



**ROLE OF PLATELETS IN THE PROCOAGULANT ENVIRONMENT
IN BLOOD**

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Role of platelets in the procoagulant environment in blood

Abstract

Platelets contribute to coagulation through their own response and through interaction with other cells, especially monocytes. The project investigated the mechanisms whereby platelets expose a procoagulant surface and induce tissue factor (TF) in monocytes.

A whole blood flow cytometric assay to measure phosphatidylserine (PS) exposure on platelets was established and validated. Using this assay, it was demonstrated that collagen is the primary agonist in eliciting platelet PS exposure, individuals' platelets vary in their response to collagen, and not all express PS despite maximal activation. Collagen-induced PS exposure was independent of other aspects of platelet activation. ADP enhanced the procoagulant effect of collagen, but could not induce PS exposure on its own. The potentiating effect of ADP was mediated mainly through P2Y₁₂ and was required for the formation of microparticles. Procoagulant microparticles were enriched in CD42b and P-selectin, but had reduced ability to bind fibrinogen. Thrombin could instigate PS exposure only where there was platelet-platelet contact. These data suggest that at a wound site, platelet PS exposure is stimulated by subendothelial collagen at the same time as coagulation is initiated by TF. It is not dependent on initial thrombin generation but thrombin and ADP perpetuate thrombin generation by promoting PS exposure where there is platelet-platelet contact within a thrombus.

Activated platelets caused upregulation of gene expression of monocyte TF within 2 hours, and, at a later stage, of its inhibitor, tissue factor pathway inhibitor. These effects were primarily mediated via soluble factors. Analysis of TF antigen on monocytes and monocyte-platelet aggregates by flow cytometry suggested that TF might be platelet-associated. However, detailed exploration produced no reliable evidence of TF protein, mRNA or activity in platelets. This implies that data to the contrary is probably artefactual, and that platelet-associated TF, currently a controversial subject, plays at most a small part in clotting.

Jackie A. Appleby
PhD thesis
March 2006

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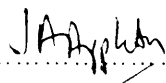
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Statement by candidate

This thesis is the result of work carried out mainly during the period of registration. It is substantially the original work of the candidate. Where it is not, this is clearly stated in the text.

A handwritten signature in black ink, appearing to read 'J Appleby', is written over a horizontal dotted line.

Jackie Appleby

March 2006

Publications arising from this thesis

Methods and Protocols “Platelets and Megakaryocytes” within the series Modern Methods in Molecular Biology, published by Humana Press. Edited by Jon Gibbins and Martyn Mahaut-Smith. *Methods in Molecular Biology* 2004; 272:225-54

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Differential contribution of P2Y₁ and P2Y₁₂ ADP receptors to GPVI-mediated phosphatidylserine exposure on platelets and microparticles. **JA Appleby**, RW Farndale, AH Goodall. *Journal of Thrombosis and Haemostasis* 2005; Volume 3, Supplement 1: OR347

Differential upregulation of tissue factor and tissue factor pathway inhibitor in monocytes by activated platelets and lipopolysaccharide. **JA Appleby**, JR Wright, RW Farndale, AH Goodall. *Journal of Thrombosis and Haemostasis* 2005; Volume 3, Supplement 1: P1528

Regulation of GPVI-mediated platelet procoagulant response and degranulation. **JA Appleby**, RW Farndale, AH Goodall. *Journal of Thrombosis and Haemostasis* 2003; Volume 1, Supplement 1: OC461

Blood borne tissue factor is present on platelets in the absence of leucocyte microparticles. Alison H. Goodall, Chris I. Jones, Dave J. Lamb, Samantha P. Tull, David A. Payne, **Jackie A. Appleby**. *Blood* 2002; 100(11; part 1) Abstract #200

Inter-donor variability in GPVI-mediated platelet procoagulant response is independent of degranulation and uninhibited by aspirin. **JA Appleby**, SP Tull, CI Jones, RW Farndale, AH Goodall. *Blood, Coagulation and Fibrinolysis* 2002; 13(8) A4; O-3)

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Monocyte activation in whole blood induced by collagen related peptide. N Li, **JA Appleby**, DJ Lamb, JR Wright, R Farndale, AH Goodall. *Thrombosis and Haemostasis* 2001; 86(1): P2707

Abbreviations

ACD: acid-citrate-dextrose
ANOVA: analysis of variance
ASA: acetylsalicylic acid (or aspirin)
AU: arbitrary units
CBA: cytometric bead array
CFR: clot formation rate
COX: cyclooxygenase
CRP: collagen-related peptide
CT: clotting time (thromboelastography parameter)
CTAD: citrate-theophylline-adenosine-dipyridamole
CTI: corn trypsin inhibitor
CV: coefficient of variation ((SD/mean) x 100%)
Cy5: cyanine-5
DEPC: diethyl pyrocarbonate
F: clotting factor (suffix 'a' denotes activated factor)
F(ab')₂: antibody antigen binding region
FS: formyl saline
GP: glycoprotein
GPRP: Gly-Pro-Arg-Pro
HBS: HEPES-buffered saline
HBS/c: HEPES-buffered saline + 2 mM CaCl₂
HEPES: 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IL: interleukin
IU: international units
LPS: lipopolysaccharide
MA: maximum amplitude or clot firmness (thromboelastography parameter)
Mab(s): monoclonal antibody (antibodies)
MCF: maximum clot firmness (thromboelastography parameter)
MFI: mean fluorescence intensity
MdFI: median fluorescence intensity
MPAs: monocyte-platelet aggregates
MP(s): microparticle(s)

MPC®: magnetic particle concentrator (DynaL Biotech)
PBS: phosphate-buffered saline
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PGI₂: prostacyclin
PI3-K: phosphatidylinositol 3-kinase
PKC: protein kinase C
PL(s): phospholipid(s)
PLC: phospholipase C
PPP: platelet-poor plasma
PRP: platelet-rich plasma
PS: phosphatidylserine
PSGL-1: P-selectin glycoprotein ligand
RANTES: chemokine (regulated on activation normal T cell expressed and secreted)
RBC: red blood cells
RPE: R-phycoerythrin
RT: reverse transcription
rTF: recombinant tissue factor
SD: standard deviation
rFVIIa: recombinant Factor VIIa
rTF: recombinant tissue factor
sTF: soluble tissue factor (extracellular domain)
SOCE: store-operated calcium entry
SPDP: 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester
TBS: Tris-buffered saline
TBS/T: Tris-buffered saline + 0.1 % Tween 20
TBS/MT: Tris-buffered saline + 0.1 % Tween 20 + 5 % Marvel dried milk
TEG: thromboelastography
TF: tissue factor
TFA: tissue factor activity
TFPI: tissue factor pathway inhibitor
TNF α : tumour necrosis factor α
TRAP: thrombin receptor activating peptide

TXA₂: thromboxane A₂

U: units

vWF: von Willebrand Factor

XL-CRP: cross-linked collagen-related peptide

5-HT: serotonin

CHAPTER 1: Introduction

1.1. Haemostasis

Haemostasis is the process of stemming blood loss following vascular injury and is therefore essential to life. It involves a fine balance between maintaining blood fluidity and clotting. Malfunction can thus take the form of excessive bleeding when haemostasis fails, or thrombosis when it is activated inappropriately, with the formation of clots or thrombi inside a blood vessel, obstructing flow. Thrombosis in the coronary arteries leading to myocardial infarction is a life-threatening feature of coronary heart disease. This is the main form of cardiovascular disease, a major cause of morbidity and mortality in the Western world and increasingly in the developing world. For example, in the UK, cardiovascular disease accounted for just under 233,000 deaths in 2003 (about 38 % of all deaths). Perhaps more importantly, it is a major contributor to premature death (just under 65,000 in 2003) and is a rising cause of morbidity, with an estimated 2.6 million adults in the UK living with heart disease (BHF Coronary Heart Disease Statistics 2005: <http://www.heartstats.org>). Thrombosis in cerebral arteries causes more than 70,000 strokes in the UK each year, leading to both death and serious disability (Intercollegiate Stroke Working Party, 2004).

Haemostasis is conventionally divided into three phases: (i) primary haemostasis; (ii) coagulation; and (iii) fibrinolysis. In the first phase, platelets adhere to specific elements, particularly collagen and von Willebrand Factor (vWF), in the subendothelium, where it is exposed at the site of injury. Adherent platelets spread and become activated, releasing soluble secondary agonists, thereby recruiting additional platelets to the developing primary plug, which is comprised of fibrinogen-linked platelets. The second phase, coagulation, is the process leading to the formation of an insoluble clot, achieved by the generation of thrombin, which stabilises the primary plug by cleaving fibrinogen to form fibrin, subsequently cross-linked by Factor (F) XIII. Finally, fibrinolysis ensures that the clot is dissolved after the vessel damage has been repaired. The three

phases are not sequential, but rather overlap both temporally and functionally, with aspects of one phase playing a part in another. In addition, there are numerous links between haemostasis and other processes, for example, inflammation (Weyrich *et al*, 2003) and apoptosis (Greeno *et al*, 1996).

The main players in all three phases of haemostasis are the endothelium, plasma proteins and blood cells, particularly platelets. Although it has become clear that leukocytes are also significant (Lowe *et al*, 1985), the importance of platelets in all phases of haemostasis has long been recognised. It is with platelets, and their role in promoting the second phase of haemostasis, coagulation, both in their own right and in their interaction with leukocytes, particularly monocytes, that this thesis will be concerned. This is of significance not only in understanding normal haemostasis, but also thrombosis, particularly arterial thrombosis, where platelets are thought to be especially influential.

1.2. Coagulation

1.2.1. Classical enzyme cascade model of coagulation

The classical enzyme cascade, or waterfall, model of coagulation emerged in 1964 (MacFarlane, 1964; Davie & Ratnoff, 1964). The model envisaged a series of conversions of zymogen to active protease, culminating in the generation of thrombin, which cleaves fibrinogen to form fibrin monomers, which then polymerise to form an insoluble fibrin clot. The basic outline of the traditional model is shown in Figure 1.1; for clarity, a number of alternative and inhibitory pathways and feedback loops are omitted from this diagram. The series of reactions was at one time thought to follow either the intrinsic route initiated by the contact system or the extrinsic route initiated by tissue factor (TF). The routes converged on the activation of FX and the subsequent generation of thrombin. Apart from showing the cascade of proteolytic reactions, the diagram also highlights the calcium-dependency of many of the reactions as well as the

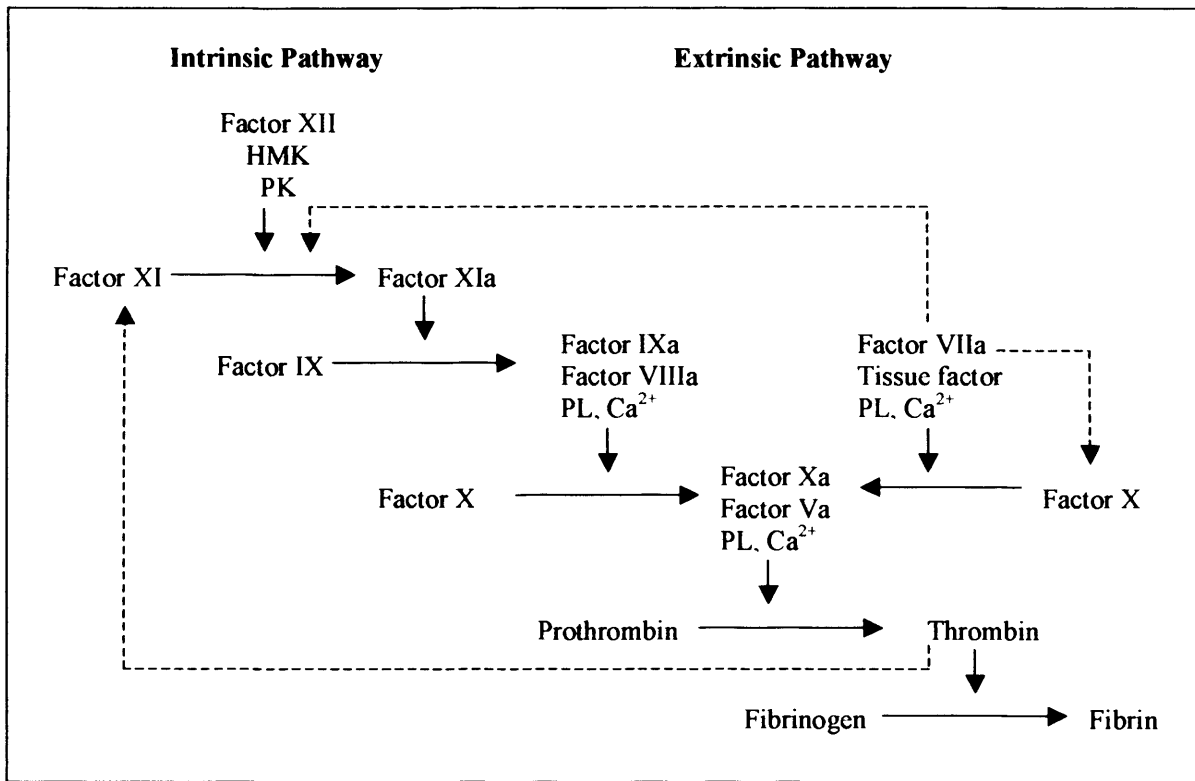


Figure 1.1. The cascade model of coagulation (adapted from Hoffman & Monroe, 2001). HMK: high molecular weight kininogen; PK: pre-kallikrein. The dashed lines show important modifications to the original scheme.

importance of anionic phospholipids (PL), especially phosphatidylserine (PS), required for the assembly of some of the complexes and for enhancing their activity (Bach *et al*, 1986; Thiagarajan & Tait, 1990; Andree & Nemerson, 1995).

Although the cascade model has served well in many respects, it has in recent years been modified as knowledge has increased. Unsurprisingly, the system has turned out to be more complex than the “simple pattern” originally envisaged. Revision was prompted by a number of clinical observations which could not be explained by the earlier version. For example, patients with a FXII deficiency do not suffer from the bleeding problems which one would predict from the cascade model. There was also a striking contrast between the bleeding problems of those with a FXI deficiency, which are often mild, compared with the severe bleeding suffered by those with a FIX deficiency (haemophilia B) (Hoffbrand & Pettit, 1993). Thus, although initially, based on *in vitro* models of contact activation, the intrinsic pathway was thought to be ascendant, TF is now regarded as the main initiator of coagulation (Rapaport & Rao, 1995). It is clear that there are not in fact separate intrinsic and extrinsic pathways since the TF/FVIIa complex can activate FIX as well as FX (Østerud & Rapaport, 1977), thus explaining the mild FXI deficient phenotype as FXI is not the sole means of activating FIX. Another deviation from the original scheme is the reported ability of FVIIa to activate FX independent of TF (Monroe *et al*, 1998). In addition, thrombin is not only one of the products generated downstream as described by the cascade model, but it also plays a complex role in the earlier stages of coagulation through the cleavage of FVIII from its carrier protein vWF (Hamer *et al*, 1987; Pieters *et al*, 1989), and its activation of FXI (Oliver *et al*, 1999) and FV (Suzuki *et al*, 1982). It is also said to contribute to its own generation through its induction of PS exposure in platelets (Thiagarajan & Tait, 1990), so forming catalytic sites for the tenase (van Dieijen *et al*, 1981) and prothrombinase complexes (Rosing *et al*, 1985). In addition, thrombin is important in the later stages of coagulation through its activation of FXIII, which cross-links polymerised fibrin, as well as featuring in its own inhibition, via activated protein C, and in

fibrinolysis, making it central to all aspects of haemostasis. A multi-faceted enzyme with a long evolutionary history (Di Cera, 2003), thrombin also has functions unrelated to haemostasis, such as myoblast survival (Chinni *et al*, 1999) and angiogenesis (Rickles *et al*, 2003).

In recent years the emphasis has been placed on stages of the coagulation process rather than pathways, involving a network of reactions rather than a waterfall: (i) *initiation* via TF, followed by (ii) *augmentation* or *amplification* via the intrinsic pathway (Camerer *et al*, 1996) and (iii) *propagation*, and finally, (iv) *shut down*. Initiation is thought to result in the formation of small quantities of FXa by the TF/FVIIa complex leading to the generation of thrombin. This thrombin is insufficient to cleave fibrinogen throughout the primary plug, but is enough to activate FXI on activated platelets (Naito & Fujikawa, 1991; Greeno *et al*, 1996; Walsh, 2001) and convert FIX to FIXa. It can also activate some FV and FVIII before inhibition of TF/FVIIa/FXa by tissue factor pathway inhibitor (TFPI). By the time TFPI has closed down the TF/FVIIa/Xa complex, the intrinsic route has been primed sufficiently to take over, resulting in a great amplification of thrombin generation.

1.2.2. Cell-based model of coagulation

In a more recent development, emphasis on plasma proteins as regulators of coagulation has switched to the role played by vascular cells. Although the importance of the coagulation proteins is acknowledged, it is now recognised that cells, far from being mere passive surfaces on which the important action takes place, play a significant role in the control of coagulation. This view has been most clearly expounded by Hoffman and Monroe (Hoffman & Monroe, 2001), depicted in Fig.1.2, but has gained wider acceptance, although there is debate over some aspects, such as the presence of platelet receptors for coagulation factors (Heemskerk *et al*, 2002), and it has been criticised for not dealing with spatial clot growth (Ovanesov *et al*, 2005).

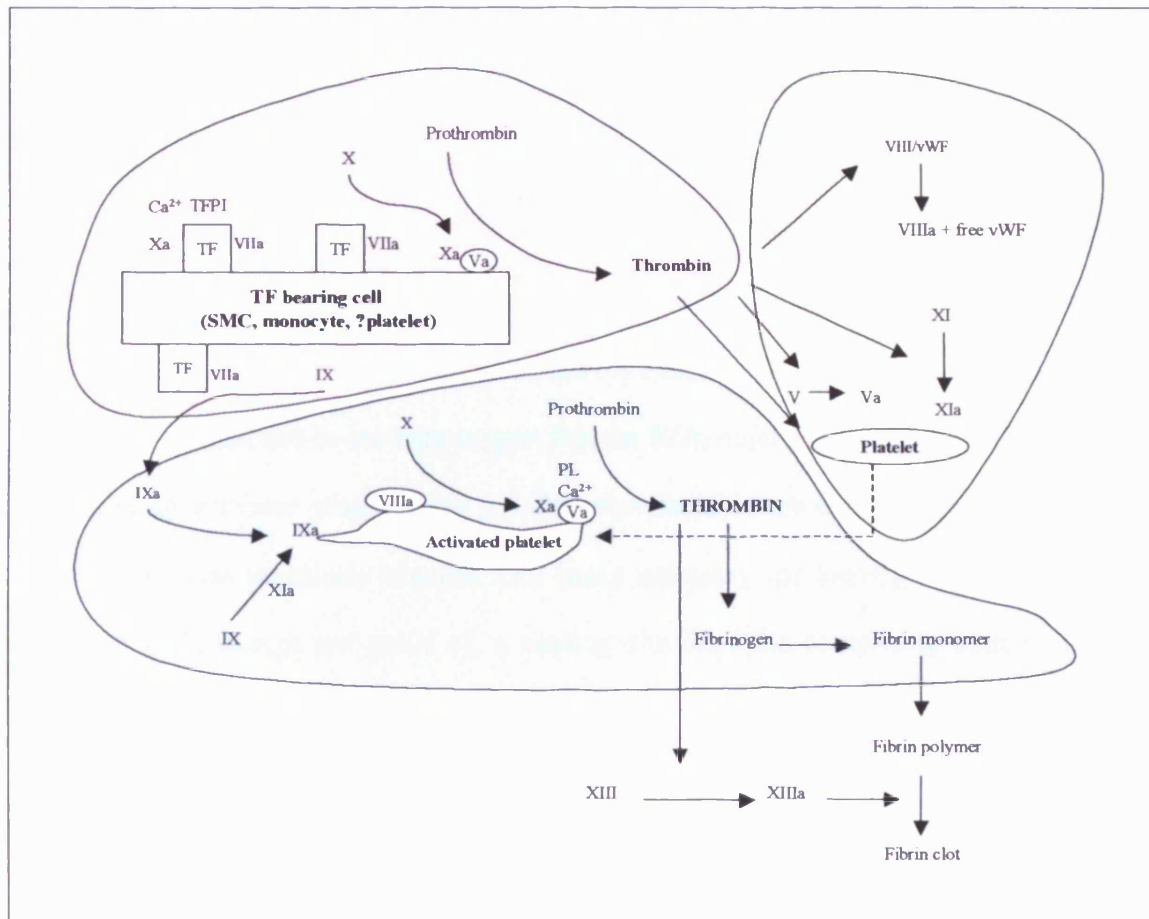


Figure 1.2. Cell-based model of coagulation. Reactions in the pink-shaded box show the initiation phase of coagulation; in yellow, the amplification phase; and in green, the propagation phase. Adapted from Hoffman & Monroe, 2001.

Cellular regulation can be achieved in various ways. Evidence is accumulating that proteins as well as phospholipids (PLs) in the cell membrane have a part to play. As previously stated, activation of FXI occurs on activated platelets; the binding site for FXI has been identified as GPIb α (Baglia *et al*, 2002). There are also persistent, though not universally accepted, reports of other non-lipid receptors (or perhaps more properly, binding proteins, since they may not transmit a signal) for coagulation factors FVIIIa, FIXa and FX being involved in the regulation of coagulation. For example, Walsh and co-workers suggest that the differential ability of Annexin V to inhibit FXa generation on activated platelets and 1:3 PS /phosphatidylcholine (PC) vesicles is indicative of factors other than negatively charged PLs being necessary for binding of the tenase complex, consistent with, though not proof of, a binding site for FIXa comprising both protein and PL (London *et al*, 1996). However, others (Heemskerk *et al*, 2002) feel that there is insufficient evidence to be sure that such binding proteins do play a significant role in FXa generation. Similarly, it has been proposed that non-lipid receptors play a part in the prothrombinase complex. The integrin $\alpha_{IIb}\beta_3$ has been shown to bind prothrombin independently of PLs (Byzova & Plow, 1997), although blocking this interaction with an $\alpha_{IIb}\beta_3$ antagonist only delays, not reduces, thrombin generation (Butenas *et al*, 2001). Some workers have proposed that the prothrombinase complex consists of FXa bound to effector cell protease receptor-1 as well as PL and FVa (Bouchard *et al*, 1997), although the involvement of this receptor was not demonstrated in other studies (Briedé *et al*, 2001).

Whether or not proteins do have a significant part to play, it is clear that PLs do. Since PS is necessary for coagulation to occur (this will be discussed in more detail later), it is clear that control can be exerted both in the way in which PS is exposed and possibly also in how it operates, as there is some evidence that it acts differently in different circumstances. For instance, although it is generally regarded as procoagulant, on endothelial cells PS has been shown to perform an

anticoagulant function (Ravanat *et al*, 1992), implying that factors other than mere presence are involved.

1.3. Tissue Factor (TF)

1.3.1. Main initiator of coagulation

Now that the intrinsic pathway has been subordinated, TF is recognised as the main initiator of coagulation (Bächli, 2000). The realisation that TF/FVIIa complex could activate FIX as well as FX was supported by clinical observations, not only by the phenotypes of FXI and FIX deficiencies (mild and severe, respectively), but by the life-threatening bleeding experienced by (at least some) patients with rare FVII deficiency (Rizza, 1994) which resembles haemophilia.

1.3.2. Structure and function

A 47 kDa transmembrane glycoprotein, TF is a member of the cytokine receptor family and consists of 263 amino acids, of which residues 1-219 comprise the extracellular domain (so-called soluble TF (sTF)), 220-242 the transmembrane domain and 243-263 the cytoplasmic domain (Morrissey *et al*, 1987). The complete sequence of the gene, in which a number of transcription factor binding sites, including AP-1 and AP-2, were identified, was published in 1989 (Mackman *et al*, 1989). Later work suggested that the transcription factor NF- κ B is also important, at least in monocytic cells, since it seems that regulation of TF gene expression may be cell-specific (Mackman, 1997). The structure of the TF gene, which is located on chromosome 1, mRNA and protein is illustrated in Fig.1.3. Recently, an alternatively spliced form of TF, which lacks the transmembrane domain, having exon 4 spliced directly to exon 6, has been detected (Bogdanov *et al*, 2003).

TF has no protease activity itself; rather, it acts as a co-factor for FVIIa, enhancing its activity by four orders of magnitude on its own and more than a millionfold in the presence of

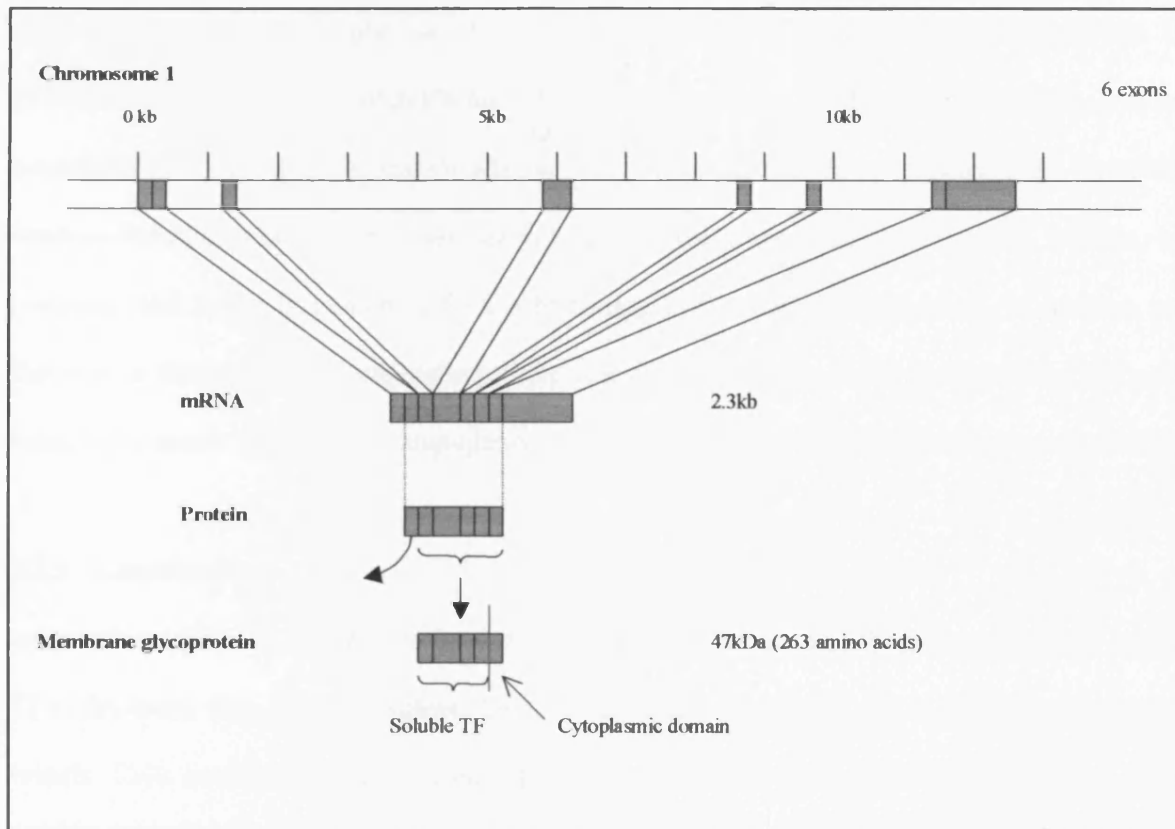


Figure 1.3. Diagram showing the structure of the human TF gene, mRNA and protein. The plasma membrane is denoted by the red line. Adapted from Edgington *et al*, 1991.

anionic PLs (Morrissey, 2001). Exposed TF forms a complex with FVIIa either by capturing the small amount of FVIIa in plasma (about 1 % of the total) or by cleavage of bound FVII. The TF/FVIIa complex activates both FX and FIX, FX being the preferred substrate under most *in vitro* conditions, although it is not known whether this is also the case *in vivo*. Since FIXa and FVIIIa catalyse the activation of FX when assembled into the tenase complex on a PL surface, both reactions lead to the formation of FXa, which triggers the start of coagulation. In addition to its function as the initiator of coagulation, there is also an expanding literature describing TF's other roles, for example in signalling, angiogenesis and metastasis (for a review see Chen *et al*, 2001).

1.3.3. Localisation

Immunohistochemical studies (Drake *et al*, 1989a) have demonstrated the presence of high levels of TF in the brain, skin, placenta, spleen, lung, myocardium, gut mucosa and in the adventitia of blood vessels. Cells constitutively expressing TF include fibroblasts (Zacharski *et al*, 1973) and smooth muscle cells (Schechter *et al*, 1997). It has recently been reported that TF is synthesised and secreted by pancreatic islets cells (Moberg *et al*, 2002), which may form a link between diabetes and coronary heart disease (Nilsson *et al*, 2005). TF has also been found at high levels in atherosclerotic plaques (Wilcox *et al*, 1989) where it presumably contributes to the thrombogenicity of the plaque (Toschi *et al*, 1997). The conventional view has been that, as a protection against unwanted TF activity and coagulation, none of the cells normally in contact with coagulation proteins in plasma produce TF constitutively. Thus TF only becomes accessible to these proteins to initiate coagulation when exposed in the adventitia following damage to the vessel wall. The tissue distribution is therefore thought to constitute a 'haemostatic envelope'.

1.3.4. TF induction in leukocytes

Nevertheless, it has been well documented that some blood cells can be induced express TF under certain circumstances. There is lively debate over the presence of TF in neutrophils (Østerud *et al*, 2000; Nemerson, 2000; Nakamura *et al*, 2004; Østerud, 2004) but monocytes are accepted to express TF in response to a number of inflammatory stimuli such as lipopolysaccharide (LPS) (Rivers *et al*, 1975), immune complexes and activated complement C5a. There is some disagreement over the role of other inflammatory agents such as interleukin (IL)-1 β and tumour necrosis factor α (TNF α). Some claim these also as inducers of monocytic TF (Conkling *et al*, 1988) whilst others regard them as having only an enhancing effect, the discrepancy probably being explained by differences in experimental conditions with monocytes perhaps becoming activated during isolation (Østerud, 2001). Following stimulation with LPS, monocytes are also able to shed TF⁺ microparticles which can disseminate procoagulant activity (Satta *et al*, 1994). Since TF upregulation in monocytes is connected with inflammation, and its appearance is a relatively slow process, as it has to be synthesised *de novo* (Semeraro *et al*, 1983), it is unclear whether it has a role to play in normal haemostasis or whether it reflects the monocyte's part in wound healing. Pathologically, however, it is likely that monocyte TF is important in diseases with a thrombotic component such as sepsis (Østerud & Flaegstad, 1983). TF is also implicated in atherosclerosis, which has a strong inflammatory component, being present in plaques, associated with monocytes and macrophages, foam cells, endothelial cells and smooth muscle cells (Thiruvikraman *et al*, 1996). Apoptotic monocytes and lymphocytes also contribute TF-bearing microparticles to the plaque (Mallat *et al*, 1999). Rupture of such plaques therefore exposes highly procoagulant contents.

In addition to the effect of soluble inflammatory mediators, cell-cell interactions appear to be important. According to some workers, monocyte TF activity is regulated by granulocytes acting in concert with platelets (Halvorsen *et al*, 1993). That platelets influence TF in monocytes has been

recognised for thirty years (Niemetz & Marcus, 1974), although the exact mechanism by which they do so remains to be elucidated. Their effect consists either of the induction of TF in monocytes or of their enhancement of TF activity when it has been induced by another agent (Pellegrini *et al*, 1998). It has been asserted that platelets induce TF by binding to monocytes via P-selectin (Celi *et al*, 1994; Amirkhosravi *et al*, 1996), as P-selectin binding to PSGL-1 causes the transcription factor NF- κ B, one of the transcription factors controlling expression of TF (Mackman, 1995), to be translocated from the cytoplasm to the monocyte's nucleus. However, others have been unable to replicate this finding (Weyrich *et al*, 1996), although monocyte MCP-1 and IL-8 were upregulated by thrombin-activated platelets. Upregulation was not directly dependent on P-selectin, although tethering by P-selectin was required to achieve the close proximity necessary for the chemokine RANTES (regulated on activation normal T cell expressed and secreted; CCL5) to exert its effects. A similar mechanism, where P-selectin tethering was necessary but signalling was via another molecule, was reported for the release of platelet activating factor by monocytes (Elstad *et al*, 1995). In addition, there is some evidence that binding of CD40L on platelets to CD40 on monocytes can induce TF on the latter (Lindmark *et al*, 2000). Thus whether platelets upregulate monocyte TF through direct contact or via soluble mediators is still an open question.

1.3.5. Blood-borne TF

Despite the notion of TF being sequestered from coagulation factors, there have been reports for some years of TF being detected in the plasma of healthy subjects (Fukuda *et al*, 1989; Albrecht *et al*, 1996). Elevated levels of TF antigen have been found in patients suffering, for example, from sepsis (Green *et al*, 2002), disseminated intravascular coagulation (Gando *et al*, 1998), paroxysmal nocturnal haemoglobinuria (Liebman & Feinstein, 2003), angina (Misumi *et al*, 1998) and diabetes (Koyama *et al*, 1994; Zumbach *et al*, 1997). Increased activity in pathological states has also been measured by some workers, for example, in sickle cell disease (Key *et al*, 1998) and sepsis (Green

et al, 2002). It is not always clear whether measurements are being made of soluble TF or TF associated with microparticles, nor whether antigen is matched by activity. These two questions may be related in that a phospholipid environment, perhaps provided by microparticles, is necessary for TF activity. In a seminal paper (Giesen *et al*, 1999), it was reported that TF participated in the build-up of a thrombus in a situation where it could only have originated from the blood. The TF appeared to be associated with microparticles, possibly leukocyte-derived, which clustered around platelets in the forming thrombus. The significance of this paper lies in its demonstration of the involvement of blood-borne TF in thrombus propagation at the site of injury, with coagulation being initiated by TF not exposed at the site of injury as previous dogma had dictated.

The origin of blood-borne TF, be it soluble or microparticle-associated, is hotly debated. It is speculated that sTF (that is, the extracellular domain) may arise from the shedding of TF from the cell surface in a manner comparable with the shedding of certain cytokine receptors, such as IL-6R (Rose-John & Heinrich, 1994), with which TF shares a resemblance. Alternatively, TF may be released into the blood embedded in microparticles. These would be most likely to arise from leukocytes, particularly monocytes, which are known to be capable of synthesizing TF in response to certain stimuli including LPS, which also induces vesiculation (Satta *et al*, 1994). There is some evidence to suggest that leukocytes are indeed the source of TF⁺ microparticles (Rauch *et al*, 2000), though other groups report finding TF⁺ microparticles from red cells, platelets and granulocytes (Biro *et al*, 2003). It is also possible that microparticles shed during apoptosis could carry TF (Mallat *et al*, 1999). In addition, there is a report that pancreatic islet cells can release microparticles containing active TF (Moberg *et al*, 2002).

It might be expected that were this circulating TF constitutively active, there would be general coagulation. However, low levels of TF activity (TFA) in plasma from healthy donors have been reported (Key *et al*, 1998) as have low levels of thrombin (Berckmans *et al*, 2001), which at

these levels may act as an anticoagulant. Even if this is the case, it is still possible that there is some inactive TF in circulation as it can exist in an 'encrypted' form.

1.3.6. TF activity: encryption and de-encryption

It has long been realised that TF antigen and activity are not necessarily the same thing. In one study, although TF was found only on the surface of monocytes and was capable of binding FVII/FVIIa and accessible to inhibition by antibodies, its procoagulant activity on intact cells was much less than that of disrupted cells, implying a regulatory role for the membrane, possibly by its PL composition (Drake *et al*, 1989b). This has led to the notion of TF encryption – defined as “post-translational suppression of TF procoagulant activity on the cell surface” (Bach, 2006) - when TF is detectable but not fully active. The exact mechanism whereby TF is de-encrypted is not fully understood. It appears that the cytoplasmic domain is not required (Wolberg *et al*, 2000; Carson & Bromberg, 2000), which suggests that signalling is not involved. Experiments using the calcium ionophore A23187 to raise intracellular calcium concentration were consistent with a role for PL in the regulation of TF activity (Bach & Rifkin, 1990), as was a later study which showed TF activity rendered manifest after disruption of fibroblast cell membranes with detergent (Carson, 1996). At least in the endothelial cell-line ECV304, TF/FVIIa activity may be regulated by translocation to caveolae (Sevinsky *et al*, 1996). However, other workers have shown that there are also PS-independent mechanisms of TF de-encryption (Wolberg *et al*, 1999). It has also been suggested that dimerisation of TF is involved (Bach & Moldow, 1997). More recent papers concerning the role of lipid rafts in the regulation of PS exposure give rise to the speculation that they might also be involved in the de-encryption of TF (Kunzelmann-Marche *et al*, 2002).

1.3.7. Inhibition of TF by TFPI

The principal inhibitor of TF is TFPI, which is synthesised primarily and constitutively by endothelial cells, but found in three pools: the endothelium (50 - 80%), plasma (10 – 50%, mostly complexed with lipoproteins) and platelets (2.5%) (Bajaj *et al*, 2001). Other cells which have been shown to produce TFPI are monocytes (Ott *et al*, 2001) and platelets (Novotny *et al*, 1988; Bajaj *et al*, 1990). A Kunitz-type serine protease inhibitor, TFPI inhibits TF/FVIIa most efficiently in the presence of FXa, to which it binds via the second Kunitz domain, promoting binding of its first Kunitz domain to TF/FVIIa. Inhibition by TFPI occurs shortly after the formation of the TF/FVIIa/FXa complex, so that thereafter FIXa and FXa can only be generated on the platelet surface by the tenase complex and bound FXIa as coagulation enters the amplification phase.

1.4. Role of phospholipids in coagulation

1.4.1. PL enhancement of procoagulant activity

It is apparent from the above that PLs, in the presence of Ca^{2+} , play a key role in several of the reactions of the coagulation process: in the encryption and de-encryption of TF and the activity of TF/FVIIa complex, as well as the tenase and prothrombinase complexes. For completeness, it should be mentioned that PLs are also important in some anticoagulant reactions, such as the inactivation of FVa by activated protein C. Their procoagulant role has been recognised from at least 1949 (Ware & Seegers, 1949), but, despite progress regarding the nature of this role, much remains to be done. Although kinetic studies show that reaction rates of all three complexes are greatly enhanced in the presence of PLs (Rosing *et al*, 1980), it is not clear exactly how this is achieved. It is possible that it is due to the increased substrate concentrations caused by their binding to PLs and by the confining of sequential reactions to the same membrane. Some groups believe that it is the negative charge carried by PLs, particularly PS, which is important, and indeed there are data to suggest that there is an optimal charge for supporting procoagulant activity

(Bangham, 1961). Other data, however, show that the introduction of stearylamine to give a PS-containing membrane an overall positive charge had little effect on prothrombin-converting activity (Rosing *et al*, 1988). Since phosphatidyl-beta-lactate, which differs from PS only by an amino group in the polar head, was sensitive to this treatment, the implication was that the amino group of PS is important in the interaction with coagulation proteins. Others have also argued that PS has a specific role, basing this view on experiments showing that a soluble form of PS known as C6PS binds to and induces conformational changes in prothrombin, FXa and FVa as summarized in (Lentz, 2003).

The mixture of PLs, as well as the presence of Ca^{2+} , appears to be of critical importance in the activity of coagulation complexes. For example, experiments using vesicles with varying proportions of PC and PS demonstrated appreciable TF/FVIIa activity with PC alone which was enhanced when PS was added (Bach *et al*, 1986). It has been found that a mixture of neutral PC and anionic PS is a more potent catalyst of prothrombinase activity than either PL alone (Pei *et al*, 1993). The most effective combination is PS - phosphatidylethanolamine (PE) - PC, and the least effective PS-sphingomyelin mixtures (Smeets *et al*, 1996). When membrane asymmetry collapses, this combination is achieved through the exposure of PE as well as PS and the decrease of sphingomyelin in the outer layer (see Fig 1.4). Although various negatively charged PLs enhance the activity of coagulation complexes when mixed with PC, PS, and in particular phosphatidyl-L-serine, is the most effective anionic PL, optimally at a concentration of 10 – 15 %mol (Zwaal *et al*, 1998).

1.4.2. PLs in the plasma membrane

The source of the PLs which participate in coagulation is, of course, the plasma membranes of the cells of the vasculature. PLs make up about half of the mass of most mammalian plasma membranes, the remainder being membrane proteins. Of these PLs, 50-60% contain choline (PC

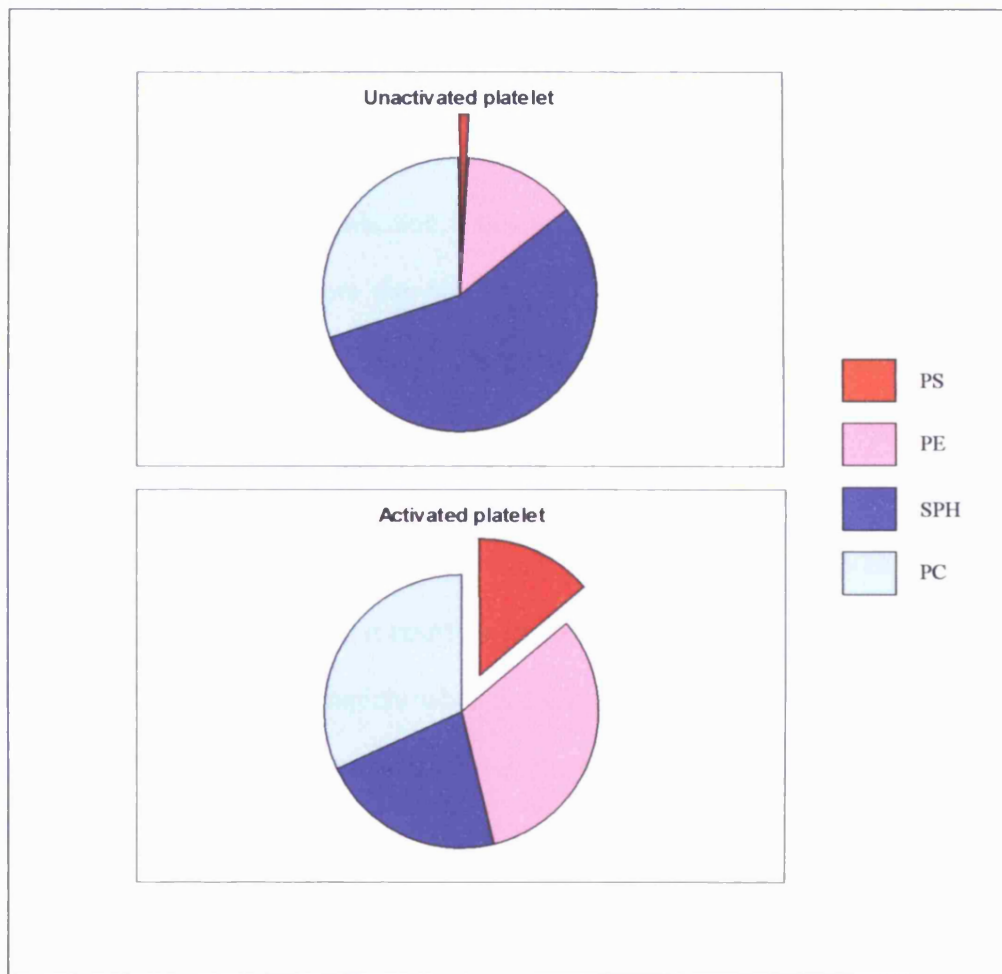


Figure 1.4. Change in PL composition of outer leaflet of platelet plasma membrane following activation. The proportion of aminophospholipids PS and PE are increased at the expense of choline-phospholipids sphingomyelin (Sph) and PC when platelets are activated by strong agonists. Adapted from Zwaal *et al*, 2004.

and sphingomyelin) whilst the others are the aminophospholipids, PS and PE (Zwaal, 1978). PS content varies between 8 and 14 mol% depending on the type of blood cell - in platelets about 10 % (Heemskerk *et al*, 2002). The distribution of these PLs in the unactivated cell is asymmetric, with the choline-containing PLs being on the outside of the membrane and the aminophospholipids sequestered on the inside. This distribution is maintained by an ATP-dependent translocase which rapidly transports PS and PE from the outer to the inner leaflet of the plasma membrane in conjunction with an ATP-dependent floppase which slowly transports both amino and choline PLs in the opposite direction (Zwaal & Schroit, 1997). This is shown diagrammatically in Fig.1.5. The asymmetric arrangement means that when cells are resting or undamaged the procoagulant PLs are not in contact with coagulation proteins. Interestingly, as noted previously, the TF/FVIIa complex is active with PC alone, which suggests it could be active on the surface of unstimulated cells.

This situation can change rapidly when the cell is activated: the activity of the translocase and floppase ceases and a scramblase, as yet unidentified, then operates to randomise the distribution of all PLs with the result that more PS and PE are exposed on the cell surface (Fig.1.5). Activation of the scramblase requires a persistent rise in cytosolic Ca^{2+} concentration, which simultaneously inhibits the translocase (Heemskerk *et al*, 2002). This process occurs in all blood cells but is particularly notable in platelets (Beyers *et al*, 1982) and is usually accompanied by the formation of microparticles (Comfurius *et al*, 1990). Both the microparticles and the 'parent' platelet thus have an optimal PL composition for the prothrombinase and tenase complexes (Sims *et al*, 1988). The importance of the redistribution of PLs is demonstrated by the bleeding problems suffered by patients with Scott syndrome, where aminophospholipid transport is defective (Weiss *et al*, 1979). Conversely, coagulant activity could be detected on the resting platelets of a patient suffering from 'inverse Scott syndrome', or Stormorken syndrome, with permanently exposed PS (Stormorken *et al*, 1995).

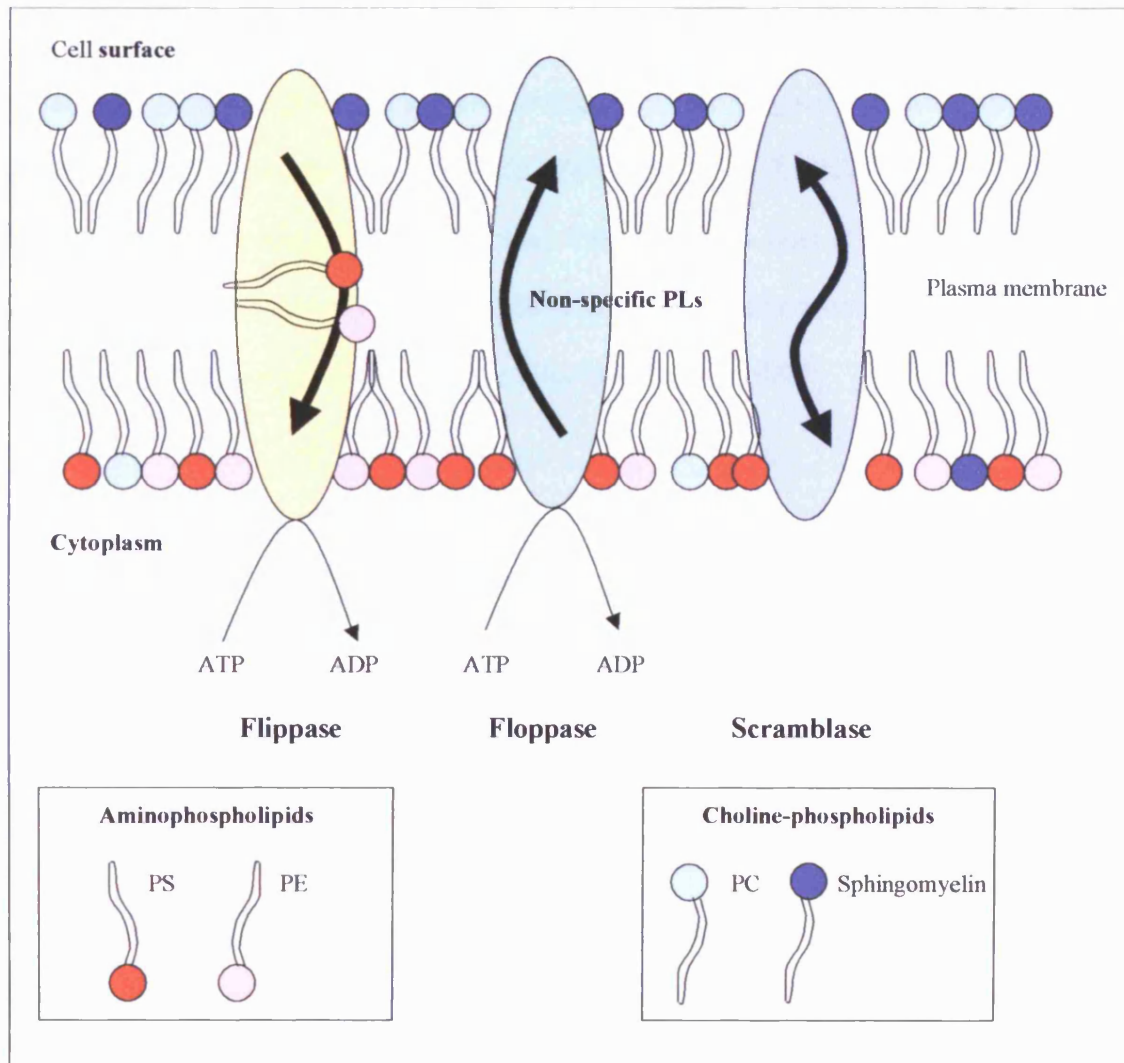


Figure 1.5. Transmembrane lipid transportation. Aminophospholipids PS and PE are transported inwards by a flippase and both aminophospholipids and choline-phospholipids are transported slowly outwards by a floppase, maintaining the PL distribution shown in the diagram. Both movements are against a gradient and ATP-dependent. A rise in cytosolic calcium activates a scramblase which transports PLs in both directions, destroying membrane asymmetry within minutes. Adapted from Zwaal *et al*, 2004.

Exposure of PS is also a hallmark of apoptosis (Martin *et al*, 1995). This makes apoptotic cells a potential source of procoagulant activity (Greeno *et al*, 1996) and raises the question as to whether there are any mechanistic links between apoptosis in nucleated cells and PS exposure in platelets. It has also been proposed that PS marks cells out for phagocytosis and is therefore part of the clearance mechanism for apoptotic cells (Fadok *et al*, 1992; Fadok *et al*, 1998; Fadok & Henson, 2003) and possibly also for platelets. That this is the case is lent weight by the low platelet count found in patients with inverse Scott syndrome despite normal platelet production and asplenia blocking the normal clearance route (Stormorken *et al*, 1995).

1.5. Platelets

Although the provision of PLs by platelets plays an important part of coagulation, their role in haemostasis is much wider-ranging and is set out in more detail below.

1.5.1. Structure and function

Platelets ‘bud off’ from precursor multinucleate megakaryocytes and circulate for between five and ten days before being cleared from the circulation in the spleen. Despite their small size, they play a pivotal role in all phases of haemostasis, and their structure is adapted to this (for a review see Hartwig, 2002). When unstimulated, platelets are a discoid shape (dimensions $\sim 3\mu\text{m} \times 0.5\mu\text{m}$) but the plasma membrane is invaginated, forming an open canicular system which allows the platelet a much greater potential surface area. Although lacking a nucleus, platelets have a full complement of other organelles: mitochondria, lysosomes and endoplasmic reticulum. There are also two types of specialised granule in the platelet cytoplasm: α -granules which contain proteins such as cytokines, plasminogen activator inhibitor-1, platelet factor 4, β -thromboglobulin, fibrinogen, FV and vWF, and P-selectin in their membrane; and smaller dense granules whose contents of low molecular weight molecules include high concentrations of ATP, ADP, calcium and serotonin (5-HT)

(Holmsen & Weiss, 1979). The cytoskeleton is a prominent feature of the platelet, maintaining its shape when resting, often in conditions of high shear, and playing an active role in its activation. The major proteins comprising the cytoskeleton are spectrin and actin, the most abundant of the platelet proteins (about 20 % of the total protein). Actin filaments are cross-linked by filamin, which also links actin to the cytoplasmic tail of the receptor glycoprotein (GP) Ib α (CD42b) via actin binding protein, but in the resting platelet about 60% of actin exists as a pool of unpolymerised actin bound to β 4-thymosin (Hartwig, 1992).

Since they are anucleate, platelets cannot transcribe mRNA, but they do contain in their cytoplasm numerous transcripts derived from their parent megakaryocyte. Recently, the presence in platelets of a functional spliceosome capable of processing pre-mRNA has been described (Denis *et al*, 2005). Platelets possess the machinery for protein synthesis and there have been reports of this occurring (Kieffer *et al*, 1987; Lindemann *et al*, 2001). Nevertheless, the majority of proteins found in the platelet are presumed to derive from the megakaryocyte in which they were either synthesised or, in the case of FV, endocytosed from plasma (Camire *et al*, 1998). However, a number of proteins found in platelet granules, such as fibrinogen and albumin, have been shown to be acquired from plasma, by endocytosis or possibly uptake via GPIIb/IIIa by platelets themselves as well as megakaryocytes (Harrison *et al*, 1989; Handagama *et al*, 1993).

Activation causes profound changes in platelet morphology. Platelets adhering to the site of injury in the vessel wall undergo a rapid shape change as they spread on the surface, becoming spherical and then extending pseudopodia. This entails a reorganisation of the cytoskeleton, with the assembly of new actin filaments, following cleavage of actin filaments by gelsolin, which is activated during platelet activation, and uncapping of actin barbed ends (Hartwig, 1992). Exposure of barbed ends on preexisting actin filaments allows the assembly of new filaments, tapping into the large pool of unpolymerised actin stored in complex with β 4-thymosin. Cytochalasins prevent this

reorganisation by binding to the exposed barbed ends (Fox & Phillips, 1981) and can therefore be used as experimental tools to prevent actin polymerisation.

These activation changes are mediated by an array of receptors on the platelet surface. The receptors can be assigned to several families and some of the more important are listed in Table 1.1. Platelet receptors of the integrin superfamily include the fibrinogen receptor $\alpha_{IIb}\beta_3$ (or GPIIb/IIIa), the collagen receptor $\alpha_2\beta_1$, the vitronectin receptor $\alpha_v\beta_3$, and the fibronectin receptor $\alpha_5\beta_1$. Typically, these integrins require activation to become functional. The most numerous platelet receptor, with around 60,000 copies per platelet is $\alpha_{IIb}\beta_3$, followed by GPIb, a member of the leucine-rich repeat family found uniquely on platelets and megakaryocytes, with about 25,000 copies per platelet (Coller *et al*, 1983). The seven transmembrane G-protein coupled receptor family is the major agonist receptor family. The main members of this family on platelets are the thrombin receptors PAR1 and PAR4 (Coughlin, 2000), the ADP receptors P2Y₁ and P2Y₁₂ and the thromboxane (TXA₂) receptor. Other important platelet receptors of this class are the prostaglandin I₂ receptor, which binds PGI₂ released by endothelial cells to maintain platelets in a resting state, and a number of chemokine receptors (Clemetson *et al*, 2000). The collagen agonist receptor GPVI is a member of the immunoglobulin superfamily. Although it has recently been detected on endothelial cells (Sun *et al*, 2003), there have been no reports of it having been found on any blood cells other than platelets (Jandrot-Perrus *et al*, 2000). Another receptor with immunoglobulin domains, CD31 or PECAM-1, is reported to regulate platelet responses involving GPVI (Patil *et al*, 2001; Cicmil *et al*, 2002). Other members of the FcR family found on platelets are the Fc receptors Fc γ RIIA (CD32) and Fc ϵ RI. Of the many other platelet receptors so far identified, two relevant to this thesis are CD40L, the ligand for CD40 which is expressed on the surface of activated platelets (Henn *et al*, 1998) and P2X₁ which is a ligand-gated cation channel responsible for rapid Ca²⁺ influx. The ligand in question was once thought to be ADP until it was realised that most ADP preparations are contaminated with ATP, which is now regarded as the actual ligand (Mahaut-Smith

Family, receptor	No. per platelet	Principal ligand	Principal function
<i>Integrins</i>			
$\alpha_{IIb}\beta_3$ (GPIIb/IIIa)	~80,000	Fgn, vWF, Fn, Vn	Firm adhesion, aggregation
$\alpha_v\beta_3$	50-100	Vn	Adhesion
$\alpha_2\beta_1$ (GPIa/IIa)	<1,000	Collagen	Adhesion
$\alpha_5\beta_1$	<1,000	Fn	Adhesion
$\alpha_6\beta_1$	<1,000	Laminin	Adhesion
<i>Leucine-rich repeat</i>			
GPIb-IX	~25,000	vWF, thrombin	Initial adhesion
GPV	~12,000	vWF, thrombin	Initial adhesion
<i>Selectins</i>			
P-selectin (CD62P)	800 (unstimulated) 9,600 (activated)	PSGL-1	Adhesion to leukocytes
<i>Seven transmembrane</i>			
PAR1	~2,500	Thrombin	Aggregation, secretion
PAR4		Thrombin	Aggregation, secretion
P2Y ₁	~250	ADP	Shape change, transient aggregation
P2Y ₁₂	~500	ADP	Potential of secretion, sustained aggregation
TXA ₂ receptor (TP)	~2,000	TXA ₂	Amplification of activation
PGI ₂ receptor (IP)	~90 low, ~2,750 high affinity	PGI ₂	Inhibition of aggregation
PAF receptor	300	PAF	Aggregation
CXCR (various)		Chemokines	Aggregation
CCR (various)		Chemokines	Aggregation
β -adrenergic receptor		Epinephrine	Enhancement of aggregation
5-HT receptor		5-HT	Amplification of activation
<i>Immunoglobulin superfamily</i>			
GPVI	~1,000	Collagen	All aspects of platelet activation
Fc γ RIIA (CD32)		IgG Fc	Immunological defence
PECAM-1 (CD31)		PECAM-1	Regulation of collagen response
CD47		TSP	Regulation of $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$.
<i>Miscellaneous</i>			
P2X ₁	~80-130	ATP	Thrombus formation at high shear
CD36 (GPIV)	12-19,000	TSP, ?collagen	Fatty acid transporter

Table 1.1. Principal platelet receptors. Fgn: fibrinogen, Fn: fibronectin, PAF: platelet activating factor, TSP: thrombospondin, Vn: vitronectin. Compiled from McEver & Martin, 1984, Neri Serneri, 1984, Armstrong, 1996, MacKenzie *et al*, 1996 and Clemetson, 2002.

et al, 2000). The roles of those receptors most pertinent to this study will be described in more detail below.

1.5.2. Platelet activation responses

Although the focus of this study is on the procoagulant response, primary haemostasis and coagulation are so entwined that the one can only be fully understood in the light of the other, and it could be argued that the distinction is in any case false. Both aspects therefore form a relevant backdrop to this project. Platelet activation entails a complex network of interactions and intracellular signalling pathways which are not yet fully elucidated. Calcium signalling appears to be common to both components of platelet activation. The concept of Ca^{2+} thresholds for different responses has been in coin for some time (Crawford & Scrutton, 1994), with $\alpha_{\text{IIb}}\beta_3$ activation and shape change requiring the smallest change in $[\text{Ca}^{2+}]_i$ and PS exposure the greatest. Thus both conformational change in GPIIb/IIIa, which results in the binding of fibrinogen and consequent platelet aggregation, and secretion of α -granule contents with P-selectin exposure are triggered at lower internal Ca^{2+} concentrations than PS translocation. Agonists differ in their ability to induce calcium fluxes, but the data showing ADP to be comparable with or stronger than collagen in this regard indicate that other factors are involved in platelet activation, since in terms of end-points, ADP and TXA_2 are regarded as weak, and collagen and thrombin as strong agonists (Crawford & Scrutton, 1994). It seems that it is both the change in cytosolic calcium concentration and its duration which determine the level of activation response (Heemskerk *et al*, 2002).

1.5.2.1. Adhesion

Platelet activation starts when platelets adhere to constituents in the subendothelium exposed following damage to the vessel endothelium. The nature of the ligand-receptor interaction is determined by the flow conditions. At high shear, the initial interaction is mediated by the

interaction between collagen-bound vWF and GPIb $_{\alpha}$ (Ruggeri, 1997; Ruggeri, 2003), which causes the platelet to slow down to the extent that other receptors can also participate in tethering the platelet to the wound. Firmer adhesion is maintained by the binding of vWF to $\alpha_{IIb}\beta_3$. Activation of the latter may be by calcium signals induced by GPIb (Nesbitt *et al*, 2002), and the platelet starts to spread. Under conditions of lower shear, initial adhesion is thought to involve collagen, fibronectin and laminin, mediated by GPVI and/or integrins such as $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$.

1.5.2.2. Aggregation

Conformational changes in $\alpha_{IIb}\beta_3$ allow it to bind soluble fibrinogen in the plasma. Since fibrinogen has dual binding sites, it can form bridges between activated platelets. The latter become activated either by having adhered to the subendothelium themselves, or through binding soluble agonists released by other platelets which are adherent or from damaged endothelial or red cells. Chief among the soluble agonists is ADP, released from the dense granules upon activation (Holmsen & Weiss, 1979) and found at high concentrations in red blood cells (RBC). Another, TXA₂, is generated from PLs in the plasma membrane via the cyclooxygenase pathway and is also released and able to activate passing platelets. Both these and other soluble agonists, such as epinephrine and 5-HT, are also able to bind to receptors on the platelet which released them, thus enhancing the original activation. Although the thrombus grows primarily via fibrinogen bridges between platelets, vWF can also link platelets, and P-selectin has been reported to stabilise platelet aggregates by means of an as yet unidentified receptor (Merten & Thiagarajan, 2000), possibly GPIb (Romo *et al*, 1999), or even PSGL-1. There have been conflicting reports about the presence of PSGL-1 on platelets (Frenette *et al*, 2000; Sperandio *et al*, 2003). Recent advances in technology have allowed real-time imaging of the formation of a thrombus in animal models. These show that the majority of platelets pass by a wound too quickly to adhere and that there is also a rapid

dissociation of those that do adhere (Falati *et al*, 2002). The thrombus subsequently forms around a small nucleus of platelets which do manage to establish firm adhesion.

1.5.2.3. Degranulation

Degranulation of activated platelets constitutes a targeted delivery system of a wide range of substances which influence the course of haemostasis from aggregation and coagulation to fibrinolysis. Secretion requires more potent activation than either shape change or the conformational change in $\alpha_{IIb}\beta_3$ which allows aggregation and whether ADP can achieve degranulation is controversial (Janes *et al*, 1994; Cattaneo *et al*, 2002). The secretome has recently been analysed using proteomic techniques; over 300 proteins were identified (Coppinger *et al*, 2004). In addition to proteins in the α -granules, it includes several nucleotides and ions stored in the dense granules. The release of ADP and ATP can lead to high local concentrations of these agonists which both recruit additional platelets into a thrombus and reinforce activation in the originating platelet. It has been suggested that the release of agonists from granules which have gathered together in the centre of the platelet following activation gives them a lengthy passage to the outside, thus prolonging the time over which ADP can activate other platelets compared with direct release from the plasma membrane (Fogelson & Wang, 1996). Degranulation also leads to the translocation of P-selectin to the platelet surface. This allows platelets to interact with monocytes and other leukocytes which express P-selectin glycoprotein ligand (PSGL-1). There are also internal pools of $\alpha_{IIb}\beta_3$, the TXA_2 receptor and $P2Y_1$ (Nurden *et al*, 2003) resident in the membranes of the platelet granules which are translocated to the surface on degranulation.

In one of several links between degranulation and coagulation, the platelet releasate includes partially activated FV (Wyshock *et al*, 1994) and FXI (Tuszynski *et al*, 1982). It has been reported that a sub-population of predominantly young platelets (identified by the binding of thiazole orange to mRNA in reticulated platelets) activated by convulxin and thrombin together,

known as COAT platelets, express high levels of α -granule-derived FV and that these preferentially bind FXa, making them highly procoagulant (Alberio *et al*, 2000).

1.5.2.4. Platelet PS exposure

The provision of anionic PLs on the platelet surface has been recognised as a major contributor to procoagulant activity for many years (Bever *et al*, 1982) and is often referred to as the platelet procoagulant response. Requiring high and sustained calcium fluxes, it has long been apparent that not all agonists are able to achieve this. Physiological agonists capable of inducing high Ca^{2+} concentrations are thrombin, collagen, and particularly a combination of both, and the membrane attack complex of complement (C5b-9) (Sims *et al*, 1988). By common agreement, the calcium ionophore A23187 is the most potent platelet agent in this regard, and as such has proved useful as a tool in dissecting out mechanisms (Bassé *et al*, 1993), although its physiological relevance must be questionable. Cytoplasmic calcium concentration is raised by the release of Ca^{2+} from the endoplasmic reticulum and entry of extracellular calcium triggered by depletion of intracellular stores, known as store-operated calcium entry (SOCE), mediated by the actin cytoskeleton (Rosado *et al*, 2000; Kunzelmann-Marche *et al*, 2001). Although a rise in $[\text{Ca}^{2+}]_i$ is necessary for PS exposure, it is not sufficient (Siljander *et al*, 2001), which implies that other signals must be operative. This was graphically illustrated recently when it was shown that a Scott syndrome patient's platelets failed to expose PS despite showing normal calcium mobilisation (Munnix *et al*, 2003).

PS exposure has also been shown to occur after a fall in intracellular pH (Stout *et al*, 1997). Interestingly, acidification also occurs in apoptosis (Meisenholder *et al*, 1996), or programmed cell death, which exhibits other similarities to the platelet procoagulant response in terms of PS exposure (Martin *et al*, 1995). It is not known whether scramblase activation due to acidification,

rather than a rise cytosolic Ca^{2+} concentration, is of physiological significance, although it may play a part in the exposure of PS seen in platelets stored for transfusion (Shapira *et al*, 2000).

The process of PS exposure is usually accompanied by the formation of microparticles (MPs), although it has been possible to separate the two phenomena by means of non-physiological agonists (Dachary-Prigent *et al*, 1995; Bucki *et al*, 1998) suggesting that there are two distinct though closely linked underlying mechanisms. The MPs which bud off are themselves procoagulant, possibly more so than the 'parent' platelets (Sims *et al*, 1988; Sims *et al*, 1989). It is not clear what normal function, if any, procoagulant platelet-derived microparticles perform, although a recent paper has described finding small numbers in the circulation of healthy subjects and speculated that they might perform an anticoagulant function by generating low levels of thrombin which activates the anticoagulant protein C (Berckmans *et al*, 2001). There have, however, been numerous reports of elevated numbers of MPs, derived from platelets and leukocytes, in the blood of patients with a variety of pathological states, such as diabetes (Diamant *et al*, 2002), acute coronary syndromes (Mallat *et al*, 2000), sepsis (Joop *et al*, 2001), patients undergoing coronary bypass surgery (Nieuwland *et al*, 1997) and paroxysmal nocturnal haemoglobinuria (Hugel *et al*, 1999).

1.5.3. Platelet agonists and receptors

As mentioned previously, agonists differ in their ability to evoke these activation responses, implying that they act via distinct pathways. Some of these agonists and their mode of action will now be reviewed in more detail. The principal agonist receptors mentioned in this project, together with an outline of their signalling pathways, are shown in Fig.1.6.

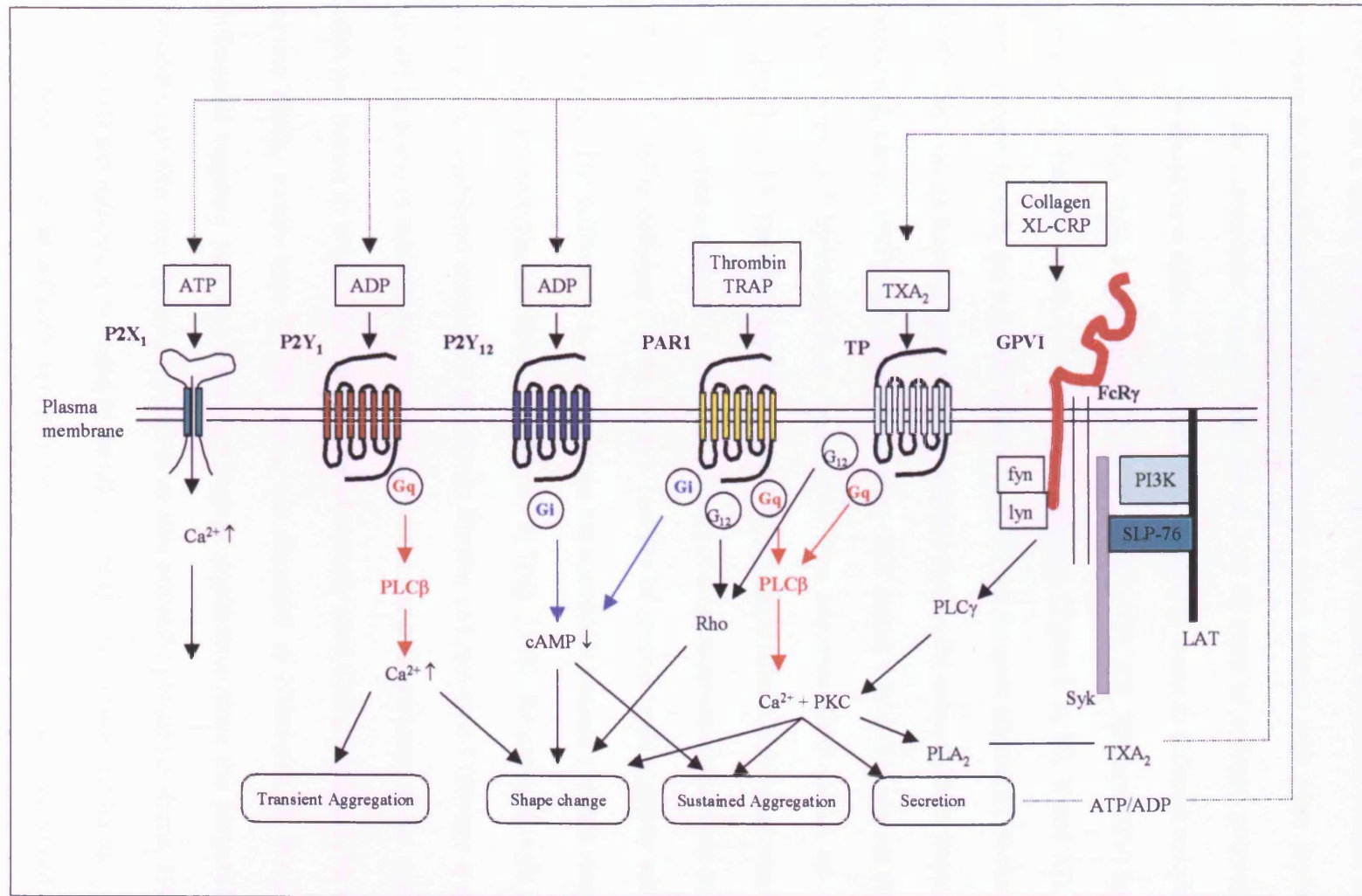


Figure 1.6. Principal platelet agonist receptors mentioned in the text and their signalling pathways. Adapted from Woulfe et al, 2002 and Gachet, 2001.

1.5.3.1. Collagen and collagen receptors

Collagens are a family of closely related proteins synthesised by smooth muscle cells which can aggregate to form filaments, fibrils or meshworks which interact with other proteins to provide support in the extracellular matrix. There are at least 28 types of collagen polypeptide chains (α chains) produced from different genes which combine to give rise to different morphological forms (Farndale, 2006). Nine of these types (I, III, IV, V, VI, VIII, XII, XIII and XIV) have been found expressed in the vessel wall. Of the fibrillar collagens (Types I, II, III, V and XI), which are the most common form in the subendothelium, I and III are the most abundant (Prockop & Kivirikko, 1995). The network forming type IV is the principal form in the subendothelial basement membrane (Stevens & Lowe, 1997). Collagens have repeat GXY motifs where G is glycine and X and Y are often proline and hydroxyproline respectively. The sequence GPO makes up about 10% of collagens I and III. The GXY repeat sequence forms a single helix which, when associated with two other chains, forms a superhelix. The cross-linking of these monomeric structures results in fibrillar collagen. Fibrillar collagen *in vivo* usually consists of several types together with other matrix components. The differences in collagen types are significant because platelets react differently to different collagen types and structures (Alberio & Dale, 1998); for example, $\alpha_2\beta_1$ is essential for adhesion to solubilised monomeric but not to fibrillar collagen type I (Savage *et al*, 1999). This should be borne in mind in experimental work since the collagen preparations used vary and are often not human in origin (for example, the commonly used Collagenreagent Horm® consists of equine fibrils, mostly type I). The issues are discussed in Nieswandt & Watson, 2003. The differential response may also have pathological significance since the proportion of different collagens can alter over the development of an atherosclerotic plaque (Ooshima, 1981; van Zanten *et al*, 1994) and collagen is degraded by metalloproteinases into monomeric forms.

Several platelet collagen receptors have now been identified and sequenced. The two main receptors are the integrin $\alpha_2\beta_1$ and GPVI; CD36 appears to play a lesser role and it is possible that

there are other collagen receptors. Over the past few years a two-receptor model has been developed which posits initial binding to $\alpha_2\beta_1$, which recognises sequences in collagens I, III, IV and VI, with signalling then occurring through GPVI (Barnes *et al*, 1998). More recently, this model has been questioned, with suggestions that GPVI might also be involved in adhesion as well as signalling. This has given rise to some lively debate, not least because some studies have examined mouse platelets, which may or may not be applicable to humans. A recently published study has demonstrated a complex pattern of cross-talk between GPIb, GPVI and $\alpha_2\beta_1$ in thrombus formation (Siljander *et al*, 2004) and even more recently, a unifying model in which GPVI activation can either precede or follow integrin adhesion has been put forward (Auger *et al*, 2005).

In recent years, the development of synthetic peptides which form helices due to their GPO repeats and which can then be cross-linked has allowed the study of individual receptors in relation to platelet activation. The GPO-containing peptide was termed collagen-related peptide (CRP) and the cross-linked form XL-CRP, and it has been shown to be specific for GPVI (Knight *et al*, 1999). Since amongst blood cells GPVI is found only on platelets, this peptide has been useful for specifically activating platelets in whole blood. Later, the same group synthesised another peptide which specifically activates $\alpha_2\beta_1$ (Emsley *et al*, 2004). In addition to synthetic peptides, the snake venom convulxin is also apparently an agonist specific for GPVI, although questions have been raised about its specificity since aggregation due to convulxin was partially blocked by an antibody against $\alpha_2\beta_1$ (Jandrot-Perrus *et al*, 1997) and it has recently been shown to bind to GPIb (Kanaji *et al*, 2003). All these agents have allowed study of the contribution of particular collagen receptors to platelet activation, although the *in vivo* situation is likely to be far more complicated given the complex nature of the subendothelium and the effects of flow.

A strong platelet agonist, collagen gives rise to the full array of platelet activation events: shape change, arachidonate release, aggregation, secretion and procoagulant response. As collagen is immobilised with repetitive recognition sequences, this facilitates clustering of receptors,

characteristic of integrin signalling. Since much of this project is concerned with activation of platelets by XL-CRP, an outline of the signalling pathway through GPVI is given here; in any case, the role of $\alpha_2\beta_1$ as a signalling receptor is debated. GPVI is unusual among platelet agonist receptors in being a member of the immunoglobulin superfamily and recognises collagens I and III (Knight *et al*, 1999). It is associated with the Fc receptor γ -chain and signalling proceeds through a tyrosine kinase-based pathway, via phosphorylation of the ITAM motif of Fc γ (Gibbins *et al*, 1996) and recruitment of syk, LAT SLP-76 and phosphatidylinositol 3-kinase (PI3-K) (Gibbins *et al*, 1998), to the activation of phospholipase C (PLC) γ and then protein kinase C (PKC), which results in the elevation of cytosolic calcium. There have been suggestions that GPIb signalling might follow a similar route through FcR γ or FcRIIa, but this, and even whether GPIb signals at all, is still open to debate (Jackson *et al*, 2003).

1.5.3.2. ADP/ATP and purinergic receptors

A weak agonist, capable on its own of causing only shape change and aggregation, or at most only partial degranulation (Janes *et al*, 1994), ADP released from the dense granules of the platelet is an important secondary agonist augmenting platelet activation by stronger agonists which cause degranulation (Cattaneo *et al*, 1990; Trumel *et al*, 1999). Being soluble, ADP can act on the platelet from which it was released and to recruit additional platelets to a growing thrombus. ADP-induced aggregation is reversible at physiological Ca^{2+} concentrations; the irreversible aggregation seen in citrated blood is due to the release of TXA_2 and subsequent activation of platelets via thromboxane receptors leading to a second wave of aggregation which is easily visible in Born aggregometry at intermediate concentrations of ADP (Packham *et al*, 1989). The importance of ADP in haemostasis is illustrated by the effectiveness as antithrombotic drugs of ADP receptor inhibitors such as clopidogrel (CAPRIE Steering Committee, 1996), and the bleeding problems suffered by those with ADP receptor defects (Nurden & Nurden, 2001).

Adenine nucleotides interact with P2 receptors, which are found on many cell types. For a recent review, see Gachet, 2001. These receptors are divided into G-protein-linked P2Y receptors and ligand-gated P2X ion channels. Of the P2Y receptors, platelets possess two, P2Y₁ and the recently cloned P2Y₁₂ (Hollopeter *et al*, 2001). The former is linked to the Gα_q protein and the latter is Gα_i-coupled. Study of the respective roles of these two receptors has been facilitated by the existence of specific inhibitors, MRS2179 and AR-C69931MX. P2Y₁ activation causes shape change and is necessary for platelet aggregation in response to lower, but not higher, concentrations of ADP (Hetherington, Department of Cardiovascular Sciences, University of Leicester, UK, unpublished data), its signalling pathway leading via PLCβ to the release of calcium from internal stores. However, this response is transitory and requires signalling through P2Y₁₂ for sustained activation, achieved through inhibition of cAMP, a major inhibitor of platelet activation.

ADP receptor inhibitors have been used to show that P2Y₁₂ is important in the procoagulant response of platelets to thrombin receptor activating peptide (TRAP) in platelet-rich plasma (PRP) (Storey *et al*, 2000). More recently, it has been demonstrated that only the P2Y₁₂ receptor is involved in PS exposure following stimulation by thrombin (Leon *et al*, 2003). Although both studies included collagen as an agonist, neither addressed the procoagulant response mediated specifically by GPVI and Leon *et al* were surprisingly unable to see a significant increase in PS exposure in response to collagen.

ATP, which it is now clear activates P2X₁, is released along with ADP from the platelet dense granules. Because this receptor is easily desensitised, it has proved difficult to study, which may account for the failure of some workers to discern any role for P2X₁ in haemostasis (Jin *et al*, 1998). Investigation has also been hampered by the lack of a specific antagonist, although some relatively selective antagonists are now becoming available (North, 2002). However, others have reported that, if the receptor is prevented from being desensitised, shape change follows a rapid influx of Ca²⁺ mediated by P2X₁ (Rolf *et al*, 2001) and that it may be implicated in the regulation of

response to collagen (Oury *et al*, 2001). Moreover, there have been reports of over-expression of the receptor in mice leading to thrombotic complications, with evidence of co-operation between P2X₁ and GPVI and a role for the receptor in PS exposure (Oury *et al*, 2003). Studies using P2X^{-/-} mice have shown reduced thrombus formation following laser-induced vascular injury under conditions of high shear, although the bleeding time was normal in most of the knock-out mice. This suggests that, whilst it may be relatively unimportant in primary haemostasis, P2X₁ does have a significant part in pathological thrombus formation (Hechler *et al*, 2003).

1.5.3.3. Thromboxane and its receptor

Thromboxane and prostaglandins such as prostacyclin (PGI₂), collectively known as prostanoids, are oxygenated metabolites of arachidonic acid, with cyclooxygenase (COX) being the rate-limiting step in their synthesis from membrane PLs. Acetylsalicylic acid (ASA or aspirin) permanently inhibits both COX-1 and, to a lesser extent, the inducible COX-2. TXA₂ is the major arachidonic acid metabolite in platelets, and is produced in response to platelet activation by other agonists such as thrombin and ADP. In contrast to PGI₂, which is an anti-aggregatory agent synthesised mainly by endothelial and smooth muscle cells, TXA₂ acts as a secondary agonist itself via the TXA₂ receptor TP. TP is a seven transmembrane protein, mediating shape change, aggregation and degranulation by means of signal transduction via G proteins of the G α_q family, PLC β activation and phosphatidylinositol metabolism (Vezza & FitzGerald, 2002).

1.6. Involvement of other blood cells in coagulation

The presence of leukocytes in arterial thrombi and red cells in venous thrombi has been observed for very many years, but in both cases these cells were initially thought to have been passively incorporated into the developing thrombus. However, in addition to their vital role in modifying the spatial distribution of other cells within the vessel so that platelets travel adjacent to the wall

(Goldsmith *et al*, 1999), it has become increasingly clear that there are specific mechanisms which recruit them to the clot. Though this is particularly the case with leukocytes, a recent paper has shown that ICAM-4 on red cells binds activated GPIIb/IIIa, which may be relevant to thrombosis at low shear (Hermans *et al*, 2003). Red cells also possess a scramblase which allows them to expose PS on their surface. Since erythrocytes make up about 99% of total blood volume, even a small number of procoagulant cells could have a significant impact on clotting (Kuypers *et al*, 1996).

Far more attention has been paid to the involvement of leukocytes in coagulation especially because of their ability to express TF. TF-bearing monocytes and neutrophils have been identified within thrombi obtained from patients undergoing thrombectomy (Himber *et al*, 2002). The role of both inflammatory mediators and cell-cell cross-talk in upregulating monocyte TF has been outlined in §1.3.4. Recent real-time studies of the formation of a thrombus in a mouse model have shown that fibrin deposition as the thrombus grows is dependent on the recruitment of TF- and PSGL-1- (CD162) positive monocyte-derived microparticles which bind to activated platelets in the thrombus (Falati *et al*, 2003). Studies where bone marrow has been exchanged between wild-type and low-TF mice suggest that, whilst subendothelial TF is responsible for the initiation of coagulation, incorporation of such blood-borne TF into the clot is essential for propagation of the thrombus (Furie & Furie, 2004). However, the importance of leukocyte TF in driving thrombus formation has been dismissed (Day *et al*, 2005), although this publication gave rise to lively correspondence (Hathcock & Nemerson, 2005; Niemetz, 2005). The nature of the leukocyte contribution to coagulation is therefore a topical area of research in which platelets are a key component (Polgar *et al*, 2005).

1.7. Aims of the project

Platelets are central to the coagulation process, both through changes to the platelet itself following activation and through their interaction with leukocytes, particularly monocytes, localising,

amplifying and sustaining the coagulant response at the injury site. The overall aim of this project was to explore the ways in which platelets influence the procoagulant environment in blood with particular reference to (i) provision of negatively-charged phospholipids, especially PS, on the platelet surface and (ii) the induction of TF and other procoagulant mediators in monocytes and other cells.

The specific aims were:

- To develop a whole blood flow cytometric assay for the detection of procoagulant (PS exposing) platelets and platelet-derived microparticles.
- To delineate the role of primary and secondary agonists and their receptors in platelet PS exposure.
- To explore the relationship between PS exposure and microparticle formation.
- To examine the relationship between PS exposure and other aspects of platelet activation.
- To examine the role of platelet activation in clot formation.
- To investigate the effect of platelet activation on expression of TF mRNA, antigen and protein in monocytes, platelets, and monocytes-platelet aggregates.
- To investigate the possibility that platelets contribute to blood-borne TF.

CHAPTER 2: Materials and methods

2.1. Materials

2.1.1. Reagents

Except where stated, standard reagents (analytical grade or molecular grade for PCR) were supplied by Sigma (Poole, UK) or BDH (Poole, UK). Details of more specialised reagents and buffer recipes are given in the text as they arise. Summaries of suppliers' details and buffers are given in Annexes 1 and 2.

2.1.1.1. Agonists

Platelet agonists used were Collagenreagent Horm® equine fibrillar collagen (Nycomed, München, Germany), collagen-related peptide (CRP), generously provided by Drs. Richard Farndale and Graham Knight (Department of Biochemistry, University of Cambridge, UK) and cross-linked in-house as described below (§2.1.2), the calcium ionophore A23187 (Sigma), ADP (Sigma), thrombin receptor activating peptide (TRAP) SFLLRN (Calbiochem) and human α -thrombin (Sigma) at various concentrations as indicated in the results. ADP, TRAP and thrombin were prepared at appropriate dilutions and aliquotted prior to storage at -80°C to maintain reproducibility. Monocytes were directly activated with LPS from Sigma.

2.1.1.2. Inhibitors

Inhibitors used were the PI3-K inhibitors wortmannin and LY294002 (both Alomone Labs, Jerusalem, Israel) and the actin polymerisation inhibitor Cytochalasin D (Sigma). Platelet activation by ATP or ADP was prevented by the ATPase/ADPase apyrase (Grade VII; Sigma). The $\alpha_{IIb}\beta_3$ blocking antibodies RFGP56 (Cymbus Biotechnology, Chandlers Ford, UK) and ReoPro® (Centocor BV, Leiden, The Netherlands) were used to prevent platelet-platelet aggregation in some experiments. The P-selectin blocking antibody 9E1 (R & D Systems, Abingdon, UK) was used to

prevent P-selectin-PSGL-1 interaction between platelets and monocytes. TF activity was inhibited by antibodies HTF-1 (BD Pharmingen, Oxford, UK) and #4508 (American Diagnostica, Greenwich, CT, USA).

2.1.2. Cross-linking CRP

The potency of collagen-related peptide (CRP) as an agonist depends on its being cross-linked prior to use. Lyophilised monomeric CRP (Gly-Cys-Hyp-(Gly-Pro-Hyp)₁₀-Gly-Cys-Hyp-Gly-NH₂; M_r3280) was stored at -20°C in the presence of silica gel desiccant. After being slowly brought to room temperature, the peptide was dissolved in 0.1 M NaHCO₃ to give a concentration of 10 mg/mL (3.049 mM) and the solution flushed with nitrogen gas to prevent the formation of disulphide bonds. The cross-linking agent 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester (SPDP) was dissolved in ethanol dried with molecular sieve 3A (Sigma) to give a 15.6 mg/mL (50 mM) solution. The SPDP solution was then added to CRP in the molar ratio of SPDP to CRP of 1.5:1.0 (30 µL 50 mM SPDP to 330 µL 3.049 mM CRP). The solution was left at room temperature for 1 h. The CRP was then transferred to a microdialyser (0.5 mL capacity, QuixSep part # QS3-0500; Triple Red Limited, Long Crendon, UK) and dialysed twice with rapid stirring for 2 h against 500 mL 0.01 M acetic acid at room temperature using Cellu.Sep® H1 dialysis membrane (nominal M_r cut-off 1000; Triple Red Limited). The cross-linked CRP (XL-CRP) was transferred to a microtube with two 100 µL washes of 0.01 M acetic acid and the concentration adjusted to 5 mg/mL with 0.01 M acetic acid, assuming full recovery of peptide. Each batch was titrated in a platelet flow cytometric assay using P-selectin as the activation marker. Since the activity of each batch varied, a single batch was used for each set of experiments. The cross-linked CRP was stored at 4°C. At the stock concentration of 5 mg/mL, the peptide was stable for 6-12 months under these conditions whereas more dilute solutions were stable for approximately 1 h kept on ice. Diluted solutions were therefore prepared immediately before use.

2.1.3. Antibodies used in this study

Antigen	Conjugate used	Clone	Supplier	Use
CD42b	-RPE	HIP1	BD Pharmingen (Oxford, UK)	Flow cytometry
CD45	-RPE-Cy5	T29/33	DakoCytomation (High Wycombe, UK)	Flow cytometry
CD14	RPE-Cy5	TÜK4	Serotec (Oxford, UK)	Flow cytometry
CD62P	-FITC	AK-6	Serotec	Flow cytometry
CD62P	Unconjugated	9E1	R & D Systems (Abingdon, UK)	Blocking
CD11b	-FITC	ICRF44	Serotec	Flow cytometry
CD54	-FITC	15.2	Serotec	Flow cytometry
CD41/61	Unconjugated	RFGP56	Cymbus Biotechnology (Chandlers Ford, UK)	Blocking
CD41/61	-FITC	RFGP56	Cymbus Biotechnology	Flow cytometry
CD41/61	-FITC	PAC-1	Becton Dickinson	Flow cytometry
CD142	-FITC	4508CJ	American Diagnostica (Greenwich, CT, USA)	Flow cytometry
CD142	Unconjugated	4508	American Diagnostica	Flow cytometry Western blotting Blocking
CD142	Unconjugated	4509	American Diagnostica	Flow cytometry Western blotting
CD142	-RPE	HTF-1	BD Pharmingen	Flow cytometry
CD142	Unconjugated	HTF-1	BD Pharmingen	Western blotting Blocking
CD142	Unconjugated	TF9 10H10	Calbiochem (Nottingham, UK)	Western blotting
Fibrinogen	FITC	Rabbit polyclonal	DakoCytomation	Flow cytometry

Table 2.1. Details of used antibodies used in this study. RPE: R-phycoerythrin. Cy5: cyanine-5

2.2. Methods

2.2.1. Blood collection

The procedure for blood collection was standard for all experiments and was designed to minimise activation of platelets prior to analysis. Donors denied having taken aspirin or other platelet-affecting drugs in the previous fortnight. Except where stated, blood from the antecubital vein of apparently healthy volunteers was drawn, with minimal stasis, using a 21G butterfly needle. In all cases, a lightly applied tourniquet was removed after insertion of the needle. Blood was collected into 3 mL Vacutainers™ (Becton Dickinson, Oxford, UK) spray coated with EDTA or 4.5 mL Vacutainers™ containing 0.105 M sodium citrate (1:9 v/v). On some occasions, blood was collected into Vacutainers™ containing either citrate-theophylline-adenosine-dipyridamole (CTAD) or 143 international units (IU) lithium heparin or into siliconised glass Vacutainers™ with no anticoagulant before transfer into hirudin. For all experiments, the first tube of blood collected was discarded, assays being performed using blood from subsequent tubes. Cell counts were performed using a Coulter® A^C-T diff™ Hematology Analyser (Beckman Coulter™, High Wycombe, UK). Blood was collected in accordance with ethical permission granted by the local Ethics Committee of the University Hospitals of Leicester NHS Trust. Most experiments, both platelet and monocyte, were performed on whole blood which was used within 10 min of collection.

Expired platelets (more than 5 days old) were obtained from the Blood Bank of the Leicester Royal Infirmary and kept at room temperature.

2.2.2. Preparation of platelets and plasma

2.2.2.1. *Platelet-rich and platelet-poor plasma*

Although wherever possible assays were carried out in whole blood to minimise artefactual activation of platelets, some techniques, such as light aggregometry (see below), are inherently unsuitable for use with whole blood. When required, platelet-rich plasma (PRP) was prepared by

centrifugation of citrated whole blood at 163 g for 20 min at room temperature and gentle removal of the platelet suspension in the upper layer of blood, avoiding disturbance of red cells or white cells in the 'buffy coat' immediately above the red cells. Platelet-poor plasma (PPP) was prepared by centrifugation of PRP in a microfuge at 7200 g for 10 min.

Plasma was prepared by centrifugation of citrated blood at 1811 g for 30 min at 4 °C. If the plasma was to be used to dilute blood or PRP already at room temperature, centrifugation was also performed at room temperature to avoid activating the platelets. For some studies, to minimise the number of microparticles, plasma was 'double-spun' by further centrifugation of the cell-free supernatant in a microfuge at 7200 g for 10 min at room temperature or filtered in a 0.2 µm filter. When not used immediately, plasma was stored at -80 °C.

2.2.2.2. *Washed platelets*

For use in Western blotting and the tissue factor activity (TFA) assay, platelets were isolated from plasma by washing. Washed platelets for Western blotting were prepared by collecting blood into CTAD Vacutainers™. PRP was prepared by centrifugation at 163 g for 20 min at room temperature. Platelets were pelleted by centrifugation of PRP at 804 g for 15 min in the presence of 200 ng/mL PGI₂ (Sigma; final concentration) to prevent platelet activation. The supernatant was removed and the platelets resuspended using a pastette, without forming bubbles, in a small volume of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)-buffered saline pH6.0 (HBS; 150 mM NaCl; 5 mM KCl; 1 mM MgSO₄; 10 mM HEPES; pH 6.0 using NaOH). The platelet suspension was then diluted in a larger volume of HBS pH 6.0 and centrifuged, again in the presence of 200 ng/mL PGI₂, at 515 g for 15 min. The platelets were again washed in HBS pH 6.0, centrifuged with PGI₂ and the supernatant removed. The washed platelets were resuspended in a small volume of HBS pH 7.4, counted in the Coulter® A^C·T diff™ Hematology Analyser and the count adjusted as

required with HBS pH 7.4. Aliquots of washed platelets were centrifuged at 7200 g for 10 min, the supernatant removed and the pellet stored at -80 °C until required.

For the TFA assay, minor modifications were made to the washing protocol. Platelets in PRP or buffer were pelleted by centrifugation at 1800 g for 20 min and were washed only once in HBS pH 6.0. After washing, platelets were resuspended in Tris-buffered saline (TBS; 20 mM Tris; 137 mM NaCl; 38 mM 1 M HCl; pH 7.6), the count adjusted to $1 \times 10^9/\text{mL}$ and 500 μL aliquots containing 5×10^8 washed platelets stored at -80 °C until use.

2.2.3. Isolation of monocytes

2.2.3.1. *Density gradient method*

Where it was not important to exclude lymphocytes from the cell suspension (in flow cytometry, where cells could be distinguished by surface marker and light scatter characteristics), monocytes were isolated from whole blood using a density gradient method. After warming 3 mL Histopaque®-1077 (Sigma) to 37 °C in a 15 mL tube, 3 mL citrated whole blood was gently layered on top and centrifuged at 400 g for 30 min at room temperature. Centrifugation causes erythrocytes and granulocytes aggregated by polysucrose to sediment, and leaves mononuclear cells as an opaque band at the plasma-Histopaque® interface. The upper layer was aspirated to within 0.5 cm of the opaque interface and discarded. The opaque layer was carefully transferred to a clean 15 mL centrifuge tube and 10 mL PBS added. The cell suspension was gently mixed, then centrifuged at 250 g for 10 min and the supernatant removed and discarded. The pellet was resuspended gently in 5 mL PBS and centrifuged at 250 g for 10 min, the supernatant being discarded. The last washing step was repeated and the cells finally resuspended (in PBS, autologous plasma or cell culture medium RPMI 1640 as indicated) at the desired concentration as determined in the Coulter® A^CT diff™ Hematology Analyser.

2.2.3.2. Isolation of monocytes using magnetic beads

In order to obtain a relatively pure sample of monocytes for reverse transcription (RT)-PCR, CD14-positive cells were isolated from whole blood using Dynabeads® M-450 CD14 beads (DynaL Biotech), coated with RMO 52 monoclonal antibody against CD14, according to the manufacturer's instructions. Briefly, 100µL (4×10^7) beads were washed twice in ice-cold Dulbecco's-phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} (Invitrogen)/0.3 % BSA (w/v) (PBS/BSA) and resuspended in 1 mL citrated blood in a sterile 2 mL microtube. The beads and blood were incubated while rotating gently at 4 °C for 10 min, except where indicated. According to the manufacturer's instructions, the recovery of CD14⁺ cells can be increased by lengthening the time of incubation, but in some instances (for example, where the mRNA of interest has a short half-life, as is the case with TF mRNA), the advantages of so doing are outweighed by the possibility of degradation of the target mRNA. This was the rationale behind the compromise choice of the normal incubation period of 10 min. The CD14⁺ cells/beads were isolated by placing the microtube on the magnetic Dynal magnetic particle concentrator (MPC®). The supernatant was removed and the beads washed three times in ice-cold PBS/BSA. The CD14⁺ cells/beads were resuspended in 500 µL PBS/BSA.

2.2.4. Flow cytometry

2.2.4.1. General methods

Samples were analysed on a Coulter® EPICS® XL-MCL™, equipped with an air-cooled, software-controlled 15 mW argon ion laser operating at 488 nm. The fluorescence of FITC, RPE and RPE-Cy5 was detected by using 525 ± 20 nm, 575 ± 20 nm and 680 ± 20 nm bandpass filters respectively. The flow cytometer was aligned daily using Flow-Check™ Fluorospheres (Beckman Coulter™). Unless otherwise stated, signals were detected at logarithmic gain. Where two or more colour analysis was performed, the signal was corrected electronically for overlap. Except where indicated,

5000 events of interest were analysed. Data were analysed and the cytometer controlled with System II™ Version 3.0 software (Beckman Coulter™).

The optimum concentration of all antibodies used was determined by titration. Buffers were filtered with a 0.2 µm filter prior to use to reduce background signal. Negative controls for conjugated monoclonal antibodies (Mabs) were set at approximately 2% using the appropriate volume of a similarly conjugated irrelevant isotype-matched Mab. Unconjugated primary antibodies were detected with a secondary, FITC-conjugated Rabbit anti-Mouse F(ab')₂ fragment (DakoCytomation). Samples incubated with this F(ab')₂ fragment alone served as negative controls in two-layer assays. Assays were routinely performed in duplicate.

2.2.4.2. Basic platelet flow cytometry assays

Sample preparation

Within 10 min of collection, or after incubation with agonists or inhibitors as indicated in the results, 5 µL citrated whole blood (or for some experiments, PRP) was added to polystyrene tubes containing HBS, appropriately diluted antibodies and agonist and/or inhibitor (where used) to bring the final volume to 50 µL. When using the rabbit polyclonal antibody against fibrinogen, the negative control sample was prepared in exactly the same manner as the test sample except for the addition of 6 mM EDTA (final concentration); since the binding of fibrinogen to $\alpha_{IIb}\beta_3$ is calcium-dependent, any antibody binding in the presence of EDTA is assumed to be non-specific. When thrombin was used as an agonist, the peptide Gly-Pro-Arg-Pro (GPRP) (Sigma; 0.5mg/mL final concentration) was added to prevent the formation of a clot by blocking fibrin polymerisation (Michelson, 1994).

When using directly conjugated antibodies, samples were incubated at room temperature for 20 min after which the reaction was stopped by the addition of 500 µL 0.2% formyl saline (FS: 0.2% formyl saline; 0.85% (w/v) saline). Where two layers of antibody were used, blood was

incubated with the primary, unconjugated antibody for 15 min before the addition of the secondary, fluorescently-labelled antibody and the reaction stopped as above following a further 15 min incubation. In both cases, samples were allowed to incubate for 10 min at room temperature after the reaction was stopped to allow re-equilibration of the antibodies in the larger volume of buffer, since 0.2 % formaldehyde is insufficient for complete fixation of the antibody-antigen interaction. Prior to analysis in the flow cytometer, samples were further diluted 1:10 in 0.2 % FS; this second dilution step was to avoid coincident signals resulting from two or more cells passing through the laser beam simultaneously, an artefact which can occur if cell concentrations are too great.

Platelets were identified using the pan-platelet marker CD42b-RPE. Where single staining was used to detect activation markers, a separate sample was labelled with CD42b-RPE in order to validate the identity of the events analysed in parallel samples. The platelet activation markers used were activated $\alpha_{IIb}\beta_3$ using antibody PAC-1 or an antibody against bound fibrinogen, or CD62P (P-selectin). The presence of TF on the platelet surface was also investigated by flow cytometry.

Analysis of platelet surface markers

The flow cytometric analysis of platelets in whole blood has been previously described (Shattil *et al*, 1987). Briefly, platelets were distinguished from other blood cells on the basis of their size (forward scatter) and granularity (side scatter) characteristics. An electronic gate was set around this population to select events for further analysis (Fig.2.1). Where single colour analysis was used, it was ensured that the events in this gate were more than 95% positive for the platelet-specific marker CD42b-RPE. Platelets in separate samples were analysed by single colour analysis for activation markers or surface antigens using FITC- or RPE- conjugated antibodies to obtain both the percentage positive and median fluorescence. Samples labelled simultaneously with antibodies conjugated to different fluorophores were analysed by combinations of parameters as indicated in the results.

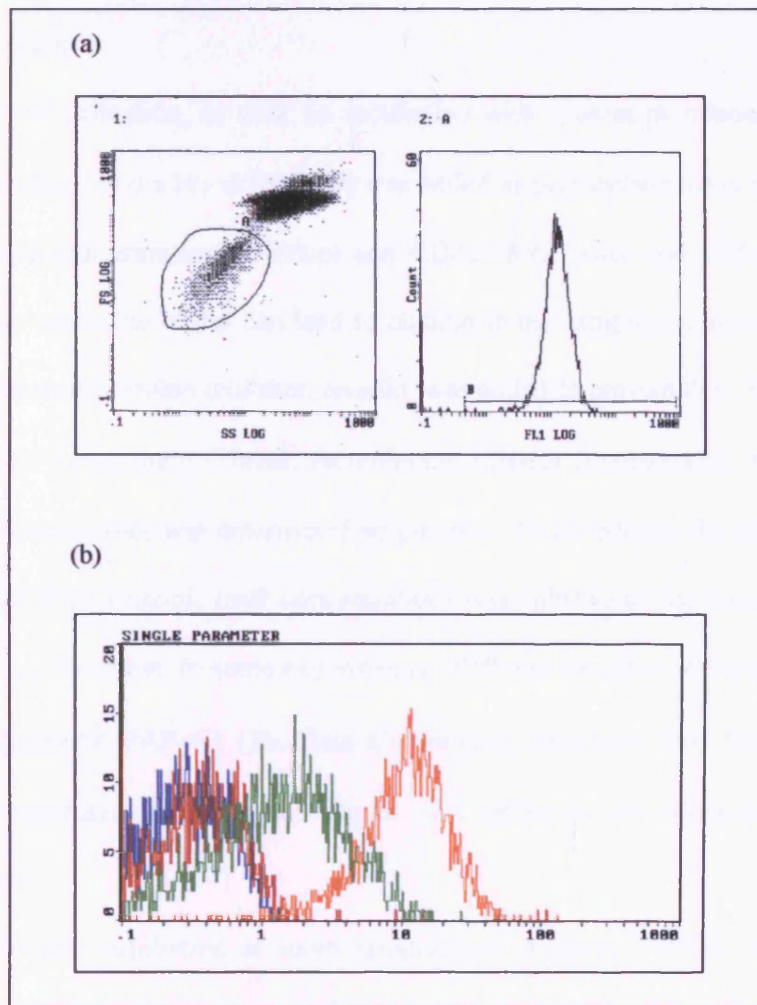


Figure 2.1. Flow cytometric analysis of platelet surface markers. (a) Platelets in whole blood were distinguished on the basis of their forward and side scatter characteristics and gated electronically (first histogram). The distribution of fluorescence on gated platelets is shown in the second histogram. All events in the range covered by the 'cursor' are considered positive for the marker under consideration, in this case, CD42b. The position of the cursor was set to include only the highest 2% labelled by an isotype- and fluorescence-matched negative control antibody. (b) Overlay of second histogram traces where platelets were labelled with an anti-P-selectin antibody, showing negative control (blue), unstimulated platelets (red) and platelets activated with 3×10^{-6} M ADP (green) and 1×10^{-4} M TRAP (orange),

2.2.4.3. Procoagulant platelet assay

Sample preparation

Within 10 min of collection, or after an incubation with agonist or inhibitor as indicated in the results, 5 μ L citrated whole blood (or PRP) was added to polystyrene tubes containing HBS with 2 mM CaCl_2 (final concentration) (HBS/c) and CD42b-RPE, with and without agonist. Since the presence of calcium in the buffer can lead to clotting in the sample, rendering it unusable for flow cytometry, the direct thrombin inhibitor, hirudin, was added to prevent this. The concentration used, 5 μ L 100 antithrombin units (U)/mL recombinant hirudin (Pentapharm, Basel, Switzerland; 10 U/mL final concentration), was determined empirically. To investigate the effect of thrombin in the experiment, GPRP (0.5mg/mL final concentration) was substituted for hirudin to prevent clotting without inhibiting thrombin. In some experiments, PRP was stirred at 900 rpm at 37 °C in a Platelet Aggregation Profiler® PAP-4D (Bio/Data Corporation, Horsham, PA, USA), with and without inhibitors and agonists, before a sub-aliquot was added to test tubes containing reagents as described above.

After 10 min incubation at room temperature, Annexin V-FITC (BD Pharmingen) was added, bringing the final volume to 50 μ L. After an additional 10 min incubation at room temperature, a 1:100 dilution was made by sub-aliquotting into HBS/c. Analysis on the flow cytometer was carried out immediately.

As a negative control for Annexin V binding, a tube was set up exactly as above except that HBS without calcium was used for both the initial reaction mixture and the final 1:100 dilution. The rationale for this was that Annexin V binding to phosphatidylserine (PS) is highly calcium-dependent so that a sample without calcium was appropriate to determine non-specific binding. An irrelevant, RPE-conjugated isotype-matched IgG₁ monoclonal antibody was analysed in a separate sample as a negative control for CD42b binding.

Flow cytometric analysis of procoagulant platelets and platelet-derived microparticles

Platelets in whole blood were identified initially on the basis of their forward and side scatter (size and granularity) characteristics. These events were then analysed for positivity for the pan-platelet marker CD42b-RPE. Only CD42b-positive events were subsequently analysed for Annexin V binding. Further analyses were made to determine the size of PS-exposing cells. This analysis will be described in greater detail in Chapter 3.

2.2.4.4. Monocyte flow cytometric assays

Sample preparation

Flow cytometry was used to investigate surface antigens on monocytes either in whole blood or isolated by density gradient (§2.2.3). Within 10 min of collection or after an incubation with agonist or inhibitor as indicated in the results, 5 µL citrated whole blood was added to polystyrene tubes containing HBS, appropriately diluted antibodies and agonist as described in the results. In some experiments, antibodies were incubated with 50 µL aliquots of PBS containing 1×10^5 isolated monocytes. Monocytes have on their surface a large number of Fc receptors which could lead to a significant amount of non-specific antibody binding. Therefore, an irrelevant, unconjugated mouse antibody clone MOPC31C (Sigma; 200 µg/mL final concentration) to block the receptors was added to all tubes except where a secondary antibody was necessary as the secondary would have bound to MOPC31C as well as the primary antibody of interest. To prevent the formation of monocyte-platelet aggregates in the test tube, the P-selectin blocking antibody 9E1 was added at a final concentration of 6 µg/mL. In all cases, samples were allowed to incubate with antibodies at room temperature for 30 min followed by the addition of 500 µL 0.2% FS and analysis after 10 min.

Monocytes form only a small proportion of cells in whole blood, numbering $0.2 - 0.8 \times 10^3/\mu\text{L}$ whole blood compared with $4.2 - 5.9 \times 10^6$ red cells and $150 - 400 \times 10^3$ platelets/ μL whole blood. In order to distinguish monocytes more easily, their proportion in a whole blood sample was in some experiments effectively increased by the selective lysis of red cells using Optilyse® C lysing solution (Immunotech, Miami, FL, USA). Where samples were prepared using Optilyse® C, 50 μL whole citrated blood was incubated with antibodies in 50 μL HBS as described above. Following incubation, 250 μL Optilyse® C lysing solution was added to the sample; it was mixed and allowed to incubate for 10 min at room temperature, after which 250 μL PBS was added. Having been allowed to rest for at least 10 min, the samples were analysed.

Antibodies used to identify cells were the pan-leukocyte marker CD45-RPE-Cy5, CD14-RPE-Cy5 for monocytes and CD42b-RPE for platelets. TF on the surface of monocytes or monocyte-platelet aggregates (MPAs) was investigated using several anti-TF antibodies, the details of which are given in Table 2.1.

Flow cytometric analysis of monocytes and monocyte-platelet aggregates

Two methods were used for analysing antigens on monocyte and monocyte-platelet aggregates. When assaying whole blood without lysis of red cells, detection of monocytes was triggered by positivity for the leukocyte marker (CD14- or CD45-RPE-Cy5) as previously described (Li *et al*, 1999). Monocytes were further distinguished on the basis of size and granularity (at linear gain) in a second histogram. A combination of size and granularity as well as CD14 positivity is necessary to identify monocytes since CD14 is also present on a small subset of granulocytes.

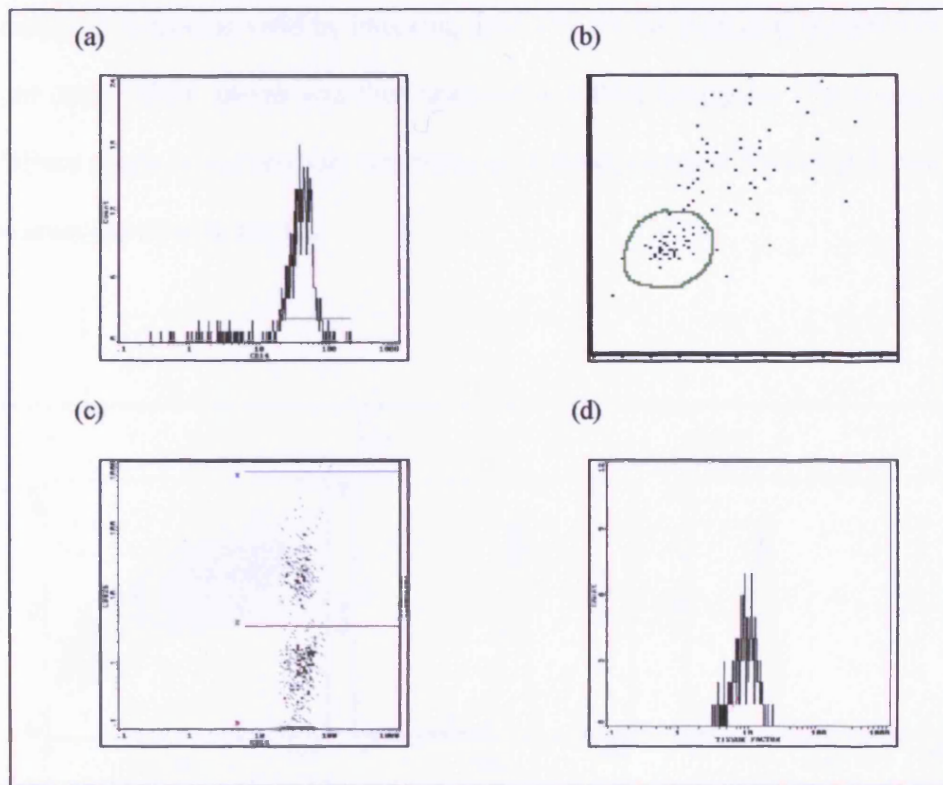


Figure 2.2. Three-colour flow cytometric monocyte-platelet aggregate assay. CD14 positive events (a) were identified as monocytes on the basis of their FS and SS characteristics (b). Monocytes were then divided (c) into those which were free monocytes, being CD42b negative (lower gate), and those which were monocyte-platelet aggregates, being CD42b positive (upper gate). Free monocytes and MPAs were analysed separately for TF antigen, illustrated in (d).

When MPAs were being investigated, these were identified as those events in the initial gate which were positive for both the leukocyte marker (CD14- or CD45-RPE-Cy5) and the platelet marker CD42b-RPE. Free leukocytes were those events positive for only the leukocyte marker. MPAs and free monocytes could be further investigated for the presence of TF by investigating the binding of FITC-labelled anti-TF Mab 4508CJ in the CD42b⁺ and CD42b⁻ populations respectively. This three-colour assay is illustrated in Fig.2.2.

Where the proportion of monocytes in a sample had been increased, either through their isolation from whole blood or by lysing red cells using Optilyse® C lysing solution, monocytes

were identified on the basis of their forward and side scatter characteristics, detected at linear gain, the gate being confirmed as valid by checking positivity for the monocyte marker CD14-RPE-Cy5. Antigen on these CD14⁺ events was then analysed in a third histogram. The analysis is shown in Fig.2.3. Where single colour analysis was being performed, events in the initial forward/side scatter gate were analysed directly for TF.

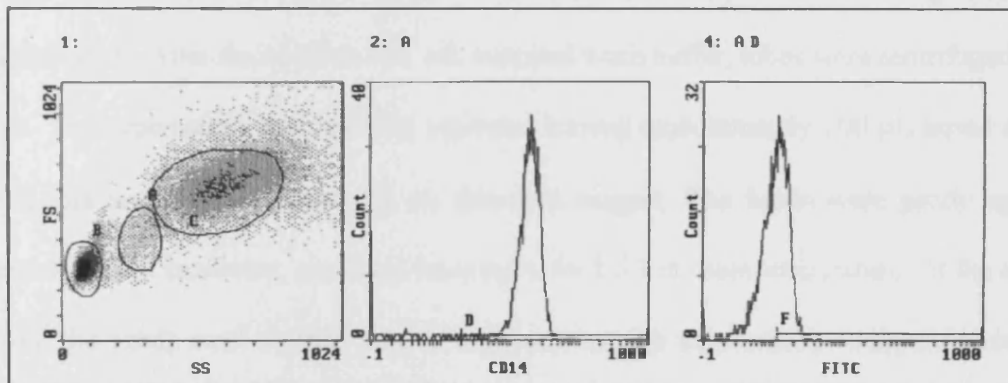


Figure 2.3. Optilyse[®] C assay. Red cells in whole blood were lysed with Optilyse[®] C after incubation with antibodies, and leukocyte subsets distinguished on the basis of their forward and side scatter characteristics (first histogram). Monocytes were further identified by their positivity for CD14 (second histogram) and analysed for TF (third histogram).

2.2.4.5. *BD[™] Cytometric Bead Array (CBA) human inflammation kit*

The release of cytokines from activated platelets was investigated using a multiplexed flow cytometric assay kit (BD Biosciences) to quantify IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF α simultaneously. Citrated plasma was prepared from whole blood stimulated with 50 μ g/mL XL-CRP or 200 ng/mL LPS (as a positive control) and PRP stimulated with 50 μ g/mL XL-CRP. Samples were incubated at 37 °C for 10 min and 2 h before preparing plasma by centrifugation at 1800 g for 30 min at room temperature. Untreated plasma controls were prepared at 0 min, 10 min

and 2 h. Plasma was filtered using a 0.2 μm filter to reduce cell debris and microparticles, and stored at -80°C prior to use.

The assay was performed according to the manufacturer's instructions. Briefly, the standards were reconstituted and serial dilutions made in the assay diluent. Capture beads for each cytokine were mixed and vortexed before transferring 50 μL of the mixed bead suspension to test tubes. The standard dilutions and test plasma samples (50 μL per tube) were added to the appropriate test tubes containing the capture beads and incubated, protected from light, for 1.5 h at room temperature. After the addition of 1 mL supplied wash buffer, tubes were centrifuged at 200 g for 5 min. The supernatant was carefully aspirated leaving approximately 100 μL liquid above the pellet. To this mixture was added 50 μL detection reagent. The beads were gently agitated to resuspend them and incubated, protected from light, for 1.5 h at room temperature. At the end of the incubation, the beads were washed and, having removed the supernatant, resuspended in 300 μL wash buffer before analysis. The flow cytometric assay protocol was set up according to the manufacturer's instructions using supplied set up beads.

The capture beads for each reagent are distinguished by their different levels of autofluorescence through FL4, with the fluorescence through FL2 indicating the amount of cytokine captured. The data were saved as listmode files and analysed using BD™ Cytometric Bead Array Analysis Software (BD Biosciences) which calculates the cytokine concentration from the standard curve.

2.2.5. Platelet aggregometry

Aggregation was measured using the light impedance method of Born (Born & Cross, 1963) using a Platelet Aggregation Profiler® PAP-4D. Siliconised glass test tubes containing 450 μL PRP and a magnetic stir bar were warmed for 3 min at 37°C before being transferred to wells containing magnetic stirrers set to rotate at 900 rpm. Aggregation following the addition of agonist was

measured as the percentage of maximum light transmission compared with autologous PPP, which was set at 100% light transmission. In some experiments, PRP was incubated with inhibitors prior to the addition of agonist. Where PS exposure was being investigated by flow cytometry, aliquots were removed from the aggregometry tubes into test tubes containing the appropriate buffer and antibodies.

2.2.6. Thromboelastography (TEG)

The monitoring of the kinetics of clot formation and firmness was performed with a Whole Blood Haemostasis Analyser model ROTEM®05 Coagulation Analyser (Sysmex, Milton Keynes, UK) using rotation thromboelastography with software Version 2.95. In accordance with generally agreed practice (Luddington, 2005), blood was allowed to rest for 30 – 60 min before assay.

In the studies on platelet PS exposure in Chapter 3, clotting was initiated and platelets activated by adding 300 µL citrated whole blood to a cup containing 20 µL 200 mM CaCl₂ (final concentration 11.8 mM), 10 µL 414 pg/mL recombinant TF (final concentration 12.2 pg/mL; Hemoliance RecombiPlastin®; Instrumentation Laboratory, Warrington, UK) and 10 µL HBS or XL-CRP (final concentrations 150 ng/mL, 500 ng/mL, 1.5 µg/mL, 5 µg/mL and 50 µg/mL) or TRAP (final concentrations 3×10^{-5} M and 6×10^{-5} M). A very low concentration of TF was used to avoid masking the effects of platelet activation (Sorensen *et al*, 2003). Immediately after the blood was added and mixed, but before recording the clotting reaction on the ROTEM®, 5 µL of the reaction mixture was subsampled into test tubes set up to analyse PS and P-selectin expression as described above in §§2.2.4.2 and 2.2.4.3; the time lag in so doing did not exceed 10 s, the maximum time permitted by the manufacturer's protocol.

When studying platelet and blood-borne TF (Chapter 5), rTF was omitted from the reaction mixture and corn trypsin inhibitor added to minimise contact activation (final concentration 54 µg/mL). Clotting was initiated by recalcification with 30 µL 200 mM CaCl₂ (final concentration

16.7 mM), and platelets were activated with 14 µg/mL XL-CRP. To assess the TF-dependent component of clotting, 8 µL anti-TF Mab 4508 (final concentration 22 µg/mL), or in control experiments an irrelevant Mab, MOPC31C, was added to the blood. All reagents were added to the RoTEM® cup prior to addition of 300 µL citrated whole blood. Blood and reagents were mixed by pipetting gently before starting the test.

Clot firmness is monitored in terms of obstruction by the growing clot of a rotating pin and is measured as amplitude, which is 0 mm until the clot forms and maximum at 100 mm, defined as infinite firmness. Parameters measured (Fig.2.4) were the clotting time (CT; the period from measurement start until the recognisable start of clot formation, defined as when a 2 mm amplitude occurs), clot formation time (CFT; the time taken to reach an amplitude of 20 mm), maximum clot firmness or maximum amplitude (MCF or MA; the maximum vertical expansion/amplitude of the thromboelastograph), clot formation rate (CFR; the largest clot formation in degrees until the MCF is reached, defined by the largest angle during the rise of the curve, and describing the dynamics of clot formation), alpha angle (the angle between the centre line and a tangent to the curve through the 2 mm amplitude point – the start of clot formation - indicative of rate of clot formation), and the time taken to achieve various percentages of lysis (fibrinolysis as a percentage of MCF). The MCF that the clot attains is only measurable if clotting is allowed to proceed to the point at which lysis occurs; otherwise, it only indicates the amplitude at that time point.

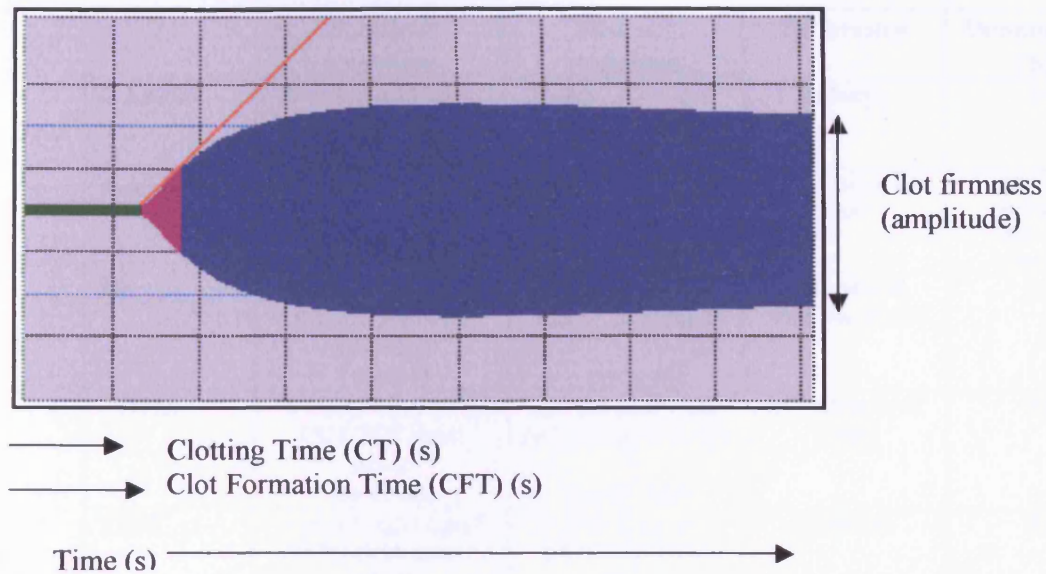


Figure 2.4. TEG parameters. Image obtained from the RoTEM® in which the green line on the left shows the clotting time to an amplitude of 2 mm, the clot formation time to an amplitude of 20 mm, shown by the purple area, whilst the course of subsequent clot firmness to maximum amplitude and then lysis is depicted by the blue area. The red line gives the α angle, and is a tangent to the curve at an amplitude of 2 mm, giving an indication of clot kinetics.

2.2.7. RT-PCR

2.2.7.1. Materials

All reagents were of molecular grade and were purchased from Sigma unless otherwise stated. EAhy 926 cDNA, used as a positive control in some experiments, was prepared by Dr. Samantha Tull (Department of Cardiovascular Sciences, University of Leicester, UK).

Primer details are given in Table 2.2. In-house primers were designed, and published sequences checked, using Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>). Gene specificity was verified using the BLAST database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Oligonucleotides were purchased from Sigma-Genosys and Gibco.

	Forward sequence	Reverse sequence	Reference	Product size (bp)
GAPDH	5'-AGA ACA TCA TCC CTG CCT C-3'	5'-GCC AAA TTC GTT GTC ATA CC-3'	In-house	350
P-selectin	5'-TGC TCA GAA CTA CAT GT-3' (Exon 13)	5'-AGG ACT CGG GTC AAA TG-3' (Exon 16)	(Johnston <i>et al</i> , 1990)	381 +trans membrane 261 –trans membrane
TF	5'-GAC AAT TTT GGA GTG GGA ACC C -3' (Exon 2)	5'-CAC TTT TGT TCC CAC CTG-3' (Exon 4)	(Khajuria & Houston, 2000)	310
CD11b	5'-ACG CAA TGA CCT TCC AAG AG-3' (bp 41-63)	5'-TCT CGT ACC ACT TTG CGG AT- 3' (bp 667-686)	(Hickstein <i>et al</i> , 1989)	646
TFPI	5'-GAT ACG GAG TTG CCA CCA CT-3' (Exon 4)	5'- GTT CAT ATT GCC CAG GCA TC-3' (Exon 6)	In-house	368
IL-1β	5'-TTG AAG CTG ATG GCC CTA-3' (Exon 2)	5'-TGC TCA GGT CAT TCT CCT-3' (Exon 3)	(Huang <i>et al</i> , 1995)	204
IL-8	5'-GCT TTC TGA TGG AAG AGA GC-3' (Exon 2)	5'-GGC ACA GTG GAA CAA GGA CT-3' (Exon 3)	(Yamaoka <i>et al</i> , 1998)	605
TNFα	5'-TAG ATG GGC TCA TAC CAG GG-3' (Exon 1)	5'-TCT CTA ATC AGC CCT CTG GC-3' (Exon 3)	In-house	373

Table 2.2. Sequences and sources of primers used for RT-PCR.

2.2.7.2. Prevention of contamination

Separate work areas and equipment were used for the extraction of mRNA and performing PCR. Pipettes were irradiated with UV light after use. All plasticware was certified by the manufacturer as DNase/RNase free and was either sterile or autoclaved before use. Filter tips were used and were autoclaved for 40 min at 122°C prior to use. Buffers were prepared using autoclaved diethyl pyrocarbonate (DEPC) - treated water (0.1% DEPC) to protect RNA against degradation. Controls for contamination were run at the RT and PCR stages as described below. Primers were designed to span introns so that genomic DNA could readily be detected by their larger than expected size in the ethidium bromide stained gels.

2.2.7.3. Preparation of platelets for RT-PCR

Platelet mRNA was isolated essentially according to a previously published method (Vanags *et al*, 1997). Briefly, PRP was prepared from whole blood collected into a Vacutainer™ containing CTAD. A small aliquot was removed to obtain a platelet count in the Coulter® A^CT diff™ Hematology Analyser. PRP containing 10⁹ platelets was transferred to a sterile 15mL tube and was centrifuged at 804 g for 20 min to pellet the platelets. The supernatant was removed and the platelets lysed by the addition 1 mL lysis/binding buffer (100mM Tris-HCl, pH 8.0; 500mM lithium chloride; 10mM EDTA; 1% (w/v) lauryl sulfate lithium salt; 5mM dithiothreitol).

2.2.7.4. Preparation of monocytes for RT-PCR

Monocytes were isolated from citrated whole blood using Dynabeads® CD14 M-450 as described in §2.2.2.2. Following removal of the PBS/BSA in which they were resuspended, monocytes were lysed by the addition of 1 mL lysis/binding buffer and CD14 beads removed using the MPC®.

2.2.7.5. Extraction of mRNA from lysed platelets and monocytes

Total mRNA was extracted from platelet and monocyte lysates using magnetic Dynabeads® Oligo (dT)₂₅ (DynaL Biotech) in conjunction with the MPC®. For each extraction, 100 µL beads were washed in, and resuspended in, an equal volume of lysis/binding buffer prior to use. The beads were added to the cell lysate and mixed on a rotor for 5 min at room temperature. The beads and attached mRNA were washed twice in Wash buffer A (10 mM Tris-HCl, pH 8.0, 150 mM LiCl, 1 mM EDTA, 0.1% LiDS) followed by two washes in Wash buffer B (10 mM Tris-HCl, pH 8.0, 150 mM LiCl, 1 mM EDTA). The beads were finally resuspended in 20 µL ultrapure H₂O.

2.2.7.6. RT-PCR

Resuspended beads with attached mRNA were reverse transcribed immediately using AMV reverse transcriptase (Roche Diagnostics, Lewes, UK). Each RT-positive reaction was set up as follows: 9.57 μ L ultrapure H₂O, 7.50 μ L AMV buffer (Roche Diagnostics), 3.75 μ L dNTPs (Roche Diagnostics; dATP, dCTP, dGTP and dTTP diluted 1:10 in ultrapure H₂O), 0.93 μ L RNasin (RNase inhibitor; Promega UK, Southampton, UK), 15.00 μ L mRNA, 0.75 μ L AMV-RT. Each RT-negative reaction (to check for genomic DNA contamination) was set up as: 3.44 μ L ultrapure H₂O, 2.50 μ L AMV buffer, 1.25 μ L dNTPs, 0.31 μ L RNasin, 5.00 μ L mRNA. The reaction tubes were incubated at 42°C for 1 h in a Stratagene® RoboCycler (Stratagene, Amsterdam, The Netherlands).

The resultant cDNA was further amplified using *Taq* polymerase (Promega UK). Reactions were set up as 42 μ L ultrapure H₂O, 5 μ L 10x 'AJ (Alec Jefferies) buffer' (10x buffer: 450 mM Tris pH 8.8, 110 mM (NH₄) SO₄, 45 mM MgCl₂, 2 mM dATP, 2 mM dCTP, 2 mM cGTP, 2 mM dTTP, 1.1 mg/mL BSA (Roche Diagnostics), 67.21 mM β -mercaptoethanol, 44 μ M EDTA), 1 μ L each of 10 pmol/ μ L forward and reverse primer and 1 μ L template cDNA. As a control for contamination, a water blank was run for each PCR, 1 μ L H₂O being substituted for the template cDNA. All PCRs started with a 3 min initial denaturing time at 94°C after which 1 μ L per reaction *Taq* polymerase (diluted 1:4 in 10x 'AJ buffer') was added. All PCRs finished with a 4 min final extension at 72°C. PCR cycling conditions were as shown in Table 2.3.

Primers	Denaturing temp	Denaturing time (min:s)	Annealing temp	Annealing time (min:s)	Extension temp	Extension time (min:s)	No. of cycles
GAPDH	94°C	0:45	60°C	0:45	72°C	0:45	25
P-selectin	94°C	0:45	47°C	0:45	72°C	0:45	35
TF	94°C	0:30	57°C	1:00	72°C	2:00	35
TFPI	94°C	1:15	64°C	1:15	72°C	1:15	30
CD11b	94°C	0:45	62°C	0:45	72°C	0:45	35
IL-1β	94°C	0:45	52°C	0:45	72°C	0:45	35
IL-8	94°C	0:45	60°C	0:45	72°C	0:45	30
TNFα	94°C	0:45	56°C	0:45	72°C	0:45	30

Table 2.3. PCR cycling conditions.

PCR products were visualized on a 2% agarose (ICN Biomedicals, Inc, Basingstoke, UK) gel stained with ethidium bromide (1 μ g/mL final concentration) for 10 min, rinsed in distilled water and photographed using a GDS 5000 imaging system (Ultra Violet Products, Cambridge, UK). Where densitometry was used, gels were visualized on an Alpha Innotech Corporation transilluminator and analysed using Alpha Ease™ Version 3.24 software (Alpha Innotech Corp.; GRI, Braintree, UK). The density of the band of interest was compared with that of GAPDH bands obtained by PCR from the same samples, after deduction of background.

2.2.8. Western blotting

Washed platelets stored at -80°C were thawed and lysed into Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK) containing 10% (v/v) β -mercaptoethanol and immediately heated at 95°C for 5 min. Samples were kept on ice until lysis to prevent degradation of proteins. Proteins from platelet samples were separated on a 4-15% Tris-HCl Ready Gel (Bio-Rad) run at 200 V for 35 min in a Mini Protean II system (Bio-Rad). In order to check the molecular mass of transferred proteins, 5 μ L Novex™ Multimark® protein standard (Invitrogen) was loaded on each gel. Proteins were

then transferred to a Hybond C Extra nitrocellulose membrane (Amersham Pharmacia, Little Chalfont, UK) in a MiniBlot system (Bio-Rad) at 100 V for 1 h. Protein transfer was checked by staining the membrane in 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid (Sigma). Membranes were washed in TBS-T (TBS + 0.1 % Tween 20) and stored in TBS at 4°C until use.

Detection was performed by blocking in TBS-MT (TBS-T with 5% (w/v) dried skimmed milk (Marvel; Premier Foods, St.Albans, UK) for 1 h, followed by incubation for 1 h in the primary antibody (diluted in TBS-T as indicated). Membranes were washed in TBS-T, blocked again for 30 min, washed in TBS-T and incubated for 1 h in the horseradish peroxidase (HRP) – conjugated secondary antibody (Dako, High Wycombe, UK), diluted 1:2000 in TBS-MT. After further washing, in TBS-T followed by TBS, protein bands were visualized using an electrochemiluminescence (ECL) kit (Amersham Pharmacia) according to the manufacturer's instructions and Kodak X-OMAT XAR-5 film (Sigma), developed using an Agfa Curix 60 (Agfa-Gevaert Ltd., Brentford, UK). Exposure times were as indicated.

2.2.9. Lowry protein assay

Protein concentrations in samples for Western blotting were determined by Lowry protein assay. Dilutions of 400 µg/mL bovine serum albumin (Sigma) were made in order to construct a standard curve. After freeze-thawing, cell lysates were serially diluted in 0.4 M NaOH and vortexed. Duplicate aliquots of 15 µL of each cell sample and standard curve dilution and blanks of 0.4 M NaOH were added to a 96-well plate (Nunc). To each well 15µL of Lowry A (0.5 M NaOH; 100 mg/mL Na₂CO₃; 2 mg/mL N⁺/K⁺ tartrate; 0.5 mg/mL CuSO₄) was added and the mixture incubated for 10 min at room temperature. Immediately before use, Folin & Ciocalteu's reagent was diluted 1:14 in distilled H₂O; 60 µL was added to each sample. The plate was incubated for 10 min at 37°C and absorbance read at 620 nm in an Anthos 2010 microplate reader (Anthos Labtec Instruments Ges. M.b.H., Salzburg, Austria) with Stingray Version 1.5 software (Dazdaq Ltd., Ringmer, UK).

The protein concentrations of the samples were then read from the standard curve using GraphPad Prism® version 3.02 for Windows (GraphPad Software Inc., San Diego, CA, USA).

2.2.10. TFA assay

Washed platelets were prepared as described in §2.2.2.2 and stored at -80 °C in 500 µL aliquots each containing 5×10^8 platelets in TBS. For use as a positive control, monocytes were isolated from peripheral blood by density gradient as described in §2.2.3, resuspended in RPMI 1640 medium without phenol red and incubated with 200 ng/mL LPS for 4h at 37°C. Following incubation, they were centrifuged at 250 g for 10 min, resuspended in TBS and stored in aliquots of 5×10^6 cells/mL at -80°C. Before use, platelets and monocytes were lysed by freeze-thawing three times at 37 °C and -80 °C. Monocytes were further lysed by sonication for 10 min in a Decon FS 100 Frequency Sweep sonicator (Decon Ultrasonics Limited, Hove, UK) and resuspended at a concentration of 5×10^5 /mL in warmed TBS. Aliquots of lysed preparations containing 1×10^8 platelets and 5×10^4 monocytes (100 µL per well) were added to duplicate wells of a 96-well microplate (Nunc™, Invitrogen Ltd., Paisley UK). Cell numbers were chosen on the basis of preliminary experiments to give absorbance values which fell on the linear part of the standard curve.

Recombinant human TF (rTF; Hemoliance® RecombiPlasTin, Instrumentation Laboratory), diluted in 20 mL ddH₂O, was used as the standard and a 1/100 dilution was assigned an arbitrary unitage of 1 arbitrary unit (AU). The TF chromogenic assay measures TF in a purified system consisting of FVIIa (the First International Standard for Blood Coagulation FVIIa concentrate, 89/688, National Institute of Biological Standards and Control); purified FX (98/754, National Institute of Biological Standards and Control); calcium; a chromogenic substrate specific for FXa, S2765 (Chromogenix AB, Milan, Italy; Quadrachem, Epsom, Surrey); recombinant hirudin (Pentapharm, Basel, Switzerland); and lysed cells as the source of TF under examination. A

combined reagent consisting of FVIIa (0.15 IU/mL final concentration), CaCl_2 (8 mM final concentration), hirudin (50.6 $\mu\text{g/mL}$) and S2765 (0.53 mM) was prepared and kept on ice throughout the experiment. Immediately before use, purified FX at a final concentration of 0.063 IU/mL was added and 100 μL of the ice-cold mixture was transferred to the appropriate wells containing either standards or test samples. Colour was developed for 60 min at 37 °C and absorbance at 405 nm read using an Anthos 2010 microplate reader (Anthos Labtec Instruments Ges. M.b.H., Salzburg, Austria) with Stingray Version 1.5 software (Dazdaq Ltd., Ringmer, UK).

Negative controls consisted of identical samples incubated with a combined reagent lacking chromogenic substrate. The specificity of the TF activity was checked by pre-incubating cell samples with the anti-human TF Mab HTF-1 (PharMingen) at a concentration of 10 $\mu\text{g/mL}$, shown in preliminary experiments (in collaboration with Samantha Tull, Division of Chemical Pathology, University of Leicester, UK) to block the activity of 4 AU rTF.

2.2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism® versions 3.02 and 4.01 for Windows (GraphPad Software Inc., San Diego, CA, USA). Unless otherwise stated, results are presented as mean values \pm standard deviation (SD). Where the data were normally distributed, differences between two groups were determined by paired or unpaired t-tests, whilst differences between two or more groups were determined by one-way analysis of variance (ANOVA) followed by the appropriate post test (for example, Tukey's multiple comparison). For non-parametric data, Wilcoxon matched pairs tests, Mann-Whitney tests, and Kruskal-Wallis were the respective equivalents for normally distributed data. Relationships between independent variables were determined using the Pearson correlation coefficient. Results were considered significant where $p < 0.05$.

CHAPTER 3: Platelet PS exposure

3.1. Introduction

The exposure of procoagulant, negatively-charged phospholipids, particularly PS, on the surface of suitably activated platelets is the most obvious and widely studied aspect of platelets' contribution to the procoagulant environment in blood since it was first described over twenty years ago (Beyers *et al*, 1982). A number of different methods have been used to investigate the phenomenon, including prothrombinase assays and amidolytic assays where excess concentrations of TF, FVIIa and FX leave PS as the limiting factor for the production of FXa. More recently, flow cytometric assays have been developed, measuring either FV or PS on the platelet surface. Many of these assays have involved a degree of sample manipulation, some using washed or gel-filtered platelets, whilst others have used PRP; such preparation can introduce artefact. In addition, there have been varying approaches to the analysis of flow cytometric data, with many workers focussing exclusively on the size of the particles, regarding the platelet procoagulant response as inextricably linked with vesiculation. However, some would maintain that the two processes, though related, are in fact separate (Dachary-Prigent *et al*, 1993) and in any event it is arguable that the negatively charged surface is more significant for coagulation than the size of the particle concerned.

The first phase of this project therefore aimed to establish a flow cytometric assay for the detection of PS on the surface of both platelets and platelet-derived microparticles in the more physiological milieu of whole blood. This assay then served as a tool for investigating the level of procoagulant platelets circulating in healthy subjects and in expired platelets and was also used in conjunction with platelet antigen assays to characterise the formation of procoagulant platelets, as well as some of the mechanisms underlying the exposure of PS.

3.2. Results

3.2.1. Platelet PS exposure assay

A two-colour whole blood flow cytometric assay was developed to detect PS-exposing, procoagulant platelets and platelet-derived microparticles. The details of the assay are set out in the methods (§2.2.4.3). The assay depends on the high avidity of Annexin V for anionic phospholipids (Tait *et al*, 1989; Thiagarajan & Tait, 1990). The binding of Annexin V to PS is calcium dependent and therefore the HBS buffer used included 2mM CaCl₂. Conversely, the negative control was set where 2% platelets were Annexin V positive in a sample diluted in buffer which did not contain CaCl₂. Flow cytometric assays for surface antigens generally include a fixation step after labelling the antigens with antibodies. The percentage of formaldehyde routinely used in our laboratory is very low (0.2%) so that the reaction is stopped (or greatly slowed) more by dilution than fixation. However, preliminary experiments indicated that when 0.2% FS was used with calcium-containing buffer, Annexin V binding was very high. This was possibly due to the plasma membrane being slightly permeabilised, allowing the highly PS-avid Annexin V to bind to both internal and external PS. Alternatively, it has been suggested by others who have also observed this effect that it may be due to inhibition of the aminophospholipid translocase (Dörmann *et al*, 1998). For the present studies, the reaction was therefore stopped (or at least greatly slowed) entirely by dilution in calcium-containing HBS, thus maintaining the calcium concentration so that Annexin V binding was not altered.

The addition of calcium to buffer containing citrated blood risks allowing the blood to clot, which would make it unsuitable for assay by flow cytometry, relying as it does on a suspension of cells. The direct thrombin inhibitor hirudin was therefore added to prevent this. The concentration of hirudin used was determined empirically, but later shown to be able to inhibit a final concentration of 0.16 U/mL thrombin (Dr. R.K. Singh, Department of Cardiovascular Sciences, University of Leicester, UK, personal communication).

The assay was first established using whole blood activated by the calcium ionophore A23187 since this agonist is well known as a potent agent of PS exposure in platelets (Sims *et al*, 1989). The flow cytometric analysis is illustrated in Fig.3.1. Platelets were distinguished from RBC and leukocytes on the basis of forward and side scatter (size and granularity respectively), shown in the first histogram. The initial gate did not impose a lower limit on forward scatter so as to avoid the exclusion of microparticles. However, it was found necessary to employ a discriminator on the forward scatter in order to eliminate electronic signal 'noise'. The discriminator imposes a threshold below which data is ignored, thus in this instance excluding the smallest events. It was set at the lowest level (AU = 1) allowed by the software and experiments were carried out to evaluate the effect of this on the number of PS-positive events detected (see below).

The identity of the events in the initial gate as either intact platelets or platelet-derived microparticles was confirmed by their positivity for the pan-platelet marker CD42b-RPE (second histogram). Although levels of CD42b are reduced on activated platelets in suspension (Michelson & Barnard, 1987), there are still sufficient receptors available to make this a suitable platelet marker, although the assay could as well be performed using RPE-conjugated antibodies against CD42a (GPIX) or CD61 (GPIIb/IIIa). In the third histogram, only CD42b-positive events were further analysed for PS on their surface using Annexin V-FITC. Additional histograms allowed further analysis of the size of PS- and CD42b- positive events. Thus, CD42b-RPE is plotted against Annexin V-FITC in histogram 4; the size of all CD42b-positive events, two populations of platelets and platelet-derived microparticles, is shown in histogram 5; histogram 6 illustrates the size of Annexin V-positive platelets and microparticles; CD42b fluorescence is plotted against size in histogram 7. An eighth histogram displays the size distribution of all CD42b-positive, PS-positive events.

In general, results from the assay were expressed as the percentage of platelets positive for Annexin V rather than the median fluorescence intensity (MdFI) because of the difficulty in

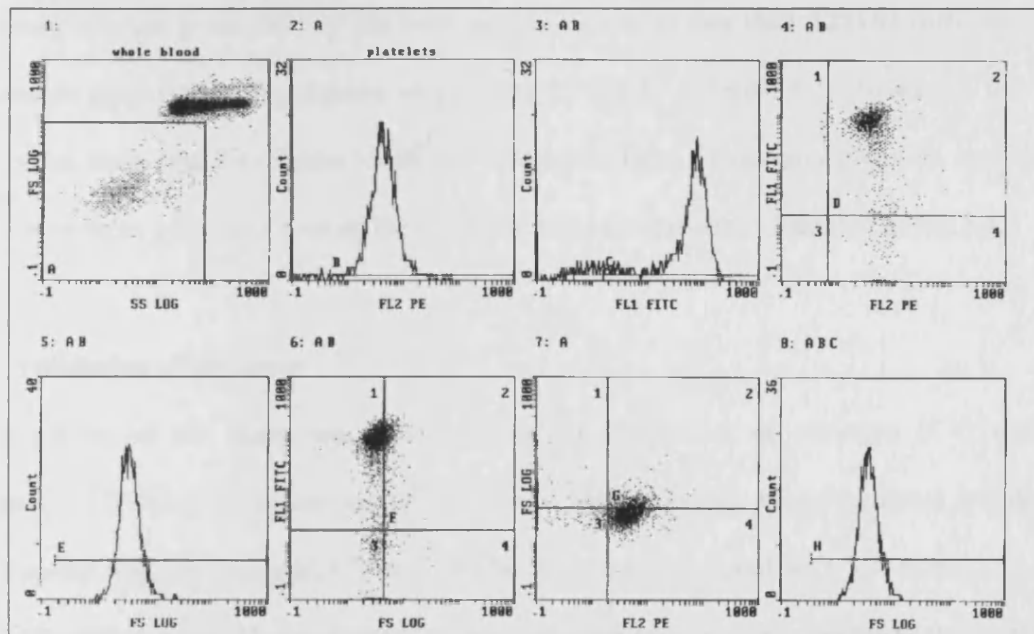


Figure 3.1. Flow cytometric analysis in platelet PS exposure assay. Platelets in whole blood were distinguished from red cells and leukocytes initially on the basis of their forward and side scatter characteristics (first histogram), and then on CD42b-RPE positivity (second histogram). These were then analysed for Annexin V-FITC binding (third histogram). Further histograms show different combinations of these parameters. In the fourth histogram, Annexin V fluorescence is plotted against CD42b fluorescence; the size distribution of all CD42b-positive events is displayed in Histogram 5, and of all Annexin V-positive events in Histogram 8; and the dot-plot in Histogram 6 shows the size of all platelets in relation to PS exposure. The histograms shown are representative of blood activated by 10 μ M A23187.

interpreting the data given the fact that activation by agonists other than A23187 sufficient to elicit PS exposure gave rise to populations of platelets of widely different size. However, the median fluorescence, reflecting the relative levels of PS on the surface of individual platelets, was examined in relation to these populations using the combinations of parameters illustrated in Fig.3.1.

3.2.2. Validation of the assay

Reproducibility of the assay was calculated as the coefficient of variation (CV; defined as $(SD/mean) \times 100\%$) of the percentage of Annexin V-positive events. In unstimulated samples set up in 10 separate reaction mixtures, CV was 14.9%. In samples activated with 500 ng/mL XL-CRP, it was 12.2% respectively. The percentage positive in each case was low (unstimulated mean 2.8%, range 2.0-3.3%; stimulated mean 8.8%, range 7.6-11.1%), which tends to yield high CVs so these figures were acceptable. The CV of the same sample analysed 10 times was 8.9%, indicating that most of the variation was attributable to that inherent in the flow cytometer.

The time within which the assay needed to be performed was examined both in terms of the stability of blood before its addition to the assay tube and the stability of the reaction mixture prior to analysis. The former was examined by setting up reaction mixtures using citrated blood which had been left undisturbed at room temperature for 0, 1.5, 2, 4, 6 and 24 h. Blood from two separate donors was activated with 500 ng/mL and 10 μ g/mL XL-CRP. Although the unstimulated control samples showed negligible change over 24 h, all the stimulated samples from both donors showed, for reasons that are unclear (perhaps a release of ADP), a temporary rise in response to XL-CRP between 2 and 6 h (Fig.3.2). Thereafter, the percentage of platelets positive for PS remained similar to that in blood used immediately after phlebotomy. To avoid the possibility of an artefactual rise in the number of PS-exposing platelets, blood was routinely used immediately after being drawn.

The stability of samples after final dilution is shown in Fig.3.3. Although there was a slight rise in the percentage of platelets positive for Annexin V after 30 min in the stimulated platelets

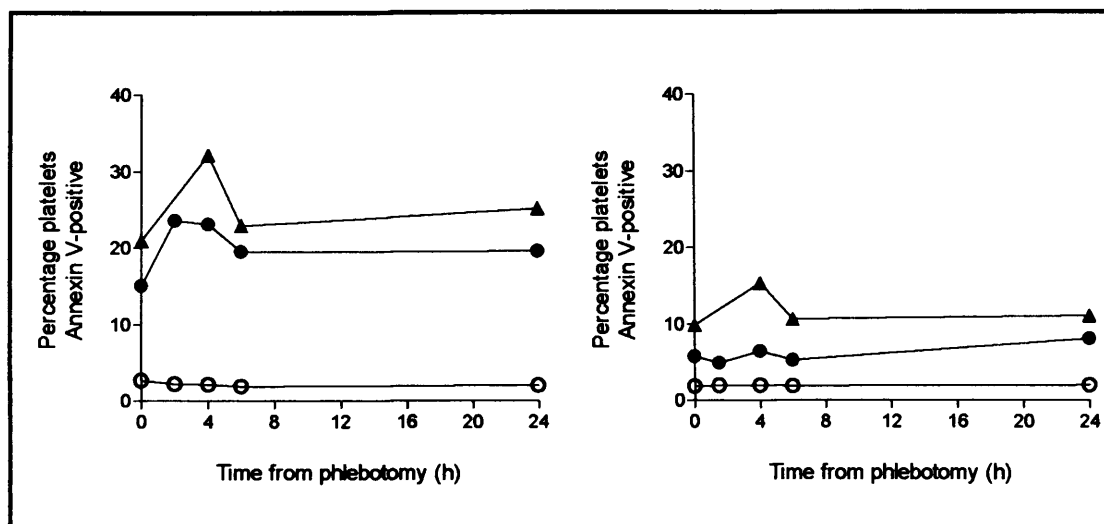


Figure 3.2. Stability of whole blood prior to assay for PS exposure. Citrated whole blood from two different donors, shown on separate graphs, was left undisturbed at room temperature before addition to reaction tubes containing buffer (open circles) or 500 ng/mL (closed circles) or 10 µg/mL XL-CRP (closed triangles) at the concentrations shown for the PS exposure assay at the times indicated.

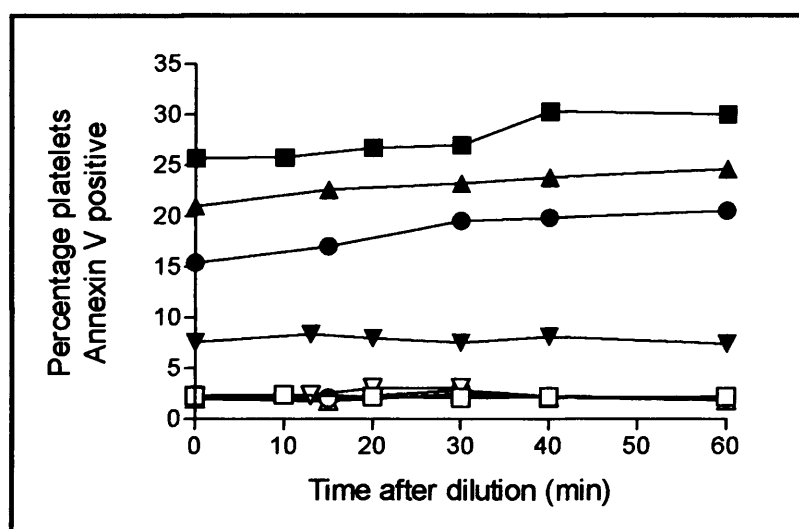


Figure 3.3. Stability of samples after reaction stopped by dilution. Platelets from 4 different donors were activated with 500 ng/mL XL-CRP (closed symbols; open symbols are unstimulated samples) and assayed for PS exposure. After dilution in HBS/c, samples were analysed in the flow cytometer at the time points indicated.

from one donor, samples were generally relatively stable over one hour. In all subsequent experiments, samples were analysed within this time.

There were concerns about the stability of dilute solutions of XL-CRP beyond an hour after dilution from the 5 mg/mL stock solution, possibly due to absorption onto plastic tubes from the weak solution. Therefore, the agonist was diluted immediately before use on each occasion. As this introduced another source of variability to the assay, this step was examined by making three separate serial dilutions of the 5 mg/mL stock solution down to 5 µg/mL and this was used to activate platelets from the same donor on the same occasion (final concentration 500 ng/mL). The CV was 8.96%.

The inclusion of some form of anticoagulant was imperative for a flow cytometric assay in citrated blood recalcified by the addition of 2mM CaCl₂ to the HBS. To determine whether the inhibition of thrombin by hirudin might be affecting the results, experiments were carried out where hirudin was replaced by the peptide GPRP. This prevents clotting by blocking fibrin polymerisation and has been shown to be compatible with examining thrombin activation of platelets by flow cytometry (Michelson, 1994). In two separate experiments there was no apparent difference in the percentage of Annexin V-positive events detected (Fig. 3.4).

In order to ascertain that inter-donor variability in platelet procoagulant response to XL-CRP was not simply due to inter-assay variability, platelets from 5 different donors were activated with 500 ng/mL XL-CRP on 2 or 3 separate occasions spanning intervals of between 3 days and 3 months. Although there was some change in the response on each of these occasions (possibly due to the deterioration of the XL-CRP preparation over time), the relative ranking of donors as high or low donors remained fairly constant (see Fig.3.5). There was very little variation in the low PS exposure seen on unstimulated platelets.

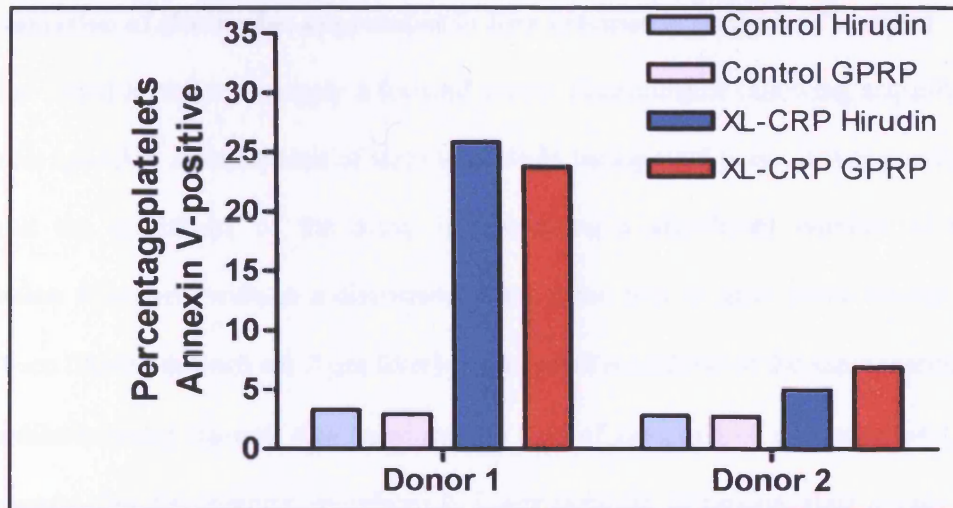


Figure 3.4. Replacing hirudin with GPRP has no effect on Annexin V binding. Clotting was prevented by the addition of 10 U/mL hirudin or 0.5 mg/mL GPRP to reaction tubes of unstimulated platelets or platelets activated with 500 ng/mL XL-CRP before assay for PS expression. Data are means of 2 separate experiments using blood from different donors.

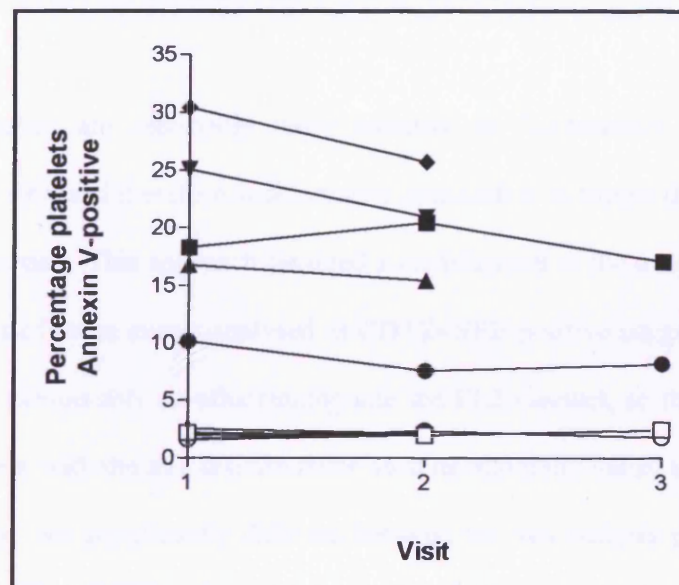


Figure 3.5. Inter-assay variation in PS exposure assay. Blood from 5 different donors was assayed for PS exposure on 2-3 separate occasions at intervals of between 3 days and 3 months. Open symbols are unstimulated samples; closed symbols are platelets activated with 500 ng/mL XL-CRP.

3.2.3. Evaluation of alternative approaches to flow cytometric analysis of samples

Since it was found necessary to apply a forward scatter discriminator (allowing acquisition of data only above a specified arbitrary unit of size) to exclude background noise, it was possible that this would limit the usefulness of the assay by excluding a significant number of very small microparticles. However, without a discriminator the noise was so great (even though all buffers used had been filtered through a 0.2 μm filter) that in parallel analysis of the same samples with and without a discriminator showed that there was no loss of detection of procoagulant (Annexin V positive) events. On the contrary, significantly lower numbers of procoagulant events in samples activated with 10 μM A23187 were detected when the discriminator was lifted compared with when it was in place (Fig.3.6). Using beads of known size (Molecular Probes, Paisley, UK), it was demonstrated that, on the settings employed in this assay, events smaller than the smallest 1 μm diameter bead were included in the analysis (Fig.3.7). The forward scatter measurement of these beads was approximately 10 arbitrary units (AU) compared with approximately 3 AU for microparticles.

Flow cytometers are reportedly more sensitive to fluorescence than to light scatter (Horstman & Ahn, 1999) and therefore an alternative approach is to trigger detection by gating first on CD42b-positive events. This approach required a modification of the analysis since the forward scatter characteristics of some events analysed as CD42b-RPE positive suggested that they were in fact red blood cells, presumably autofluorescing into the FL2 channel, so these were excluded by their size. In any event, with the size discriminator on at its minimum value, the number of Annexin V-positive events was not significantly different between the two analysis protocols (Fig.3.8). As previously, fewer procoagulant events were counted with the size discriminator removed than when it was in place, whether the initial gating was based size (2101.0 ± 391.8 vs. 683.3 ± 51.5 ; $p = 0.0291$) or fluorescence (2043.0 ± 302.0 vs. 656.0 ± 244.6 ; $p = 0.0158$).

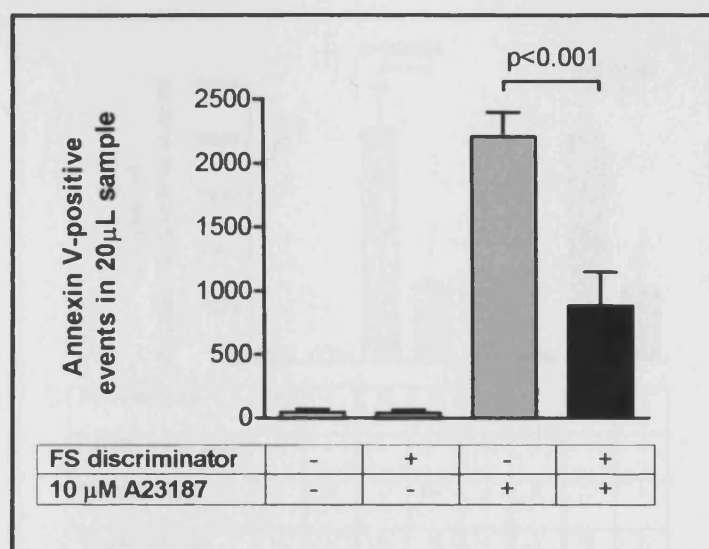


Figure 3.6. Effect of applying an forward scatter (size) threshold on the number of procoagulant events detected. A fixed volume of sample (20µL) activated by 10 µM A23187 was analysed in the PS exposure assay and the number of events positive for Annexin V compared with and without the imposition of a discriminator which set a minimum FS (size) threshold for the collection of data in the initial FS/SS histogram. Experiments were carried out on blood from 3 different donors.

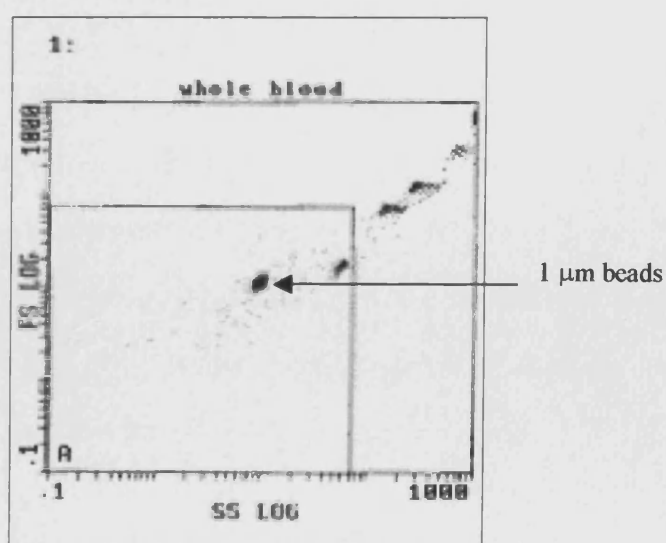


Figure 3.7. Particles smaller than 1 µm in diameter can be detected in the PS exposure assay. Calibrated beads of known size were suspended in Isoton flow cytometry buffer and analysed on the PS exposure assay. The smallest beads, 1 µm in diameter, are indicated.

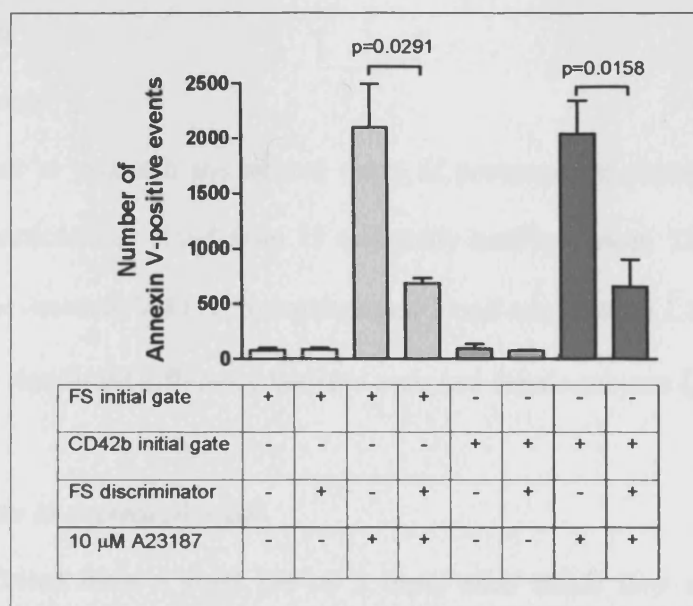


Figure 3.8. Effect of initial gating on FS/SS compared with triggering detection with CD42b-RPE. Platelets in a fixed volume of sample (20 μ L) were analysed for PS exposure, gating first either on FS/SS (FS) or on CD42b-RPE positivity (GPIIb), with the FS discriminator on and off, unstimulated control or activated with 10 μ M A23187 (n=3).

3.2.4. *Ex vivo* data

3.2.4.1. *Normal range*

The assay was used to establish the normal range of procoagulant platelets or platelet-derived microparticles in unstimulated blood from 15 apparently healthy donors. The mean percentage of platelets positive for Annexin V-FITC in unstimulated blood ranged from 1.3% to 3.9% (mean \pm SD 2.5 \pm 0.6%), with no significant difference between male and female subjects ($p=0.61$) (Fig.3.9).

3.2.4.2. *PS exposure in expired platelets*

Platelets for transfusion have a shelf life of 5 days, after which they are prone to bacterial contamination and therefore discarded (Perrotta & Snyder, 2002). Expired platelets from concentrates obtained from the Blood Bank at Leicester Royal Infirmary were stored at room temperature and examined for PS exposure by flow cytometry for each of the 5 days following the date of expiry (day 5). As shown in Fig. 3.10, the percentage of platelets positive for Annexin V rose steadily from 15.0 \pm 0.8 % on day 6 to 51.8 \pm 13.7% on day 10, by day 9 being significantly higher than immediately after expiry. From the gradual increase in the percentage of procoagulant platelets, starting from a percentage considerably higher than the mean of 2.5% in the normal range mentioned above, it can be speculated that even before expiry there are above-normal numbers of PS-exposing platelets. It is interesting to note that the platelets' size changed very little over the course of the experiment despite the changes in their membrane, implying that under these conditions, PS exposure was not associated with microparticle formation.

3.2.5. *Inter-donor variability in procoagulant response to XL-CRP*

The platelet procoagulant response to an intermediate concentration of XL-CRP was investigated in thirty normal, healthy donors. There was a greater than 8-fold difference between the percentage of platelets positive for Annexin V-FITC in the lowest and highest responders in the group as shown

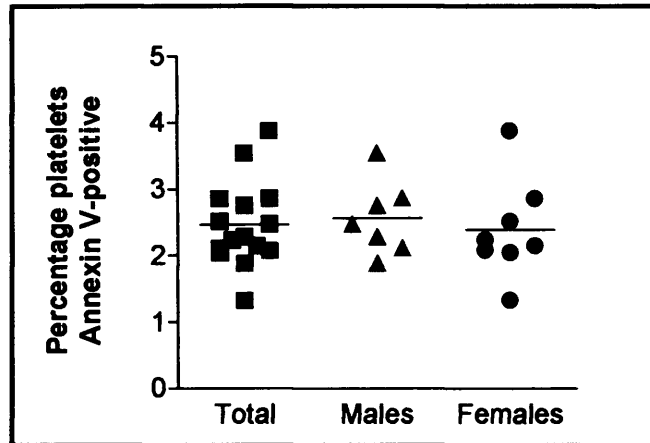


Figure 3.9. Circulating platelet-derived (micro)particles positive for Annexin V-FITC in healthy volunteers. Lines show the median values. There was no significant difference between males and females ($n=15$; 7 males and 8 females; $p=0.61$).

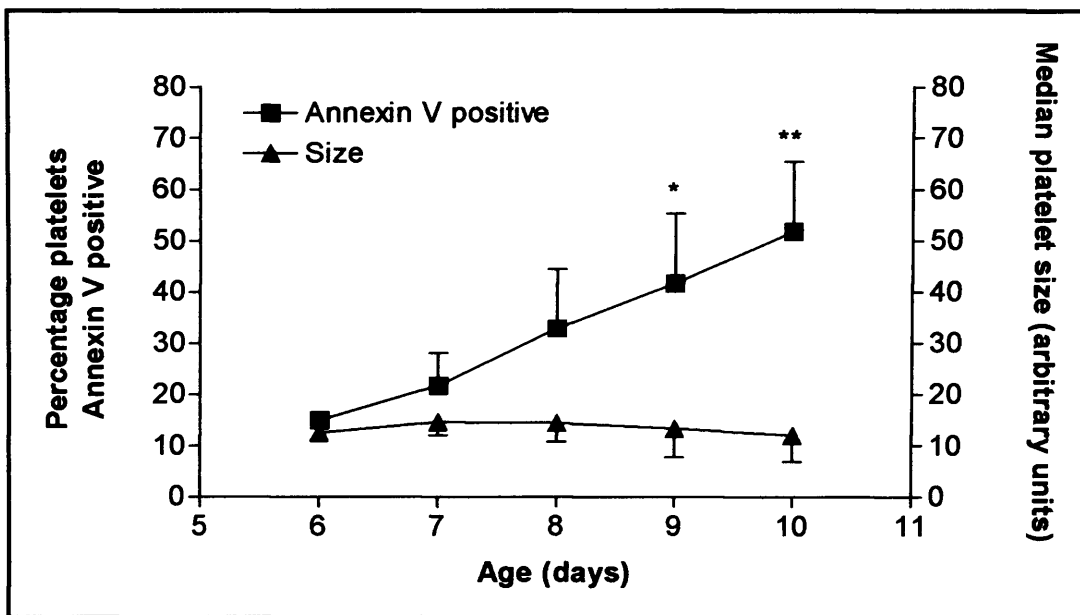


Figure 3.10. PS exposure and size of expired platelets. The percentage of platelets exposing PS as measured by Annexin V-FITC binding and the mean platelet size of expired platelets was investigated using the procoagulant platelet assay. The data are shown as mean \pm SD ($n=3$). * $p<0.05$ vs. day 6; ** $p<0.001$ vs. day 6.

in Fig.3.11 (mean \pm SD: 17.14 \pm 7.01; range: 3.95-34.35%). This variability was not merely in terms of sensitivity but also in the maximum response to activation, as illustrated in Fig.3.12, where at the highest concentration of XL-CRP, 100 μ g/mL, the range of the percentage of platelets which were Annexin V-positive was 10.4-51.0%. The majority of donors maintained their relative response throughout the concentration range. The dose-response curves for XL-CRP (Figs.3.15 and 3.37) and the variability seen when using Horm® collagen (Fig.3.32), also suggested that there was considerable variation between donors in the ability to expose PS.

3.2.6. Characterisation of procoagulant platelets

3.2.6.1. PS exposure and microparticle formation

Figure 3.13 shows the size and Annexin V-binding characteristics of platelets activated by increasing concentrations of the calcium ionophore A23187. The right-hand quadrants of each histogram included all but the smallest 2% of unactivated platelets and the upper quadrant limit was set by the Annexin V negative control. It can be seen that at the lowest concentration of A23187 (2 μ M), there was a distinct population of high Annexin V-binding, small platelets, which may be termed microparticles, although there is no specific evidence that they have in fact budded off from their parent platelets as opposed to being platelets which have drastically contracted. At intermediate concentrations of A23187, a separate population of less fluorescent platelets, also smaller than the majority of resting platelets, is visible but then, as the concentration rises, combines with the smaller, highly PS-positive population. Until the concentration exceeds 4 μ M, there is still a significant number of Annexin V-negative platelets in the intact platelet size region of the histogram and some of the Annexin V-positive platelets are also of similar size. At 10 μ M A23187, there is a single population of highly Annexin V-positive platelets/microparticles, consisting of virtually all the platelets in the original sample. The relationship between the median population size and PS exposure in platelets treated with A23187 is illustrated graphically in

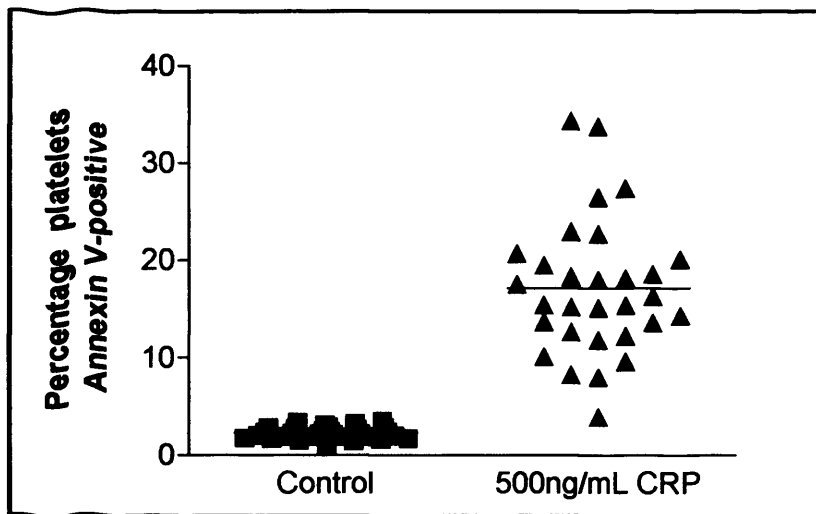


Figure 3.11. Variability in donor procoagulant response to XL-CRP. Platelets from normal, healthy donors were activated by 500 ng/mL XL-CRP and PS exposure detected using Annexin V-FITC by flow cytometry (n=30).

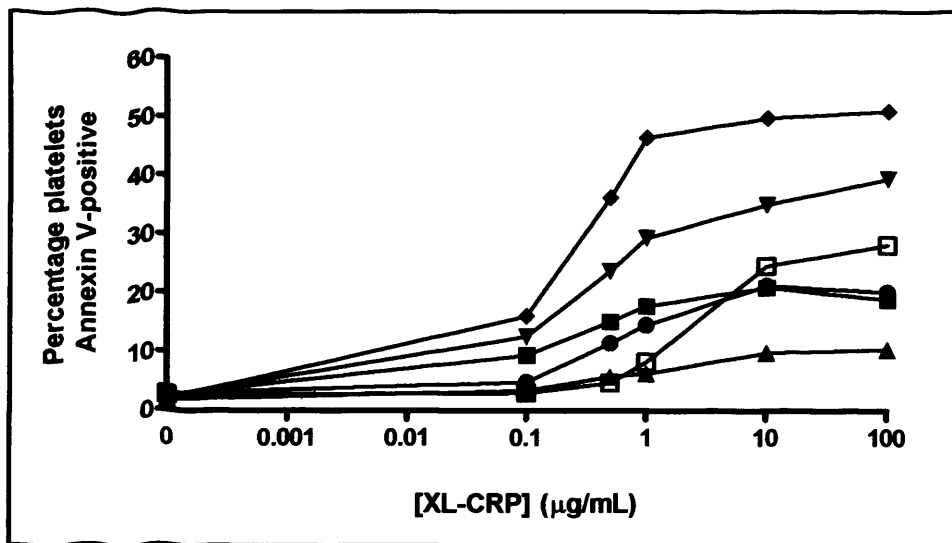


Figure 3.12. Variability in donor procoagulant response to XL-CRP extends over a range of concentrations. Platelets from six separate donors were activated with 0-100 $\mu\text{g/mL}$ XL-CRP and PS exposure detected using Annexin V-FITC by flow cytometry. Each symbol represents an individual donor. (Data are from Fig.3.29, combined for clarity).

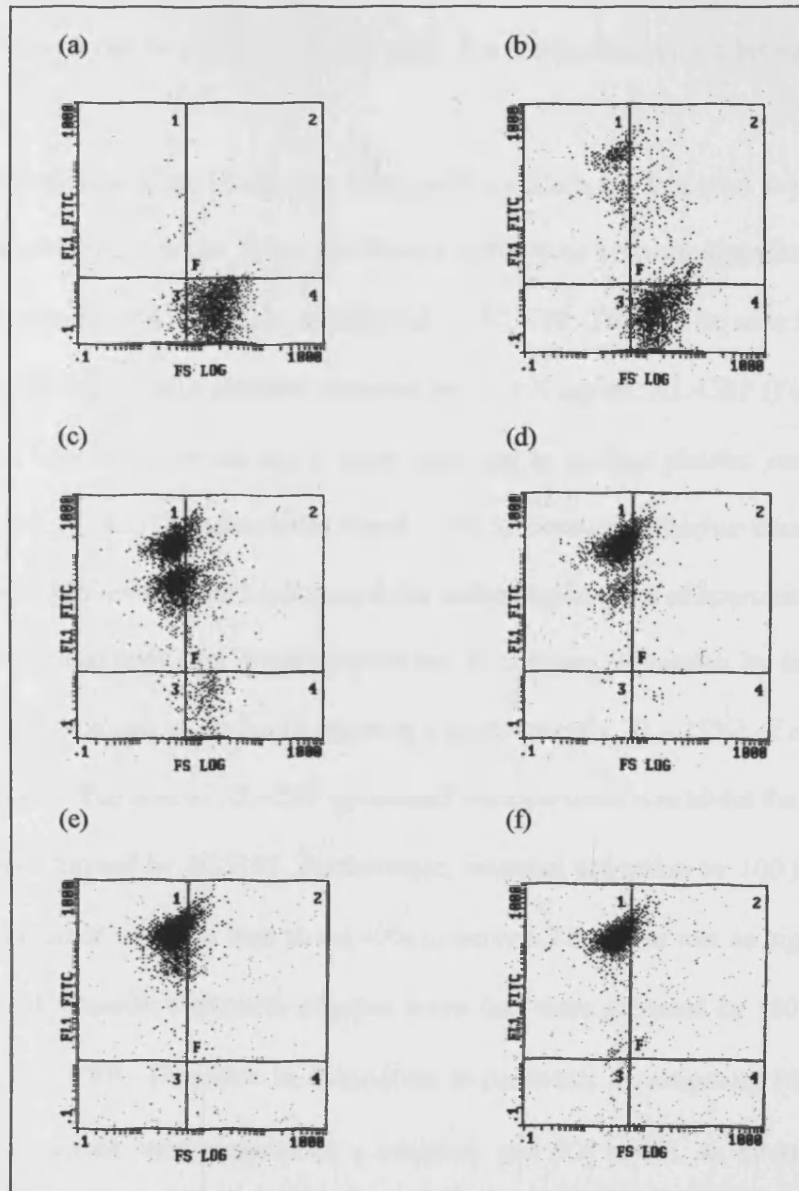


Figure 3.13. Size (forward scatter) vs. PS exposure in platelets treated with varying concentrations of A23187. Platelets were treated with 0, 2, 4, 6, 8 and 10 μ M A23187 (dot-plots a-f respectively). Forward scatter is on the x-axis and fluorescence (Annexin V-FITC) on the y-axis. Not all donors display the two-stage PS exposure illustrated here, but dot-plots (a) and (f) are typical of the 4 donors tested over this concentration range.

Fig.3.14. Here it can be seen clearly that there is a reciprocal relation between size and Annexin V positivity.

Although there are similarities between this pattern and that seen in platelets stimulated with more physiological agonists, there are distinct differences between signalling via agonist receptors compared with direct Ca^{2+} influx, as effected by A23187. This can be seen in dose-response curves of size and PS exposure in platelets activated by 0 - 100 $\mu\text{g/mL}$ XL-CRP (Fig.3.15). Comparing this figure with Fig.3.14, one can see a lesser decrease in median platelet size in XL-CRP-activated platelets than in A23187-stimulated blood. This is because at higher concentrations of A23187, nearly all CD42b^+ events were microparticles with a median size of approximately 3 AU. More than 95% of these microparticles were PS-positive. In contrast, activation by higher concentrations of XL-CRP never caused more than a minority (approximately 20 – 25%) of all CD42b^+ events to be microparticles. The size of XL-CRP-generated microparticles was about the same (approximately 3 AU) as those formed by A23187. Furthermore, maximal activation by 100 $\mu\text{g/mL}$ XL-CRP caused a subpopulation of no more than about 40% to express PS. There was no significant increase in the percentage of Annexin V-positive platelets when they were activated by 100 $\mu\text{g/mL}$ compared with 10 $\mu\text{g/mL}$ XL-CRP. Therefore in subsequent experiments investigating PS exposure induced by XL-CRP, 10 $\mu\text{g/mL}$ was regarded as a maximal, and 500 ng/mL an intermediate, concentration. This PS^+ subpopulation consisted, not just of microparticles, but of both platelet microparticles (smaller than the smallest 2 % of resting platelets) and platelets. This is further illustrated in Fig.3.16, which shows the effect of activating platelets with a high concentration of XL-CRP (10 $\mu\text{g/mL}$). The size distribution of all Annexin V-positive platelets reveals two distinct PS-positive populations, of platelets and microparticles. PS^+ platelets and microparticles both expressed high and low levels of PS similar those seen in microparticles at 2-6 μM A23187 in Fig.3.13 (b)-(d). This pattern is typical of all XL-CRP-activated platelets investigated and demonstrates clearly

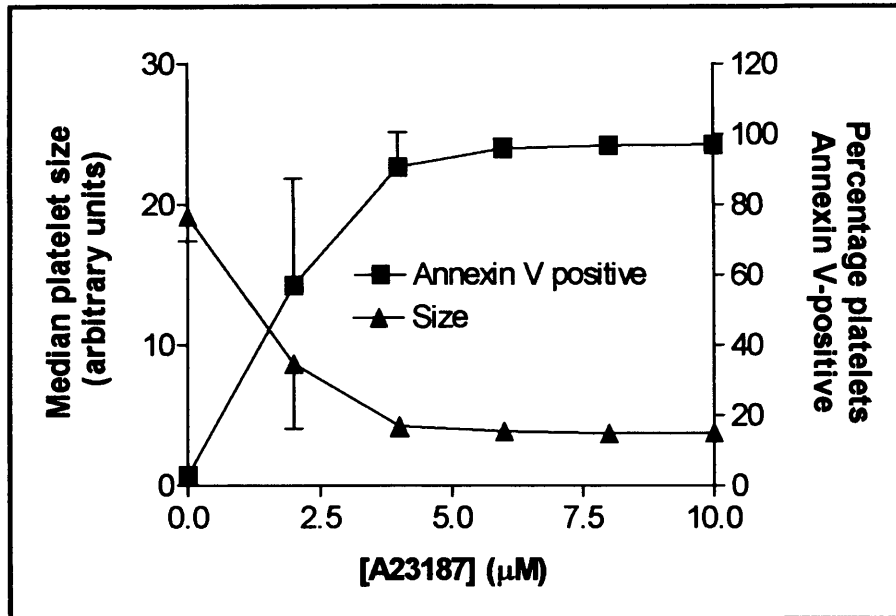


Figure 3.14. Percentage of platelets positive for Annexin V-FITC and mean platelet size after activation by 0 – 10 μM A23187. Measurements were made using the platelet PS exposure flow cytometry assay. Data are shown as means \pm SD (n=3).

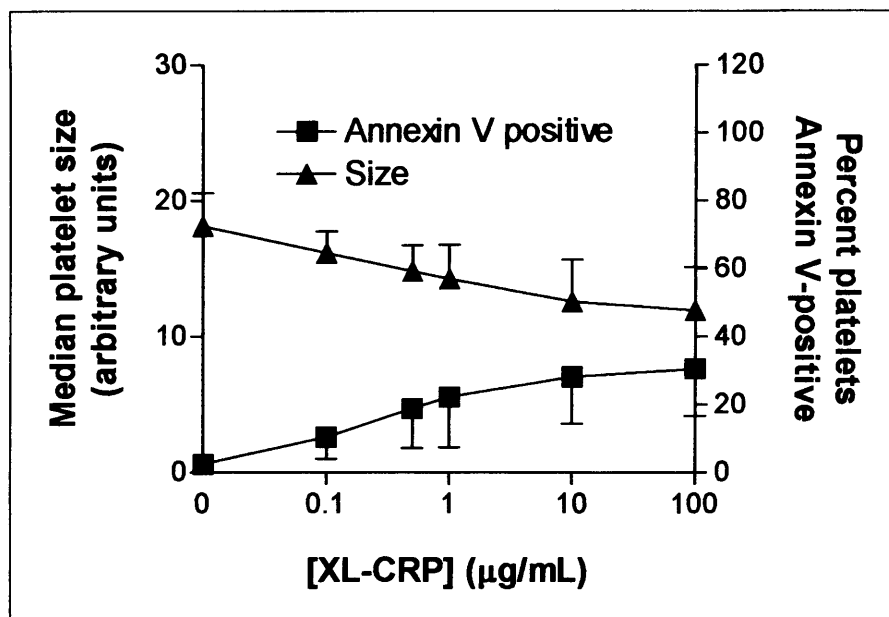


Figure 3.15. Percentage of platelets positive for Annexin V-FITC and mean platelet size after activation by 0 – 100 $\mu\text{g/mL}$ XL-CRP. Measurements were made using the platelet PS exposure flow cytometry assay. Data are shown as means \pm SD (n=7-8).

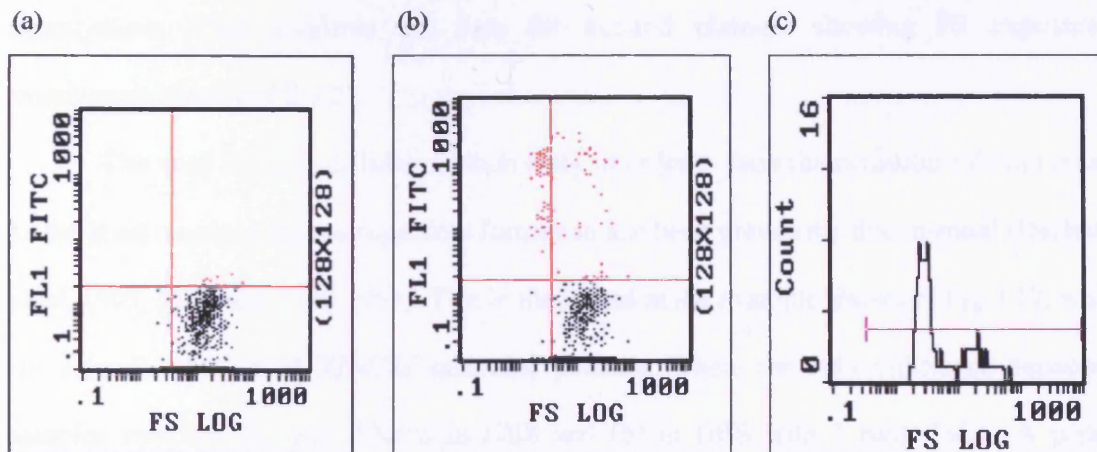


Figure 3.16. Annexin V binding and size characteristics of platelets stimulated by 10 µg/mL XL-CRP. The two right-hand quadrants include ~98% unstimulated platelets (a). Of the Annexin V-positive events in blood stimulated with 10 µg/mL XL-CRP, shown in red in the two upper quadrants (b), those weakly positive (just above the horizontal line on the y-axis), in the upper right-hand quadrant, are the same size as unstimulated platelets. The strongly Annexin V positive events, however, also include platelet-derived microparticles (upper left-hand quadrant) as well as platelets. (c) Histogram shows the size distribution of Annexin V-positive platelets, two populations being clearly visible.

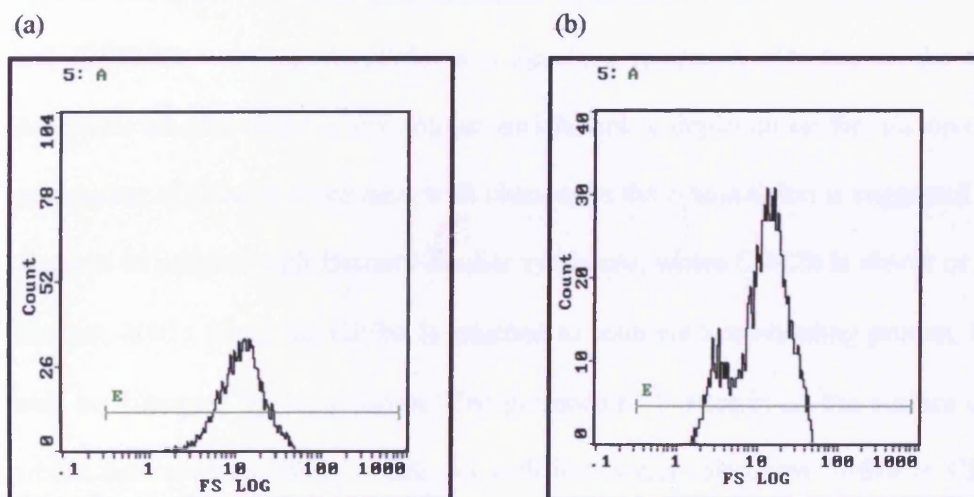


Figure 3.17. Extracellular calcium is required for the formation of microparticles. Platelets were activated with 500 ng/mL XL-CRP in the absence (a) and presence (b) of 2mM CaCl₂. The histograms show the size distribution of all CD42b-positive events. Note the difference in scale on the y-axis.

that, although all microparticles are positive for PS, microvesiculation and PS exposure are not synonymous. This confirms the data for expired platelets showing PS exposure without microvesiculation (§3.2.4.2).

The need for extracellular calcium entry in order to raise the cytosolic calcium concentration to the level required for microparticle formation has been previously documented (Dachary-Prigent *et al*, 1995; Martinez *et al*, 1999). This is illustrated in the example shown in Fig.3.17, which shows the size distribution of XL-CRP-activated platelets, where the only difference between the two samples was that (a) was diluted in HBS and (b) in HBS with 2 mM CaCl₂. A population of platelet-derived microparticles - the smaller peak on the left - was formed only in the latter. A comparison of PS positivity in calcium-free and calcium-containing buffer was not possible because the calcium-dependence of Annexin V binding.

3.2.6.2. Antigen expression on procoagulant platelets and platelet-derived microparticles

The role of platelet-derived microparticles released from collagen-activated platelets at the site of a wound will depend on their characteristics. Expression of some key antigens - CD42b, P-selectin and GPIIb/IIIa - on microparticles was therefore compared with that on the remnant platelets, to determine whether there is any antigen enrichment or depletion on the microparticles. The possible association of CD42b expression with changes in the cytoskeleton is suggested by the large size of platelets in patients with Bernard-Soulier syndrome, where CD42b is absent or reduced (Nurden & Nurden, 2001). Since the GPIIb is attached to actin via actin-binding protein, its expression might well be disrupted by vesiculation. The presence of P-selectin on the surface of the microparticle would determine its ability to interact with leukocytes (discussed further in Chapter 4). Likewise, possession of functional GPIIb/IIIa receptors would allow microparticles to interact with each other or other platelets.

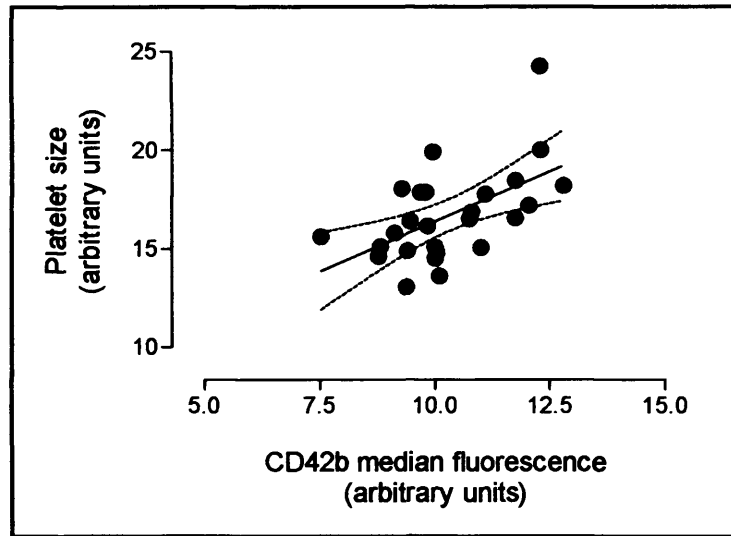
CD42b (GPIb α)

Estimates suggest that there are approximately 25,000 copies of CD42b on the average platelet surface (Berndt *et al*, 1985). As illustrated in Fig.3.18a, when unstimulated platelets from 26 donors were examined, the copy number indicated by the median fluorescence of bound monoclonal antibody correlated strongly with the size of the platelet ($r = 0.55$; $r^2 = 0.31$; $p = 0.0033$). When comparing the platelet and microparticle population as a whole, the mean value of the MdFI fell by 23.1% from 10.30 ± 1.29 to 7.94 ± 1.35 compared with a 15.9% reduction in the mean platelet size, there was still a strong correlation between CD42b surface expression and size ($r = 0.72$; $r^2 = 0.52$; $p < 0.0001$) in platelets stimulated by an intermediate concentration of XL-CRP (500 ng/mL; see Fig.3.18b). Combining the data for stimulated and unstimulated platelets yields an even stronger correlation ($r^2 = 0.5578$; $p < 0.0001$).

However, a slightly different pattern emerges on closer examination of dot-plots displaying individual events rather than the overall mean, which merges the two populations of platelets and microparticles. In blood stimulated with a maximal (10 μ g/mL) concentration of XL-CRP (see §3.2.6.1 and Fig.3.15), which forms a larger, more visible subpopulation of microparticles, it can be seen from the dot-plot (Fig.3.19) that microparticles (events in the bottom right quadrant) are relatively strongly positive for CD42b. Interestingly, the pattern of red Annexin V-positive dots suggests that it is predominantly the most highly CD42b-positive of the platelets which express PS, whilst all microparticles express PS. Examined in three donors (Fig.3.20), this seems to be the case since there is a much greater difference in the mean CD42b-RPE fluorescence (20.2 ± 3.0 vs. 13.9 ± 3.2) than there is in size (12.7 ± 1.2 vs. 3.1 ± 0.1). As a crude measure of whether the surface density of antigen is greater on microparticles than on remnant platelets, one can assume that the size (in AU) represents the diameter of a spherical activated platelet. Thus the mean surface area of remnant platelets assuming a mean size of 13 AU is:

$$4\pi^2 = 4\pi(6.5)^2$$

(a)



(b)

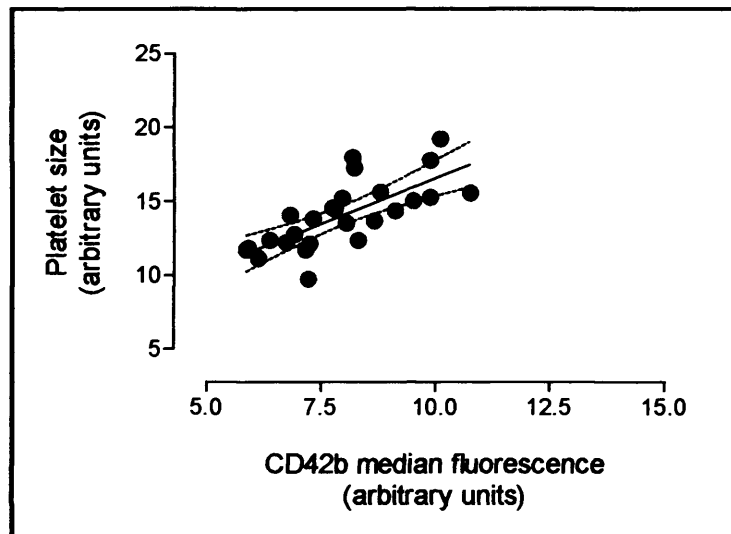


Figure 3.18. Correlation of CD42b expression and platelet size in platelets (a) unstimulated and (b) activated by 500 ng/mL XL-CRP (n=26). Data are taken from Histogram 2 of the PS exposure assay (see Fig. 3.1) performed on blood from separate donors. Mean platelet size was 16.69 ± 2.36 in resting and 14.03 ± 2.31 in stimulated platelets. Size and CD42b MFI were significantly correlated in both unstimulated and activated platelets ($r^2 = 0.3073$; $p=0.0033$ and $r^2 = 0.5214$; $p<0.0001$, respectively).

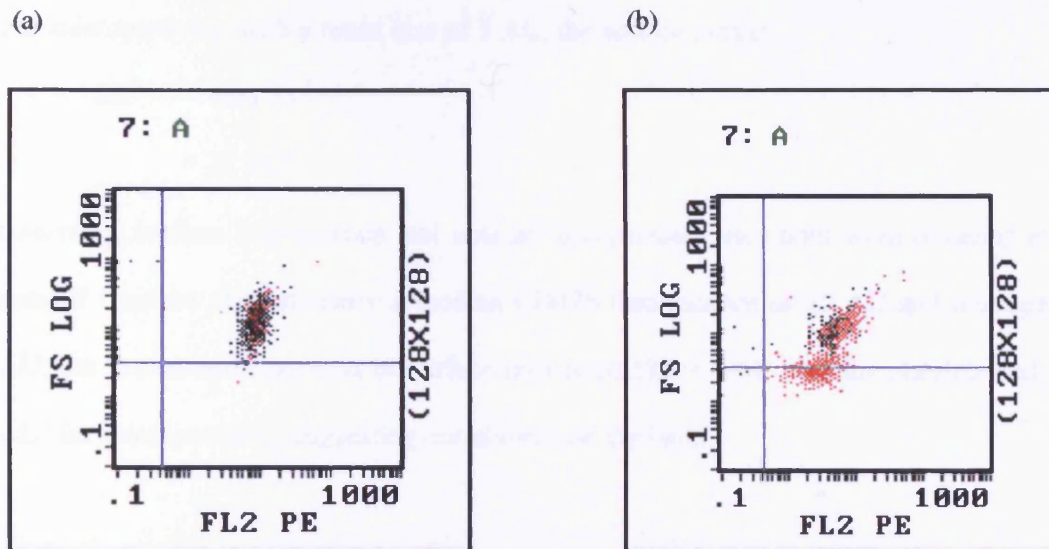


Figure 3.19. CD42b expression against platelet size. CD42b-RPE fluorescence against platelet size (both in arbitrary units) in resting platelets (a) and platelets activated by 10 $\mu\text{g/mL}$ XL-CRP (b). The upper right quadrant represents platelets which are larger than the smallest 2% of unactivated platelets; those below are microparticles. Red dots represent platelets which are Annexin V-positive.

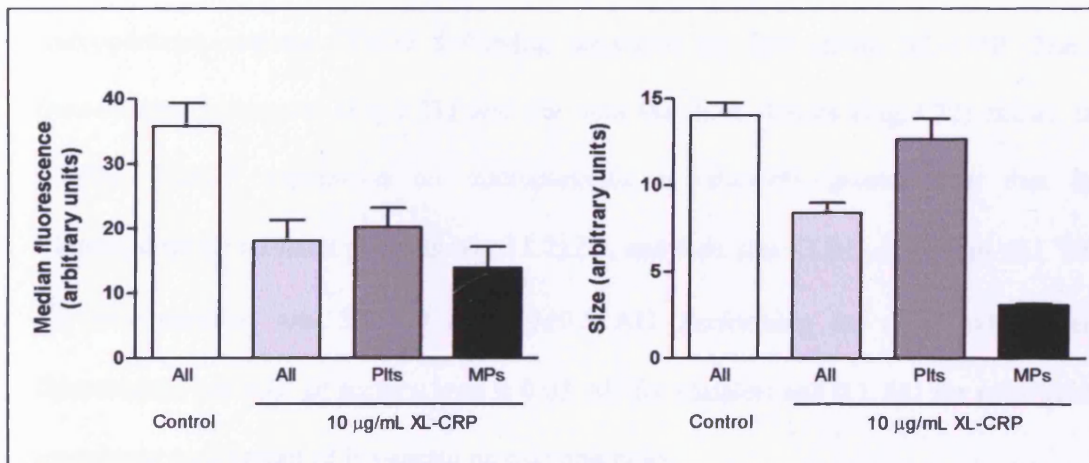


Figure 3.20. Collagen-generated platelet-derived microparticles are enriched in CD42b compared with the remnant platelets. Platelets were activated with 10 $\mu\text{g/mL}$ XL-CRP in HBS with 2 mM CaCl_2 and assayed for CD42b (GPIb) by flow cytometry. Graphs show the MdFI (left-hand panel) and platelet and microparticle size (right-hand panel). Data are from 3 separate experiments.

$$= 531 \text{ AU}^2$$

For microparticles, with a mean size of 3 AU, the surface area is:

$$\begin{aligned} 4\pi r^2 &= 4\pi(1.5)^2 \\ &= 28 \text{ AU}^2 \end{aligned}$$

Assuming median fluorescence and size are comparable since both were obtained at logarithmic gain, if remnant platelets carry a median CD42b fluorescence of 20 AU and microparticles of 14 AU, the fluorescence per unit of surface area is $20/531 = 0.04/\text{AU}^2$ for platelets and $14/28 = 0.5/\text{AU}^2$ for microparticles, suggesting enrichment on the latter.

CD62P (P-selectin)

Translocation of P-selectin from the α -granules was measured using a single colour flow cytometric assay where the extracellular calcium necessary for microparticle formation was supplied by using HBS supplemented with 2 mM CaCl_2 . As with the PS exposure assay, hirudin was added to prevent clotting and samples were diluted as described. Over 95% of platelets and platelet-derived microparticles express CD62P following activation by 500 ng/mL XL-CRP. The size versus fluorescence histogram (Fig.3.21) and the data for three donors (Fig.3.22) shows that, as with CD42b, CD62P expression on microparticles is relatively greater than that on platelets. Fluorescence on remnant platelets was 11.7 ± 2.3 , and their size 13.8 ± 1.4 , both in AU. The same data for microparticles was 5.3 ± 1.0 and 3.7 ± 0.5 AU. Performing the same calculation as above, fluorescence per AU^2 of surface area is 0.02 AU for platelets and 0.1 AU for microparticles, again suggesting enrichment of P-selectin on microparticles.

Fibrinogen binding and GPIIb/IIIa expression

The comparative ability of platelets and microparticles to bind fibrinogen, which would imply the presence of activated GPIIb/IIIa on their surface, was analysed using a FITC-labelled polyclonal

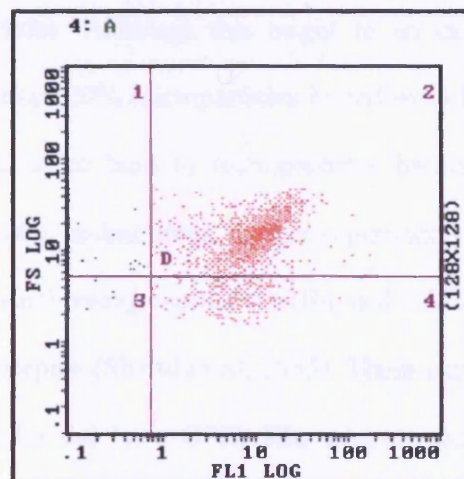


Figure 3.21. CD62P expression against platelet size. CD62P fluorescence against platelet size (both in arbitrary units) in platelets activated by 500 ng/mL CRP. Events in quadrant 2 (upper right) are platelets which are larger than the smallest 2% of unactivated platelets; those below are microparticles. Red dots represent platelets which are P-selectin positive.

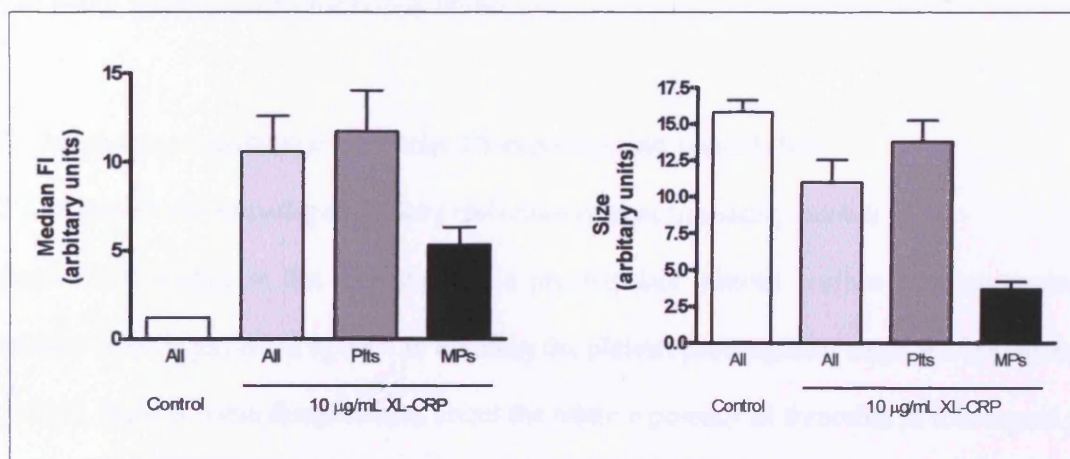


Figure 3.22. Collagen-generated platelet-derived microparticles are enriched in P-selectin (CD62P) compared with the remnant platelets. Platelets were activated with 500 ng/mL XL-CRP in HBS with 2 mM CaCl_2 and assayed for P-selectin (CD62P) by flow cytometry. Graphs show the MdFI (left-hand panel) and platelet and microparticle size (right-hand panel). Data are from 4 separate experiments.

antibody against fibrinogen. As shown in Fig.3.23, fibrinogen was found predominantly on platelets rather than microparticles. Although this might to an extent be due to the small size of the microparticles, only about 20% microparticles bound even low levels of fibrinogen. To determine whether fibrinogen failed to bind to microparticles because they lacked GPIIb/IIIa or because GPIIb/IIIa was present but unfunctional, further experiments were carried out using the monoclonal antibodies RFGP56, which recognises GPIIb/IIIa, and PAC-1, which is directed against the active conformation of the receptor (Shattil *et al*, 1985). These experiments, shown in Fig.3.24, indicated that whilst microparticles did have GPIIb/IIIa receptors on their surface, since they were >95% positive for RFGP56, these were not in a conformation capable of binding fibrinogen, as indicated by the low levels of binding of PAC-1 (4.2%). This could either be because the receptor was not activated or because some change, for example disruption of the cytoskeleton, prevented it from binding fibrinogen. The intact platelets, on the other hand, were >79% positive for PAC-1, reflected by their being >94% positive for bound fibrinogen.

3.2.7. Mechanisms underlying platelet PS exposure and vesiculation

3.2.7.1. Central role of collagen in the production of procoagulant platelets

Although early studies in the formation of a procoagulant platelet surface have recognised the importance of collagen as an agonist in eliciting the platelet procoagulant response (Thiagarajan & Tait, 1990), there is some disagreement about the relative potency of thrombin in this regard. Some have reported an increase in prothrombinase activity following platelet activation by 0.1 U/mL thrombin (Sims *et al*, 1989), whilst others have detected very little Annexin V binding to thrombin-activated platelets (Ramström *et al*, 2003). Some of these differences are likely to reflect differences in experimental procedure in terms of both platelet preparation and the measurement of the endpoint. Unless otherwise stated, the results presented in this section were carried out in whole blood with minimal manipulation, thus reducing the chances of artefactual activation.

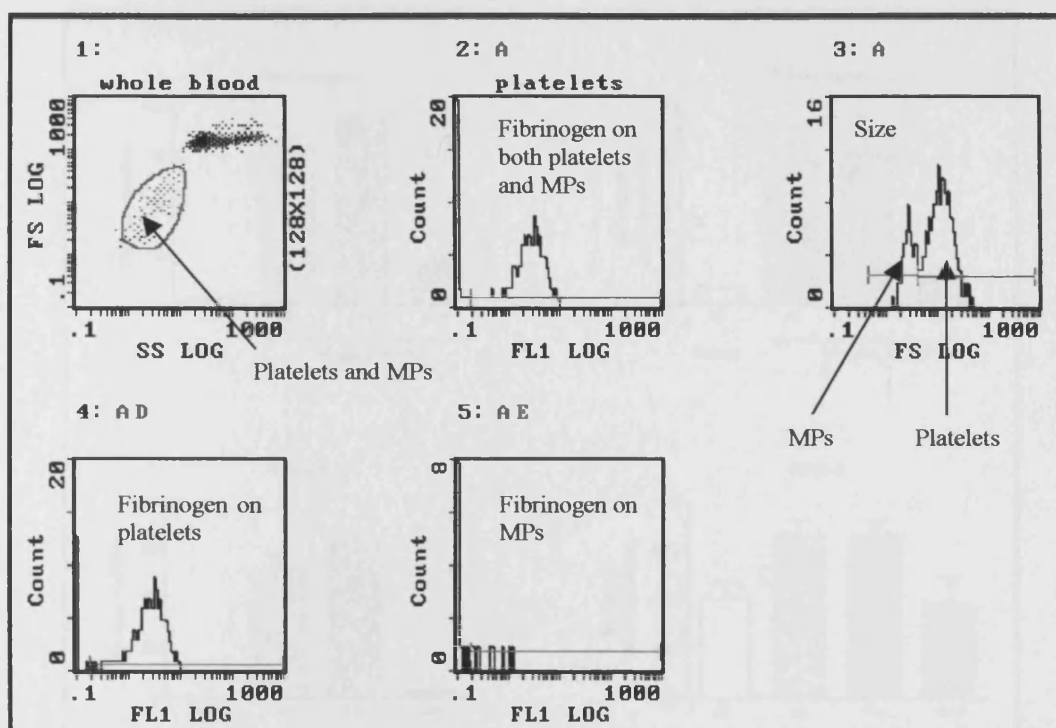


Figure 3.23. Differential fibrinogen binding on platelets and platelet-derived microparticles. Platelets were activated in HBS containing 2 mM CaCl_2 by 10 $\mu\text{g/mL}$ XL-CRP and bound fibrinogen detected using a FITC-conjugated polyclonal antibody by flow cytometry. The first histogram shows the platelet and platelet-derived microparticle gate based on FS/SS characteristics, with the next showing bound fibrinogen on the entire population. The third histogram shows the size distribution of the platelet/microparticle population and the following two histograms the bound fibrinogen on platelets and platelet microparticles respectively.

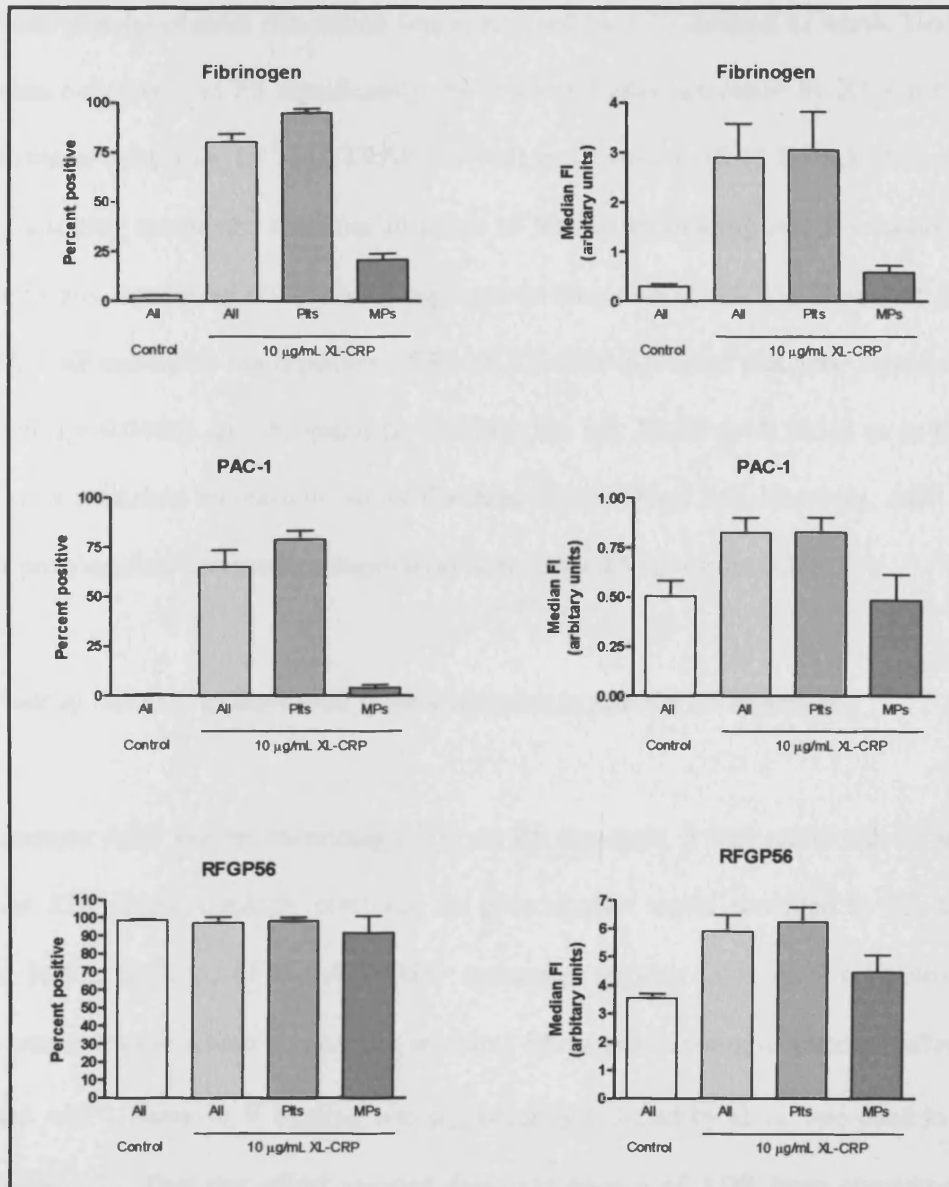


Figure 3.24. Collagen-induced platelet-derived microparticles have predominantly non-active GPIIb/IIIa receptors which do not bind fibrinogen. Platelets were activated with 10 µg/mL XL-CRP in HBS with 2 mM CaCl₂ and assayed for bound fibrinogen, activated GPIIb/IIIa and GPIIb/IIIa by flow cytometry. Graphs show percentage positive (left-hand panel) and MFI (right-hand panel). Experiments were performed on blood from 3-4 individual donors.

Where platelet-platelet interaction was minimised by 1:10 dilution of whole blood, platelets in suspension only exposed PS significantly above control after activation by XL-CRP. High final concentrations of ADP (3×10^{-5} M), TRAP (10^{-4} M) and thrombin (0.32 U/mL), all concentrations capable of eliciting maximum response in terms of fibrinogen binding and P-selectin expression, failed to have any significant effect on PS exposure on their own (Fig.3.25). However, ADP, TRAP and thrombin all enhanced the exposure of PS on XL-CRP-activated platelets, significantly in the case of ADP ($p=0.0486$) and thrombin ($p=0.0230$), but not TRAP ($p=0.1051$) as in this instance there was only a marked increase in two of the three donors (Fig.3.26). However, ADP was unable to evoke a procoagulant surface in combination with either TRAP or thrombin.

3.2.7.2. Role of released endogenous soluble agonists in platelet PS exposure

ADP

Since exogenous ADP had an enhancing effect on PS exposure, it was reasonable to suppose that endogenous ADP might similarly reinforce the procoagulant signal provided by XL-CRP. When performed in the presence of the ATP/ADP scavenger apyrase (at a final concentration of 80 $\mu\text{g/mL}$, a concentration which completely inhibited fibrinogen binding to platelets after activation with 10 μM ADP), Annexin V binding was significantly reduced by about one third in 6 different donors (Table 3.1). That this effect resulted from the release of ADP from platelets rather than erythrocytes was confirmed by performing similar experiments using PRP instead of whole blood in 2 separate experiments.

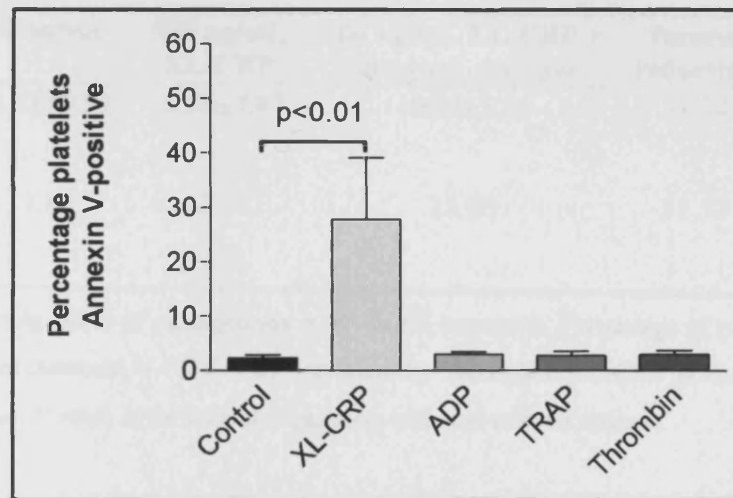


Figure 3.25. Collagen is the only single agonist capable of eliciting platelet PS exposure. Platelets were activated by high concentrations of XL-CRP (10 $\mu\text{g/mL}$), ADP (3×10^{-5} M), TRAP (10^{-4} M) and thrombin (0.32 U/mL, with GPRP substituted for hirudin). Only XL-CRP produced a significant increase in the percentage of Annexin V-positive platelets, as measured by flow cytometry, compared with control ($p<0.01$; $n=3$).

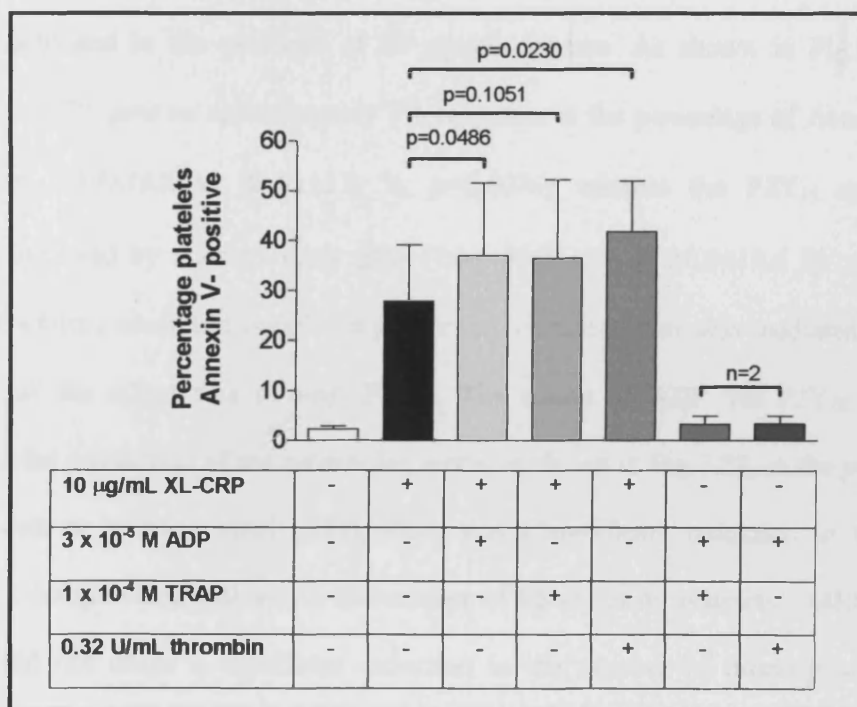


Figure 3.26. ADP and thrombin enhance the procoagulant effect of XL-CRP. Platelets were analysed for PS exposure by flow cytometry after activation with combinations of agonists as indicated. When stimulated by thrombin, GPRP was substituted for hirudin in order to prevent clotting. Data are from 3 separate experiments, except as indicated.

	Control	500 ng/mL XL-CRP	500 ng/mL XL-CRP + 80 µg/mL apyrase	Percent reduction	<i>p</i>
Whole blood (n=6)	2.57±0.59	15.96±7.87	10.93±5.86	31.52	<0.05
PRP (n=2)	1.84	22.78	15.60	31.52	N/A

Table 3.1. Enhancing effect of endogenous ADP on PS exposure. Percentage of platelets in whole blood and PRP positive for Annexin V-FITC after activation by 500 ng/mL XL-CRP in the presence and absence of 80 µg/mL apyrase. *P* value is for activated platelets with and without apyrase.

In order to determine which ADP receptors were involved in mediating the enhancing effect of secreted ADP, platelets were activated with a high concentration (10 µg/mL) of XL-CRP in the presence of specific competitive antagonist of P2Y₁ (10 µM MRS 2179), or the P2Y₁₂ (3 x 10⁻⁷ M AR-C69931MX), or both. As a control, to ensure that all ADP effects had been eliminated, a further sample was activated in the presence of 80 µg/mL apyrase. As shown in Fig.3.27, the P2Y₁ inhibitor MRS 2179 gave an approximately 7% reduction in the percentage of Annexin V-positive platelets (from 39.7±16.8 to 36.9±15.8 %; *p*=0.0076) whereas the P2Y₁₂ antagonist (AR-C69931MX) inhibited by approximately 50% (from 39.7±16.8 to 20.0±12.6 %; *p*=0.0179). This indicates that whilst a small but significant proportion of PS exposure was mediated through P2Y₁, the majority of the effect was through P2Y₁₂. The action of ADP via P2Y₁₂ appears to be principally on the production of microparticles, since, as shown in Fig.3.28, in the presence of AR-C69931MX (with or without MRS 2179), there was a significant reduction in the number of procoagulant microparticles, but not in the number of PS-exposing platelets. MRS 2179, on the other hand, did not cause a significant reduction in the number of procoagulant platelets or microparticles, although as noted above there was a small but significant reduction in the percentage of PS-positive events when they were considered together.

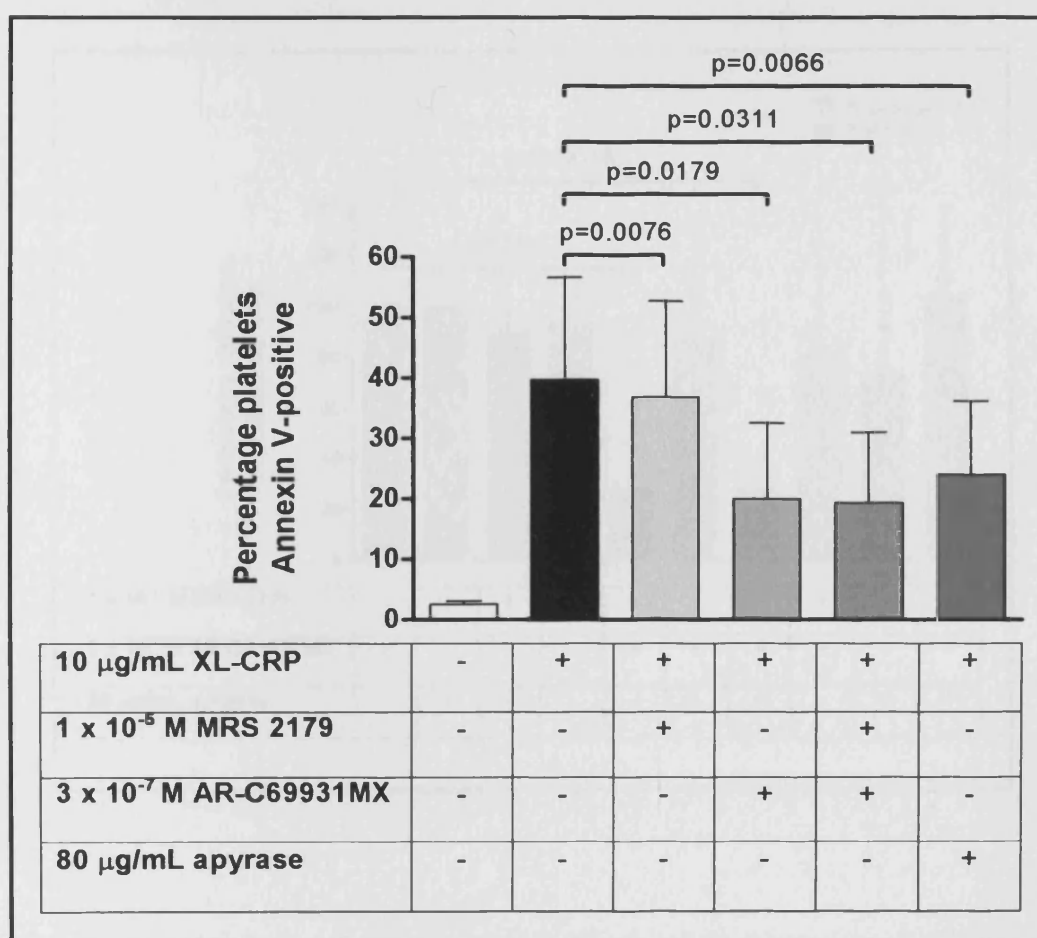
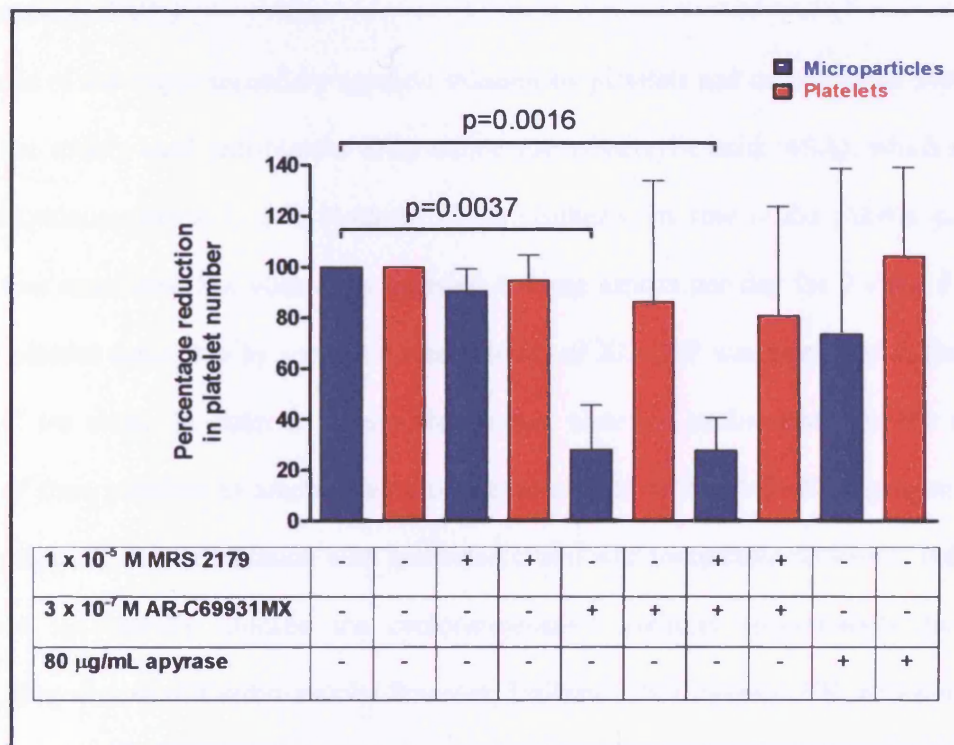


Figure 3.27. Contribution of P2Y₁ and P2Y₁₂ receptors to PS exposure in response to platelet activation by XL-CRP. Platelets were activated with 10 $\mu\text{g/L}$ XL-CRP in the presence or absence of the ADP receptor inhibitors MRS 2179 and AR-C69931MX or the ADP scavenger apyrase. PS exposure was measured by flow cytometry using Annexin V-FITC. Data are from 4-5 separate experiments performed using blood from different donors.

(a)



(b)

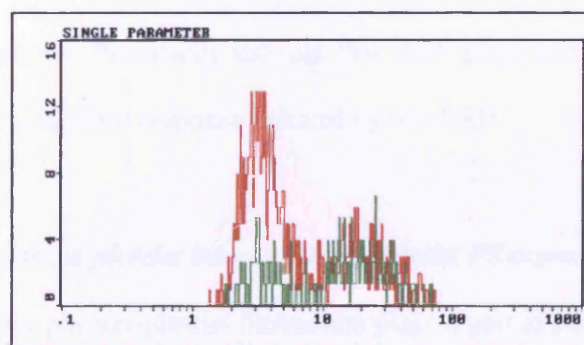


Figure 3.28. Differential impact of ADP receptor inhibition on procoagulant platelets and platelet-derived microparticles. (a) Percentage reduction in PS exposing microparticles and platelet-activated blood after inhibition by MRS 2179, AR-C69931MX and apyrase (final concentrations as indicated). Data are from 4 separate experiments using different donors. (b) Multigraph overlay showing XL-CRP activated Annexin V-positive platelets with (red trace) and without (green trace) AR-C69931MX as a function of size.

Thromboxane A₂ (TXA₂)

TXA₂ is one of the major secondary agonists released by platelets and its metabolic pathway is the target of the widely used anti-platelet drug aspirin (acetylsalicylic acid; ASA), which irreversibly acetylates cyclooxygenase-1, thus blocking TXA₂ synthesis. Its role in the platelet procoagulant response was examined. Six volunteers ingested 150 mg aspirin per day for 7 days. PS exposure following platelet activation by various concentrations of XL-CRP was measured at the beginning and end of the week. In order to ensure that donors were not aspirin-resistant, the aggregation response of their platelets to arachidonic acid was measured in the PAP-4D aggregometer. In all cases, aggregation after stimulation with arachidonic acid was completely abolished, indicating that aspirin had successfully blocked the cyclooxygenase-1 pathway (experiments performed by C.I.Jones, Department of Cardiovascular Sciences, University of Leicester, UK; data not shown). In only two out of six donors was there a reduction in PS exposure following aspirin treatment at any concentration of XL-CRP (Fig.3.29); in the other four, the response was nearly identical. This variation in donor response meant that the reduction did not reach significance when the data were considered as a whole. The results indicate that ADP plays a more important role than TXA₂ in reinforcing the procoagulant response induced by XL-CRP.

3.2.7.3. Role of platelet-platelet interaction on platelet PS exposure

To examine whether platelet-platelet interaction plays a part in PS expression in platelets, a slightly different experimental set-up was employed. PRP was prepared so that aggregation in the sample could be monitored in the PAP-4D aggregometer. Samples were stirred at 900 rpm at 37 °C; at exactly 20 s after the addition of the agonist, subsamples were transferred into Annexin V binding assay flow cytometry reaction mixtures and analysed as previously described. This time interval allowed some aggregation but spared sufficient single platelets for flow cytometric analysis. In

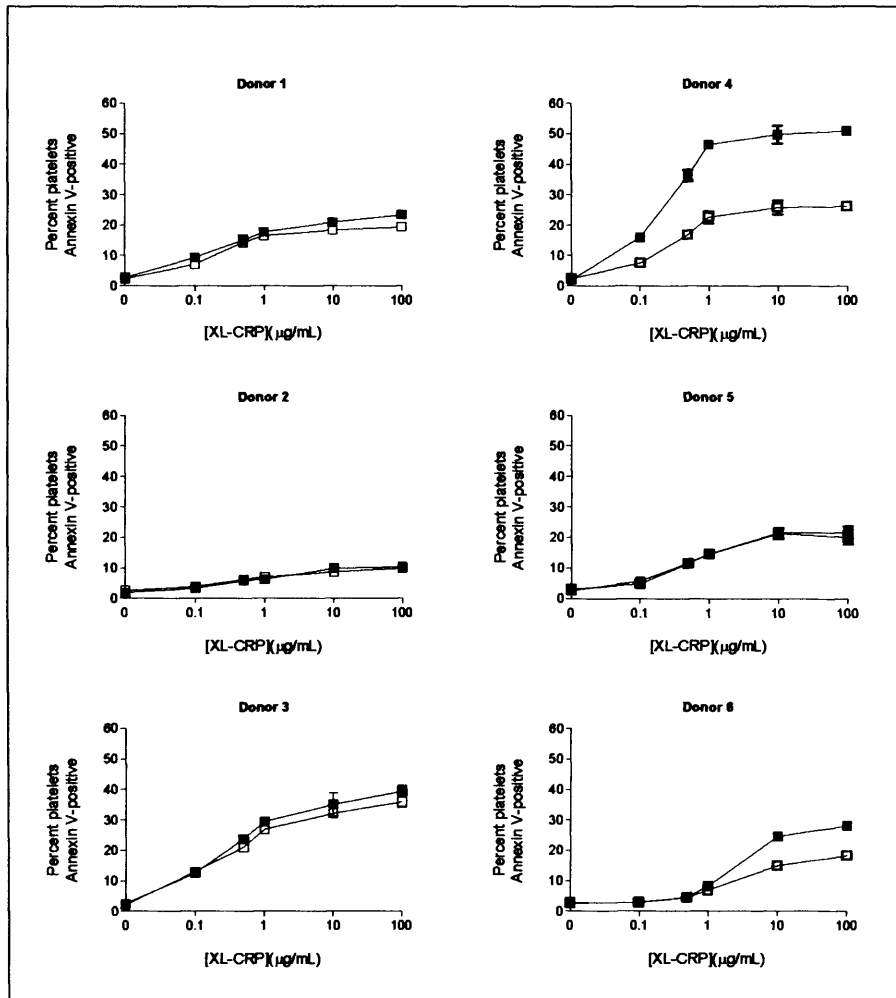


Figure 3.29. Thromboxane does not have a significant role in platelet procoagulant response. Platelets from six donors were assayed for PS exposure by flow cytometry following activation by different concentrations of XL-CRP before (closed symbols) and after (open symbols) taking 150 mg ASA for 7 days.

order to block platelet aggregation in certain samples, the GPIIb/IIIa blocking antibody ReoPro® (at a final concentration of 42 µg/mL) was added 3 min prior to the addition of the agonist.

As shown in Fig.3.30, stirring increased the percentage of procoagulant platelets in both the XL-CRP and TRAP-activated samples, although the increase was significant only in the case of the latter (from 9.7 ± 1.9 to 15.8 ± 3.6 %, $p=0.1899$; and from 3.2 ± 0.3 to 7.6 ± 0.3 %, $p=0.0056$ respectively). There was also a significant increase in PS positive platelets in the non-stirred samples activated with both agonists compared with the non-stirred control. In the case of the TRAP-activated platelets, this was in contrast with the results achieved when platelets were activated in suspension in which cell-cell contact was minimal (see Fig.3.25).

To further demonstrate that this enhancement in PS exposure was indeed due to platelet-platelet interaction, PRP was similarly activated, with and without stirring, in the presence and absence of the GPIIb/IIIa antagonist ReoPro®. In all activated samples, the percentage of Annexin V-positive platelets was significantly reduced, although not abolished, by the addition of ReoPro® (Fig.3.31) by 62.7 ± 19.9 % ($p=0.0320$; $n=3$) and 60.1 ± 22.8 % ($p=0.0436$) in XL-CRP activated samples with and without stirring and 73.0 ± 4.2 % ($p=0.0011$) in stirred TRAP-stimulated samples. In unstimulated samples, only about 2 % of platelets bound Annexin V, and this percentage was not reduced by ReoPro®. The light aggregometry traces, summarised in Fig.3.32, of the same samples indicated that although ReoPro® at this concentration (42 µg/mL, compared with 20 µg/mL said to produce near-saturation binding to surface GPIIb/IIIa receptors (Ammar *et al*, 1997)) significantly reduced aggregation, there was still some residual aggregation in the XL-CRP-activated PRP which was still apparent even at higher concentrations of inhibitor (data not shown). It is possible that this is due to the XL-CRP forming cross-linked aggregates.

Rapid exposure of PS was also seen when platelets were activated by a more complex collagen, in similar experiments allowing platelet-platelet interaction using fibrillar Horm® collagen as the agonist. Increasing percentages of platelets were positive over this time, rising from

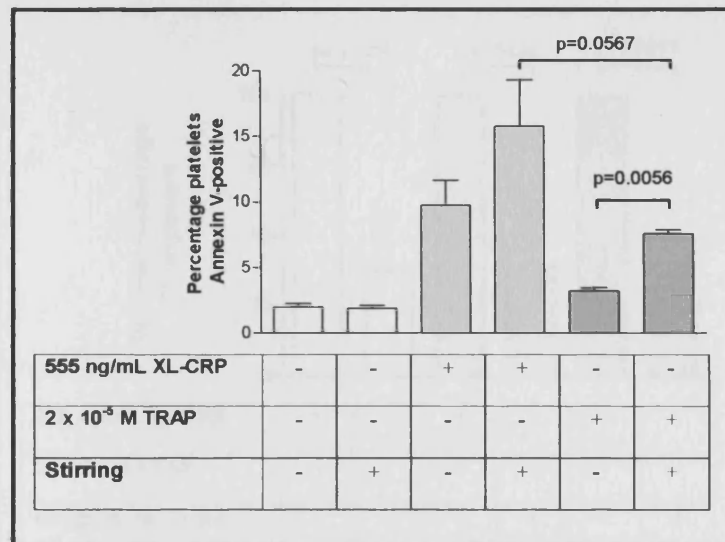


Figure 3.30. Effect of cell-cell interaction on PS exposure. Subaliquots of PRP activated by XL-CRP and TRAP at 37°C in the PAP-4D aggregometer, with and without stirring, were removed for PS assay in the flow cytometer 20s after the addition of agonist. Data are from 3 separate experiments using blood from different donors.

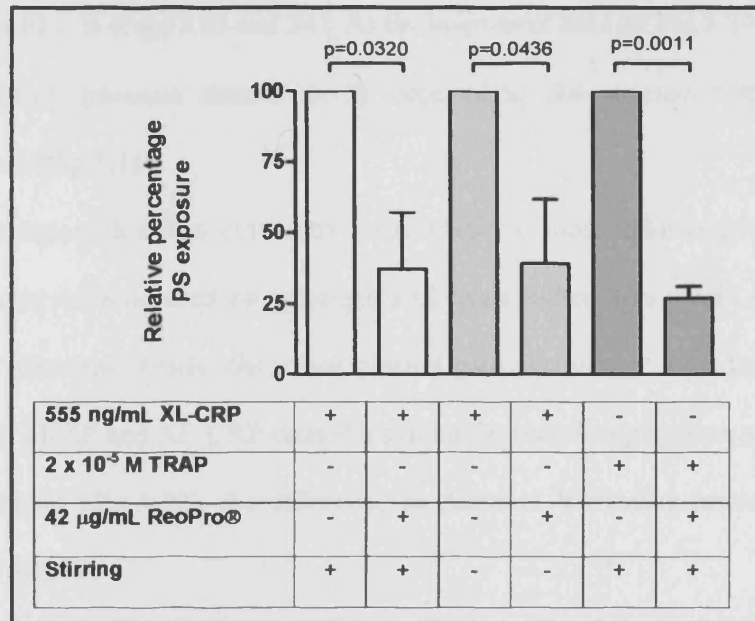


Figure 3.31. Effect of inhibiting platelet-platelet interaction on PS exposure in XL-CRP- and TRAP-stimulated platelets in stirred and unstirred systems. The relative PS exposure following platelet activation by XL-CRP and TRAP in the presence and absence of the GPIIb/IIIa inhibitor ReoPro®, with and without stirring in the PAP-4D light aggregometer. Subsamples were transferred to flow cytometry test tubes to be assayed for Annexin V binding. Data are from 3 separate experiments using blood from different donors.

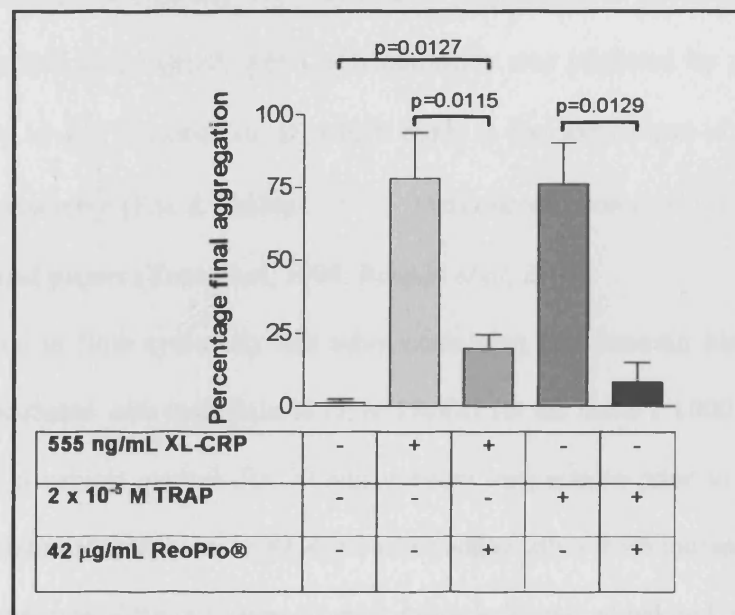


Figure 3.32. Inhibition of platelet aggregation by ReoPro® as indicated by light aggregometry. The extent of platelet-platelet aggregation with and without ReoPro® after activation by XL-CRP and TRAP. Data are from 3 separate experiments using blood from different donors.

1.8±0.6 % to 17.8±10.1 % (Figs.3.33 and 34). As the large error bars on Fig.3.34 indicate, there was considerable variation between donors (n=5), resembling the varying response to XL-CRP previously observed (Fig.3.11).

It could be argued that flow cytometry assay of stirred subsamples is open to artefact in that the fluorescence may be measured on aggregated platelets rather than single platelets. However, two points refute this view: firstly, the initial platelet gate excludes at least large aggregates; and secondly, although TRAP and XL-CRP caused a similar degree of aggregation as determined by the PAP-4D aggregometer (Fig.3.32), the difference in Annexin V binding approached significance ($p=0.0567$) (Fig.3.30).

3.2.7.4. Role of actin polymerisation in platelet PS exposure

Since cytoskeletal reorganisation is involved in the regulation of GPIIb/IIIa (Shattil & Newman, 2004) and has been reported to be necessary for store-mediated calcium entry in platelets (Rosado *et al*, 2000), required for PS exposure, the role of actin polymerisation in the procoagulant platelets and microparticles was investigated. Actin polymerisation was inhibited by preincubating whole blood or PRP with 10 µM cytochalasin D, which binds to the barbed end of actin filaments thus preventing their reassembly (Fox & Phillips, 1981). The concentration used (10 µM) was chosen on the basis of published papers (Yano *et al*, 1994; Rosado *et al*, 2001).

Whole blood in flow cytometry test tubes containing the Annexin binding assay reaction mixture was preincubated with cytochalasin D, or DMSO (at the same 1:1000 dilution as used for cytochalasin D) as a vehicle control, for 30 min at room temperature prior to the addition of XL-CRP. An intermediate concentration of XL-CRP was used to allow both increases and decreases in Annexin V to be apparent. After a further 10 min, Annexin V was added and, after another 10 min incubation, the samples were diluted and analysed on the flow cytometer. In this experimental set-up, with cells in suspension with minimal cell-cell interaction, cytochalasin D caused a significant

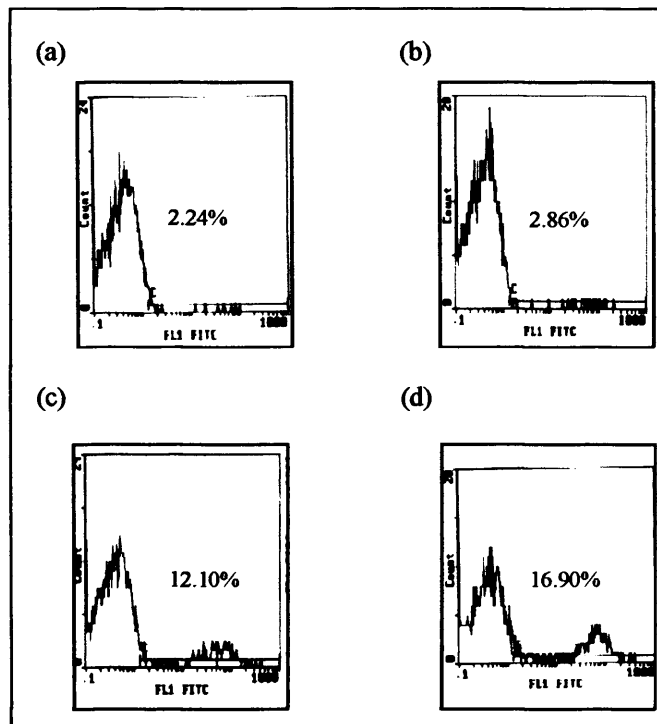


Figure 3.33. Procoagulant microparticle production after collagen activation. Percentage of platelet-derived particles positive for Annexin V-FITC as determined by flow cytometry before (a) and 15, 30 and 45s [(b) to (d) respectively] after addition of 4.4 $\mu\text{g/mL}$ fibrillar Horm® collagen. PRP was stirred at 37°C. The histograms are representative of 5 independent experiments.

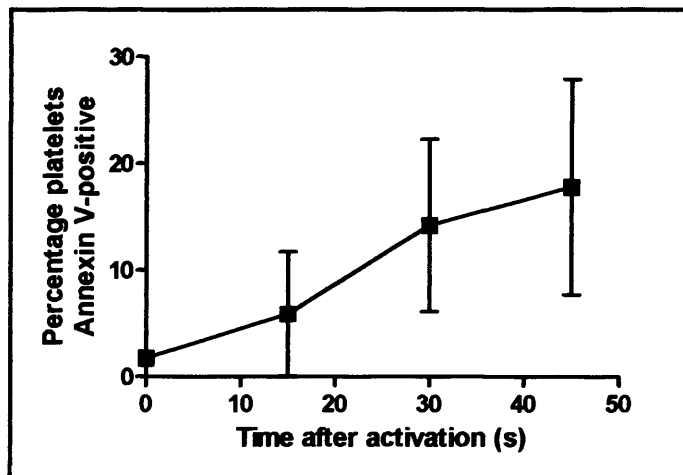


Figure 3.34. Percentage of platelets binding Annexin V-FITC after activation by fibrillar collagen. PRP was stirred at 37°C and activated with 4.4 $\mu\text{g/mL}$ fibrillar Horm® collagen. 5 μL aliquots were removed at 15s intervals and examined in the platelet PS exposure flow cytometric assay. The percentage of platelets and platelet-derived microparticles positive for Annexin V-FITC from 5 separate experiments is shown (mean \pm SD).

reduction, from 16.1 ± 9.8 % to 8.6 ± 4.5 % ($p=0.011$; $n=7$), in the percentage of platelets positive for PS; this was not seen in the blood treated with the vehicle control (Fig.3.35).

However, in an experimental set-up where platelet-platelet interaction was allowed, there was a stark contrast in the results. The experiment was conducted in a similar fashion to the stirring experiments described previously (§3.2.7.3), except that PRP was preincubated with cytochalasin D for 30 min at 37°C prior to the addition of XL-CRP. Subsamples were transferred to flow cytometry PS exposure assay reaction mixtures 20s after activation. As shown in Fig.3.36, under these conditions, more treated platelets exposed PS than their untreated counterparts (31.6 ± 15.2 vs. 16.2 ± 7.4 %; $p=0.02$; $n=6$), whilst the DMSO vehicle had no significant effect (15.0 ± 6.8 %; $p=0.2416$; $n=6$).

3.2.8. Relationship of procoagulant response to other aspects of platelet activation

Differences in the potencies of various agonists in respect of degranulation and procoagulant response in platelets have led to speculation that degranulation and PS exposure occur via different mechanisms (Dachary-Prigent *et al*, 1996). In this study, this question was addressed more closely through comparing P-selectin and PS exposure following activation through the collagen receptor, GPVI, using XL-CRP.

A dose-response curve (Fig.3.37) demonstrates clearly that stimulation of platelets via GPVI results much more readily in P-selectin expression than PS exposure. Maximum P-selectin expression, measured by flow cytometric assay, was achieved at $1 \mu\text{g/mL}$ XL-CRP, whereas maximal PS exposure required $10 \mu\text{g/mL}$, as has been observed previously (Fig.3.15). It is also notable that whilst at maximal stimulation nearly 100% platelets are positive for P-selectin, only a sub-population of platelets bound Annexin V even at the highest concentrations. Although the percentage of XL-CRP activated platelets positive for PS was increased by the addition of ADP, TRAP or thrombin (Fig.3.26), the percentages positive were still only 37.0 ± 14.6 , 36.6 ± 15.7 and

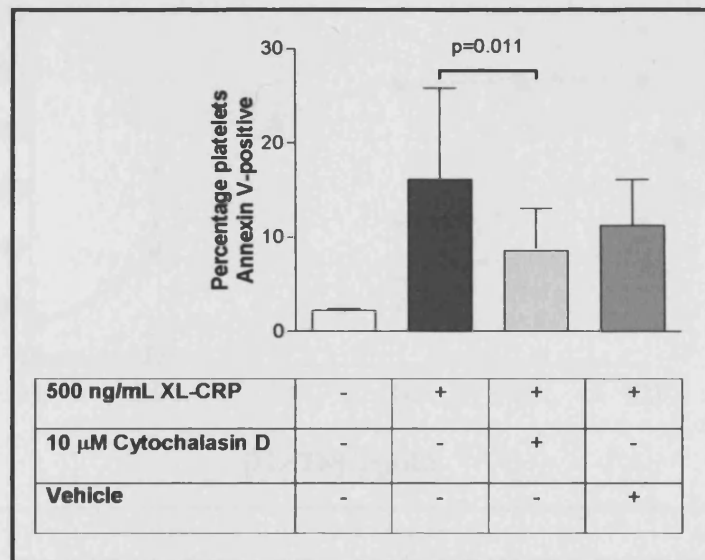


Figure 3.35. Inhibition of actin polymerisation reduces PS exposure in XL-CRP-activated platelets in suspension. Whole blood diluted 1:10 in PS exposure assay reaction mixture were preincubated with 10 μ M cytochalasin D for 30 min prior to the addition of XL-CRP. Annexin V binding was then measured by flow cytometry. Data are from 7 separate experiments using blood from different donors, of which 3 included a vehicle control.

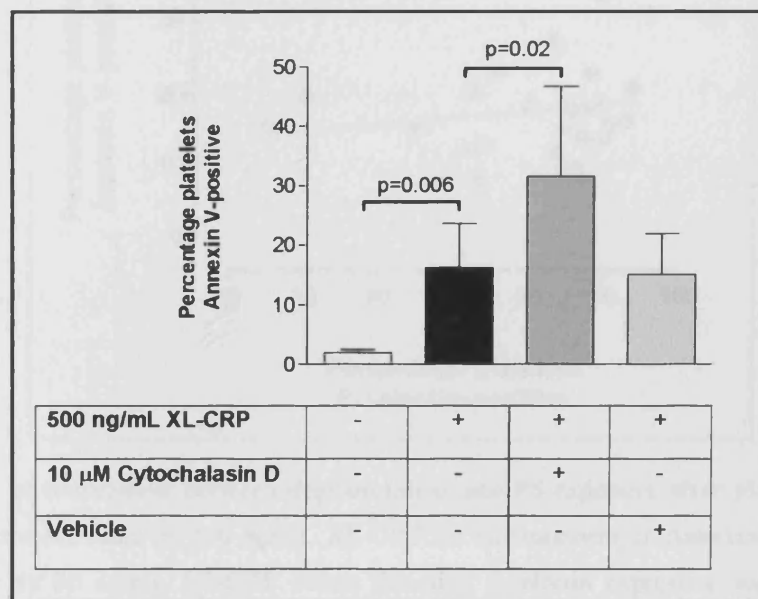


Figure 3.36. Inhibition of actin polymerisation potentiates PS exposure in XL-CRP-activated stirred platelets. PRP was preincubated with 10 μ M Cytochalasin D for 30 min prior to being activated whilst stirring in the PAP-4D light aggregometer. After 20 s, subsamples were transferred to flow cytometry test tubes to be assayed for Annexin V binding. Experiments were performed on blood from 6 separate donors.

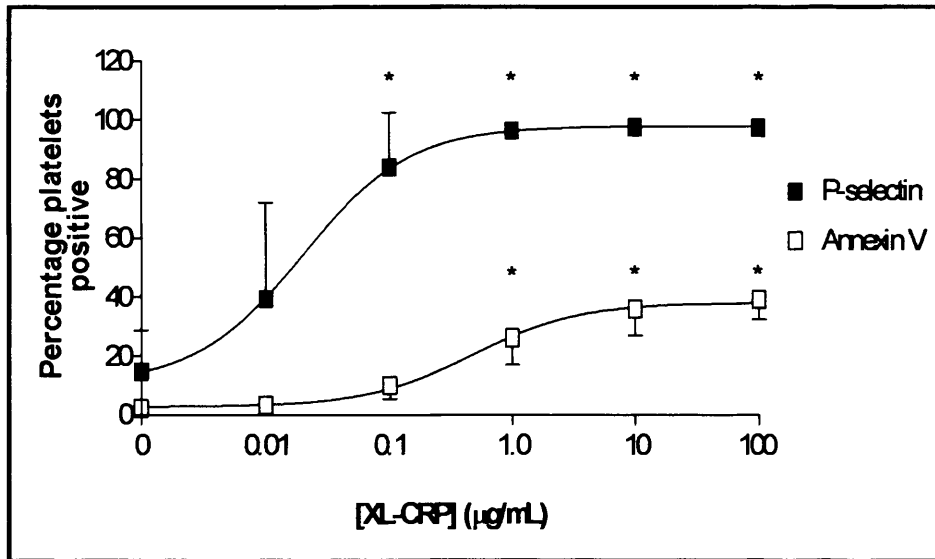


Figure 3.37. Percentage of platelets positive for P-selectin and Annexin V-FITC after stimulation by various concentrations of XL-CRP. P-selectin exposure was measured in a whole blood platelet assay, whilst PS exposure (Annexin V-FITC binding) was measured in the platelet PS exposure assay. * $p < 0.001$ vs. control. Data are from 4 separate experiments using blood from different donors.

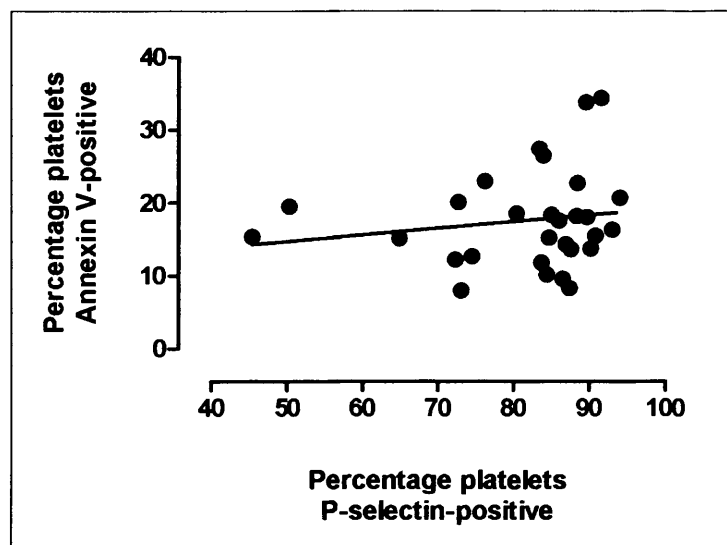


Figure 3.38. Lack of correlation between degranulation and PS exposure after platelet activation via GPVI. Platelets were activated by 500 ng/mL XL-CRP for measurement of Annexin V-FITC binding by flow cytometry or by 30 ng/mL XL-CRP before detecting P-selectin expression using a FITC-labelled monoclonal antibody in a flow cytometric assay. Data are from 29 different donors measured on separate occasions. The Pearson correlation coefficient, r , was 0.1598 and $p = 0.4077$.

41.7±10.2 respectively. On the other hand, more than 95% of platelets activated with high concentrations of the calcium ionophore A23187 were capable of exposing PS and forming microparticles in all the donors examined during this project (Fig.3.14).

Independent pathways are also suggested by the fact that, in a study of 25 normal donors, there was no correlation between the procoagulant and secretion responses to concentrations of XL-CRP expected to produce an intermediate effect (500 ng/mL for PS exposure and 30 ng/mL for degranulation; data for 30 ng/mL XL-CRP was provided by Nashat Qamar, Department of Cardiovascular Sciences, University of Leicester). The Pearson coefficient of correlation, r , was 0.1598 and the corresponding p -value 0.4077 (Fig.3.38). There was similarly no correlation between the P-selectin median fluorescence and the percentage of Annexin V-positive platelets (Pearson coefficient of correlation, r , was 0.0209; $p=0.9144$; data not shown).

The effect of ADP released from dense granules affected PS exposure, fibrinogen binding and α -granule release differently. Its potentiating effect on PS exposure, mediated through both P2Y₁ and especially P2Y₁₂, has been demonstrated in Fig.3.27. Inhibition of P2Y₁₂ with AR-C69931MX also resulted in reduced fibrinogen binding (median fluorescence 66.1±31.1 vs. 104.9±20.0 AU; $p=0.0224$; $n=4$), as determined by flow cytometric measurement of the fluorescence of anti-fibrinogen antibody to XL-CRP activated platelets (Fig.3.39). Inhibition of P2Y₁ by MRS 2179 had no effect on fibrinogen binding (101.5±22.0 vs. 104.9±20.0 AU; $p=0.1645$). In combination with AR-C69931MX, fibrinogen binding shown by the median fluorescence of the anti-fibrinogen antibody was still lower (57.4±32.2 AU; $p=0.0169$) (Fig.3.39). In contrast, there was no positive feedback detected through either receptor from ADP release on the P-selectin translocation in response to XL-CRP (Fig.3.40).

Since the above data reinforced the hypothesis that degranulation and procoagulant responses are effected through different signalling pathways, it was decided to investigate the role of phosphatidylinositol 3-kinase (PI3-K) in the two responses. PI3-K occupies an important early

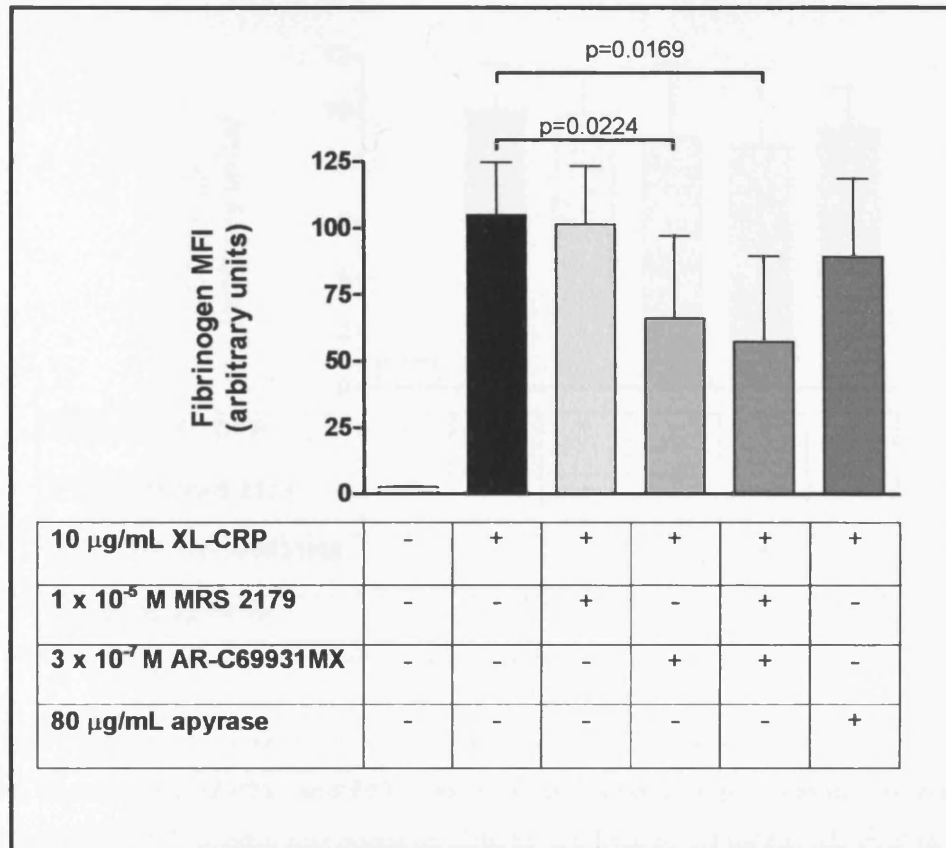


Figure 3.39. Contribution of P2Y₁ and P2Y₁₂ receptors to fibrinogen binding in response to platelet activation by XL-CRP. Fibrinogen binding to platelets activated by 10 µg/mL XL-CRP in the presence and absence of ADP receptor inhibitors was measured by flow cytometry using a FITC-conjugated polyclonal antibody raised against fibrinogen. Data are expressed as mean median fluorescence giving a relative measure of bound fibrinogen (n=4-5).

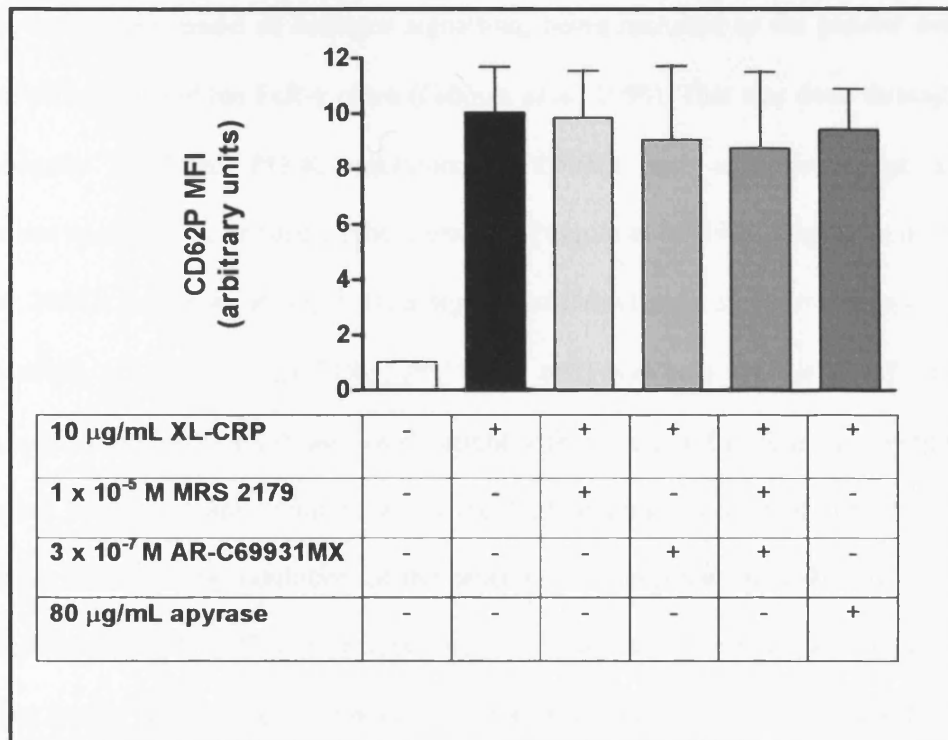


Figure 3.40. Contribution of P2Y₁ and P2Y₁₂ receptors to P-selectin expression in response to platelet activation by XL-CRP. P-selectin expression on platelets activated by 10 µg/mL XL-CRP in the presence and absence of ADP receptor inhibitors was measured by flow cytometry using a FITC-conjugated monoclonal antibody raised against P-selectin. Data are expressed as mean median fluorescence giving a relative measure of degranulation (n=4-5).

position in the current model of collagen signalling, being recruited to the platelet membrane by association with LAT and the FcR- γ chain (Gibbins *et al*, 1998). This was done through the use of two structurally unrelated PI3-K inhibitors, LY294002 and wortmannin, at a range of concentrations spanning those used in the literature (Pasquet *et al*, 1999; Lagrue *et al*, 1999; Barry & Gibbins, 2002). As shown in Fig.3.41, a significant reduction in the percentage of Annexin V-positive platelets (11.0 ± 6.5 vs. 26.3 ± 9.1 ; $p=0.0075$; $n=3$) was only seen at 1 μ M wortmannin, a very high concentration of inhibitor which might affect platelet function in non-specific ways. When diluted 1:40, the same dilution as in the highest concentration of inhibitor, the DMSO vehicle, rather than causing inhibition of the procoagulant response, actually increased it slightly but significantly ($p=0.047$). This is unsurprising given the known stimulatory effect of DMSO on platelets, but it may have caused an underestimation of the inhibitory effect of the LY294002 and wortmannin. DMSO diluted 1:400 did not affect PS exposure. The percentage of platelets positive for P-selectin was not reduced by inhibition of PI3-K (data not shown). However, median fluorescence was lower, though not abolished, at both the higher concentrations of both inhibitors (6.6 ± 3.3 and 4.9 ± 2.3 vs. 9.2 ± 2.2 AU, $p=0.0436$ and 0.0272 , for 25 and 250 μ M LY294002 respectively; and 6.0 ± 2.9 and 2.6 ± 1.1 vs. 9.2 ± 2.2 AU $p=0.0327$ and 0.0017 for 100 nM and 1 μ M wortmannin respectively; $n=4$) (Fig.3.42), albeit with the same caveat as above. Taken together, the data suggest that PI3-K plays a relatively minor role in *both* the procoagulant and degranulation responses.

There were also differences in the role of actin polymerisation in degranulation compared with PS 'flip'. In cells in suspension, cytochalasin D inhibited PS exposure (Fig.3.35), but in a similar experimental set-up, P-selectin expression was enhanced by inhibition of cytoskeletal rearrangement when platelets were activated by a sub-maximal concentration of 10 ng/mL XL-CRP (Fig.3.43). The percentage of degranulated platelets was significantly increased from $39.0 \pm 20.2\%$ to $62.7 \pm 17.5\%$ ($p=0.0043$; $n=7$) and the median fluorescence was also greater on the treated platelets

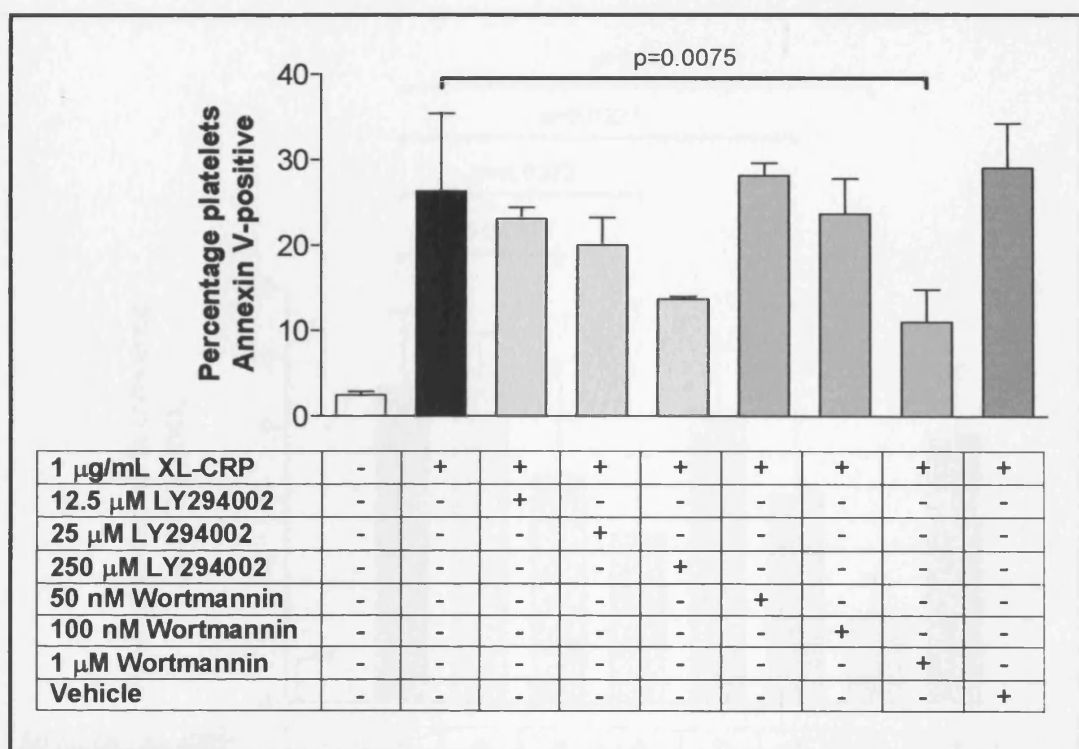


Figure 3.41. PS exposure in XL-CRP activated platelets is mediated through PI3-K. Whole blood was incubated with inhibitor or vehicle for 15 min at 37°C prior to activation of platelets by 1 µg/mL XL-CRP. Subsamples were then analysed for PS exposure by flow cytometry using Annexin V-FITC. Experiments were performed on blood from 4 individual donors.

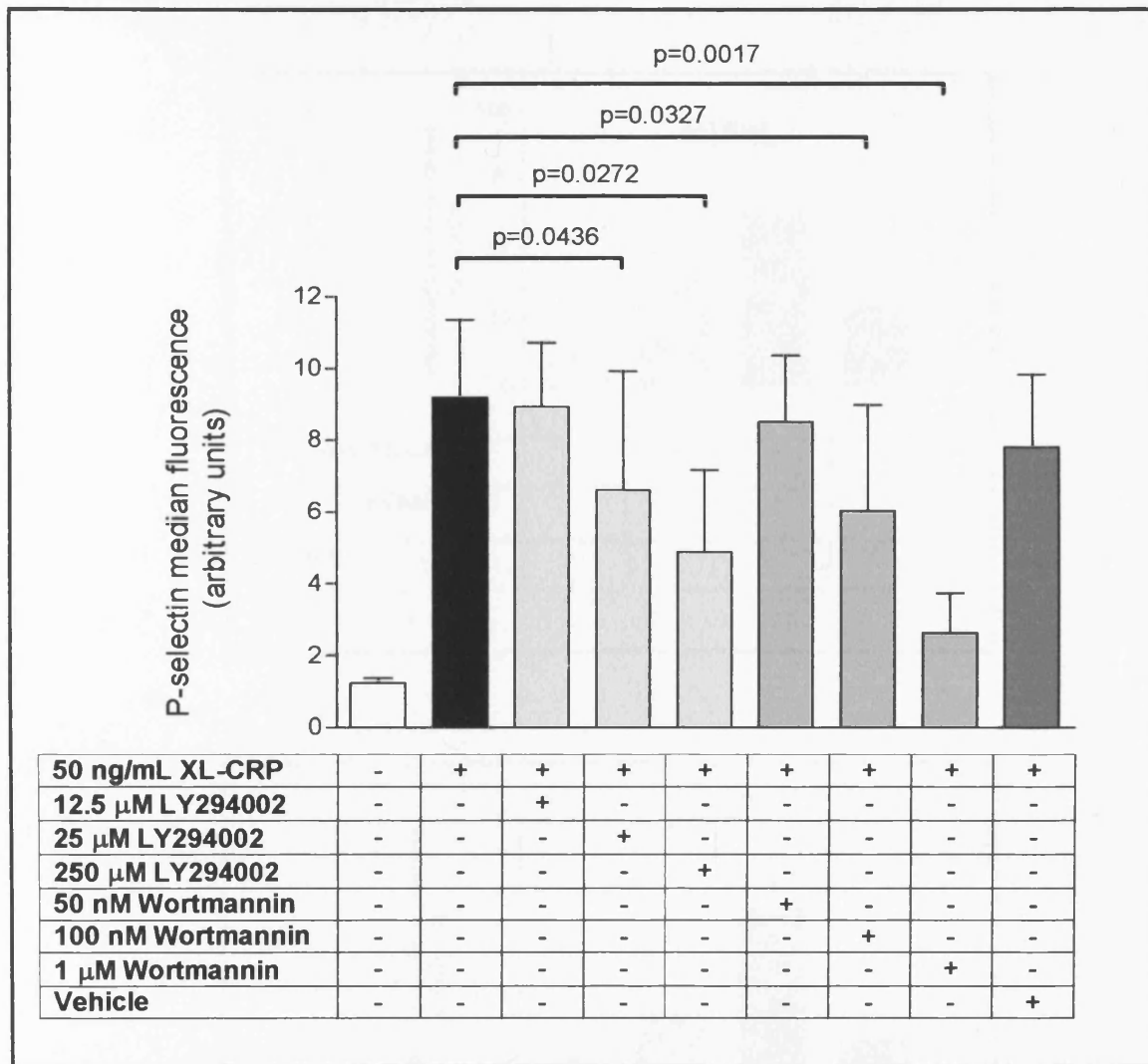
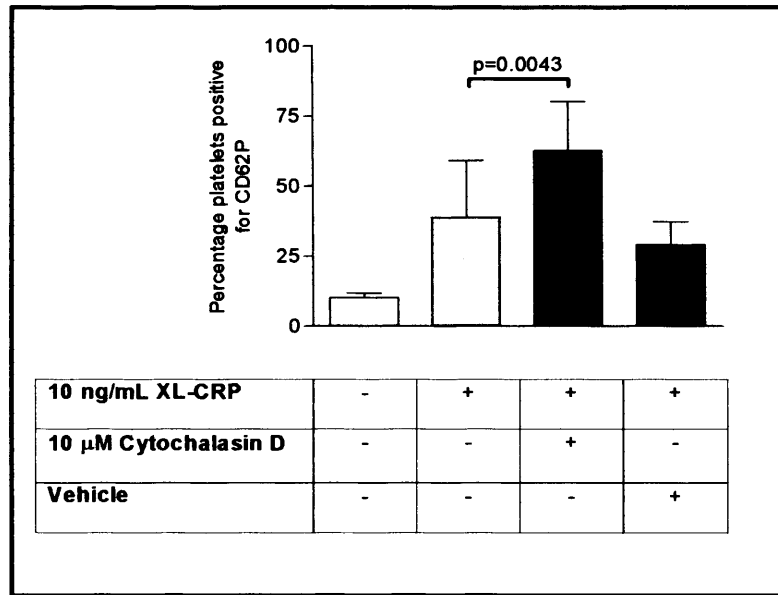


Figure 3.42. P-selectin expression is reduced by PI3-K inhibition. Whole blood was incubated with inhibitor or vehicle for 15 min at 37°C prior to activation of platelets by 50 ng/mL XL-CRP. Subsamples were then analysed for P-selectin expression by flow cytometry. Experiments were performed on blood from 4 individual donors.

(a)



(b)

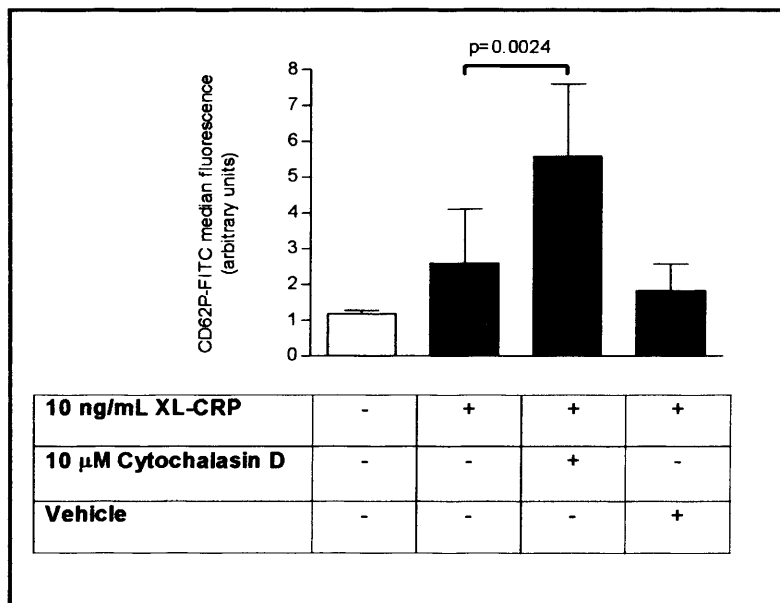


Figure 3.43. Inhibition of actin polymerisation potentiates P-selectin expression in XL-CRP activated platelets. Platelets in whole blood were preincubated with 10 μM cytochalasin D or vehicle for 30 min prior to activation with 10 ng/mL XL-CRP. Platelets were assayed for P-selectin expression by flow cytometry (a) percent positive and (b) median fluorescence (n=7).

(2.6 ± 1.5 versus 5.6 ± 2.0 ; $p=0.0024$; $n=7$). There was no effect in the vehicle control ($p=0.2237$).

This enhancement was not apparent at higher concentrations of agonist (data not shown).

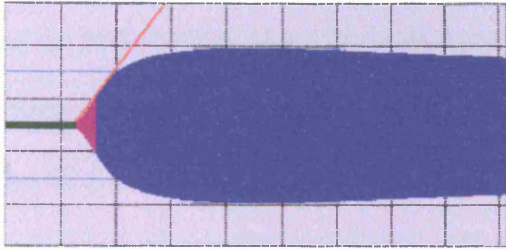
3.2.9. Role of PS exposure in coagulation as demonstrated by TEG

The variability in platelet procoagulant response to collagen has been highlighted previously (§3.2.5). To establish whether this variability in PS exposure has an impact on clot formation, PS exposure was measured by flow cytometry in samples whose clot formation characteristics were monitored by TEG. TEG measures clotting time and the kinetics of clot formation and dissolution by recording the degree of resistance to a rotating pin immersed in the growing or lysing clot. Aliquots of recalcified citrated whole blood with a low concentration of TF (12.2 pg/mL), with or without agonist, were removed from samples immediately prior to being read on the RoTEM® as described in methods (§2.2.6). These subaliquots were analysed for PS and P-selectin expression by flow cytometry and the data compared with the RoTEM®05 Whole Blood Haemostasis Analyser-generated parameters.

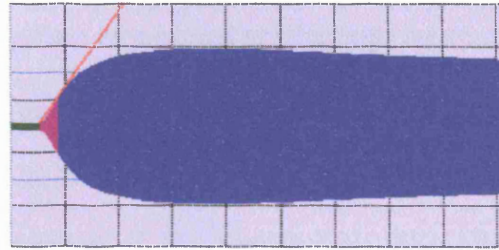
Clotting times were shortened by platelet activation, shown by the shortening of the horizontal green line in the middle of the RoTEM® traces (the dotted vertical lines indicate 10 min intervals). The reduction in clotting time was significant where platelets had been activated by 6×10^{-5} M TRAP and concentrations of XL-CRP of 5 µg/mL and above. Clotting time was reduced from 593.1 ± 149.8 s to 337.7 ± 34.4 s for 6×10^{-5} M TRAP ($p=0.0300$) or 283.5 ± 40.8 s ($p=0.0254$) and 263.0 ± 46.9 s for 5 µg/mL and 50 µg/mL XL-CRP ($p=0.0012$) respectively (Fig.3.44). The clotting time in blood activated by 50 µg/mL XL-CRP was almost significantly shorter than that in the high concentration (6×10^{-5} M) TRAP-activated blood ($p=0.0576$). The percentage of Annexin V-positive platelets with each agonist concentration was in line with previous results: a concentration-dependent increase in the case of XL-CRP and a much lower increase (albeit a significant one at 3×10^{-5} M, probably due to cell-cell interaction in the relatively undiluted blood)

(a)

i.



ii.



(b)

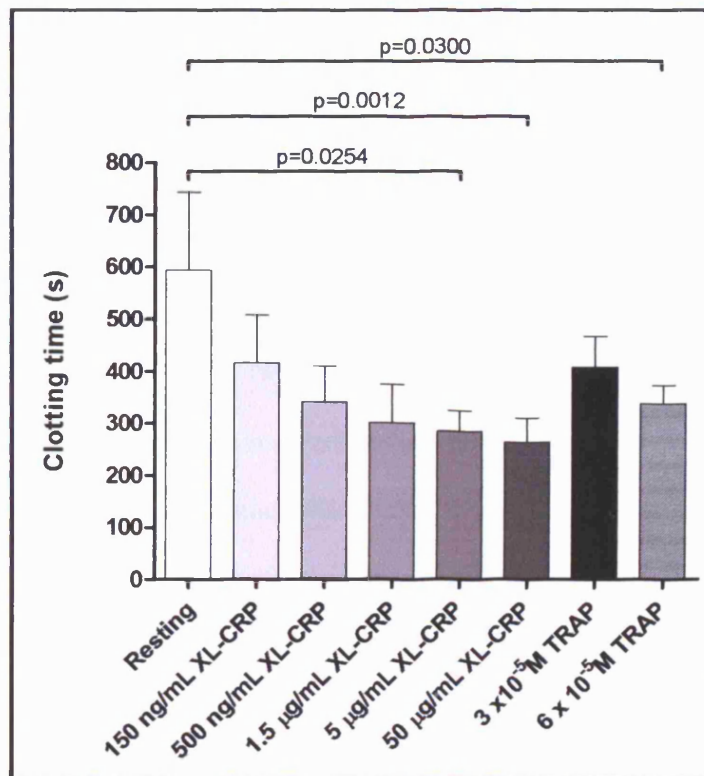


Figure 3.44. Clotting times are shortened by platelet activation. (a) Typical RoTEM® traces (green line is clotting time) for (i) unstimulated blood and (ii) blood activated by 5 µg/mL XL-CRP in the presence of 12.2 pg/mL rTF. (b) Platelets were activated in the presence of 12.2 pg/mL rTF by various concentrations of XL-CRP and TRAP as shown and clotting times measured by thromboelastography in the RoTEM® Whole Blood Haemostasis Analyser. Experiments were performed on blood from 3-9 separate donors.

with TRAP (Fig.3.45). The percentage of PS-positive platelets appears slightly lower than expected in blood activated with 5 $\mu\text{g/mL}$ XL-CRP because, due to the limited number of channels available on the RoTEM®05, blood from the same donors could not be analysed at all agonist concentrations. Plotting the clotting time against the percentage of procoagulant platelets (Fig.3.46) shows that as the level of PS+ platelets moves from 0 to about 8 %, there is a significant effect on clotting time (red line). Above this, there was only a slight shortening of the clotting time (blue line). This indicates that clotting is dependent on some PS being available, but it does not require all platelets to be procoagulant.

The percentage of P-selectin positive platelets differed little with agonist concentrations, being >90% except for the 150 ng/mL XL-CRP sample (Fig.3.47a), but agonists varied in the extent of the degranulation they caused (Fig.3.47b). The scatter plot of clotting time against P-selectin median fluorescence (Fig.3.48) indicates a 2-phase response and suggests that P-selectin, or at least degranulation, does play a part in early clot formation (red line) but beyond a certain low level, its effect is rather small (blue line). It is possible that this reflects the relationship between PS exposure and P-selectin expression rather than being a result of degranulation, although some releasates, such as FV, might well influence the speed of clotting in addition to the lipid environment in the outer platelet membrane. This relationship, covering only activated samples, is plotted in Fig.3.49. As demonstrated previously, this shows a very weak relationship between the extent of degranulation (P-selectin fluorescence) and the percentage of PS-positive platelets.

Not only the clotting times were shortened by the presence of activated platelets; the clot formation time (time taken to achieve an arbitrarily specified clot firmness of 20 mm) was also reduced significantly from 181.5 ± 52.8 s to 136.5 ± 21.1 s and 106.0 ± 16.1 s in the presence of 5 $\mu\text{g/mL}$ XL-CRP ($p=0.0216$) and 6×10^{-5} M TRAP ($p=0.0211$) respectively. There was a clear trend in the case of the lower agonist concentrations (Fig.3.50). There was little discernible pattern in the effect of activation on maximum clot firmness, although maximum amplitude was significantly

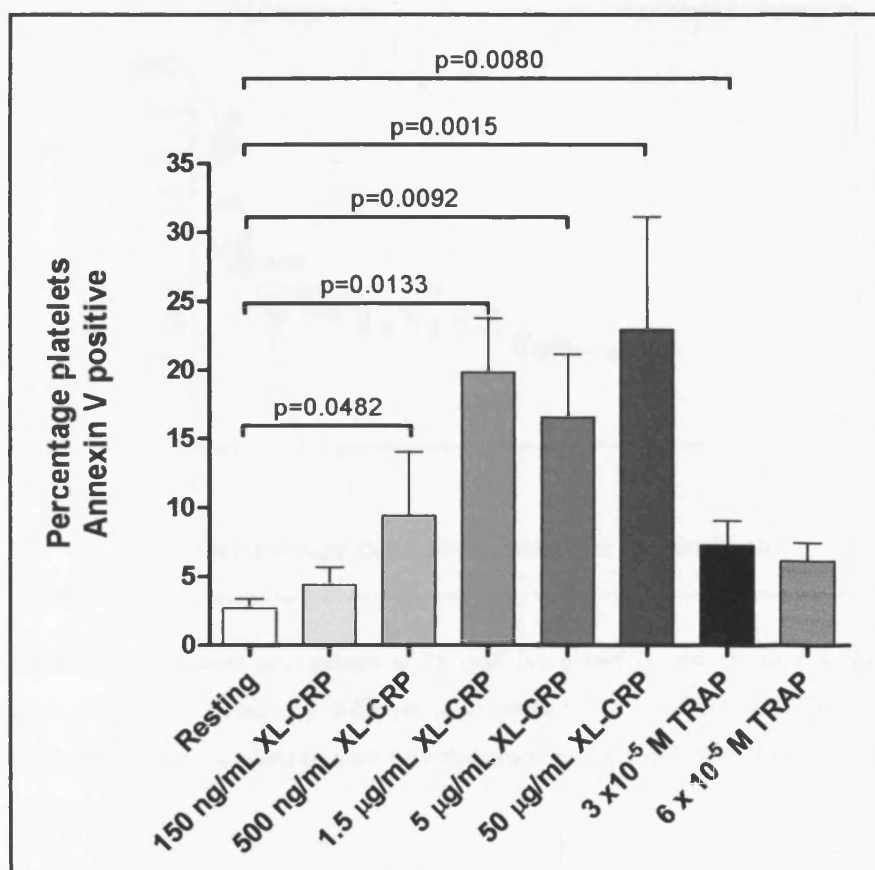


Figure 3.45. PS exposure in samples subsampled prior to clotting analysis in the RoTEM® Whole Blood Haemostasis Analyser. Recalcified citrated whole blood with 12.2 pg/mL TF with agonist as indicated was subsampled immediately prior to positioning the RoTEM® rotating cup onto the pin. Subsamples were analysed for PS exposure by flow cytometry using Annexin V-FITC. Experiments were performed on blood from 3-9 separate donors.

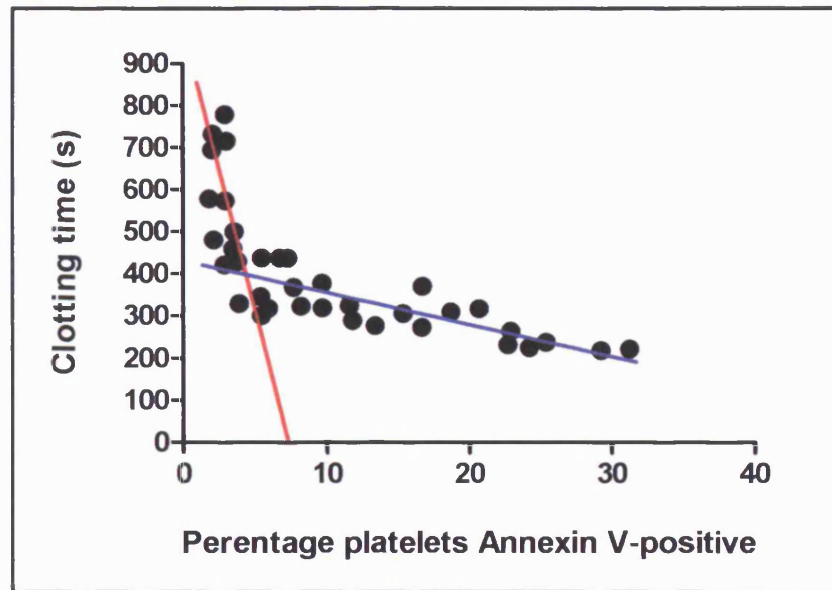


Figure 3.46. Relationship between percentage of PS-positive platelets and clotting times. Percentage of Annexin V-positive platelets, activated by different concentrations of XL-CRP or TRAP (see Fig.3.44), as shown by flow cytometry, and the clotting time as determined in the RoTEM® Whole Blood Haemostasis Analyser (3-9 donors; n=36).

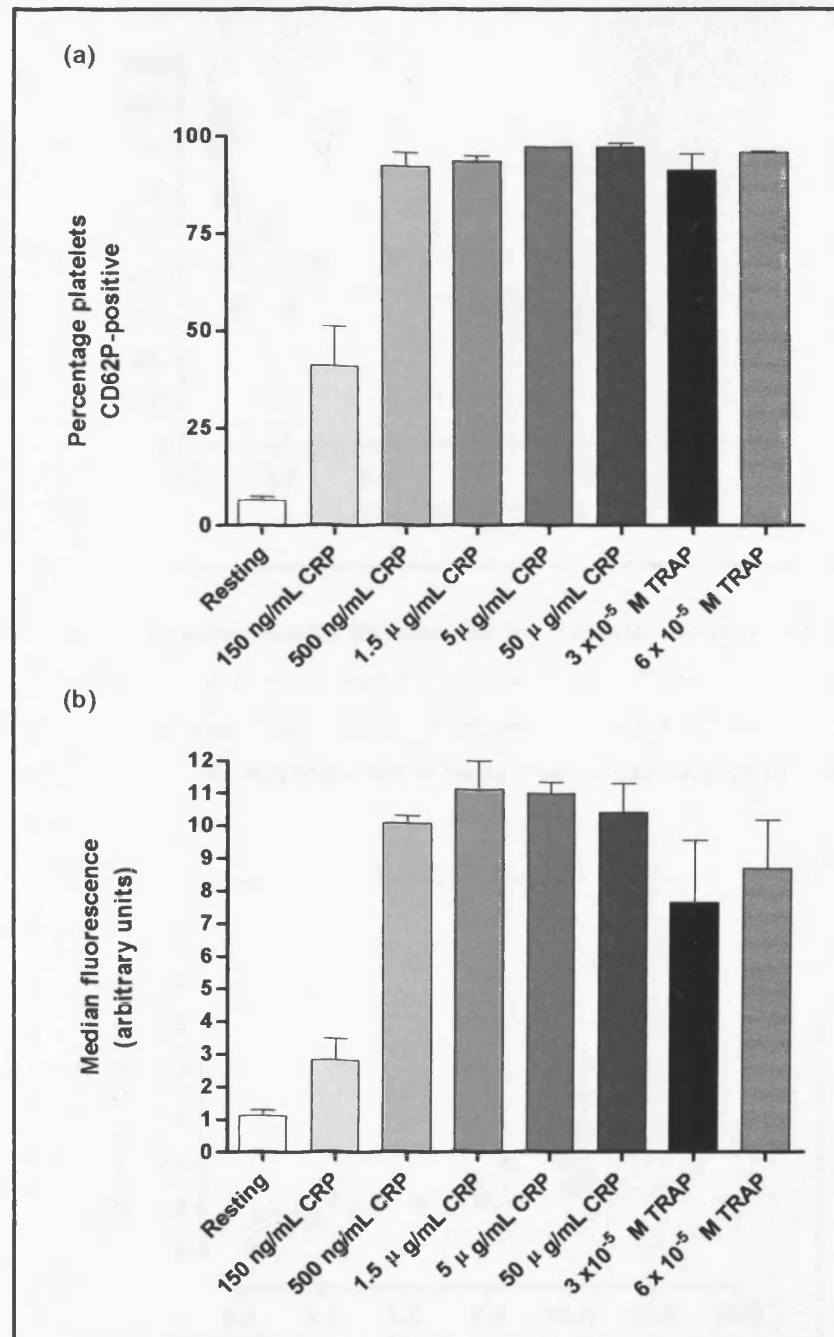


Figure 3.47. Percentage platelets positive for P-selectin (a) and median fluorescence intensity (b) in samples subsampled prior to clotting analysis in the RoTEM® Whole Blood Haemostasis Analyser. Recalcified citrated whole blood with 12.2 pg/mL TF with agonist as indicated was subsampled immediately prior to positioning the RoTEM® rotating cup onto the pin. Subsamples were analysed for P-selectin expression by flow cytometry. Experiments were performed on blood from 3-9 separate donors.

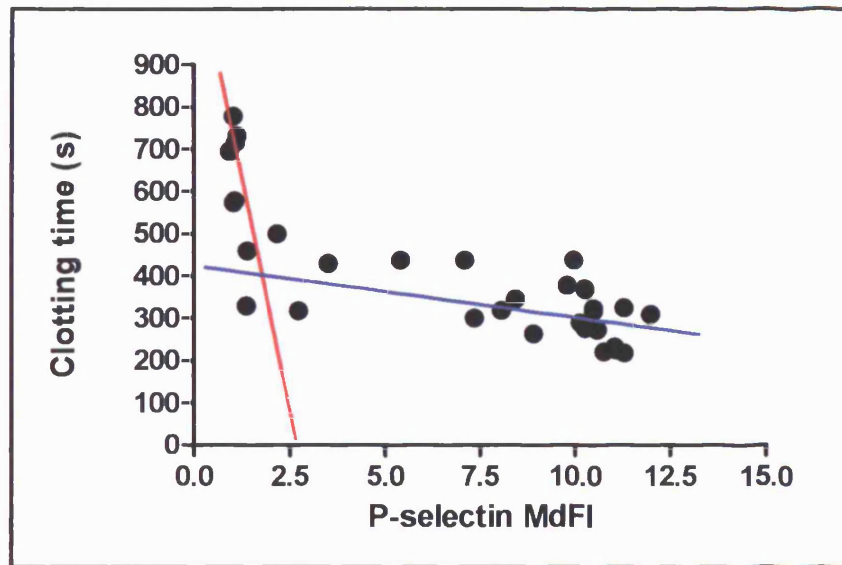


Figure 3.48. Relationship between median fluorescence on P-selectin positive platelets and clotting times. Amount of P-selectin expression as indicated by the median fluorescence intensity on platelets activated by different concentrations of XL-CRP or TRAP (see Fig.3.46), as shown by flow cytometry, and the clotting time as determined in the RoTEM® Whole Blood Haemostasis Analyser (3–9 donors; n=36).

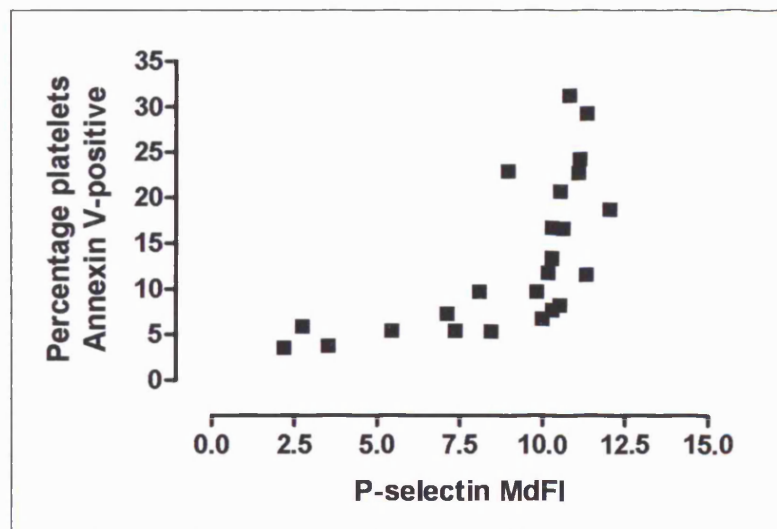


Figure 3.49. Relationship between platelet P-selectin expression and PS exposure. P-selectin median fluorescence and PS exposure were measured by flow cytometry in subsamples taken from recalcified citrated whole blood with 12.2 pg/mL TF with agonist as indicated in Figs.3.45 and 3.47 immediately prior to positioning the RoTEM® rotating cup onto the pin (3–9 donors; n=36).

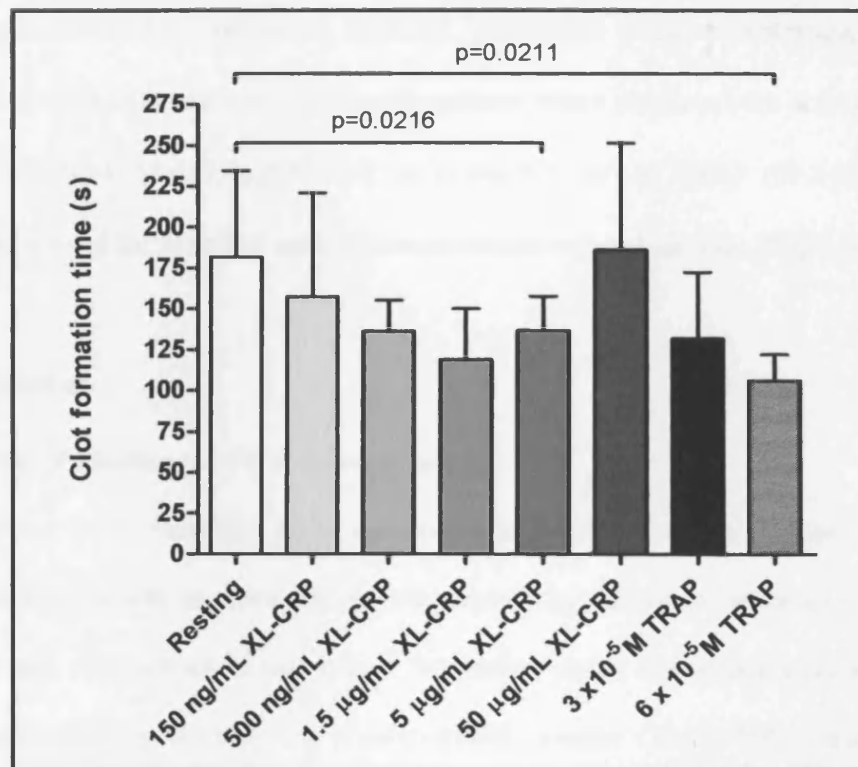


Figure 3.50. Effect of platelet activation on clot formation time. RoTEM® Whole Blood Haemostasis Analyser generated clot formation time for recalcified citrated blood samples with 12.2 pg/mL TF activated by different concentrations of XL-CRP and TRAP. Data are from 3-9 separate donors.

greater in blood activated by 500 ng/mL XL-CRP, but not any other concentration, and 6×10^{-5} M TRAP. The clot formation rate was significantly greater where platelets were activated by 5 μ g/mL XL-CRP (63.8 ± 3.0 vs. 57.6 ± 7.8 ; $p=0.0162$; $n=3$) and 6×10^{-5} M TRAP (69.2 ± 3.0 vs. 57.6 ± 7.8 ; $p=0.0234$), but a trend for the other agonist concentrations was not obvious (Fig.3.51).

3.3. Discussion

3.3.1. Annexin V binding (or PS Exposure) assay

The whole blood flow cytometric assay developed to detect PS on the surface of platelets and platelet-derived microparticles represents an extension of the method developed by Abrams and co-workers (Abrams *et al*, 1990) in that it is a two colour assay identifying platelets and platelet-derived microparticles by the use of a platelet-specific marker CD42b-RPE simultaneously with Annexin V-FITC. The focus of the assay is on the procoagulant nature, in terms of PS exposure as determined by Annexin V binding, of the cells with their size being a secondary consideration. The general approach could be extended by using the appropriate forward/side scatter characteristics and specific cell-surface marker to look at PS on the surface of cell types other than platelets. The method involves minimal handling of blood and, with the proviso discussed below, includes analysis of all cells present in the sample. Many alternative methods, whether looking at platelets or exclusively at microparticles, use centrifugation for their isolation and this may introduce artefact, possibly artificially generating microparticles. When preparing PRP, platelets may be activated by centrifugation. Additionally, even if they are not activated, a considerable number are precipitated along with red cells and leukocytes and it is possible that a particular population of platelets of larger or denser platelets might be excluded from PRP and hence not analysed. The assay gave satisfactory CVs and, importantly, sufficient inter-assay reproducibility for the consideration of variability in donor response. As a tool for examining some of the underlying mechanisms of PS exposure, the assay gave credible results.

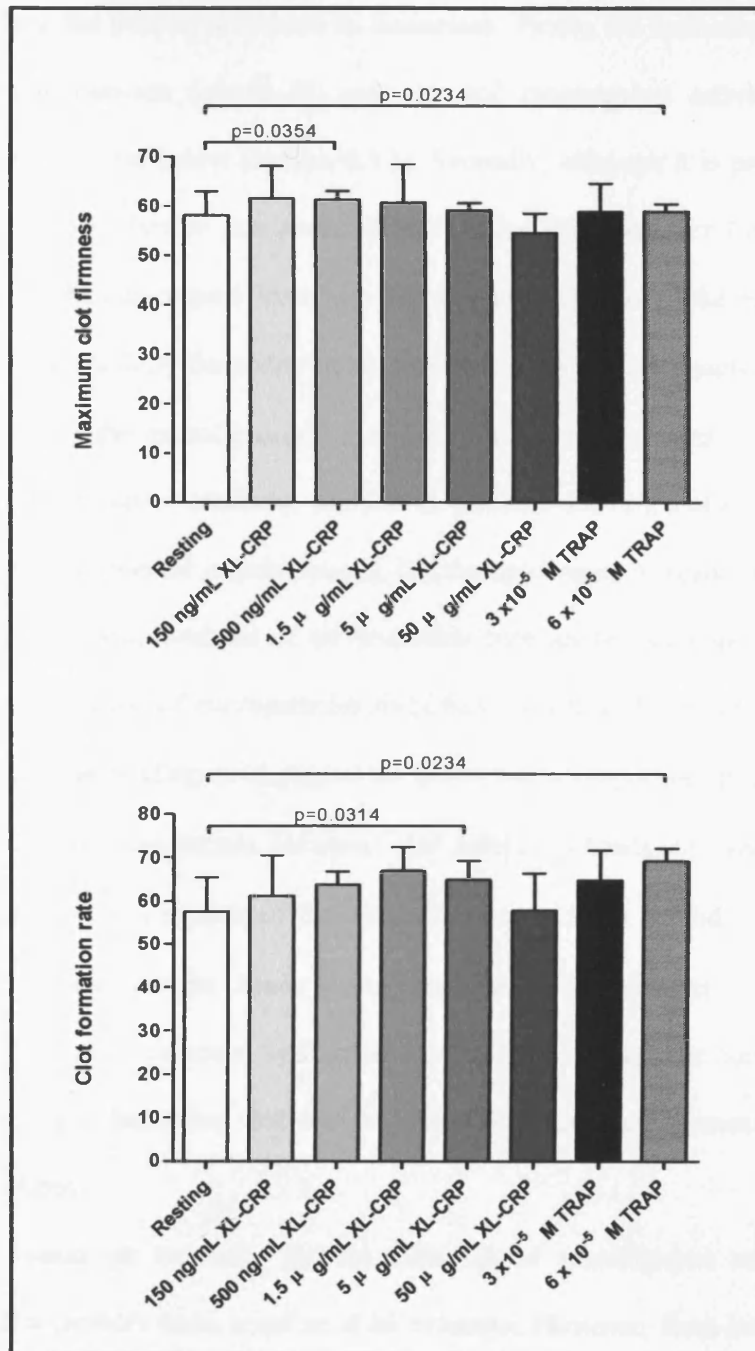


Figure 3.51. Effect of platelet activation on maximum clot firmness and clot formation rate. Maximum clot firmness and clot formation rates were measured by thromboelastography in the RoTEM® Whole Blood Haemostasis Analyser in recalcified citrated blood samples with 12.2 pg/mL TF activated by various concentrations of XL-CRP and TRAP (3-9 donors). * $p < 0.05$ versus resting control.

Nevertheless, the method does have its limitations. Firstly, the method assumes that there is a direct relationship between anionic PL exposure and procoagulant activity. Whether this is justifiable will be discussed below (section 3.3.7). Secondly, although it is probable that the vast majority of PS-positive platelets are analysed, it is clear that there are limits on the reliable detection of procoagulant microparticles which may in fact carry most of the procoagulant activity. The limits are imposed both by the ability of the flow cytometer to detect particles below 0.5 μm in diameter (according to the manufacturer's specification) and by the need to exclude noise. The application of a discriminator, confining analysis to particles above a specified threshold (in this case, size), is seldom mentioned in publications, but the appearance of scatter plots often indicates its existence. Even allowing analysis of all detectable particles by not imposing a discriminator implies missing a proportion of microparticles since they are generally regarded as falling into the 0.1 μm – 1 μm size range (Dignat-George *et al*, 2004) and a proportion of these would thus be undetectable by most flow cytometers. However, size calibration beads indicated that the cytometer used in the present study was capable of detecting sub-1 μm particles but did not show the absolute limit of detection since smaller beads were unavailable. Experiments with and without a discriminator and triggering detection by fluorescence failed to increase the number of Annexin V-positive events detected suggesting that the analysis used in the assay presented here is optimal within these limitations.

For this reason, as an assay for the detection of procoagulant *microparticles*, flow cytometry, though commonly used, is not an ideal technique. However, there are also drawbacks to alternative methods. This is reflected in the fact that there is as yet no standard protocol and the definition of microparticle is itself open to debate, some defining by reference to the unactivated platelet population (Storey *et al*, 2000) or A23187-activated platelets (Dachary-Prigent *et al*, 1993) and some by reference to both a specific size and PS exposure (Shet *et al*, 2004). For this reason, a working party to study the issue has recently been set up by the International Society for

Thrombosis and Haemostasis, who have published a series of papers discussing the relative merits of various methods of detecting microparticles (Jy *et al*, 2004). These methods vary not only in sample preparation but also in capture and the end point measured. For example, some assays use immobilised Annexin V to capture procoagulant microparticles in plasma, with anionic PL content being estimated via a prothrombinase assay, thus emphasizing activity (Hugel *et al*, 2004), but possibly underestimating the microparticle content of the sample since some may escape capture, due perhaps to interference by soluble antigen, and hence not be measured. Another approach is to use an ELISA technique, capturing platelet-derived MPs with an anti-GPIX Mab and detecting with a biotinylated anti-GPIb Mab, which might overcome the limitation in the detection of the smallest particles but could be confounded by the presence of soluble glycoproteins such as GPIb (Nomura, 2004).

In contrast with other published reports (Berckmans *et al*, 2001), the present study was unable to detect the presence of appreciable levels of platelet-derived microparticles in the peripheral blood of normal subjects. In addition, despite reports in the literature of raised numbers of microparticles in patients undergoing surgery (Nieuwland *et al*, 1997), data from this laboratory using the described assay found only insignificant levels in the blood of patients immediately following carotid endarterectomy (Payne *et al*, personal communication). There are several possible explanations for these discrepancies. There are technical differences in the preparation of samples, although in both the papers cited, microparticles were detected by flow cytometry. In the published studies, microparticles were not investigated in whole blood as described here, but were isolated from plasma by centrifugation. In addition to the possibility of forming microparticles through shear, their numbers were also concentrated by being resuspended in a volume of buffer smaller than the original. As regards the surgery patients, blood samples were obtained directly from the pericardial cavity in the Nieuwland study, whereas for this project peripheral blood was drawn from the carotid endarterectomy patients from the arterial line (Payne *et al*, 2004). It might be expected

that the number of microparticles might be lower in blood collected from a location distant from the site of trauma since they would be cleared from the circulation by adhering to other cells. This has in fact been demonstrated elsewhere, albeit by an ELISA method of measuring microparticles (Osumi *et al*, 2001). In addition, microparticles may be phagocytosed, since PS is apparently a signal for clearance (Fadok *et al*, 1992).

3.3.2. *Ex vivo* data

The levels of microparticles or procoagulant platelets found in the unactivated blood of healthy subjects were extremely low and no difference between males and females was observed. Low numbers (a mean of $237 \times 10^6/L$ – that is, approximately 1% of the average platelet count) platelet-derived microparticles have been reported in healthy donors previously (Berckmans *et al*, 2001). The technical issues relating to the measurement of circulating microparticles have already been discussed (§3.3.1). In the paper cited, it was suggested that these microparticles support low levels of thrombin generation, which, far from being procoagulant, may be anticoagulant, by activating Protein C, though this has not been demonstrated.

The increasing percentage of procoagulant platelets in expired platelets is not a new finding. The rising trend suggests that PS exposure is already higher before expiry than the basal levels found in normal healthy donors, and this was found in a study of Annexin V binding to platelets from day 0 to day 6 of storage (Metcalf *et al*, 1997; Shapira *et al*, 2000). The presence of microparticles in platelet concentrates has also been reported (Divers *et al*, 1995), but PS exposure on these was not examined. The data in this project suggest that the size of the Annexin V-positive particles started within the normal range of resting platelets and declined only marginally over the time covered and that the procoagulant events were therefore more likely to be intact platelets rather than microparticles. This demonstrates that PS exposure and vesiculation are not necessarily inextricably linked. However, the complete absence of procoagulant microparticles, which was not

seen in agonist-activated platelets, implies that the mechanisms leading to PS exposure might be different. One possibility in the case of expired platelets is that it is due to exhaustion of ATP, required for translocase activity. Whether higher numbers of PS-positive platelets in transfused products is of any clinical significance, either because of increased clearance or increased procoagulant activity, remains to be determined.

3.3.3. Variability in donor procoagulant response to collagen via GPVI

The platelet procoagulant response to XL-CRP showed considerable variability – approximately 8-fold at an intermediate concentration of 500 ng/mL. This variability was maintained over the minimum to maximum concentration range indicating that sensitivity is linked to response, so that more sensitive platelets appear to be also high responders and vice versa. An early paper reporting such variation in XL-CRP induced prothrombinase activity related this to platelet GPVI content (Furihata *et al*, 2001). However, GPVI content was measured in lysed platelets and may therefore have been taking into account internally located receptor. It has recently been reported (Suzuki *et al*, 2003) that approximately one third of the total GPVI content is located in the α -granules and open canicular system, from where it is externalised upon activation. Thus surface expression is not constant and may be underestimated if detection relies on antibody binding to inaccessible receptors on unstimulated platelets. However, there is no indication of whether internal stores of GPVI can play a role in prothrombinase activity. In a paper published in collaboration with this laboratory, it was suggested that variation in several aspects of platelet response to XL-CRP was linked to possession of a particular allele in GPVI, the lower frequency allele being associated with lower response (Joutsu-Korhonen *et al*, 2003). Function was found to be linked to surface expression of GPVI, but it seems improbable that receptor expression is responsible for all the variability seen here since, even allowing for some undetected receptor in the open canicular system and α -granules when measured on unstimulated platelets, the variation in response is much greater than in reported

expression (Chen *et al*, 2002; Joutsu-Korhonen *et al*, 2003; Best *et al*, 2003). Since the frequency of homozygotes for the 'b' allele in 1153 volunteers was 2%, it is unlikely that this accounts for all the variability seen in the 30 donors in this study.

3.3.4. Characterisation of platelet-derived microparticles

It is well known that rearrangement of PLs in the platelet membrane is linked to microvesiculation (Sims *et al*, 1989; Chang *et al*, 1993) and the two processes are sometimes regarded as being synonymous. However, it has been demonstrated that, although closely related, the two are in fact independent events (Dachary-Prigent *et al*, 1995) although in that study synthetic agents were used to generate the effect. A rare bleeding disorder, Castaman defect, where PS exposure is normal but microparticle formation is impaired, further illustrates their independence (Castaman *et al*, 1997). The scatterplots presented in the present study demonstrate clearly that using XL-CRP, a more physiological agonist than A23187, PS was exposed on the surface of platelets which were within the same size range as their unstimulated counterparts. It cannot, however, be excluded that these are the remnants of larger platelets from which microvesicles have been shed or platelets yet to vesiculate, that is, distinguished by time not pathway. It has been suggested that calpain plays a role in microvesiculation (Fox *et al*, 1991). In a study on platelets adherent to immobilised fibrinogen, microvesiculation could be prevented by the inhibition of calpain-mediated disruption of the cytoskeleton (Briedé *et al*, 1999). This suggests that calpain activation does not occur in all XL-CRP-activated platelets which expose PS and that these do not shed microparticles. Furthermore, the fact that inhibition of the P2Y₁₂ receptor particularly reduced microparticle formation suggests that ADP reinforcement of the original stimulus via this receptor has an important role in microvesiculation.

Whilst largely confirming what is already known about microvesiculation (Pasquet *et al*, 1996), the data presented here illustrate clearly the requirement for extracellular calcium. The

scatterplots in Fig.3.13 also demonstrate the heterogeneity of response within the platelet population to the same agonist under the same conditions. This variability is not just in terms of whether or not a platelet undergoes PS 'flip', but also in terms of the extent of PS exposure. As the concentration of A23187 increases, the various populations of differently sized and procoagulant platelets/microparticles merge to form one highly PS-positive population of microvesicles. This is also apparent from the tight error bars in Fig.3.14 for high concentrations of A23187. When activated by a more physiologically relevant agonist, XL-CRP, a similar pattern of differing levels of PS exposure obtains, with distinct populations of highly and less procoagulant microparticles as well as procoagulant platelets. The possible existence of two populations of PS-positive platelets, though activated not by collagen alone but by collagen-and-thrombin, has been postulated previously (Dachary-Prigent *et al*, 1993), but not demonstrated as clearly as here. The authors in the work cited did not observe this phenomenon when A23187 was the agonist, but this is may be due to the concentration used. It is not clear what distinguishes the different populations but this is discussed further in sections 3.3.5.1 and 3.3.6.

Studies on antigen expression have previously shown that GPIb is expressed on the microparticle surface and indeed this marker has been used for the flow cytometric detection of microparticles for many years (Sims *et al*, 1988). This finding has been confirmed in the present project and extended with the observation that microparticles appear to be more strongly positive for CD42b and conversely, that it is the most highly CD42b-positive platelets which are Annexin V-positive following activation by XL-CRP. The reason for this is unclear. Of the other antigens investigated, P-selectin was found to be present on the surface of all Annexin V-positive events whether they were platelets or platelet-derived microparticles. This is in line with previous findings where microparticle antigen expression reflected that of the parent cell although that of exosomes released from platelets did not (Heijnen *et al*, 1999). Like CD42b, P-selectin seemed to be enriched on microparticles compared with platelets, which would perhaps render them more capable of

interacting with leukocytes and may be significant in view of the current interest on the effect of such microparticles on thrombus formation (Polgar *et al*, 2005), which will be discussed at greater length in Chapters 4 and 5.

Interestingly, very little bound fibrinogen was detected on the surface of microparticles produced following XL-CRP activation. This was not because microparticles lacked the receptor GPIIb/IIIa since >95% bound the antibody RFGP56. Earlier studies have shown conflicting results when examining the presence of functional GPIIb/IIIa and bound fibrinogen to microparticles and the suggestion has been that the agonist involved and the signalling it engenders is of critical importance. For example, in one study, activation by complement C5b-9 did not result in fibrinogen binding to microparticles whereas activation by thrombin plus collagen did (Sims *et al*, 1989). In a later paper, fibrinogen binding to microparticles was reported not to have occurred after activation by ADP, A23187 or thrombin (Nomura *et al*, 1992). It was suggested that this was due to disruption of GPIIb/IIIa caused by the alterations in the cytoskeleton which take place during microvesiculation. In neither of these studies was the effect of collagen alone on the binding of fibrinogen to microparticles examined. In this study, the data show that whilst microparticles formed after XL-CRP stimulation do possess GPIIb/IIIa receptors, many of these are in a non-functional conformation (shown by the low PAC-1 Mab binding) and so do not bind fibrinogen, and those that are functional bind very low levels. No clearly defined subpopulation of fibrinogen-positive microparticles is evident from the scatterplot, although the relatively low numbers may make this difficult to detect. It is possible that additional activation by thrombin might cause conformational change and hence fibrinogen binding on microparticles, but this was not examined further.

3.3.5. Mechanisms of PS exposure

3.3.5.1. Collagen is the principal procoagulant agonist

Where cells are in suspension, with minimal cell-cell contact, the data presented here show clearly that collagen in the form of XL-CRP is the only agonist capable of eliciting a procoagulant response in platelets. This suggests that the statement, “Thrombin in conjunction with collagen is the physiological platelet agonist for procoagulant phosphatidylserine exposure and consecutive membrane shedding,” found in a recent review (Morel *et al*, 2004) is not entirely correct. A previous report (Siljander *et al*, 2001), using gel-filtered platelets rather than whole blood, has suggested that XL-CRP at a concentration of 2.5 µg/mL is able to cause only minimal PS exposure in platelets in suspension. This could be due to the small number of donors involved, who could all have been ‘low-responders’ since, as shown in the present study, there is considerable variation in donor procoagulant response to XL-CRP, and in our hands, 2.5 µg/mL XL-CRP is a sub-maximal concentration in this context. Stirring has been regarded as necessary for PS exposure following platelet activation by collagen and thrombin (Rosing *et al*, 1985), but it is clear that, at least with XL-CRP, this is not the case.

Although studies on the relative potency of different agonists date back over ten years, and have identified collagen as the major physiological procoagulant agonist when acting alone, several, unlike the present study, have also shown thrombin to be active, albeit only slightly (Sims *et al*, 1989; Dachary-Prigent *et al*, 1993). However, the overall picture regarding the platelet procoagulant efficacy of thrombin is somewhat confused. This may be due to differing experimental conditions: most workers have used gel-filtered platelets which have undergone several centrifugations rather than whole blood and in some cases the concentration of platelets in the assay has exceeded that found in whole blood. Another study which showed TRAP as producing considerable Annexin V binding was performed in stirred samples of PRP (Storey *et al*, 2000). All of these procedures would make platelet-platelet interaction likely with consequent enhancement of PS exposure (see

further below). Moreover, the endpoint in some papers was prothrombinase activity (Sims *et al*, 1989) or thrombin generation (Dorsam *et al*, 2004) rather than PS exposure, which may also account for some of the differences in result. In another instance where PS exposure following activation by TRAP has been reported, non-anticoagulated blood was used and the percentage of platelets positive for Annexin V was very low, variable and presumably not significant since there is no indication to the contrary in the text (Ramström *et al*, 2003). Thus the discrepancies between data relating to the efficacy of thrombin in achieving PS exposure are probably related to technical differences, in particular the effect of platelet-platelet interaction, and do not invalidate the conclusion drawn in the present study that neither TRAP nor thrombin are capable on their own of changing PL orientation in the platelet membrane. It cannot be excluded, however, that, although the concentration of thrombin used (0.32 U/mL) is regarded as a high concentration for flow cytometric assays in this laboratory, causing maximum activation of GPIIb/IIIa and degranulation, an effect would have been seen had the concentration been raised further.

The question arises as to how far XL-CRP can be considered an adequate replacement for native collagen. There are practical difficulties in reproducing collagen activation as found *in vivo*, not least because of the complexity of the subendothelial matrix, as discussed in the Introduction (§1.5.3). The commonly used Horm® fibrillar collagen is problematic in flow cytometry since it cross-links platelets, quite apart from its being equine, not human. However, to gain some information regarding the comparison between XL-CRP and Horm® collagen, both were used in a stirred system and subsamples assayed a short interval after activation so that aggregation had not progressed beyond the point where flow cytometric analysis would be inappropriate. Although stirring will probably have enhanced the procoagulant response, PS exposure following activation by both agonists was evident after only 15s when there will have been little platelet-platelet contact, suggesting that the collagen alone was capable of eliciting ‘flip’. Given in addition published data regarding the pre-eminence of GPVI as a collagen signalling receptor (Nieswandt & Watson, 2003;

Moroi & Jung, 2004), regardless of the exact nature of the interplay between $\alpha_2\beta_1$ and GPVI during adhesion, which has yet to be fully elucidated, it seems reasonable to use XL-CRP as a substitute for collagen in studying the platelet procoagulant response.

In contrast, there is general agreement that, when used singly, ADP is ineffective as a procoagulant agonist and that was the case here also. It is well known that ADP released from the dense granules following stimulation with another agonist has an enhancing effect on many aspects of platelet activation (Gachet, 2001). In this study it was shown that joint activation by ADP and XL-CRP significantly increased the percentage of procoagulant platelets, but no effect was seen if ADP was added when either TRAP or thrombin was the agonist. This is not in agreement with early data which showed no additive effect in prothrombinase activity by adding ADP to collagen, but did when a combination of thrombin plus ADP was used (Sims *et al*, 1989). However, the previous discussion regarding technical differences applies here also.

Although the isolated contributions of collagen and thrombin are the subject of some disagreement, as noted above, the fact of the combination of thrombin and collagen leading to an enhanced procoagulant response is well established. This was also found under the experimental conditions in this project, although the difference was significant with thrombin, not TRAP, plus XL-CRP. This may suggest a role for PAR-4 and possibly GPIb (or another thrombin receptor), although further work would be necessary to establish this, particularly as the number of donors in the experiment was only 3 and significance might have been achieved had there been more. During the writing of this thesis, a paper has appeared showing a role for both PAR receptors in enhancing platelet prothrombinase activity in response to collagen (Dorsam *et al*, 2004).

In recent years, the concept of COAT-platelets (COLlagen And Thrombin-activated) has attracted considerable interest since they were first described in 2000 (Alberio *et al*, 2000). These are a sub-population of platelets which, on dual stimulation via GPVI and with thrombin (or TRAP at room temperature (Szasz & Dale, 2003)) apparently bind much higher levels of, *inter alia*, FV,

fibrinogen and fibronectin by a mechanism involving the transglutaminase mediated conjugation of serotonin to proteins released from the α -granules (Dale *et al*, 2002). It has also been reported that such dual activated platelets also bind higher levels of FVIII, FIX and FX as well as FV (Kempton *et al*, 2005).

No investigation into the nature of dual activated cells was undertaken in the present study, but the results presented here do impinge on the issue of COAT platelets. In the original COAT platelet paper (Alberio *et al*, 2000), flow cytometric assays of gel-filtered platelets indicated, as found here (and see the discussion above) that there was no Annexin V binding to platelets activated with an admittedly low concentration of thrombin. In their study, 0.5 nM thrombin was used, which is approximately 0.08 U/mL, compared with 0.32 U/mL used in the present work. However, unlike the results presented here, activation via GPVI, using convulxin rather than XL-CRP, produced only a very small percentage of PS positive platelets even at a high concentration. The existence of subpopulations of platelets has been remarked upon previously (Fig.3.16 and §3.3.4), but in the present project, these were seen when platelets were activated solely by XL-CRP, specifically in the *absence* of thrombin since the assay contained the direct thrombin inhibitor hirudin. This suggests that the mechanism behind the generation of COAT-platelets might not be quite as described in that it may be, as the data here show, that collagen generates the subpopulation whilst thrombin acts in another fashion, perhaps in activating the transglutaminase-mediated conjugation of serotonin, since it is a known activator of the transglutaminase FXIII (Muszbek *et al*, 1999).

3.3.5.2. Role of secondary soluble agonists in PS exposure

Both exogenous ADP and that released from the dense granules was shown to enhance the procoagulant effect of XL-CRP, as it does other activation responses (Nieswandt *et al*, 2001; Atkinson *et al*, 2003). Although the specific ADP receptors involved in potentiating the Annexin V

binding to TRAP-activated platelets in PRP have already been studied (Storey *et al*, 2000; Leon *et al*, 2003), the contribution of the different ADP receptors to PS exposure mediated through GPVI has not been addressed, though a recent paper has examined the effect of inhibition of P2Y₁ and P2Y₁₂ after platelet activation by type I collagen and thrombin on thrombin generation rather than PS exposure (Dorsam *et al*, 2004). Use of the ADP scavenger apyrase resulted a reduction in the percentage of platelets expressing PS, and the fact that this effect was similar in PRP indicated that the ADP was primarily platelet-derived, although red cell release might also play a part. As previously found with TRAP and ADP, most of the potentiating effect of ADP on GPVI-mediated membrane change was through P2Y₁₂. The effect of inhibiting ADP activation of P2Y₁₂ (but not P2Y₁) was mainly on the production of PS positive microparticles, leaving the PS⁺ platelets virtually unaffected. Perhaps ADP potentiation through P2Y₁₂ is critical for the calpain activation necessary for vesiculation. In contrast to previous results with different agonists, a small but significant contribution from P2Y₁ was also found. A recently published study has a bearing on both these aspects (Takano *et al*, 2004), since these investigators found a role for both P2Y₁ and P2Y₁₂ in platelet microparticle production by collagen-activated platelets, but this study defined microparticles solely on size and did not consider PS exposure.

Until a specific inhibitor for P2X₁ becomes available it is not possible to directly investigate the role of this purinogenic receptor in enhancing collagen-induced PS exposure. Such a role seems plausible in view of previous studies implicating this calcium ion channel in the regulation of collagen activation of platelets (Oury *et al*, 2001). The reduction in thrombus size in P2X₁^{-/-} mice (Hechler *et al*, 2003) and, conversely, the generation of a prothrombotic phenotype in a transgenic mouse in which the receptor is over-expressed (Oury *et al*, 2003) lend weight to this suggestion. It is probable that any effects would be in synergistic interaction with other receptors, especially P2Y₁ and P2Y₁₂ (Mahaut-Smith *et al*, 2004).

Of the other soluble secondary agonists, only the role of TXA₂ was investigated in this project. PS exposure in platelets incubated with aspirin has previously been shown to be little affected when activated by TRAP (Storey *et al*, 2000) or adherent to collagen (Heemskerk *et al*, 1997). The present study differs from these in having donors ingest ASA before assay of whole blood where platelets were activated specifically through GPVI. Although this regime resulted in no significant reduction in the percentage of PS positive platelets overall, there was variation in the inhibition seen in the 6 donors used, with two donors showing inhibition but the others little effect. This might be worthy of further investigation, as it might have implications for the use ASA as anti-platelet therapy.

3.3.5.3. Role of platelet-platelet interaction in PS exposure

The influence of platelet-platelet interaction on coagulation has been recognised for some years. An early paper, measuring prothrombin activity, regarded stirring as essential (Rosing *et al*, 1985) even when platelets were activated by collagen plus thrombin. The GPIIb/IIIa blocking antibody ReoPro® has been shown to prolong the activated clotting time in normal controls (Ammar *et al*, 1997) and to inhibit prothrombinase activity in PRP and PS exposure in thrombin-activated, gel-filtered platelets (Pedicord *et al*, 1998). GPIIb/IIIa has also been implicated in microvesiculation, which may be related to PS exposure (Gemmell *et al*, 1993). The data presented here show that PS exposure is potentiated by platelet-platelet interaction and, conversely, inhibited by ReoPro®. This is in line with findings in a study very similar to this one in that it involved the flow cytometric assay of whole blood (Furman *et al*, 2000), but here the results show specifically that signalling through GPVI is enhanced via GPIIb/IIIa, whereas in the earlier study a combination of thrombin plus collagen was used to activate the platelets so that it was not clear which signals were being amplified.

In the case of XL-CRP, platelet-platelet contact was not necessary for PL rearrangement since it occurred in cells in suspension. Added to this, ReoPro® did not completely abolish Annexin V binding, although the ReoPro® did not entirely eliminate XL-CRP-induced aggregation either, possibly indicating an aggregation mechanism other than fibrinogen-GPIIb/IIIa bridging. However, no significant PS exposure was seen in TRAP-activated platelets in suspension or in the absence of stirring. With TRAP, ReoPro® did abolish aggregation as detected in the light aggregometer and also reduced PS exposure to the level seen in the control. It is interesting to note that when the samples were stirred, thrombin promoted PS exposure to the same extent as TRAP (data not shown). Some workers have argued that stimulation through PAR-1 and PAR-4, with a possible contribution from another thrombin receptor such as GPIb, is necessary for PS exposure (Dörmann *et al*, 2000; Dorsam *et al*, 2004). Whilst there may indeed be a contribution from several receptors, the data here indicate that activation through PAR-1 in conjunction with potentiation via GPIIb/IIIa is capable of causing membrane scrambling without the participation of other thrombin receptors. As discussed previously, the incidence of platelet-platelet interaction may account for the contradictory reports regarding the ability of thrombin or TRAP to cause PS exposure. In some, the preparatory procedures would inevitably involve a degree of platelet contact, although this does not account for all of the differences observed since some workers who have used washed platelets have not seen PS exposure or procoagulant activity in response to thrombin or TRAP (Goodwin *et al*, 1994).

How GPIIb/IIIa influences PL rearrangement has not been fully elucidated. When platelets were activated by A23187, thus circumventing outside-in signalling, blockade of GPIIb/IIIa by Mab c7E3 (Fab fragment of chimeric human-mouse Mab 7E3; ReoPro®) had substantially less effect than in thrombin-activated platelets, indicating that outside-in signalling probably contributes to PS exposure and is interfered with by GPIIb/IIIa antagonists (Pedicord *et al*, 1998). However, the same workers did not find any reduction in calcium mobilisation in the presence of ReoPro® (*ibid.*). In

the experimental set-up employed here, fibrinogen would have been bound to platelets in suspension (demonstrated in other experiments at these concentrations of TRAP and XL-CRP). This would have initiated outside-in signalling through GPIIb/IIIa, yet this was insufficient to induce PS exposure in the absence of stirring in TRAP-activated platelets. Stirring also enhanced PS on the surface of XL-CRP activated platelets. This may imply that aggregation itself, rather than mere fibrinogen binding, promotes clustering of the GPIIb/IIIa receptor and hence enhances the strength of outside-in signalling for PL rearrangement (Shattil & Newman, 2004). It is also possible that signalling through some other receptors is involved.

3.3.5.4. Role of actin polymerisation in PS exposure and degranulation

There was an interesting contrast in the results of preincubating platelets with the actin polymerisation inhibitor cytochalasin D in stirred and unstirred samples, the treatment potentiating and inhibiting the procoagulant response respectively. Inhibition of Annexin V binding by cytochalasin D in HEL cells activated by A23187 and thapsigargin in a static system has been reported previously (Kunzelmann-Marche *et al*, 2001). Similarly, Annexin V binding in collagen- but not thrombin-activated platelets, also in a static system, was reduced by cytochalasin B (Diaz-Ricart *et al*, 2002). Cytochalasins were found to render platelets unresponsive to collagen (Nakano *et al*, 1989). However, in an earlier study, PS expression in platelets stimulated by A23187 was independent of actin polymerisation (Gaffet *et al*, 1995). It has also been reported that cytochalasin D inhibited various aspects of platelet activation only via $\alpha_2\beta_1$ activation not GPVI, but PS exposure was not investigated (Inoue *et al*, 1999). The effect of cytochalasin D on PS exposure in a stirred system has not previously been addressed. It is not clear why such conflicting effects should be observed. It is tempting to speculate that the reason may be related to the role of the cytoskeleton in the regulation of the affinity of GPIIb/IIIa for fibrinogen and consequent signalling (Buensuceso *et al*, 2003), which, as seen above, potentiates PL rearrangement. As noted above, the cytoskeleton

also plays a part in the regulation of store-mediated calcium entry. This regulation has been reported to be modulated by fibrinogen binding to GPIIb/IIIa which attenuates store-mediated calcium entry by inhibiting the polymerisation of the actin cytoskeleton (Rosado *et al*, 2001). If this is the case, it seems surprising that blocking fibrinogen binding with ReoPro® would inhibit PS exposure as reported here and elsewhere.

The role of actin reassembly in degranulation is similarly unclear. Previous workers were surprised to find potentiation of P-selectin expression in ADP-stimulated, unstirred PRP by cytochalasin H (Natarajan *et al*, 2000). As demonstrated here, this was also the case when a much stronger agonist, XL-CRP, was used to activate platelets in whole blood. However, washed platelets activated by purified type I collagen, but not thrombin, showed significantly less P-selectin translocation when treated with cytochalasin B (Diaz-Ricart *et al*, 2002). There is no obvious explanation of why this happens, or of the relationship between increased degranulation and decreased PS exposure in unstirred samples. One might expect that increased release of ADP from dense granules (if this parallels α -granule release) would operate in an opposite direction. In sum, the role of actin polymerisation as revealed by the use of cytochalasin inhibitors requires further work to elucidate the connections between the cytoskeleton, GPIIb/IIIa and outside-in signalling with the resultant effects on different aspects of platelet activation.

3.3.6. Relationship of PS exposure to other aspects of platelet activation

Activation of platelets through GPVI leads to degranulation (at least of α -granules since dense granule secretion was not explicitly examined in this project, although ADP was shown to be secreted) at much lower concentrations than PS exposure. Agonists such as thrombin or TRAP which are able to elicit a strong degranulation response are unable when acting in isolation to cause PL rearrangement. Similarly, there was no correlation between an individual's degranulation and procoagulant response to XL-CRP. The initial report describing Scott syndrome mentions that

defective PS exposure was not accompanied by defective secretion (Weiss *et al*, 1979). A more recent paper has also shown that abciximab inhibits platelet procoagulant activity but not granule release which again suggests distinct pathways (Ilveskero & Lassila, 2003). This all implies that regulation of P-selectin and PS expression are separate, as has been recognised for many years (Thiagarajan & Tait, 1990; Dachary-Prigent *et al*, 1996; Ilveskero & Lassila, 2003). That this is the case is highlighted in this study because differences were seen following activation via a single specific receptor.

The dose-response curves also illustrate an interesting feature of these aspects of platelet activation in that whilst nearly all platelets will degranulate on maximal stimulation, only a subset of platelets is capable of exposing PS. Recent work has demonstrated that when fibrillar collagen is used as an agonist, only a limited number of platelets may be activated because of the restricted number of binding sites available on the collagen. In contrast, XL-CRP binds and dissociates so that all platelets can be stimulated (Jarvis *et al*, 2004). Since at concentrations greater than 1 $\mu\text{g/mL}$, XL-CRP had caused more than 95% platelets to express P-selectin, the restricted percentage of PS positive platelets could not have been due to lack of availability of agonist. Combinations of agonists were also unable to produce PS exposure in 100% of platelets. On the other hand, platelets treated with calcium ionophore A23187 were nearly all Annexin V-positive, showing that inability to express PS is not related to membrane composition or unresponsiveness to cytosolic calcium concentration, but rather to the signalling mechanisms leading to PL translocation. The observation that only a subpopulation exposes PS after activation has been alluded to previously in connexion with COAT platelets (§3.3.5.1) which were examined with platelets in suspension, but others have reported almost universal PS exposure in platelets adherent to collagen (Heemskerk *et al*, 1997). Whether these differences between platelets in suspension and adherent are of physiological significance is uncertain, particularly in view of the fact that the collagen used in adhesion studies was equine not human and not the complex mixture found in the subendothelium. Even if it exists

only *in vitro*, it is not clear what distinguishes this subpopulation of PS-exposing platelets. It is possible that there is a decline in platelet responsiveness to collagen over the lifetime of the platelet, as described in canine platelets (Alberio *et al*, 2002).

The role of ADP in augmenting PS exposure in XL-CRP activated platelets has been discussed previously. Parallel experiments examining the role of P2Y₁ and P2Y₁₂ in fibrinogen binding and P-selectin expression following an initial stimulus via GPVI revealed further differences between PL flip and secretion. Whilst ADP potentiated fibrinogen binding as well as PS expression, primarily through P2Y₁₂, it had no effect on P-selectin translocation. Previous studies have shown that AR-C69931MX does inhibit degranulation, but these studies have investigated platelets activated by TRAP not XL-CRP (Leon *et al*, 2003; Klinkhardt *et al*, 2003). Although a decrease in P-selectin expression in response to collagen has been shown when the ADP pathway was blocked (Storey *et al*, 2000), the results presented are surprising both in that a high concentration of collagen (8 µg/mL) induced relatively modest P-selectin expression, and that this appeared to be almost entirely mediated by TXA₂ and ADP. It could be argued that the use of a higher concentration of AR-C69931MX in the current study might have been more effective in blocking P2Y₁₂ but one would have expected degranulation to be inhibited before fibrinogen binding in view of the fact that milder activation is required for the latter. As noted above, contrasts were also seen between the effect of preventing actin polymerisation on PS exposure and α-granule degranulation in platelets in suspension, inhibitory and potentiating respectively. Thus the data here support the proposition that there are independent pathways from GPVI to degranulation and PS exposure.

Where the differences in signalling for degranulation and PS exposure occur is unclear. Although PI3-K is an important early component in GPVI signalling leading to activation of PLCγ2 (Gibbins *et al*, 1998), it has been shown that it can be by-passed (Pasquet *et al*, 1999) so it is a potential candidate for the point of divergence. Inhibition with wortmannin and LY294002

suggested that degranulation was more dependent on PI3-K than PS exposure, but since there was a similar pattern of incomplete inhibition, both processes appear to be mediated by this pathway, though only partially. It is possible that the involvement of PI3-K in PS exposure is principally due to its participation in the ADP receptor-mediated pathway. Further work would be necessary to determine the possible differences between the secretion and procoagulant pathways.

3.3.7. Relationship of platelet PS exposure to coagulation as demonstrated by TEG

Although, as noted previously, the necessity of negatively charged PLs for coagulation has long been known, the detailed relationship between platelet membrane reorganisation and coagulation is less clearly defined. An early *in vitro* study found a direct relationship between the PS content of lipid vesicles and procoagulant activity (Connor *et al*, 1989), but a later study which assayed the ability of the washed platelets of 14 volunteers to support tenase and prothrombinase activity found no significant correlation between the increase in PS exposure and either parameter (Sumner *et al*, 1996). The study was limited by the small number of donors examined and also the analysis related procoagulant activity to the increased PS on activated platelets without considering the number of platelets so activated. Thus there is no account taken of the total amount of PS available. A variant of this criticism can also be levelled at the analysis employed in this project in that the percentage of positive platelets is taken as a measure not the relative fluorescence. Because of the subpopulations involved, it is difficult to establish a single measure so this reservation should be borne in mind when reviewing data. It has also been reported that where liposomes contained more than about 12% PS, procoagulant activity went down (Zwaal *et al*, 1998), a reminder both that there is an optimal level of PS and also that PLs play a part in fibrinolysis as well as coagulation. In considering the broader issue of thrombotic disease, both aspects must be taken into account.

TEG is a well-established method of investigating the kinetics of clot formation and the strength of the resultant clot. However, the system is a static one, as indeed is flow cytometry, and

this may limit its usefulness. There has to my knowledge been no previous study simultaneously measuring PS exposure and P-selectin expression by flow cytometry and clotting parameters by TEG. There was a clear effect of platelet activation on shortening the clotting time and the clot formation time. However, the influence of platelet activation on clot formation rate and the maximum firmness of the clot was less obvious since there was a significant increase respectively only at particular agonist concentrations without an obvious trend. In general, these data are in line with those in a recent paper which, using a fluorogenic method to measure the endogenous thrombin potential, found that platelet activation influenced the initial kinetics of thrombin generation but not the total thrombin generating capacity (Vanschoonbeek *et al*, 2004).

The data in this project do not give a clear indication as to how platelet activation affects clotting times. The percentage of PS-positive platelets in TRAP-activated platelets was similar to that in platelets stimulated with 150-500 ng/mL XL-CRP and clotting times were also similar. The plot of clotting time against percentage Annexin V-positive platelets suggests that any increase of PS exposing platelets above approximately 7% is associated with only a very slight shortening of the clotting time, but that clotting times would be greatly extended were fewer than about 2% of platelets to express PS. In other words, these data support the view that PS exposure is necessary for coagulation but that the amount required is relatively low with further increases not resulting in any further reduction in clotting time. This is in accord with data from Sumner *et al*, 1996, using a different experimental set-up (a chromogenic amidolytic assay for FXa activity using gel filtered platelets) and contrasts with the earlier reports of a linear relationship between PS content and procoagulant activity (Connor *et al*, 1989). A simple relationship between PS exposure and clotting *in vivo* seems unlikely given the complexity of the system. For example, anionic PLs are necessary for the assembly of protein C anticoagulant enzyme complexes, but at approximately ten times the concentration required for procoagulant activity (Morel *et al*, 2004). This suggests that variation in the ability to expose PS might not be a simple risk factor for cardiovascular events. A rather more

subtle study into the precise composition of the outer platelet membrane and specific components of the coagulation process would be warranted.

The relationship between P-selectin MFI (which gave better differentiation between TRAP and XL-CRP than the percent of P-selectin positive platelets) and clotting time was similar pattern to that between PS and clotting time. Experiments using TEG in which P-selectin was blocked using Mab 9E1 (see Chapter 4, §4.2.8) revealed a slight prolongation of clot formation time but not clotting time and did not affect the strength of the clot. It is possible that other components of the releasate, expressed in parallel with P-selectin, also contribute to coagulation. For example, Factor V/a is also stored in the α -granules, and its expression may shorten clotting times.

This emphasizes the complexity of the role of platelets in coagulation and also raises the issue of whether assaying for PS exposure is an informative exercise. Since the provision of an anionic PL surface is necessary for coagulation to occur, understanding the mechanisms for achieving this is important for understanding the process and designing therapeutic drugs. Although it is arguable that assays which measure activity are more pertinent than the measurement of PS by flow cytometry, the complexity of the system requires consideration of several different activities. Flow cytometry has the advantage of allowing analysis of platelets with minimal manipulation whereas most other methods require sample preparation. For example, platelets have to be washed in order to measure calcium flux and although a fluorogenic substrate has been developed for monitoring thrombin generation, this can only be used in PRP entailing centrifugation and leave only a subset of platelets for analysis. As is so often the case, problems need to be approached from different angles with each approach contributing to the overall picture.

3.3.8. Concluding discussion

This part of the study has demonstrated that, whilst other agonists have an enhancing effect, it is the activation of platelets by collagen acting through GPVI (though not excluding a role for other

collagen receptors) which is responsible for eliciting PS exposure. Thrombin can only cause PL rearrangement when there is platelet-platelet interaction.

This project therefore postulates an adjustment to the cell-based model of coagulation as expounded by Hoffman and co-workers (Hoffman, 2003), by emphasizing the importance of the exposed subendothelium in providing not only TF but also the stimulus for anionic PL exposure on platelets. Although in a recent exposition of the cell-based model, platelet adhesion to the site of injury is alluded to, it is only regarded as an instigator of *partial* activation which is furthered by the initial thrombin burst (Monroe & Hoffman, 2005). The influence of collagen in providing the necessary platelet lipid environment is therefore underestimated the Hoffman model. The data in this study suggest that thrombin will only cause platelets in the thrombus to become procoagulant, thus limiting this effect to those platelets recruited to the thrombus and not to passing platelets which fail to aggregate. This appears to be one of a number of mechanisms for promoting thrombin generation within the emerging clot, such as the ability of fibrin to promote platelet procoagulant activity (Béguin *et al*, 1999) and the inability of ADP alone to promote PS exposure.

The data presented in this study have implications for antithrombotic drug therapy. Clopidogrel, whose metabolites inhibit P2Y₁₂, can reduce PS exposure through reducing enhancement by ADP. Similarly, GPIIb/IIIa inhibitors also exert some of their antithrombotic properties through the reduction of PS exposure, although there is evidence to suggest that different blockers may have different potencies in this regard (Goto *et al*, 2003). Aspirin, on the other hand, is relatively ineffective as an anticoagulant agent, despite its undoubted usefulness in inhibiting other aspects of platelet activation. However, there may be differences in donor response which could be investigated in order to provide an appropriate prescribing regime for a particular individual. The data suggest that the window for reducing PS exposure may be relatively small. Complete abolition might produce the bleeding phenotype of a Scott syndrome patient, whilst a smaller reduction may have little or no effect. Moreover, the separate pathways indicated for PS

exposure and degranulation have implications for drug therapy, as treatment which might reduce one aspect may not deal with the other.

3.3.9. Further work

The work in this section of the study could be extended by:

- Examining the procoagulant response under conditions of flow.
- Characterising in more detail the signalling pathways from GPVI to secretion and PS exposure
- Relating the effect of various modulators of the basic GPVI response (i.e. ADP, TXA₂, outside-in signalling etc) to changes in intracellular calcium and the role of calpain. The end-points to be considered are PS exposure and microparticle formation as well as degranulation.
- Examining the characteristics of the subpopulation of PS-positive platelets, for example, by measuring Annexin V-binding on reticulated (young) platelets or other multiple staining in flow cytometry. PS-positive and -negative platelets can also be separated using magnetic beads for further analysis of differences (e.g. phosphorylation status by Western blotting).
- Measuring thrombin or FXa generation and/or binding of FV or FVIII to the platelet surface to see whether this correlates to Annexin V binding in all cases.
- Dissecting out the relationship between the composition of the outer leaflet of the platelet plasma membrane and the entire coagulation pathway, that is taking into account the activity of the tenase and prothrombinase complexes but also the anticoagulant complexes such as the protein C.
- Determining whether the differences between donors are due to differences in signalling through GPVI, to differences in PL composition and/or oxidation. There are indications that procoagulant activity is linked, not just to PS exposure but also to lipid peroxidation, which would be reflected in the binding of Annexin V. Therefore inter-donor differences could be

linked to antioxidant defence systems in the platelets and blood, such as glutathione, superoxide dismutase (SOD) and vitamin E.

CHAPTER 4: Procoagulant interaction between platelets and monocytes

4.1. Introduction

In addition to the changes which occur in the platelet itself, addressed in the previous chapter, the interaction between platelets and leukocytes also has an impact on the procoagulant environment in blood, as has been recognised for many years (Semeraro *et al*, 1981). This interaction, which forms a link between the processes of inflammation and coagulation, can be indirect, through the action of soluble mediators, or direct, through the formation of heterotypic aggregates. The formation of such aggregates has been implicated in a number of pathological conditions associated with a procoagulant environment, such as angina and coronary artery disease (Ott *et al*, 1996; Furman *et al*, 1998).

Of the leukocyte sub-types, monocytes preferentially form aggregates with platelets compared with neutrophils, whilst the participation of lymphocytes is minimal (Rinder *et al*, 1991b). In addition, since macrophages are heavily implicated in the development of atherosclerotic plaques (Fuster *et al*, 2005), interactions which render them more procoagulant are of considerable clinical interest. It is therefore the monocyte-platelet interaction which forms the focus of this study. Leukocyte-platelet aggregation is mediated by platelet P-selectin and PSGL-1 on the leukocyte (Larsen *et al*, 1989) and therefore involves only activated platelets. These have been reported to enhance the procoagulant activity of monocytes in a variety of ways, including through the induction of TF or through its de-encryption (Celi *et al*, 1994; Halvorsen *et al*, 1993; Østerud, 1995; Østerud, 2001). They may also cause the induction and/or release of a number of cytokines (Weyrich *et al*, 1996). However, some workers have failed to confirm monocyte TF activity resulting from interaction with activated platelets (Weyrich *et al*, 1996), nor is it yet clear whether particular effects result from direct contact or whether close proximity and soluble factors will suffice. This section of the project examines the formation of monocyte-platelet aggregates and

their relationship to the induction of both TF and TFPI, and the role of direct cell-cell contact compared with soluble factors in mediating this response.

4.2. Results

4.2.1. Platelet-monocyte flow cytometric assays

The percentage of monocytes with adherent platelets (MPAs) and antigen present on either single monocytes or MPAs was determined by a three-colour flow cytometric assay performed in whole blood, with minimal manipulation, to reduce artefact. The assay is described in Chapter 2 §2.2.4.4. MPAs were those events which were positive for both CD14 and CD42b, whilst single monocytes were CD14 positive but CD42b-negative. Some of the flow cytometry experiments in §§4.2.2 and 4.2.8 were performed by Dr. Nailin Li (Karolinska Institute, Stockholm, Sweden).

4.2.2. Role of P-selectin and GPIIb/IIIa in the formation of MPAs

The formation of MPAs in citrated whole blood in response to stimulation of platelets by XL-CRP at 37 °C was followed over 4 h using flow cytometry. The concentration of XL-CRP was chosen by titration of each cross-linked batch, measuring by flow cytometry its ability to promote MPA formation in whole blood, examples of which are given in Fig.4.1. Initial titrations covering 0 – 500 µg/mL XL-CRP showed that 100 µg/mL was sufficient to give maximal activation (Fig.4.1a) and titrations over a narrower range (0 –150 µg/mL) indicated that 10 µg/mL was adequate. However, batch-to-batch variation meant that optimum concentrations to yield maximum MPA formation varied. The role of P-selectin and GPIIb/IIIa was evaluated by incubating some samples with their respective blocking antibodies, Mabs 9E1 and RFGP56, both at final concentrations of 25 µg/mL (Fig.4.2). The concentrations of both blocking antibodies were chosen on the basis of data previously published in collaboration with this laboratory (Li *et al*, 1999). Note that the initial time point was 5 min after the addition of XL-CRP rather than immediately after drawing blood, and

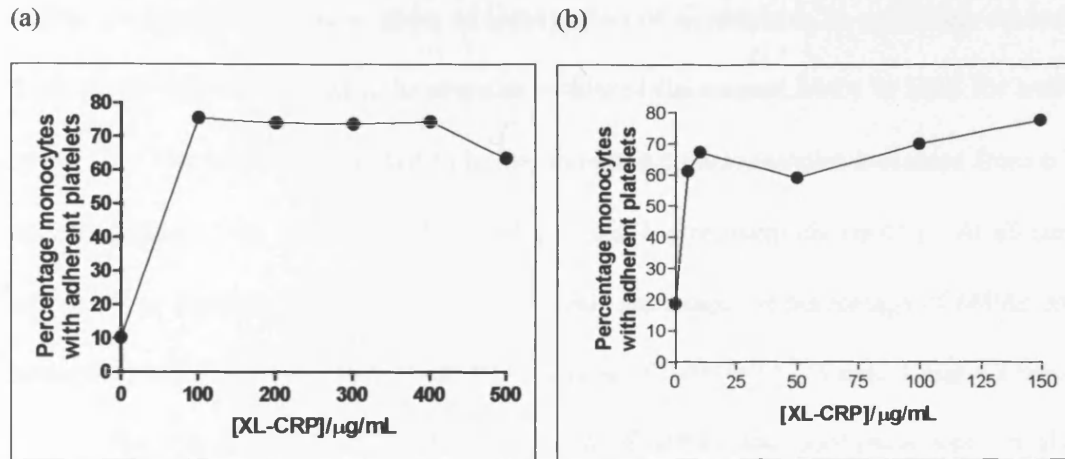


Figure 4.1. Titration of XL-CRP. Whole blood was incubated for 1 h at 37 °C with (a) 0 - 500 $\mu\text{g/mL}$ XL-CRP and (b) 0 - 150 $\mu\text{g/mL}$ XL-CRP and analysed for the formation of MPAs using flow cytometry to identify CD42b-positive monocytes.

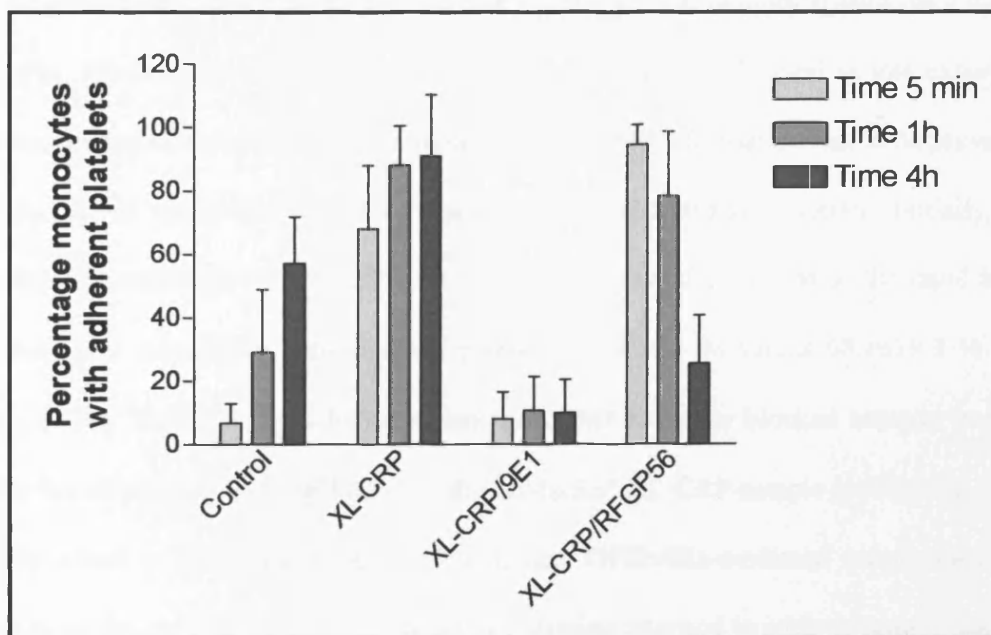


Figure 4.2. Effect of platelet activation on the formation of monocyte-platelet aggregates. Platelets in whole blood were maximally activated with XL-CRP, with and without the P-selectin blocking antibody 9E1 (25 $\mu\text{g/mL}$) or the GPIIb/IIIa blocking antibody RFGP56 (25 $\mu\text{g/mL}$), and incubated for 4 h at 37 °C. At the time points indicated, subsamples were analysed by flow cytometry to determine the percentage of monocytes with adherent platelets. Experiments were performed using blood from 5-10 individual donors (mean \pm SD).

before treatment. This was to allow an initial effect of treatment to be measured. At each time point, 5 μ L blood was transferred to appropriately diluted fluorescent Mabs in HBS for analysis by flow cytometry. The percentage of MPAs in the untreated control samples increased from 6.7 ± 6.1 % at 5 min to 29.2 ± 19.7 % and 57.1 ± 14.9 % at 1 h and 4 h respectively ($n=10$). At all time points the activation of platelets with XL-CRP significantly increased the percentage of MPAs compared with controls ($p < 0.01$) to 68.1 ± 19.9 %, 88.0 ± 12.3 % and 91.0 ± 19.2 % (5 min, 1 and 4 h respectively).

The role of P-selectin in the formation of MPAs was confirmed since at all time points MPAs in samples activated with XL-CRP in the presence of 9E1 were significantly diminished ($p=0.0035$, $p=0.0003$ and $p=0.0033$, respectively; $n=5$). The percentage of MPAs was indistinguishable from that in the 5 min control (8.0 ± 8.5 %, 10.5 ± 10.8 %, 9.9 ± 10.45 % versus 6.7 ± 6.1 %; $p=0.30$, 0.71 and 0.86 for 0 h, 1 h and 4 h 9E1-treated samples against the 5 min control, respectively). These results confirmed that the concentration of 9E1 used in this experiment (25 μ g/mL) was appropriate for all further experiments where MPA formation was to be prevented.

The role of GPIIb/IIIa in MPA formation followed a different pattern. Initially, blocking platelet-platelet interaction with RFGP56 in XL-CRP- activated blood led to the rapid increase in the percentage of monocytes with attached platelets (94.6 ± 6.3 % versus 68.1 ± 19.9 % at 5 min; $p=0.0046$; $n=10$). However, by 4 h the percentage of MPAs in the blocked samples had fallen to 25.4 ± 15.3 % compared with 91.0 ± 19.2 % in the unblocked XL-CRP sample ($p < 0.0001$).

The effect of blocking P-selectin-PSGL and GPIIb/IIIa-mediated interactions was also examined from the point of view of the number of platelets attached to each monocyte, shown semi-quantitatively by the CD42b-RPE mean fluorescence intensity, as plotted in Fig. 4.3. The number of platelets attached to monocytes in control samples remained fairly steady throughout (MFI 12.3 ± 2.9 , 22.3 ± 9.8 , 27.5 ± 10.9 AU at 5 min, 1 and 4 h respectively). Thus, although incubation without the addition of an agonist caused a gradual increase in the percentage of monocytes with attached platelets, the number of platelets adhering to each monocyte was relatively low. Activation

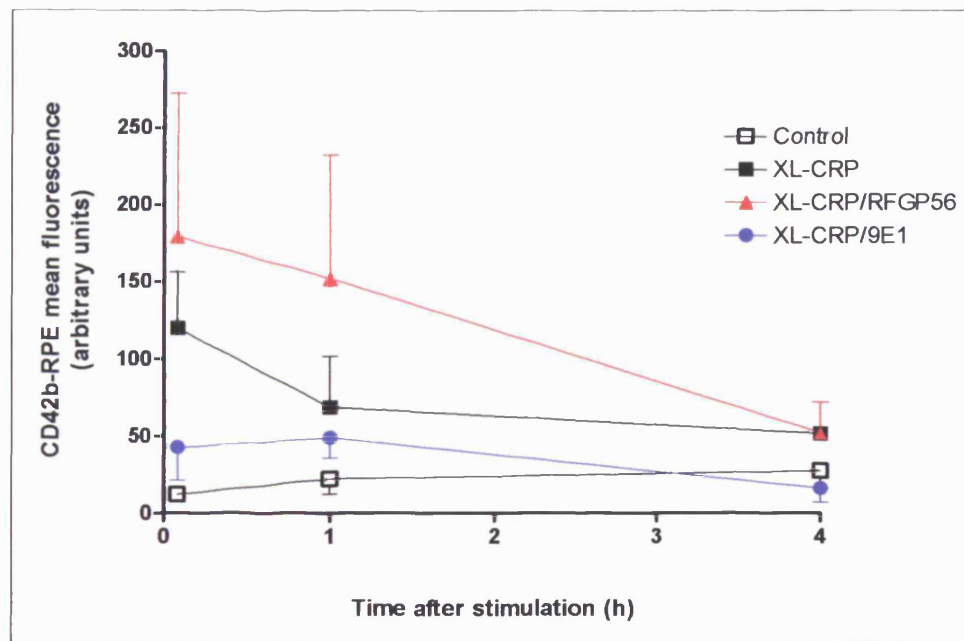


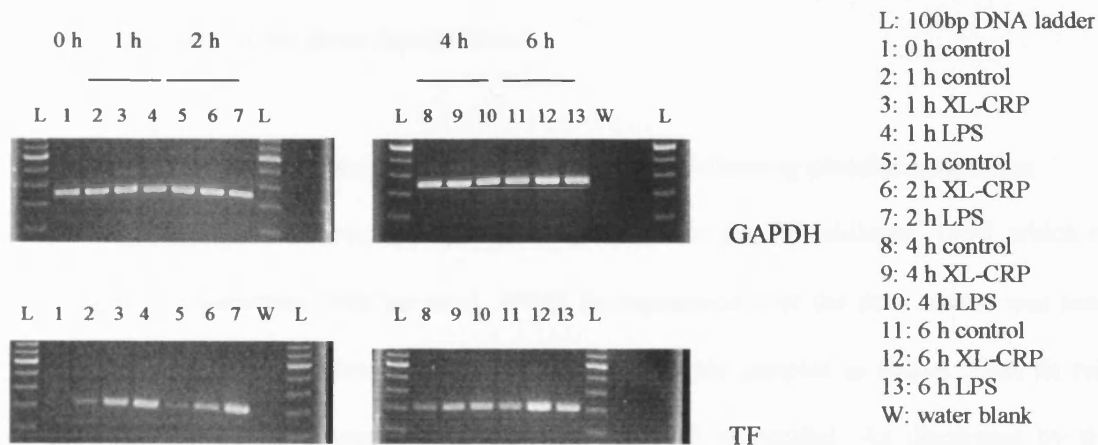
Figure 4.3. Relative numbers of platelets adhering to monocytes as indicated by CD42b-RPE fluorescence on MPAs. Platelets in whole blood were maximally activated with XL-CRP, with and without the GPIIb/IIIa blocking antibody RFGP56 (25 $\mu\text{g/mL}$) or the P-selectin blocking antibody 9E1 (25 $\mu\text{g/mL}$), and incubated for 4 h at 37 $^{\circ}\text{C}$. At the time points indicated, subsamples were analysed by flow cytometry to determine the percentage of monocytes with adherent platelets. The mean fluorescence intensity of CD42b-RPE on CD14^{+} monocytes gives a semi-quantitative measure of the number of platelets attached to each monocyte. Experiments were performed using blood from 4 individual donors (mean \pm SD).

of platelets with XL-CRP initially increased the number of platelets attached to aggregates by about 10-fold compared with control (120.1 ± 36.1 vs. 12.3 ± 2.9 AU; $p=0.0074$; $n=4$). However, CD42b-RPE MFI in XL-CRP activated samples fell to 68.7 ± 32.9 AU at the end of the first hour and further to 51.7 ± 20.5 AU by the end of the experiment ($p=0.0099$ 4 h compared with 5 min). Blockade of platelet-platelet aggregation with RFGP56 caused more platelets to adhere to monocytes compared with untreated XL-CRP samples for the first hour (179.4 ± 92.9 vs. 120.1 ± 36.1 AU at 5 min and 152.3 ± 80.5 vs. 68.7 ± 32.9 AU at 1 h; $p=0.2774$ and 0.1571), but by 4h there were significantly fewer adherent platelets than at 5 min (MFI 52.2 ± 19.9 AU; $p=0.0521$). Indeed, at 4h there was little difference between any of the samples. On the few MPAs present in Mab 9E1-treated blood, the number of attached platelets was about the same as in control samples.

4.2.3. Induction of monocyte TF gene expression following platelet activation

The effect of platelet activation on TF gene expression in monocytes was monitored using RT-PCR. The concentration of XL-CRP used was 50 $\mu\text{g/mL}$, which had been shown by titration to maximally stimulate platelets as measured by MPA formation, as described in §4.2.2. Use of the platelet-specific agonist XL-CRP ensured that monocytes themselves were not directly activated. Monocytes were extracted from whole blood incubated at 37 °C at 0, 1, 2, 4 and 6 h after the addition of XL-CRP. As a positive control, monocytes were activated by LPS. The intensity of the PCR product bands was measured by densitometry and compared with GAPDH products amplified from the same cDNA. A representative set of gels is shown in Fig.4.4a. The data for 3 individual donors are displayed graphically in Fig.4.4b and indicate that platelet activation results in a significant increase in TF mRNA after only 1 h of incubation, this increase being sustained over the next 5 h, though the variability in results with such a small sample number meant that significance was only additionally achieved at 4 h. Although LPS appeared to elicit a greater increase in monocyte TF expression over the first 4 h, there was never a significant difference between the

(a)



(b)

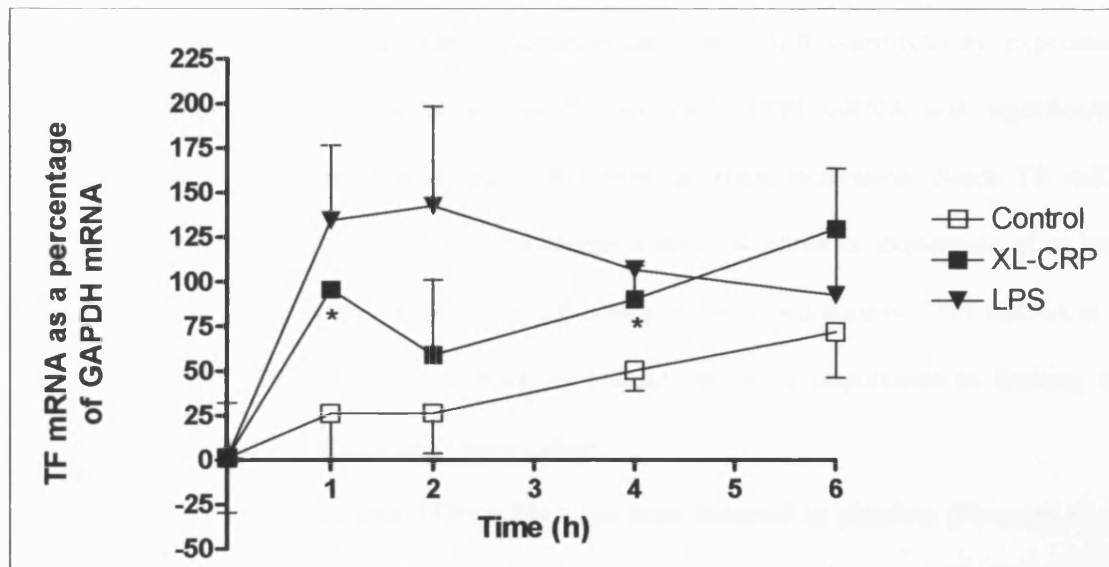


Figure 4.4. Induction of monocyte TF mRNA following platelet activation with XL-CRP. Platelets in whole blood were activated with 50 $\mu\text{g/mL}$ XL-CRP and incubated at 37 $^{\circ}\text{C}$ with regular mixing. Monocytes, also in whole blood, were activated with 200 ng/mL LPS as a positive control. At 0 h, 1 h, 2 h, 4 h and 6 h monocyte mRNA was extracted and RT-PCR performed using TF primers. (a) shows typical gel pictures for GAPDH and TF mRNA and (b) the densitometry data for 3 separate donors. * is $p < 0.05$ for XL-CRP-activated platelets versus control.

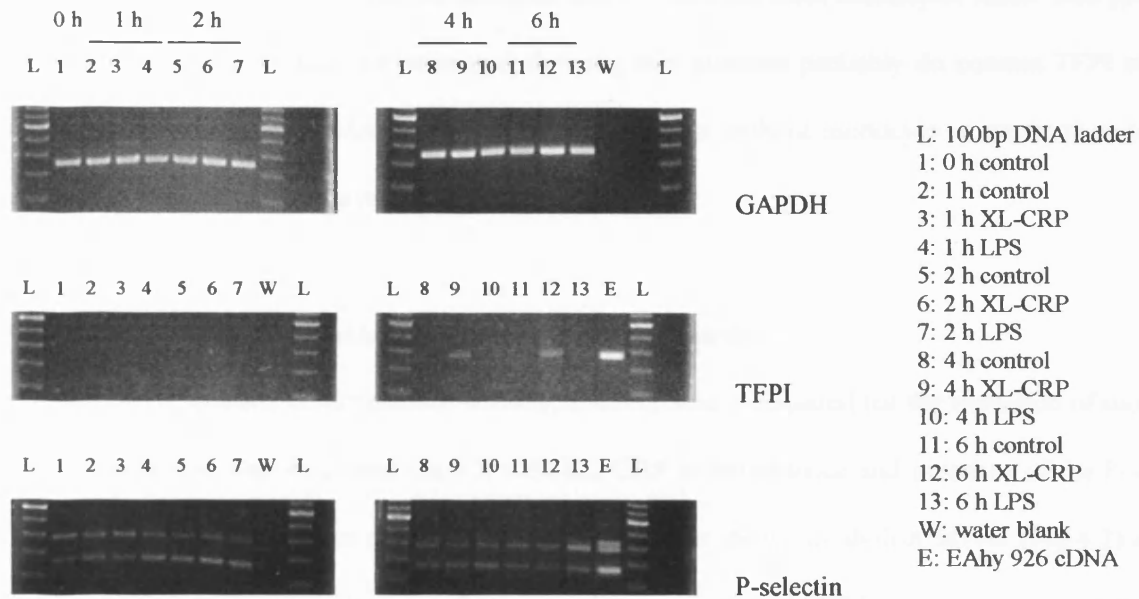
response to LPS and XL-CRP. In the 0 h control monocytes, TF mRNA was detectable, at a low level, in only one of the three donors tested.

4.2.4. Induction of monocyte TFPI gene expression following platelet activation

Since the procoagulant environment might be affected by the TF inhibitor, TFPI, which reportedly expressed by monocytes (McGee *et al*, 1994), its expression over the time course was measured by RT-PCR of the mRNA extracted from the same monocyte samples as above. Thus its relationship to platelet-induced TF expression could be determined in parallel. As illustrated by the gels in Fig.4.5a and the accompanying densitometry data (Fig.4.5b), and in contrast to TF, by 6 h TFPI mRNA was not increased above the control by LPS. As expected, there was a strong band visible in the positive control EAhy 926 (an endothelial cell line which constitutively expresses TFPI). However, where platelets were stimulated by XL-CRP, TFPI mRNA was significantly more abundant than in the control at 4 and 6 h following initial activation. Since TF mRNA was significantly upregulated after 1 h, this represents a delay in inhibitor expression of at least 1 and possibly 3 h. There was some variability in the timing of the appearance of TFPI mRNA in the three donors examined. Whether this variability is widespread or of importance in limiting thrombus growth would need to be tested in a larger cohort.

It has been reported that TFPI mRNA has been detected in platelets (Novotny *et al*, 1988) and a megakaryocytic cell line (Bajaj *et al*, 1990) so it was necessary to establish that the TFPI was indeed of monocytic origin. The presence of platelet mRNA was therefore investigated by amplifying cDNA using primers for P-selectin, which was assumed to be indicative of platelet presence, their being the only blood cells thought to express it (McEver *et al*, 1989). However, the gel for P-selectin in Fig.4.5a was typical and shows low but detectable levels of P-selectin in all the sample lanes. It might have been expected that more intense bands would be present in samples with larger numbers of platelets attached to the monocytes, such as in the XL-CRP stimulated

(a)



(b)

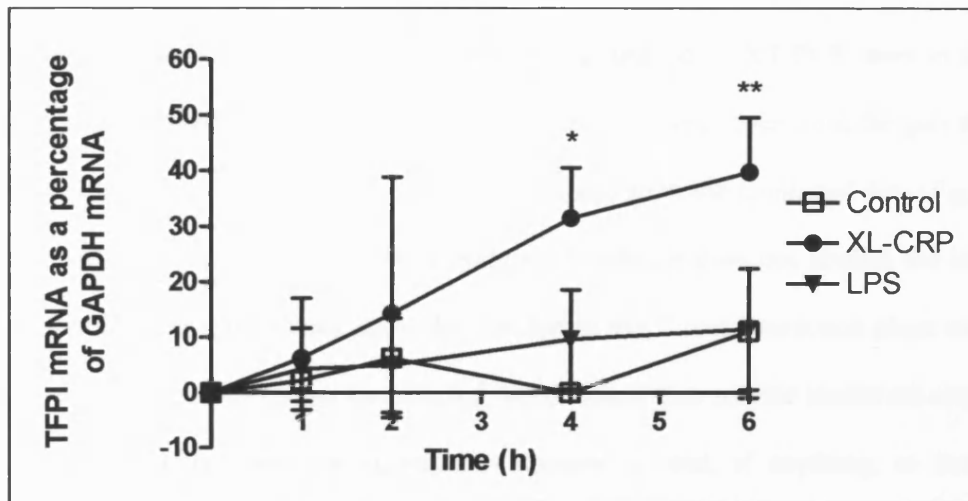


Figure 4.5. Induction of monocytes TFPI mRNA following platelet activation with XL-CRP. Platelets in whole blood were activated with 50 $\mu\text{g}/\text{mL}$ XL-CRP and incubated at 37 $^{\circ}\text{C}$ with regular mixing. At 0 h, 1 h, 2 h, 4 h and 6 h monocyte mRNA was extracted and RT-PCR performed using TFPI primers. Blood stimulated with 200 ng/mL LPS served as a comparison and cDNA from EAhy 926 endothelial cells was a positive control. (a) shows typical gel pictures for GAPDH TF and P-selectin mRNA and (b) the densitometry data for 3 separate donors. * is $p < 0.05$ and ** $p < 0.01$ for XL-CRP-activated platelets versus control.

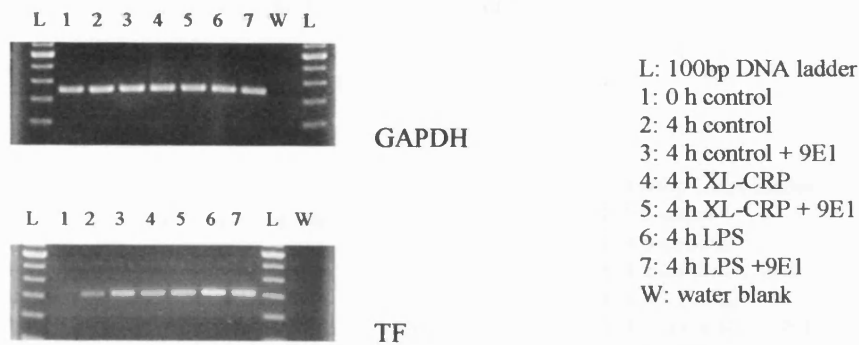
samples, but there was no clear pattern observable. However, since the presence of TFPI is independent of P-selectin, it can be deduced that it is derived from monocytes rather than platelets. In Chapter 5 (§5.2.4) data are presented showing that platelets probably do contain TFPI mRNA, although the difficulty in obtaining cDNA from platelets without monocyte contamination made it impossible to give a definitive answer.

4.2.5. Role of soluble mediators in monocyte TF induction

To determine whether direct platelet-monocyte interaction is required for the induction of monocyte TF, whole blood was incubated for 4 h with XL-CRP in the absence and presence of the P-selectin blocking antibody 9E1. The concentration used had been shown to abolish MPAs (Fig.4.2) and the 4 h time point was chosen because there was clear upregulation of TF gene expression at this time (Fig.4.4). Blood was stimulated with LPS as a positive control, or was incubated without an agonist, both in the absence and presence of 9E1. Although the method of RT-PCR used in this project allowed only semi-quantitative analysis of PCR products, it is clear, both from the gels themselves, of which a representative example is shown in Fig.4.6a, and from the combined data (Fig.4.6b), that blocking the attachment of platelets to monocytes via P-selectin does not abolish the induction of TF in the monocyte. The data do not allow the conclusion that direct interaction plays no role at all in this since small differences cannot be detected, but do imply that soluble mediators are important. In fact, blocking platelet-monocyte aggregation appears to lead, if anything, to increased TF expression, which would suggest that direct interaction has an inhibitory effect.

In similar experiments, shown in Fig.4.7, it was found that blocking P-selectin-PSGL-1 platelet-monocyte interaction also had no discernible effect on TFPI expression. Again, the P-selectin expression was independent of TFPI expression and so TFPI could be assumed to be monocytic in origin. The data therefore lead to the same conclusion as for TF, namely that direct heterotypic contact is not essential for monocyte TFPI induced by platelet activation. In fact, as was

(a)



(b)

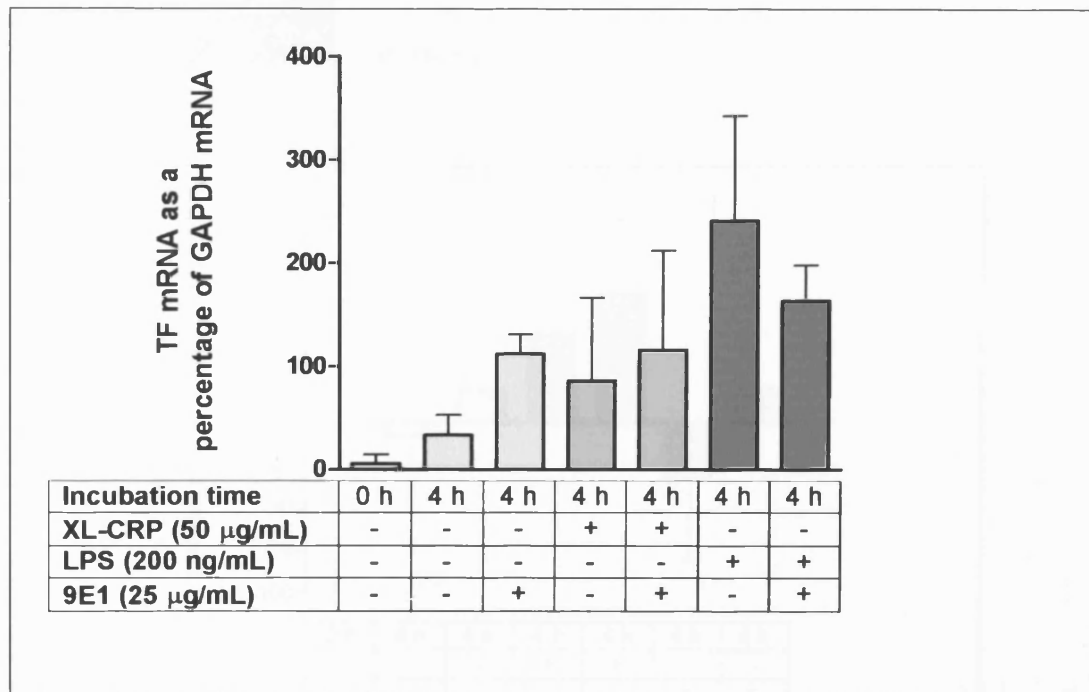
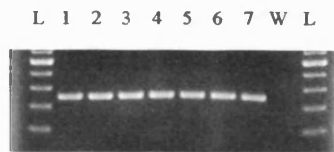
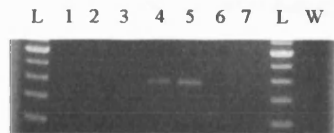


Figure 4.6. Blocking P-selectin-PSGL interaction between monocytes and platelets does not abolish upregulation of monocyte TF. Platelets in whole blood were activated with XL-CRP in the absence and presence of P-selectin blocking antibody 9E1 (25 μ g/mL). After 4 h incubation at 37 °C, mRNA was extracted and RT-PCR performed using GAPDH and TF primers. (a) Representative gels for GAPDH and TF PCR products; and (b) densitometry data for 3 separate donors.

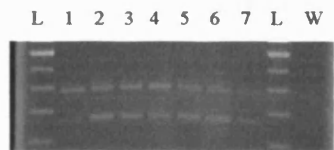
(a)



GAPDH



TFPI



P-selectin

L: 100bp DNA ladder

1: 0 h control

2: 4 h control

3: 4 h control + 9E1

4: 4 h XL-CRP

5: 4 h XL-CRP + 9E1

6: 4 h LPS

7: 4 h LPS + 9E1

W: water blank

(b)

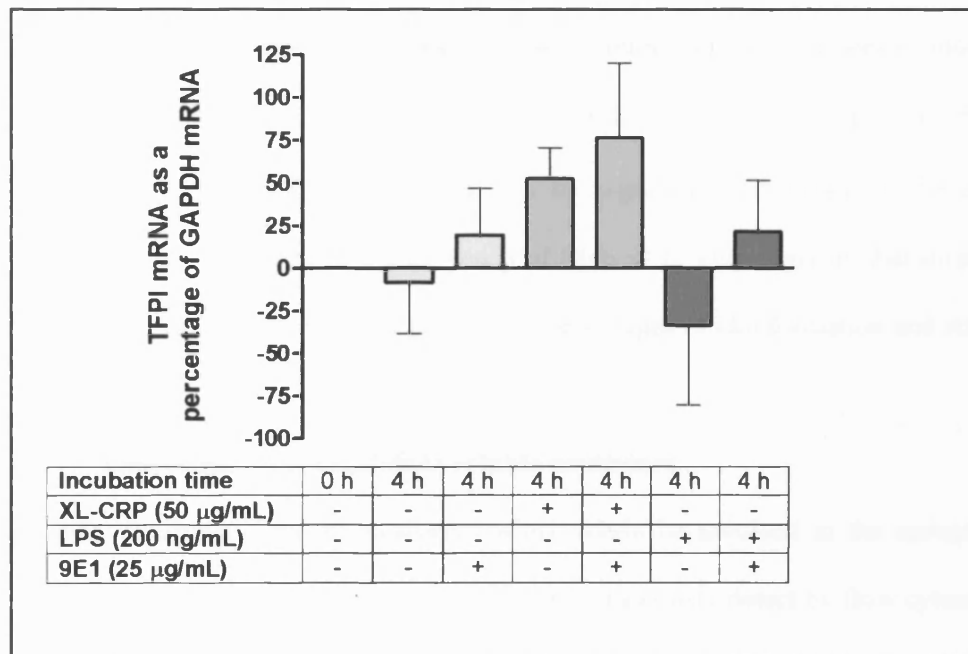


Figure 4.7. Blocking P-selectin-PSGL interaction between monocytes and platelets does not abolish upregulation of monocyte TFPI. Platelets in whole blood were activated with XL-CRP in the absence and presence of P-selectin blocking antibody 9E1. After 4 h incubation at 37 °C, mRNA was extracted and RT-PCR performed using GAPDH and TF primers. (a) Representative gels for GAPDH, TFPI and P-selectin PCR products; and (b) densitometry data for 3 separate donors.

the case with TF, blocking the formation of MPAs appears to increase TFPI gene expression. It is also interesting to note that monocyte TFPI was not upregulated in response to LPS by 4 h, in contrast to the rapid induction of TF in LPS-stimulated monocytes (Fig.4.4).

4.2.6. Effect of blocking P-selectin mediated monocyte-platelet interaction on clot formation

To determine whether direct interaction between platelets and monocytes influences the dynamics and structure of clot formation, clotting time, clot formation time and maximum clot formation (maximum amplitude) were measured by TEG. Platelets in whole blood were activated by 14 $\mu\text{g/mL}$ XL-CRP (which had been shown to cause maximal MPA formation – see Fig.4.1) in the presence and absence of 28 $\mu\text{g/mL}$ Mab 9E1, which blocks MPA formation. Platelet activation significantly shortened the clotting time (661.3 ± 184.6 s vs. 329.8 ± 63.02 s; $p=0.0201$; $n=4$), but blocking P-selectin-mediated interaction had no effect (Fig.4.8). However, blocking direct platelet-monocyte contact slightly, but significantly, prolonged the clot formation time (168.5 ± 42.1 s vs. 133.3 ± 32.2 s; $p=0.007$; $n=4$). There was no significant difference in the maximum amplitude (maximum clot strength) in the presence of Mab 9E1. This suggests that direct monocyte-platelet interaction does not play a large part in the early stages of clot formation and structure.

4.2.7. Identification of candidate soluble mediators

In order to identify soluble mediators which might be involved in the upregulation of TF and/or TFPI in monocytes, a CBA kit was used to simultaneously detect by flow cytometry the presence of IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF α in plasma. This particular combination was chosen since a number of these cytokines have been reported to be released either by activated platelets or monocytes, though not necessarily over the timescale relevant to this study. Some, such as TNF α (Mackman, 1995), are known to induce TF in monocytes. Plasma was prepared after activation by XL-CRP of platelets in either PRP or whole blood following an incubation of 10 min or 2 h. Plasma

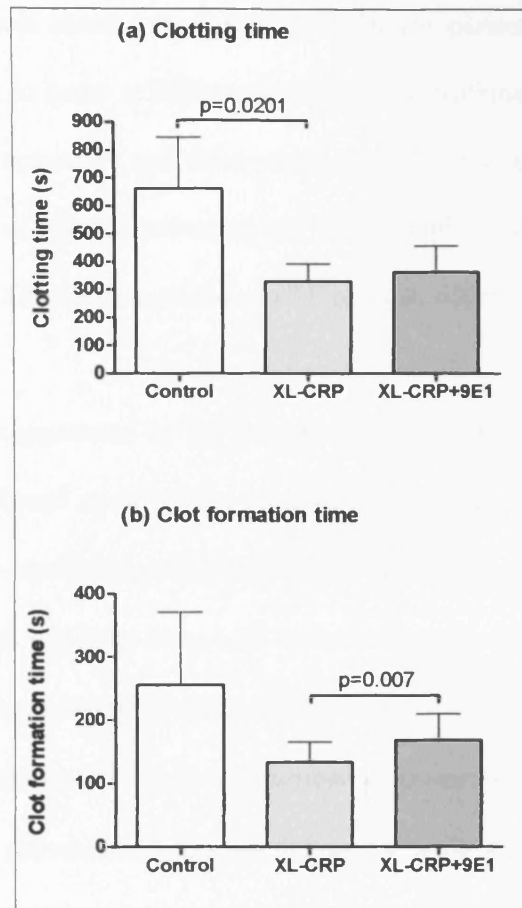


Figure 4.8. Blocking P-selectin mediated monocyte-platelet interaction extends clot formation time. Platelets were activated by 14 $\mu\text{g/mL}$ XL-CRP in the presence and absence of 28 $\mu\text{g/mL}$ P-selectin blocking antibody 9E1 and clotting times and clot formations time measured by thromboelastography in the RoTEM® Whole Blood Haemostasis Analyser. Data are from 4 individual donors.

was also prepared from whole blood activated by LPS for comparison. The rationale for choosing these incubation times was in order to differentiate between cytokines released immediately from platelet granules following activation and those which might be synthesised either in the platelet or in the monocyte as a result of platelet activation and which might be detectable in plasma after 2 h, still leaving sufficient time for the upregulation in TF or TFPI which had been shown to occur by 4 h (Figs.4.4 and 4.5).

The standard curves generated by the manufacturer's software are shown in Fig.4.9. There was no IL-10 or IL-12p70 found in any of the samples investigated. Fig.4.10 shows the data where cytokines were recorded at levels above the level of the negative control. Of these, TNF α was, as expected, seen in the plasma collected from LPS-treated whole blood samples after 2 h incubation. Although the difference between this and the control was great, it did not achieve significance, presumably because of variation in the small number of donors (1206 ± 669 vs. 7.0 ± 4.6 pg/mL; $p=0.0890$; $n=3$). However, activation of platelets did not cause the release of TNF α above the level of the controls either directly from platelets or indirectly from monocytes. The same was also true of IL-6, which was found in plasma from LPS stimulated blood compared with control after 2 h (822 ± 357 vs. 0 pg/mL; $p=0.0574$). Plasma isolated from whole blood incubated with LPS for 2 h had a concentration of IL-1 β of 471 ± 394 vs. 3.7 ± 6.5 pg/mL ($p=0.1722$). Only low levels of IL-1 β were seen in other samples, although the hint of an increase in the plasma derived from whole blood incubated with XL-CRP (15.6 ± 15.0 vs. 3.7 ± 6.5 pg/mL; $p=0.3250$) for 2 h may argue for this to be examined in a larger number of donors. A different pattern was seen for IL-8: after 2 h, there was a significant difference between the control and XL-CRP activated plasma derived from PRP (2.3 ± 2.3 vs. 8.5 ± 0.6 pg/mL; $p=0.0414$), although the levels were very low compared with the whole blood derived plasma samples. At this time, levels were raised in all the whole blood derived plasma samples, with XL-CRP levels being intermediate between the control and LPS-stimulated samples (1065 ± 765 , 211 ± 147 and 1819 ± 1173 pg/mL respectively). The variation in this small

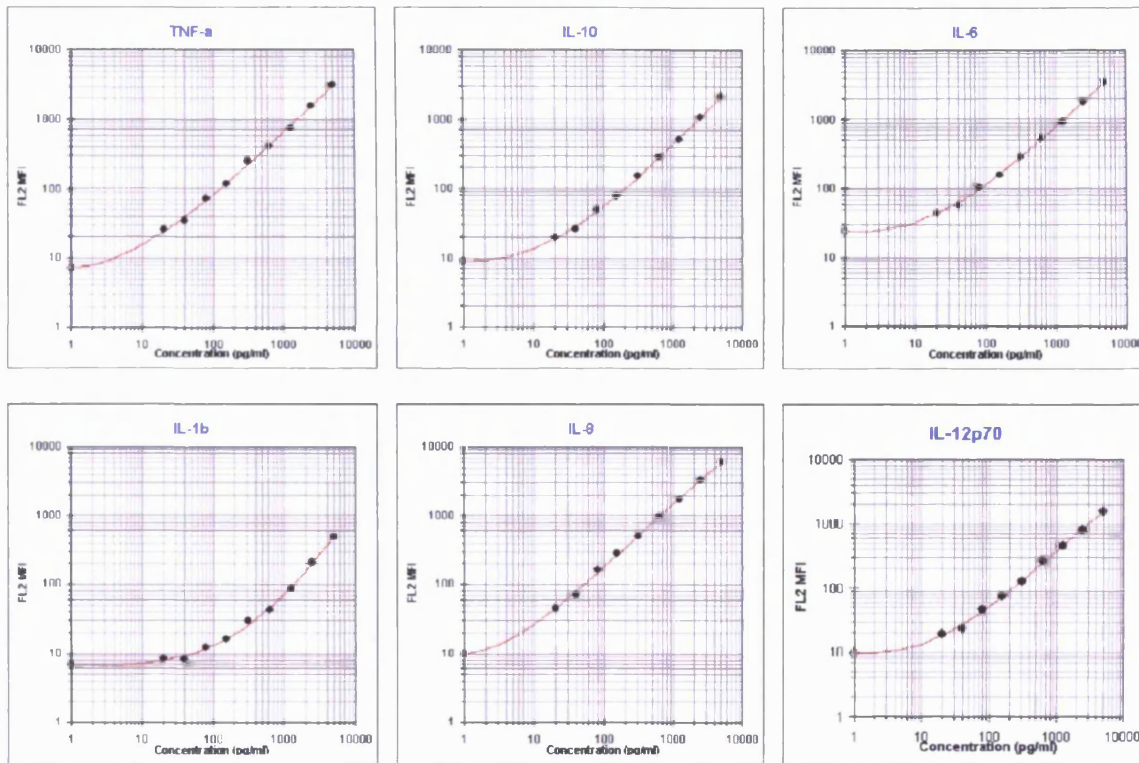


Figure 4.9. Standard curves for CBA assay. Standard curves for the cytokines IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF α were compiled by Becton Dickinson software from standards supplied in a kit used according to the manufacturer's instructions.

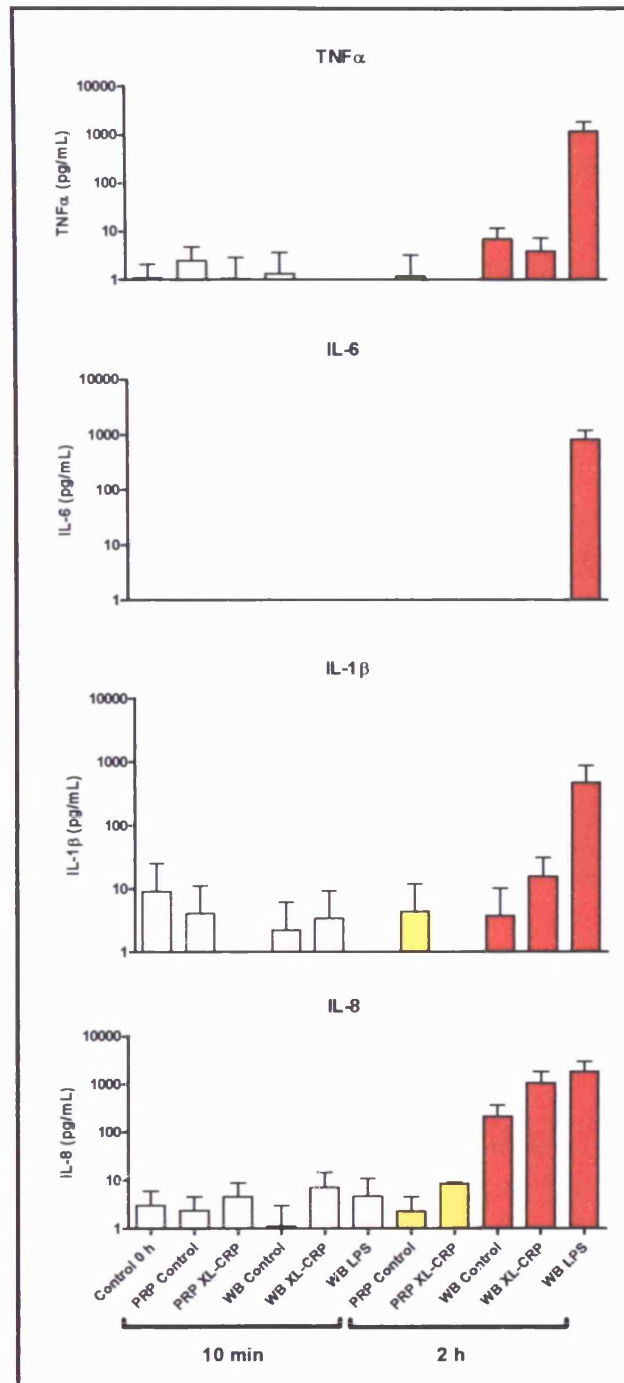
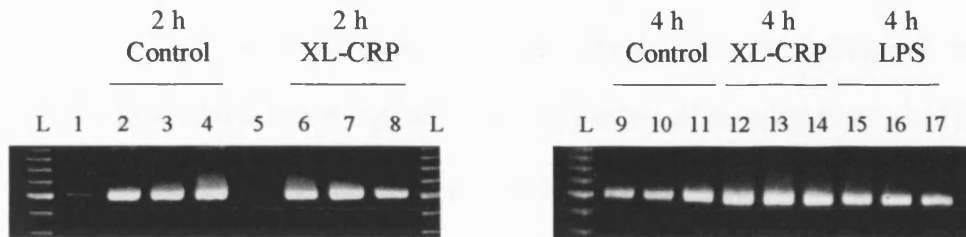


Figure 4.10. Cytokine secretion by platelets and monocytes following platelet stimulation. Plasma was isolated from whole blood and PRP of three separate donors following activation with 50 $\mu\text{g/mL}$ XL-CRP and incubation for 10 min and 120 min. Plasma was assayed simultaneously for IL-1 β , IL-6, IL-8, IL-10, IL-12p70 and TNF α using a cytometric bead array. Levels of IL-10 and IL-12p70 were below the detection limit throughout. Note that the concentrations are plotted on a log scale. Plasma from whole blood stimulated with 200 ng/mL LPS served as a positive control. Data are shown as means \pm SD for three individual donors.

(a) IL-8

(b) TNF α (c) IL-1 β 

L: ladder

1: C₀2: C₂3: C₂ + 9E14: C₂ + RFGP56

5: Water blank

6: XL-CRP₂7: XL-CRP₂ + 9E18: XL-CRP₂ + RFGP569: C₄10: C₄ + 9E111: C₄ + RFGP5612: XL-CRP₄13: XL-CRP₄ + 9E114: XL-CRP₄ + RFGP5615: LPS₄16: LPS₄ + 9E117: LPS₄ + RFGP56

Figure 4.11. Upregulation of IL-8, TNF α and IL-1 β in monocytes is not dependent on direct monocyte-platelet interaction. Platelets in whole blood were activated with 150 μ g/mL XL-CRP in the absence and presence of P-selectin blocking antibody 9E1 and GPIIb/IIIa blocking Mab RFGP56 (both at 25 μ g/mL). After 2 and 4 h incubation at 37 °C, monocyte mRNA was extracted and RT-PCR performed using IL-8, TNF α and IL-1 β primers. Gels are for (a) IL-8, (b) TNF α and (c) IL-1 β PCR products.

number of donors was probably responsible for these differences not reaching significance, but the results do suggest that this experiment could usefully be performed on plasma from more donors.

To determine whether direct monocyte-platelet contact influenced monocyte gene expression of IL-8, TNF α and IL-1 β , RT-PCR was performed on mRNA extracted from monocytes in blood where platelets were activated by XL-CRP in the presence of the P-selectin blocking Mab 9E1 (and also the GPIIb/IIIa blocking Mab RFGP56). In all cases there was an increase in gene expression during the course of the incubation both in the controls and the XL-CRP stimulated samples. Blocking direct monocyte-platelet interaction did not abolish expression of any of the three cytokines (Fig.4.11), indicating that direct contact was not essential for their upregulation.

4.2.8. TF surface antigen on monocytes and MPAs

Flow cytometric data obtained by a three-colour assay using anti-TF Mab 4508CJ for TF antigen on the surface of monocytes and MPAs are shown in Fig.4.12. The data are presented as the number of TF-negative and -positive events (mean \pm SD) as composite bars in order to indicate the proportion of each within a given population, whilst simultaneously showing the total number of events in this population. Under all experimental conditions, the proportion of TF-positive events was greater amongst MPAs than in single monocytes. It is notable that where >100 total MPAs were analysed at the first time point, in XL-CRP stimulated blood with and without GPIIb/IIIa blockade by RFGP56, 86% and 71% respectively of the total were TF-positive. In none of the samples was there any clear increase in the number of TF-positive events despite the upregulation of the gene shown in Fig.4.4, though it is possible that translation and surface expression would take longer than the 4 hours examined. It was also notable that in samples activated with XL-CRP in the presence of Mab RFGP56 in which the number of MPAs fell over the first hour, there was no increase in the number of free monocytes positive for TF which would reflect the fall in the number of TF-positive MPAs. In other words, once dissociated from MPAs, monocytes appeared to lose their TF. This and the

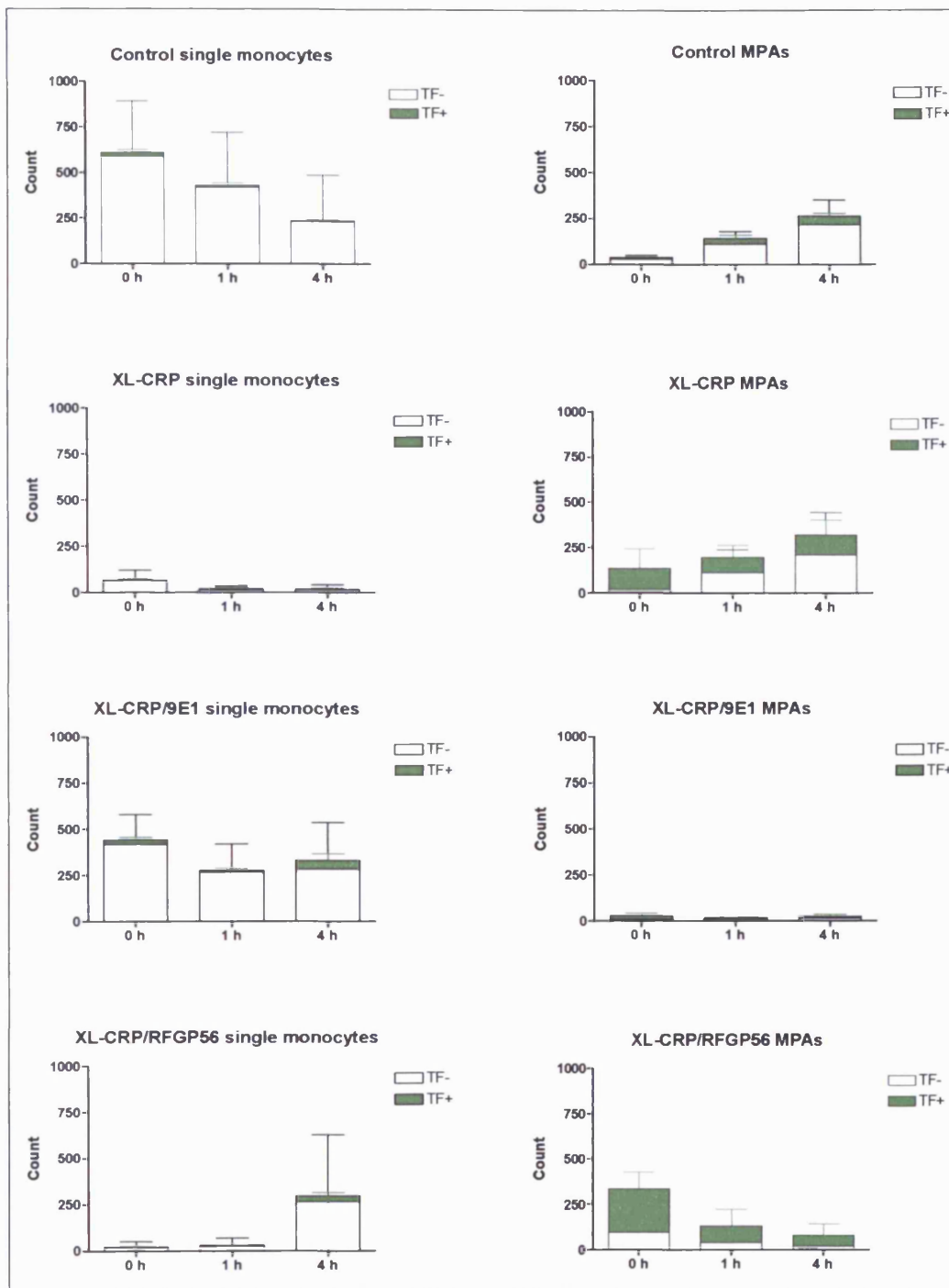


Figure 4.12. Effect of platelet activation on the percentage of monocytes and monocyte-platelet aggregates with TF on their surface. Platelets in whole blood were maximally activated with XL-CRP, with and without the P-selectin blocking antibody 9E1 or the GPIIb/IIIa blocking antibody RFGP56 (both at 25 $\mu\text{g/mL}$) and incubated for 4 h at 37 $^{\circ}\text{C}$. At 5 min, 1 h and 4 h, subsamples were analysed by flow cytometry to determine the percentage of monocytes (a) and MPAs (b) positive for TF. Experiments were performed using blood from 5-10 individual donors.

rapid surface expression on MPAs led to the suspicion that the antibody was detecting antigen on platelets as well as on monocytes.

To examine this possibility, the amount of TF on MPAs was related to the number of platelets involved in the aggregates, by plotting the MPA TF MFI against CD42b MFI (Fig.4.13). Data were from 4 independent experiments from those reported above and covered all conditions in time and activation plus blocking. Linear regression showed that there was a significant positive correlation between the two ($p=0.0422$), although the association was not a strong one ($r^2=0.1127$).

4.2.9. Short timescale increases in monocyte TF surface antigen following platelet activation

In the light of the data above suggesting that the antigen detected by the antibody might be platelet-associated, the rapid appearance of TF on MPAs was examined in more detail by taking subsamples from whole blood incubated with 150 µg/mL XL-CRP at intervals of 30 s, 1, 2, 3, 4, 5, 10, 30 and 60 min. Subsamples were transferred into flow cytometry tubes for assay for MPA formation and TF antigen. As shown in Fig.4.14, there was an immediate increase from the initial level of 2.9 ± 2.5 % to a peak of 58.1 ± 34.2 % at 3 min, followed by a fall in the percentage of MPAs over the next 7 minutes (Fig.4.14a). The percentage of MPAs then rose steadily from about 26 to 59 % between 10 and 60 minutes. Within 30s of activation, the percentage of MPAs which was TF positive rose from just below 30 % to nearly 80 % before gradually falling to about 19 % at the end of 60 min, which is in the second quartile of the 1 h data shown in Fig.4.12. In contrast, the percentage of TF⁺ single monocytes remained relatively unchanged at about 20 % over the time course, a situation which continued over the next 3 h as indicated in Fig.4.12. Immediately after activation, the amount of TF on MPAs appeared to be higher than that on single monocytes although by 30 min, the MdFI on the two classes of monocyte was little different.

The three sets of data for MPAs and TF antigen on free and aggregated monocytes need to be considered in conjunction with each other. Since the system was a closed one with an

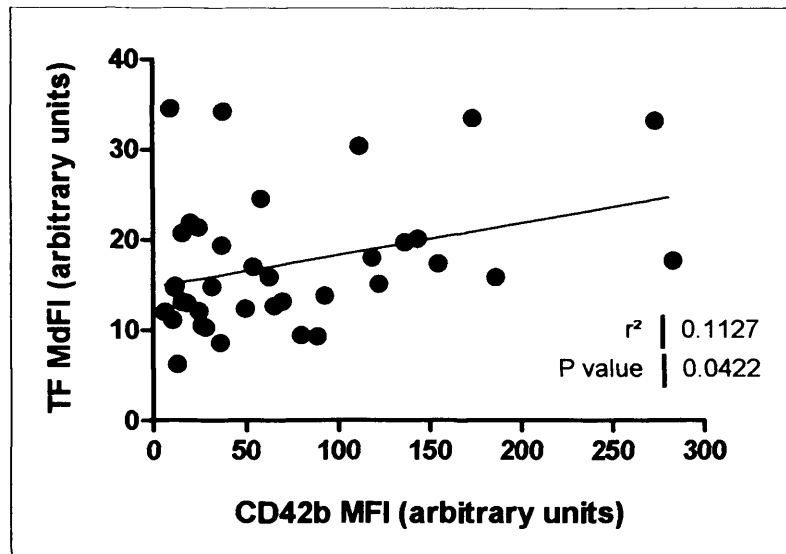


Figure 4.13. Correlation between MPA surface TF antigen and platelet burden. The mean fluorescence of CD42b-RPE and median fluorescence of CD142-FITC (TF) on MPA was determined by flow cytometry. CD42b fluorescence was indicative of the platelet load on monocytes. There was a significant positive correlation between surface TF and the number of platelets aggregated with monocytes ($p=0.0422$; $r^2=0.1127$; data from 4 independent experiments).

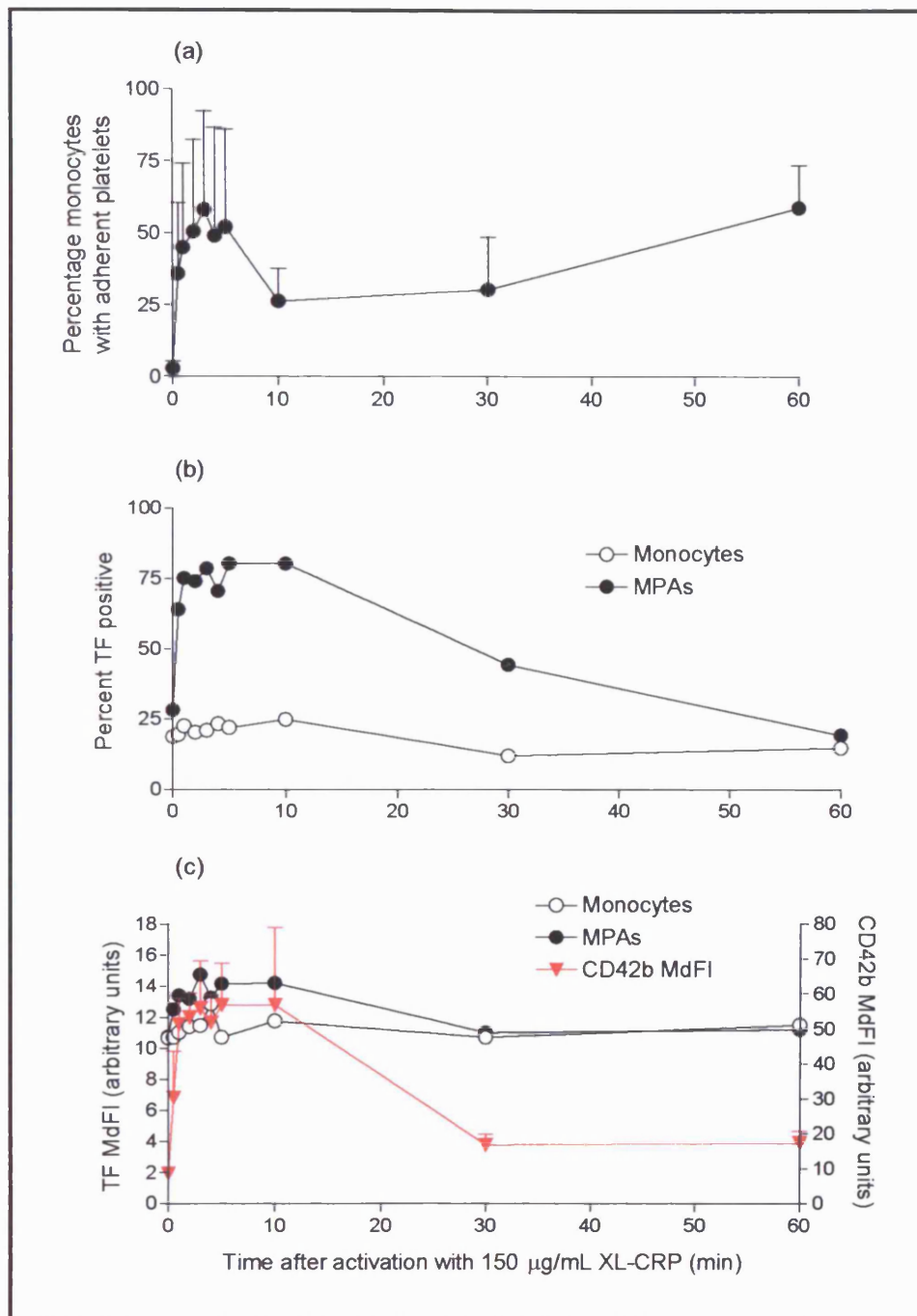


Figure 4.14. Formation of MPAs and TF surface antigen in 60 min following platelet activation. Platelets in whole blood were activated by 150 $\mu\text{g/mL}$ XL-CRP and subsamples analysed by flow cytometry to determine (a) the percentage of free monocytes and MPAs; (b) the percentage of monocytes and MPAs positive for TF; and (c) TF antigen on free monocytes and MPAs and (in red) the CD42b MdFl on MPAs. Data are from 2–3 independent experiments.

unchanging number of monocytes, MPAs forming after activation involve cells which previously had no attached platelets, and, conversely, where the percentage of MPAs falls, former aggregate members are included in the single monocyte data. Thus the rapid rise in TF⁺ MPAs is not simply due to the adhesion of platelets to monocytes which already have TF antigen on their surface, but implies that within 30 s MPAs have by some means become newly TF positive. Further supporting the hypothesis that the Mab #4508CJ was binding to an antigen on the platelet surface, the pattern of change in TF antigen on MPAs was paralleled by the number of adherent platelets as indicated by CD42b MdFI on the MPAs (Fig.4.14c, red line).

4.3. Discussion

4.3.1. Role of P-selectin and GPIIb/IIIa in the formation of MPAs

In contrast with at least one previous report (Rinder *et al*, 1991a), few monocyte-platelet aggregates were found in unstimulated samples. This may be due to methodological differences. Data from our laboratory suggest that unless a P-selectin blocking antibody is added to the flow cytometric reaction mixture (as was done in the present study), MPAs are formed *in vitro*, leading to an overestimate of their *in vivo* numbers. The data presented here confirm the importance of the P-selectin-PSGL-1 interaction in the formation of MPAs since they were completely abolished if platelets were activated in the presence of the Mab 9E1, as reported previously (Larsen *et al*, 1989; Fernandes *et al*, 2003).

Although the role of GPIIb/IIIa in monocyte-platelet aggregation has been investigated previously, *in vitro* studies have generally been over a short timescale following activation (Scholz *et al*, 2002; Steiner *et al*, 2003). As was the case here, these workers observed increased MPA formation 1-30 minutes after stimulation, presumably because prevention of platelet-platelet aggregation favours monocyte-platelet interaction. This project has extended this work by following monocyte-platelet interaction over 4 h, showing that GPIIb/IIIa has a role in stabilising MPAs over

a longer timescale, demonstrated in this project by the significant reduction in MPAs between 1 h and 4 h of the activation of platelets in the presence of the blocking Mab RFGP56. Studies into the effects of the GPIIb/IIIa antagonist abciximab in patients have generally reported a reduction in monocyte-platelet interaction (Neumann *et al*, 1999; Furman *et al*, 2005). Since these data were collected between 12 and 24 hours after abciximab administration, they are not inconsistent with the possibility that the antagonist causes an initial, transient increase in MPAs followed by their gradual dissolution.

It should be noted that a distinction can be made between the platelet load carried by monocytes, as determined by the fluorescence intensity of the platelet markers, and the percentage of monocytes with any adherent platelets. Divergent results may be obtained according to the measure used. Thus Steiner and co-workers (Steiner *et al*, 2003) noted an increase in the *percentage* of monocytes with attached platelets whilst at the same time recording a fall in the *number* of platelets attached to each monocyte as a result of GPIIb/IIIa blockade. In other cases, only the platelet mass attached to the monocytes was affected (reduced) by abciximab, the percentage positive remaining much the same (Neumann *et al*, 1999). This is probably attributable to the inhibition of platelet-platelet aggregation such that only single platelets, not aggregates, are able to adhere to monocytes. The latter reported a correlation between the number of platelets aggregated with a monocyte and the upregulation of MCP-1, but whether this is also the case for other effects is unclear. The data in this project differ from both these inasmuch as the significantly higher percentage of MPAs seen in samples activated in the presence of RFGP56 at 5 min (Fig.4.2) was not accompanied by any significant reduction in the number of adherent platelets (Fig.4.3). Although Neumann *et al* (*ibid*) measured MPAs 15 min after activation with ADP, which did not coincide with any of the time points chosen here, Steiner *et al* (*ibid*) monitored MPA formation and TF for 1 h after stimulation with TRAP so that the 5 min and 1 h points are comparable. At 1 h the data are in agreement, with no significant difference between the control and blockaded samples.

However, though the 5 min data in this project diverge from Steiner *et al* in being higher than the control XL-CRP samples, the data are very variable so that more work is required to clarify the effect of GPIIb/IIIa.

It is uncertain what the consequences of this pattern of MPA formation and dissolution might be. It has been suggested that the initial boost given to MPA formation and consequent prothrombotic reaction might be one reason for some of the adverse effects of anti- GPIIb/IIIa therapy (Zhao *et al*, 2004). However, it is possible that prolonged monocyte-platelet contact may be necessary for some effects to occur, and these may be attenuated by these antagonists. This will be discussed further in the context of TF below.

4.3.2. Induction of monocyte TF gene expression following platelet activation

The induction of monocyte TF by activated platelets was investigated using semi-quantitative RT-PCR. The isolation of monocytes from peripheral blood was achieved through the use of anti-CD14 monoclonal antibody-coated magnetic beads, which has two possible disadvantages. Firstly, monocytes isolated in this way may or may not have platelets attached, particularly after incubation for an hour or more; and, secondly, the binding of the antibody to CD14 (the LPS receptor) might lead to activation of the monocyte, and indeed it is possible that the preparation process causes some activation by other means. However, both potential problems were addressed by the use of controls at each time point and not merely at the start of the experiment. The addition of the P-selectin blocking antibody 9E1 would have minimised the number of MPAs extracted in these samples and should ensure that the cDNA was overwhelmingly monocytic in origin. It should also be mentioned that some have criticised the use of GAPDH as a house-keeping gene; alternatives are ribosomal 18S or actin. However, GAPDH appeared to be fairly constant under these conditions.

Variable amounts of TF mRNA were detected in the freshly isolated monocytes of the three donors examined here, as has been noted elsewhere (McGee *et al*, 1994; van der Logt *et al*, 1994),

although some have been unable to detect any mRNA in unstimulated samples (Osnes *et al*, 1996; Neumann *et al*, 1997a; Steiner *et al*, 2003; Lindmark *et al*, 2000). The ability of activated platelets to induce TF mRNA in human monocytes has been reported previously (Lindmark *et al*, 2000; Steiner *et al*, 2003). However, these workers used TRAP as an agonist and it is possible that monocytes as well as platelets were directly activated since monocytes also possess PAR-1 receptors (Colognato *et al*, 2003), which have recently been shown to be a possible route of monocyte activation (Schaffner *et al*, 2005). Although an earlier paper demonstrated upregulation of monocyte TF in response to P-selectin (Celi *et al*, 1994), the P-selectin used was purified from platelet membranes and not in its native state. Since there have been no reports of the presence of GPVI on any circulating blood cells other than platelets, the experiments here using the GPVI-specific agonist XL-CRP unequivocally demonstrate the influence of platelet activation on TF gene expression in monocytes.

4.3.3. Effect of platelet activation on monocyte TFPI gene expression

The data presented here indicate that platelet activation leads to the induction of TFPI in monocytes after 4 h. It was important to ascertain that the TFPI mRNA found was indeed associated with monocytes rather than platelets. To this end, P-selectin mRNA was amplified from the same cDNA in the expectation that it would serve as a platelet marker. However, it was found in approximately the same quantities in all the samples (Fig. 4.5), even in those in which the P-selectin blocking antibody had been employed, greatly reducing the number of contaminating platelets attached to monocytes. Similarly, there was no distinct increase in samples where there would have been a large number of adherent platelets. Although it is conceivable that P-selectin mRNA could be of endothelial cell origin, this is highly unlikely to be the case since endothelial cells are present in blood only at very low levels. Moreover, as endothelial cells constitutively express TFPI, the TFPI mRNA would have been expected to follow the P-selectin pattern were it of endothelial origin. This

seems to suggest that P-selectin mRNA might emanate at least in part from monocytes. In support of this theory, it has been reported that P-selectin is differentially expressed in dendritic cells compared with macrophages and cDNA array images show low levels of P-selectin in the 'parent' monocytes (Baltathakis *et al*, 2001). Since the levels of P-selectin were more or less constant when the platelet load was varying, the inference is that the TFPI was upregulated in the monocyte as a result of platelet activation. As was the case with TF, the presence of, if anything more, TFPI mRNA when direct platelet-monocyte interaction was blocked (reinforcing monocytic origin) suggests that soluble mediators are responsible for this, at least to some extent.

The literature regarding the interaction between platelets and monocytes in terms of TFPI upregulation is sparse. In one study, co-culture of thrombin-activated platelets with monocytes was found to have no effect on monocyte TFPI mRNA, whereas LPS led to a five-fold increase (Steiner *et al*, 2003). Although at first sight this appears to contradict the findings presented here, the timescales involved were different, being 24 h compared with 6 h in the present study. It is also possible that the different agonists may account for the discrepancies, since, as noted earlier, monocytes as well as platelets possess PAR-1 receptors. Since both XL-CRP and TRAP are strong platelet agonists, one would not expect there to be a great difference in any effects of platelet activation mediated by soluble agonists released from the platelets, implying the importance of the primary agonist.

As to the physiological significance of the data here, it is possible that platelet induced upregulation of first TF and then TFPI in monocytes is a restraint mechanism in normal thrombus formation. Co-localisation of TF and TFPI on monocytes would seem a logical arrangement, and conversely, separation of TF and TFPI in atherosclerotic plaques has been posited to contribute to their thrombogenicity (Crawley *et al*, 2000).

4.3.4. Mode of action of platelets on monocytes

Allowing for the insensitivity of the assays, it is still clear from both the surface antigen and gene expression experiments that there is no absolute requirement for cell-cell interaction for TF upregulation. The data presented here do not suggest a major role for P-selectin-PSGL interaction in the induction of monocyte TF or TFPI, nor of IL-8, IL-1 β or TNF α , although some influence cannot be ruled out since the techniques employed do not allow discrimination of small changes. In fact, TF and TFPI gene expression appeared to be slightly enhanced in the presence of the P-selectin blocking antibody 9E1. Since blocking P-selectin virtually abolished the formation of MPAs, it can also be deduced that monocyte-platelet interaction mediated through other pathways, such as CD40-CD40L, are also relatively unimportant in upregulating monocyte TF or TFPI. However, soluble CD40L, released following cleavage of surface-expressed CD40L and thought to derive predominantly from platelets (Andre *et al*, 2002), might induce monocyte TF. Although CD40L, expressed on activated platelets (Henn *et al*, 1998), has been shown to upregulate monocyte TF mRNA and antigen (Lindmark *et al*, 2000; Amirkhosravi *et al*, 2002), possibly via oxidative stress (Sanguigni *et al*, 2005), in neither of the former cases was the interaction an absolute requirement.

Previous reports have not been unanimous on the effect of activated platelets on monocyte TF. Although purified P-selectin has been found to induce TF in monocytes (Celi *et al*, 1994), the effect was small compared with that of activated platelets which suggests that other factors may be involved. Others failed to find any increase in TF activity after monocytes were incubated with activated platelets, purified P-selectin or RANTES (Weyrich *et al*, 1996). The data in this project support the earlier findings in showing that platelets do influence monocyte TF expression, but imply a predominantly soluble mediator mode of action rather than a P-selectin-PSGL-1 route.

Of the possible platelet-induced soluble mediators, 12-HETE has already been implicated (Pellegrini *et al*, 1998). Cytokines have long been known to influence monocyte TF gene

expression. Candidate cytokines were investigated in this project using a kit which simultaneously measures the plasma concentrations of six cytokines. The range available at the time was limited but that chosen included five cytokines which have been implicated in coagulation. Since IL-1 β has been shown to induce TF in monocytes (Osnes *et al*, 1996) and this cytokine is reportedly synthesised by thrombin-activated platelets (Lindemann *et al*, 2001), it was anticipated that this would also play a role when platelets were activated by collagen. Although TNF α is recognised as inducing TF in endothelial cells (Tijburg *et al*, 1991), its effect on monocytes is disputed (Conkling *et al*, 1988; Osnes *et al*, 1996). Both IL-6 and IL-8 have been linked with an increase in monocyte procoagulant activity in mononuclear cells when incubated with the recombinant cytokines (Wada *et al*, 1993; Neumann *et al*, 1997a), although some workers have been unable to detect such an effect of IL-6 (Schwager & Jungi, 1994). In contrast, IL-10, a precursor transcript of which has been found in platelets (Lindemann *et al*, 2001), appears to have an inhibitory effect on monocyte TF activity (Ramani *et al*, 1993; Osnes *et al*, 1996).

In agreement with previous results using the same commercial kit, the levels of all cytokines in control plasma were either undetectable (limit 2.5 pg/mL) or very low (Reitsma & Rosendaal, 2004). IL-10 and IL-12p70 were unchanged throughout. Although there have been several reports of both these cytokines being induced in monocytes by LPS, this may have required longer stimulation than that applied here (Faas *et al*, 2002). It would therefore seem that any increase in TF would be unopposed by IL-10 over the timescale examined here. IL-6 and TNF α plasma concentrations were only raised in LPS stimulated samples and are therefore unlikely to be involved in platelet-initiated TF or TFPI induction, at least over 4 h. No significant increase in plasma IL-1 β levels were seen in any of the samples despite a seemingly large increase in the LPS-stimulated plasma. Although there have been reports of platelet IL-1 β activity following thrombin and collagen activation (Hawrylowicz *et al*, 1989), this was thought to be surface-associated. Lindemann and co-workers did find IL-1 β in the supernatant of thrombin-activated platelets (Lindemann *et al*, 2001),

but this required synthesis and by 2 h little mature or pro-IL-1 β had accumulated so the results here were not grossly inconsistent with this. Concentrations were reportedly only raised above control after 4 h, which, like this data, implies that IL-1 β is not involved in the platelet-induced increase in TF or TFPI gene expression seen at or before 4 h.

Increased levels of IL-8 were seen in all the samples after 2 h, although the only change that achieved significance in so small a number of donors was that between the control and XL-CRP activated PRP-derived plasma. This suggests that IL-8 is released from activated platelets, although the presence of contaminating leukocytes as a source cannot be ruled out. Although some authors have mentioned the presence of IL-8 in platelet α -granules (Boehlen & Clemetson, 2001; Gear & Camerini, 2003), the author has been unable to trace this to a particular reference, nor find it listed in any of the recent proteomics studies (Coppinger *et al*, 2004; McRedmond *et al*, 2004). As far as the author can ascertain, therefore, this is the first actual data to support the assertion of the existence of IL-8 in platelets. It is probably acquired from megakaryocytes, which have been shown to release IL-8 (Takeuchi *et al*, 1999), or possibly acquired from plasma. However, no IL-8 mRNA was detected in platelets (Power *et al*, 1995).

The ability of thrombin-activated platelets to signal synthesis of IL-8 in monocytes has been published previously (Weyrich *et al*, 1996; Neumann *et al*, 1997b; Waehre *et al*, 2004). The results presented here are consistent with that finding in that higher concentrations of IL-8 were found in plasma prepared from XL-CRP activated whole blood than PRP, implying an additional, non-platelet source. Thus it would seem that platelets might influence monocyte activity both through the secretion of IL-8 from their own granules and in promoting release from monocytes. Although IL-8 is primarily chemotatic rather than directly procoagulant in function, exposed neutrophils become activated, leading to a range of responses including the respiratory burst (Baggiolini *et al*, 1989). The release of superoxide may well have procoagulant consequences (Heistad, 2003).

However, RT-PCR did not demonstrate a role for XL-CRP-activated platelets in upregulating monocyte gene expression of IL-1 β , IL-8 or TNF α , since mRNA was also detected at approximately the same levels in control samples following incubation. This contrasts with data previously reported (Neumann *et al*, 1997b) which showed, by Northern blot, increased IL-8 and IL-1 β gene expression in monocytes incubated for 2 h with thrombin-activated platelets which appeared to be dependent on direct interaction. However, in contrast to the present study, blocking P-selectin-PSGL-1 interaction reduced (though it did not abolish) this effect. Although Neumann and co-workers minimised the effect of soluble mediators by using washed, fixed platelets for co-incubation with monocytes, they could not exclude the possibility of soluble factors influencing monocytes before fixation.

4.3.5. Effect of platelet activation on monocyte and MPA TF surface antigen

The presence of TF antigen on the monocyte or MPA surface was investigated using a flow cytometric assay essentially as described previously (Li *et al*, 1999). For reasons that are unclear, in activated samples the number of monocytes detected using this technique fell to low levels. This did not appear to be due to gross loss of monocytes in the sample, for example, because of adhesion to the test tube, since the Ac•T diff™ Haematology Analyser did not indicate any decline in numbers (data not shown). It is possible that aggregates became too large to be analysed. Gating first on single monocytes after lysis of red blood cells results in a larger number of events being analysed and is therefore a preferred method.

Both the 4-hour and short-timescale data suggested that what was being detected on MPAs was not exclusively monocyte-associated TF, but an antigen appearing on the platelets attached to monocytes which may or may not be TF. In the short timescale study, the rapid increase in the percentage of monocytes with adherent platelets was not unexpected, but the matching rise in the percentage of MPAs positive for TF over the first 5 minutes was less easy to understand. In

contrast, there was little change in the percentage of TF⁺ single monocytes in the first hour following activation. The rise was so rapid that it precluded *de novo* synthesis, implying, according to the classic view, either translocation of existing internal stores or a change in conformation to expose a new epitope. Another explanation, previously considered impossible, is that the TF is in fact on the platelets rather than the monocytes. Supporting this interpretation, TF antigen on MPAs paralleled the number of platelets in the aggregates.

The 4-hour results appeared to confirm previous findings that platelets influence monocyte TF in that a greater percentage of MPAs expressed TF compared with single monocytes (Amirkhosravi *et al*, 1996; Scholz *et al*, 2002; Steiner *et al*, 2003). However, as over the short-term, there was a relationship between the platelet load on the MPAs and TF antibody binding and monocytes which dissociated from MPAs failed to retain TF antigen, again suggesting that the antibody was at least in part binding to platelets. Earlier work using the same antibody also found that MPA formation was associated with increased TF which appeared to be associated with attached platelets (Steiner *et al*, 2003; Zhao *et al*, 2003). Indeed, in other work using Mab #4508CJ, it is unsurprising that the reported effect of GPIIb/IIIa antagonists on TF was in line with the authors' findings on its effect on MPA formation. Thus some have found increases in monocyte TF (Scholz *et al*, 2002) whilst others have found decreases (Steiner *et al*, 2003; Kopp *et al*, 2003). It is therefore questionable whether any of these studies are in fact demonstrating any effect of platelet activation on monocyte TF antigen and this needs to be addressed again using a reliable antibody. The possibility of platelet-associated TF will be explored in the next chapter.

Even with reliable antigen data, this study would have been limited by the absence of experiments to determine whether there was any TF activity on the monocyte surface. Earlier workers found that the mere presence of platelets shortened clotting times even in the absence of any other activator (Pinder *et al*, 1985) and there are other reports of the role of platelets in decrypting LPS-induced monocyte TF (Østerud, 2001). It is plausible that the upregulation of gene

expression seen in this study would at some point result in surface expression and it would be informative to know whether this was active with or without the presence of platelets.

The induction of TF in monocytes has often been considered to be a pathological phenomenon in view of its undoubted participation in inflammation and sepsis, and the marked effect of LPS (Østerud, 1995). However, the upregulation of monocyte TF by platelets demonstrated here and elsewhere is suggestive of a physiological function whereby thrombin generation is maintained within the thrombus, aiding clot stability and promoting wound healing. This would be unsurprising from an evolutionary point of view, since it seems unlikely that TF induction by platelets would not have a normal function. The timescale of TF gene expression demonstrated here, being significantly upregulated within 1 h of platelet activation, is consistent with this hypothesis, though surface expression of active TF remains to be demonstrated.

4.3.6. Concluding comments

Platelet-monocyte interaction contributes to the regulation of the procoagulant environment in blood through, *inter alia*, the induction of TF in the monocyte and the later upregulation of its inhibitor TFPI. Collagen-activated platelets also cause an increase in the plasma concentration of cytokines such as IL-8 which may also be procoagulant in effect. These interactions appear to be driven primarily through the action of soluble mediators such as cytokines whose action is localised. It is interesting to note, on the basis of data in the previous chapter (Fig.3.33), that inhibition of ADP receptors did not significantly reduce degranulation (demonstrated by P-selectin expression) in response to collagen, so drugs based on blocking this pathway may not inhibit platelet procoagulant interaction with monocytes if it is mediated by granule contents. The effects on gene expression occur over a longer timescale than would be expected for initial clot formation, so their physiological role would seem to be in the stabilisation of the clot. In the context of an atherosclerotic plaque, the interaction could contribute to pathological thrombogenicity.

Certain results presented above, such as the extremely rapid appearance of TF on MPAs and the apparent loss of TF on monocytes which became dissociated from platelets, were suggestive of TF being on platelets instead or as well as on monocytes. Following the seminal paper by Nemerson's group in USA (Giesen *et al*, 1999), this possibility seemed worthy of further investigation, as detailed in the next chapter.

4.3.7. Further work

The project could be extended by:

- Real-time PCR to confirm the differences in the timing of TF and TFPI gene expression and the effect of blocking the P-selectin-PSGL-1 interaction shown by semi-quantitative RT-PCR
- Re-examination of monocyte and MPA surface TF antigen using a different TF Mab and using the Optilyse© C method of preparing and analysing samples
- Western blotting to show TF and TFPI protein as a result of mRNA upregulation
- Activity assays for TF and TFPI to determine the activation status of any TF or TFPI antigen
- More detailed consideration of the role of soluble mediators as possible mediators of TF and TFPI upregulation of a wider range of cytokines. For example, isolated monocytes could be incubated with possible mediators e.g. RANTES (Kameyoshi *et al*, 1992) and subsequent TF and TFPI mRNA expression, antigen and activity measured
- Investigation into donor variation in the timing of TF and TFPI upregulation suggested by the data here

CHAPTER 5: Platelet Tissue Factor

5.1. Introduction

As indicated in the previous chapter, several strands of data led to the suspicion that platelets might themselves have TF on their surface. Hitherto, the only blood cells thought capable of expressing TF were monocytes, and then only induced as a result of inflammatory mediators. However, the publication at about this time of the seminal paper from Nemerson's group regarding blood-borne TF (Giesen *et al*, 1999) made a platelet-associated TF hypothesis more plausible than previously thought. This chapter sets out the work done to examine this hypothesis.

5.2. Results

5.2.1. Flow cytometric assays to detect TF on the platelet surface

Since the initial observations regarding possible platelet-associated TF were made using flow cytometry, it was logical to start the investigation with the same technique. The analysis depended on the antibody used. As the then only available conjugated TF Mab was labelled with FITC, it could be used simultaneously with RPE-CD42b and RPE-Cy5-CD14 in a three-colour assay which could distinguish between platelets and monocytes and measure TF on both. In some experiments, where unconjugated TF antibodies were used, so that FITC-conjugated secondary antibodies were necessary, or for an initial screen, platelet TF was initially examined in a single colour assay in which platelets were identified on the basis of their forward and side scatter. All experiments used whole blood except where stated.

5.2.1.1. Flow cytometry using anti-TF Mab #4508CJ

With a view to ensuring that any TF detected was associated with platelets and not with monocytes or monocyte-derived microparticles which might be attached to the platelets, the analysis of the three-colour flow cytometric assay was as shown in Fig. 5.1. Platelets were initially identified on

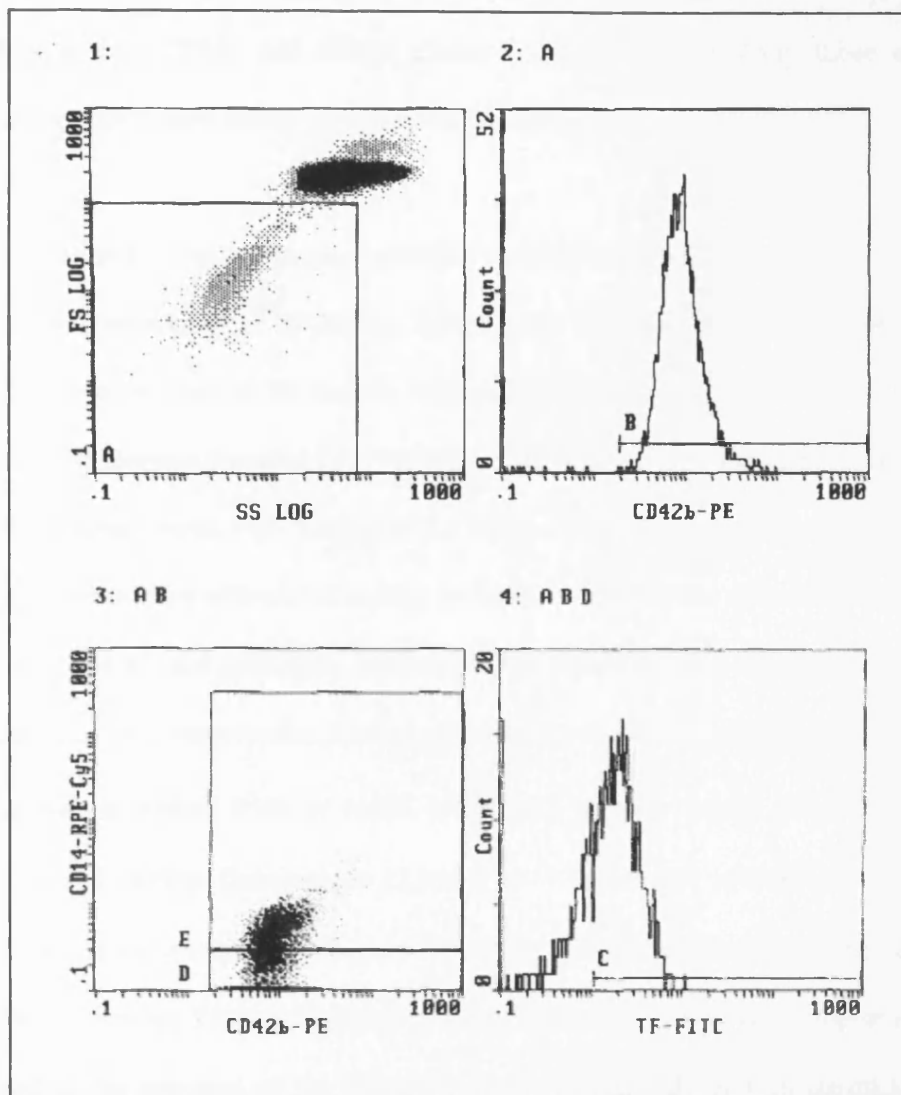


Figure 5.1. Three-colour, whole blood flow cytometric platelet TF assay. Platelets were identified on the basis of forward and side scatter (histogram 1) and also by their positivity for CD42b-RPE in the (histogram 2). They were then subjected to CD14-RPE-Cy5 vs. CD42b-RPE analysis (histogram 3) with only CD42b⁺/CD14⁻ events being further analysed for TF (histogram 4).

the basis of size and CD42b positivity (first and second histograms). CD42b-positive events were then divided into CD14⁺ and CD14⁻ events (third histogram). Only those events which were negative for CD14 were further analysed for TF antigen.

TF antigen detected on unstimulated platelets from normal donors

Unstimulated platelets from 10 healthy donors were analysed for TF using this assay. As shown in Fig. 5.2a, platelets from all the donors were positive for TF using the anti-TF Mab #4508CJ from American Diagnostica (range 64.8 - 96.0%; mean 80.1%). The median fluorescence ranged from 1.3 to 4.1 arbitrary units, with a mean of 2.17 (Fig. 5.2b). In a representative sub-set of experiments (n=5), platelets were confirmed as resting by being < 10% for the activation marker P-selectin (data not shown). To test the specificity of the antibody, samples from eight of the normal donors were incubated with antibodies in the presence of 6 µg/mL sTF (an approximately 10-fold molar excess). Binding was abolished both in terms of percent positive (range 1.8 – 8.0%; mean 3.6 ± 2.1 ; $p < 0.0001$) and median fluorescence (2.2 ± 0.8 vs. 1.1 ± 0.1 AU; $p = 0.005$; n=3) (Fig. 5.2). Overlaid traces (Fig. 5.2c) illustrate the complete inhibition of antibody binding in the presence of sTF. As confirmation that any TF was on platelets and not adherent monocytes, samples from 3 donors were incubated in the presence of the P-selectin blocking antibody 9E1 (6 µg/mL), but there was no significant reduction in either the percent positive ($80.1 \pm 8.0\%$ vs. $76.0 \pm 7.1\%$; $p = 0.3585$) or median fluorescence for TF (2.2 ± 0.8 vs. 1.8 ± 0.1 AU; $p = 0.3035$).

In order to demonstrate further the specificity of the blocking of antibody binding by sTF, a dose response curve was constructed (Fig. 5.3). This showed a progressive reduction in binding of the antibody 4508CJ, demonstrated by falling median fluorescence (from 3.6 to 1.4 arbitrary units) and percentages of positive platelets (from 80.6 % to 8.2 %), as the concentration of sTF increased. Maximum blocking was achieved at approximately 1.2 µg/mL. The binding of another monoclonal antibody, the platelet marker CD42b, in a separate single colour flow cytometric assay was

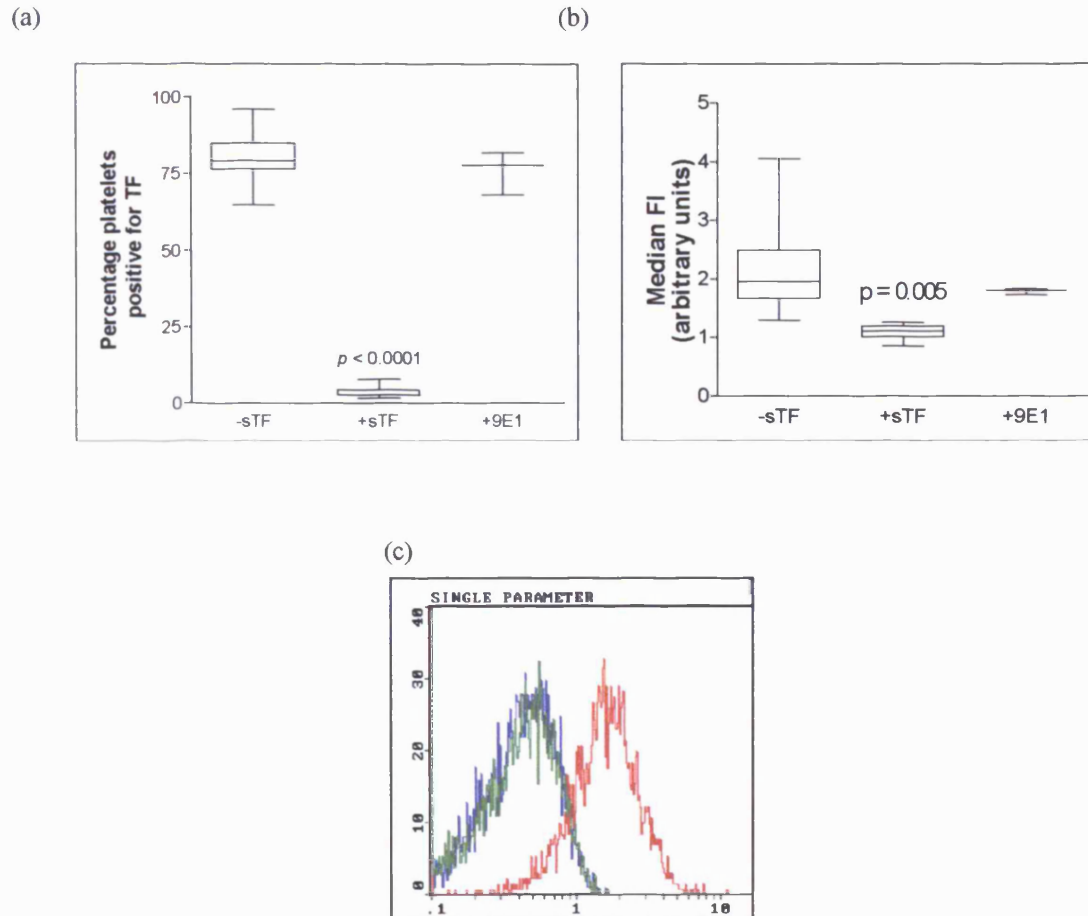


Figure 5.2. Percentage of unstimulated platelets from healthy donors positive for TF with and without blocking by sTF and anti-P-selectin Mab 9E1. The platelet TF assay was used to find the (a) percentage of platelets positive for and (b) the median fluorescence of anti-TF Mab #4508CJ with and without (i) 6 $\mu\text{g/mL}$ sTF and (ii) 6 $\mu\text{g/mL}$ Mab 9E1. The range of data is shown, whilst the box shows the 25th and 75th percentiles with the internal line being the median. The results are from 3-10 separate donors. (c) is a multigraph from a representative experiment showing the isotype control (blue), the trace with Mab #4508CJ without sTF (red) and with sTF (green).

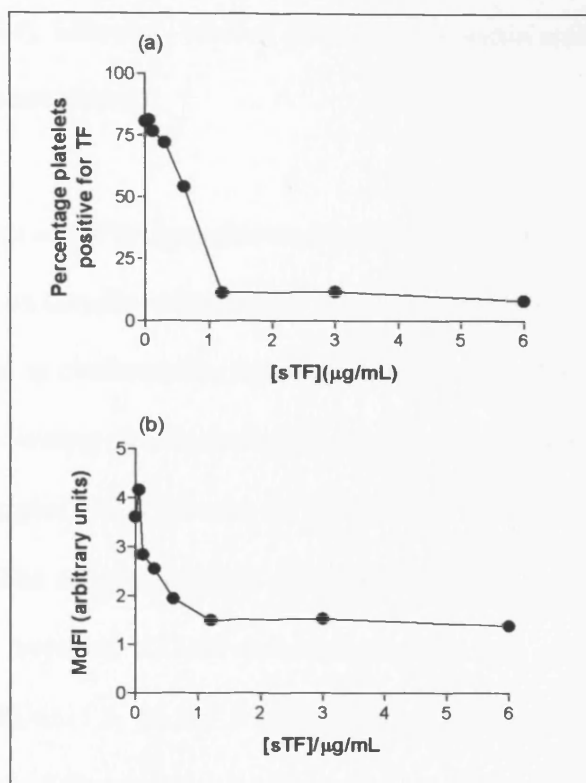


Figure 5.3. Anti-TF Mab 4508CJ binding to platelets is blocked by sTF in a dose-dependent manner. (a) percentage of unstimulated platelets positive for and (b) median fluorescence (MdFI) of 4508CJ, measured by flow cytometry after platelets were incubated with antibodies in the presence of 0 – 6 µg/mL sTF.

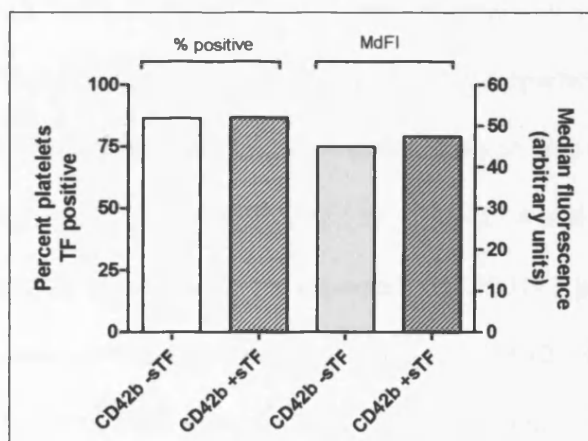


Figure 5.4. Binding of CD42b to platelets is unaffected by sTF. Percentage platelets positive for and median fluorescence of CD42b measured by flow cytometry after platelets were incubated with antibody in the presence or absence of 6 µg/mL sTF (Means; n=2).

unaffected by sTF (Fig.5.4). Likewise, binding of an anti-P-selectin antibody was also unaltered in the presence of sTF (data not shown).

Effect of platelet activation on TF antigen detected by Mab #4508CJ

Although TF was found on unactivated platelets, it was possible that it was also stored in internal granules or that a change in conformation upon activation would affect antibody binding, so that changes might be seen following platelet activation. To assess this, platelets were strongly activated by 10^{-3} M TRAP, 1.15 $\mu\text{g/mL}$ XL-CRP and 10 μM A23187 and analysed by three-colour whole blood flow cytometry. The only significant difference between the percentages of TF-positive platelets (Fig.5.5a) was between A23187-activated platelets and control, TRAP and XL-CRP samples (19.2 ± 18.1 vs. 71.4 ± 11.5 , 82.3 ± 7.5 and 70.8 ± 15.8 % respectively; $p=0.0206$, 0.0037 and 0.0018 ; $n=4$). Significant differences were also apparent in the MdfI data (Fig.5.5b), again, between A23187-stimulated platelets and control, TRAP and XL-CRP samples (1.3 ± 0.1 vs. 2.3 ± 0.1 , 2.9 ± 0.4 and 2.1 ± 0.5 AU respectively; $p=0.0003$, 0.0036 and 0.0310). In addition, there was significantly more TF antigen on TRAP-activated platelets than on both the controls and XL-CRP-stimulated platelets (2.9 ± 0.4 vs. 2.3 ± 0.1 and 2.1 ± 0.5 respectively; $p=0.0428$ and 0.0049). The concentration of A23187 used was known to produce platelet microparticles (see Chapter 3) and the differences in TF surface expression might be attributable simply to size differences, plotted in Fig. 5.5c. The sizes, in arbitrary units, were 13.0 ± 1.6 , 12.1 ± 1.4 , 12.7 ± 1.4 and 3.8 ± 0.2 in control, TRAP, XL-CRP and A23187 samples respectively. As expected, the A23187 platelets were considerably smaller than any of the other samples ($p=0.0020$, 0.0017 and 0.0012 vs. control, TRAP and XL-CRP samples respectively). The difference between the TRAP and XL-CRP-activated platelets, though small, was also significant ($p=0.0109$). It is tempting to speculate that the MdfI is related to size, but linear regression analysis was not appropriate since the data fell into two distinct populations (Petrie & Sabin, 2000).

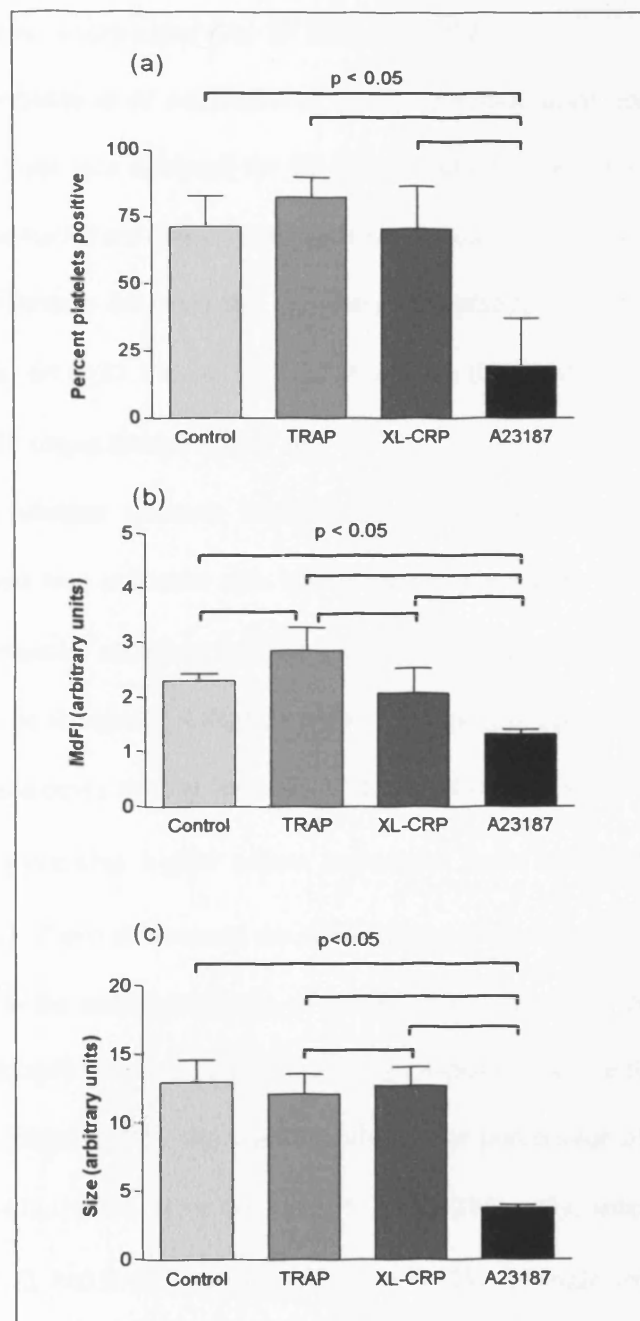


Figure 5.5. Activation of platelets did not significantly affect binding of Mab #4508CJ to platelets. Platelets were activated by final concentrations of 10^{-3} M TRAP, $1.15 \mu\text{g/mL}$ XL-CRP or $10 \mu\text{M}$ A23187 and examined for surface TF antigen using Mab #4508CJ in a three-colour whole blood flow cytometric assay. (a) percent platelets positive for TF; (b) median fluorescence; and (c) platelet size. All control samples were $< 7\%$ positive for P-selectin. Arrows indicate $p < 0.05$. Data are from 4 individual donors.

Validity of flow cytometric assay using anti-TF Mab #4508CJ

Lest the particular combination of antibodies in the three-colour assay led to artefact, platelets in whole blood and PRP were also analysed for TF using a single colour flow cytometric assay where platelets were distinguished from other blood cells on the basis of forward and side scatter. There was no significant difference between the percentage of platelets positive for TF in any of the assays ($69.1 \pm 16.8\%$ vs. $69.6 \pm 23.1\%$ vs. $59.1 \pm 27.0\%$ in the three-colour assay, single colour assay in whole blood and PRP respectively) (Fig. 5.6).

To determine whether calcium concentration or choice of leukocyte marker affected #4508CJ binding, blood was collected into Vacutainers® containing EDTA for comparison with citrated blood and in parallel samples, the pan-leukocyte marker CD45 was substituted for CD14, the assay being otherwise the same. A slightly higher percentage of platelets were TF-positive when CD45 was used as a leukocyte marker instead of CD14 (64.0 ± 7.4 vs. $53.4 \pm 9.9\%$; $p=0.0184$; $n=3$; Fig.5.7). The MdfIs were also higher where leukocytes were stained with CD45 (1.9 ± 0.2 vs. 1.6 ± 0.2 AU; $p=0.0466$). These differences are possibly due to discrepancies in colour compensation (adjusting for overlap in the emission spectra of the fluorochromes used) but the data were in broad agreement that unstimulated platelets bind anti-TF Mab #4508CJ. A role for calcium in the binding of Mab #4508CJ was suggested by the significantly higher percentage of TF⁺ platelets in EDTA compared with citrate ($72.7 \pm 12.5\%$ vs. $53.4 \pm 9.9\%$; $p=0.0386$; $n=3$), although the MdfIs were not significantly different (2.1 ± 0.5 vs. 1.6 ± 0.2 AU; $p=0.1387$). A single experiment examining the effect of other anticoagulants also suggested a role for calcium in that binding was less in lithium heparin and hirudin anticoagulated blood in which calcium concentrations would be physiological (Fig.5.8).

Although the basic observation that unstimulated platelets display TF antigen was not challenged by these results, it is notable that in this experiment, using a different batch of #4508CJ,

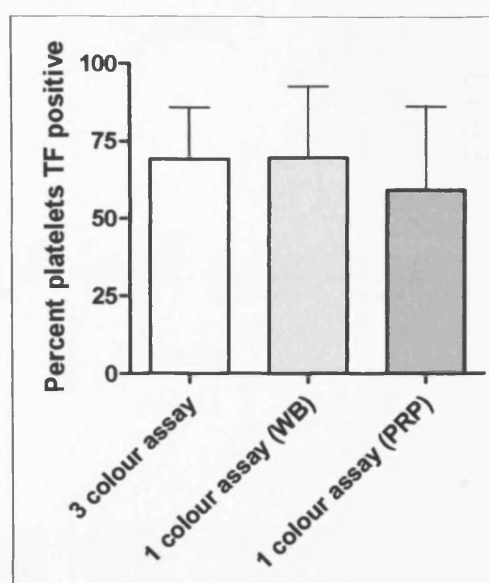


Figure 5.6. The percentage of platelets positive for TF was not influenced by the type of flow cytometric assay. Platelets were analysed for TF by flow cytometry using the whole blood three-colour assay described in Fig. 5.1 and a single colour assay for whole blood or PRP. There was no significant difference between any of the assays. Data are from three independent experiments using different donors.

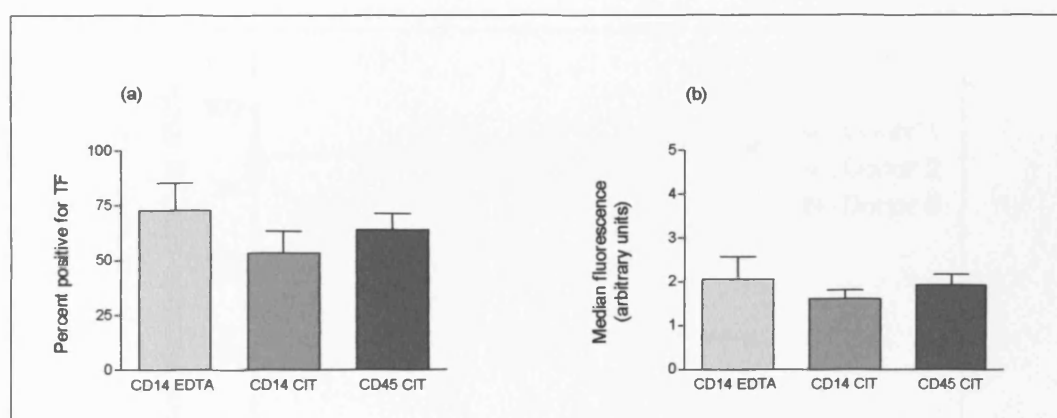


Figure 5.7. Effect of calcium concentration and leukocyte marker on platelet TF antigen. Platelets in citrated (CIT) or EDTA whole blood were analysed for TF, detected by Mab #4508CJ, by flow cytometry using the whole blood three-colour assay described in Fig. 5.1 using either CD14 or CD45. (a) percent TF positive and (b) median fluorescence. Data are from three separate donors.

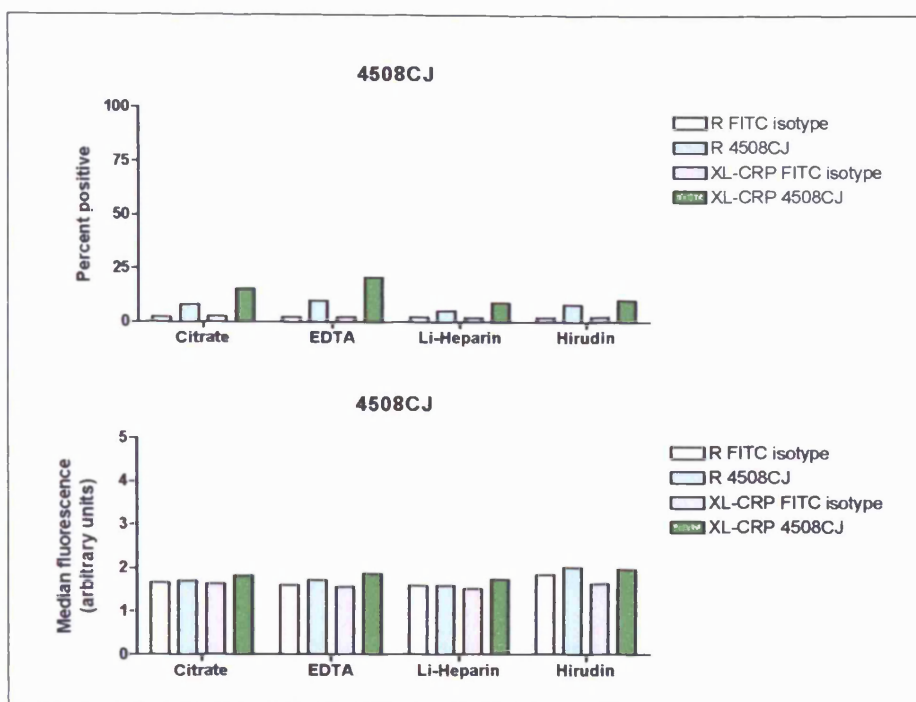


Figure 5.8. Effect of different anticoagulants on binding of anti-TF Mab #4508 to platelets. Blood was collected into Vacutainers® containing sodium citrate, EDTA, 17 IU/mL lithium heparin or into unanticoagulated Vacutainers® before being immediately transferred into hirudin (100 U/mL final concentration). Antibody binding to unstimulated platelets and platelets activated with 50 ng/mL XL-CRP was analysed by whole blood single colour flow cytometry. The results are from a single experiment.

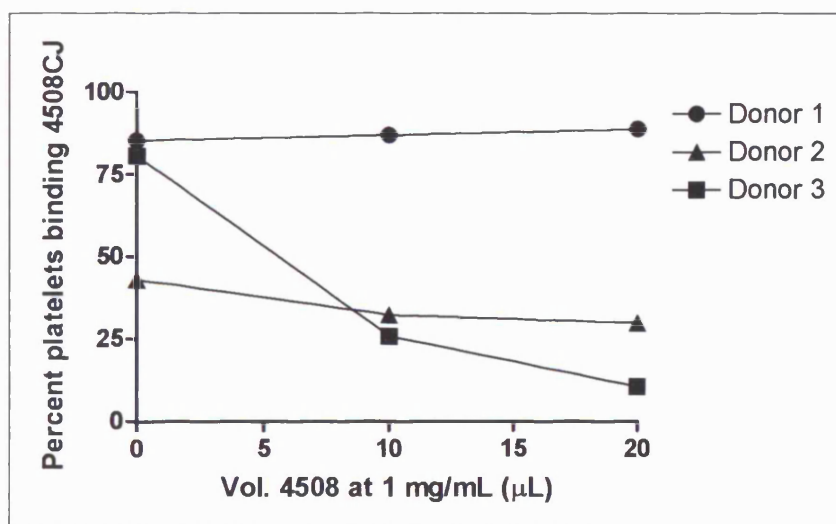


Figure 5.9. Inconsistency of blocking of FITC-labelled 4508 by unconjugated 4508. Whole blood was incubated with anti-TF Mab 4508CJ in the presence of the indicated volumes of 4508 and binding of the labelled Mab determined by flow cytometry.

the binding was considerably less than with a previous batch using blood from the same donor (8% vs. 79% positive for TF respectively). Further doubts about the reliability of Mab #4508CJ emerged. Following up anecdotal evidence that the unconjugated Mab #4508 failed to block the binding of its conjugated counterpart, it was found that there was considerable variation in the inhibition of Mab #4508CJ binding by unconjugated #4508 at a 60-fold molar excess (Fig.5.9). The batch of conjugated Mab used for all three donors was the same as that employed in the study of healthy donors (Fig.5.2).

To examine further the specificity of Mab #4508CJ, its ability to bind to monocytes stimulated with 200 ng/mL LPS for 7 h. Monocyte TF antigen was assessed using the Optilyse® C method of identifying monocytes in flow cytometry, in which red cells are lysed leaving leukocytes clearly identifiable as three distinct populations. Events classed as monocytes on the basis of their forward and side scatter characteristics were checked for CD14-positivity, and these were further analysed for TF antigen with Mab #4508CJ. Although the Mab 4508CJ bound to LPS-stimulated monocytes (48.0% and 8.4%; n=2), the binding was not completely blocked by 3 µg/mL sTF (reduced to 40.7% and 5.7% respectively). It was therefore decided to investigate platelet TF antigen using a different anti-TF antibody.

5.2.1.2. Flow cytometry using anti-TF Mab HTF-1

At the time of performing these experiments, there was only one commercially available conjugated anti-TF other than #4508CJ, namely, RPE-conjugated HTF-1 from BD Pharmingen. Because the antibody was labelled with RPE, it could not be used without adjustment in the three-colour flow cytometric assay described previously since the CD42b used in the assay was also RPE-conjugated. The initial experiments were therefore conducted using a single colour whole blood assay, platelets being distinguished on the basis of forward and side scatter. There was no HTF-1 binding to platelets evident using this assay whether the platelets were unstimulated or activated with TRAP,

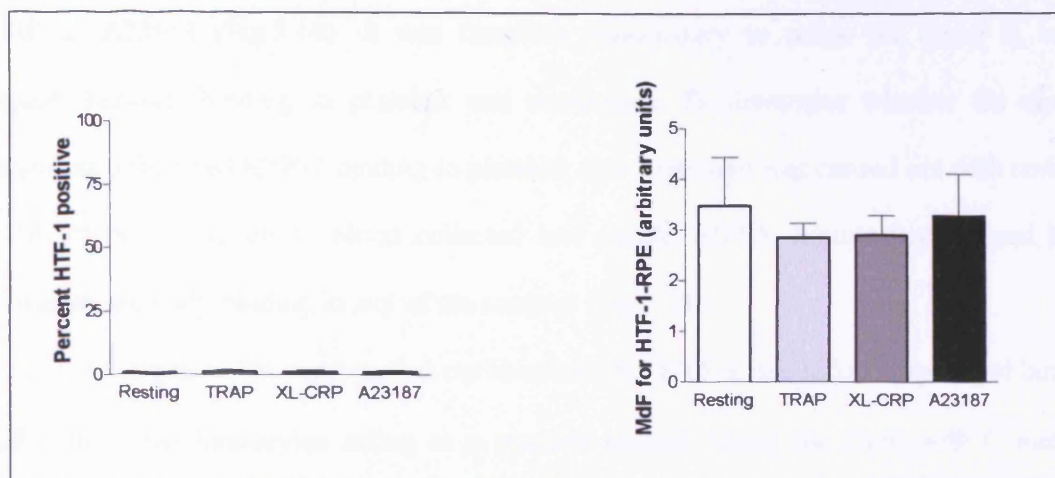


Figure 5.10. Anti-TF Mab HTF-1 did not bind to unstimulated or stimulated platelets. Platelets were activated by final concentrations of 10^{-3} M TRAP, 1.15 $\mu\text{g/mL}$ XL-CRP or 10 μM A23187. Whole blood flow cytometry using RPE-conjugated HTF-1 did not reveal any binding to unstimulated or TRAP-, XL-CRP- or A23187-stimulated platelets. Data was from 4 independent experiments using blood from different donors.

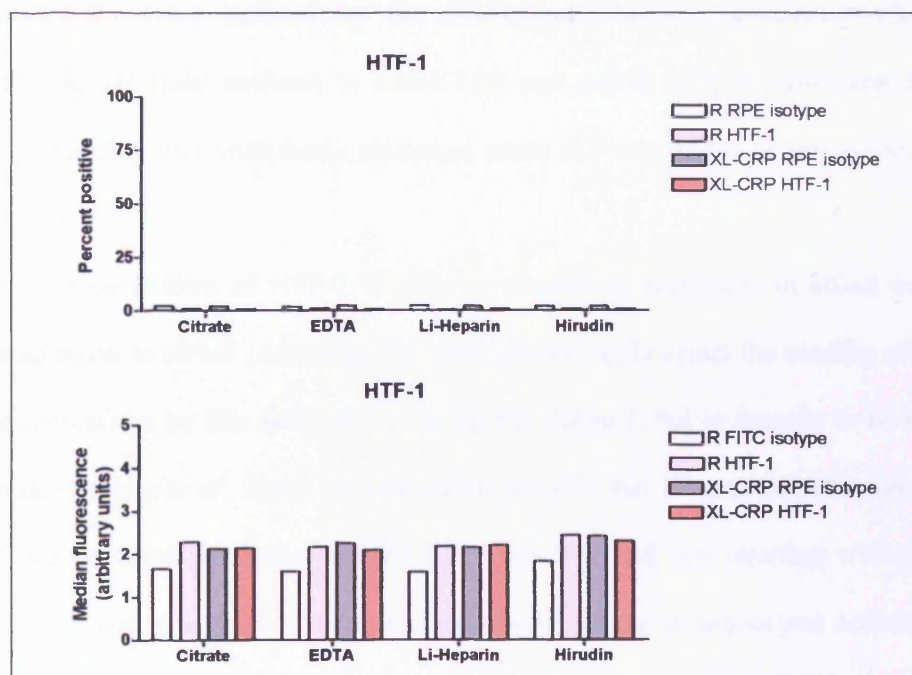


Figure 5.11. Effect of different anticoagulants on binding of anti-TF Mab HTF-1 to platelets. Blood was collected into Vacutainers® containing sodium citrate, EDTA, 17 IU/mL lithium heparin or into unanticoagulated Vacutainers® before being immediately transferred into hirudin (100 U/mL final concentration). Antibody binding to unstimulated platelets and platelets activated with 50 ng/mL XL-CRP was analysed by whole blood single colour flow cytometry. The results are from a single experiment.

XL-CRP or A23187 (Fig.5.10). It was therefore unnecessary to refine the assay in order to distinguish between binding to platelets and monocytes. To determine whether the choice of anticoagulant influenced HTF-1 binding to platelets, an experiment was carried out with resting and XL-CRP activated platelets in blood collected into citrate, EDTA, lithium heparin and hirudin. There was no antibody binding in any of the samples (Fig.5.11).

Several experiments were carried out to ensure that HTF-1 was indeed capable of binding to TF, LPS-stimulated monocytes acting as a positive control. Using the Optilyse® C method of analysing monocytes, there was very little HTF-1 binding to monocytes stimulated with LPS for 5 – 7 h in two out of three donors (data not shown). In the third donor, approximately 70 % monocytes were TF-positive after 7 h, but there was no distinct positive population, just a slight shift of the entire monocyte population. Following the protocol used by the suppliers for testing the antibody, whereby monocytes were isolated by the Histopaque® density gradient method and then resuspended in RPMI 1640 medium, to which LPS was added, 37.9 % monocytes bound HTF-1 after 4 h. This binding was completely abolished when sTF was added at the antibody incubation stage (Fig. 5.12).

The different pattern of HTF-1 binding to monocytes incubated in blood compared with those activated when in RPMI 1640 suggested that plasma might affect the binding of the antibody. The epitope recognised by this antibody is not clearly defined, but is thought to be near the FVII binding site (Kirchhofer *et al*, 2000). It is therefore possible that TF expressed on monocytes in the presence of plasma would immediately bind FVII, which would thus interfere with the binding of the antibody. This was examined by comparing antibody binding to monocytes activated by LPS in whole blood with those stimulated in RPMI 1640, in the absence and presence of 60 µg/mL recombinant FVIIa (rFVIIa; Novo Nordisk, Crawley, UK). This concentration of rFVIIa completely abolished antibody binding to monocytes in the absence of plasma, but did not affect binding in its presence (Fig.5.13). The histograms also illustrate the different binding patterns seen on isolated

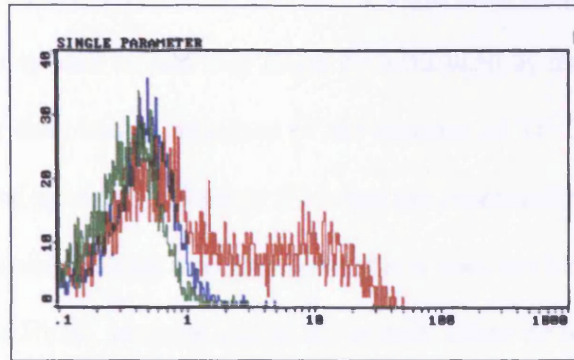
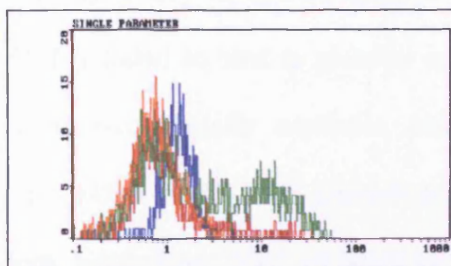


Figure 5.12. Anti-TF Mab HTF-1 binds specifically to TF on LPS-stimulated monocytes. Monocytes were isolated by the Histopaque® density gradient method, resuspended in RPMI and incubated for 4 h in the presence of 200 ng/mL LPS. Cells were stained with RPE-conjugated HTF-1 in the presence and absence of 30 μ g/mL sTF for analysis by flow cytometry. Blue trace shows the isotype control, red and green traces HTF-1 binding to LPS-activated monocytes in the absence and presence of sTF respectively.

(a)



(b)

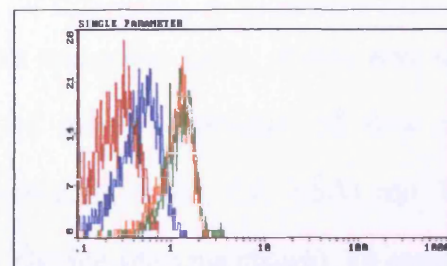


Figure 5.13. Anti-TF Mab HTF-1 binds at or near the FVIIa binding site. Monocytes were activated with 200 ng/mL LPS at 37 °C for 4 h before being stained for TF using Mab HTF-1 in the absence (green) and presence of 60 μ g/mL rFVIIa (orange). Control monocytes are shown in red and the isotype control in blue. (a) Monocytes were isolated using Histopaque® and resuspended in RPMI 1640 before activation. Antibody binding in the absence of plasma was completely abolished by rFVIIa. (b) Monocytes were activated in whole blood before being analysed by flow cytometry using OptiLyse® C to remove red cells. Antibody binding in plasma was unaffected by rFVIIa.

monocytes and those in whole blood: in the former a distinct population of monocytes was strongly positive for TF, whereas in the latter, there was just a slight shift in the entire population. These results suggest that failure to bind an antibody might be influenced by the environment in which the antibody was used rather than being indicative of the absence of TF. This raises the question of whether HTF-1 would bind to washed platelets. This was not examined but one can speculate that if TF is on unstimulated platelets in such a conformation that it does not bind FVII, then HTF-1 would bind, whilst if it does bind FVIIa, washing would not in itself allow the antibody to bind. Moreover, since the binding of FVII to TF is calcium-dependent, one would expect dissociation in the presence of EDTA to allow antibody binding; this did not happen (Fig.5.11). To ensure that the concentration of EDTA was sufficient to dissociate the TF/FVIIa complex, an experiment was carried out in which citrated PRP was incubated with HTF-1 in the presence of 6 mM and 9 mM EDTA. In neither case was there any binding to platelets.

5.2.1.3. Flow cytometry using unconjugated anti-TF Mabs

Because HTF-1 failed to bind to platelets in the flow cytometric assays, studies were undertaken using several commercially available, unconjugated anti-TF antibodies. In flow cytometric experiments, #4508 and #4509 (American Diagnostica, Stamford, CT, USA) and TF910H10 (Calbiochem, Nottingham, UK) all failed to bind to platelets (data not shown). To ensure that the antibodies did indeed recognise TF, they were also used with FITC-labelled secondary antibodies to detect TF on monocytes incubated with LPS for 7 h. TF9 10H10 bound to a limited extent, but 4509 did not (data not shown). Though it is possible that this failure to bind to monocytes in whole blood is related to FVII/a binding, this is unlikely since the binding sites of 4508 and 4509 are said by the manufacturer to be in the same region (amino acids 1 –25) and the TF9 10H10 data sheet states that the antibody does not block binding of FVII/a to TF.

5.2.2. Western blotting

Since according to the manufacturers, the epitopes recognised by all the antibodies used in flow cytometric assays are in the extracellular domain, this would suggest that had TF been present on the platelet surface, they could reasonably be expected to have detected it. However, since TF is known to exist in an encrypted form (Drake *et al*, 1989b), it was possible that encryption (suppression of activity) of any TF on the platelet surface could have prevented antibody binding. Therefore TF was sought using Western blotting of denatured platelet lysates in which the conformation of any TF would be altered.

The protein content of platelet lysates was determined by Lowry assay, and showed that 1×10^6 platelets from three donors contained an average of approximately 1.3 μg protein (Fig.5.14). Gels were loaded with lysates from 5×10^7 platelets, each containing about 65 μg protein, and the proteins resolved using SDS-PAGE. Western blotting of whole platelet lysates from three different donors using Mab #4508 (Fig.5.15) showed that whilst the antibody did bind to the recombinant TF (rTF) positive control (24 ng), it also bound to other proteins on the blot, including lysozyme in the Multimark® protein molecular weight marker. Particularly strong bands were seen in all the platelet lanes at a molecular weight slightly higher than that of the rTF, but others bands, both higher and lower, were also visible. To investigate the nature of the bands close to the size of the rTF, blots were incubated with Mab #4508 in the absence and presence of rTF. Although in the blocked sample the band in the positive control lane unsurprisingly disappeared, the bands running at the slightly larger molecular weight were unaffected by exogenous sTF (Fig.5.16).

Immunoblotting using Mab #4509 (Fig. 5.17) showed that this antibody bound much more strongly to proteins other than TF. After 15 min exposure of the film, an extremely faint band could be seen in the rTF positive control. Whilst TF9 10H10 bound strongly to rTF, it also bound non-specifically to many other proteins in the platelet lysates, including one with a molecular weight just higher than the rTF (45–47 kDa) (Fig.5.18). Confirmation of the lack of specificity of these

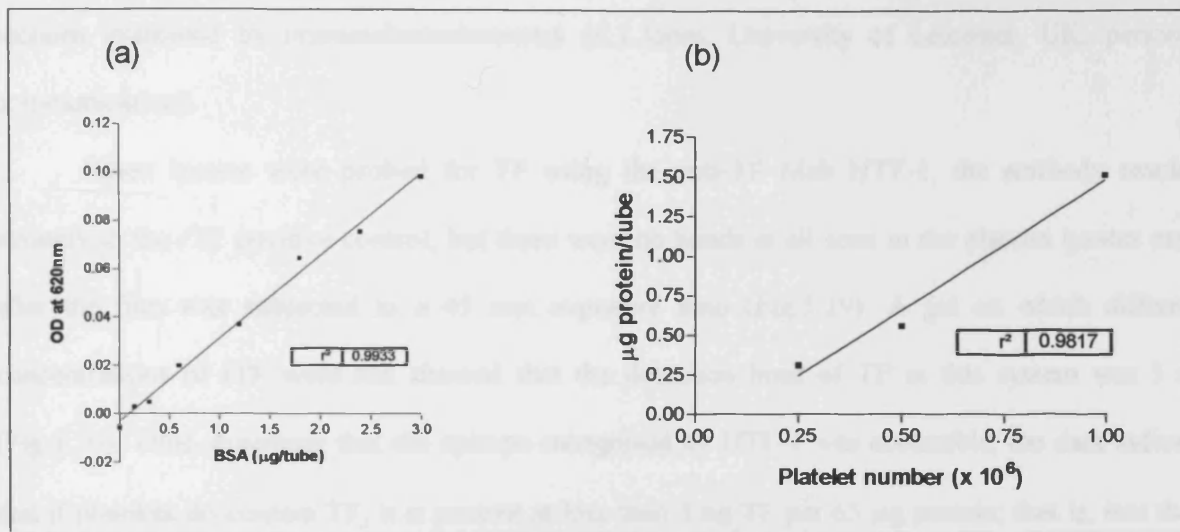


Figure 5.14. Protein content of platelets determined by Lowry assay. A standard curve (a) was constructed from serial dilutions of BSA, and platelet protein content determined by reading off the OD_{405nm} of lysates of 1×10^6 , 0.5×10^6 and 0.25×10^6 platelets from individual donors against the standard curve. A representative example of the protein content of different numbers of platelets from one donor is shown in (b).

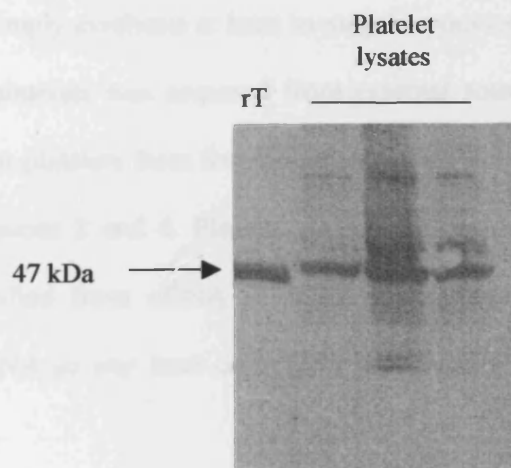


Figure 5.15. Non-specific binding of anti-TF Mab #4508 to platelet lysates. Western blotting using anti-TF Mab 4508 showed a strong band at the expected 47 kDa position for rTF (24 ng). In platelet lysate lanes, bands at slightly larger than 47 kDa and at approximately 100 kDa, as well as fainter bands of other sizes, were present. There was also a band present in the lysozyme position of the marker lane.

antibodies was given by the fact that they also bound non-specifically to proteins in thrombus sections examined by immunohistochemistry (C.I.Jones, University of Leicester, UK, personal communication).

When lysates were probed for TF using the anti-TF Mab HTF-1, the antibody reacted strongly to the rTF positive control, but there were no bands at all seen in the platelet lysates even after the film was subjected to a 45 min exposure time (Fig.5.19). A gel on which different concentrations of rTF were run showed that the detection limit of TF in this system was 3 ng (Fig.5.20). Thus, assuming that the epitope recognised by HTF-1 was accessible, the data indicate that if platelets do contain TF, it is present at less than 3 ng TF per 65 µg protein; that is, less than 0.005 % of total protein.

5.2.3. No TF mRNA detectable in platelets

Despite the contradictory nature of the data regarding the presence of TF in platelets using Western blotting and flow cytometry, the presence of TF mRNA was investigated by RT-PCR. The presence of message would imply synthesis at least in megakaryocytes, whilst its absence would suggest that any TF found in platelets was acquired from external sources. Oligo (dT)₂₅ beads were used to extract mRNA from platelets from four donors and the cDNA produced by RT was amplified using TF primers from exons 2 and 4. Platelet mRNA was demonstrably present in the samples as P-selectin was amplified from cDNA from all four donors (Fig.5.21a). However, there was no detectable TF mRNA in any lane other than the positive control of LPS-stimulated monocytes (Fig.5.21b).

5.2.4. TFPI mRNA in platelets

Several cell types, such as monocytes and endothelial cells, which express TF also express TFPI (Østerud *et al*, 1995). There is an early report of TFPI mRNA being present in platelets (Novotny *et*

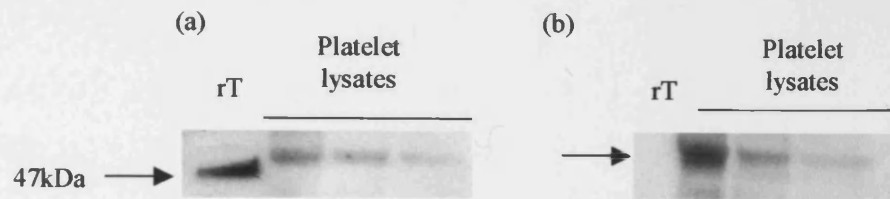


Figure 5.16. Effect of blocking 4508 with rTF. Membranes with transferred platelet lysates from three individual donors were incubated with 2 $\mu\text{g/mL}$ Mab #4508 without (a) and with (b) preincubation in 14.5 $\mu\text{g/mL}$ rTF before incubation with antibody.

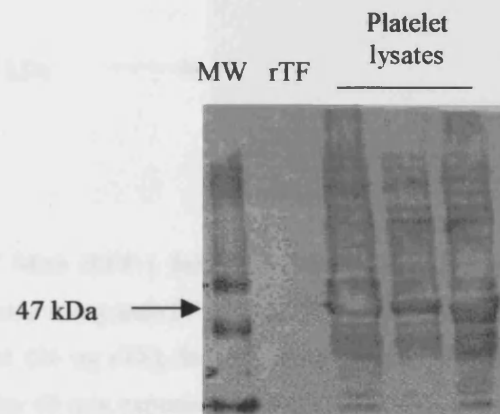


Figure 5.17. Non-specific binding of anti-TF Mab #4509 to platelet lysates. Western blotting using anti-TF Mab 4509 after 5 min exposure revealed extensive non-specific binding to platelet lysate proteins and proteins in the molecular weight marker lane (MW). Extremely faint binding to rTF was seen after 15 min exposure (not shown).

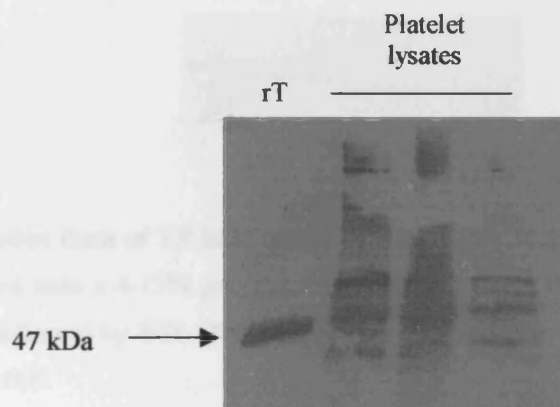


Figure 5.18. Non-specific binding of anti-TF Mab clone TF9 10H10 to platelet lysates. Western blotting using anti-TF Mab TF9 10H10 after 12 min exposure. Although the antibody bound to rTF (24ng), there was extensive non-specific binding to platelet lysate proteins.

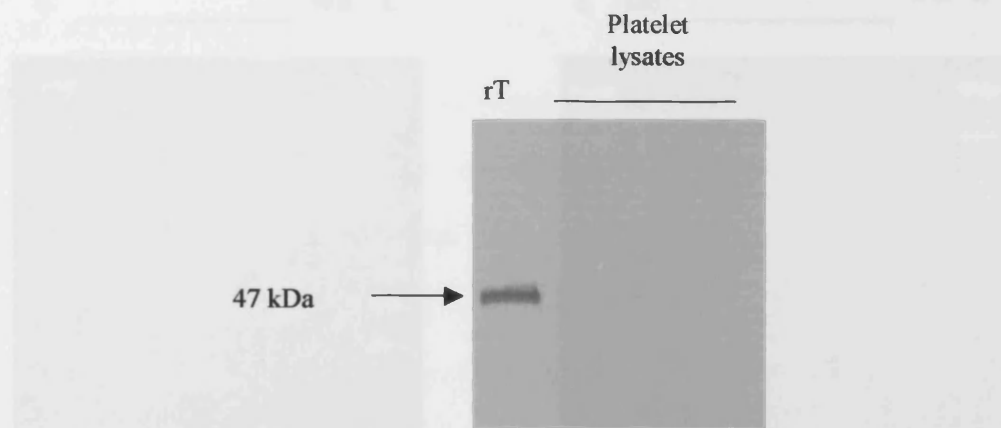


Figure 5.19. Anti-TF Mab HTF-1 failed to detect TF in platelet lysates. Western blotting analysis of whole cell platelet lysates using anti-TF Mab HTF-1 showed a strong band at the expected 47 kDa position for the positive control (24 ng rTF), but no bands were visible in lysates from 3 individual donors. This image was obtained after 45 min exposure.

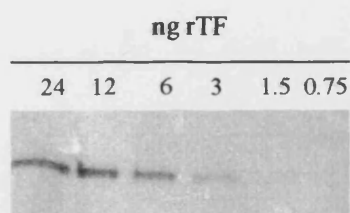


Figure 5.20. Detection limit of TF in Western blotting using Mab HTF-1. Serial dilutions of rTF (24ng-0.75ng) were loaded onto a 4-15% gel. Detection was using anti-TF Mab HTF-1 and an HRP-conjugated rabbit anti-mouse followed by ECL. This picture was obtained after 50 min exposure. No clear bands could be seen below 3ng rTF.

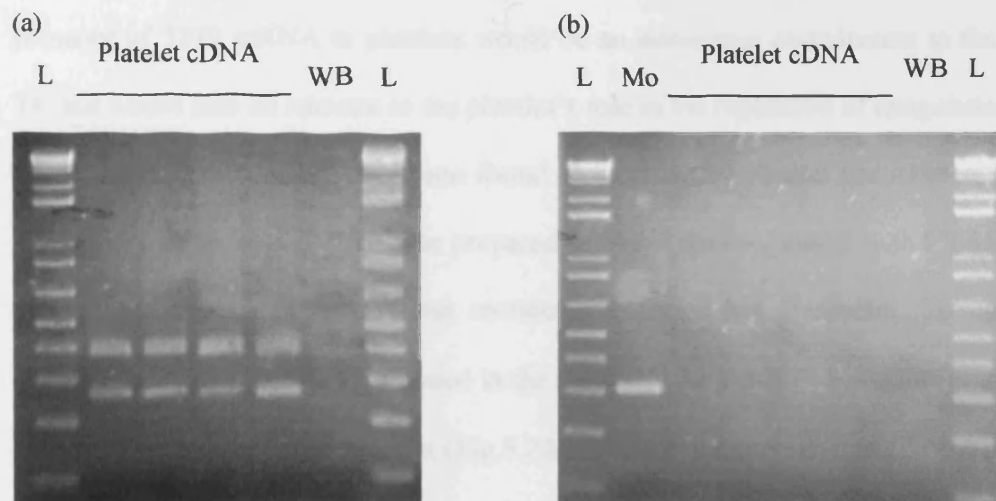


Figure 5.21. No TF mRNA present in platelets. Platelet mRNA was extracted from 1×10^9 platelets from 4 donors using Oligo (dT)₂₅ beads and after RT, the cDNA was amplified using (a) P-selectin and (b) TF primers. cDNA from LPS-stimulated monocytes (Mo) served as a positive control. PCR products were visualised on a 2 % agarose gel stained with ethidium bromide.

al, 1988) but there is no indication of the purity of the total RNA used, although a similar finding in the megakaryocytic cell line MEG-01 would appear to corroborate this (Bajaj *et al*, 1990). The presence of TFPI mRNA in platelets would be an interesting complement to the studies of platelet TF, but would also be relevant to the platelet's role in the regulation of coagulation.

Since TFPI mRNA has been found in monocytes, platelet preparation aimed to minimise leukocyte contamination. PRP was prepared in blood anticoagulated with CTAD in order to avoid platelet activation and hence limit monocyte adhesion via P-selectin. To check for leukocyte contamination, the cells were counted in the Coulter® AcT diff™ Hematology Analyser; it showed a zero differential monocyte count (Fig.5.22a). Flow cytometric analysis of CD14-stained PRP also revealed no monocytes either in terms of size and granularity or CD14 positivity (Fig.5.22b). However, amplification of platelet cDNA using primers for CD11b, which is constitutively expressed on most leukocytes, including monocytes, revealed bands in platelet lysates from three out of four donors (Fig.5.23a). When TFPI mRNA was amplified in the same cDNA, bands were only visible in the lanes in which CD11b were also present (Fig.5.23b), which might lead to the conclusion that the TFPI was monocytic in origin. However, the situation is further complicated by the fact that in unstimulated monocytes no TFPI was detected (final lane Fig.5.23b). The explanation does not appear to lie in uneven loading since the P-selectin amplified from the same lysates (Fig.5.21) were relatively similar in intensity.

5.2.5. Tissue Factor Activity (TFA) in platelets (amidolytic assay)

To approach the issue of the possible presence of platelet-associated TF from another angle, platelet TFA was measured in an amidolytic assay. Platelet lysates consisting of 1×10^8 platelets from 6 donors were introduced into a purified system measuring FXa activity in cleaving a chromogenic substrate, S2765. FVIIa, FX and calcium, together with hirudin to inhibit any traces of thrombin in the system, were added to the cells in excess, leaving TF in the cells as the limiting factor. The

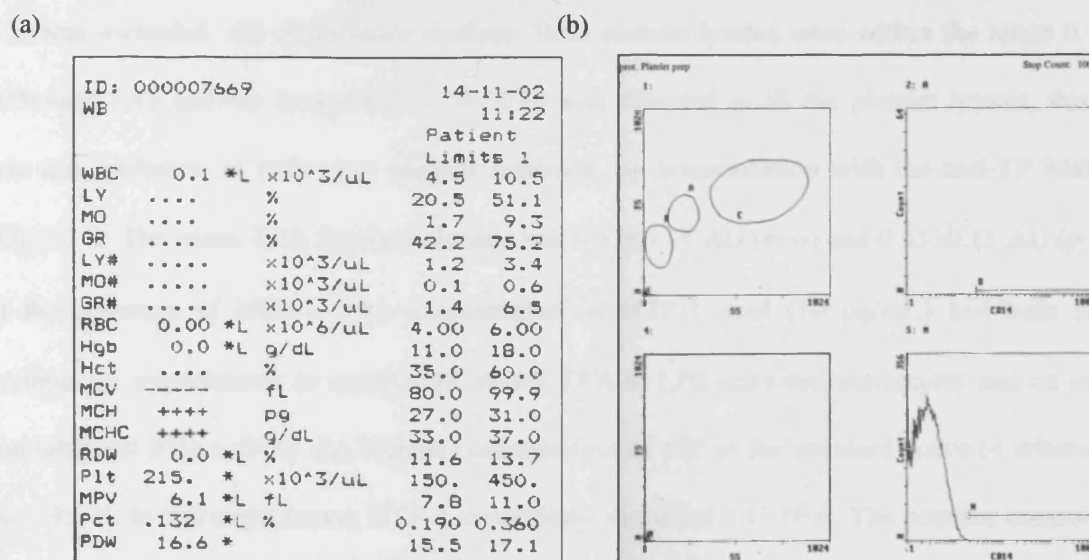


Figure 5.22. Monocyte contamination of platelet preparations for RT-PCR cannot be detected by flow cytometry or haematology analyser. Platelets isolated for mRNA extraction were counted in the AcT Diff Haematology analyser, which gave a zero count for monocytes in a typical report (a). PRP was also stained with CD14 and analysed by flow cytometry (b), but no monocytes were detected by either forward and side scatter or CD14 positivity.

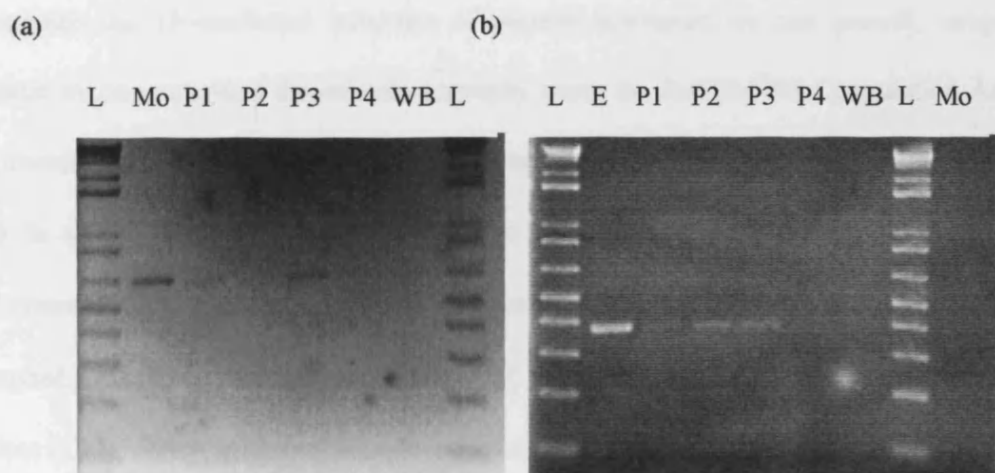


Figure 5.23. Platelets cannot be shown conclusively to contain TFPI mRNA. Platelet mRNA was extracted from 1×10^9 platelets from 4 donors (P1-4) using Oligo (dT)₂₅ beads and after RT, the cDNA was amplified using (a) CD11b and (b) TFPI primers. cDNA from Eahy 926 cells (E) and monocytes (Mo) served as positive controls. PCR products were visualised on a 2% agarose gel stained with ethidium bromide.

standard curve was only linear over the range 0 - 0.8 arbitrary units (AU) ($r^2=0.9993$) and therefore 1 U was excluded. All absorbance readings from platelet lysates were within the range 0 – 0.8 U. Although FXa activity (range 0.34 – 0.72 U) was detected in all the platelet lysates, this activity was not inhibited, or only very slightly inhibited, by preincubation with the anti-TF Mab HTF-1 (Fig.5.24). The mean TFA for the 6 donors was 0.57 ± 0.14 AU ($n=6$) and 0.55 ± 0.15 AU ($p=0.0888$) in the presence of HTF-1. The concentration of HTF-1 used (10 $\mu\text{g/mL}$) had been shown in preliminary experiments to maximally inhibit TFA in LPS-activated monocytes and on each plate run inhibited FXa activity the highest concentration of rTF in the standard curve (4 arbitrary units) by > 95 %. In this experiment, HTF-1 completely abolished 1 U TFA. The positive control gave an absorbance above the range of the standard curve and could therefore only be described as > 1 U, which was reduced to 0.22 U in the presence of HTF-1, an inhibition of at least 78 %. Thus most, if not all, the FXa activity seen in platelet lysates was not due to TF.

5.2.6. Whole blood thromboelastography

To examine the TF-mediated influence of platelet activation on clot growth, coagulation was estimated by computerised thromboelastography using the RoTEM®05 Coagulation Analyser. The experimental conditions for TEG were chosen to match those previously described (Zillmann *et al*, 2001). In all the assays, 0.3 mL citrated whole blood was recalcified with 30 μL 200 mM CaCl_2 (final concentration 16.8 mM) with and without XL-CRP (14 $\mu\text{g/mL}$), and the anti-TF Mab #4508 (22 $\mu\text{g/mL}$). To minimise the contribution of the contact pathway of coagulation, corn trypsin inhibitor (CTI), which inhibits FXIIa (Rand *et al*, 1996), was added at a concentration of 54 $\mu\text{g/mL}$, determined by titration (Fig.5.25). Data are shown graphically in Fig.5.26 and typical thromboelastograms in Fig.5.27. In unactivated blood, in the absence of exogenous TF, neither clotting time (551.3 ± 110.2 s vs. 554.3 ± 137.9 s; $p=0.9071$; $n=3$) nor the clot formation time (230.3 ± 126.7 s vs. 199.3 ± 71.2 s; $p=0.4752$) was inhibited by the presence of anti-TF Mab #4508.

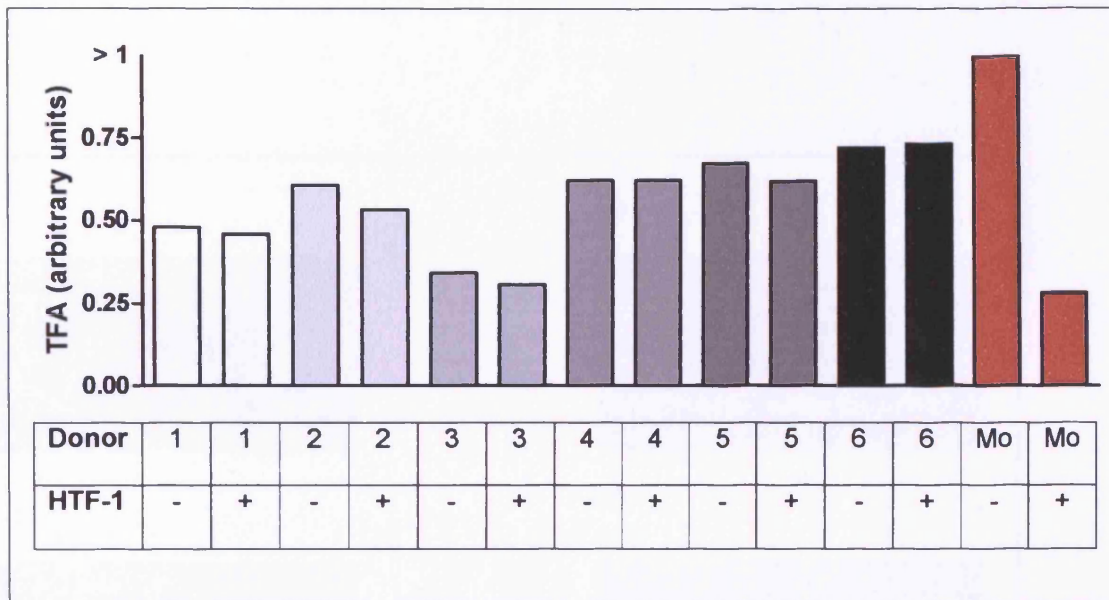


Figure 5.24. FXa activity detected in platelet lysates is not TF-dependent. Platelets from 6 donors were washed and lysed and incubated for 1 h at 37 °C in the presence of saturating concentrations of FVIIa, FX (0.15 IU/mL and 0.063 IU/mL respectively), 50.6 µg/mL hirudin and 8mM CaCl₂ together with 0.53 mM S2765, a chromogenic substrate for FXa. Generation of FXa was measured by absorbance at 405 nm read against a standard curve prepared from rTF. LPS-treated monocytes acted as a positive control. The specificity of TF activity was ascertained by pre-incubating samples with 10 µg/mL anti-TF Mab HTF-1.

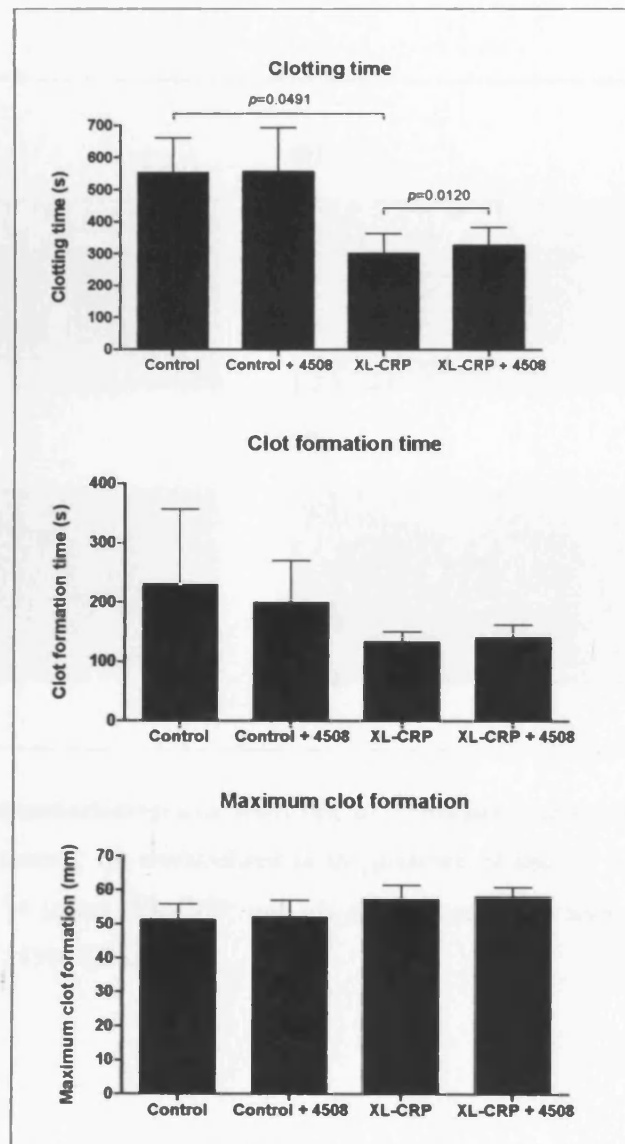


Figure 5.26. Effect of anti-TF Mab #4508 on clot formation. Whole blood was recalcified with 16.8 mM CaCl_2 in the presence of 54 $\mu\text{g/mL}$ CTI but without exogenous rTF. Clot formation in control and XL-CRP-stimulated (14 $\mu\text{g/mL}$) samples was measured in the absence and presence of anti-TF Mab #4508 (22 $\mu\text{g/mL}$) by thromboelastography. Data are from 3 independent experiments.

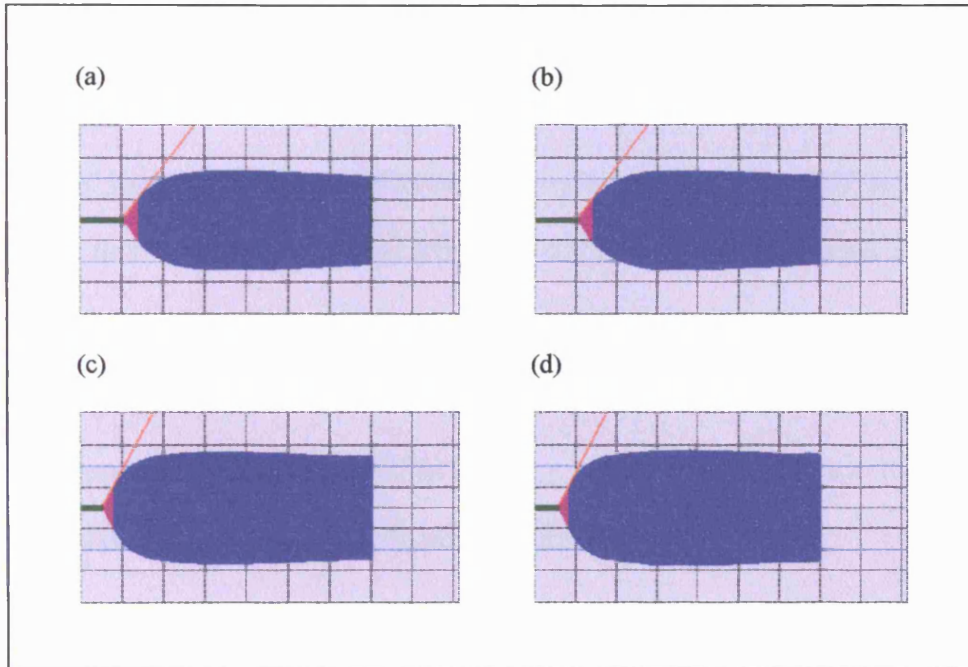


Figure 5.27. Typical thromboelastograms from one of 3 independent experiments as detailed in the legend to Fig.5.26: (a) control; (b) unstimulated in the presence of anti-TF Mab #4508 (22 $\mu\text{g/mL}$); (c) platelets activated with 14 $\mu\text{g/mL}$ XL-CRP; and (d) platelets activated with 14 $\mu\text{g/mL}$ XL-CRP in the presence of anti-TF Mab #4508 (22 $\mu\text{g/mL}$).

Maximum clot formation was higher in the presence of Mab #4508 and the difference, though small, approached significance (51.2 ± 4.7 vs. 52.0 ± 5.0 ; $p=0.0577$; $n=4$). Thus coagulation could not be shown to be dependent on blood-borne TF using this technique. Where platelets were activated by $14 \mu\text{g/mL}$ XL-CRP, clotting time was shortened to $300.0 \pm 61.3\text{s}$ ($p=0.0491$). This decrease was reduced slightly but significantly by Mab #4508 (CT: $324.7 \pm 57.2\text{s}$; $p=0.0120$). An irrelevant Mab MOPC31C made no significant difference to the clotting time in XL-CRP-activated blood. The clot formation time was also diminished (CFT: $133.0 \pm 17.7\text{s}$; $p=0.0599$) but this was unaffected by Mab #4508 ($140.0 \pm 21.9\text{s}$; $p=0.5381$). Maximum clot formation was greater where platelets were activated (57.3 ± 4.0 ; $p=0.0577$), an increase which was not TF-dependent (58.0 ± 3.0 ; $p=0.4226$). A concentration of $10 \mu\text{g/mL}$ Mab #4508 had been shown to inhibit the activity of 145 ng/mL rTF in the amidolytic TFA assay by $> 90\%$ (data not shown).

5.3. Discussion

5.3.1. Immunological detection of platelet TF antigen

When using anti-TF Mab #4508CJ, TF was consistently detected, to varying degrees, on the unstimulated platelets of healthy donors. Significantly, the binding of the antibody Mab #4508CJ was completely abolished by sTF and in many cases this would be taken as convincing evidence of its specificity. However, further work, prompted by anecdotal misgivings about this antibody, cast doubt on its specificity; the conclusion was therefore inescapable: that data produced with this antibody were unreliable.

The study was therefore extended to include three other anti-TF antibodies. None of these bound to platelets in flow cytometric assays. All were tested for their ability to bind to LPS-activated monocytes. American Diagnostica Mab #4509 failed to bind, whilst TF9 10H10 (Calbiochem) did bind, but only slightly. When used in Western blotting experiments, both #4509 and TF9 10H10 displayed problems of non-specificity. Only one Mab, HTF-1, gave consistent and

clean results in both flow cytometry and Western blotting but the antibody did not recognise TF in platelets under any of the conditions tested. Thus this project did not produce unequivocal evidence of the presence of TF antigen in platelets and indeed was more supportive of its absence.

The presence of TF in platelets has become a highly controversial issue. Before this experimental work was completed, a paper was published reporting the appearance of TF on the surface of activated, but not unstimulated, platelets (Zillmann *et al*, 2001). A subsequent paper from the same group showed TF located in platelet α -granules (Müller *et al*, 2003). This initial paper was followed shortly afterwards by another (Siddiqui *et al*, 2002) which confirmed the initial data in this project, finding TF on both resting and, to a greater extent, on activated platelets. However, these results are in direct contradiction with immunological studies by other groups (Drake *et al*, 1989a; Monroe *et al*, 1997; Monroe *et al*, 1998; Butenas *et al*, 2005; del Conde *et al*, 2005). Another recent paper described TF present on stored platelets (Perez-Pujol *et al*, 2005), but the percentage positive was extremely low (2.2 % initially, rising to a maximum of 5.5 % on day 7, but falling again to 4.3 % on day 11).

It is not easy to reconcile these divergent data. It is probable that technical issues relating to the antibodies account for some of the differences. The earliest study of TF localisation (Drake *et al*, 1989a) used immunohistological assays which may have been less sensitive than flow cytometry. Flow cytometric studies which have found TF on the platelet surface have generally used the same antibody, #4508CJ, clone VD8 (Albrecht *et al*, 1992), from American Diagnostica (Zillmann *et al*, 2001; Siddiqui *et al*, 2002; Camera *et al*, 2003; Leon *et al*, 2004), though Camera *et al* replicated their results with two other antibodies as well as FITC-labelled rFVIIa (*ibid.*). Indeed, Mab #4508CJ, which until recently was the only directly conjugated anti-TF Mab available commercially, has been used extensively in studies of TF expression on monocytes and microparticles, to give single examples from several groups (Nguyen *et al*, 1999; Lindmark *et al*, 2000; Joop *et al*, 2001; Lee *et al*, 2002; Scholz *et al*, 2002; Steiner *et al*, 2003). Based on the results

in the present study, it is possible that at least some of the TF ascribed to monocytes might have been due to antibody binding to adherent platelets. Indeed, it is probable that this is the case for the flow cytometric data for MPAs in Chapter 4 (§4.2.4). TF-positive events in platelet concentrates have been detected using Mab HTF-1 (Perez-Pujol *et al*, 2005) but these were very small in number, and the authors themselves thought that the TF may well have been associated with residual leukocytes in the platelet concentrates. Those who have failed to find TF on platelets have used the American Diagnostica Mab #4509 and a variety of in-house antibodies (Monroe *et al*, 1997; Butenas *et al*, 2005; del Conde *et al*, 2005). Workers detecting TF in Western blots performed on platelet lysates have used a polyclonal antibody against human brain TF from American Diagnostica (Siddiqui *et al*, 2002), or Calbiochem anti-TF Mab (probably clone TF9 10H10 since this is the only TF antibody currently in the Calbiochem catalogue) (Camera *et al*, 2003).

Thus TF on platelet surfaces has been detected primarily by one particular Mab, #4508CJ, shown in this project to be unreliable. All the antibodies used for which data is available indicate that they recognise the extracellular domain of TF and therefore have a reasonable prospect of binding to surface-expressed TF. There is no clear reason why some antibodies failed to identify TF on platelets. None of the antibodies recognise epitopes in the exon 5 region missing in alternatively spliced TF (Bogdanov *et al*, 2003), which would make them unable to bind to TF if it were present in this form. It is possible that only certain epitopes are accessible, particularly as TF is known to exist in an encrypted form. Perhaps platelet-associated TF exists in a different form from that in other locations.

The epitopes of the anti-TF Mabs commercially available are not clearly defined. Nevertheless, the epitope of HTF-1 is known to include residues which make contact with the catalytic area of FVIIa (Kirchhofer *et al*, 2000) and its ability to inhibit procoagulant activity was influenced by the concentration of FVIIa, suggesting that it competes with FVIIa in binding to TF

(Carson *et al*, 1987). That this was indeed the case was demonstrated clearly in this project. Data from the TFA assay shows that HTF-1 is capable of blocking TF but preliminary experiments had indicated that pre-incubation with cells gave better inhibition, attributed to its competing with FVIIa. The implication of this is that the antibody is probably unsuitable for use in the presence of FVII – in practical terms, in the presence of plasma – if the TF detected is active and able to bind FVII. However, since HTF-1 did not bind to platelets even when they were in blood anticoagulated with EDTA, it is probable that blocking of the epitope by bound FVIIa was not the reason for the absence of antibody recognition because the formation of the TF-FVIIa complex is calcium dependent (Banner *et al*, 1996). Although this antibody has been used in whole blood and identified circulating TF (Balasubramanian *et al*, 2002a), it is not clear whether or not this TF was active (with bound FVIIa) since fibrin had formed before TF was visible, nor is its precise location apparent. The authors of this paper also mentioned that the antibody did not bind to resting or activated washed platelets. Taken as a whole, the data therefore suggest that it is possible, but unlikely, that the failure of HTF-1 to bind to platelets was a consequence of its epitope.

It is improbable that epitope position accounts for the differing binding characteristics of Mabs #4508 and #4509 either. According to the supplier's technical data sheets, both antibodies are directed against overlapping epitopes in the first 25 amino acids of TF, but the former bound to platelets whilst the latter did not. Though this does not rule out the possibility of a conformational explanation, it makes it less likely. It is interesting to note that, unlike all the other antibodies used in this study, TF9 10H10 does not inhibit TF activity (Morrissey *et al*, 1988) and therefore binds to a very different epitope, in locus I (Ruf *et al*, 1991). It did not, however, bind to platelets, although it also displayed only slight binding to LPS- treated monocytes.

The data presented here suggest that there might also be problems with some of the other antibodies as well as with Mab #4508CJ. In Western blots, Mab #4509 bound to many platelet proteins, but only weakly to rTF. The antibody is not specifically recommended for Western

blotting by the supplier, so it is possible that it does not recognise TF under reducing conditions as used here. However, since the antibody – at least, this particular batch – did not bind to LPS-stimulated monocytes when checked in control experiments, its inability to bind to platelets is uninformative and casts some doubt on the absence of TF on platelets reported by others using it (Monroe *et al*, 1997). Western blotting also demonstrated issues of non-specificity with TF9 10H10, although it did bind strongly to the positive control (Fig.5.18). Non-specific binding was also extensive when this antibody was used in immunohistochemical studies in our laboratory (C.I.Jones, personal communication). Close inspection of a blot of platelet and HUVEC lysates using this antibody in the literature reveals other unidentified bands (Camera *et al*, 2003). The only antibody used which did not raise concerns about specificity was HTF-1; and this did not bind to platelet proteins under any of the conditions used either in flow cytometry or Western blotting.

Some workers have detected platelet TF antigen using ELISAs employing different antibody combinations (Zillmann *et al*, 2001; Siddiqui *et al*, 2002). The amount found in platelet lysates by Zillman *et al* (*ibid.*) was described as “considerable”, and amounted to 56 ± 12 pg/mg. On the basis that platelets on average contain approximately 0.4 pg protein per platelet (Hartwig & DeSisto, 1991), which is of the same order of magnitude as that determined by Lowry assay (Fig.5.14), the number of platelets comprising 1 mg protein is 1×10^9 pg / 0.4 pg = 2.5×10^9 platelets. Thus each platelet, according to this assay, contains a mean of $56 / 2.5 \times 10^9 = 2.2 \times 10^{-8}$ pg TF, which is only about 5.6×10^{-6} % of total platelet protein. The amount in platelet releasates found by Siddiqui *et al* (*ibid.*) was even lower, about 30 pg/mg protein in the releasate from an unspecified number of platelets. Thus, even if such measurements are reliable, the amount of TF available from platelets is very low, although local concentrations in the presence of numerous platelets could reach levels of functional significance.

Taken as a whole, the data in this study do not support the view that there is TF associated with unstimulated or stimulated platelets and suggest that its immunological detection may well be

the result of non-specific antibody binding. There does, however, remain the puzzling ability of sTF to block binding of the antibody which has been the most successful in recognising antigen on platelets. Presumably, this is due to a shared epitope. Further examination of this question would require the use of a panel of TF antibodies recognising different and defined epitopes.

5.3.2. Platelets do not contain TF mRNA

To complement the antigen studies, RT-PCR was used to determine whether platelets contain TF mRNA. If they were found to do so, this would imply that megakaryocytes, and possibly also platelets, synthesize TF. If they did not, any TF protein present in platelets must be acquired from other cells or plasma, for which there is ample precedent, for example, with fibrinogen (Harrison *et al*, 1989). However, TF mRNA, if present at all, was below detectable levels in all the donors examined (Fig 5.21). The question has not been extensively examined in the literature. Zillmann *et al* did not look for TF mRNA in platelets but found it to be undetectable in megakaryocytes (Zillmann *et al*, 2001). Of the previously published reports regarding platelet TF mRNA, one agreed with the data here in failing to detect any (Fink *et al*, 2003), but the other showed appreciable amounts in resting platelets (Camera *et al*, 2003).

It is possible that the existence of alternatively spliced transcripts accounts for these differing results. However, the only published TF splice variants are one which involves the omission of exon 5 (Bogdanov *et al*, 2003) and a very recently published one found in tumour cells involving alternative splicing of the first intron between exons 1 and 2 (Chand *et al*, 2005). The latter involves addition rather than omission and the Fink *et al* primers (Fink *et al*, 2003), spanning exon 1 and 2, would still have amplified any of the alternatives present. The Camera *et al* primers span exons 5 and 6 (Camera *et al*, 2003) and therefore would not have amplified alternatively spliced TF lacking exon 5, implying that it is the full length transcript which was present. The primers used in this project span exons 2 to 4 and would therefore have picked up all known

transcripts. Nevertheless, it is possible that other variants exist which would explain the divergent results.

Another potential explanation for the failure to amplify TF mRNA is the method of extraction, using Oligo(dT)₂₅ beads rather than total RNA. This might lead to the loss of TF mRNA if the copy number is low or if the poly-A tail has been degraded in the cytoplasm. However, both Camera *et al* and Fink *et al* (*ibid.*) extracted total RNA (despite the latter describing total RNA extraction as mRNA extraction in their methods) using essentially the same protocol, but the former amplified TF mRNA whilst the latter did not. It should also be noted that TF mRNA has a short half-life, at least in endothelial cells and monocytes, in which half-lives of between 48 min and 2 h have been reported (Gregory *et al*, 1989; Scarpati & Sadler, 1989), which could affect recovery depending on the method of preparation. However, the method of mRNA extraction used in this project was quick (under 1 h) and certainly fast enough to detect TF mRNA in monocytes, as demonstrated in Chapter 4.

Additionally, the discrepancies may be related to the amount of mRNA extracted, reverse transcribed and subjected to PCR. Direct comparisons are not easily made since authors give different measures of loading. Camera *et al* reverse transcribed 100 ng total RNA, whilst in this study 1×10^9 platelets were used. If one takes the amount of total RNA from half the PRP obtained from 50 mL whole blood as being 20 μ g (Newman *et al*, 1988), one can estimate the number of platelets required to obtain 100 ng RNA. Assuming approximately 20 mL of PRP containing about 250×10^3 platelets/ μ L is produced, this means that 20 μ g RNA requires 5×10^9 platelets. Thus each platelet contains 4 fg RNA, so that to obtain 100 ng RNA one would need approximately 2.5×10^7 platelets. Although only an estimate, this does suggest that using fewer platelets initially was not the reason for the lack of product. From the published information, it is not clear how much cDNA was subjected to PCR or product loaded onto agarose gels by Camera *et al*, so no comparison could be made. It is possible that the very low levels of TF mRNA found by Camera *et*

al (about 2.16×10^{-7} fg per platelet) would not be visible were too little loaded onto the gel. Fink *et al* varied platelet numbers between 10,000 and 600,000 (considerably fewer than the other workers mentioned), but although at all numbers they detected $\beta 3$ and $\alpha 2$ integrin, they were unable to detect TF in any sample (Fink *et al*, 2003). Both they and Camera *et al* used real-time RT-PCR so the loading of gels was not an issue.

It is more likely that the discrepancy is due to leukocyte contamination. As demonstrated in Chapter 4, even unstimulated monocytes may contain low levels of TF mRNA, even if this is only due to activation during sample preparation. In addition to small numbers of leukocytes being present in the plasma, if platelets become activated during isolation, they are likely to have some adherent monocytes or monocyte fragments. As shown in Fig.5.22, this is not always apparent from either hemocytometers or even flow cytometry, since the amplification process of PCR means that even very small quantities of mRNA become detectable (Fig.5.23). Monocytes contain approximately 25 fg mRNA/cell – 12,500 fold higher than the 0.002 fg found in platelets (Wicki *et al*, 1989). Therefore, in addition to taking precautions to minimise platelet activation during preparation, a leukocyte specific mRNA should be amplified to exclude contamination. Although Camera *et al* did check in this way for such contamination (Camera *et al*, 2003), the markers chosen were CD2 and CD20, both of which are lymphocyte markers. Although a very recent paper has suggested that TF can be induced in a subset of B lymphocytes by phorbol myristate acetate (Mechiche *et al*, 2005), this is not relevant to unstimulated blood. Thus CD2 and CD20 seem to be irrelevant markers, particularly as lymphocytes do not readily form aggregates with platelets (Rinder *et al*, 1991). A pan-leukocyte marker such as CD45 would be a better option, or the CD11b used in this project, as this is expressed by most leukocytes, including the TF-competent monocytes. The second approach to ensuring platelet purity employed by Camera *et al* (*ibid.*) was to perform RT-PCR on the discarded leukocytes from the platelet preparation. This approach may be flawed since the leukocytes assayed might not include those attached to platelets (possibly only

fragments), which would be most likely to be monocytes in which TF can be more readily induced, especially following interaction with platelets. Fink *et al* went to considerable lengths to avoid contamination and were able to do so using laser assisted microdissection and manipulation; after this, they found no TF transcripts (Fink *et al*, 2003).

5.3.3. Platelet TFPI mRNA

The data in this project relating to platelet TFPI mRNA are illustrative of the difficulties in obtaining sufficiently pure samples to confidently ascribe a transcript to a particular cell type when dealing with such a sensitive technique as PCR. As noted above, this is especially relevant to the debate on whether platelets contain TF mRNA. In the case of TFPI, message was only evident in samples in which CD11b was also amplified. Although this would suggest that the mRNA emanated from leukocyte contamination, it should be noted that it was not amplified in the monocyte cDNA. Furthermore, the data in Chapter 4 (Fig.4.5) show that monocyte TFPI mRNA is not upregulated for at least 4 h in unstimulated blood and takes at least 2 h to appear even where platelets have been strongly stimulated with XL-CRP. On balance of probabilities, therefore, platelets do contain low levels of TFPI mRNA. Further work is necessary to determine whether TFPI protein and activity are also to be found in platelets and whether it has any role in the regulation of coagulation.

5.3.4. Platelet TF activity

Immunological assays generally give no information as to whether any TF they detect is functional. To approach the question of whether or not platelets carry TF from this arguably more important perspective, TFA in lysed platelets was investigated using an amidolytic assay measuring FXa activity. TFA in whole blood containing both unstimulated and stimulated platelets was examined using thromboelastography. In neither assay was appreciable TF-specific activity shown. Although

FXa activity was seen in lysed platelets, this was not blocked by Mab HTF-1. Whilst XL-CRP-stimulated platelets shortened clotting times by about 44 %, only approximately 8 % of this enhancement of coagulation was inhibited by anti-TF Mab #4508. Neither the clot formation time nor the maximum strength of the clot was altered by the presence of Mab #4508. In unstimulated blood, clotting was not inhibited by #4508. The effectiveness of both the antibodies, HTF-1 and #4508, had been demonstrated in the amidolytic assay.

As with other aspects of platelet-associated TF, there is also controversy regarding platelet TFA. Previous amidolytic results detected no activity on the surface of unactivated platelets (Zillmann *et al*, 2001; Siddiqui *et al*, 2002). That no active TF was found on the surface of unstimulated platelets is unsurprising; were it otherwise, the body would be liable to massive thrombosis. However, the latter group did record FXa activity in the supernatants of both resting and activated platelets, suggesting that TF released from platelets is active whilst that expressed on the surface is not. The authors do not state whether FXa activity was associated with the surface of activated platelets nor is there mention of testing the TF-dependence of this activity.

High concentrations of FVIIa such as those used here have been found to activate FX independently of TF (Monroe *et al*, 1997). That is indeed the implication of the results in this study, where exposure of PS in lysed platelets was associated FXa activity which was not inhibited by Mab HTF-1. Zillmann *et al* (*ibid.*) reported FXa activity only when platelets were activated in the presence of neutrophils, and this activity was blocked by an anti-TF Mab. This data is therefore not inconsistent with that presented in this study, since here only isolated platelets were tested in the amidolytic assay.

Whole blood assays, whilst obviously more physiological, have the disadvantage that any TF activity cannot be localised to a particular cell. Where there was no intentional platelet activation and clotting was initiated merely by recalcification, addition of Mab #4508 did not prolong the clotting time or retard clot growth (Fig.5.26). Since there was no exogenous TF in the

system, this supports the view that there is no functional blood-borne TF. The results obtained by Zillmann *et al* using thromboelastography to demonstrate TF presentation by activated platelets (Zillmann *et al*, 2001) could not be fully replicated here, though in this project XL-CRP was used instead of collagen. Here, the anti-TF antibody #4508 did prolong clotting times, but only slightly and not substantially as Zillmann *et al* reported. This suggests that when platelets are activated, the resultant acceleration of clotting is not predominantly TF-dependent. Since both studies employed corn trypsin inhibitor to block FXIIa (Rand *et al*, 1996), the inability of an anti-TF Mab to completely abolish clotting implies that there may be another, TF-independent route to thrombin generation. This was also observed by Butenas *et al* (Butenas *et al*, 2005) who postulated that FXI, which has been found in platelets and is released in active form (Tuszynski *et al*, 1982), could be responsible for this. The action of FXIa in thrombin generation in the absence of TF has recently been described (Wiolders *et al*, 2004) and its possible activity in the context of thromboelastography would warrant further investigation.

In recent correspondence discussing the presence of active TF in blood, Bogdanov *et al* (Bogdanov *et al*, 2004) stressed the importance of shear in the appearance of TF on platelets and suggested that the absence of flow might account for differences in results. Although shear clearly is an important factor, there are discrepancies in result even amongst static studies, both immunological and activity-based.

5.3.5. Blood-borne TF

There has been much recent debate about blood-borne TF, regarding its nature, source and activity. Although reports of immunological detection of TF in plasma date back over 10 years (Koyama *et al*, 1994), it is not clear what is being detected in these assays: soluble TF, truncated forms of TF, TF attached to cells or microparticles - or even artefact. Early studies tended to use ELISAs (Koyama *et al*, 1994; Suefuji *et al*, 1997) which do not discriminate between microparticles and

soluble TF. Though the discovery of alternatively spliced TF has fuelled research into soluble TF (Szotowski *et al*, 2005), TF-bearing microparticles have been the focus of more recent attention (Sturk-Mauelin *et al*, 2003). The cells from which these microparticles derive have not been clearly defined. The likely candidates are monocytes and endothelial cells, since they are both capable of expressing TF, and it is also possible that some are shed from atherosclerotic plaques (Mallat *et al*, 1999).

Platelet involvement has been posited by some of the participants in this debate. The evidence regarding the existence of TF antigen on platelets is contradictory, and the data presented in this project show that much of this is unreliable. Nevertheless, even if platelets do not normally carry TF, it is plausible that under certain circumstances it could be transferred to them via microparticles from monocytes or other leukocytes to platelets via the PSGL-1 - P-selectin interaction (Rauch *et al*, 2000; del Conde *et al*, 2005). However, the precise role of platelets has not been delineated. It is noteworthy that in PSGL-1 negative mice, minimal TF and fibrin was observed even though platelets accumulated at the site of injury (Falati *et al*, 2003), which implies the absence platelet-associated TF. Even if one accepts the view postulated in this project, that it is unlikely that TF is present on resting or activated platelets, this is not incompatible with recently published data showing the assimilation into the membrane of activated platelets via P-selectin of TF- and PSGL-1-positive microparticles arising from lipid rafts in monocytes (del Conde *et al*, 2005). Such fusion might not occur if no measures were taken to induce microvesiculation. The data in this project do, however, suggest that there are not large numbers of platelets circulating which have acquired TF prior to participating in thrombus formation. The circulation of previously activated platelets which could have acquired TF by this method would be possible since platelets have been shown to circulate following activation (Michelson *et al*, 1996).

Studies using real-time imaging have shown that TF-bearing microparticles participate in thrombus build-up (Falati *et al*, 2002; Falati *et al*, 2003). However, it is not obvious that leukocyte-

derived microparticles would be available on a consistent basis were this transfer necessary for normal haemostasis, given the nature of their formation and that clearance is thought to be mediated by the PS which is frequently on a microparticle surface (Fadok *et al*, 1992). The microparticles used by del Conde *et al* for transfer to platelets were generated by LPS treatment (del Conde *et al*, 2005), and in another paper detailing the transfer of TF⁺ microparticles from leukocytes to platelets, TNP-1 cells were treated with TNF α (Rauch *et al*, 2000). Although microparticles were not intentionally created in the studies of thrombus build-up in a living mouse, it is possible that these were generated by the laser-induced injury to the vessel wall (Falati *et al*, 2003). Microparticles have been isolated from normal plasma but this has often involved concentration steps (Berckmans *et al*, 2001; del Conde *et al*, 2005), which leaves open the question of whether sufficient would be available to assist in thrombus formation in normal circumstances. It is possible, therefore, that such TF-bearing microparticles are of more importance in inflammatory states than in healthy individuals, but this requires further investigation.

Some have argued that blood-borne TF, whatever its source, is a necessary element of thrombus build-up because the minute amounts of FXa and thrombin generated initially at the endothelial wound could not diffuse to the surface of activated platelets to give rise to the thrombin burst which leads to fibrin formation (Giesen *et al*, 1999; Balasubramanian *et al*, 2002b; Walsh, 2003). Similarly, it is thought that blood-borne TF would explain the ability of blood to clot in the absence of vascular injury and indeed, in a rabbit model, TF antibodies were found to inhibit formation of venous thrombi or venous thrombosis (Himber *et al*, 2003). On the other hand, Butenas and Mann maintain that there are no reliable data supporting the presence of significant levels of functional TF in normal plasma since in their experimental system as little as 20 fM (approximately 1 pg/mL) is sufficient to significantly accelerate clot formation (Butenas *et al*, 2005). In agreement with this, a recent paper found that blood-borne TF did not affect clot growth rate (Ovanesov *et al*, 2005) and another recent paper has emphasized the importance of the vessel

wall in initiating coagulation (Day *et al*, 2005). Other workers suggest that whilst vessel wall TF is the principal initiator of coagulation, blood-borne TF is responsible for its propagation (Chou *et al*, 2004).

The TEG data in this study do not support a major role for TF in coagulation in a model in which the only TF present was blood-borne. Considered in conjunction with data from the FXa activity assay (Fig.5.24), which showed minimal TFA even in lysed platelets, platelet-associated TF does not appear to have an important role in coagulation, at least in the absence of shear.

The data in this study have not shown TF-specific activity associated with platelets and imply that a TF-independent path to coagulation, possibly via FXI, is operative. Since these assays were performed either on isolated platelets with no possible interaction with leukocytes, or in circumstances where leukocyte microvesiculation is minimal, these data are not inconsistent with the leukocyte-platelet TF transfer theories. They do, however, lend weight to the argument that transfer of TF from leukocytic microparticles is important principally in inflammatory situations. They furthermore suggest that platelets themselves are not a significant source of blood-borne TF, though they may well be recipients of leukocyte TF and, as demonstrated in Chapter 4, they could contribute to this TF source through their interaction with monocytes.

5.3.6. Further work

Although during the course of this project there has been an increasing literature on the subject of platelet TF, because there is continuing controversy, additional data is still valuable. Because of the continuing discrepancies between immunological data, it would be useful to investigate the presence of TF antigen on platelets using a panel of antibodies with clearly defined epitopes. Comparing binding with those antibodies already in use would establish whether the discrepancies in data arise for conformational reasons or artefact. Such a panel of antibodies would also be useful in characterising the nature of the TF antigen detected in blood. Real-time PCR and use of primers

from different exons would reinforce the data on the presence of TF mRNA in platelets and perhaps reconcile conflicting results.

The development of a satisfactory method for identifying microparticles, as discussed in Chapter 3, would also aid consideration of whether these do play a role in thrombus growth in healthy people. The nature of the role of activated platelets in coagulation could be further elucidated by the use of a FXIa inhibitor.

CHAPTER 6: Summary and conclusions

6.1. Summary

Validation tests indicated that the flow cytometric assay for measuring PS exposure on platelet and platelet-derived microparticles was a reasonably robust tool for investigating the mechanisms underlying the provision of a procoagulant surface. The data thus obtained show clearly that collagen is the main single agonist in initiating PL rearrangement, although ADP and thrombin, but not TXA₂, play important enhancing roles. Platelet-platelet contact was also shown to facilitate PS exposure and this effect was abolished by the GPIIb/IIIa inhibitor ReoPro®. The evidence also suggested that PS exposure is independent of other aspects of platelet activation. Considerable variation in PS expression in response to XL-CRP was shown in normal donors. Although the creation of a small percentage of PS positive platelets was shown to accelerate clotting, beyond this point there was no direct relationship between PS exposure and the dynamics of clot formation.

Platelet activation was shown to lead to upregulation of both TF and TFPI gene expression in monocytes, with the inhibitory TFPI appearing later than TF. These effects were mediated primarily through soluble mediators since preventing the formation of monocyte-platelet aggregates by blocking PSGL-1-P-selectin interaction did not abrogate either TF or TFPI mRNA expression. When monitoring clot formation by thromboelastography, the prevention of MPA formation slightly lengthened clot formation time, but direct monocyte-platelet interaction did not otherwise impinge on the parameters measured. Flow cytometry showed increased TF surface antigen on MPAs, but subsequent experiments testing the specificity of the antibody used showed that this data, and by implication much published data using the same antibody, to be unreliable. Activated platelets released, and caused monocytes to release, IL-8. Although the procoagulant IL-8, TNF α and IL-1 β were upregulated in incubated whole blood, this could not be ascribed specifically to platelet activation but was also not blocked by preventing PSGL-1-P-selectin ligation.

Experiments pursuing the possibility that TF was present on platelets demonstrated the unreliability of the antibody used here and most commonly in published studies of this kind. Other antibodies did not detect any TF on the platelet surface or in lysates. In addition, TF mRNA was undetectable, although TFPI mRNA was evident. Although FXa activity was found in platelet lysates, it was not TF-dependent. Where platelets were unstimulated, blood-borne TF was not found to play a part in clotting. A very small (< 10 %) of the shortening of clotting time caused by XL-CRP-activated platelets, as measured by TEG, was attributable to TF. Since clotting was accelerated by activated platelets in the presence of both CTI and an anti-TF Mab, this suggests that platelets contribute other factors to coagulation. A recent paper has put forward the hypothesis that polyphosphate released from platelet dense granules promotes activation of FV and inhibits TFPI and hence promotes clotting (Smith *et al*, 2006). Alternatively, it may be that FXIa performs this role.

6.2. Conclusions

Taken together, these data can be integrated into and contribute to current theories of coagulation, as illustrated in Fig.6.1. The data stress the importance of collagen in the provision of an appropriate PL surface. Collagen would probably be exposed at the site of injury at the same time as TF. PS exposure would therefore be effected at the same time as coagulation was initiated and does not rely on the first thrombin generated to activate platelets as stated in many expositions of coagulation. Though this point is now acknowledged (Monroe & Hoffman, 2005), the activation due to collagen is referred to as partial, requiring thrombin for full expression. Whilst thrombin does enhance the procoagulant effect of collagen and may also contribute increased binding of coagulation factors to sub-populations of platelets (Kempton *et al*, 2005), collagen of itself is capable of eliciting a strong response. Given the data showing that clotting is not further accelerated once approximately 7% platelets express PS, it is questionable whether thrombin is essential for PS

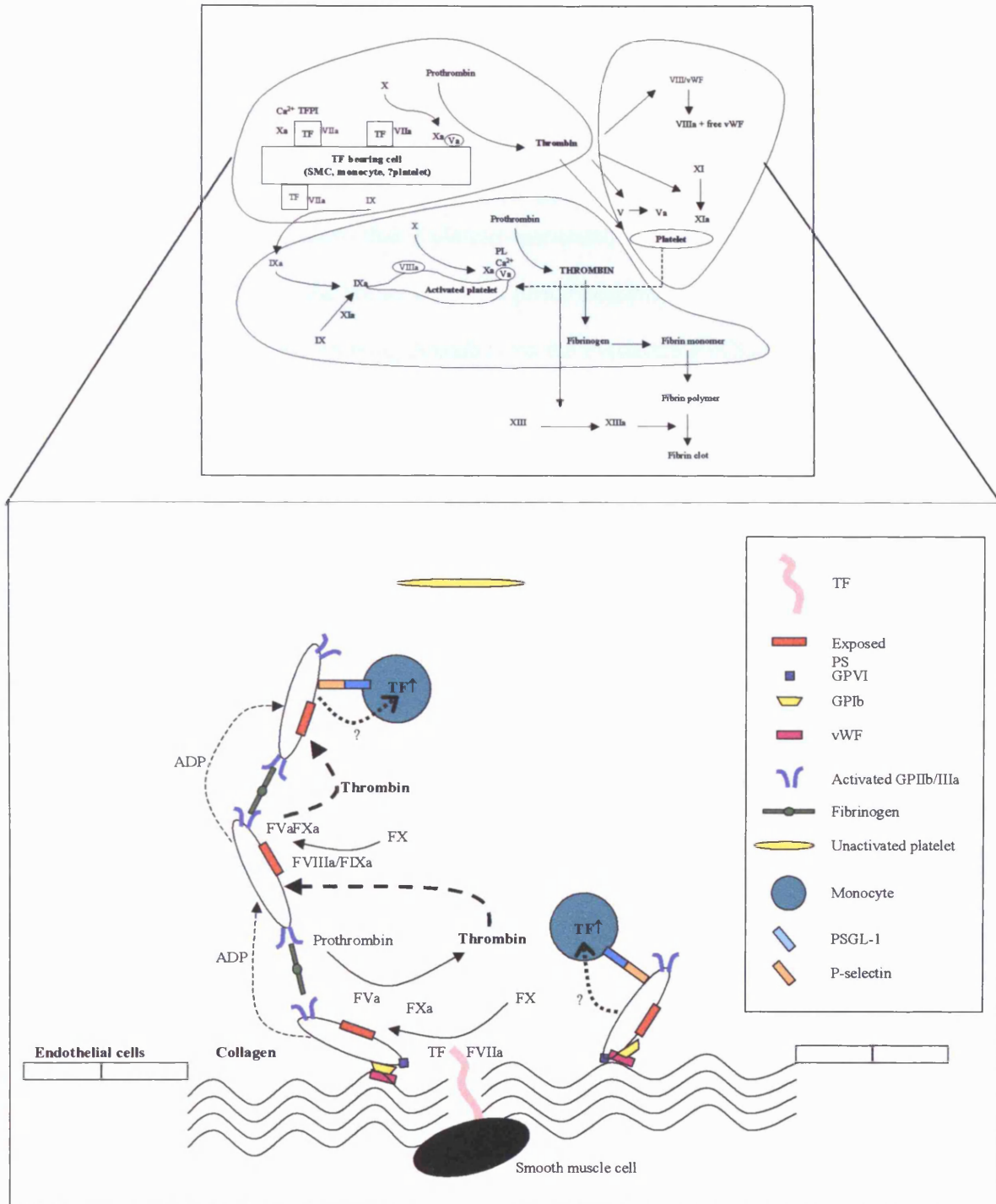


Figure 6.1. Revised cell-based model of coagulation. Initiation of coagulation and PS exposure occur simultaneously after exposure of collagen and TF in the subendothelium. Thrombin, either from the initial phase of coagulation or the thrombin burst, causes PS exposure only on platelets in contact with other platelets. Soluble factors and ADP released from activated platelets enhances the procoagulant response and upregulate TF in monocytes. Adapted from Hoffman & Monroe, 2001 and Gibbins, 2004.

exposure in the first phase of coagulation. However, once a thrombus has started to form and platelets are in contact with each other, thrombin and ADP released from activated platelets would then enable PS to be exposed on platelets distal from the collagen at the site of the wound. The need for contact before thrombin can cause PS exposure would help to confine clot formation to the area of injury. The data presented show that if platelet-associated TF plays any part at all in clotting, it is minimal, implying that TF in the vessel wall is of prime importance. Although monocytes and other leukocytes are recruited to the growing thrombus via the P-selectin-PSGL-1 interaction, it is soluble mediators which upregulate TF and, later, TFPI in monocytes. This presumably stabilises, and then limits, the clot.

The data in this project also have implications for anti-thrombotic therapy. They indicate that there is a relatively small window for altering coagulation through altering PS exposure. It is clear that, whilst aspirin is effective in damping down other aspects of platelet activation, it has little impact on the procoagulant response. In contrast, anti-ADP receptor agents, such as clopidogrel, would reduce PS exposure. Inhibitors of GPIIb/IIIa would similarly reduce PS exposure. Though they might also temporarily increase the number of monocytes with attached platelets, in the longer term, they would appear to reduce the longevity of MPAs. However, it is not clear what the consequences of such MPA promotion and dissolution would be, particularly given the evidence that soluble factors mediate at least some aspects platelet-monocyte interaction. The complexity of the situation is also illustrated by the role of P-selectin in coagulation. Although this project shows that many procoagulant aspects of monocyte activation are not prevented by blocking P-selectin, TEG experiments showed that clot formation was slightly influenced by Mab 9E1. A recent paper has reported that P-selectin causes monocytes to express PS, thereby enhancing thrombin generation (del Conde *et al*, 2005), and it has also been shown to promote monocyte vesiculation (Hrachovinova *et al*, 2003). It may be these aspects of its activity rather than its effect

on TF gene regulation that have led to animal models showing promising results from targeting P-selectin either to inhibit or enhance coagulation (Cambien & Wagner, 2004).

The role of platelets in the regulation of the procoagulant environment in blood is multifaceted. Suggestions on how to take the work of this project forward have been made at the end of the relevant chapters, but perhaps the key theme is investigating the precise contribution of elements of the secretome to coagulation. Understanding how platelets affect thrombus formation, and subsequent lysis, is crucial to the prevention and treatment of thrombotic disorders.

ANNEX 1: suppliers' addresses

Alomone Labs	Jerusalem, Israel
Alpha Laboratories Ltd.	Eastleigh, UK
American Diagnostica	Greenwich, CT, USA
Amersham Pharmacia	Little Chalfont, UK
BD Biosciences	Oxford, UK
BDH	Poole, Dorset, UK
Beckman Coulter™	High Wycombe, UK
Becton Dickinson, UK	Oxford, UK
Bender MedSystems	Vienna, Austria
Biogenesis	Poole, UK
Bio-Rad	Hemel Hempstead, UK
Calbiochem	Nottingham, UK
Centocor BV	Leiden, The Netherlands
Chromogenix	via Quadrantech Diagnostics Ltd., Epsom, UK
Cymbus Biotechnology Ltd.	Chandlers Ford, UK
DakoCytomation	High Wycombe, UK
Dynal Biotech	Wirral, UK
Enzyme Research Laboratories	Swansea, UK
Enzyme System Products	Livermore, CA, USA
Gibco BRL (Invitrogen)	Paisley, UK
ICN Biomedicals, Inc	Basingstoke, UK
Immunotech	Miami, FL, USA
Instrumentation Laboratory UK	Warrington, UK
Invitrogen	Paisley, UK
Membrane Filtration Products, Inc	Braine-l'Alleud, Belgium
Molecular Probes	Eugene, Oregon, USA
Novagen (Merck Biosciences)	Nottingham, UK
Novo Nordisk Pharmaceuticals	Crawley, UK
Nycomed	München, Germany
Pentapharm GmbH	Basel, Switzerland
BD Pharmingen (BD Biosciences)	Oxford, UK

Promega UK	Southampton, UK
R & D Systems	Abingdon, UK
Roche Diagnostics	Lewes, UK
Serotec	Oxford, UK
Sigma	Poole, Dorset, UK
Sigma-Genosys	Cambridge, UK
Stratagene® Europe	Amsterdam, The Netherlands
Sysmex	Milton Keynes, UK
Triple Red Limited	Long Crendan, UK

ANNEX 2: buffers

HBS:	150mM NaCl; 5mM KCl; 1mM MgSO ₄ ; 10mM HEPES; pH to either 6.0 or 7.4 using NaOH as required
HBS/c:	HBS + 2mM CaCl ₂
0.2 % FS:	0.2% v/v formaldehyde in 0.85% w/v isotonic saline solution
PBS:	0.01M phosphate buffer; 2.7mM KCl; 137mM NaCl; pH 7.4
D-PBS:	2.67mM KCl; 1.47mM KH ₂ PO ₄ ; 138mM NaCl; 8.1mM Na ₂ HPO ₄ ·7H ₂ O
TBS:	20mM Tris; 137mM NaCl; 38mL 1M HCl; pH 7.6
TBS/T:	TBS + 0.1% Tween 20
TBS/MT:	TBS + 0.1% Tween 20 + 5% Marvel dried milk

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