together it appears that the increase in phospholipid dependent procoagulant activity is due to lipid peroxidation product-adducts (possibly MDA-adducts) altering the net charge on the cell surface, which allows phospholipids including and other than PS to participate in the propagation of thrombin generation. Additionally it is possible that oxidation of phospholipid other than PS might enhance the effect of PS.

### 7.1.4 T-lymphoblastoid procoagulant activity: Basal State

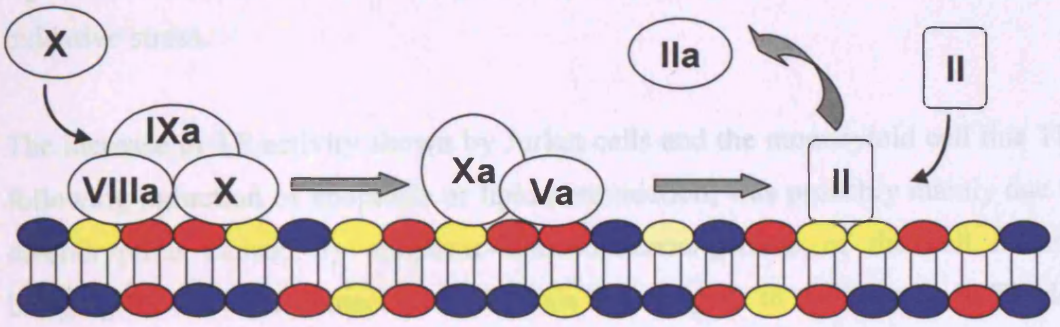
The present study leads to the conclusion that T-lymphoblastoid basal procoagulant activity is due to the synergistic effect of apoptosis and lipid peroxidation. Apoptosis causes the translocation of PS to the outer membrane whilst the lipid peroxidation of PS due to the release of cytochrome c from mitochondria into the cytoplasm enhances its catalytic efficiency. Induction of apoptosis by staurosporine can enhance the procoagulant activity of T-lymphoblastoid cell lines mainly due to the exposure of PS. Lipid peroxidation also enhances procoagulant activity, but in this case it is prossibly due to MDA-adducts altering the net charge on the cell-surface. This explanation for the procoagulant activity of T-lymphoblastoid cells in the different assays for basal and pathological states is shown in Figure 7.1.

The differences in the antioxidant capacity of the cell lines and their consequent ability to resist oxidative stress due to apoptosis and/or lipid peroxidation would also contribute to the different activities of the cells in both basal and pathological states.

A. T-Lymphoblastoid PCA: Basal state.




B. T-Lymphoblastoid PCA: Effect of Apoptosis.




C. T-Lymphoblastoid PCA: Effect of Lipid Peroxidation.

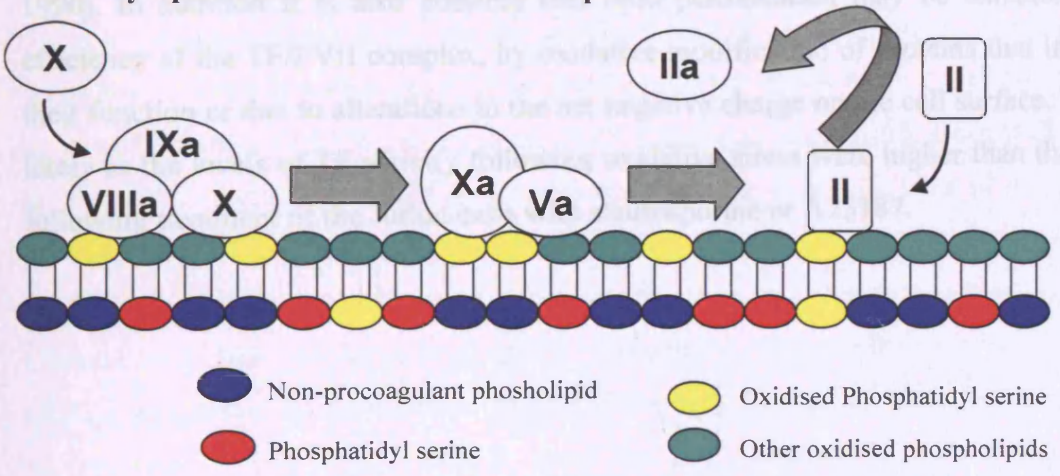



***Figure 7.1: The procoagulant activity (PCA) of T-Lymphoblastoid cells in (A) basal and the pathological states (B) apoptosis and (C) lipid peroxidation.***

### 7.1.5 T-lymphoblastoid procoagulant activity: The role of tissue factor

The basal procoagulant activity supported by the T-lymphoblastoid cells in the FXa generation, prothrombin activation and thrombin generation assays could not be attributed to the presence of TF. This was because addition of an inhibitory anti-TF antibody to these assay systems did not reduce the observed procoagulant activity. Of the T-lymphoblastoid cell lines investigated only Jurkat cells were shown to have TF procoagulant activity that was a third of that observed with the monocytoid cell line THP-1. As a consequence the cell lines, CEM-CCRF, Molt-4 and A3.01 did not show a significant increase in TF clotting activity following the induction of apoptosis or oxidative stress.

The increase in TF activity shown by Jurkat cells and the monocytoid cell line THP-1, following induction of apoptosis or lipid peroxidation, was probably mainly due to TF de-encryption caused by apoptotic-induced rearrangement of the cell membrane, bringing PS in closer contact with TF. This is analogous to the increase in TF clotting activity following treatment with the calcium ionophore, A23187 (Bach & Rifkin, 1990). In addition it is also possible that lipid peroxidation may be enhancing the efficiency of the TF/FVII complex, by oxidative modification of proteins that increase their function or due to alterations in the net negative charge on the cell surface. This is likely as the levels of TF activity following oxidative stress were higher than that seen following treatment of the Jurkat cells with staurosporine or A23187.

## 7.2 Possible clinical relevance of T-lymphoblastoid procoagulant activity

The possible *in vivo* relevance of the ability of the cell-surface of malignant T-lymphoblastoid cells to support phospholipid dependent coagulation reactions mainly relates to the increased risk of thromboembolism (TE) in patients suffering from cancer undergoing chemotherapy. However, the data reported in this thesis concerning the enhancement of this procoagulant activity due to apoptosis and lipid peroxidation also has implications for the thrombotic events associated with atherosclerotic plaque rupture. More tentatively the increase in phospholipid-dependent procoagulant activity after exposure to oxidative stress might contribute to the increase in the incidence of TE with increasing age.

### 7.2.1 Malignancy

Acute lymphoblastic leukaemia (ALL) is the most common malignancy of children, constituting nearly one-third of all childhood cancers. The introduction of intensive, multi-agent and risk-adapted regimens has resulted in a considerable improvement in the cure rates that currently reach 80%; nevertheless, resistant ALL remains the leading cause of cancer-related death among children (Pui & Evans, 1998). The reported incidence of thromboembolism (TE) in childhood ALL varies from 1.1 to 36.7% with an overall average of 3.2% (Priest *et al*, 1982; Castaman *et al*, 1990; Eckhof-Donovan *et al*, 1994; Mitchell *et al*, 2003). This is much higher than that seen in the general paediatric population, with a incidence of 0.0007 – 0.0014% for deep vein thrombosis (DVT) and 0.053% for pulmonary embolism (PE) per 10,000 hospital admissions (van Ommen *et al*, 2001; Mongale *et al*, 2001). The wide variation in the reported incidence of ALL associated TE seems to be related to the definition of TE (symptomatic versus asymptomatic), diagnostic methods used for the detection of TE, study design (prospective versus retrospective analysis), and ALL treatment protocols. Also the confounding effects of other inherited or acquired thrombophilic defects, such as antithrombin, protein C and protein S deficiencies, and the factor V Leiden, prothrombin gene and methylenetetrahydrofolate (C677T) reductase mutations as well as antiphospholipid antibodies will play their part in the incidence of TE.

In studies comparing symptomatic and asymptomatic TE there is increased incidence of TE associated with the use of central venous line catheters (David & Andrew, 1993; Massicotte *et al*, 1998; Wermes *et al*, 1999) and the use of asparaginase. Comparison of German Co-operative Study Group for Childhood Acute Lymphoblastic Leukaemia (COALL) and Berlin-Frankfurt-Munster (BFM) protocols illustrates the importance of chemotherapy schedule on the incidence of TE. Despite a similar treatment time frame and similar ethnic population, the incidence of TE is 10 times higher in children receiving treatment in the BFM 90/95 ALL study compared to those receiving therapy on the COALL 92/97 protocol (Nowak-Gottl *et al*, 1999; Mauz-Korholz *et al*, 2000; Nowak-Gottl *et al*, 2001).

Most of the studies investigating the risk categorisation of individual ALL patients suffering from TE do not compare the incidence of TE in leukaemia subtypes or risk categories (Priest *et al*, 1982; Pui *et al*, 1985; Miniero *et al*, 1987; Castaman *et al*, 1990; Mitchell *et al*, 1994; Silverman *et al*, 2001; Kankirawatana *et al*, 2002; Mitchell *et al*, 2003). Of the few that do, the suggestion is that the incidence of TE might be higher in patients with T-ALL (Giordano *et al*, 2000; Giordano *et al*, 2003). In a study investigating children with ALL treated using Dana Farber Cancer Institute (DCFI) protocols, the results indicate that patients classified as high risk (HR-ALL) seem to have at least a four times higher risk of developing TE compared to those with standard risk (SR-ALL) (Athale *et al*, 2003). The impact of risk stratification and immunophenotype of ALL on the development of TE is important to analyse, as most of the current therapies use more aggressive therapy to treat HR-ALL patients, including those with T-ALL, compared to SR-ALL patients. The development of TE also has the potential to adversely affect the outcome from ALL by discontinuation or delays in anti-cancer therapy like asparaginase. Thus the data reported in this thesis regarding malignant T-ALL cell line phospholipid-dependent procoagulant activity, and the effect of apoptosis and/or lipid peroxidation suggests a possible mechanism or risk factor for TE in patients with T-ALL. In effect, more aggressive therapy leads to increased apoptosis and consequently a more procoagulant environment.

### 7.2.2 Atherosclerosis

Three cellular components of the circulation, monocytes, platelets, and T-lymphocytes, together with two cell types of the artery wall, endothelial and smooth muscle cells, interact in multiple ways in concert with low-density lipoprotein (LDL)-cholesterol in the generation of atherosclerotic lesions.

The current model of atherosclerosis establishes a number of consecutive stages in the development of the disease (Glass & Witztum, 2001; Libby, 2001). Stage (i), lesion initiation characterised by recruitment of inflammatory leucocytes by activated endothelial cells and accumulation of extracellular lipids in the intima. Stage (ii) involves smooth muscle cell (SMC) migration and proliferation accompanied by synthesis of extracellular matrix and transformation of recruited macrophages to lipid laden foam cells owing to accumulation of oxidised low-density lipoproteins (oxLDL). Stage (iii) is lesion progression characterised by matrix degradation by metalloproteinases and weakening of the fibrous cap, and finally stage (iv) plaque rupture and thrombus formation at the site of the lesion. Less frequently thrombus formation at the site of an atherosclerotic plaque can be a result of superficial erosion of endothelium without breakdown of the plaque fibrous cap.

The formation of the occlusive thrombus at the site of the lesion is the major cause of complications of atherosclerosis such as stroke and myocardial infarction, which are often fatal. In some cases, the thrombus can be resorbed, and the plaque can evolve to advanced fibrous plaque (sometimes calcified) leading to significant stenosis and symptoms of stable angina pectoris.

### *7.2.2.1 Coagulation and atherothrombosis*

A major role in determining the thrombogenicity of atherosclerotic lesions and generation of acute coronary syndromes has been ascribed to the TF dependent pathway of blood coagulation (Toschi *et al*, 1997; Badimon *et al*, 1999; Hasenstab *et al*, 2000). In contrast to the normal vessel wall, high levels of TF mRNA and protein were found in atherosclerotic lesions, and TF expression level positively correlated with plaque severity (Moreno *et al*, 1996) and vulnerability (Ardissino *et al*, 1997). Furthermore addition of TF enriched atheroma fragments to blood led to thrombus formation (Toschi *et al*, 1997) which could be substantially inhibited by TFPI (Badimon *et al*, 1999).

Although the TF dependent pathway is undoubtedly responsible for initiation of thrombus formation, alone it is unlikely to be responsible for the occlusive thrombus formation. This is supported by evidence from clinical and epidemiological studies, especially of haemophilia A. Haemophilia A patients show a reduced risk of myocardial infarction (Triemstra *et al*, 1995; Sramek *et al*, 2001). Although haemophiliacs have lower numbers of atherosclerotic plaques (Bilora *et al*, 2001), there were no significant differences in atherosclerosis risk factors between haemophiliacs and normal individuals (Rosendaal *et al*, 1990) so that the differences in atherogenesis can not account for the reported five fold reduction of mortality from myocardial infarction in haemophilia A patients (Triemstra *et al*, 1995). It is thought that it is impaired coagulation that prevents the occlusive thrombus formation at the site of lesion. In direct comparison epidemiological studies (Rosendaal, 1992; Pan *et al*, 1997) reported that increased levels of FVIII positively correlated with coronary heart disease and carotid atherosclerosis. Although it is unclear whether FVIII elevation is a cause or consequence of disease progression, there is a consensus that elevated levels of FVIII is a risk factor for atherothrombosis. It is reported that the intrinsic pathway significantly enhances thrombogenicity of atherosclerotic lesions after removal of the endothelial layer and exposure of smooth muscle cells and macrophages to blood flow (Ananyeva *et al*, 2002).

#### *7.2.2.2 Accumulation of oxidised LDL and atherothrombosis*

Plasma lipoproteins participate in atherogenesis at all atages of lesion development. The predominant role belongs to LDL, because its oxidation into ox-LDL is considered to be the most atherogenic hallmark of atherosclerosis (Glass & Witztum, 2001). In contrast, high density lipoproteins (HDL) are believed to be an anti-atherogenic factor (Mertens & Holvoet, 2001). It is also thought that the LDL/HDL balance may also reflect the coagulation status of blood: increased concentrations of LDL have a procoagulant effect (Moyer *et al*, 1998) whereas HDL was shown to be anticoagulant (Griffin *et al*, 1999). Plasma lipoproteins can provide a phospholipid surface for the assembly of the macromolecular complexes of the coagulation cascade. It has been reported that triglyceride rich lipoproteins can enhance thrombin generation (Barrowcliffe *et al*, 1984). In particular, oxLDL and very low-density lipoprotein effectively support prothrombinase activity (Moyer *et al*, 1998). LDL also supports the activity of the extrinsic and intrinsic tenase complexes (Khrenov *et al*, 2002).

#### *7.2.2.3 Pathological changes induced by oxLDL in the cell that might contribute to atherothrombosis*

The accumulation of oxLDL in the plaque also induces pathological changes in all the cell types that compose the vessel wall, making it highly thrombogenic. It has been demonstrated that oxLDL induces TF expression in endothelial cells (Fei *et al*, 1993) and macrophages (Brand *et al*, 1994) and upregulates TF expression in vascular smooth muscle cells *in vitro* (Penn *et al*, 2000). In fact, the procoagulant activity of oxLDL treated cells in the extrinsic pathway is thirty fold higher for cultured human macrophages (Schuff-Werner *et al*, 1989), twenty-four fold higher for macrophages isolated from atherosclerotic plaques (Tipping *et al*, 1989), six fold higher for cultured smooth muscle cells (Penn *et al*, 2000), and four fold higher for endothelial cells (Weis *et al*, 1991).

The accumulation of oxLDL could also enhance the ability of the cells that make up the atherosclerotic lesion to provide a phospholipid suitable for the assembly and activity of the tenase and prothrombinase complexes. When human macrophages and human aortic smooth muscle cells were exposed to ox-LDL, it enhanced their ability to support the tenase and prothrombinase complexes. This increase in intrinsic pathway procoagulant activity was related to formation of additional FVIII binding sites and a more efficient assembly of the tenase complex, probably due to increased exposure of PS on the outer surface of oxLDL treated cells (Wintergerst *et al*, 2000). Immuno-histochemical studies on human atherectomy specimens show colocalisation of FVIII with macrophages and smooth muscle cells in the atheromatous regions, with massive deposits of oxLDL, supporting potential involvement of the intrinsic pathway in thrombus formation *in vivo*.

#### *7.2.2.4 Apoptotic events and atherothrombosis*

One of the reasons for the increased thrombogenicity of the plaque could be the enrichment of the advanced lesion with apoptotic cells that have translocated their PS from the inner to the outer leaflet of the cell membrane. Sustained accessibility of PS at the surface of apoptotic cells is likely to be an important factor that defines the plaque thrombogenic potential. Induction of apoptosis by oxLDL has been demonstrated in vitro for smooth muscle cells (Bachem *et al*, 1999), endothelial cells (Sata & Walsh, 1998) and macrophages (Wintergerst *et al*, 2000). Another phenomenon that contributes to thrombogenicity of the plaque is the shedding of microparticles by apoptotic macrophages, lymphocytes and possibly smooth muscle cells (Mallat *et al*, 1999; Schecter *et al*, 2000). Such microparticles not only contain high amounts of PS, but also represent a major source of functionally active TF (Bombeli *et al*, 1999) suggesting that they might participate in both intrinsic and extrinsic pathways. The presence of microparticles may explain why the highest levels of TF are found in the acellular lipid rich core of atherosclerotic plaques, which represents the most thrombogenic part of the plaque (Toschi *et al*, 1997).

### *7.2.2.5 Atherothrombosis and overexpression of LDL receptor-related protein*

The LDL receptor related protein (LRP) is another potential link that connects atherosclerosis and coagulation. This multi-ligand endocytic receptor possesses the ability to bind and internalise a number of ligands, including lipoproteins and coagulation factors (Herz & Strickland, 2001). LRP is overexpressed in atherosclerotic plaques and there is a strong correlation between LRP mRNA levels and atherosclerotic plaque progression (Handschug *et al*, 1998). In atherogenesis, LRP functions as one of the scavengers that mediate the influx of oxLDL into macrophages, which converts them to foam cells. LRP also potentially downregulates coagulation via internalisation of components of the intrinsic pathway, FVIII, FIXa and FXa (Neels *et al*, 2000) as well as internalisation of the TF/FVIIa complex (Hamik *et al*, 1999). However, the anti-thrombogenic potential of LRP is apparently insufficient to decrease thrombogenicity of the plaque in the extrinsic pathway, despite enhanced accumulation of TFPI in atherosclerotic tissues (Crawley *et al*, 2000). This may be related to severe impairment of lysosomal degradation mechanisms in the cells that comprise atherosclerotic lesions (Li *et al*, 1998) which would result in insufficient degradation of the internalised coagulation factors. Thus it is unclear whether LRP-internalised but not lysosomally degraded components of the tenase complex (FVIII and FIXa) can subsequently contribute to plaque thrombogenicity.

### *7.2.2.6 T-lymphocytes and atherothrombosis*

Complications of atherosclerosis are the most common causes of death in Western societies, and rupture of an atherosclerotic plaque is considered a precursor event to arterial thrombosis (Fuster *et al*, 1992; Rauch *et al*, 2001; Corti & Badimon, 2002). Three cellular components of the circulation, monocytes, platelets, and T-lymphocytes, together with cells of the artery wall (endothelial and smooth muscle cells) interact with low-density lipoprotein-cholesterol in generating atherosclerotic lesions. The presence of immunocompetent T-lymphocytes, many of which are activated, and mononuclear

phagocytes marks one of the earliest detectable atherosclerotic events that persist throughout atherogenesis (Ross, 1999). T-lymphocytes can make up nearly 20% of the cell population of a plaque (Jonasson *et al*, 1986; Hansson *et al*, 1989) Within this T-cell population, 70% were found to be CD4+ and the remainder were CD8+ (Hansson, 1997). Activated T-cells and macrophages most heavily infiltrate the 'shoulder region' of the fibrous cap of the plaque, which is the point of greatest vulnerability for plaque disruption (Libby *et al*, 2002).

T-cells that express anionic phospholipid but have no TF activity would not, on their own, be expected to be procoagulant. To participate in coagulation the T-cells could provide a procoagulant surface *in vivo*, which would be involved in the amplification and propagation phases of the cell-based model of haemostasis (Hoffman & Monroe, III, 2001). A rupture of the fibrous cap would not have much of a deleterious consequence were it not for the ensuing thrombin generation and thrombus formation. However, the release of TF from the lipid core activates the clotting cascade. The TF appears to be monocyte/macrophage associated and T-cells have been shown to induce macrophages to express TF (Mach *et al*, 1997). Thus one could envisage the scenario where there is plaque rupture, with large numbers of T-lymphocytes that have been exposed to oxidative stress, as well as undergoing apoptosis, in close proximity to TF bearing cells of the monocytes/macrophage lineage that are also exposing PS and probably oxidised phospholipids. The T-cells could potentiate and amplify the procoagulant response leading to arterial thrombosis (Jonasson *et al*, 1986; Libby *et al*, 1999).

### 7.2.3 Ageing

The average life expectancy of humans has increased dramatically over the past century. Increasing age is considered as a contributory risk factor for thrombosis and evidence for this is that the incidence of venous thrombosis increases with age (Morrell & Dunnill, 1968; Kakkar *et al*, 1970). As the age of an individual advances beyond adolescence, undesirable changes in gene expression or function become increasingly

prominent, altering normal physiological processes. The annual incidence of venous thromboembolism is 1 to 2 cases per 1000 persons (Anderson, Jr. *et al*, 1991; Nordstrom *et al*, 1992), for children the annual incidence is less than 5 per 100,000 children and increases to more than 400 per 100,000 adults older than 80 years (White, 2003). There are many theories relating to the mechanisms of ageing. One such theory is centred on shortening telomeres, the ends of chromosomes, which were once considered to simply protect against DNA breakdown. Telomere shortening has been reported in atherosclerosis and the risk of premature myocardial infarction (Samani *et al*, 2001; Brouilette *et al*, 2003). However, the theory that currently has the most evidence supporting it, and the least number of apparent contradictions, is the 'oxidative stress theory' (Harman, 1956). This theory is based on observations of progressive accumulation of molecular damage to many tissues. The theory suggests that excesses of reactive oxygen and nitrogen species determine maximum lifespan and also account for many of the diseases of advanced age.

It is well accepted that the cellular levels of oxidatively modified macromolecules increase with age in all species studied, especially in the later part of the lifespan (Beckman & Ames, 1998). In general, oxidative modifications to proteins decrease their structural or enzymatic activities. Oxidative damage also accrues in DNA, altering both the cellular and mitochondrial genomes, which are not always effectively repaired. Finally, cellular and subcellular membranes become oxidatively damaged, stimulating the propagation of a free radical generating cascade that catalyses the production of additional ROS. The increased generation of ROS with ageing is often accompanied by a reduction in endogenous antioxidant activities.

Much is known about the effect of age on the immune function of T-lymphocytes (Linton & Dorshkind, 2004). T-lymphocytes are important components of adaptive immunity that are generated from the maturation of bone marrow derived progenitors within the thymus. One of the earliest descriptions of age-associated alterations in the immune system was the marked reduction in mass and cellularity of the thymus with ageing. In addition, secretion of thymus-derived hormones, some of which are essential

for the development and differentiation of T-lymphocytes can no longer be detected in humans aged over 60. Ageing is accompanied by a decrease in the amount of interleukin-2 produced by T-lymphocytes and hence a poorer response to novel antigens. Likewise the generation of cytotoxic $CD8^+$ T-lymphocytes, essential for the killing of virally infected or tumour cells, is temporarily delayed, and the overall cytotoxic activity reduced and of shorter duration.

Ageing is additionally accompanied by increased numbers of 'memory' T-lymphocytes, believed to be a result of clonal expansion subsequent to encountering specific antigens. Consequently, there are decreased numbers of 'naïve' T-lymphocytes in aged individuals. T-lymphocytes from aged animals and humans also show an increased incidence of autoreactivity. Also, some of the age-associated alterations in the function of B-lymphocytes have now been attributed to the inability of T-lymphocytes to appropriately modulate B-lymphocyte activation and differentiation, and this may partially explain the inability of aged individuals to be effectively vaccinated with protein antigens. Thus much is known about the effect of age on the immune function of T-lymphocytes. However, little is known about the effect of ageing and associated oxidative damage on the procoagulant activity supported by T-lymphocytes (and other circulating blood cells) and this could be an area of further research which is addressed in section 7.5.4.

## 7.3 Conclusions

A number of conclusions can be drawn from this study.

- There are differences between the basal activities of the malignant T-cell lines when they are substituted for phospholipid in phospholipid-dependent assays. Also there are differences between the activities of each cell line when used in the different assay systems.

- Malignant T-cells appear to be better able to support the assembly and function of the intrinsic tenase complex than the prothrombinase complex in this static *in vitro* system.

- The APTT-like assay can be affected by the presence of cell-surface associated TF as demonstrated by the addition of an inhibitory anti-TF antibody to the assay system.

- The binding of annexin $A5^{FITC}$ and the anti-PS antibody 3G4 along with their inhibitory effect on basal malignant T-cell procoagulant activity indicates that this activity is mainly due to the exposure of anionic phospholipid. This is further confirmed by the fact that the calcium ionophore A23187, which induces PS exposure, increases the procoagulant activity of the cells. It was also observed that 3G4 does not inhibit phospholipid dependent activity as effectively as annexin A5. Together, this is evidence that the PCA of the cell lines is due to both PS exposure and oxidised phospholipids.

- Following treatment with staurosporine (to cause apoptosis) and $H_2O_2/CuSO_4$ (to induce lipid peroxidation) malignant T-cell procoagulant activity was dramatically enhanced by two different but synergistic mechanisms: (i) the exposure of PS and (ii) by the net charge of the cell-surface being altered by lipid peroxidation product-adducts (possibly MDA-adducts).

- These findings suggest a possible mechanism whereby malignant T-cells might contribute to TE associated with chemotherapy for childhood T-ALL, and to atherothrombosis. It is also possible that this phospholipid PCA described using malignant T-cells may also apply to other cells.

## 7.4 Further Work

The possible further work that might be undertaken is described in the following sections. It involves the further characterisation of T-lymphoblastoid procoagulant activity, the role of T-cells in atherothrombosis and in TE associated with malignancy and/or infection, as well as possibly being a risk factor for the increased incidence of TE with ageing.

### 7.4.1 Further characterisation of T-lymphoblastoid procoagulant activity

The data shown in this thesis suggests that malignant T-cells are better able to support intrinsic FXa generation than the other coagulation complexes. To see why this is the case it would be interesting to investigate the binding and distribution of coagulation factors on the cell surface.

The data has shown that malignant T-cells can be procoagulant, but the question of whether it is the whole or a sub-set of the population contributing to this activity has not been answered. The possible role of microparticles in promoting this procoagulant activity also needs to be addressed.

The results reported in this study were all obtained under static conditions. It would be interesting to investigate the effect of flow conditions on the procoagulant activity of malignant T-cells as well as any possible interactions with other circulating blood cells under flow conditions.

### 7.4.2 Malignancy

As reported in section 7.2.1 malignancy, especially chemotherapy of, is associated with an increased risk of TE. An area of future work would be to investigate the effect of the different T-ALL chemotherapy regimens on T-ALL procoagulant activity using the

phospholipid dependent assays described in this thesis. In combination with the study of chemotherapy regimens it would be of clinical interest to attempt to relate the immunophenotype of children with T-ALL with the incidence of TE, in order to attempt to identify if there is a sub-set of patients with an increased risk of TE.

### 7.4.3 Atherothrombosis

To further investigate any possible role for T-cells in atherothrombosis it would be of value to compare and contrast the ability of T-cells obtained from atherosclerotic plaques to support activation of the tenase and prothrombinase complexes with that shown by malignant or normal T-cells. The atherosclerotic plaques could be obtained from patients undergoing carotid endarterectomy and this would also allow us to look at the formation of coagulation complexes *in situ*.

### 7.4.4 Ageing

The possibility that the procoagulant activity of T-cells and other circulating blood cells increases with age of the individual could be investigated by measuring the procoagulant activity of cells over various age ranges. Any observed changes could then be related to markers of lipid peroxidation in these individuals, and would also answer the question whether normal T-cells can become procoagulant.

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