The importance of platelets and complement in material-induced leukocyte activation *in vitro*

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Chemical Engineering and Applied Chemistry
University of Toronto

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A mes parents, et à Robert

The importance of platelets and complement in material-induced leukocyte activation in vitro.

PhD. 2001

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Abstract

The role of material, platelets, and complement on material-induced leukocyte activation was investigated using an *in vitro* bead (45 μm) assay with polystyrene (PS), polyethylene glycol-immobilized polystyrene (PS-PEG), and PS-PEG-NH_2. Blood contact with beads activated leukocytes (tissue factor (TF) expression, CD11b upregulation and association with platelets), resulting in increased procoagulant activity. In the bulk, activation was independent of material surface chemistry: activation of adherent leukocytes was material-dependent.

In blood, tissue factor expression on monocytes, but not CD11b upregulation on leukocytes in the bulk, was independent of material surface area. Material-induced TF expression, but not CD11b upregulation, required the presence of platelets. In fact, TF expression was dependent on the association between platelets and monocytes. Both anti-IIb/IIIa and anti-P-selectin reduced TF expression. Inhibiting complement alone with sCR1 (a specific complement inhibitor) was without effect on material-induced TF expression. However, combining sCR1 and the platelet antagonist anti-GPIIb/IIIa significantly reduced material-induced TF expression in blood.

Complement inhibition with sCR1 was only effective in reducing CD11b upregulation in the bulk in the presence of a material that strongly activated complement.

such as PS-PEG, but not with the moderate activator PS. When effective, complement inhibition with sCR1 only partially reduced material-induced CD11b upregulation in the bulk, suggesting that other mechanisms exist. Platelet activation was identified as the other mechanism contributing to material-induced CD11b upregulation in blood. Inhibiting both complement (with sCR1) and platelets (with anti-GPIIb/IIIa) blocked material-induced CD11b upregulation. The presence of pyridoxal-5-phosphate alone also significantly reduced both TF expression and CD11b upregulation in the bulk: this is believed to be associated with its C1q and platelet inhibitory activities.

Both adsorbed complement products and adherent platelets mediated leukocyte adhesion. TF expression on adherent monocytes was strongly dependent on the presence of adherent platelets, while adsorbed complement products regulated CD11b upregulation on adherent leukocytes.

In vitro material-induced leukocyte activation, associated with the expression of procoagulant activities, appeared to be secondary to both complement and platelet activation. Blood-material compatibility may be greatly improved with a material that minimally activates both platelets and complement, or by the use of therapeutic agents, such as P5P, that simultaneously inhibit platelets and complement.

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Abbreviations

aPTT	activated prothrombin time
ATIII	antithrombin III
BSA	Bovine serum albumin
C1-INH	C1 inhibitor
EDTA	Ethylenediaminetetraactic acid
EFW	Endotoxin free water
ELISA	Enzyme linked immunosorbent assay
EU	Endotoxin units
FBS	Fetal bovine serum
FDA	U.S. Food and drug administration
FITC	Fluorescein isothiocyanate
FL	Fluorescent
FSC	Forward side scatter
FVII	Factor VII
GP	Glycoprotein
GPIIb/IIIa	Glycoprotein IIb/IIIa
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HMWK	High molecular weight kininogen
HTB	Hepes tyrode's buffer
ICAM-1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IIb/IIIa	GPIIb/IIIa
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MPs	Microparticles
NAAGA	N-acetyl aspartyl glutamic acid
P5P	Pyridoxal-5-phospate
PAF	Platelet activating factor
PBS	Phosphate buffer saline
PE	R-phycoerythrin
PE-Cy5	R-phycoerythrin-cytochrome 5
PEG	Polyethylene glycol
PEO	Polyethylene oxide
PET	Polyethylene terephtalate

PMA	Phorbol 12-myristate 13-acetate
PPACK	D-phenylalanyl-L-propyl-arginyl chloromethyl ketone
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Polystyrene
PSGL-1	P-selectin glycoprotein ligand-1
PTT	Prothrombin time
sCR1	Soluble complement receptor Type 1
SEM	Scanning electron microscopy
SSC	Side size scatter
TAT	thrombin-antithrombin III complex
TF	Tissue Factor
TFPI	tissue factor pathway inhibitor
vWF	von Willebrand factor
XPS	X-Ray photoelectron spectroscopy

Glossary

C1q	Classical complement protein. Circulates in plasma as a subunit of the first component of complement C1. Participates in the activation of the classical complement pathway by antigen-antibody complexes.
CD11b	Leukocyte integrin receptor. Binds ICAM-1, fibrinogen, Factor X and iC3b. CD11b is constitutively expressed on leukocytes and is upregulated upon leukocyte activation.
Coagulation	System of plasma proteins involved in the generation of thrombin and fibrin.
Complement	System of plasma proteins participating in the inflammatory response. Complement is activated by the presence of antigen-antibody complexes and any foreign/non-self material.
Endotoxin	Also called lipopolysaccharide (LPS). Endotoxins are part of the outer membrane of gram-negative bacteria and are released upon death or lysis of the bacteria. Endotoxin is a common contaminant in water and materials. Blood cells, such as leukocytes, become activated in the presence of endotoxin.
GPIIb/IIIa (or IIb/IIIa)	Platelet glycoprotein receptor. Binds fibrinogen (primary ligand) and enables platelet aggregation. Blocking the GPIIb/IIIa receptor is seen as a useful means to block platelet aggregation and hence prevent thrombus formation.
iC3b	Inactivated complement protein, generated upon complement activation. Binds/adsorbs to cells or materials and promotes leukocyte adhesion.
Leukocytes	Nucleated white blood cells. Participate in the inflammatory and immunologic response. Neutrophils and monocytes are the major cellular components of the inflammatory response triggered by the presence of a material.
L-selectin	Leukocyte receptor. Initiates rolling of leukocytes on cells and materials prior to adhesion. Shed upon leukocyte activation.
Microparticles	Small particles generated upon vesiculation of cellular membranes.
NAAGA	Non-specific complement inhibitor. Blocks both the classical and alternative pathway of complement activation.
P5P	Co-enzyme form of vitamin-B_6. Non-specific agent with both platelet and classical complement inhibitory activities.
Pentamidine	Antiprotozeal drug. Non-specific complement inhibitor (serine protease inhibitor). Blocks classical complement

	activation by inhibiting C1s.
Platelets	Small anuclear blood cells which main role is to preserve the integrity of the vessel wall through the formation of platelet plug via platelet aggregation (clot, thrombus).
P-selectin	Platelet receptor expressed upon platelet activation. P-selectin mediates the formation of platelet-leukocyte aggregates by binding to its ligand on leukocytes.
Reopro	GPIIb/IIIa antagonist. Commercial name of a chimeric antibody to GPIIb/IIIa.
SC5b-9	Complement protein generated upon activation of the terminal pathway. Indicates the extent of complement activation generated by a foreign surface.
sCR1	Specific complement inhibitor. Blocks the activation of the alternative pathway of complement.
Thrombus	Composed of fibrin and blood cells (platelets, red blood cells and leukocytes). May form in and obstruct (partially or entirely) any part of the cardiovascular system.
Tissue factor (TF)	Transmembrane glycoprotein receptor. TF initiates the extrinsic pathway of coagulation. It is expressed constitutively on tissue barriers between the body and the environment. Leukocytes, such as monocytes and macrophages, also synthesize and express TF on their membrane upon activation.

• Herein, non-specific implies that these agents have other anti-inflammatory properties than the mentioned anti-complement activities.

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Chapter 1

Thesis scope and hypotheses

I. Introduction

Blood-material interaction triggers a complex series of events including protein adsorption, platelet and leukocyte activation/adhesion, and activation of the complement and coagulation cascade. In the wide variety of blood-contacting devices, no material has been found yet to be truly blood-compatible. Despite anticoagulant and anti-platelet activation therapy, thrombosis and thromboembolism continue to occur in cardiovascular devices[1,2]. When assessing the thrombotic properties of biomaterials, most studies focus on platelets and/or Factor XII activation. However, while platelets are a key cellular component to thrombosis, material-induced leukocyte activation may also be an important player in cardiovascular material failure, possibly by contributing directly to thrombin production (thrombogenesis) and/or by releasing procoagulant mediators.

In vitro[3-6] material-induced leukocyte activation, as measured by increased degranulation, oxidative burst and CD11b upregulation, has been reported with little difference among materials, regardless of chemistries and protein coatings. Monocytes were also recently shown to express procoagulant activity, through tissue factor (TF), during cardiopulmonary bypass[7,8] and simulated extracorporeal circulation[9,10]. There is however very little known on monocyte expression of TF following material contact and material-induced leukocyte activation with CD11b upregulation: both have potential procoagulant activities but have rarely been studied simultaneously. The mechanisms of material-induced leukocyte activation are also unclear: platelets, complement, released inflammatory mediators, adsorbed proteins, and material chemistry may all have a role in leukocyte activation. The role of complement on leukocytes has been extensively studied but its relative contribution to material-induced leukocyte activation is uncertain.

Improvements in the blood compatibility of cardiovascular materials will be difficult unless we can achieve a better understanding of all relevant interactions with biomaterials. Our underlying premise is that this requires understanding of the mechanisms of leukocyte activation as well as defining the impact of this activation. While many investigations on leukocyte activation have focused on one factor (usually material chemistry or complement),

we have undertaken a more global approach where the effects of complement, platelets and material were assessed on parameters of leukocyte activation that can be linked to procoagulant activity. While complex, this approach allowed us to better understand the multifactorial aspects of blood-material interactions. Such an understanding was expected to help in the design of better biomaterials and/or lead to novel therapeutic approaches that effectively reduce thrombotic complications with cardiovascular devices. The objectives of my thesis were to:

1. evaluate tissue factor expression and other membrane-associated procoagulant activities on leukocytes following blood material-contact (Chapter 3);
2. characterize some of the mechanisms of material-induced leukocyte activation associated to procoagulant activity:
    a) role of platelets (Chapters 4 and 6)
    b) role of complement activation (Chapters 5 and 6).

II. Thesis scope and format

An *in vitro* assay with small beads (45 μm) was used to assess the role of material, complement and platelets on material-induced leukocyte activation that may be associated with procoagulant activity. Our previous experience has shown that little difference appears to exist among materials of different chemistries[5;6]. Therefore, we focused on two materials, polystyrene and polyethylene glycol-immobilized polystyrene, as model of blood activating surfaces. Material-induced leukocyte activation was characterized by flow cytometry and both leukocytes in the bulk and adherent to the beads were studied. Our parameters of leukocyte activation that are linked to procoagulant activity were TF expression, CD11b upregulation and leukocyte association with platelets. To assess the role of complement in material-induced leukocyte activation, the complement inhibitors, sCR1, pentamidine, NAAGA, and pyridoxal-5-phosphate were chosen. Two platelet antibodies, anti-GPIIb/IIIa and anti-P-selectin, were selected for our studies on the role of platelets in material-induced leukocyte activation. *In vitro* experiments were performed with either PPACK or heparin as anticoagulants.

Each chapter is presented as an independent unit. Chapter 2 provides an overview of the current knowledge on the different components of blood-material interactions

(coagulation, complement, leukocytes and platelets) as well as a review of leukocyte procoagulant activities. Chapters 3 to 6 present our investigations on the effect of material, platelets, and complement on material-induced leukocyte activation in whole blood or isolated cells. Chapter 7 provides a synopsis of the lessons learned and offers perspectives for future work. Chapter 8 summarizes the conclusions.

III Hypotheses

The primary hypothesis of this work was that material-induced leukocyte activation results in the expression of membrane associated procoagulant activities. As illustrated in Figure 1 (next page), leukocyte activation occurs in both the circulating blood and on the surface. Adherent activated leukocytes have an important role, by contributing directly to localized intravascular thrombosis.

Different hypotheses were then formed to assess material-induced leukocyte activation and the mechanisms involved. In **Chapter 3**, two hypotheses were tested. (1) In whole blood, the presence of a biomaterial induces significant tissue factor expression and other membrane-associated procoagulant activities on leukocytes. (2) Material-induced leukocyte activation is independent of material chemistry. In **Chapter 4**, we hypothesized that leukocyte activation in the bulk was independent of platelets while adherent platelets played a role in monocyte adhesion and subsequent activation. In **Chapter 5**, our hypothesis was that inhibiting complement activation reduced leukocyte activation and adhesion independently of the material. Finally, in **Chapter 6**, we hypothesized that, in whole blood, inhibiting both platelet and complement activation reduced material-induced leukocyte activation.

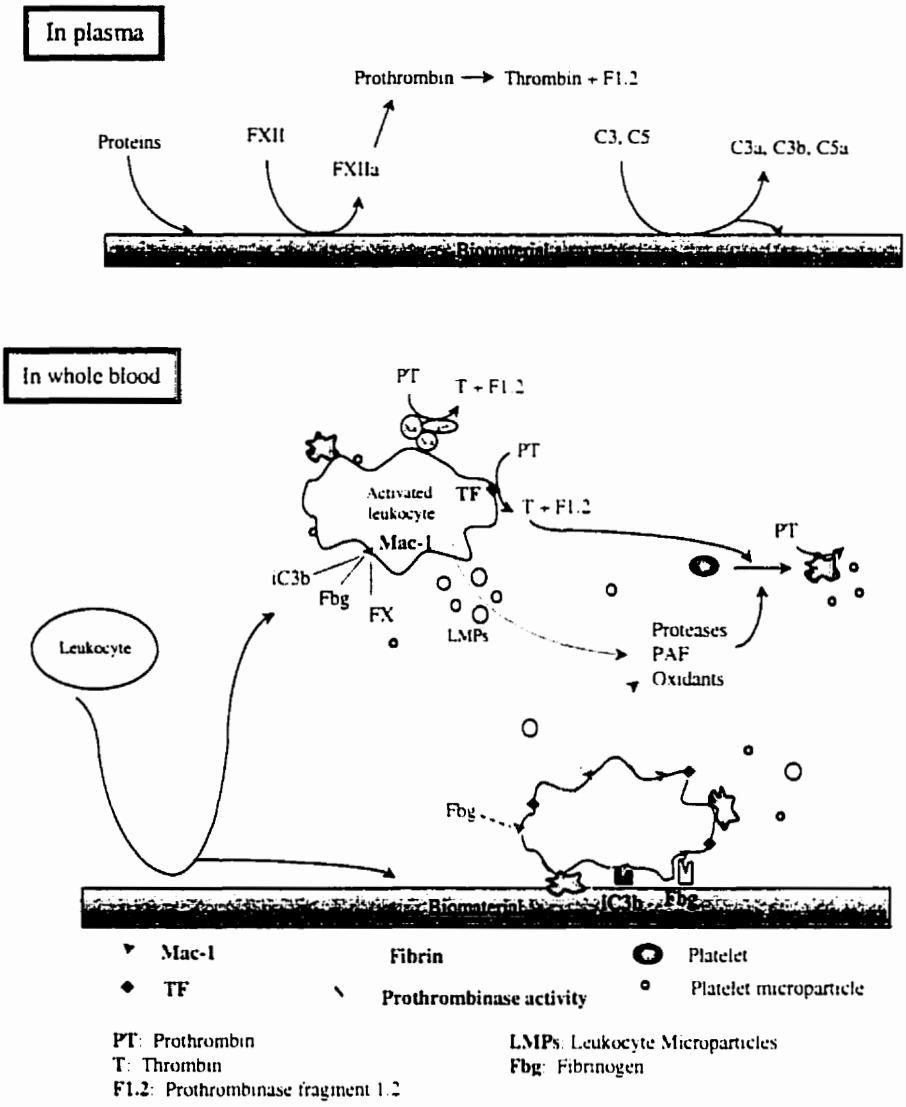

Figure 1: Blood-material interactions, leukocyte activation and potential leukocyte procoagulant activities. Following material contact, leukocytes become activated. Monocytes synthesize and then transport tissue factor (TF) to the cell surface. TF is a transmembrane glycoprotein initiating the extrinsic pathway of coagulation. An integrin leukocyte adhesion molecule, CD11b is expressed on monocytes, neutrophils and natural killer cells and is upregulated following activation. CD11b can bind ICAM-1, iC3b, fibrinogen and Factor X, the latter two being most important for the procoagulant properties of leukocytes. The membranes of activated leukocytes will also provide negatively charged phospholipids for the assembly of the prothrombinase complex (Factor Xa - Factor Va). Inflammatory mediators such as proteases, oxidants, and the platelet activating factors can also be released and result in platelet activation as

well as further activation of the leukocytes. Similarly to platelets, leukocytes also generate microparticles.

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Chapter 2

Thrombosis and Biomaterials

I Introduction

Biocompatibility is defined as "the ability of a material to perform with an appropriate host response in a specific application". Biocompatibility of blood contacting devices or hemocompatibility relates in part to the thrombogenic and inflammatory response induced by the materials, but also to their toxicity, their immunogenicity, and to the level of hemolysis. Although no material has been found truly biocompatible, many cardiovascular devices function with low or acceptable risks of complications[1]. Hemolysis, toxic, and immunologic reactions have usually been dealt with earlier in the development of a material to be used for cardiovascular devices and are rarely an issue with their use. However, thrombotic and thromboembolic complications, as well as bleeding risks associated with the necessary anticoagulant therapy remain of serious concern with cardiovascular devices. Clinical manifestations of the bioincompatibility of cardiovascular devices are numerous such as sudden and complete obstruction of stents within weeks[2]; acute and subacute thrombotic occlusion in medium sized grafts (4-6 mm)[3]; embolic complications with artificial hearts[4], catheters[5], and prosthetic valves[6,7]; thrombotic complications during cardiopulmonary bypass[3] and angioplasty[8]. Larger vascular grafts also remain thrombogenic for many years, but fewer thrombotic complications are observed as high flows disperse the clotting factors. However, occasional embolic episodes occur as high flows may dislodge the thrombotic deposits. Even if the risk of thrombotic complication appears to be low (varying between 2 and 10% depending on the device), they may have fatal outcomes and the cost associated with the new intervention is not negligible. Furthermore, these thrombotic complication with cardiovascular devices also occur despite the use of antiplatelet and anticoagulant therapies underlining the inherent thrombogenicity of the materials. Material thrombogenicity is further illustrated by the acute failure of small diameter vascular grafts despite the strong anticoagulant regimen. The many years of intensive research on materials have not yet produced a material, which has proven suitable for this application.

To improve the blood compatibility of cardiovascular devices, surface modifications, such as attachment of antithrombotic agents, increase of the material hydrophilicity with polyethylene oxide (PEO), and substitution of hydroxyl groups, have been considered but their success has been limited. Despite more than 20 years of research, coating surfaces with polyethylene glycol or PEO, which reduces protein adsorption and prevents platelet adhesion, has remained at the laboratory stage. Different methods of heparin coatings have been developed and have actually been able to reach the commercial stage in cardiopulmonary bypass[9]. However, reports on the improvement of *in vivo* biocompatibility have been mixed[10-16]. Heparinized cardiopulmonary bypass circuits appear to partially reduce the inflammatory response associated with cardiopulmonary bypass[17;18]. But to date, heparin coating has not yet been proved to significantly reduce the number of postoperative complications, improve patient outcome, or reduce hospital stay[16;19;20]. This illustrates another limit of our understanding of blood-material interactions: we do not know the levels at which the inflammatory and thrombosis response induced by the device alter the normal hemostasis and result in harmful consequences.

Since chemical surface modification has not brought solution to the biocompatibility issue of cardiovascular devices, different pharmacological approaches are now being investigated. Complement inhibition with the use of sCR1[21] or anti-C5a antibody[22], serine protease inhibitors such as aprotinin[23;24], platelet receptor antagonists such anti-GPIIb/IIIa[25], and cytokine antibody[26] have been tested to reduce thrombotic complications with cardiopulmonary bypass. While the results are promising by showing a partial reduction of the inflammatory or the thrombotic response to cardiovascular devices, it is as yet too early to conclude on the overall improvement of the device biocompatibility. Each compound only affects one of the players in the blood compatibility response and this might not be sufficient to result in clinical benefits. The use of these inhibitors and antibodies will also provide valuable information on the mechanisms involved in thrombotic complications of cardiovascular devices.

II Important components in biocompatibility of cardiovascular devices

Under normal hemostatic conditions, blood contacts an endothelium with anticoagulant and antithrombotic properties. The use of cardiovascular device represents the introduction of a foreign surface in the circulation, without the properties of the endothelium.

Blood-material interactions trigger a complex series of events including protein adsorption, platelet and leukocyte activation/adhesion, and the activation of complement and coagulation (Figure 1).

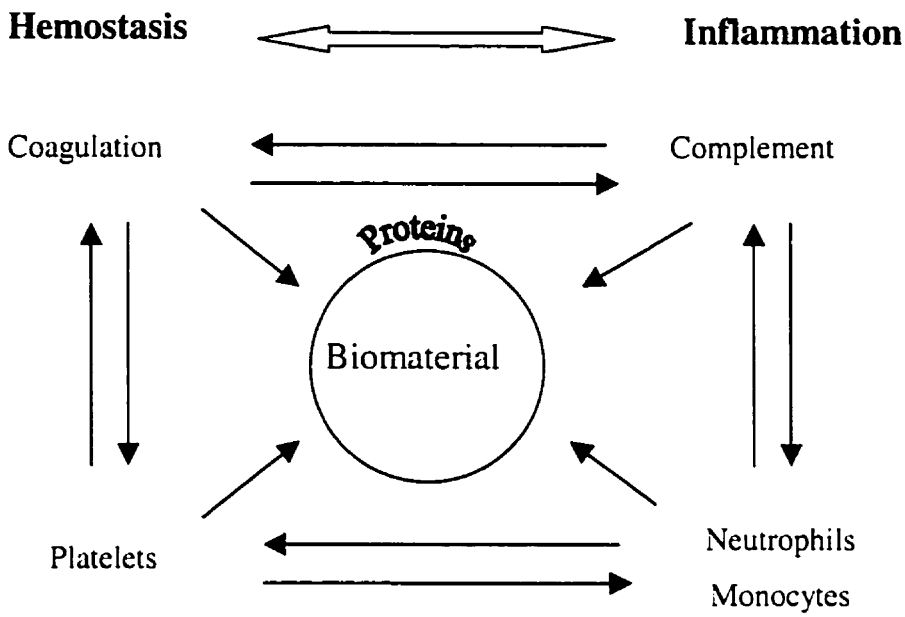


Figure 1: Schematic of blood-material interactions

1) Coagulation cascade

Blood coagulation involves a series of proteolytic reactions resulting in the formation of a fibrin clot. Thrombin is formed following a cascade of reactions where an inactive factor becomes enzymatically active following surface contact or after proteolytic cleavage by other enzymes; the newly activated enzyme then activates another inactive precursor factor. Initiation of clotting occurs either intrinsically by surface-mediated reactions, or extrinsically through tissue factor expression by cells. The two systems converge into the common pathway resulting in the formation of fibrin clot upon action of thrombin on fibrinogen. Then, Factor XIII, activated by thrombin, crosslinks and stabilizes the fibrin clot into an insoluble fibrin gel (Figure 2).

a) The intrinsic pathway

The intrinsic pathway is initiated by contact activation of high molecular weight kininogen (HMWK), prekallikrein and Factor XII: these molecules require contact with (negatively charged) surfaces for zymogen activation *in vitro*[27]. Factor XII is activated by

adsorption, FXIIa converts prekallikrein into kallikrein and with HMWK as a cofactor activates Factor XI to Factor FXIa. Factor XIa activates Factor IX to Factor IXa. Following a cascade of reactions involving among others the intrinsic tenase complex (Factor IXa-Factor VIIIa), prothrombin is cleaved into thrombin.

The importance of the intrinsic pathway to normal blood coagulation remains speculative, as the occurrence of negatively charged surfaces *in vivo* is limited. Collagen present in the subendothelium after vessel injury may provide the surface required for this reaction[28]. Under physiologic conditions, the lack of relevance of the contact activation system is also underscored by the fact that deficiencies of the contact proteins, HMWK, prekallikrein and Factor XII, have not been associated with abnormal bleeding[1;27;29].

b) The extrinsic pathway

The physiological initiator of coagulation is tissue factor, which is expressed on damaged cells at the site of vascular injury. Plasma Factor VII (FVII) binds to tissue factor (TF) on the cell membranes and requires activation to FVIIa to form the extrinsic tenase complex: TF-VIIa complex. FVII is activated by trace amounts of thrombin, FIXa, FXa and TF-VIIa complex, the latter being more likely physiologically relevant as small amounts of TF-FVIIa are present extravascularly *in vivo*[30;31]. Picomolar concentrations of FVIIa circulate normally in blood and are also thought to serve as a primer in the initiation of the coagulation cascade by allowing direct formation of TF-FVIIa complex upon TF exposure[32]. TF-FVIIa complex on cell membranes cleaves Factor X into Factor Xa in the presence of calcium. The prothrombinase complex can then assemble on the membrane and generate thrombin (common pathway of the coagulation cascade). FX is not the only physiological substrate of the TF-FVIIa complex. The TF-FVIIa complex also activates FIX[33]. As illustrated in Figure 3, a new view of the blood coagulation cascade, centered on TF dependent pathway, is now considered dominant.

The extrinsic and intrinsic pathways are not independent from each other. When coagulation is initiated by a TF-dependent pathway, the intrinsic tenase remains important, as production of FXa by FIXa-FVIIIa complex has been shown to significantly contribute to thrombin generation[34]. It actually appears that extrinsic tenase TF-FVIIa is responsible for the onset of coagulation while the intrinsic tenase is the major player in the propagation phase[35]. The activation of FX by FIXa is also all the more important than tissue factor pathway inhibitor (TFPI) will reduce the production of FXa by TF-VIIa complex[30;31].

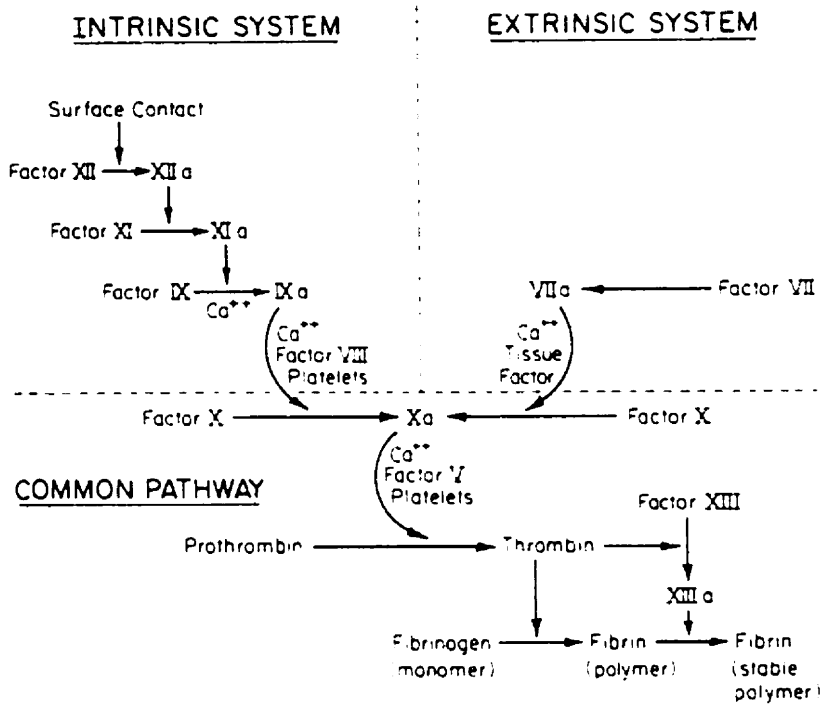


Figure 2: The blood coagulation cascade (old view)[36]

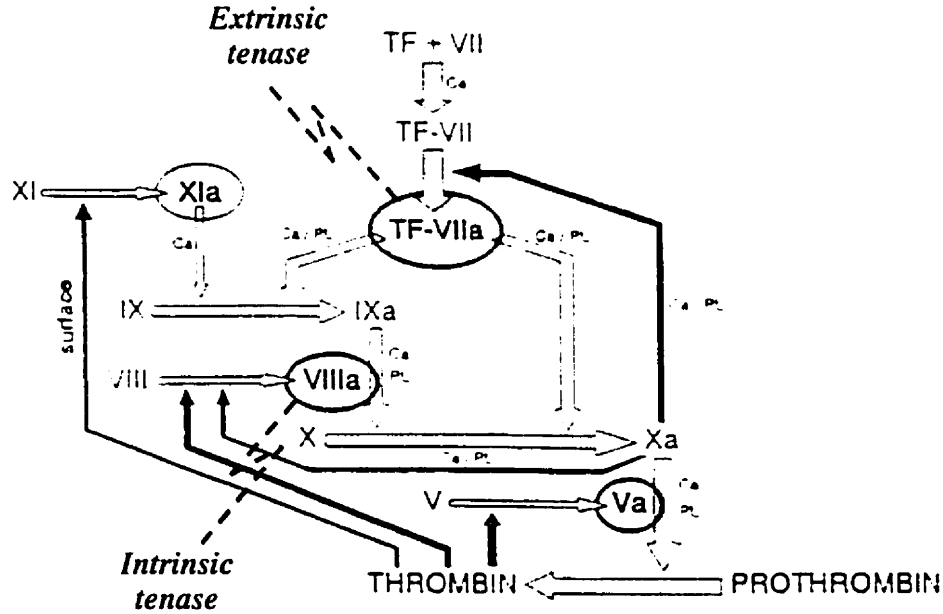


Figure 3: The revised blood coagulation cascade[30]

c) Physiologic inhibitors of coagulation

Activated coagulation factors are serine proteases. Plasma contains several protease inhibitors, such as α_1-protease inhibitor, α_2-macroglobulin, heparin cofactor II and antithrombin III, to modulate and inhibit their activity. Antithrombin III is the most important[37]: it neutralizes its target enzymes, FXa and thrombin, by forming a complex with the enzyme in which the enzyme's active site is blocked. In the absence of heparin, complex formation occurs at a relatively slow rate. However, in the presence of the polysaccharide heparin or naturally occurring heparan sulfate (on the endothelium), inhibition rates rise significantly and antithrombin is also able to inhibit FIXa, FXIa and FXIIa[38].

While TF-VIIa is not efficiently inhibited by antithrombin, it has its own inhibitor: the tissue factor pathway inhibitor (TFPI). Plasma TFPI is not the major intravascular pool of TFPI. A larger pool exists on the luminal surface of the vascular endothelium, which is released by a bolus injection of heparin. Platelets also carry 10% of the total TFPI in blood and release their TFPI following stimulation by thrombin and other agonists[39]. Activated monocytes may also release TFPI[40]. TFPI has two inhibitory sites, one for FXa and one for TF-VIIa complex. Inactivation of FXa through binding to TFPI in solution is required for inhibition of the complex TF-VIIa. The initial binding of FXa to TFPI is relatively slow[41] and may not be able to prevent thrombosis when TF-FVIIa complex are being formed at a high rate[31]. Leukocyte elastase also cleaves TFPI and impairs its ability to inhibit both FXa and TF-VIIa complex. Upon exposure of a complex FXa/TFPI/TF-VIIa to elastase, FXa and TF-VIIa activities are restored[42].

The endothelium also participates in the regulation of thrombosis via thrombomodulin. Thrombin binds to thrombomodulin on endothelial cells and this complex activates protein C[43]. Protein C is a vitamin K-dependent protein and activated protein C inactivates FVa and FVIIIa. Protein S, also a vitamin K-dependent protein, is a necessary cofactor for activated protein C. More than half of the protein S in plasma is bound to the C4b-binding protein (a regulatory protein of the complement system) and is not functionally active.

d) Biomaterials and coagulation pathways

While under physiological conditions, the role of FXII activation is questionable, in the presence of a cardiovascular device: activation of FXII may occur as the artificial surface represents the required (negatively charged) surface.

Protein adsorption is the first event in blood-material interactions, and adsorption of the contact phase proteins may result in activation of the intrinsic cascade. Earlier studies had focused on protein adsorption on glass or biomaterial surfaces with isolated protein solutions or diluted plasma, and showed how fibrinogen would be replaced over time by HMWK (the Vroman effect), suggesting a possible role of the intrinsic pathway in material thrombosis[44]. Recent studies using whole blood or plasma have provided new insights on the adsorption and activation of contact phase proteins. FXII adsorption has been observed in moderate amounts on materials used in vascular grafts[45] and hemodialysers[46], however it was not found in its activated form[47]. Although HMWK and prekallikrein may be adsorbed on the material surface, the lack of FXIIa on the material surface will stall the initiation of coagulation through the intrinsic pathway. In some instance, *in vitro* activation of FXII and kallikrein has been reported with biomaterials[48-50], however no test was performed to determine if such activation resulted in any significant activation of coagulation. Other studies have actually shown that only minute amounts of thrombin or thrombin-antithrombin III complex (TAT) are generated when biomaterials are incubated with undiluted plasma alone[51-53]. Furthermore, higher levels of adsorbed kallikrein and Factor XII on biomaterials do not correlate with TAT formation[51], suggesting that the contact phase proteins play little role in the activation of coagulation. In fact, a study by Hong et al[52] showed that the presence of leukocytes is required for activation of the coagulation cascade; the requirement of a TF-dependent pathway of initiation of coagulation may thus also apply to biomaterials. *In vivo*, a small increase of FXIIa is observed during cardiopulmonary bypass[54;55], but it appears to be in response to the surgical intervention and the establishment of extracorporeal circulation does not further increase FXIIa levels[55]. Furthermore, no significant correlation between FXIIa and thrombin generation have been observed[55]. *In vivo* results with hemodialysis also failed to show any significant increase of FXIIa[56]. The fact that a patient with a severe FXII deficiency showed similar level of thrombin generation during cardiopulmonary bypass than normal patients[57] casts further doubts on the role of Factor XII in the initiation of coagulation with biomaterials. Taken all together, these studies suggest that activation of the contact phase proteins do not play a role in the activation of coagulation by biomaterials.

While the tissue factor pathway of blood coagulation has become the focus in haematology and has led to the revised version of the coagulation cascade, the biomaterials

community has been slow to recognize its importance, still referring to the older model of coagulation with the separated intrinsic and extrinsic pathways and thinking that the extrinsic pathway is not directly related to blood-material interactions[1;58;59;60]. However, blood contact with a material represents a potential stimulus to induce TF expression by monocytes, resulting in the initiation of blood coagulation. Indeed, monocyte TF expression has been observed *in vivo* during or after cardiopulmonary bypass[61-63]. TF expression in thrombosis and blood-material interactions is reviewed below in section III. The role of leukocytes (most likely due to TF on monocytes) in activation of the coagulation by biomaterials has recently been highlighted in the study by Hong et al[52]. However there has been no study yet to determine if material-induced TF expression is responsible for the initiation of the coagulation cascade. More research is required to define the role of the extrinsic pathway of coagulation and biomaterials. This thesis is directed to this objective.

The time scale between initiation of the coagulation cascade by contact phase activation versus TF expression may also have an impact on the role of the two pathways in thrombotic complications due to cardiovascular devices. As it is part of the protein adsorption "reaction", contact phase activation occurs during the first few minutes of blood contact with a material. On the other hand, to be expressed on monocytes, TF requires synthesis and thus a minimum of 60 minutes (in a normal patient) would elapse before this pathway could significantly contribute to thrombin generation. It appears also that thrombin generation by FXIIa on materials is also dependent on flow[64]: supported by collective experimental evidence, the mathematical model used in Basmadjian's study[64] demonstrated that under very low (near stagnant) and high shear conditions, little thrombin was generated from the activation of the intrinsic pathway. Thus, the relative contribution and the role of the intrinsic and extrinsic pathways in thrombin generation likely depends on the flow situation. This adds another level of complexity to the understanding/characterization of thrombin generation with cardiovascular devices.

e) Anticoagulants and biomaterials

To prevent the formation of thrombi during cardiopulmonary bypass, hemodialysis and angioplasty, anticoagulants are routinely administered. One of the most commonly used anticoagulants is heparin[38]. Heparin binds to antithrombin III (ATIII) via a unique pentasaccharide sequence, and causes a conformational change in the reactive centre of ATII, thereby accelerating the rate of ATIII-mediated inactivation of several clotting

enzymes (thrombin, FXIa, FXa, and FIXa). Heparin promotes the formation of the complex thrombin-ATIII (TAT) by binding to both proteins. On the other hand, to inactivate FXa, heparin needs to bind only to ATIII. ATIII forms a 1:1 irreversible complex with coagulation enzymes, the heparin then dissociates and can be reused. One limitation of heparin is that it is unable to inactivate thrombin bound to fibrin or to surfaces, and to inhibit FXa within the prothrombinase complex[65].

Following implantation of cardiovascular devices, such as vascular grafts, stents and artificial valves, warfarin is the most commonly used oral anticoagulant. It interferes with the vitamin K cycle and thus impairs the biological function of vitamin K–dependent coagulation proteins (prothrombin, FVII, FIX, and FX)[38].

Other anticoagulants of interest in the use of cardiovascular devices[65] are the tick anticoagulant peptide (TAP) and antistatin which binds to FXa even within the prothrombinase complex; hirudin, a leech-derived protein, a potent thrombin inhibitor; and D-Phe-Pro-ArgCH_2Cl (or PPACK), a peptide that directly inactivates thrombin by interacting with the active site of thrombin. Both PPACK and hirudin are able to inactivate fibrin-bound and surface-bound thrombin[65;66].

When studying blood-material interactions *in vitro*, heparin is usually the anticoagulant of choice as it is the most widely used with cardiovascular devices. However, heparin also possesses some anti-complement activity[67]. PPACK and hirudin, which appear to be more specific than heparin, may then be used especially when the mechanisms of cell activation are studied. However, the associated high cost restricts their use.

2) Complement

The complement system plays an important role in the body defence mechanisms against infection and "non-self" elements. The complement system consists of more than twenty plasma proteins that function either as enzymes or as binding proteins. Complement activation is initiated by the classical or the alternative pathway and the terminal pathway is common to both (Figure 4). Both pathways contain an initial enzyme that catalyses the formation of the C3 convertase, which in turn generates the C5 convertase allowing the assembly of the terminal complement complex.

Specific complement products (C3b, C4b, iC3b) bind to particles, surfaces, bacteria, and immune complexes in a process called opsonization[68], which facilitates their uptake by

inflammatory cells. Activation of complement may also result in cell lysis when the terminal attack complex is inserted into the cell membrane. Complement activation also releases C3a, C4a and C5a, which are anaphylatoxins. These peptides are humoral messengers that bind to specific receptors on neutrophils, monocytes, macrophages, mast cells and smooth muscle cells. They induce a variety of cellular responses such as chemotaxis, vasodilatation, cell activation and cell adhesion[69]. They are responsible for the systemic effects of complement activation.

a) Classical pathway[29;70]

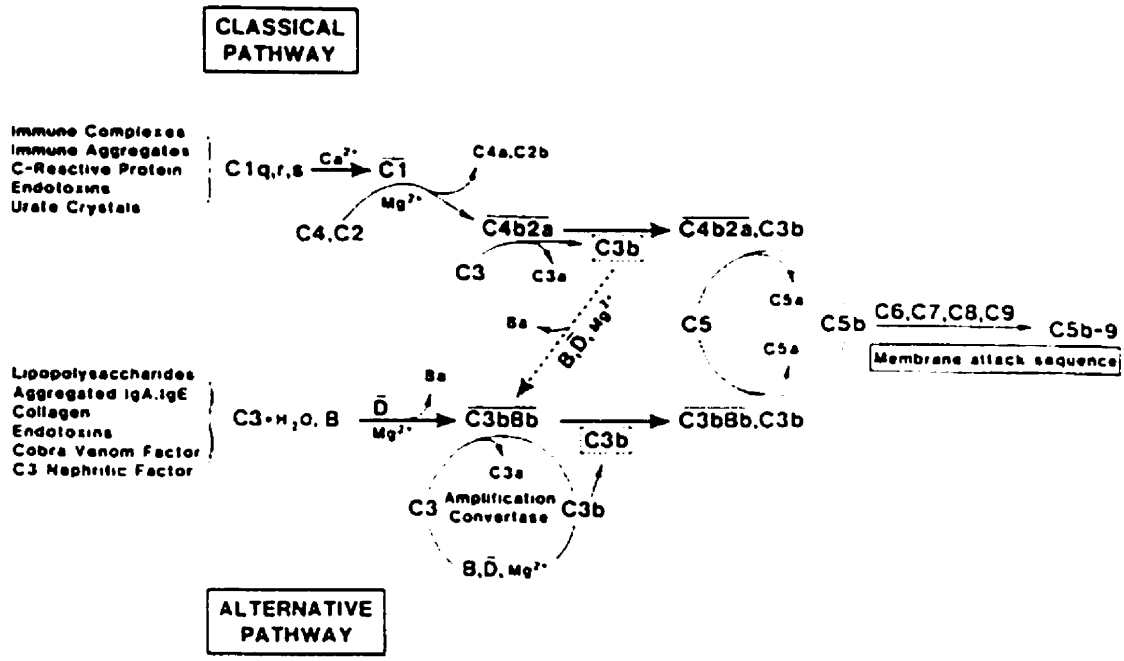
The classical pathway is triggered by antigen-antibody complexes that bind the C1 complex (C1q, C1r, C1s) by its C1q component. This activates C1s, which is then able to cleave the C4 complement protein into C4a and C4b. C4b attaches to its target surface via its exposed metastable thioester binding site. It is important to note that C4b does not bind efficiently to membrane surfaces and the fluid phase C4b is rapidly inactivated by the loss of its binding site. C2 binds to the attached C4b and is cleaved by C1s, releasing C2a. The classical C3 convertase, C4bC2b, is thus formed and can cleave C3 into C3a (anaphylatoxin) and C3b. The combination of C4bC2b and C3b becomes the C5 convertase, which cleaves C5 into C5a (anaphylatoxin) and C5b. C5b is the first component of the terminal complex and has high affinity for C6. C5bC6 then binds C7, C8 and up to twelve molecules of C9 and this forms the terminal complement complex C5b-9. If C5b is attached to a biological surface, the terminal complement complex (also called the membrane attack complex, mC5b-9) inserts itself into the lipid layers resulting in cell damage and/or lysis. In the absence of a biological membrane, the complex binds to protein S to create SC5b-9 in the fluid phase.

b) Alternative pathway[29;70;71]

The activation of the alternative pathway does not require antibody or immune complexes and is activated by any foreign surfaces, such as fungal, bacterial polysaccharides, lipopolysaccharides, particles and biomaterial surfaces. Complement activation via the alternative pathway occurs spontaneously at a low rate. Spontaneous hydrolysis of the internal thioester group of C3 occurs in the fluid phase, generating $C3.H_2O$. This hydrolysed C3 can bind and activate Factor B and cleave another C3 molecule into C3a and C3b. The alternative C3 convertase, C3bBb, is formed. In the absence of a surface to support binding of C3b, little complement activation occurs.

In the presence of a surface, covalent binding of C3b to hydroxyl or amine groups on the surface may occur via its carbonyl group in the C3b thioester binding site. Attachment of C3b to a surface favors binding of Factor B and Factor D to C3b. Factor D cleave Factor B into Ba and Bb, and the alternative C3 convertase, C3bBb, is formed again. This attached C3 convertase is able to generate more C3b, resulting in a positive amplification loop. Properdin acts to stabilize the C3 convertase. The clustering of C3b on the surface allows the formation of the alternative C5 convertase, C3bBbC3b, and C5 can be cleaved. The assembly of the TCC follows as for the classical pathway.

a)



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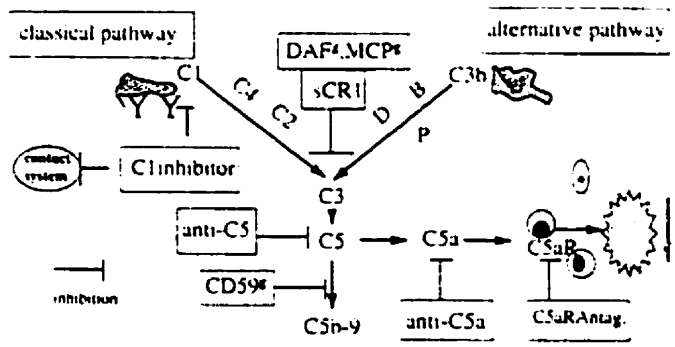

Figure 4: Pathways of complement activation[72] (a) and inhibition[73] (b)

c) Regulatory molecules of complement activation

The various molecules involved in the regulation of complement activation are illustrated in Figure 4b. The classical pathway is regulated by two specific mechanisms[29]: C1 inhibitor, a plasma protein, binding C1s or C1q and inhibiting the enzymatic activity of the C1 complex; and the C4b-binding protein inhibiting C4b bound to a membrane or a surface.

Factor I is a proteolytic enzyme that binds C4b and C3b, generating iC4b and iC3b, which are further degraded into smaller fragments. C4b-binding protein is a cofactor of Factor I and augments the degradation of C4b by Factor I. Factor H is a cofactor of Factor I for the degradation of C3b. Factor H is also able to displace Bb in the C3 convertase to promote C3b inactivation by Factor I[74].

On cell surfaces, the C3 and C5 convertase activity are regulated by three integral membrane proteins[74]. The decay-accelerating factor (DAF), found on all peripheral blood cells, destabilizes the C3 convertase by promoting the release of factor Bb. The membrane cofactor protein (MCP), expressed on leukocytes and platelets, favours the dissociation of Factor B and promotes C3b association with Factor I. The complement receptor type 1 (CR1), present on all blood cells except platelets, acts like Factor H and displaces Bb from the C3 convertase and facilitates inactivation by Factor I.

To prevent lysis of "bystander" blood cells, membrane proteins called homologous restriction factors limit the ability of the terminal complex to properly assemble on autologous cells[74]. Two proteins have been characterized: CD59 and the C8 binding protein (also called MAC inhibiting protein). CD59, found not only on blood cells but in many cells such as hepatocytes and epithelial cells, binds to C8 and C9 and inhibits C9 polymerization. Little is known about the C8 binding protein, which is believed to bind C8 and C9.

d) Interactions of complement and coagulation cascade

Although the coagulation and complement cascades are discussed as separate entities, the two cascades appear to interact significantly to modulate each other's activity[29;74]. Factor XIIa and kallikrein are known to cleave C1s[29] and thus have the capacity to trigger classical complement activation. Thrombin activates C3, C5, C6 and Factor B; kallikrein cleaves C5 and factor B; and Factor XIIa also cleaves C3. The activity of thrombin on C3 and C5 may actually explain the higher background levels of C3a and C5a in serum versus plasma. Table 1 outlines the various interactions between complement and coagulation factors.

Table 1: Interactions of complement and coagulation systems[29;74]

Protein	Type of interaction
Thrombin	Proteolysis of C3, C5, C6 and factor B
Factor XIIa	Proteolysis of C1r, C1s and C3
Kallikrein	Proteolysis of C1, C5 and Factor B
Antithrombin III	RBC protection from lysis by mC5b-9
Bb	Proteolysis of prothrombin
C3bBb	Proteolysis of prothrombin
C1 inhibitor	Inactivates FXIIa and kallikrein

e) Complement activation and biomaterials

Complement activation is part of the inflammatory response induced by the biomaterial. Among others, complement activation is known to occur during cardiopulmonary bypass and hemodialysis[74-77], and with catheters and prosthetic vascular grafts[78;79]. It is recognized that, both in the short and long term, complement activation plays a role in the clinical sequelae associated with the use of cardiovascular devices such as leukopenia, hypotension and pulmonary injury[80;81].

The presence of a biomaterial is believed to activate complement via the alternative pathway. Biomaterials are usually classified as "activating" or "non-activating" surfaces[71]. On a non-activating surface, negatively charged groups such as carboxyl and sulfate, sialic acid and bound heparin appear to promote high affinity association between bound C3b and Factor H. On the other hand, an activating surface is usually characterized by the presence of nucleophiles such as hydroxyl and amino groups: these groups will allow covalent binding of C3b and promote formation of the C3 and C5 convertase[2;70]. However, even in the absence of these activating groups on the surface, some biomaterials, such as polyacrilonitrile, are able to activate complement, suggesting that the mechanisms of material-induced complement activation due to nucleophilic groups is not the whole story. A newer hypothesis has been developed where emphasis is now on interaction of Factor H with the surface: an activating material is then defined as its capacity to bind Factor B rather than Factor H[70]. As discussed earlier, binding of Factor H would lead to C3b inactivation by Factor I and thus terminate the propagation of the complement cascade.

Some activating materials generate high levels of both C3b and C5b-9, while others will generate high C3b level but little C5b-9. Why the efficiency of C5 convertase formation

relative to that of C3 formation differs from one activating surface to another is not well understood. However, even low amounts of C5b-9 are able to activate leukocytes[71] and thus a low terminal complement activating material may still induce a significant inflammatory response. The question remains as to which are the appropriate levels of complement activation that the host can accept without deleterious effect. We also have to determine if, in the long term, the host will be able to differentiate/discriminate between moderate and high complement activating surfaces.

The hypothesis that material-induced complement activation occurs exclusively via the alternative pathway has also been challenged. Reports of complement activation via the classical pathway during cardiopulmonary bypass[18,82] and a delay in complement activation observed with C4-deficient patients undergoing hemodialysis[83] suggest that classical activation plays a role in material-induced activation. The presence of immune complexes may allow for a rapid onset of complement activation, and then subsequently the alternative pathway becomes activated. *In vitro* studies have also demonstrated activation of the classical pathway by some biomaterials[84-86].

While some of the consequences of complement activation are well understood, the mechanisms of material-induced complement activation have not yet been fully characterized.

3) Platelets

a) Platelet biology

The platelet's main role in hemostasis is to preserve the integrity of the vascular wall through formation of a platelet plug. Platelets are anuclear, disc shaped cells with a diameter of 3-4 μm. They are derived from megakaryocytes in the bone marrow and circulate at an average concentration of $200x10^6$ cells/mL, with individual platelet concentrations ranging from 150 to $400x10^6$ platelets/mL.

Platelets respond to minimal stimulation and become activated when they contact any thrombogenic surface such as injured endothelium, subendothelium and artificial surfaces. Platelet activation is initiated by the interaction of an extracellular stimulus with the platelet surface. This interaction involves the coupling of the agonist to specific receptors on the platelet plasma membrane[87]. Plasma proteins such as thrombin and fibrinogen; vascular wall products such as collagen; and molecules derived from inflammatory cells (i.e: leukocytes)

or platelets, such as platelet activating factor (PAF) or cathepsin G, are all potent platelet activators. A list of known platelet receptors and their specific agonist/ligand is presented in Table 2.

Activation results in at least four physiologic responses [88]. (1) A platelet release reaction in which biologically active compounds stored in intracellular platelet granules are secreted into the microenvironment, such as platelet factor 4, thrombospondin, β-thromboglobulin, ADP and serotonin. (2) P-selectin (previously referred to as GMP-140 or PADGEM) is released and expressed on the platelet membrane after α granule secretion. P-selectin is a cell-surface glycoprotein belonging to the selectin family and plays an important role in mediating adhesion of activated platelets to neutrophils, monocytes and a subset of lymphocytes[89,90]. (3) The platelet eicosanoid pathway is initiated, resulting in the liberation of arachidonic acid from platelet phospholipids and in the synthesis and release of prostaglandins and thromboxane B_2. (4) Platelet activation is characterized by a drastic shape change, which promotes platelet-platelet contact and adhesion. The rearrangement of the platelet membrane during activation also promotes the association of the tenase and prothrombinase complexes on its phospholipids. (5) In addition, platelet activation results in the formation of platelet microparticles, which are particularly rich in factor Va, platelet factor 3 and phospholipid-like procoagulant activity (phosphatidylserine)[91,92]. Platelet microparticles (PMPs) are formed from the surface membrane through exocytotic budding. Their physiologic role remains unclear but *in vitro* results have shown that they can bind and adhere to fibrinogen and fibrin, and coaggregate with platelets[93,94]. The procoagulant activity of platelet microparticles, generated both *in vitro* and *in vivo*, has also been demonstrated[95-97].

Table 2: Platelet receptors [87;98]

a. Platelet activating receptors	
Receptor	*Ligand/Agonist*
Thrombin receptor	Thrombin
Thromboxane A_2 receptor	TxA_2, PGH_2, PGG_2
V_1A receptor	Vasopressin
PAF receptor	Platelet activating factor
$5HT_2$ receptor	Serotonin or 5-OH tryptamine
α_2 receptor	Epinephrine
ADP receptor	ADP, ATP
PGE_2 receptor	PGE_2
C1q receptor	C1q
b. Platelet inhibiting receptors	
Receptor	*Ligand/Agonist*
A_2 receptor	Adenosine
PGI_2 receptor	PGI_2, PGE_1
PGD_2 receptor	PGD_2
c. Platelet adhesion receptors	
Receptor	*Ligand/Agonist*
GPIa/IIa or VLA-2	Collagen
GPIb/IX or GPIb	Von Willebrand factor (vWF), thrombin
GPIc/IIa or VLA-5	Fibronectin
GPIc'/IIa or VLA-6	Laminin
GPIIb/IIIa	Collagen, fibrinogen, fibronectin, vitronectin, vWF
GPIV or GPIIIb	Collagen, thrombospondin,
GPVI	Collagen
Vitronectin receptor	Vitronectin, thrombospondin
PECAM-1	Heparin
Fcγ-RII	Immune complexes
ICAM-2	LFA-1
P-selectin	Sialyl-Le^x
Leukosialin, sialophorin	ICAM-1

GP: glycoprotein; VLA: very late antigen

Among the different platelet adhesion receptors (Table 2), GPIb and GPIIb/IIIa have the highest density on platelets. GPIb (CD42) is a leucine-rich glycoprotein receptor and approximately 25000 receptors are present on the platelet surface[88;98]. It is complexed one to one with GPIX but the function of the latter remains unknown. GPIb is a long molecule, making it more susceptible to conformational change upon shear stress. GPIb mediates platelet interaction with von Willebrand factor (vWF). It will not bind plasma vWF unless the antibiotic ristocetin or the snake venom botrocetin is present. On the other hand, GPIb will bind to adsorbed or immobilized vWF on a surface. Shear stress is an important factor in platelet adhesion to vWF as it induces the required conformational change of vWF to bind GPIb. Thrombin also binds to GPIb but the functional significance of this binding has not been elucidated. GPIIb/IIIa (CD41/CD61) is an integrin receptor and is constitutively expressed on platelets. GPIIb/IIIa is the dominant platelet receptor with 40 to 80 thousand receptors present on the surface of a resting platelet. Another 20 to 40,000 are present inside the platelets, in α granules membranes and in the membranes lining the open canalicular system[98]. They are translocated to the platelet membrane during the release reaction. Upon platelet activation, a conformation change also occurs resulting in the exposure of the fibrinogen-binding site. Binding of fibrinogen to GPIIb/IIIa leads to platelet aggregation as well as formation of platelet-leukocyte aggregates, via crosslinking of GPIIb/IIIa on two different platelets by fibrinogen and crosslinking between GPIIb/IIIa and Mac-1 (on leukocyte) by fibrinogen. Other adhesive glycoproteins containing RGD sequences can also bind to activated GPIIb/IIIa: vWF, thrombospondin, fibronectin and vitronectin. Since GPIIb/IIIa mediates platelet aggregation, its inhibition has generated much interest in the control of prothrombotic states[99;100]. An antibody against GPII/IIIa (7E3) has been developed and has entered clinical trials with angioplasty[101], myocardial infarction[102] and unstable angina[102;103].

b) Platelets and biomaterials

Platelet activation (platelet release, platelet microparticle formation, P-selectin expression, aggregation) and adhesion is known to occur during cardiopulmonary bypass[20;104], hemodialysis[105;106], as well as with vascular grafts and catheters[101;107-109]. Without any doubt, the thrombotic complications associated with cardiovascular devices are linked to their ability to activate platelets. Adherent platelets[110] and circulating platelet

microparticles generated by material contact[96;97] have actually been shown to be procoagulant.

Association between platelets and leukocytes via P-selectin also occurs in the presence of cardiovascular devices[8] and has become a relatively new parameter to study biocompatibility. However the implications of this association are mostly unknown: they may directly or indirectly contribute to thrombin generation (see section 3) or participate in the removal of platelets from the circulation since several platelets can be bound to each neutrophil or monocyte.

Following contact with the layer of adsorbed proteins on the artificial surface, platelets will either adhere or bounce off[111], most likely depending on their state of activation and the ligands present at the interface[112]. Platelet adhesion on surfaces is mediated by GPIIb/IIIa and fibrinogen and interaction with GPIb/IIa and vWF can also occur[36;113-115]. However, the absence of significant platelet adhesion does not preclude platelet activation as shown by the generation of platelet microparticles with the polyvinyl alcohol hydrogel both *in vitro*[116] and ex vivo[117]. Indeed, animal studies have shown that, despite the absence of platelet adhesion, blood contact with some hydrogel surfaces[118;119], NHLBI reference materials and silastic[120] appears to activate platelets, resulting in their removal from the circulation. Furthermore, the rate of platelet consumption has been reported to be related to the water content of hydrogels[119].

The mechanisms of platelet activation by biomaterials are not yet totally understood. Even in the presence of heparin, small levels of thrombin generation are generated and may activate platelets. Complement activation can also lead to platelet activation in many ways. Platelets possess a receptor for C1q that has been shown to induce GPIIb/IIIa activation, P-selectin expression and procoagulant activity[121]. It is currently not known how classical complement activation activates platelets, but *in vitro* results support a role for C1[122]. Insertion of C5b-9 in platelets has also been associated with increased platelet procoagulant activity[91]. However, during *in vitro* blood-material contact, inhibiting complement activation has led to conflicting results on the role of complement in platelet activation[123-126]. Further research is needed to fully understand how complement activation via the classical or alternative pathway participates in material-induced platelet activation.

4) Leukocytes

a) Leukocyte biology

Circulating leukocytes are composed of neutrophils, monocytes, lymphocytes, basophils, and eosinophils. Only neutrophils and monocytes in blood but not after they emigrate into tissues will be addressed in this review as they are the major players in the classic inflammatory response with cardiovascular devices. In this section, the focus will be on their role as inflammatory cells as their procoagulant activities are discussed extensively in the next section.

Neutrophils or polymorphonuclear leukocytes (PMN) are the most abundant white blood cells, representing 40 to 60% of the leukocyte population (3 to $5x10^6$ neutrophils/mL), while monocytes represent 5% with a concentration of 0.2 to $1x10^6$ monocytes/mL. Both monocytes and neutrophils originate from stem cells in the bone marrow. Under normal circumstances, neutrophils have a very short half-life in blood (8 to 20 hours) but under inflammatory stimuli, such as LPS or cytokines, their viability can increase up to three fold [127] and their role may be even more active and central. Monocytes enter the circulation for a short period (36 to 104 hours) and they migrate into tissues where maturation and differentiation occur and they become macrophages[128]. They may also be deposited on injured blood vessels and later differentiate into macrophages. It is important to note that when neutrophils and monocytes are recruited in tissues during inflammation, their half-life increases to up to a few days.

As for platelets, monocytes and neutrophils have receptors, which upon ligand binding, trigger cell activation. Monocytes and neutrophils possess receptors for different complement products and other pro-inflammatory mediators such as platelet activating factor (PAF) and cytokines. Platelet release, such as β thromboglobulin and PDGF, has also been reported to activate neutrophils *in vitro*[129-131]. Other neutrophil and monocyte activating stimuli include bacteria and their products and cell adhesion. A list of the most relevant receptors involved in the inflammatory response is presented in Table 3.

As for platelet activation, leukocyte activation results in several physiological responses. (1) Upon activation, changes in expression of membrane receptors occur on neutrophils and monocytes: upregulation of CD11b by translocation from intracellular granules[132], down regulation of L-selectin by shedding[132], synthesis and expression of tissue

factor (TF)[133]. (2) Leukocyte activation results in release of inflammatory mediators. Neutrophils contain three types of granules (gelatinase, specific and azurophil granules) and their contents may be released upon activation: among others, elastase, cathepsin G and lactoferrin are important inflammatory mediators. Activated monocytes and neutrophils[i] also release cytokines such as IL-1, IL-6, IL-8, TNFα, G-CSF and GM-CSF. Arachidonic acid metabolites, such as leukotriene B_4 and platelet activating factor (PAF), are produced and released by activated neutrophils. The released inflammatory mediators have various properties: they may be chemoattractant for leukocytes, promote adherence to endothelial cells, and further activate platelets or leukocytes. (3) Activation may also result in the onset of the oxidative burst whereby neutrophils and monocytes release oxidants, such as O_2^- and H_2O_2. These products damage tissues and activate cells[134]. (4) Activated neutrophils and monocytes have also an increased adhesive capacity on endothelium and other surfaces[135].

Depending on its concentration and character, the presence of an inflammatory stimulus does not always result in direct leukocyte activation. Instead a certain stimulus may exercise a priming effect on leukocytes, whereby in the presence of a new stimulus, an enhanced activation response is observed[136].

Table 3: Leukocyte receptors in acute/immediate inflammatory response[136;137]

a. Complement receptors		
Receptor	*Ligand*	*Function*
C1q R	C1q, MBP	Enhance phagocytosis, respiratory burst
CR1	C3b > C4b > iC3b	Immune adherence, phagocytosis
CR3 or CD11b	iC3b, fibrinogen, FX, ICAM-1	Phagocytosis, respiratory burst, adhesion
CR4 or CD11c	iC3b, fibrinogen	Adhesion, phagocytosis
C3a R	C3a	Chemotaxis, degranulation, respiratory burst
C5a R	C5a	Chemotaxis, degranulation, respiratory burst
b. Other receptors (R)		
Receptor for		*Function*
TNFα, IL-1		Degranulation, respiratory burst
IL-8		Chemotaxis, degranulation, respiratory burst
PAF, LTB_4		Strong activation
GM-CSF, G-CSF, IFNγ		Weak activation, priming

[i] Neutrophils had long been thought to be unable to synthesize cytokines but progress in molecular biology has shown otherwise[127;301].

Leukocyte adhesion to the endothelium is an important means by which neutrophils and monocytes participate in the inflammatory response. Adherent leukocytes have been shown to be more activated than their counterpart in the bulk[138;139] but the level of activation of adherent leukocytes depends on the surface[140]. The adhesion molecules involved in the process of leukocyte adhesion to blood vessels is briefly presented as they are relevant to adhesion on cardiovascular devices. Leukocyte adhesion molecules are divided into three main families[141]: the selectins, the integrins and the immunoglobulin superfamily. The selectin family regroups three integral membrane glycoproteins: L-selectin is expressed by neutrophils, monocytes and lymphocytes; E-selectin is expressed by cytokine-activated endothelial cells; P-selectin is expressed by activated platelets and endothelial cells. The integrins are cell surface receptors composed of $\alpha_x\beta_x$ heterodimers and each subfamily shares the same β subunit: the β_1 subfamily corresponds to the very late antigens (VLA) mostly important in migration mechanisms and on lymphocytes; the β_2 subfamily corresponds to the leukocyte integrins and β_3 to the cytoadhesins (GPIIb/IIIa, vitronectin receptor). The three members of β_2 integrins are CD11a/CD18 or LFA-1, CD11b/CD18 or CR3 or Mac-1, and CD11c/CD18 or CR4 or p150,95. Among others, the immunoglobulin family contains some of the ligands for the leukocyte β_2 integrins. ICAM-1, the counter-receptor for CD11a and CD11b, is upregulated on endothelial cells following hours of cytokine stimulation. ICAM-2 is another ligand for CD11a and is constitutively expressed on endothelial cells. The mechanism of leukocyte adhesion to endothelial cells, a three-step mechanism, has been well-characterized[142]. Step 1: L-selectin is involved in the initial rolling of leukocytes on endothelium. Step 2: The rolling stage enables leukocytes to slow their movement and sample the local environment, and they may become activated due to local stimulation and additional interaction between receptor/ligand. Step 3: CD11/CD18 mediates the firm adhesion. With activated neutrophils and monocytes, a functional upregulation is observed for CD11a, while for CD11b and CD11c a quantitative and functional upregulation occurs, the upregulation for CD11b being more rapid and important than for CD11c. It is important to note that the functional change of CD11b upon leukocyte activation and/or adhesion can occur despite no measurable increase in CD11b surface expression. The functional change is conformational involving receptor phosphorylation and resulting in increase binding affinity for certain ligands (such as fibrinogen and Factor X)[240].

A quantitative increase in CD11b expression on leukocytes does not imply increased adhesion, unless it is accompanied by functional change in the receptor[302].

b) Leukocyte activation and biomaterials

Contact with cardiovascular devices *in vivo* activates both neutrophils and monocytes. Indicators of leukocyte activation such as L-selectin shedding and CD11b upregulation on leukocytes have been widely observed following angioplasty[143-146], hemodialysis[105;147;148] and cardiopulmonary bypass[20;22;149-152] (for an extensive review of studies on the expression of leukocyte adhesion molecules with *in vitro* cardiopulmonary bypass, see Asimakopoulos et al[153]). Degranulation with the release of elastase and lactoferrin[154-158] and the presence of cytokines[152;159;160], such as IL-1 and TNFα, have been associated with extracorporeal circulation and further demonstrate leukocyte activation. Activation of the respiratory burst is also a common trait with hemodialysis[147;161].

The mechanisms of material-induced leukocyte activation remain unknown. Whether they are directly activated by contact with a foreign surface, via complement activation, kallikrein or platelet activation has not been fully determined. *In vitro* and *in vivo* investigations with protease inhibitors[158;162-165], complement inhibitors[21;22;125;166-169] and anti-platelet agents[101] suggest that they all play a role, but no one inhibition has led to consistent results with a significant reduction of material-induced leukocyte activation. Material-induced leukocyte activation may be mediated by several factors and inhibition of one pathway of activation may not be sufficient to result in a significant impact on leukocyte activation.

Material-induced leukocyte activation also results in increased adhesion. *In vivo* studies have found activated leukocytes adhering to stents[170;171], oxygenators[172] and hemodialysis membranes[173;174]. Material-activated leukocytes also adhere to the endothelium, such as at the anastomoses of a vascular graft or in the lung during extracorporeal circulation. The mechanisms of leukocyte adhesion on artificial surfaces remain unclear, but it appears to be mediated in part by the complement product iC3b[69;128]. This is supported by *in vitro* work showing that inhibition of complement activation *in vitro* significantly reduced leukocyte adhesion[139;175-177]. On the other hand, fibrinogen also appears to play an important role in leukocyte adhesion to material[178;179]. The presence of platelets on the surface may also mediate leukocyte adhesion but conflicting reports exist on

the requirement for platelets in leukocyte adhesion[180;181]. As the biomaterial is larger than a micro-organism and can not be engulfed by leukocytes, adherent neutrophils and monocytes undergo a frustrated phagocytosis whereby they release their battery of potent oxygen metabolites and proteolytic enzymes[128]. Material characteristics and proteins at the interface appear to modulate the level of activation of adherent leukocytes[139;182;183].

The response of leukocytes following contact with biomaterials has been well characterized *in vivo* and *in vitro*. However, the mechanisms that regulate material-induced leukocyte activation are not well understood, preventing significant reduction in the inflammatory response induced by cardiovascular devices. To bring further understanding in this domain, this thesis aims at defining the role of material, complement and platelets in material-induced leukocyte activation.

5) Other important factors in blood-material interactions

a) Flow

Fluid dynamics affect the growth of thrombi and the deposition of fibrin. The composition difference between arterial and venous thrombi is one example of how fluid dynamics affect thrombosis, although the underlying mechanisms are still not well understood. Flow determines the rates of transport of cells and proteins to the surface. As platelets are an important part of the thrombus, the effect of shear on platelets has been studied extensively. Higher shear results in higher platelet deposition and lower fibrin deposition, while at lower shear the inverse is true[184]. High shear, such as the ones observed at stenotic plaques, is also able to induce platelet aggregation even in the absence of any other exogenous factors[185;186]. Conflicting results have been obtained on the effect of flow on leukocyte adhesion while little is known of its effect on leukocyte activation. High shear has been shown to either reduce, increase, or leave unchanged leukocyte adhesion on different substrates[180;187-193]. These conflicting results may be explained by differences in experimental conditions: the presence of red blood cells[188], platelets[180;191;194] and plasma proteins[180]; the surface studied[191] and the state of leukocyte activation[192].

When studying the effect of flow on cells, and even proteins, the presence of red blood cells are of paramount importance as they affect transport phenomena[195]. Also, hypotheses using mathematical modeling especially with blood cells have to be regarded with caution, as cells have properties that solid particles do not have (flexibility, receptors on

a long chain) and the models are likely inconsistent with the reality as demonstrated by Tempelman et al[196].

As for the effects of flow on the coagulation cascade, it has been less studied. Current knowledge is limited to Factor Xa generation initiated by the extrinsic pathway and thrombin generation initiated by the intrinsic pathway (with biomaterials). Factor Xa generation by the complex TF:VIIa increases with shear rate (and shear stress)[197;198]. For thrombin generation by the intrinsic pathway, modeling has identified three types of reactions[64]: at low flow, a significant amount of thrombin is produced after a long lag time (over 10 hours); at moderate flow, significant thrombin generation is produced in a short time (within minutes); at high flow, low levels of thrombin are produced within seconds.

While the importance of flow has been recognized, our current understanding of its mechanisms is limited mostly to platelets. Many *in vitro* and *in vivo* flow models are available and have been successfully used to assess antithrombotic drugs in whole blood[199]. More fundamental research is required on blood coagulation, leukocytes and flow. Previous research with flow has focused on isolated cells or proteins, which is far from the *in vivo* situation. The use of more physiological experimental conditions (e.g; presence of red blood cells, plasma proteins) should result in significant advances in our knowledge on the effect of mechanical factors on thrombosis and hemostasis.

b) Endotoxin

Endotoxins, also called lipopolysaccharide (LPS), are the component of the outer membrane of gram-negative bacteria and are released into the circulation upon disruption of the intact bacteria (death, cell lysis)[200]. Endotoxin is commonly found everywhere in our environment and it is the most significant pyrogen in parenteral drugs and medical devices. Endotoxins are also present in the digestive system. Their presence in the blood stream may cause septic reactions with a variety of symptoms such as fever, hypotension, nausea, shivering and shock[201]. High concentrations can lead to serious complications such as disseminated intravascular coagulation (DIC), endotoxin shock and adult respiratory distress syndrome (ARDS). Endotoxins are known to activate complement, the kinin system, leukocytes, platelets and endothelial cells[200;201] and are the "enemy" of both *in vitro* and *in vivo* study of blood-material interactions. *In vivo*, they may lead to the complications mentioned above, while *in vitro*, the presence of this contaminant may affect the outcome of the study.

FDA regulates the acceptable level of endotoxin contamination with medical devices to be 0.5 endotoxin units/mL[202]. There have been few reports of endotoxin contamination with the use of cardiovascular devices. During cardiopulmonary bypass and extracorporeal membrane oxygenation, the presence of endotoxins has been observed *in vivo*[203:204]. They appear to originate mostly from the gut[205-207] rather than from the materials and are believed to be a reaction to the surgical procedure. During hemodialysis, endotoxin contamination is also an issue and the dialysate is usually the source[201:208]. While endotoxin contamination may be present *in vivo* in some patients and studies, there has been no investigation showing a significant correlation between the magnitude of endotoxin contamination and postoperative complications[204:209].

On the other hand, endotoxin contamination during *in vitro* work may be much more common, as sterile conditions are not always available and the laboratory-working environment contributes to their presence. The most conspicuous source of endotoxin may actually be the water since distilling and deionizing columns do not remove endotoxin. Endotoxin has an effect on platelets only at high concentration (over 1 μg/mL equivalent to 5000 EU/mL)[210], while leukocytes have been reported to be activated by endotoxin concentrations as low as 0.01ng/mL (equivalent to 0.05 EU/mL)[211:212]. When studying blood-material interactions, endotoxin may be contained in buffers and/or on materials, and its priming and activating effect on leukocytes may affect the observed results. However, it is important to consider that all the studies performed on the effect of endotoxin on leukocyte activation used purified strains of endotoxin while the endotoxin present in laboratory materials and buffers are of an environmental nature. Purified endotoxins are much more potent than environmental endotoxins[213] and even among purified endotoxins, their activity might be different[211:214]. Contrary to a purified strain of endotoxin[208], the presence of relatively high levels of environmental endotoxins (100 EU/mL) was shown to have little impact on the leukocyte response to hemodialysis[215].

III Leukocyte activation and procoagulant activities

Neutrophils and monocytes have long been recognized for their essential role in the inflammatory response through the release of lysosomal enzymes, cytokines, reactive oxygen species and lipid mediators[216:217]. Until recently, platelets were usually considered as the key

cellular player in thrombus formation. However, there is now evidence that leukocytes, neutrophils and monocytes in particular, may also play an important role in thrombogenesis.

Circulating leukocytes, like endothelial cells and platelets, express little or no procoagulant activity under normal physiological conditions. Inflammatory disorders, such as glomerulonephritis, allograft rejection reaction or the generalized Schwartzman reaction, lead to pathological conditions that stimulate the procoagulant activity of leukocytes resulting in fibrin deposition and localized intravascular coagulation[218], illustrating the implication of leukocytes in thrombosis. Surgery or contact with a foreign material such as oxygenators and vascular grafts also represent an inflammatory stimulus that may lead to the expression of procoagulant activities on leukocytes. Indeed, monocyte TF expression has been observed with cardiopulmonary bypass[61,63] and ventricular assist devices[62,62,219]. The role of leukocytes in thrombosis is also illustrated by studies showing that leukopenia resulted in reduced intravascular coagulation[220-223].

Circulating monocytes and neutrophils normally roll on the endothelium. They will however adhere to damaged or stimulated endothelial cells or adherent platelets and further contribute to localized thrombogenesis. It has been suggested that neutrophil adhesion may well play an important role in the initiation and propagation of venous thrombosis[224]. In fact, in patients undergoing hip replacement, intravenous administration of lignocaine, a substance preventing leukocyte adhesion *in vivo*, during the first 6 postoperative days markedly reduced the incidence of deep venous thrombosis[41]. Furthermore, it was demonstrated in a baboon model that blocking leukocyte accumulation (by blocking binding to adherent platelets via P-selectin) dramatically reduced fibrin deposition on a vascular graft[225].

All these facts confirm the procoagulant activity of leukocytes, especially neutrophils and monocytes. The different procoagulant activities may be classified as:

1. membrane-associated procoagulant activity: via tissue factor expression on the cell membrane (tissue factor dependent coagulation pathway) or via tissue factor-independent mechanisms through factor X binding to CD11b receptors leading to factor Xa generation or fibrinogen binding to CD11b; or binding of the prothrombinase complex on the membrane.
2. release of procoagulant mediators: degranulation and oxidative products have the capacity to neutralize various anticoagulant proteins and activate platelets.

3. association between platelets and neutrophils or monocytes: their interactions may lead to mutual activation and to a microenvironment protected from inhibitors.

Monocytes seem to be most essential for type 1, while neutrophils seem to be the main cause of type 2 activity. Neither lymphocytes (more particularly involved in the specific immune response), nor macrophages residing in tissues are discussed here. However it is important to recognize that T cells are able to mediate a procoagulant activity by inducing tissue factor expression on monocytes[218;226]. The object of this review is not to determine which of the two populations, monocyte or neutrophil, plays the major procoagulant role, but rather to discuss their different procoagulant abilities.

1) Membrane-associated procoagulant activity

a) The tissue factor-dependent pathway.

As mentioned previously, TF is a transmembrane glycoprotein initiating the extrinsic pathway of coagulation. TF binds factor VII/VIIa and this complex, in presence of Ca^{2+} and phospholipids, can then trigger the activation of factor X and IX. The formation of the prothrombinase complex Xa-Va on a cell-membrane results in the conversion of prothrombin to thrombin.

TF is expressed constitutively in tissue barriers between the body and the environment, such as in the granular layer of the epidermis or the respiratory mucosa; and at the boundaries between organs, such as in the adventia of arteries and venules[227]. These barriers provide a hemostatic envelope so that only vascular injury may initiate blood coagulation. However, under specific stimuli, some vascular cells can transiently express TF. The expression of TF by the different leukocyte populations has been an area of great controversy. An early study on isolated populations of lymphocytes, neutrophils and monocytes showed that, under bacterial lipopolysaccharide (LPS) stimulus, lymphocytes and neutrophils expressed TF only slightly, while monocytes showed strong TF expression[228]. Two years later, Rivers demonstrated that only monocytes were able to express TF and that positive results obtained in previous studies with lymphocytes and neutrophils were due to slight contamination with monocytes[229]. Inconsistent results on neutrophils persist. The latest study on TF expression in human leukemic cells by Hair et al[230]. using mRNA expression as well as flow cytometry with a TF antibody, failed to demonstrate TF

expression on neutrophils. On the other hand, immunohistochemical findings in human renal biopsies indicated the presence of TF in inflammatory neutrophils[231]. Recent studies have also observed TF expression on adherent neutrophils in humans[232] and animals[233;234]. Yet, there is no convincing evidence that neutrophils cannot express TF. Most of the studies have been performed on isolated cells under non-physiological conditions (room temperature, serum- or plasma-free medium), and neutrophils may express TF under different requirements than monocytes. In fact, *in vitro* results on monocyte TF expression in isolated systems and in whole blood models have shown differences in TF regulation and stimulation[235].

Despite differences in experimental protocols, TF expression on monocytes has been clearly demonstrated *in vitro* as well as *in vivo* and, unlike neutrophils, the TF expression abilities of monocytes are undisputed. Monocytes have been reported to transcribe, synthesize and transport TF to the cell membrane in response to exogenous agents such as LPS, PMA and calcium ionophore and physiological mediators such as cytokines, complement factors and LDL[133]. *In vitro* studies suggest that activation of both protein kinase C and protein tyrosine kinase are required for induction of TF mRNA[236;237]. Monocyte vesiculation (microparticle formation) has also been observed after stimulation with LPS and this phenomenon is viewed as a possible mechanism for dissemination of membrane-associated procoagulant activities[238]. Upon strong LPS stimulation, the major part of TF and phosphatidylserine-dependent procoagulant activity appears to be associated with monocyte microparticles[239]. Adhesion also enhances TF expression as monocytes adherent to surfaces consistently show higher TF expression than circulating monocytes[61;138;219]. Adherent monocytes may thus contribute to a greater extent to localized thrombogenesis than circulating monocytes.

b) TF-independent mechanisms

While monocyte procoagulant activity through TF requires synthesis, delaying activation of coagulation until its cellular expression, leukocytes have the potential to initiate the coagulation cascade in a more immediate process. Leukocytes can induce thrombin generation via factor X binding to CD11b/CD18 receptors, leading to factor Xa generation. A member of the integrin superfamily of leukocyte adhesion molecules, the CD11b receptor is expressed on monocytes, neutrophils and natural killer cells. Within minutes of activation, its cell surface expression is increased two- to ten-fold via translocation and fusion of

intracellular granules with the plasma membrane[137]. CD11b participates in the phagocytosis of opsonized particles, interacts with the complement products iC3b and C3b, and has been implicated in cell migration and diapedesis[240]. Furthermore, after appropriate stimulus, CD11b serves as a high-affinity receptor for factor X. Binding of factor X to CD11b is specific and saturable, time- and divalent cation-dependent[241]. Factor X bound to CD11b is then rapidly (under 30 minutes) proteolytically cleaved by Cathepsin G to factor Xa which can support the cell-associated formation of thrombin[242]. Although neutrophils may not be able to initiate coagulation via the extrinsic pathway (i.e they may lack TF expression), they have been reported to produce factor Xa coagulant activity, almost as efficiently as monocytes. This process of factor X activation, also observed on monocytes, is totally independent of TF since it is affected neither by neutralizing anti-TF monoclonal antibodies nor by the lack of TF surface expression.

While generation of factor Xa is the first proteolytic event that may initiate coagulation on leukocytes, additional molecular recognition is necessary to amplify the process of thrombin generation. This is provided by the membrane assembly of the prothrombinase complex (Xa-Va, Ca^{2+} and phospholipids). Assembly of the prothrombinase complex has been observed on monocytes, neutrophils, lymphocytes and platelets; the number of sites/cell, the dissociation constant and the rate of thrombin formation varied with each cell type studied[243]. The expression of prothrombinase on cell surfaces is determined by the number of sites available for the complex to form. Additional factors such as cell concentration, cellular activation, the participation of intracellular pools of factor V and its activation by cell-associated proteases also influenced prothrombinase activity on cell surfaces[243]. Platelets possess an intracellular pool of factor V. Upon activation, factor V is released during granule secretion and proteolytically activated to function as a membrane-bound cofactor of factor Xa[244]. A similar process has been described on leukocytes[245]. After observing that factor X bound to CD11b is converted to Xa, it was shown that monocytes, in particular, constitutively synthesize a membrane form of factor V/Va which is a competent receptor specific for factor Xa and coordinates the assembly of an efficient prothrombinase complex. This would imply a two-dimensional rearrangement of Xa and Va on the monocyte membrane surface. Such a mechanism of product flux between successive reaction sites of coagulation would nonetheless be quite plausible[246]. This factor Va-like protein was named effector cell protease receptor-1 (EPR-1) and identified as a cofactor for

factor Xa-catalyzed prothrombin activation [247]: in the absence of added factor Va, factor Xa bound to monocytes effectively mediated thrombin generation. This mechanism is however refuted in another study reporting that the prothrombin activity of factor Xa bound to monocytes was absolutely dependent on the addition on factor Va[248]. This study further showed that factor Va bound to the monocyte surfaces modulates factor Xa substrate specificity between factor IX and prothrombin, whereas the prothrombinase complex on platelets is highly specific for its substrate prothrombin. These disparities may result from the different experimental protocols and it is difficult to conclude on the potential existence of a monocytic mechanism of factor Xa-catalyzed thrombin generation independent of plasma factor V. Despite a greater surface expression of CD11b, neutrophils exhibit longer clotting times and less factor Xa coagulant activity than monocytes. This seems to confirm the contribution of an intracellular factor such as EPR-1 in the procoagulant activity of monocytes (independently of TF).

Similar to its recognition of factor X, CD11b recognizes fibrinogen in a calcium-dependent mechanism. Experiments with potent inhibitors against the interaction between iC3b and CD11b, as well as competitive studies with factor X, suggest that fibrinogen interaction with CD11b occurs at different intramolecular loci in the α sub-unit of Mac-1[249]. The localization of fibrinogen on the neutrophils and monocytes may facilitate a more efficient conversion to fibrin in the local generation of low levels of thrombin. Adherent leukocytes are usually more activated and thus express more CD11b; they could then contribute significantly to local fibrin deposition. This hypothesis was verified by inhibiting leukocyte adhesion by blocking interactions with platelet or endothelial P-selectin[225;250]. Both *in vivo* studies in baboons demonstrated a dramatic reduction in fibrin deposition. CD11b is not the only fibrinogen receptor expressed by leukocytes: CD11c/CD18 (p150,95), another leukocyte integrin, has also been reported to bind fibrinogen[251]. Binding of fibrinogen to its leukocyte receptors participates in intracellular signaling and activation and can induce procoagulant activities[241]. Under LPS stimulation, occupancy of CD11b by its ligand (for example fibrinogen) results in the enhancement of the procoagulant activity of monocytes through an increase in TF expression[252].

2) Release of procoagulant mediators

Neutrophils are the major component of the acute inflammatory response and upon activation they release numerous proteases and oxidative products, which in the local environment can activate platelets[199] and proteolytically inactivate several inhibitors of coagulation, including the serpins antithrombin III, heparin cofactor II, C1 inhibitor and α_2 antiplasmin[42;253]. While monocytes also release oxidative products and proteases, little research has been done in this area and they will not be further discussed.

a) Effect of proteases on proteins involved in coagulation

Elastase appears to be the most potent protease with procoagulant activities. The elastase inhibitor α_1-antitrypsin loses its inhibitory activity when oxidised. Thus within the immediate vicinity of activated neutrophils, α_1-antitrypsin can be inactivated by oxidation and elastase is then free to act on its substrates. ATIII, C1-inhibitor and presumably other plasma serpins, but not α_1-antitrypsin, have elastase-vulnerable sites on their exposed reactive centre loops[253]. The presence of free elastase results in the catalytic cleavage of the loops to the stable irreversibly inactivated forms of the serpins. This catalytic activity of elastase results in both activation of coagulation and fibrinolysis, and can be disastrous in widespread neutrophil activation, which is observed in the acute shock syndromes. The disseminated intravascular coagulation seen in such syndromes may be caused or significantly enhanced by disseminated deactivation of ATIII and α_2 antiplasmin[253].

Furthermore, elastase has an inhibitory effect on tissue factor pathway inhibitor (TFPI). As discussed above, TF is an important trigger for coagulation, but monocytes, while they promote coagulation via TF expression, will also co-express TFPI at a later point during activation[254]. The procoagulant activity of platelets is also accompanied by release of TFPI following stimulation by thrombin[255]. Neutrophil elastase proteolytically cleaves TFPI within the peptide that links Kunitz-1 and Kunitz-2, affecting its ability to inhibit FVIIa/TF and reducing its inhibition of factor Xa[42]. Kinetic studies show that while the catalytic cleavage by elastase does not reduce the initial encounter between TFPI and factor Xa, it affects the affinity of the final complex TFPI:Xa. Elastase is also able to restore TF activity from a preformed factor Xa:TFPI:factor VIIa/TF complex.

Another reported procoagulant activity of elastase is its ability to cleave vascular endothelial cell proteoglycans[256], thus inducing procoagulant activity on the endothelial cells.

This cleavage appears to be relatively selective for heparan sulfate. Along the same line, oxidation of a specific methionine in thrombomodulin by oxidative products derived from activated neutrophils blocks the formation of activated protein C, a potent endothelial-derived anticoagulant[257]. Endothelial thrombomodulin plays a significant role in hemostasis as a cofactor of thrombin-dependent activation of protein C.

Another protein released by neutrophils upon activation is lactoferrin. Lactoferrin is an iron-binding protein and it can also interact with heparin, inhibiting its anticoagulant activity. The anticoagulant effect of heparin in whole blood stimulated by various inflammatory mediators (LPS, TNF, fMLP) was inhibited, as measured by activated prothrombin time (aPTT), while increased levels of lactoferrin and platelet factor were observed[258].

While the procoagulant effect of proteases on coagulation proteins are discussed here, it is important to mention that proteases can also contribute to fibrinolysis. Thus the local inflammatory environment where the neutrophils accumulate defines in which way the proteases work.

b) Effect of leukocyte release on platelet activation

Neutrophil-derived mediators can also contribute indirectly to thrombin generation by activating platelets. Cathepsin G is one of the most studied and potent leukocyte protease able to activate platelets. Cathepsin G is a neutral serine proteinase contained in neutrophils azurophilic granules and is released upon activation. Both purified cathepsin G and the one present in supernatant of neutrophils at a physiological concentration have been shown *in vitro* to induce platelet activation[259;260], with intracellular calcium movement, thromboxane β_2 synthesis, release of dense and α granules, and activation of GPII/IIIa. The presence of proteinase 3[259;260] (also present in the azurophilic granules) or of elastase[261] appear to enhance platelet activation induced by cathepsin G. The mechanisms by which cathepsin G activates platelets are not well understood; a receptor has not yet been identified. It appears that cathepsin G cleaves and activates the platelet thrombin receptor, but an antibody against this receptor failed to inhibit cathepsin G-induced platelet activation[262].

Products of the oxidative burst, such as myeloperoxidase, hydrogen peroxide and superoxide anion, have also been reported to activate platelets[131;263;264]. Platelet activating

factor (PAF), a lipid mediator, also activates platelets following neutrophils exposure to chemotactic factors[131] or fMLP[265].

All these studies on neutrophil-induced platelet activation have been performed in-vitro and there is no direct evidence yet that such reaction takes place *in vivo*. However it seems reasonable to assume that under the appropriate pathological/inflammatory conditions, these reactions may occur. Degranulation and oxidative burst products released by neutrophils in response to an inflammatory stimulus and in the appropriate environment can contribute to thrombin formation. However, more physiological experiments (whole blood versus isolated cell suspension, *in vivo* versus *in vitro*) are required to determine the real contribution of these neutrophil procoagulant properties. The presence of potent protease inhibitors in plasma may also prevent leukocyte-induced platelet activation. However, the formation of platelet-leukocyte aggregates (as discussed below) likely offers a protective environment to the neutrophil-derived mediators so that platelet activation can ensue.

3) Platelet-leukocyte aggregates

The presence of platelet-leukocyte aggregates in thrombotic complications[8] suggests that they may have procoagulant properties[131;266]. While little is known about their function, platelet-leukocyte aggregates are believed to provide a microenvironment whereby mediators are protected from their inhibitors[262] and transcellular metabolism of arachidonic acid occurs[267], resulting in further activation of the cells associated with the aggregate and thus promoting thrombin generation. Some of the mechanisms of platelet-leukocyte aggregate formation have been characterized *in vitro*. Thrombin-activated platelets bind to monocytes and neutrophils, but only weakly to lymphocytes[90;268]: this binding is independent of temperature but dependent on the presence of Ca^{2+}. Unactivated platelets have also been shown to bind to isolated monocytes and neutrophils[269], even in the absence of calcium. However, the relevance of such interaction is questionable as unactivated platelets do not appear to significantly bind unactivated leukocytes in whole blood[270]. Monocytes also appear to bind more activated platelets and faster than neutrophils[90]. Conflicting results have been obtained on the importance of activation of leukocytes and platelets for aggregate formation. Nash[266] reports that *in vitro* formation of platelet-leukocyte aggregates requires platelet activation but not leukocyte activation. Furthermore, Rinder et al[89] found that leukocyte activation actually impaired formation of aggregates: in certain conditions, such as

PMA stimulation, neutrophil-platelet aggregates were not as stable as their monocyte counterparts and dissociated within the first hour. On the other hand, Brown et al[271] observed formation of platelet-leukocyte aggregates when either cells were activated, but platelet activation alone usually resulted in more platelets bound per neutrophils when compared to leukocyte activation alone.

The formation of platelet-leukocyte aggregates occurs via P-selectin on platelets and PSGL-1 on leukocytes[90;269;272]. Fibrinogen bridging between CD11b and GPIIb/IIIa has also been reported to be involved[273]. The predominance of one binding mechanism over the other one is not well understood and different studies result in different conclusions. The presence of both mechanisms of aggregation have been observed under static[271] and low shear conditions[274]. Under low shear, anti-P selectin alone reduced aggregates only by 30% while anti-CD11b alone had no effect[274]. However, combination of both antibodies resulted in total inhibition of platelet-leukocyte aggregates, suggesting that with low shear a more complex mechanism is involved.

Specific interactions between platelets and leukocytes also occur on surfaces where adherent platelets can be found. The mechanisms of leukocyte adhesion to adherent platelets under physiological shear have been recently characterized. Yeo et al[188] showed that P-selectin on platelets mediated the rolling of neutrophils but that a second step was required to enable firm adhesion of the neutrophils. CD11b was later identified as the ligand mediating firm adhesion on adherent platelets[275]. Firm adhesion also appears to involve leukocyte activation[276] and platelet membrane-associated PAF is partially responsible for the activation of the rolling leukocyte[277]. On the other hand, the mechanisms of leukocyte adhesion on adherent platelets in the presence of fibrin is strongly dependent on shear *in vitro*: the higher the shear stress, the more fibrinogen/fibrin is involved in leukocyte adhesion to platelets and the less leukocyte interaction with P-selectin is a prerequisite for adhesion[189;190;193;278]. These studies confirm earlier *in vivo* results with a dog shunt model, showing that an anti-Pselectin antibody partially blocked leukocyte interaction with platelets adherent to a clotted Dacron graft[225].

All the studies on the mechanism of platelet-leukocyte aggregates have been performed *in vitro* and there is yet little evidence on what the reality is *in vivo*. Platelets and leukocytes are found bound together *in vivo* but the processes by which they form and their potential functions remain speculative. The development of human and animal blocking

antibodies and the wider selection of animal antibodies for flow cytometry (an important tool in the study of platelet-leukocyte interactions) will provide new ways to study such interactions *in vivo*.

4) Are leukocyte procoagulant activities relevant to thrombus formation ?

The potential significant contribution of leukocytes to thrombosis is underscored by recent publications on the role of TF in coagulation. TF expression was shown to be able to trigger thrombin generation and platelet thrombus formation as efficiently as known thrombogenic surfaces, such as fibrillar collagen[279]. Blood-borne tissue factor also represents an important source of thrombin in thrombus formation, as anti-TF antibody reduced thrombus size by 70%[232;280]. Expression of TF by monocytes was also shown to significantly contribute to the progression of a thrombus, since adding TFPI inhibited thrombus-associated procoagulant activity[281]. Furthermore, it appears that activated monocytes expressing TF are able to generate sufficient thrombin to rapidly initiate platelet activation and aggregation[282-284]. The activated platelets then provide the sites for the assembly of tenase and prothrombinase complexes with TF-activated FXa and FIXa and thus enable the propagation phase of coagulation[282;283;285]. Even trace amounts of TF have actually been shown to activate platelets[286], further suggesting that the extrinsic pathway of activation is the initiator of the coagulation while platelets are involved in the propagation phase.

Even if not directly related to TF, the presence of leukocytes in a thrombus has been demonstrated to significantly contribute to fibrin generation both *in vitro*[190;193] and *in vivo*[225;250]. Furthermore, leukocyte depletion has been shown to reduce the levels of thrombotic complications in diseases. However, the mechanisms by which leukocytes contributed to fibrin formation were not identified. Further research is required to determine the mechanisms involved in leukocyte contribution to thrombogenesis, so that appropriate therapies can be designed.

5) Biomaterials and expression of leukocyte procoagulant activities

In recent years, studies have emerged in the biomaterials literature that examine leukocyte procoagulant activities rather than their inflammatory properties only. Following blood contact with cardiopulmonary bypass circuits or ventricular assist devices, TF

expression on monocytes has been observed *in vitro*[219;287;288] and *in vivo*[61;289]. CD11b upregulation on neutrophils and monocytes is a well-known consequence of material-induced leukocyte activation (as discussed in section II-4b). It was recently shown that CD11b, upregulated on monocytes by cardiopulmonary bypass, was able to directly activate factor X[290]. With hemodialysis membranes and oxygenators, increased levels of elastase have also been reported both *in vivo* and *in vitro* (as discussed in section II-4b) and elastase ability to inhibit TFPI in the vicinity of the implant may promote localized thrombosis. Finally, platelet-leukocyte aggregates have been observed during cardiopulmonary bypass[291], hemodialysis[292], following angioplasty[101] and stent implantation[293].

As it is a new branch of research, little is still known on the potential contribution of expression of leukocyte procoagulant activities to thrombogenesis and thrombotic complications associated with the use of cardiovascular devices. However, research on pathophysiological states of thrombosis supports the hypothesis that leukocytes can be a significant contributor to thrombogenesis and underscores the need of more research on this matter with biomaterials. This thesis studies how leukocytes become activated by *in vitro* material contact and express procoagulant activities such as TF, CD11b, leukocyte microparticle formation and association with platelets.

6) Conclusion

Both monocytes and neutrophils possess the potential to induce or contribute to thrombosis through different means: monocyte TF expression, factor X binding and activation on the adhesion receptor CD11b, fibrinogen binding on CD11b and release of mediators that may inactivate anticoagulant substances and activate platelets. While neutrophils appear to have a lower capacity to activate coagulation, there is strong evidence that they can enhance monocyte-induced procoagulant activity[294]. Platelets can also directly interact with neutrophils and monocytes, increasing procoagulant activities even further[265;295]. The potential role of leukocytes in thrombogenesis is underscored by the number of studies that have tried to minimize thrombus formation by the administration of drugs specifically targeted at leukocytes. Antibodies to block leukocyte adhesion may prove to be a reasonable therapeutic approach in the prevention of thrombus formation as illustrated in *in vivo* baboon models[225;250]. However, blocking leukocyte adhesion in a systemic approach may have deleterious consequences such as the inability to fight

infections (due to the inhibition of leukocyte recruitment to the site of infection). To circumvent such an event, blocking only leukocyte adhesion to platelets by inhibiting P-selectin/PSLG-1 interactions may be more appropriate. This would also prevent leukocytes interaction with endothelial cells via P-selectin, but other interactions via ICAM-1, iC3b/C3b would remain intact and allow leukocyte adhesion at site of inflammation/infection. Due to the important role of leukocytes in inflammation/infection/healing, any systemic approach of blocking leukocyte adhesion would have to be temporary and could then only be used during procedures such as cardiopulmonary bypass and angioplasty.

IV Conclusions

1) Thrombosis and inflammation

The molecular links between inflammation and thrombosis are undeniable. Inflammation, as characterized by the leukocyte response to a stimulus, may contribute to thrombin generation by the TF expression on monocytes; the ability of CD11b to bind Factor X and fibrinogen; the ability of released inflammatory mediators to activate platelets and block inhibitors of coagulation; and by promoting the association between leukocytes and platelets. In the last five years, many leukocyte investigators have discussed the participation of inflammatory cells in coagulation[296-299]. Thrombosis is viewed now more as a multicellular event rather than just a platelet event[300]. In certain situations, blocking leukocyte contributions to thrombin generation may appear to be a reasonable means to reduce the occurrence of thrombotic complications.

2) Perspective to contribute to the research on blood compatibility of biomaterials

The complexity of blood-material interactions explains our failure to design a material that is entirely blood-compatible. Our current stage of knowledge is far from providing us with a complete mechanism of material-induced thrombin generation. The role of Factor XII is unclear while the role of TF has not been directly assessed. Both the mechanisms of leukocyte and platelet activation by materials remain to be further elucidated. The timing of the events contributing to thrombin formation is also a complex issue. Both Factor XII activation and platelet activation are able to generate thrombin formation within minutes while thrombin generation via TF requires hours since TF has to be synthesized. The contribution of leukocyte proteases will also be affected by time since their effect will be

dependent on the presence of inhibitors and other inflammatory mediators that can potentiate their action. As illustrated in Figure 5, due to its nature, the contribution of leukocyte activation to material thrombogenicity is more likely in the longer term, compared to platelet activation. Despite already over 50 years of research on blood-material interactions, many questions remain unanswered and more fundamental research is necessary to contribute to the improvement of cardiovascular devices.

The solution to thrombotic complications associated with cardiovascular devices may not be to try to create a new material that will elicit the proper blood response but to prevent its adverse effects by blocking the final pathway responsible for the inherent thrombogenicity of the materials. Leukocytes may well be a key component in this process. Only the future will tell. In this thesis, the role of material, complement, and platelets in material-induced leukocyte activation that can be associated with some leukocyte procoagulant activities (TF, CD11b, association with platelets) were characterized *in vitro*.

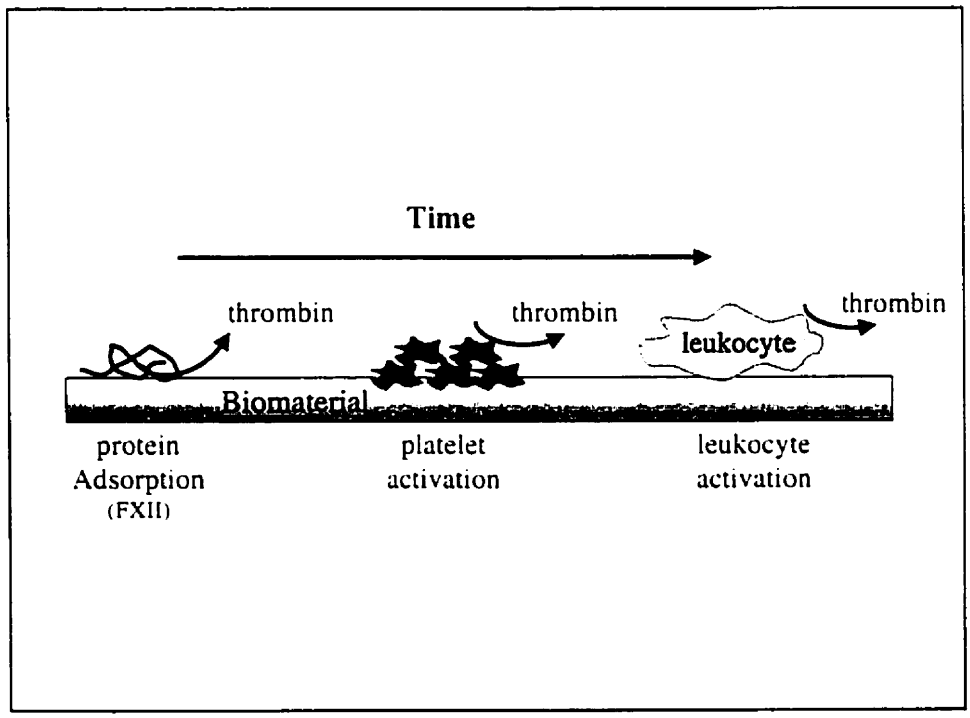

Figure 5: Perspective on the contribution of Factor XII, platelet and leukocyte activation in material thrombogenicity.

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Chapter 3

Leukocyte activation and leukocyte procoagulant activities following blood contact with polystyrene and PEG-immobilized polystyrene beads.

Abstract

Blood contact with beads (45μm) activated leukocytes in the bulk (Tissue Factor expression, CD11b upregulation, and association with platelets) independently of material surface chemistry. On the other hand, activation of adherent leukocytes was material dependent. Following blood contact with polystyrene (PS), polyethylene glycol immobilized polystyrene (PS-PEG) and PS-PEG-NH_2 beads, CD11b upregulation in the bulk, platelet-leukocyte aggregates and leukocyte adhesion were surface area-dependent while Tissue Factor (TF) expression was not. Material-induced leukocyte activation in the bulk was also independent of the beads capacity to activate platelets. However, monocyte adhesion and TF expression on beads was related to the presence of platelets on the surface. Material-induced TF expression was able to initiate the extrinsic pathway of coagulation, resulting in significant fibrin formation. Material-activated leukocytes also appeared to be able to directly activate Factor X.

While not all our markers of leukocyte activation varied with material area or chemistry, it was clear that these materials activated leukocytes in a way that resulted in increased procoagulant activity. During blood-material interactions, material-induced leukocyte activation may then contribute to thrombogenesis.

I. Introduction

Neutrophils and monocytes have long been recognized for their essential role in the inflammatory response. However, clinical evidence from certain inflammatory disorders[1] such as acute respiratory distress syndrome[2] or allograft rejection[3] suggests that neutrophils and monocytes also play a role in thrombogenesis. Inflammatory stimuli associated with surgery or biomaterial implantation may induce leukocyte procoagulant activities leading to enhanced thrombogenesis in addition to the expected inflammatory response. Increases in leukocyte procoagulant activities have been observed during cardiopulmonary bypass with CD11b upregulation[4] and tissue factor (TF) expression[5]. The clinical relevance of leukocyte activation in material-induced thrombosis has not been yet elucidated. However, the role for TF, as a trigger of coagulation, is underscored by recent studies showing the lack of significance of Factor XII (or the intrinsic pathway) in thrombin generation during blood-material contact[6-8]. The potential contribution of leukocytes to thrombin generation has also been shown in vitro and in vivo[9-11].

Interactions of blood with a biomaterial are complex and many events occur: protein adsorption, platelet and leukocyte activation and/or adhesion, as well as activation of the complement and the coagulation cascade. Despite the use of anticoagulants and anti-platelet agents, thromboembolic complications continue to occur in cardiovascular devices[12-14]. While platelets and the coagulation proteins play the central role in thrombosis, it is possible that material-induced leukocyte activation and adhesion also play an important role in cardiovascular material failure, perhaps by contributing directly to thrombin production and/or by releasing procoagulant mediators.

Both monocytes and neutrophils possess the potential to induce or contribute to thrombosis through the expression of specific membrane receptors, phospholipids, and by the release of inflammatory mediators. This study specifically focuses on leukocyte procoagulant activities via the expression of membrane receptors.. Upon activation, monocytes express tissue factor, and this initiates the extrinsic coagulation pathway. Also, leukocyte receptor CD11b is upregulated upon material contact. It binds Factor X and fibrinogen (among other molecules) and can thus favor thrombin generation. Activated leukocytes are also more adherent[15] and can adhere to damaged/stimulated endothelial cells[16], artificial surfaces[17] or platelets[18]. In thrombus formation on a surface, the presence of both platelets and fibrin has been shown to increase leukocyte adhesion[19]. Leukocytes in a thrombus do not have a

passive role, as their presence has been shown to significantly contribute to fibrin formation[11;20;21]. The leukocyte recruitment at the surface may thus further contribute to a localized thrombogenesis.

CD11b upregulation following blood contact with materials[22;23] has already been reported. However, previous studies have usually focused on the role of leukocytes in biomaterial-associated inflammation. When adherent leukocytes were studied, they were removed from the surface by incubation with a solution containing EDTA. Recently, a bead adsorption system (using 10 μm diameter beads) has been used to assess, with flow cytometry, complement[24] and platelet adhesion from platelet-rich plasma[25]. We modified this system to study leukocyte adhesion in whole blood, using larger beads (45μm diameter) to avoid stimulation of a leukocyte phagocytic response and allow enough surface for adhesion and spreading of more than one cell. The adherent cells were assessed directly since the beads (with adherent cells) were small enough to pass through the nozzle of the flow cytometer. There was then no need to dislodge the cells to determine the state of cell adhesion and activation. Non-adherent cells from the same experiment were also analysed by flow cytometry in the conventional way. A key advantage of the bead system is that it permitted direct examination of the effect of the material by varying the concentration of beads.

Using this *in vitro* bead assay, our objective was to determine how surface area and material chemistry affected material-induced leukocyte activation and the leukocyte expression of membrane-associated procoagulant activities. We hypothesized (1) that blood-contact with a material resulted in TF expression and (2) that material-induced leukocyte activation and hence expression of membrane-associated procoagulant activities was independent of material chemistry. We used TF, CD11b and the association with platelets as markers of leukocyte activation since they can be linked to thrombus formation. Three types of beads were tested to assess the influence of material chemistry: polystyrene beads as well as polystyrene beads (TentaGel) grafted with polyethylene glycol (PEG) with two different terminal groups (-OH and $-NH_2$).

II. Materials and methods

1) Reagents and antibodies

D-phenylalanyl-L-propyl-arginyl chloromethyl ketone (PPACK) was purchased from Calbiochem, San Diego. Lipopolysaccharide (LPS, Escherichia coli serotype: 0111:B4), endotoxin-free water, EDTA and paraformaldehyde from Sigma Chemical Co, St Louis, were used. Monoclonal antibodies to TF (American Diagnostica, Greenwich, CT), CD11b (Immunotech-Coulter, Marseilles, France), CD61 and CD41 (Southern Biotechnology, Birmingham, AL) were fluorescein isothiocyanate (FITC) conjugates. Monoclonal antibodies to CD45 (Caltag, Burlingam, CA), CD14 (Southern Biotechnology) and P-selectin (Monosan, Am Uden, Netherlands) were R-phycoerythrin (PE) conjugates and the R-phycoerythrin-cytochrome 5 (PE-Cy5) anti-CD45 was a generous gift from Dr. R. Sutherland (Toronto General Hospital, Canada). FACSLyse, a lysing buffer also containing parafolmadehyde was obtained from Becton Dickinson (Fullerton, CA).

For the procoagulant activity tests, citrated plasma was from George King Bio-Medical, Overland Park, KS. Factor VII, Factor X and Factor Xa were from Enzyme Research Laboratories, South Bend, IN. The monoclonal antibody to TF was from American Diagnostica, Greenwich, CT. The chromogenic substrate to detect FXa, S-2765, was purchased from diaPharma, Franklin, OH. All other chemicals were of analytical or reagent grade.

2) Beads

Polystyrene beads (PS) 45μm microspheres, Polybeads, were purchased from Polysciences, Inc. (Warrington, PA) as a 2.6% suspension in water. TentaGel beads, polystyrene beads grafted with polyethylene glycol (PEG), were obtained from Rapp Polymer, (Tübingen, Germany). TentaGel beads are prepared by grafting PEG chains (~3kD) on a polystyrene matrix and functional groups are added at the end of the PEG spacer chain[26]. To avoid reaction of both terminal hydroxyl groups of PEG with the PS matrix and additional crosslinking, anionic graft polymerization is performed. The copolymer contains approximately 70% linear PEG and 30% crosslinked PS matrix: the properties of these polymers are then highly dominated by the properties of PEG. The remaining free hydroxyl group on the immobilized PEG is then used to attach different functional groups. Figure 1 illustrates the chemical architecture of the TentaGel beads. In this study, two types of

TentaGel beads were used: one with a hydroxyl functional group (PS-PEG) and the other with an amino functional group (PS-PEG-NH_2). TentaGel beads have a dry diameter of 35 μm and they swell in phosphate buffer saline (PBS) to 45 ± 4 μm.

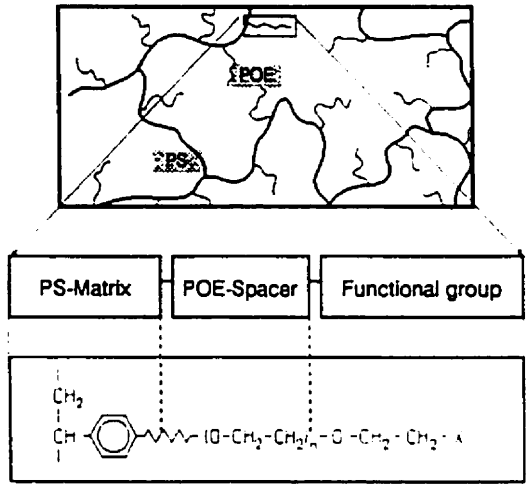


Figure 1: Architecture of the polystyrene-polyethyleneglycol polymer, from Rapp[26].

To remove endotoxin from the surfaces, all beads were washed in a series of solutions: 0.1M NaOH, 1M NaOH, 0.1M HCl, 1M HCl and 95% ethanol[27]. All washes took place in an ultrasonic bath and rinsing with endotoxin-free water (EFW) was performed between each solution. Following the last ethanol wash, beads were rinsed four times with a large volume of EFW to remove all trace of ethanol and then resuspended in EFW at a concentration of approximately $2x10^6$ beads/ml. They were stored at 4°C for a maximum of 2 weeks. Washed beads were analysed by X-ray photoelectron spectroscopy (XPS, Leybold, Max 200) utilizing MgKα X-radiation. Prior to blood contact, 10x D-PBS (Gibco BRL, Grand Island, NY; 80 g/L NaCl, 21.6 g/L Na_2HPO_4, 2 g/L glucose, 2 g/L KH_2PO_4) was added to the bead aliquots used for the experiments.

To ensure that beads were endotoxin-free, the level of endotoxin contamination on the beads for each experiment was tested using a chromogenic substrate assay that tests contamination directly on the material surface[28]. After washing, very low levels of endotoxin contamination were observed on both the TentaGel and PS beads. Even at the highest bead concentration, the residual endotoxin was equivalent to a concentration of

endotoxin contamination that was less than 0.19 EU/ml, which is below the recommended maximum FDA level of 0.5 EU/ml[29].

3) Blood-material contact

Whole blood, from donors who did not take medication within 72 hours of phlebotomy, was collected without tourniquet into a syringe preloaded with PPACK (a selective thrombin inhibitor, final concentration=120μM), after discarding the first mL so as to minimize blood activation. Beads and blood were incubated with mild rotation at 37°C on a hematology mixer (Model 14060-1, Innovative Medical System Corp, Ivyland, PA). The surface area to volume ratio was varied from 0.35 to 34 cm^{-1} by using different bead concentrations (see Table 1). Based on the 45μm bead diameter for all three types of beads, the surface area exposed to blood was approximately 0.64 $cm^2/10^4$ beads[1]. The negative control was EDTA-blood with beads (8 mM final EDTA concentration) while the positive control was blood incubated with endotoxin (5 μg/mL) (LPS serotype 0111:B4) without beads.

Table 1: Bead concentration and related surface area in blood

For a total volume (blood + beads in PBS) of 1 ml

Bead concentration (# of beads/mL x 10^4)	Bead surface area in cm^2/mL *	Volume of blood (μl)	Volume of beads (μl) #
0.55	0.32	998	2
5.4	3.2	974	26
20	12.8	900	100
54	33.4	768	232

* Equivalent to the Surface area to volume ratio: 1 mL total volume (blood + Beads in PBS)

\# Resuspended in PBS at a concentration of $2x10^6$ beads/mL

Following incubation, beads were allowed to settle and small aliquots of blood (5 to 20 μL) were removed for flow cytometry analysis of non-adherent cells. In certain cases, an additional blood sample was taken to perform a cell count using a multi-parameter, automated hematology analyser SysmexTM E-2500 (TOA Medical Electronics, Japan). To analyse adherent cells on beads in the remaining blood sample, erythrocytes were lysed for 5

[1]. Note that assuming a leukocyte has a diameter of about 10μm, its surface area is 314 μm^2, compared to the surface area of a 45 μm diameter bead which is 6361 μm^2

min with NH_4Cl buffer. Beads were washed with RPMI+10% heat inactivated fetal bovine serum (RPMI/FBS) and then incubated with the appropriate antibodies (see below) to assess the adherent cells. Figure 2 summarizes our experimental protocol. On some bead samples, SEM (Hitachi, S-520) was performed following fixation in 2.5% glutaraldehyde and dehydration in a graded series of ethanol solutions.

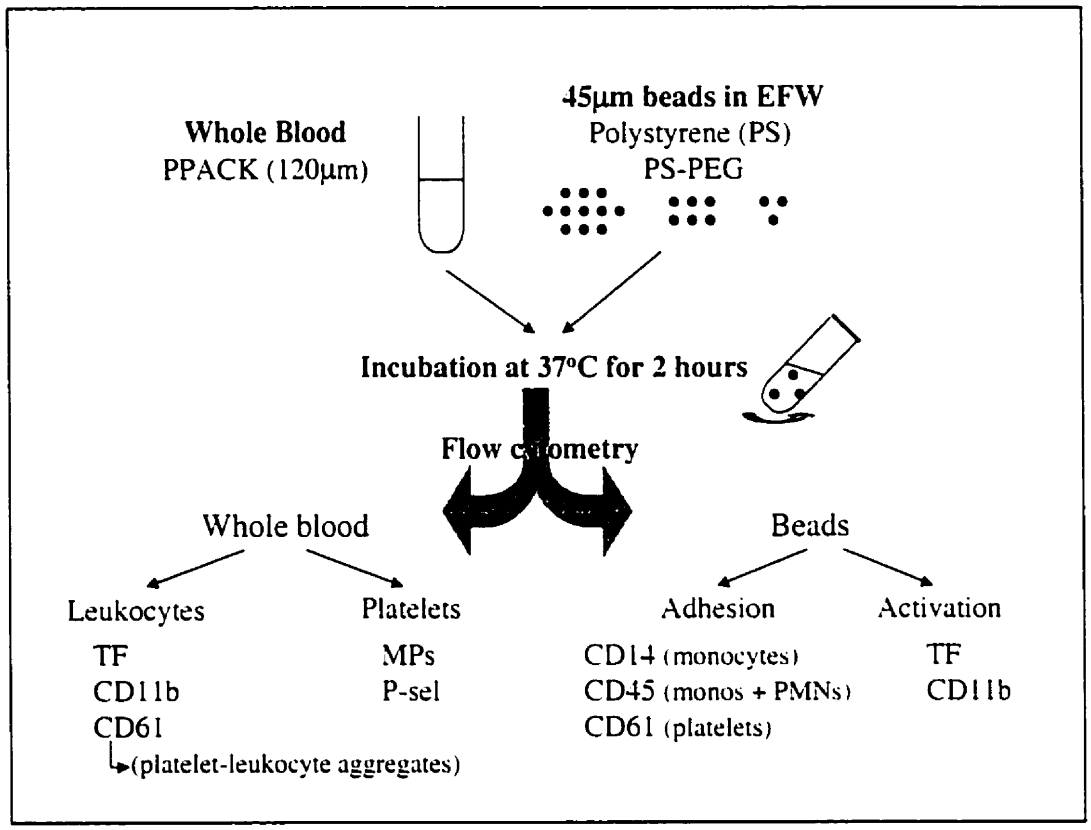


Figure 2: Schematic of experimental protocol. EFW: Endotoxin free water; PMNs: polymorphonuclear cells or neutrophils: MPs: microparticles.

4) Flow cytometry

Flow cytometric analysis was carried out on blood samples before and after exposure to beads. Small aliquots (5 to 20 μL) of blood, diluted in 50μL of Hepes Tyrode Buffer (HTB; 137 mM NaCl, 2.7 mM KCl, 16 mM $NaHCO_3$, 5 mM $MgCl_2$, 3.5 mM HEPES, 1g/L glucose, 2g/L BSA, pH 7.4) were incubated with saturating concentrations of fluorescently labeled monoclonal antibodies for 30 minutes at 4°C. After incubation, erythrocytes were

lysed using FACSlyse (Becton-Dickinson, San Jose, CA). The cells were then washed, diluted and fixed with paraformaldehyde (1% final concentration).

For adherent cells on beads, the washed beads were incubated for 30 minutes at 4°C with the fluorescently labelled antibodies. To remove non-specific binding, they were then washed in RPMI/FBS and finally diluted with 150 µL of HTB and 150µL paraformaldehyde. All samples were stored in the dark at 4°C until analysis.

Blood was assessed by dual color flow cytometry on a Becton-Dickinson FACScan flow cytometer, using CELLQuest software. To analyse leukocytes, the light scatter channels were set at a linear gain with a threshold at 200 for the forward scatter (FSC). Monocytes and neutrophils were gated using their light scatter characteristics and the CD45 versus side scatter (SSC) or CD14 versus SSC dot plots (Figure 3 a, b). The direct expression of procoagulant activity was monitored using FITC-anti TF and PE-anti CD14 (monocyte marker). The arbitrary FITC fluorescent intensity of monocytes and the percentage of monocytes staining positive for TF were recorded (Figure 3 c-e). No TF expression was observed on neutrophils. CD11b upregulation was monitored using FITC-anti CD11b and PE-anti CD45 (pan leukocyte marker). Platelet-leukocyte aggregates were characterized with FITC-anti CD61/CD41 (antibody against GPIIb-IIIa, a platelet receptor) and PE-anti CD45. The arbitrary FITC fluorescent intensities for both monocytes and neutrophils were recorded for CD11b and CD61. To analyse platelets, the light scatter channels were set on a logarithmic scale with the FSC threshold at 0. Platelet microparticles were identified by their scatter characteristics after gating on the positive events specific for GPIIb/IIIa[30]. Platelet activation was monitored by recording the arbitrary fluorescent intensities for CD61 and P-selectin. Irrelevant antibodies of the same isotype were used as controls.

For adherent cells on beads, leukocyte adhesion and activation was assessed using the antibodies FITC-anti TF or FITC-anti CD11b (for activation) with PE-anti CD14 or PE-Cy5-anti CD45 (for adhesion). Adherent platelets were also identified with FITC-anti CD61. Due to the large size of the beads and their different light scattering characteristics, the light scatter and fluorescence channels for beads differed from those of cells and new instrument settings were necessary for each type of beads (Figure 3 f-h). These were determined at the beginning of the study. Minor adjustments were sometimes required when a new batch of antibody was used. All beads had sharp forward and side light scatter histograms (indicating

minimal variation in manufactured diameter) and following blood contact an increase and broadening of the light scatter characteristics was readily observed due to cell adhesion (Figure 3 g).

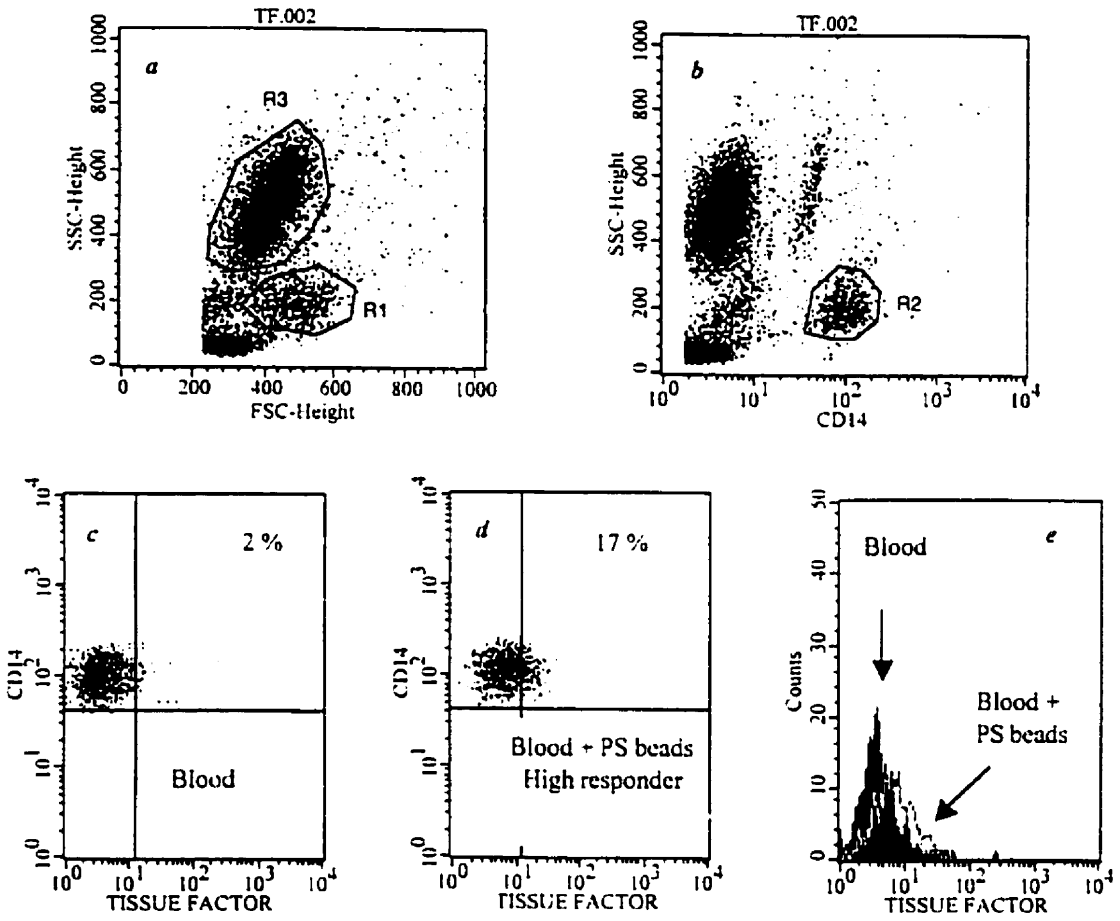


Figure 3 (a-e): Flow cytometric analysis of leukocytes after blood contact with beads for 2 hours at 37°C. [a] Dot plot showing forward (FSC) and side (SSC) scatter characteristics of leukocytes in blood. The monocyte population is gated in the region R1 and the neutrophils in R3. [b] Dot plot showing CD14 fluorescent intensity on leukocytes and side scatter. CD14 is a monocyte marker and the positive population is gated in R2. [c, d] Dot plots of CD14 and TF fluorescent intensities on monocytes (population belonging to R1 and R2) to determine the percentage of monocytes staining positive for TF expression. In (c) only 2% of monocytes in the blood incubated without beads are positive for TF. In (d) 17% of monocytes from blood from a high responder incubated with $20x10^4$ beads/mL express TF. [e] Histograms of the arbitrary fluorescent intensity of TF on monocytes. An increase in fluorescent intensity is observed following blood contact with beads.

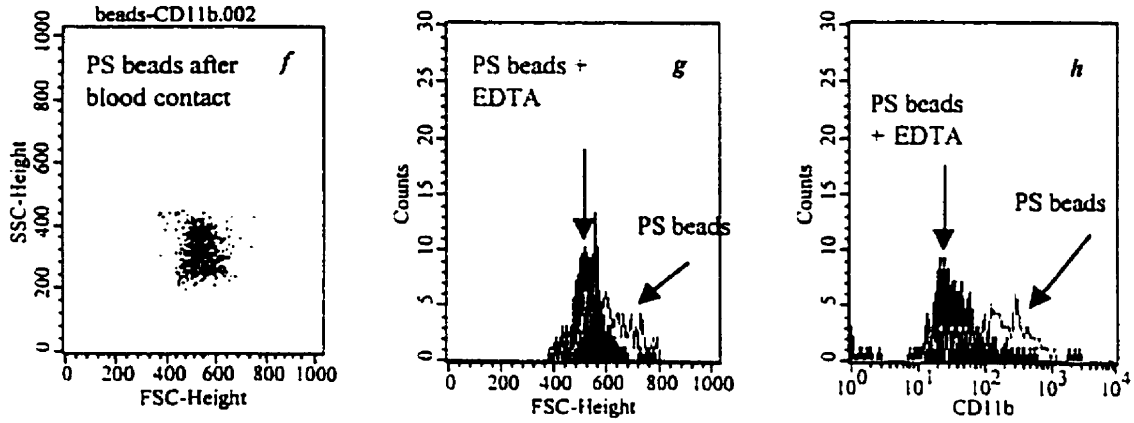


Figure 3 (f-g): Flow cytometric analysis of beads after blood contact with beads for 2 hours at 37°C. [f] Dot plot showing forward and side scatter characteristics of beads after blood contact. Gains are different so this plot and plot (a) are not comparable. [g] Histograms of the forward scatter characteristics (FSC) of beads. The filled histogram represents beads from a sample with EDTA (adhesion is inhibited by EDTA). A broadening in the FSC can be observed on beads following blood contact with 5.4×10^4 beads/mL (line histogram). This increase in size detected by the flow cytometer is due to cell adhesion. [h] Histograms of the fluorescent intensity of CD11b on beads. The filled histogram represents the background signal of the beads (beads from blood+ EDTA). CD11b upregulation is observed on beads from a sample without EDTA.

5) Procoagulant functional activity

a) Functional assay for TF

TF initiates the extrinsic pathway of coagulation. To test if TF expression on monocytes, as measured by flow cytometry, was indeed functional, the ability of leukocytes to generate thrombin was assessed. TF procoagulant activity was determined by a one-stage coagulation assay, in which cells were incubated with citrated plasma to which Ca^{2+} was added to allow for the formation of a fibrin clot.

The coagulation test was performed with a suspension of leukocytes in bulk and adherent to beads. This suspension was obtained as follows. After a 2-hour incubation of blood with beads (20×10^4 beads/mL), red blood cells were lysed with FACSlyse. The remaining cells and beads were washed twice at low speed (to remove platelets) in RPMI/FBS with EDTA. The pellet containing leukocytes and beads was then resuspended in RPMI. An aliquot of the leukocyte and bead suspension was incubated with citrated-

pooled human plasma for 2 minutes. In some cases, beads were left to settle briefly, and an aliquot of leukocytes alone were tested. The clotting time was measured automatically following the addition of 25mM $CaCl_2$ using a coagulometer (Coag Mate X2, General Diagnostics, Oklahoma City, OK). The contribution of TF to clotting was determined by incubating the samples with a monoclonal antibody directed against TF at a saturating concentration of 25 μg/mL[31;32]. Positive (LPS) and negative (no beads and beads+EDTA) controls were also tested. Results are presented as clotting times.

b) Functional assay for CD11b

To determine if the CD11b upregulation observed after blood-material contact had procoagulant activity, the ability of CD11b to activate Factor X was assessed using a chromogenic assay. The principle of the assay was to incubate activated leukocytes with Factor X in the presence of Ca^{2+} and measure Factor Xa generation.

After a 2-hour incubation of blood with beads, Factor Xa generation by leukocytes alone (no beads) was measured after RBC lysing with FACSlyse and several washes as described above. In a 96-well plate, leukocytes were incubated with 150 nM Factor X, 25mM $CaCl_2$ and 0.25 mM of S-2765 (chromogenic substrate) for 30 minutes at 37°C on a mixing platform. Acetic acid (20%) was added to stop the reaction. The plate was then read on a V-Max kinetic microplate reader (Molecular Devices, Sunnyvalle, CA) at 405 nm. A standard curve was obtained by using different concentrations of Factor Xa.

6) Statistical analysis

Analysis of variance (ANOVA) was carried out to evaluate the significance of the differences in cell activation with surface area, material chemistry and endotoxin contamination. To evaluate the significance of the differences in cell activation, analysis of variance (ANOVA) was carried out followed by the Student-Newman-Keuls test, which is a protected test for multiple comparisons[33]. A *p*-value of less than 0.05 was required for statistical significance. The number of repeat experiments was equal to or greater than three with three different donors.

III. Results

1) Surface characteristics of acid/base/ethanol washed beads

Table 2 and Figure 4 show the XPS results of washed beads: low resolution spectrum analysis (Table 2) and high resolution carbon spectra (Figure 4). In Table 2, the presence of oxygen by XPS analysis indicates that the PS beads were not pure polystyrene; there was also some silicon contamination. The higher surface oxygen (Table 2) and the large C-O peak (Figure 4) confirmed the presence of PEG in TentaGel beads. As expected, no difference between the PS-PEG and PS-PEG-NH_2 was seen in their C1s spectra.

Table 3 demonstrates the effect of washing on endotoxin contamination on the beads. As shown in Table 3, after washing, very low levels of endotoxin contamination were observed on both the TentaGel and PS beads. Even at the highest bead concentration, the residual endotoxin was equivalent to a concentration of endotoxin contamination that was less than 0.19 EU/ml, which is below the recommended maximum FDA level of 0.5 EU/ml[29].

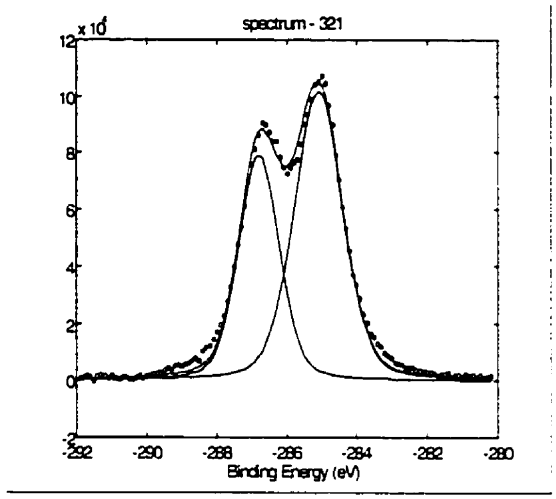
Table 2: Composition (atom %) of acid/base/ethanol washed beads from low resolution XPS analysis.

	O1s	N1s	C1s	Si2p
Polystyrene (PS)	13	0	85.8	1.2
PS-PEG	20.5	0	77.8	1.7
PS-PEG-NH_2	18.8	0.3	79.9	1.0

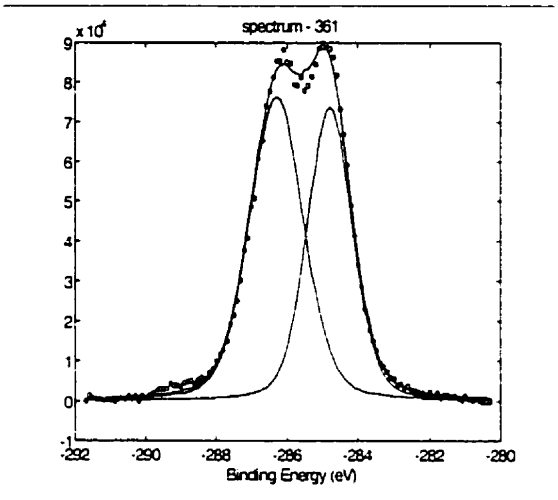
Table 3: Endotoxin amounts on beads before and after acid/base/ethanol washing.

	Before washing		*After washing*	
	EU/10^4 beads	for $54x10^4$ beads/mL equivalent EU/mL	EU/10^4 beads	for $54x10^4$ beads/mL equivalent EU/mL
Polystyrene	7 ± 3	~ 450	0.0024	0.13 ± 0.05
PS-PEG	0.4	~ 20	0.0021	0.11 ± 0.06
PS-PEG-NH_2	1.4	~ 80	0.0025	0.14 ± 0.05

n=3 ± S.D.



a)



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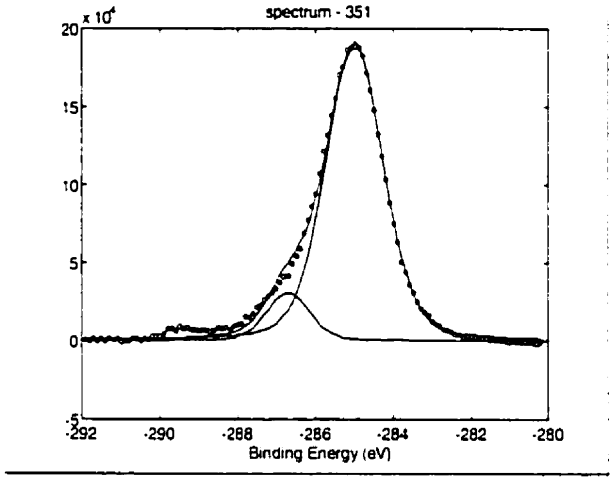

c)

Figure 4: High resolution ESCA C1s spectra of acid/base/ethanol washed beads.

a) PS-PEG, b) PS-PEG-NH_2, c) polystyrene. (A) 285 eV: C-C bonds, (B) 286.7 eV: C-O bonds. Note the larger C-O peak in the PS-PEG and PS-PEG-NH_2 spectra.

2) Effect of incubation time

Since the markers of procoagulant activities CD11b and TF have different mechanisms of expression, i.e. TF needs to be synthesized[34] while CD11b is already present in granules, the appropriate incubation time required to observe both markers was determined. PS beads with a low level of endotoxin contamination (from a different batch than all other PS beads used in the later experiments) at a concentration of $20x10^4$ beads/mL were incubated with blood for up to four hours. Figure 5 shows the effect of incubation time

on the expression of procoagulant activities on leukocytes in the bulk: TF expression, CD11b upregulation and formation of platelet-leukocyte aggregates. TF was the only marker of activation that was time-dependent: both the percentage of monocytes expressing TF and the level of expression relative to the maximum increased with time. Compared to CD11b, however, there was a high variability in the TF expression on monocytes obtained from the different donors. This is a common observation with TF; for an identical stimulus, the response of TF expression varies among individuals who can usually be separated into two groups designated as low or high responders[35].

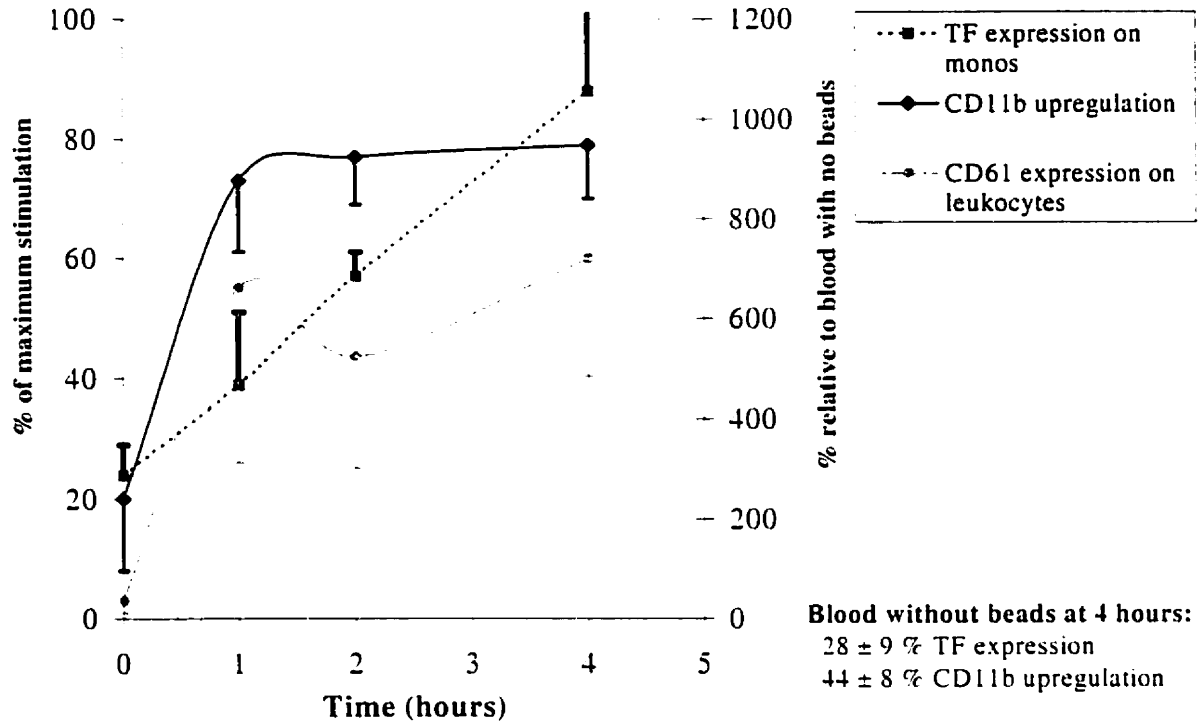

Figure 5: Time profile of leukocyte activation in the bulk after blood contact with PS beads at 37°C. TF expression and CD11b upregulation are represented as a percent relative to the maximum (LPS for 4 hours). CD61 expression on leukocytes is a measure of platelet-leukocyte aggregate formation. PS bead concentration: $20x10^4$ beads/ml; beads were not washed so there was some (low) amount of endotoxin present. N = 4 ± S.D.

Figure 6 shows that, after 4 hours of incubation at 37°C, a significant change in the light scatter characteristics of leukocytes was seen, suggesting some morphologic alteration.

Two hours was chosen as an adequate incubation time since all markers of activation were detectable with a somewhat lower variability in TF expression than that seen at 4 hours.

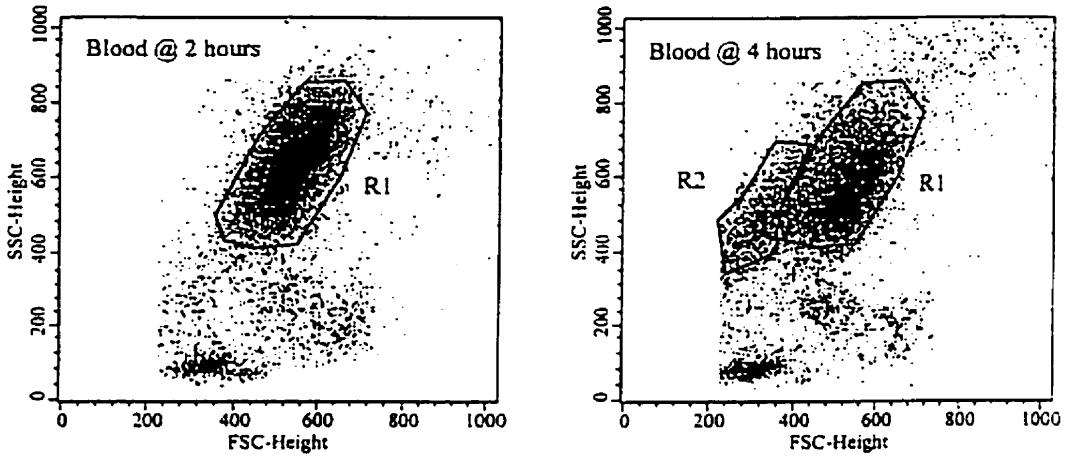

Figure 6: **Dot plot of scatter characteristics of blood without beads following a 2-hour and a 4-hour incubation at 37°C**. Note after 4-hour of incubation, the presence of a broader population of neutrophils (R1+R2). Also as illustrated in R1, neutrophils at 4 hours showed a decrease in both size (FSC) and granularity (SSC) compared to neutrophils at 2 hours.

3) Effect of surface area on leukocyte activation in the bulk

Different surface areas were obtained by varying the number of PS beads in contact with blood. Table 4 shows the effect of surface area on leukocyte activation in the bulk: TF expression, CD11b upregulation, and formation of platelet-leukocyte aggregates.

As shown in Table 4, following two hours of incubation with PS beads, there was an increase in TF expression on monocytes in the bulk regardless of bead concentration. On the other hand, CD11b upregulation steadily increased with bead concentration (surface area) to reach significant levels (compared to the no beads sample) at $20x10^4$ beads/ml (Table 4). CD11b upregulation occurred to the same extent on both monocytes and neutrophils (data not shown). The formation of platelet-leukocyte aggregates also increased with surface area, however the differences were not significant (Table 4). Compared to neutrophils, a larger CD61 signal was usually observed on monocytes both with resting and activated blood samples (data not shown). However, the relative increase in platelet-leukocyte aggregates following material contact was similar for monocytes and neutrophils.

Table 4: Leukocyte activation in blood (non-adherent cells) following contact with different washed PS bead concentrations for 2 hours at 37°C: effect of bead concentration.

Type of beads	Bead concentration (# of beads /mL x 10^4)	% of monocytes expressing TF	TF expression (%)[a]	CD11b upregulation (%)[a]	CD61 expression on leukocytes (%)[b]
No beads	0	4 ± 2	35 ± 8	51 ± 6	100
PS	0.55	7 ± 5	46 ± 10	57 ± 8	160 ± 51
	5.3	15 ± 8	51 ± 11	61 ± 8	194 ± 83
	20	19 ± 9	53 ± 11	75 ± 4 *	280 ± 134 *
	54	27 ± 24	55 ± 23	79 ± 6 *	323 ± 149 *
PS-PEG	5.3	22 ± 9 *	61 ± 15 *	63 ± 9	189 ± 70
	20	31 ± 16 *	63 ±7 *	71 ± 9 *	285 ±130 *
	54	34 ± 11 *	68 ± 6 *	80 ± 15 *	379 ± 179 *
PS-PEG-NH_2	5.3	21 ± 13	58 ± 18 *	62 ± 9	131 ± 28
	20	27 ± 11 *	60 ± 7 *	72 ± 8 *	219 ± 95
	54	31 ± 10 *	61 ± 6 *	80 ± 16 *	289 ± 171 *

n = 3 to 6 ± S.D.

[a] TF expression and CD11b upregulation are represented as a percent relative to the maximum where the maximum upregulation is determined by the fluorescent intensity of blood incubated with LPS 0111:B4.

[b] CD61 expression is represented as a percent relative to the no bead blood control; CD61 expression is a measure of platelet-leukocyte aggregate formation.

* Significantly different ($p \leq 0.05$) from no beads

4) Effect of surface area on leukocyte adhesion/activation

Figures 7 to 9 and Table 5 report the effects of surface area on leukocyte adhesion to PS beads as measured by cell count (Table 5), SEM (Figure 7), and flow cytometry (relative CD45 signal-Figure 8 and total adhesion-Figure 9). Figure 9 also shows the effect of surface area on the level of activation of adherent leukocytes with TF and CD11b signals.

In Table 5, a decrease in the number of leukocytes in the bulk shows that as the surface area (i.e., the PS bead concentration) increased, cell adhesion increased. However as the number of beads increased, fewer adherent leukocytes per bead were found on each bead by both SEM (Figure 7) and flow cytometry (Figure 8). Additional SEM pictures of leukocytes on beads may be found in Appendix E. As shown in Figure 8, since flow cytometry measures the fluorescence intensity associated with each bead, fewer cells adhering to a bead resulted in a decrease of the fluorescence associated with the bead. Thus to represent an equivalent to the total adhesion (or activation) on beads, some of the flow cytometry results in this chapter are presented as the product of the relative fluorescence to the negative control (beads + EDTA) and the number of beads per mL. As shown in Figure 9, parallel to the increase in total leukocyte adhesion (CD45), a significant increase in adherent TF and CD11b occurred as the surface area increased.

Table 5: Effect of washed PS bead concentration on leukocyte adhesion, as measured by leukocyte count, from blood incubated with beads for 2 hours at 37°C.

PS Bead concentration (# of beads/mL x 10^4)	Relative leukocyte count (%)
0	100
0.55	97 ± 5
5.3	95 ± 6
20	81 ± 5
54	78 ± 5

n = 4 ± S.D.

Leukocyte counts are expressed as a percent of leukocytes in blood relative to the number of leukocytes in blood without beads at 2 hours. Note that a sample of blood with EDTA and beads had similar leukocyte counts to that of blood without beads.

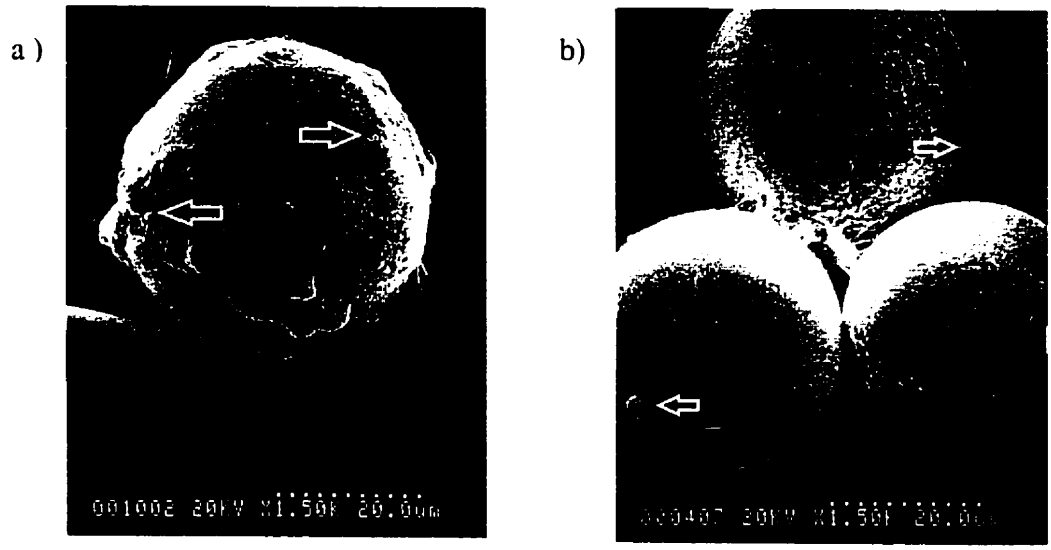

Figure 7: Scanning electron micrographs of washed PS beads for 2 hours at 37°C.
a) Bead from an experiment with $5.3x10^4$ beads/mL. Many adherent leukocytes were observed on the surface. Activated platelets are also present. b) Beads from an experiment with a higher bead concentration. Fewer leukocytes were observed (arrows).

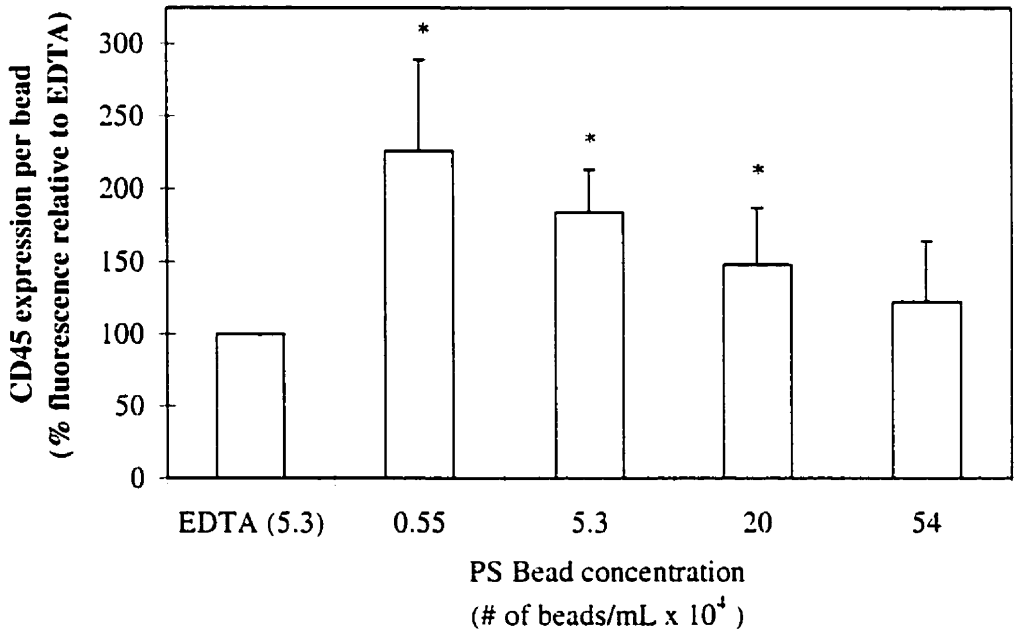

Figure 8: Changes in the CD45 fluorescent intensity (per bead) on beads incubated with blood at different concentration. Fewer leukocytes adhered per bead when high bead concentrations were used, resulting in a decrease in the CD45 signal associated with each bead. * Significantly different from beads + EDTA ($p < 0.05$).

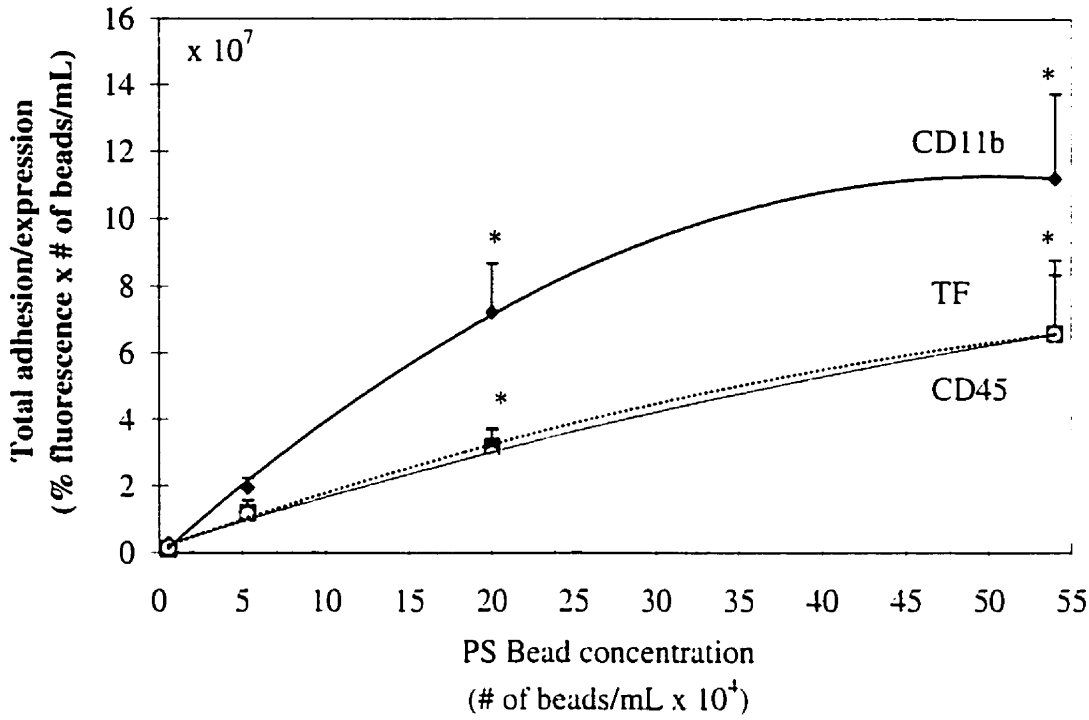

Figure 9: **Effect of bead concentration (or surface area) on the extent of leukocyte adhesion (CD45) and adherent cell activation using CD11b and TF expression on beads.** Leukocyte adhesion increased with surface area. * Significantly different from 0.55 and 5.3 x10^4 beads/mL ($p \leq 0.005$). Ordinate is explained in text.

5) Effect of material chemistry on activation and adhesion

Immobilized PEG is of interest for material biocompatibility since it is capable of resisting plasma protein adsorption and platelet adhesion[36]. Table 4 shows the effect of surface area on leukocyte activation in the bulk with PS-PEG and PS-PEG-NH_2 beads, while Figure 10 shows the effect of surface area and bead chemistry on the formation of platelet microparticles. Table 6 and Figure 11 report the effect of surface area and bead chemistry on leukocyte adhesion (Figure 11a) and adherent leukocyte activation (TF-Table 6 and CD11b-Figure 11b). In Table 6, the results on platelet adhesion are also presented.

As reported in Table 4 (page 77), PS-PEG and PS-PEG-NH_2 beads resulted in a level of leukocyte activation in the bulk that was similar to that observed with the PS beads: expression of TF was observed independently of surface area while CD11b upregulation was surface area-dependent. The presence of statistically significant differences in TF expression in the bulk with the TentaGel beads but not with PS beads may be explained by the fact that

few monocytes were adherent to the TentaGel beads (see below), thus most activated monocytes remained in the bulk. Compared to PS and PS-PEG, formation of platelet-leukocyte aggregates in the bulk was slightly (but not significantly) reduced with PS-PEG-NH_2.

As seen in Figure 10, the presence of PEG on the PS beads resulted in a significant reduction in platelet activation (in the bulk) compared to PS beads, as measured by the formation of platelet microparticles. Also, with the TentaGel beads, no P-selectin expression was observed on platelets, further confirming minimal platelet activation (data not shown).

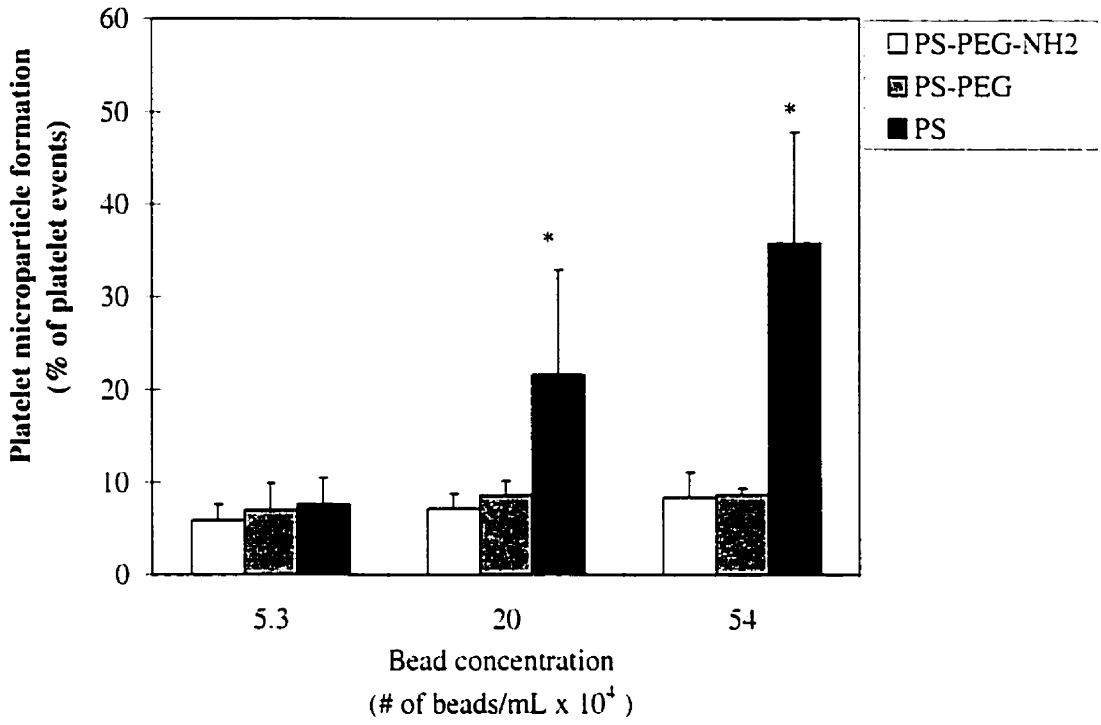

Figure 10: Effect of material chemistry on the formation of platelet microparticles in the bulk after blood contact with different concentration of beads. The presence of PEG on PS beads significantly reduced the formation of platelet microparticles. In the no beads samples, 5.3 ±1.4 % of platelet microparticles were present. * Significantly different from PS-PEG-NH_2 and PS-PEG ($p \leq 0.04$)

Table 6 shows that the absence of significant monocyte adhesion on TentaGel beads, indicated by low levels of CD14, resulted in minimal TF expression on the beads. On the other hand, as demonstrated by the CD45 signal, Figure 11a shows that neutrophil adhesion occurred on TentaGel beads. CD45 is a marker for both monocyte and neutrophil adhesion;

since little CD14 (monocyte marker) was observed on beads, this suggests that neutrophil adhesion was preponderant on TentaGel beads. In fact, despite the absence of monocytes on TentaGel beads, total leukocyte adhesion was similar for all beads since comparable CD45 levels were observed (Figure 11a). However, Figure 11b shows that the activation of adherent leukocytes, as measured by CD11b, was reduced on the PS-PEG-NH_2 when compared to both PS-PEG and PS beads. This reduction was statistically significant relative to PS beads at all bead concentrations while only at the highest bead concentration did the amino group versus the hydroxyl groups have a statistically significant effect on leukocyte activation. No CD61 (i.e., platelet adhesion) was observed on the PS-PEG-NH_2 beads while very slight platelet adhesion was found on PS-PEG (Table 6).

Table 6: Monocyte adhesion and adherent cell activation (TF expression) and platelet adhesion on washed TentaGel and PS beads.

Type of Beads	Bead concentration (# of beads/mL x 10^4)	Tissue factor (%)[a]	Monocyte adhesion (CD14, %)[a]	Platelet adhesion (CD61, %)[a]
PS	EDTA	100	100	100
	5.3	223 ± 66	213 ± 51	811 ± 226
	20	161 ± 26	161 ± 31	525 ± 193
	54	132 ± 25	133 ± 19	293 ± 92
PS-PEG	EDTA	100	100	100
	5.3	121 ± 11	117 ± 15	162 ± 41
	20	103 ± 8	124 ± 10 ♠	131 ± 38
	54	100 ± 12	101 ± 8	115 ± 18
PS-PEG-NH_2	EDTA	100	100	100
	5.3	100 ± 8	100 ± 2	112 ± 12
	20	100 ± 8	100 ± 2	108 ± 10
	54	100 ± 9	100 ± 4	103 ± 8

N = 4 to 6 ± S.D.

TF, CD14 and CD61 signals are represented as a percentage relative to the negative control ($5.3x10^4$ beads/mL + EDTA).

All values were significantly different ($p<0.05$) from the ones obtained with PS beads at the respective bead concentrations, except for the one marked by ♠.

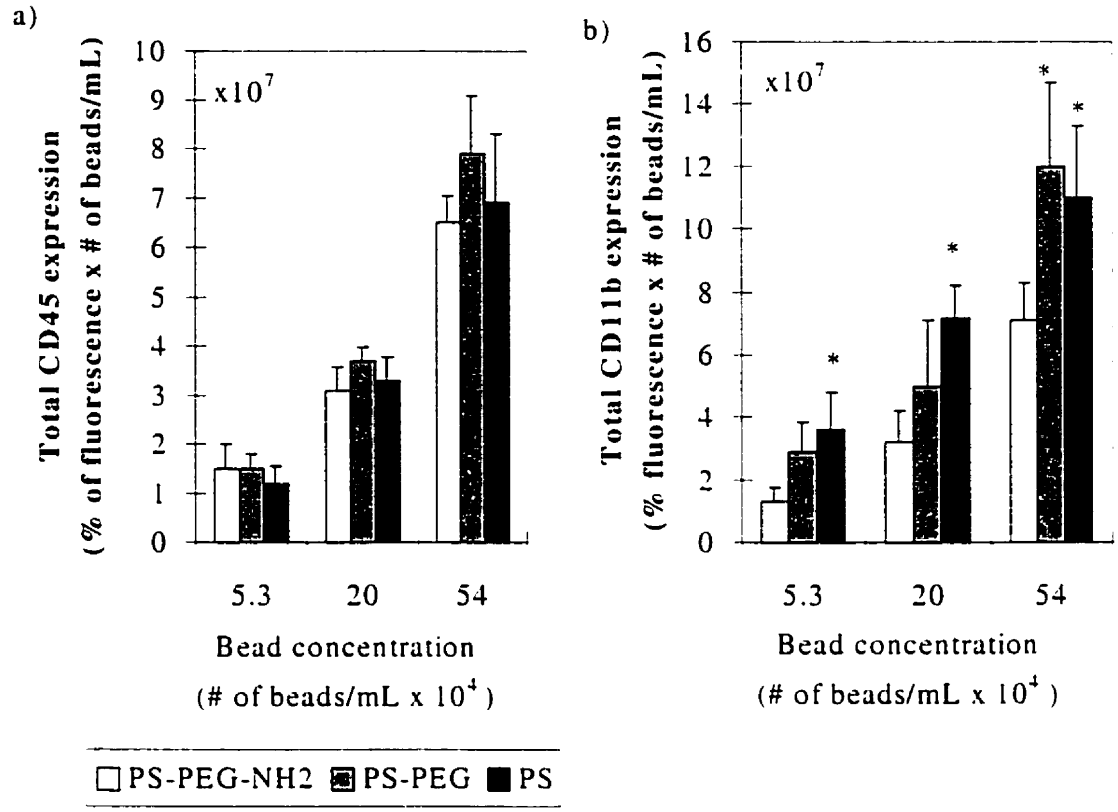

Figure 11: Effect of material chemistry on the extent of leukocyte adhesion using CD45 (a) and adherent cell activation using CD11b (b) on beads. Similar levels of leukocyte adhesion occurred on all beads. However, PS-PEG-NH_2 significantly reduced the level of activation of adherent leukocytes. * significantly different from PS-PEG-NH_2 ($p \leq 0.05$). Ordinate is explained in text.

6) Effect of endotoxin: experiments using unwashed PS beads

The level of endotoxin on unwashed PS beads, as measured by the chromogenic substrate test, was 7 ± 3 EU/10^4 beads; this was approximately equivalent to an endotoxin concentration between 2.5 and 4 EU/mL for an incubation with the lowest bead concentration. Tables 7 and 8 report the effects of endotoxin contamination of PS beads on leukocyte activation in the bulk (Table 7) and on leukocyte adhesion (Table 8) and activation of adherent leukocytes (Table 8).

As shown in Table 7, in the presence of endotoxin-contaminated PS beads, significant increases in TF expression and CD11b upregulation were observed even at low bead concentration. Most of the significant differences observed between endotoxin-

contaminated and washed beads were with TF expression. Similarly to washed beads, CD11b upregulation induced by contaminated beads depended on surface area while TF did not. A significant increase in leukocyte MPs formation was observed only in the case of the highest bead concentration: at $54x10^4$ beads/mL, contaminated beads lead to the formation of 38 ± 11 % leukocyte microparticles (data not shown).

Table 7: Effect of endotoxin contaminated (unwashed) PS beads on leukocyte and platelet activation in the bulk.

PS Bead concentration (# of beads/mL x 10^4)	TF expression (%)	CD11b upregulation (%)	CD61 expression on leukocytes (%)	Platelet MPs (%)
0	37 ± 8	51 ± 6	100	9 ± 2
0.55	64 ± 4	63 ± 11	189 ± 56	10 ± 4
5.3	91 ± 11 *	73 ± 9	371 ± 199 *	33 ± 9 *
20	97 ± 5 *	87 ± 7	453 ± 200	22 ± 12
54	60 ± 20[4]	99 ± 1 *	385 ± 182	44 ± 14

N = 4 ± S.D.

MPs = microparticles. For explanation of units, see Table 3.

[4] Few monocytes were present in blood sample.

* Significantly different from activation induced by washed (i.e., "endotoxin-free") beads ($p < 0.03$) - results presented in Table 4 (page 77) and Figure 10 (page 81).

As shown in Table 8, the presence of endotoxin on beads did not result in an increase in leukocyte adhesion: similar fluorescent intensities for CD14 and CD45 were obtained for washed and contaminated beads. On the other hand, while the TF signal did not vary with endotoxin presence, a significant increase in CD11b upregulation occurred on leukocytes adherent to contaminated beads (Table 8). As for platelet adhesion on beads, no significant difference between washed and contaminated beads was observed (data not shown).

Table 8: Effect of endotoxin contaminated (unwashed) PS beads on adherent leukocyte activation or adhesion.

PS Bead concentration (# of beads/mL x 10^4)	TF (%)	CD11b (%)	Leukocyte adhesion (CD45, %)	Monocyte adhesion (CD14, %)
EDTA	100	100	100	100
0.55	235 ± 74	1036 ± 252 *	276 ± 28	319 ± 78
5.3	242 ± 69	697 ± 177 *	210 ± 39	222 ± 46
20	189 ± 61	316 ± 73	164 ± 45	169 ± 37
54	128 ± 6	160 ± 30	143 ± 36	121 ± 9

N = 3 ± S.D

* Significantly different from activation induced by endotoxin-free beads ($p < 0.03$)

7) Measure of procoagulant activity

Table 9 shows the prothrombin time (PTT) results with the suspension of leukocytes and beads from blood incubated for 2 hours with $20x10^4$ beads/mL. Compared to the EDTA and the no beads samples, significantly shorter clotting times were observed with the samples issued from blood incubated with LPS, PS and TentaGel beads. The shorter clotting times indicated an increase in procoagulant activity (activation of the coagulation cascade leading to thrombin generation), resulting in increased fibrin formation. Adding anti-TF to the suspension of leukocytes and beads lengthened the clotting times back to the level of that with EDTA, indicating that TF was primarily responsible for the increase in procoagulant activity. Furthermore, the clotting times for PS-activated leukocytes alone were longer than when both leukocytes and beads were present, suggesting that the beads contributed to the observed procoagulant activity. On the other hand, for PS-PEG, similar clotting times were observed with the suspension of leukocytes and beads or leukocytes alone. This is in accordance with our flow cytometry results showing significant TF expression on PS beads but not on PS-PEG beads. The PTT tests demonstrated that the material-induced TF expression, measured by flow cytometry, was indeed functional and initiated the extrinsic pathway of coagulation, resulting in thrombin and fibrin formation.

Table 9: Measures of TF procoagulant activity (clotting assay) and expression (flow cytometry), following a 2-hour incubation with PS and TentaGel beads ($20x10^4$ beads/mL)

	Clotting Assay Prothrombin Time (sec)		**Flow cytometry** TF expression (%)	
	Leukos + beads	Leukos	Monos	Beads
No beads		385 ± 129	27 ± 5	
LPS		49 ± 7	100 ± 0	
Beads + EDTA	412 ± 75	N/D	17 ± 8	100 ± 0
PS	138 ± 48 *	183 ± 94 *	43 ± 14	221 ± 54 *
PS + anti-TF[a]	320 ± 47	357 ± 117		
PS-PEG	132 ± 28 *	125 ± 14 *	40 ± 5 *	129 ± 22
PS-PEG + anti-TF[a]	363 ± 87	423 ± 25		
PS-PEG-NH_2	130 ± 48 *	N/D	40 ± 9 *	100 ± 0
PS-PEG-NH_2 + anti-TF[a]	410 ± 78	N/D		

N = 3 to 4 ± S.D

[a] The suspension of leukocytes and beads was incubated with saturating concentration of anti-TF for 15 min at 4°C.

* Significantly different from no beads or beads+EDTA samples ($p < 0.05$)

N/D: not determined

Table 10 presents the results on the generation of Factor Xa by leukocytes: due to high variations, individual measurements are reported. Material-activated leukocytes appeared to be able to activate Factor X, as shown by the increase in Factor Xa concentration when compared to the leukocytes from beads + EDTA samples. Indeed, in each assay, compared to leukocytes from blood without beads, almost twice as much Factor Xa was generated by leukocytes activated by incubation with beads.

Table 10: Measure of Factor Xa generation by leukocytes following a 2-hour blood incubation with PS and TentaGel beads ($20x10^4$ beads/mL)

	Factor Xa generation (nM/L)			
	Assay 1	Assay 2	Assay 3	Assay 4
No beads	0.108	0.021	0.282	0.432
LPS	0.199	0.080		
Beads + EDTA	0.035	0.031	0.383	0.206
PS		0.048	0.586	
PS-PEG				1.123
PS-PEG-NH_2	0.195			

IV. Discussion

1) *In vitro* model

In this study, we used an in-vitro bead system to assess material-induced leukocyte activation. The small blood volume requirements, the ease in varying the surface area and the direct flow cytometric analysis of adherent cells make this bead system advantageous in studying leukocyte- (and platelet) material interactions. The surface areas tested corresponded to a surface area to volume ratio ranging from 0.3 to 34 cm^{-1}: the highest area to volume ratio being the equivalent of a tube of ~ 1.2 mm in diameter. We were able to show that some markers of leukocyte activation were surface-area and hence material-dependent - i.e., dependent on the presence of the material, although not necessarily varying with different material chemistries (as discussed below). CD11b upregulation in the bulk, platelet-leukocyte aggregates, and leukocyte adhesion were surface area dependent. Tissue factor expression, contrary to CD11b, was stimulated by the presence of a foreign material, regardless of surface area. This difference suggests that TF expression, requiring protein synthesis, and CD11b (which does not) have different mechanisms of material-induced activation. Endotoxin contamination is unlikely responsible, since at low bead concentration TF expression was seen despite undetectable levels of endotoxin.

One may also notice that the level of leukocyte activation in the no beads samples were quite high for an unstimulated control. Others[37] have also noted a significant increase

over time in CD11b upregulation in blood anticoagulated with PPACK both at room temperature and at 37°C when compared to other anticoagulants such as heparin. It is believed that the slow hydrolysis of PPACK or the fact that PPACK may not inhibit early steps in coagulation pathway are responsible for this upregulation.

2) Material Chemistry

Polystyrene and PEG-modified polystyrene (TentaGel) were only used as model blood-activating surfaces. Nevertheless, little effect of material chemistry (albeit within this limited range in chemistry) was observed on leukocyte activation in the bulk even on the markers of activation that had shown dependence on surface area: both PS and TentaGel beads caused comparable degrees of bulk CD11b upregulation, TF expression, platelet-leukocyte aggregates and leukocyte microparticle formation. These results agree with previous studies in our laboratory[28;38] that showed similar levels of bulk leukocyte activation with various surface-modified or clinically relevant cardiovascular materials. Misoph et al[39] also found that leukocyte response *in vivo* was independent of the type of circuit/material used during cardiopulmonary bypass.

On the other hand, differences appeared in the capacity of the material to support leukocyte and platelet adhesion. As expected, the presence of PEG on TentaGel resulted in the inhibition of platelet adhesion. Few monocytes were also present on the TentaGel beads. However, neutrophil adhesion still occurred. It may be that the molecular weight of PEG is too low to effectively inhibit the adsorption of the specific proteins that are ligands for leukocytes (for example the inactivated complement product iC3b). Indeed, the complement product iC3b was found adsorbed/bound on TentaGel beads (see Chapter 5). Leukocyte adhesion may also be due to specific interactions of the cell membrane with the terminal amino or hydroxyl groups. The presence of hydroxyl groups on polystyrene has been shown previously to promote leukocyte adhesion[40].

TentaGel beads were used as a PS surface modified with PEG but they also enabled us to assess indirectly the effect of platelet activation on leukocyte activation, since little platelet activation (few microparticles, little P-selectin expression) was observed with these beads. Our results indicate that leukocyte activation still occurred even in the presence of a material that minimally activates platelets. Moreover, platelet activation in the bulk did not seem to result in a significant increase in leukocyte activation since both TentaGel and PS

beads had similar levels of bulk leukocyte activation. On the other hand, the presence of platelets on the surface may be needed for monocyte adhesion and TF expression, as few activated monocytes were observed on the PS-PEG surfaces. Further experiments with isolated leukocytes and platelets are required to better characterize the role of platelets in leukocyte activation and adhesion.

Of interest was also the fact that leukocyte-platelet aggregates were present with TentaGel beads in the absence of significant platelet activation (miminal MPs formation and P-selectin expression). Platelet-leukocyte aggregates have been reported before in parallel with significant platelet activation[41;42] and therefore these aggregates are usually referred to as a platelet activation event only[43]. Our results suggest this is not always the case, and leukocyte activation may also play a role in the formation of these aggregates. The mechanisms of formation of platelet-leukocyte aggregates have been studied *in vitro* using several agonists, but conflicting results have been obtained on the role of leukocyte and platelet activation in the formation of platelet-leukocyte aggregates[18;44-47]. The mechanisms of platelet-leukocyte aggregates induced by material may also be different than that of induced by an agonist. Fewer activated platelets expressing P-selectin have been observed in blood than in platelet-rich plasma, suggesting that leukocytes rapidly bind activated platelets[48]. Thus, in our experiments with TentaGel, all activated platelets may be bound to leukocytes. However, similar levels of platelet MPs formation (also an indicator of platelet activation) were observed with and without TentaGel beads, suggesting that similar levels of platelet activation were present and thus similar levels of platelet-leukocyte aggregates should have been observed between TentaGel and the no beads sample. Two hypotheses may then be formed to explain the presence of platelet-leukocyte aggregates in the absence of significant platelet activation: (1) either little P-selectin on platelets is required to form platelet-leukocyte aggregates and the presence of TentaGel beads provide this stimulus without further activating platelets (i.e.; no formation of platelet microparticles), (2) or material-induced leukocyte activation is responsible for the formation of platelet-leukocyte aggregates observed in these experiments. Preliminary experiments with chymostatin, an inhibitor of Cathepsin G, and elastinol, an inhibitor of elastase, suggested that leukocyte release and hence leukocyte activation contributed to the formation of platelet-leukocyte aggregates (data not shown). Further research is needed to characterize the mechanisms of formation of platelet-leukocyte aggregates in the presence of a material.

3) Effect of endotoxin contamination

The presence of endotoxin is often an issue in studying leukocyte-material interactions, since leukocytes can be activated by very small amounts of endotoxin. Many have reported how LPS can prime[49] and induce leukocyte activation[50:51]. In the presence of endotoxin-contaminated beads, significant increases in leukocyte activation were observed in some cases but overall contamination did not change the conclusions obtained with "endotoxin-free" beads: CD11b upregulation in the bulk, platelet-leukocyte aggregates, and leukocyte adhesion were surface-area dependent while TF expression was not, regardless of endotoxin contamination. Others have also found that the presence of environmental endotoxins (i.e; not a purified version of LPS which is usually what had been used in studies on the effects of endotoxin on leukocytes) did not significantly affect leukocyte activation induced by *in vitro* hemodialysis[52]. It is actually known that environmental endotoxins are not as potent as purified ones[53]. Thus, while it is true that endotoxin contamination should be avoided where possible, its presence appears to only enhance the extent of material-induced activation, rather than change its qualitative character.

4) Potential procoagulant activities of material-induced leukocyte activation

In this study, leukocytes activated by contact with a material expressed features that may result in enhanced coagulation. The expression of TF on monocytes is an unequivocal procoagulant activity since TF initiates the extrinsic pathway of coagulation by binding Factor VII/VIIa. *In vivo* studies have also demonstrated that TF expression contributed to the activation of the coagulation cascade during cardiopulmonary bypass[5] and in patients with ventricular assist devices[54]. The significant increase of TF expression observed on monocytes adherent to PS beads (but not in the bulk) agrees with previous studies[31:32] showing higher levels of procoagulant activities on adherent monocytes. The presence of adherent monocytes has been reported to contribute locally to the initiation and propagation of fibrin deposition: in a baboon-shunt model, blocking leukocyte accumulation on adherent platelets inhibited the deposition of fibrin in a mechanism thought to be dependent on TF expression[11]. Using a one-stage clotting assay (PTT test), we were able to show that material-induced TF expression on monocytes resulted in fibrin formation. These results supports our hypothesis that material-activated monocytes enhance coagulation.

While TF is the most potent trigger of coagulation by leukocytes, CD11b is an additional receptor for the procoagulant proteins, Factor X, fibrinogen and fibrin, thereby favoring prothrombinase assembly on the leukocyte membrane. CD11b, when activated, can also initiate direct activation of Factor X, and both neutrophils and monocytes were demonstrated to possess such activity[55]. CD11b upregulation following material contact may then also potentially contribute to thrombin formation. However, ICAM-1 and iC3b are also ligands of CD11b and thus competitive interactions for CD11b *in vivo* may modulate this procoagulant activity. Peptides blocking CD11b have been shown to inhibit direct Factor X activation by circulating monocytes during cardiopulmonary bypass suggesting that direct Factor X activation is a significant component of the monocyte procoagulant activity[56]. The chromogenic test for Factor Xa generation showed that material-activated leukocytes appeared to be able to directly activate Factor X. However, as seen in Parrat study[56], generation of Factor Xa was highly variable. Further work is required to improve this assay and then better characterization of the procoagulant activity of material-induced CD11b upregulation can ensue.

The mechanisms of formation of platelet-leukocyte aggregates have been well characterized[18,45,57], although the functional consequences of these aggregates remains uncertain. Their procoagulant activities may be high since such an association creates a microenvironment whereby both platelet and leukocyte activation can occur in a manner that is protected from inhibitory factors in plasma. The increased presence of platelet-leukocyte aggregates induced by material contact may then provide additional means for leukocytes to express procoagulant activity.

V. Conclusion

Following contact with PS and TentaGel beads, leukocytes were activated resulting in the expression of TF, CD11b upregulation, and association with platelets. These features are recognized to be directly (TF) or indirectly involved in procoagulant activity and confirm our hypothesis that leukocytes may play a role in cardiovascular failure by contributing to thrombin formation.

As illustrated in Figure 12, the presence of an artificial surface in blood resulted in activation of bulk leukocytes with the expression of potential procoagulant activities such as TF expression, CD11b upregulation and association with platelets. None of these markers of

bulk leukocyte activation appeared to be dependent on surface chemistry, per se, suggesting that merely the presence of a foreign surface is a sufficient stimulus for leukocyte activation and expression of procoagulant activities in the bulk. Material-induced leukocyte activation in the bulk also appeared to occur independently of platelet activation. On the other hand, surface chemistry seemed to play a role in activation of adherent leukocytes, since little leukocyte activation occurred on PS-PEG-NH_2. Moreover, fewer platelets as well as monocytes adhered to the PEG-surfaces, suggesting that the presence of platelets on the surface has an effect on adhesion and/or activation of monocytes.

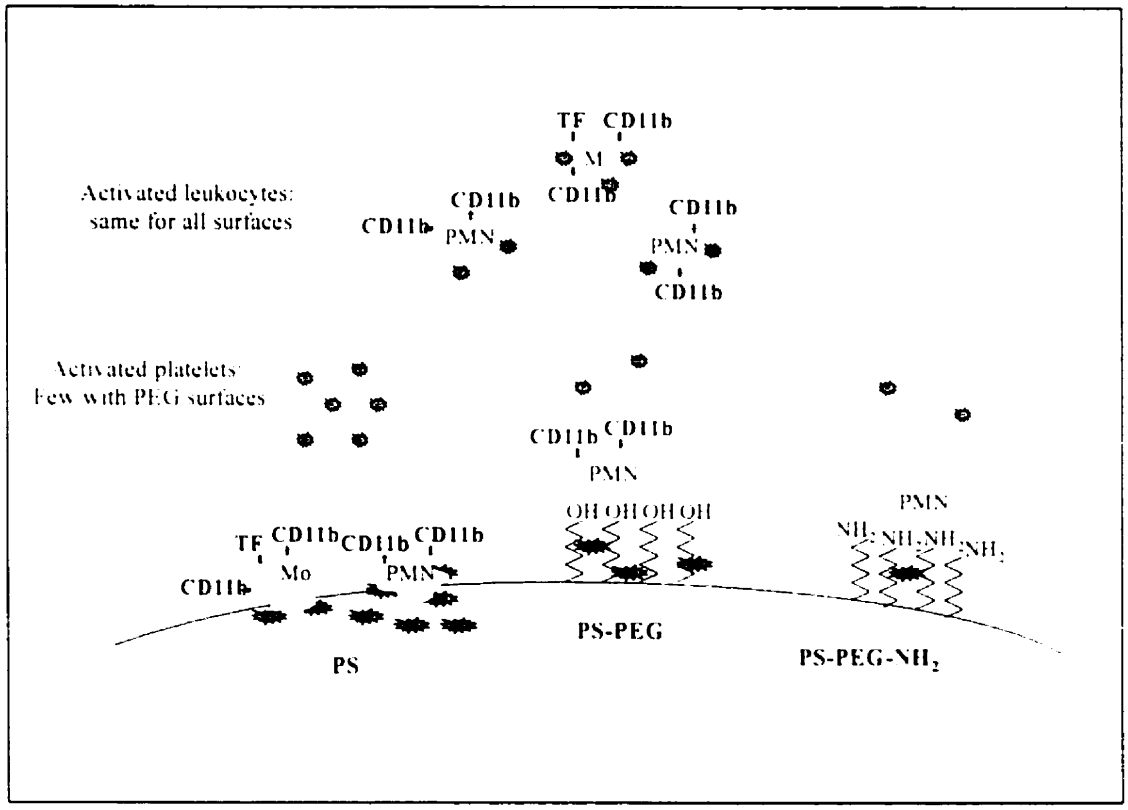

Figure 12: Material-induced leukocyte activation with polystyrene (PS) and PEG grafted polystyrene beads. Blood contact with all three bead surfaces resulted in significant leukocyte activation (TF expression and CD11b upregulation). Similar levels of platelet-leukocyte aggregates were also observed for the three different surfaces. Contact with PS beads generated many activated platelets while platelet activation remained minimal with PS-PEG and PS-PEG-NH_2. Neutrophil (PMN) adhesion occurred on all beads. However monocyte adhesion and TF expression appeared linked to the presence of adherent platelets on the beads. Leukocytes adherent to PS-PEG-NH_2 were also less activated (lower CD11b).

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Chapter 4

Role of platelets in material-induced leukocyte activation: studies with isolated cells

Abstract

Material-induced TF expression by leukocytes but not CD11b upregulation required the presence of platelets. Using isolated leukocytes resuspended in increasing concentrations of platelets in plasma (1, 12, 25, 50, 100 $x10^6$ platelets/mL), expression of TF on both adherent and non-adherent monocytes remained at background levels until $50x10^6$ platelets/mL. On the other hand, significant CD11b upregulation was observed on leukocytes in bulk and on beads regardless of platelet concentrations. The platelet dependent effect of material-induced TF expression appeared to be mediated by the formation of platelet-monocyte aggregates. Anti-P-selectin, which blocked the association between platelets and leukocytes, significantly reduced (but not entirely) material-induced TF expression. Anti-GPIIb/IIIa, a GPIIb/IIIa platelet antagonist, also partially reduced material-induced TF expression, most likely due to its inhibiting effect on the formation of platelet-monocyte aggregates. Indeed, platelet activation did not seem to play a significant role in material-induced TF expression, since similar levels of TF expression were observed with a strong (PS) and a mild (PS-PEG, PS-PEG-NH_2) platelet activator. Unlike neutrophil adhesion, monocyte adhesion and subsequent TF expression was also strongly dependent on adherent platelets in a mechanism that involved P-selectin.

The importance of platelets in material-induced TF expression appears to be mediated via the formation of platelet-monocyte aggregates. However, blocking formation of these aggregates did not entirely block material-induced TF expression, suggesting that other mechanisms, such as maybe complement activation, were involved in regulating TF expression on monocytes.

I. Introduction

Blood-material interactions are complex: blood cells (platelets, leukocytes, and red blood cells) and plasma proteins (complement, coagulation and fibrinolytic cascade) interact together to form the thrombotic and inflammatory response to the material. Despite extensive research in surface modification, there are still no truly biocompatible cardiovascular materials. Thrombotic complications continue to occur with their use, and the administration of anti-platelet therapy has not been able to solve all problems[1]. We have hypothesized that leukocyte activation, by the expression of procoagulant activities, may contribute to thrombin generation. While leukocytes are recognized for their role in inflammation, their ability to express procoagulant activities upon activation is often ignored. The thrombotic complications associated with leukocyte inflammatory disorders, such as sepsis[2], underscore the role that leukocytes may play in thrombin generation[3]. Tissue factor (TF) expression, which activates the extrinsic pathway of the coagulation cascade; CD11b upregulation, with Factor X and fibrinogen being two of CD11b ligands involved in the coagulation cascade; and exposure of phosphatidylserine, which favours the assembly of the prothrombinase complex, are all membrane-associated procoagulant activities that are present upon leukocyte activation. Leukocytes also release inflammatory mediators, such as platelet activating factor (PAF), Cathepsin G and elastase, which have been recognized for their procoagulant activities[3]. Furthermore, platelet-leukocyte aggregates[4] and leukocyte microparticles[2] have procoagulant properties.

Following contact with polystyrene and PEG-immobilized polystyrene beads, leukocytes in blood were found to express TF, upregulate CD11b, form platelet-leukocyte aggregates and release microparticles (Chapter 3). All these markers of leukocyte activation are known to potentially contribute to a procoagulant state. Despite minimal platelet activation with the PEG-immobilized PS beads, similar levels of leukocyte activation (CD11b, TF, association with platelets) were present in the bulk. On the other hand, these same beads had few adherent monocytes and little TF (correcting for the low adhesion) was observed on these beads. The latter results suggested that platelets were involved in monocyte adhesion and subsequent TF expression (at least for adherent cells). The objective of this study was to characterize the role of platelets in material-induced leukocyte activation. A role for platelets in LPS-induced TF expression was first reported in the seventy's[5] and later studies indicated that the effect of platelets was mediated by platelet-

leukocyte aggregates[6;7]. While TF expression has been reported following material contact[8-12], the mechanisms of TF expression on monocytes remains to be elucidated. Whether the role of platelets in material-induced TF expression is similar to what occurs with LPS-induced TF expression is yet unknown. Little is known also on the effect of platelets on CD11b upregulation and leukocyte adhesion in the presence of a material. Most studies on CD11b and leukocyte adhesion have focussed on the role of complement. We hypothesized that platelets contributed to material-induced TF expression.

In this chapter, the role of platelets on material-induced leukocyte activation and adhesion was assessed in heparinized plasma. Isolated mixed leukocytes were incubated with increasing concentration of platelets to characterize how the presence and number of platelets affected material-induced leukocyte activation. To further determine how platelets were playing a role in leukocyte activation, the effect of two platelet antibodies was studied in the mixed cells *in vitro* system: anti-IIb/IIIa, a GPIIb/IIIa platelet receptor antagonist, and anti-P-selectin, which inhibits the interaction between platelets and leukocytes. Figure 1 illustrates their mechanisms of action on platelets. PS and PEG-immobilized beads were used as activating agonists.

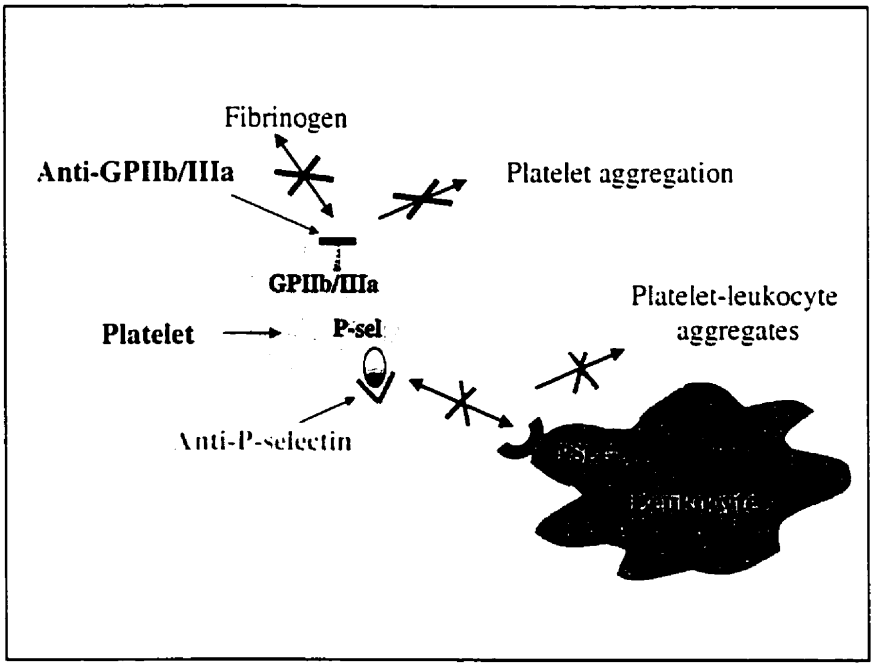

Figure 1: Platelet inhibitors. The monoclonal antibody anti-GPIIb/IIIa binds to the fibrinogen receptor on platelets GPIIb/IIIa and prevents platelet aggregation. Anti-P-selectin binds to P-selectin, expressed on activated platelets, and blocks the association between platelets and leukocytes.

II. Materials and methods

1) Reagents and antibodies

The same reagents and fluorescently labelled antibodies as in Chapter 3 were used, except for the R-phycoerythrin-cytochrome 5 anti-CD45, which was purchased from Immunotech-Coulter, Marseilles, France.

Two monoclonal antibodies against platelet receptors GPIIb/IIIa and P-selectin were used. Anti-IIb/IIIa, chimeric clone 7E3, was a generous gift from Barry Coller (Mount Sinai Hospital, New York). Anti-P-selectin antibody, clone WAPS 12.2, was purchased form Zymed laboratories, San Francisco, CA.

2) Beads

As in Chapter 3, 45μm beads were used: polystyrene (PS) and TentaGel beads, polystyrene beads immobilized with polyethylene glycol with a hydroxyl (PS-PEG) or amino (PS-PEG-NH_2) functional group. They were cleaned from endotoxin contamination as described previously (Chapter 3), and resuspended in endotoxin free PBS. In all experiments, the residual endotoxin on the beads was equivalent to a concentration of endotoxin contamination that was less than 0.1 EU/ml, which is below the recommended maximum FDA level[13].

3) Leukocyte isolation

Whole blood from normal human volunteers, who were medication-free for the last 72 hours, was drawn into syringes preloaded with anticoagulant (5 Units/mL heparin) after discarding the first millilitre. Platelet-rich plasma was removed after a centrifugation at 100g for 10 minutes, and the buffy coat was diluted (1:1) in RPMI + 10% heat inactivated foetal bovine serum (RPMI/FBS) with EDTA (5 mM final concentration). 20 mL of blood cells was applied on top of a two-layer density gradient consisting of 10 mL of FicollPaque and 10 mL of Polymorphprep[14]. After centrifugation at 510g for 25 minutes, mononuclear cells and neutrophils appeared as two separate bands. The separated cells were washed at low speed (100g) to remove platelets. Provided platelet contamination was less than $1x10^6$/mL, leukocytes were resuspended together in RPMI to reach a final concentration of $5x10^6$ neutrophils/mL and $1x10^6$ monocytes/mL when 25% platelet-poor or platelet-rich plasma was added. Cell viability as determined by trypan blue was greater than 98%.

4) Heparinized plasma

Autologous plasma was prepared from the platelet-rich plasma (PRP) by centrifugation for 20 minutes at 1000g. Platelet-poor plasma (PPP) was then removed and ultracentrifuged for 3 minutes. The plasma was set aside at room temperature[1] during the leukocyte isolation procedure (approximately 3 hours).

5) Leukocyte-material contact

Mixed leukocytes, resuspended in platelet-poor plasma or platelet-rich plasma (25% final concentration), were incubated with only one concentration of beads ($20x10^4$ beads/mL – equivalent to a surface area to volume ratio of $13cm^{-1}$) for 2 hours at 37°C on a haematology mixer. Prior to adding the leukocytes, the platelet-rich plasma concentration was adjusted to $400x10^6$ platelets/mL. The different platelet concentrations (25, 50, 75 $x10^6$ platelets/mL) were obtained by diluting platelet-rich plasma with the appropriate amount of platelet-poor plasma.

Experiments of mixed leukocytes with platelets and platelet antibodies (anti-IIb/IIIa or anti-P-selectin) were performed only at the highest platelet concentration ($100x10^6$ platelets/mL). Saturating concentration of anti-IIb/IIIa (25 µg/mL) or anti-P selectin (20 µg/mL) was added to the leukocytes immediately before the PRP. Anti-IIb/IIIa saturating concentration had been determined previously by Gemmell (personal communication, 1999). For anti-P-selectin, the same saturating concentration as the one in Evangelista study[15] was used, after verifying its total blocking effect on the formation of platelet-leukocyte aggregates in whole blood. To confirm that the presence of the few contaminating platelets in the platelet-poor plasma sample did not significantly affect leukocyte activation, anti-IIb/IIIa (25 µg/mL) was also added to the platelet-poor plasma samples.

For the negative control, EDTA (8 mM final concentration) was added to leukocytes and beads, while the positive control was leukocytes incubated with endotoxin (5 µg/mL equivalent to 25000 EU/mL) without beads.

Following incubation, beads were allowed to settle and aliquots were taken for flow cytometry analysis of non-adherent cells and for a cell count using CellDyne 1600 (Abbott

[1] In this case, storage at room temperature is better, as too many temperature changes have been shown to activate complement[66].

Laboratories, IL). To analyse adherent cells on beads, the cell supernatant was removed and beads were washed once with RPMI/FBS and then incubated with the appropriate antibodies.

6) Flow cytometry

Flow cytometric analysis was carried out on leukocyte suspension and beads as before. Briefly, small aliquots (30 µL) of leukocytes or beads, diluted in 50µL of HTB were incubated with saturating concentrations of fluorescently labelled monoclonal antibodies for 30 minutes at 4°C. After a 30-minute incubation, samples were diluted and fixed with paraformaldehyde (1% final concentration). Samples were analysed on a Becton Dickinson FACScan flow cytometer (Mountain View, CA), using CELLQuest software.

7) Statistical analysis

All results are reported as means ± SD. To evaluate the significance of the differences in cell activation, analysis of variance (ANOVA) was carried out followed by the Student-Newman-Keuls test, which is a protected test for multiple comparisons[16]. A p-value of less than 0.05 was required for statistical significance. The number of repeat experiments was equal to or greater than three with three different donors.

III. Results

1) Effect of platelet concentration on leukocyte activation

At first, only PS beads were used. Table 1 shows the effect of platelet concentration on leukocyte adhesion (CD45 and CD14 signals) and the level of activation of adherent leukocytes (TF and CD11b signals) on PS beads. Platelet adhesion (CD41) is also reported in Table 1 and shows that the higher the platelet concentration, the higher the number of adherent platelets on PS beads. Monocyte adhesion (CD14) significantly increased with platelet concentration while neutrophils (CD45)[11] adhered to PS beads independently of the various platelet concentrations (or of the number of platelets adherent to the beads). As seen in Table 1, TF expression on adherent monocytes was dependent on the presence of platelets: significant TF expression on beads was only observed when significant platelet adhesion

[11] CD45 is present on both neutrophils and monocytes. During our experiments, monocyte concentration was set to be 20% that of neutrophils, and CD45 signals on beads is interpreted to be an indirect measure of neutrophil adhesion.

occurred (at 50 and 100x10^6 platelets/mL). On the other hand, CD11b upregulation on beads was not significantly affected by platelet concentration.

Table 1: Effect of platelet concentration on leukocyte adhesion and activation on PS beads incubated with isolated leukocytes in platelet-rich plasma.

Platelet concentration (x10^6 plts/mL)	Platelet adhesion (CD41, %)[a]	Leukocyte adhesion (CD45, %)[a]	Monocyte adhesion (CD14, %)[a]	TF on beads (%)[a]	CD11b on beads (%)[a]
PS + EDTA	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
< 1	91 ± 11 #	152 ± 22 *	139 ± 19 #	108 ± 4 #	178 ± 32 *
12	107 ± 10 #	137 ± 20	148 ± 23 #	99 ± 14 #	185 ± 18 *
25	121 ± 11 #	137 ± 18	150 ± 23 #	102 ± 6 #	208 ± 65 *
50	181 ± 40 *#	173 ± 30 *	159 ± 22 #	128 ± 11 *	261 ± 85 *
100	232 ± 40 *	164 ± 53 *	226 ± 73 *	135 ± 14 *	245 ± 40 *

N = 3 to 5 ± S.D.

[a] TF, CD14, CD45, CD11b and CD41 signals on beads are represented as a percentage relative to PS+EDTA

* Significantly different from PS + EDTA ($p \leq 0.05$)

Significantly different from 100x10^6 platelets/mL ($p < 0.05$)

Figure 2 and Table 2 show the effect of platelet concentration on leukocyte activation in the bulk: TF expression and CD11b upregulation (Figure 2) and formation of platelet-leukocyte aggregates (Table 2). As represented in Figure 2, only background TF expression was present on monocytes resuspended in platelet poor plasma (≤ 1x10^6 platelets/mL). As the concentration of platelets increased, TF expression and the number of monocytes expressing TF increased to reach a significant level at 50x10^6 platelets/mL. On the other hand, CD11b was significantly upregulated on bulk leukocytes independently of platelet concentration. It is also important to note in Figure 2 that the level of activation of leukocytes without beads incubated with platelet-poor or platelet-rich plasma was similar.

Table 2 shows that formation of platelet-leukocyte aggregates was directly related to platelet concentration. Higher levels of CD41 were usually observed on monocytes, suggesting that more platelets were bound to monocytes or that the platelets adherent to

monocytes were more activated. Table 2 also reports platelet activation: similar levels of platelet microparticle formation were observed at all platelet concentrations.

The only two parameters of material-induced leukocyte activation in the bulk to vary with platelet concentration were TF expression and platelet-leukocyte aggregates. As seen in Figure 3, a positive correlation between platelet-monocyte aggregates and TF expression on monocytes was actually observed (Pearson correlation: $R = 0.64$, $p < 0.001$), suggesting that the role of platelets in TF expression may be related to the association between platelets and monocytes.

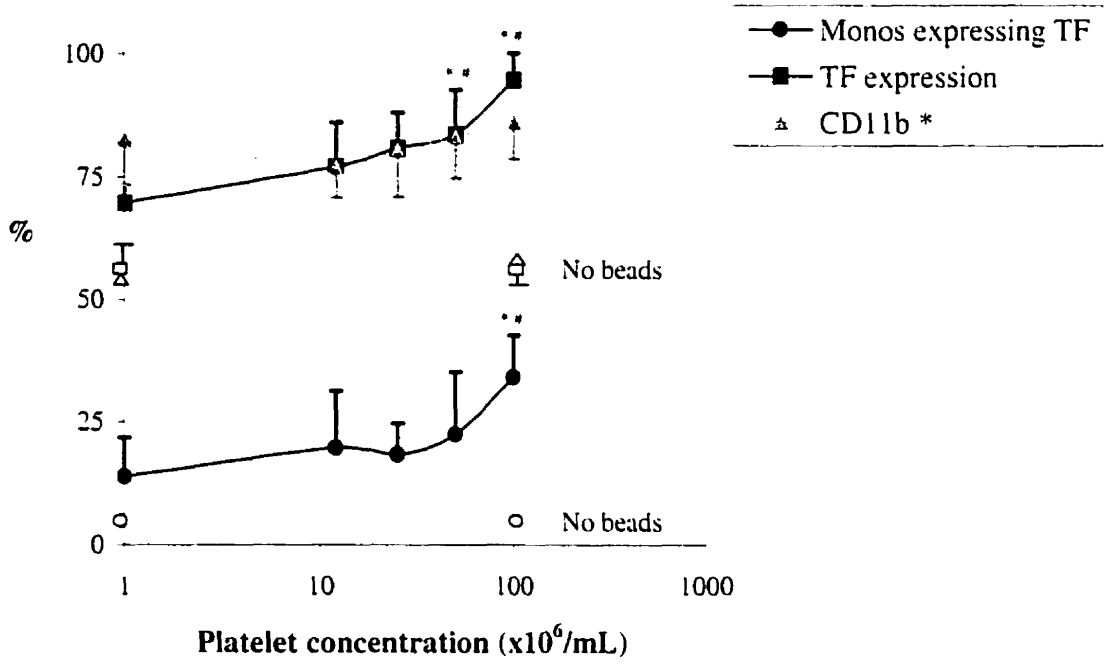

Figure 2: Effect of platelet concentration on leukocyte activation in the bulk induced by PS beads. Material-induced TF expression on monocytes but not leukocyte CD11b upregulation was dependent on the presence of platelets. TF expression and CD11b upregulation are represented as percentage relative to the maximum (determined by the fluorescent intensity of leukocytes incubated with LPS). * Significantly different from the no beads samples. # Significantly different from $1x10^6$ platelets/mL. ($p < 0.04$) N = 4 to 6 ± S.D.

Table 2: Effect of platelet concentration on the formation of platelet-leukocyte aggregates and platelet microparticles induced by PS beads with isolated leukocytes in PRP.

Platelet concentration ($x10^6$ plts/mL)	Neutrophil-Platelet Aggregates (FL units)[a]	Monocyte-Platelet aggregates (FL units)[a]	Platelet microparticles (%)
No beads	24 ± 13 #	30 ± 14 #	6 ± 2 #
< 1	29 ± 10 #	32 ± 4 #	Not applicable
12	45 ± 16	52 ± 9 #	30 ± 8
25	45 ± 18	73 ± 21 #	22 ± 5
50	58 ± 20	108 ± 56 #	22 ± 4
100	89 ± 32 •	176 ± 91	23 ± 8

N = 4 to 6 ± SD

[a] Arbitrary fluorescent CD41 intensity on neutrophils and monocytes

Significantly different from $100x10^6$ platelets/mL ($p < 0.05$)

• Significantly different from monocyte-platelet aggregates ($p < 0.05$)

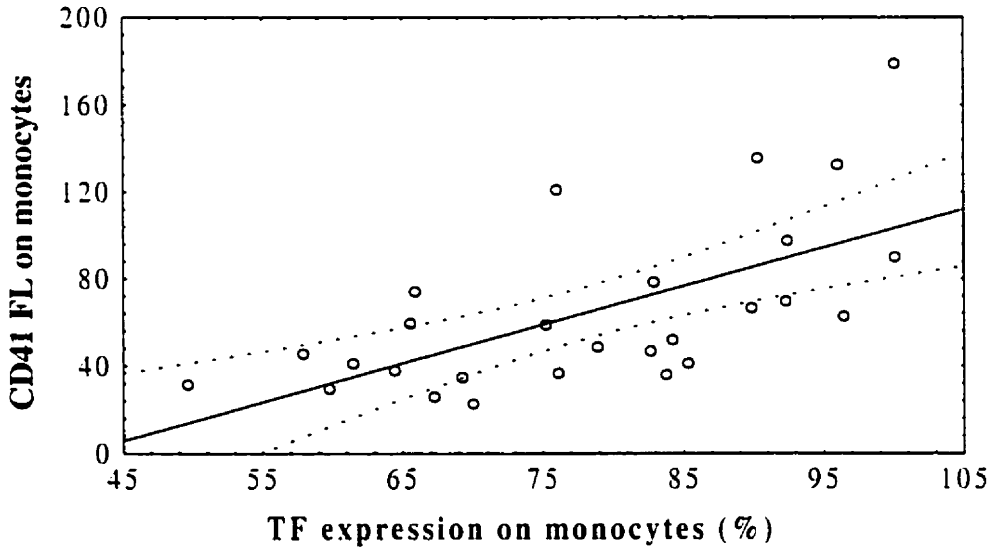

Figure 3: Correlation graph between TF expression on monocytes and monocyte-platelet aggregates (CD41 signal on monocytes) after incubation of PS beads with isolated leukocytes and platelets in plasma. (Pearson R =0.61; $p \leq 0.001$; N = 28)

2) Effect of anti-IIb/IIIa and anti-P-selectin on leukocyte activation induced by PS

The presence of platelets was found to have an effect on TF expression on monocytes in the bulk and adherent to PS beads but not on CD11b upregulation. To identify the mechanisms by which platelets were involved in TF expression, the platelet antibodies, anti-IIb/IIIa and anti-P-selectin, were used. Mixed leukocytes were incubated with platelet-rich plasma (final concentration: $100x10^6$ platelets/mL).

First, the effects of anti-IIb/IIIa and anti-P selectin on platelet activation and adhesion are presented in Table 3. Then, the effects of anti-IIb/IIIa and anti-P-selectin on leukocyte adhesion are shown in Figure 4 (monocyte adhesion and TF) and the first two columns of Table 4 (total leukocyte adhesion and CD11b). The antibodies effects on leukocyte activation in the bulk are in Figure 5 (TF) and Table 4 (CD11b, platelet-leukocyte aggregates).

As shown in Table 3, anti-IIb/IIIa totally blocked material-induced platelet activation (platelet adhesion and platelet activation in the bulk). On the other hand, in the presence of anti-P-selectin[iii], significant platelet adhesion and platelet activation were still observed.

Table 3: Effect of platelet inhibitors on adhesion and activation of platelets from PRP incubated with PS beads and isolated leukocytes.

	On beads	In bulk	
Conditions	**Platelet adhesion (CD41, %)[a]**	**Platelet microparticles (%)**	**P-selectin expression on platelets (%)[b]**
No beads	Not applicable	6 ± 2	100 ± 0
Beads + EDTA	100 ± 0	7 ± 2	Not measured
PS + PRP	250 ± 48 *	20 ± 7 *	161 ± 25 *
+ anti IIb/IIIa	108 ± 14 #	6 ± 1 #	99 ± 21 #
+ anti P-selectin	189 ± 39 *#	22 ± 9 *	135 ± 12 *
PS + PPP + anti -IIb/IIIa	88 ± 11 #	Not applicable	Not applicable

N = 4 to 6 ± S.D.

[a] Represented as a percentage relative to beads+EDTA (CD41) or to no beads (P-selectin).

* Significantly different from beads + EDTA ($p < 0.05$)

Significantly different from PS+PRP ($p < 0.05$)

[iii] Anti-P-selectin blocks the formation of platelet-leukocyte aggregates but not the expression of P-selectin by platelets, unless the expression is measured by flow cytometry using the same clone as the blocking antibody.

As shown in Figure 4, adding anti-IIb/IIIa or anti-P-selectin to platelet-rich plasma significantly reduced monocyte adhesion, suggesting that adherent platelets and/or monocyte-platelet interactions played a role in monocyte adhesion. The reduced monocyte adhesion observed in the presence of anti-IIb/IIIa or anti-P-selectin resulted in background level of TF expression on the beads (Figure 4). On the other hand, similar levels of total leukocyte (neutrophil) adhesion were observed regardless of platelet conditions (CD45, Table 4). As for CD11b on beads (Table 4), significant levels of CD11b were observed with anti-IIb/IIIa both with platelet-rich and platelet-poor plasma. However, the presence of anti-P-selectin in PRP significantly reduced (but not entirely) CD11b on beads.

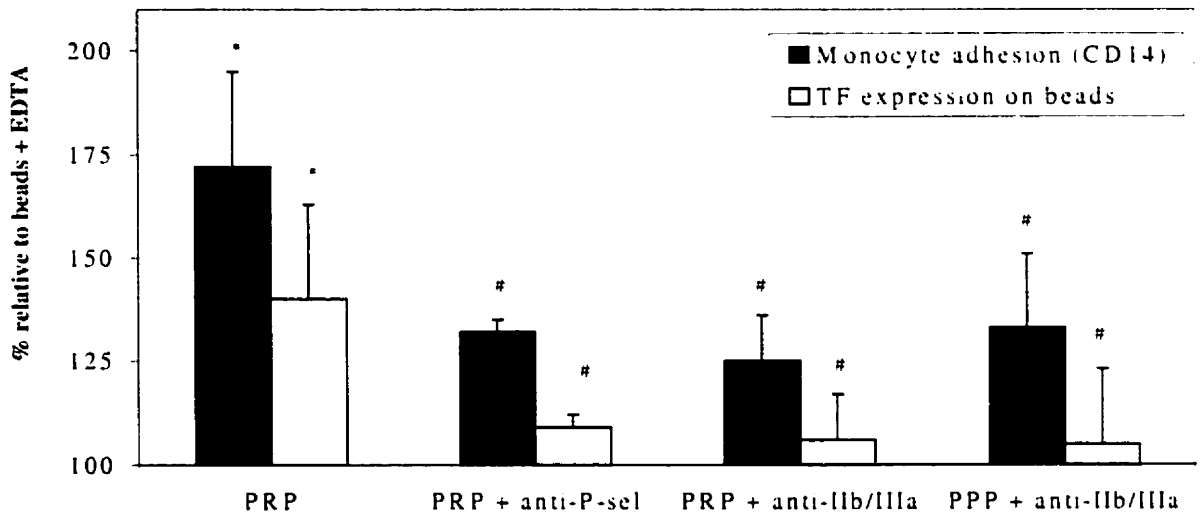

Figure 4: Effects of anti-IIb/IIIa and anti-P-selectin on monocyte adhesion and TF expression on PS beads with isolated leukocytes. The presence of anti-IIb/IIIa in PRP significantly reduced monocyte adhesion and hence TF expression on beads. CD14 and TF are expressed as a percentage relative to beads+EDTA. * Significantly different from PS+EDTA ($p < 0.05$). # Significantly different from PS+PRP ($p < 0.05$). N = 3 to 6 ± S.D.

In the bulk, as seen in Figure 5, in the presence of anti-IIb/IIIa or anti-P selectin, both TF expression and the number of monocytes expressing TF were significantly (but not entirely) reduced. On the other hand, as seen before, significant CD11b upregulation on bulk leukocytes was observed independent of platelet conditions (Table 4). As expected, anti-P selectin blocked platelet-leukocyte interactions (levels similar to no beads and PPP + anti-IIb/IIIa samples) (Table 4). Anti-IIb/IIIa also significantly reduced (but not entirely) platelet-leukocyte interactions.

Table 4: Effect of platelet inhibitors on leukocyte adhesion and activation induced by PS beads with isolated leukocytes in platelet-rich plasma.

	On beads		In bulk	
Conditions	**Leukocyte adhesion (CD45, %)[a]**	**CD11b on beads (%)[a]**	**CD11b upregulation (%)[b]**	**Platelet-leukocyte aggregates (FL units)**
No beads	N/A	N/A	55 ± 11	31 ± 7
PS + EDTA	100 ± 0	100 ± 0	16 ± 2	37 ± 11
PS + PRP	149 ± 31 *	297 ± 123 *	85 ± 5 *	87 ± 34 *
+ anti-IIb/IIIa	145 ± 26 *	270 ± 72 *	85 ± 8 *	51 ± 10 #
+ anti-P-selectin	128 ± 21 *	177 ± 13 #	83 ± 9 *	36 ± 7 #
PS + PPP + anti-IIb/IIIa	132 ± 17 *	213 ± 81 *	85 ± 8 *	35 ± 14 #

N = 3 to 6 ± S.D.

[a] Represented as a percentage relative to beads+EDTA

[b] CD11b upregulation is represented as percentage relative to the maximum (maximum expression determined by the fluorescent intensity of leukocytes incubated with LPS).

* Significantly different from PS+EDTA or no beads ($p < 0.05$)

Significantly different from PS+PRP ($p < 0.05$)

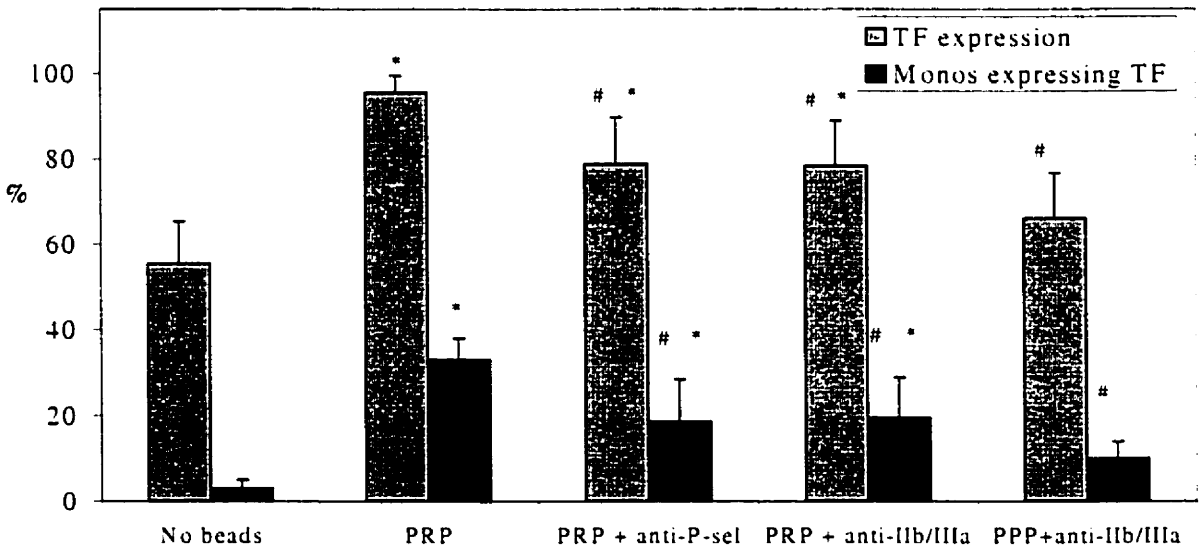

Figure 5: Effects of anti-IIb/IIIa and anti-P-selectin on TF expression in the bulk with isolated leukocytes and PS beads. In PRP, both anti-IIb/IIIa and anti-P-selectin reduced PS-induced TF expression. Legend is explained in Figure 2. * Significantly different from no beads ($p < 0.05$) # Significantly different from PS+PRP ($p < 0.05$). N = 3 to 6 ± S.D

3) Effect of platelets on leukocyte activation induced by PEG- immobilized PS beads

To ensure that our results on the effect of platelets on material-induced leukocyte activation were not specific to PS beads, some experiments were performed with PEG-immobilized PS beads. Although given the low platelet adhesion/activation, significant differences were not expected.

Table 5 (first four columns) shows the effect of platelets on leukocyte adhesion and activation of adherent leukocytes. As expected, since PS-PEG beads support minimal platelet adhesion, minimal monocyte adhesion and hence minimal TF expression was observed on the beads. These results further demonstrate the role of platelets in monocyte adhesion. As with PS beads, CD11b on PS-PEG and PS-PEG-NH_2 beads was similar with and without platelets, while anti-P-selectin in PRP reduced CD11b on beads. On the other hand, while anti-IIb/IIIa had no effect on CD11b on PS beads, its presence in PRP reduced CD11b on PS-PEG beads. In accordance with our results in blood (Chapter 3), lower levels of CD11b were observed with PS-PEG-NH_2 when compared to PS-PEG or PS beads.

Table 5 also shows the effect of platelets on TF expression on monocytes and CD11b upregulation in the bulk (last two columns). In platelet-rich plasma, significant TF expression was observed with both PS-PEG and PS-PEG-NH_2. As with PS beads, the presence of anti-P-selectin or anti-IIb/IIIa (to a lesser extent) reduced TF expression induced by both PS-PEG and PS-PEG-NH_2 beads, while they had no effect on CD11b upregulation. In platelet-poor plasma, a background degree of TF expression was observed, while CD11b was significantly upregulated.

Table 5: Effect of platelets and platelet inhibitors on leukocyte activation (adherent and bulk) induced by PS-PEG and PS-PEG-NH_2 beads in isolated leukocytes in platelet-rich plasma.

		On beads				In bulk	
Type of beads	**Conditions**	**Platelet (CD41, %)[a]**	**Monocyte (CD14, %)[a]**	**TF (%)[a]**	**CD11b (%)[a]**	**TF expression (%)[b]**	**CD11b upregulation (%)[b]**
	No beads	N/A	N/A	N/A	N/A	52 ± 3 #	58 ± 8
PS-PEG	PRP	125 ± 11	122 ± 18	111 ± 10	352 ± 90 *	90 ± 10 *	86 ± 9 #
	+ anti IIb/IIIa	114 ± 3	102 ± 6	108 ± 12	228 ± 66	82 ± 14 *	88 ±9 #
	+ anti P-selectin[c]	126 ± 15	115	114 ± 10	215 ± 50	75 ± 3	82 ± 1 #
	PPP	108 ± 3	111 ± 4	103 ± 14	272 ± 80	60 ± 2 #	82 ± 7 #
PS-PEG-NH_2	PRP[c]	98 ± 8	101 ± 4	97 ± 5	165 ± 28	88 ± 8	88 ± 9
	+ anti IIb/IIIa[c]	100 ± 3	102 ± 0	95 ± 2	139 ± 0	78	86 ± 1
	+ anti P-selectin[c]	101 ± 1	100 ± 0	94 ± 9	123 ± 0	75 ± 6	84 ± 10
	PPP[c]	88 ± 9	102 ± 7	96 ± 4	152 ± 8	63 ± 6	87 ± 7

N = 3 ± SD, [c] N = 2

[a] CD14, CD41, TF and CD11b signals on beads are represented as a percentage relative to beads+EDTA

[b] TF expression on monocytes and CD11b upregulation on leukocytes in the bulk are represented as a percentage of maximal stimulation (LPS for 2 hours at 37°C)

* Significantly different from beads + EDTA or no beads as appropriate ($p < 0.05$)

Significantly different from PRP conditions ($p < 0.05$)

IV. Discussion

1) *In vitro* model with isolated mixed leukocytes

Working with isolated leukocytes offers valuable advantages over whole blood when studying leukocyte activation influenced by platelets or plasma proteins. However, the preparation of isolated leukocytes may introduce artefacts that affect the outcome of the study. For example, the high levels of TF expression and CD11b upregulation on leukocytes without beads (our unstimulated sample) are most likely an unavoidable consequence of the isolation procedure. It is a well known fact that most isolation procedures prime leukocytes and upregulate leukocyte membrane receptors, such as CD11b[17;18]. However under controlled and well-chosen conditions, the deleterious effects of isolation may be minimized.

All isolation steps were carried out in polypropylene tubes at room temperature. While 4°C may intuitively appear to be an appropriate temperature for leukocyte isolation to prevent activation, leukocytes cooled during the isolation procedure significantly upregulated CD11b upon re-warming at 37°C[19;20]. Such an increase was not observed when leukocytes were prepared at room temperature[20;21]. Exposure of neutrophils to cold temperature over long period of time, such as the time required to isolate leukocytes, also impaired neutrophil function such as chemotaxis and locomotion[22], further confirming that cooling leukocytes should be avoided.

The leukocyte separation medium also affects leukocyte activation. One step separation process using a two-layer gradient was chosen, instead of a two-step procedure involving dextran-sedimentation, for its good monocyte recovery[23] and to prevent the activation seen with dextran sedimentation[17]. Red blood cell contamination of the mixed leukocyte suspension was below the recommended level of 20%[24]. Thus hypotonic erythrocyte lysis was avoided as it is also known to activate leukocytes[18]. However, in our study, platelet contamination was an issue. To remove contaminating platelets, several washes at low speed were performed. Many have reported the activation effect of washing leukocytes after isolation, especially on CD11b upregulation[18;25]. Since CD11b upregulation requires calcium[26], to minimize leukocyte activation during the washing procedure, EDTA was added during all the washing steps. Finally, as endotoxin is the bane of leukocyte scientists, tubes and reagents were routinely checked for endotoxin contamination, to ensure that following isolation, resting leukocytes would be obtained.

As platelets are smaller and more numerous compared to leukocytes, obtaining a sample free of platelets is not realistic goal. However, by performing several low speed washes, most contaminating platelets were eliminated to obtain a platelet concentration equal to or below $1x10^6$ platelets/mL. This is equivalent to 1 platelet or less for 6 leukocytes (monocytes + neutrophils). With leukocytes in platelet-poor plasma, all CD41 (platelet marker) levels on leukocytes or beads were similar to the EDTA samples. Furthermore, the presence of the few contaminating platelets in the platelet-poor plasma samples did not appear to have any significant effect on leukocyte activation or adhesion, since similar results were obtained when anti-IIb/IIIa was added to platelet-poor plasma.

To assess the effect of platelets on leukocyte activation, platelet-rich plasma but not washed or gel-filtered platelets were used. Platelet activation and ultrastructural alterations have been reported to occur upon platelet isolation[27-29]. Thus to ensure that we were studying the effect of platelets on isolated mixed leukocytes with platelets that were as close as possible to their physiological state in whole blood, platelet-rich plasma was chosen. Newer platelet washing methods, such as the ADIAGel™ platelet separation tubes, may facilitate future studies. While less thrombin specific, heparin was preferred as the anticoagulant for platelet-rich plasma, since it is more stable than PPACK over the long period of time required to isolate leukocytes from platelets.

2) Effect of platelets on leukocyte adhesion

Our results showed that monocyte but not neutrophil adhesion was strongly dependent on the presence of adherent platelets. This suggests that neutrophil and monocyte adhesion to artificial surfaces have different requirements. Difference in mechanisms of neutrophil and monocyte adhesion on endothelial cells have also been observed[30-33].

Little research has been done on monocyte adhesion on artificial surfaces in the presence of platelets. Nonetheless, in our study, monocytes adhered to the beads in a manner similar to that seen on damaged vessel wall: with a mechanism that was dependent on platelet coverage and mediated by P-selectin interaction[34].

Our results on neutrophil adhesion appear to disagree with some reports on neutrophil adhesion to artificial surfaces in the presence of platelets. Yeo et al. reported that, in the absence of adherent platelets, neutrophil adhesion on fibrinogen-coated surface did not occur[35]. In their study, neutrophil adhesion in the presence of platelets was also completely

abolished by anti-P-selectin. The presence of higher shear ($100s^{-1}$) and the absence of plasma proteins in the Yeo study may have contributed to these differences. In earlier work, we have seen that neutrophil adhesion to fibrinogen-coated surfaces under flow conditions was strongly dependent on the presence of plasma (unpublished work-Appendix B). Morley and Feuerstein[36] confirmed the effect of shear on neutrophil adhesion, using the same *in vitro* model as Yeo.: at low shear (below $50s^{-1}$), neutrophils adhered to fibrinogen-coated surfaces even in the absence of platelets. Bruil et al also observed that, in the presence of plasma and at low shear, neutrophil adhesion to polyurethane was similar with and without platelets[37].

In our experiments, neutrophil adhesion to artificial surfaces in the presence of plasma and platelets appear to follow trends similar to the ones observed with physiological *in vitro* models of injured vessel wall. At low shear rates, the presence of platelets on a fibrin surface did not significantly increase neutrophil adhesion[38]. However, at high shear, neutrophil adhesion to surfaces coated with platelets has been found to depend on P-selectin and/or CD11b[39-43]. Further studies are warranted to assess how shear will affect the role of platelets in neutrophil (and monocyte) adhesion to beads in our *in vitro* model.

As expected, anti-IIb/IIIa blocked platelet adhesion on beads. Anti-IIb/IIIa is an antibody against the platelet integrin receptor GPIIb/IIIa. Integrins are divided into three subfamilies, each with a common β subunit associating with a specific group of α subunits. The β subunits of all integrins have a high degree of homology. An antibody against one integrin may thus cross react with another integrin. Both 7E3 and c7E3, the latter being the anti-IIb/IIIa used in our study, have been reported to cross react with the leukocyte integrin CD11b[44;45]. These two antibodies were found to bind directly to CD11b and block leukocyte adhesion on fibrinogen and ICAM-1. While we did not directly assess anti-IIb/IIIa binding to leukocytes, in the absence of platelets (where fibrinogen-mediated leukocyte adhesion is likely to occur), leukocyte adhesion was similar with and without anti-IIb/IIIa. This suggested that the antibody did not interact with leukocytes. One may argue that iC3b on the surface may have mediated leukocyte adhesion, even if fibrinogen binding was blocked by anti-IIb/IIIa. However, in the presence of the complement inhibitor sCR1 (data not shown), leukocyte adhesion was also not affected by anti-IIb/IIIa, further confirming that in our experiments, anti-IIb/IIIa did not interact with the leukocyte integrin receptor CD11b. Like us, Mickelson et al also failed to show any direct binding of c7E3 on neutrophils and also observed that neutrophil adhesion to protein-coated glass was not prevented by c7E3[46]. The

two studies showing the direct interaction of anti-IIb/IIIa with CD11b were performed with cultured neutrophils[45] and monocytic cell lines[44;45] while Mickelson and we used freshly isolated leukocytes. The difference in cells most likely explains the difference in the interaction between anti-IIb/IIIa and CD11b.

Platelet microparticles are also believed to mediate leukocyte-leukocyte interactions and thus participate in neutrophil adhesion via aggregation on a surface[47;48]. However, these interactions required extremely high concentration of platelet microparticles that were not reached in our study. It is then unlikely that such a mechanism is contributing to leukocyte adhesion to the beads.

3) Effect of platelets on leukocyte activation

a) TF expression on monocytes

A role for platelets in material-induced TF expression was identified when increasing platelet concentration resulted in a significant increase of TF expression on monocytes in the bulk. TF expression and platelet-monocyte aggregates were also correlated, suggesting that the role of platelets may be via aggregate formation. However, blocking the formation of platelet-monocyte aggregates (using anti-P-selectin) significantly reduced (but not entirely) material-induced TF expression, suggesting that another pathway of activation existed. Platelet activation still occurred in the presence of anti-P-selectin and thus platelet-derived inflammatory mediators may also have contributed to TF expression. On the other hand, anti-IIb/IIIa significantly reduced platelet activation (as well as the formation of platelet-leukocyte aggregates), but was still unable to reduce TF expression to background. The inflammatory mediators released upon platelet activation appear then not to play a significant role in material-induced TF expression. This was further confirmed by the results with PEG-immobilized PS beads, which activated platelets minimally. Yet, PS-PEG still had elevated TF expression in the bulk and platelet-leukocyte aggregates. Anti-P-selectin was effective in reducing TF expression induced by all beads (PS, PS-PEG and PS-PEG-NH_2), further confirming that platelet-leukocyte interactions and not platelet activation (or more particularly platelet inflammatory mediators) were responsible for the observed platelet-dependent effect of material-induced TF expression.

A role for platelets, and more particularly platelet-leukocyte interactions, in the enhancement of LPS-induced TF expression has been previously reported[14;49]. This is not

meant to imply that material-induced TF expression is due to LPS contamination. This is highly unlikely since (1) beads and reagents were tested for endotoxin contamination routinely and (2) we have found that the low levels of environmental endotoxin present on washed beads (<0.1 EU/mL) did not induce TF expression (unpublished work). In fact, a clear distinction between LPS-induced and material-induced TF expression exists: even in the absence of platelets, LPS induces TF expression on monocytes[50], while for material-induced TF expression, platelets are required.

In our study, the presence of platelets alone without any stimulus (LPS or beads) did not result in an increase in TF expression. The role of platelets in TF expression in the absence of a stimulus appears to be an area of controversy[6,7]: some investigations show that activated platelets alone are able to induce TF expression on monocytes [51,52] while others[53,54], like us, show that platelets have a role only in the presence of a TF-inducing stimulus. It is interesting to note that the studies showing that activated platelets can induce TF expression in the absence of any other stimulus are performed using washed platelets. The others studies used platelet-rich plasma. As mentioned previously, the washing procedure most likely preactivates platelets and introduces artefacts that are not present in platelet-rich plasma. Celi et al[55] also reported that P-selectin binding alone was able to induce TF expression on monocytes. However, adherent monocytes rather than monocytes in suspension were used. Monocytes have been recognized to be stimulated to express TF upon adhesion to surfaces[56]. Thus P-selectin may have enhanced rather than induced the observed TF expression. The increase of TF induced by P-selectin alone was actually modest relative to the level induced by LPS. Indeed, at this low scale of expression, even in the absence of P-selectin, TF expression was observed. Weyrich et al[53] later contradicted these results showing that monocytes adhering to increasing concentration of immobilized P-selectin did not express increased TF activity. This further confirmed that an external stimulus and not just interaction with platelets is required for TF expression by monocytes. Whole blood studies, where the artefacts of leukocyte and platelet isolation are absent, tend to support our belief that platelet activation or P-selectin binding alone cannot induce TF expression on circulating monocytes in the absence of a stimulus such as LPS or a material. We have found that, in the absence of beads or LPS in whole blood, activating platelets with the thrombin peptide SFLLRN did not result in TF expression on monocytes (data not shown).

All our experiments were performed in the presence of neutrophils and it is possible that neutrophil activation also plays a role in material-induced TF expression. Activated neutrophils, by releasing platelet activating factor or Cathepsin G, may activate platelets, which in turn will bind and activate monocytes to express TF. A role for neutrophils in LPS-induced TF expression has indeed been reported by Osterud et al[14;57]. Further experiments, with monocytes and platelets alone, are warranted to further characterize the platelet-dependent effect on material-induced TF expression.

Our results with adherent monocytes suggest that a similar platelet-dependent mechanism may occur for TF expression on adherent monocytes. Significant platelet adhesion was required to observe TF expression on adherent monocytes. This expression also appeared to be mediated by interaction with adherent platelets via P-selectin. However, monocyte adhesion was also strongly dependent on adherent platelets and interaction with platelets, making result interpretation more complex. The level of TF expression per adherent monocyte (%TF on beads/ % CD14 on beads) was calculated (data not shown), however no significant difference was observed. The role of adherent platelets on TF expression of adherent monocytes may be more strongly demonstrated using higher platelet concentrations.

b) CD11b upregulation on leukocytes

As for TF expression, the effect of platelets and platelet activation on CD11b upregulation on leukocytes is subject to contradictory reports. Our results with platelets and anti-IIb/IIIa or anti-P-selectin, and with PEG-immobilized PS beads which support minimal platelet activation, show that platelet activation (P-selectin expression, platelet microparticles) or formation of platelet-leukocyte aggregates did not affect material-induced CD11b upregulation in the bulk. Evangelista et al also found that the presence of resting or activated platelets did not upregulate CD11b on neutrophils[58]. However, they observed that activated platelets stimulated the expression of a β_2 integrin activation-dependent epitope, suggesting that a functional change of neutrophil β_2 integrins occurred[58] (no increase of the surface expression of CD11b, but exposure of a new part of the CD11b receptor). Others have also shown that binding of P-selectin to its ligand on monocytes[59] and neutrophils[60] did not stimulate CD11b upregulation, further confirming our results that platelet/leukocyte interactions were not involved in CD11b upregulation of bulk leukocytes.

On the other hand, Jy and colleagues[47] reported that platelet microparticles significantly increased CD11b upregulation on leukocytes. This study was performed with outdated platelet concentrates and previously frozen platelet microparticles. The process of freezing platelets has been shown to affect interaction with leukocytes and result in CD11b upregulation[61], casting doubts on Jy's conclusion. Miyamoto and colleagues[62], using unfrozen platelet microparticles, later demonstrated that platelet microparticles did not upregulate CD11b on neutrophils. CD11b upregulation, dependent on platelet-leukocyte interaction via P-selectin[63], has also been reported in the presence of platelets activated with ADP. However, the observed CD11b upregulation was modest relative to agonist stimulation. Thus while this study showed that activated platelets may play a role in CD11b upregulation, the significant effect of this activation remains questionable.

While platelets had no effect on CD11b upregulation in the bulk, the presence of anti-P-selectin significantly reduced, but not entirely, CD11b on adherent leukocytes with all types of beads. This suggests that, in an isolated leukocyte system, platelet interaction with adherent leukocytes plays some role in CD11b upregulation on adherent leukocytes. Binding of P-selectin to its counterligand, PSGL-1, on adherent neutrophils has been reported to activate CD11b/CD18 by a tyrosine dependent-mechanism[15].

c) Platelet-leukocyte aggregates

Confirming previous results[64,65] with isolated leukocytes and platelets, anti-P-selectin inhibited formation of platelet-leukocyte aggregates. Anti-IIb/IIIa also significantly reduced formation of platelet-leukocyte aggregates, but not as effectively as anti-P-selectin did. The effect of anti-IIb/IIIa on platelet-leukocytes aggregates may be explained by the fact that anti-IIb/IIIa blocked material-induced platelet activation, resulting in low P-selectin expression on platelets, and thus preventing platelet-leukocyte interactions. It is also possible that anti-IIb/IIIa reduced platelet-leukocyte aggregates via its inhibition of fibrinogen binding.

V. Conclusion

Using isolated leukocytes and platelet-rich plasma, a role for platelets in material-induced TF expression but not in CD11b upregulation was identified. Material-induced TF expression required the presence of platelets, at a minimum concentration of 50×10^6 platelets/mL (about 1/3 of the normal platelet concentration). The role of platelets appeared

to be mediated mostly by platelet-leukocyte interactions via P-selectin. A role for platelet release products could not be totally eliminated and further studies using platelet supernatants are warranted to fully elucidate the mechanism of platelet-dependent material-induced TF expression. Blocking material-induced platelet activation with anti-IIb/IIIa or platelet-leukocyte interactions did not totally inhibit TF expression suggesting the existence of another mechanism, such as complement activation (see next chapter).

Platelets also played a significant role in monocyte adhesion to artificial surfaces. Monocyte adhesion was mediated by adherent platelets via P-selectin. On the other hand, neutrophil adhesion was not dependent on platelets, and the presence of adsorbed complement products and other plasma proteins were most likely responsible for neutrophil adhesion. Our experiments were performed under low shear conditions, and the proposed mechanism of leukocyte adhesion is likely limited to these conditions. There is evidence from *in vitro* models of injured vessel-walls that the mechanisms of leukocyte adhesion in the presence of adherent platelets vary with shear. Whether shear plays a role on the effect of platelets on leukocyte adhesion to biomaterials in the presence of plasma remains to be determined.

Our results provide evidence that platelets play a role in some aspects of material-induced leukocyte activation. The fact that TF expression and CD11b upregulation appear to be regulated by different mechanisms is not surprising, as TF requires protein synthesis while CD11b does not. This suggests that blocking material-induced platelet activation or designing materials that do not activate platelets is not a sufficient/satisfactory strategy in the quest for blood compatibility. Other mechanisms are involved, most likely complement activation, and have to be addressed to fully eliminate thrombotic complications with cardiovascular devices.

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Chapter 5

Role of complement in leukocyte activation induced by polystyrene and PEG-immobilized polystyrene beads

Abstract

With isolated leukocytes, inhibiting complement reduced material-induced leukocyte activation (CD11b) only with high complement activating surfaces (PS-PEG), but not with moderate activating PS. PS-PEG was a much stronger complement activator than PS, as shown by high generation of SC5b-9. Following contact with PS and PS-PEG beads, isolated leukocytes in plasma and in the absence in platelets were found to significantly upregulate CD11b, while TF expression and exposure of phosphatidylserine remained at background levels. Complement inhibition by means of sCR1 only partially reduced CD11b upregulation on PS-PEG beads, but not on PS beads. Pyridoxal-5-phosphate (P5P) was able to significantly reduce both CD11b upregulation and exposure of phosphatidylserine with PS-PEG beads, although we are uncertain as to its mechanisms of action. Pentamidine and NAAGA inhibited complement and were effective in reducing CD11b upregulation with both PS and PS-PEG. However, they also had a direct effect on leukocyte signalling mechanisms. Leukocyte adhesion occurred to similar extent on both PS and PS-PEG beads. While sCR1 and P5P blocked adhesion and activation for adherent leukocytes on PS-PEG beads, they had no effect on leukocytes adherent to PS beads.

The role of complement in leukocyte activation was material-dependent. The lack of an effect of sCR1 on leukocyte adhesion to PS beads also suggests that, with some materials, adsorbed plasma proteins, such as fibrinogen, play a more important role than complement in leukocyte adhesion. Thus, leukocyte-material compatibility may not always be resolved by a low complement activating capacity as other mechanisms appear to play a significant role in leukocyte activation and adhesion.

I. Introduction

The presence of a foreign material in blood results in the activation of the coagulation cascade, the complement system, platelets and leukocytes. These events are part of the inflammatory and thrombotic response to biomaterials. The inflammatory response associated with cardiopulmonary bypass, hemodialysis or synthetic vascular graft contributes to the development of complications such as organ/tissue injury, transient leukopenia and pulmonary dysfunction. *In vivo* leukocyte activation following contact with cardiovascular devices has been reported in many instances[1-6]. Leukocytes have long been recognized in playing a role in the inflammatory response through the release of cytokines, lysosomal enzymes, lipid mediators and reactive oxygen species[7,8]. Activated leukocytes also become more adherent to biological and artificial surfaces. Among other pathways, leukocyte activation may occur through complement activation. Generation of C3a and C5a causes chemotaxis and activation of leukocytes while adsorption of iC3b on surfaces promotes leukocyte adhesion and activation[9,10]. However, it is not clear as to whether leukocyte activation by materials is exclusively dependent on complement activation or not.

While being inflammatory cells, activated leukocytes may also be part of the thrombotic response, as they possess membrane associated procoagulant properties. Upon activation, CD11b, a leukocyte integrin receptor, is upregulated. CD11b binds to ICAM-1, iC3b, Factor X and fibrinogen, and the latter two are part of the coagulation cascade. Tissue factor becomes expressed on monocytes and will activate the extrinsic coagulation cascade. The exposure of negatively charged phospholipids (phosphatidylserine) on leukocyte membranes, where the prothrombinase complex can assemble, represents another procoagulant activity of leukocytes. Formation of platelet-leukocyte aggregates may also be a form of leukocyte procoagulant activity, as through these aggregates they further activate each other. Many inflammatory disorders such as the allograft rejection reaction or disseminated intravascular coagulation, point to the potential role of leukocytes in thrombin formation[11,12].

Thrombotic complications with cardiovascular devices are a recurrent problem. Surface modifications, and/or the use of anticoagulants and anti-platelet agents have not been able to solve material thrombotic failure. We have hypothesized that material associated leukocyte activation contributes to cardiovascular device failure by enhancing thrombin formation. We also hypothesize that leukocyte activation (and consequent thrombin

formation) is secondary to material-associated complement activation and platelet activation. In Chapter 3, we demonstrated that contact with polystyrene and PEG-grafted polystyrene beads resulted in leukocyte activation and expression of procoagulant activities: tissue factor expression, CD11b upregulation, and association with platelets occurred on leukocytes in the bulk regardless of the type/chemistry of beads. Activation of adherent leukocytes also occurred but this was, in part, material dependent. In Chapter 4, platelets were shown to play a role in tissue factor expression but not in CD11b upregulation. Increased monocyte adhesion was also observed with platelet adhesion to surfaces. The objective of this study was to characterize the role of complement activation in material-induced leukocyte activation in the absence in platelets. We hypothesized that inhibiting complement activation reduces material-induced leukocyte activation.

To focus on the role of complement in leukocyte activation and leukocyte expression of procoagulant activities, isolated leukocytes were incubated with polystyrene or PEG-immobilized polystyrene beads. The effects of complement inhibition on CD11b upregulation and TF expression of both bulk and adherent leukocytes were assessed. Since we were working in a "platelet-free" environment, we also measured prothrombinase activity on leukocytes using annexin V[1]. Various complement inhibitors were used to evaluate the role of complement. A recombinant soluble form of the complement receptor type 1 (sCR1) was used: sCR1 is a specific complement inhibitor blocking the classical and alternative convertases by binding to C3b and C4b and promoting their inactivation by Factor I[13]. N-acetyl aspartyl glutamic acid (NAAGA), a synthetic dipeptide, blocks both the classical and alternate pathways by inhibiting formation of the C3 convertase[14]. To evaluate the role of the classical pathway in material-induced leukocyte activation, pentamidine and pyridoxal-5-phosphate were used. Pentamidine, an antiprotozeal drug, is a serine protease inhibitor, which inhibits C1s, among others[15]. Pyridoxal-5-phosphate (P5P), the biologically active coenzyme form of vitamin B6 compounds, inhibits the classical pathway by modifying C1q[16]. In previous studies, pentamidine and P5P were found to block platelet adhesion on beads and reduce material-induced platelet microparticle formation[17] while sCR1 did not. Figure 1 illustrates the pathways of inhibition of the different complement inhibitors used in our study.

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[1] In the presence of platelets, as they bind to leukocytes, it is difficult to distinguish between annexin V bound to leukocytes or annexin V bound to platelets bound to leukocytes.

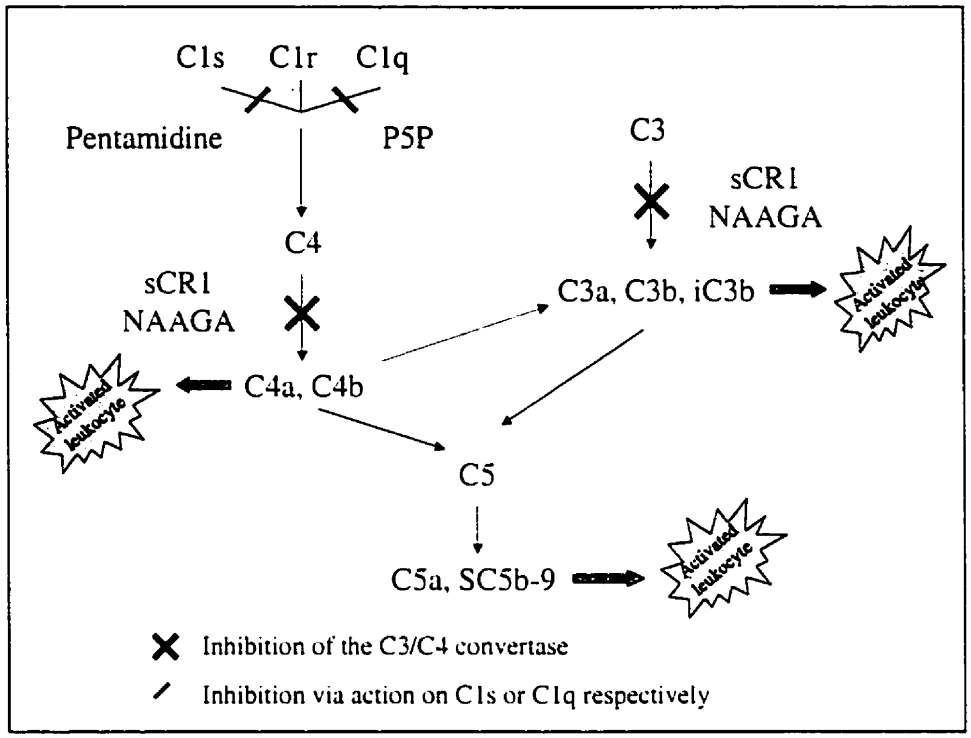

Figure 1: Pathways of inhibition of the complement inhibitors sCR1, NNAGA, pentamidine and P5P.

II. Materials and methods

1) Reagents and antibodies

Pyridoxal-5-phosphate (P5P) and N-acetyl aspartyl glutamic acid (NAAGA) were from Sigma Chemical Co, St Louis, MO. Soluble human recombinant complement receptor type 1 (sCR1) was a generous gift from Avant Immunotherapeutics, Needham, MA (Dr. Carolyn Pettey). Pentamidine was from Faulding, Canada.

R-phycoerythrin-cytochrome 5 (PE-Cy5) anti-CD45 was from Immunotech-Coulter (Marseilles, France). Mouse monoclonal antibodies against human complement iC3b and SC5b-9 were from Quidel Corporation, San Diego, CA. GAM-FITC antibody was from Dr R. Sutherland (Toronto General Hospital, Canada). A kit containing FITC-labelled annexin V and calcium binding buffer was purchased from Immunotech-Coulter. All other antibodies and reagents were similar to the ones used in Chapters 3 and 4.

2) Beads

The same 45μm beads were used as in chapter Four: polystyrene (PS) and TentaGel beads, polystyrene beads grafted with polyethylene glycol (PS-PEG). They were cleaned

from endotoxin contamination as described earlier and resuspended in endotoxin-free PBS. In all experiments, the residual endotoxin on the beads was equivalent to a concentration of endotoxin contamination that was less than 0.1 EU/ml, which is below the FDA recommended maximum level[18].

3) Leukocyte isolation

Leukocytes were separated from heparinized blood (5 U/mL) as described in Chapter 4. Provided platelet contamination was less than $1x10^6$/mL, leukocytes were resuspended together at a concentration of $5x10^6$ neutrophils/mL and $1x10^6$ monocytes/mL in RPMI with 25% plasma or serum. Cell viability as determined by trypan blue was greater than 98%.

4) Serum and heparinized plasma

Autologous plasma was prepared from the platelet rich plasma by centrifugation for 20 minutes at 1000g. Platelet poor plasma was then removed and ultracentrifuged for 3 minutes. The plasma was set aside at room temperature[ii] during the leukocyte isolation procedure (approximately 3 hours).

Pooled plasma (5U/mL heparin) and serum were prepared following standard procedures. Plasma or serum from at least four individuals was pooled. Pooled aliquots were stored at -70°C. They were thawed and ultracentrifuged for 3 minutes prior to experiments.

5) Leukocyte-material contact

Mixed leukocytes, resuspended in 25% plasma or serum, were incubated with only one concentration of beads ($20x10^4$ beads/mL – equivalent to a surface area to volume ratio of 13 cm^{-1}) for 2 hours at 37°C on a haematology mixer. The complement inhibitors were added one at a time to the leukocytes before the beads at the following concentrations: sCR1 (200 µg/mL), P5P (500 µg/mL), pentamidine (1 mg/mL) and NAAGA (20 mM). Leukocyte reactivity was also tested with PMA following incubation with complement inhibitors.

For the negative control, EDTA (8 mM final concentration) was added to leukocytes and beads while the positive control was leukocytes incubated with endotoxin (5 µg/mL)

[ii] In this case, storage at room temperature is better, as too many temperature changes have been shown to activate complement[75].

without beads.

Following incubation, beads were allowed to settle and aliquots were taken for flow cytometry analysis of non-adherent cells and for a cell count using CellDyne 1600 (Abbott Laboratories, IL). To analyse adherent cells on beads, the cell supernatant was removed and beads were washed once with RPMI/FBS and then incubated with the appropriate antibodies.

6) Flow cytometry

Flow cytometric analysis was carried out on leukocyte suspension and beads as before. Briefly, small aliquots (30 µL) of leukocytes or beads, diluted in 50µL of HTB were incubated with saturating concentrations of fluorescently labelled monoclonal antibodies for 30 minutes at 4°C. After a 30-minute incubation, samples were diluted and fixed with paraformaldehyde (1% final concentration). Samples were analysed on a Becton Dickinson FACScan flow cytometer (Mountain View, CA), using CELLQuest software.

To measure prothrombinase activity, leukocytes were incubated with FITC-annexin V in a calcium-binding buffer (50µl) for 15 minutes in the dark at room temperature. They were then diluted in HTB and analysed immediately.

To ensure that platelet contamination was kept at a minimum for all experiments, association of leukocytes with platelets and platelet adhesion on beads were monitored using expression of CD41 (GPIIb-IIIa platelet receptor). For all the isolated leukocyte experiments reported, platelet contamination was kept to a minimum ($\leq 1 \times 10^6$ platelets/mL, i.e., less than 1% of normal platelet counts): all CD41 fluorescence values on leukocytes and beads were similar to EDTA+beads levels.

7) Complement activation with beads

Pooled serum was incubated for 2 hours at 37°C on a haematology mixer with beads (20×10^4 beads/mL – equivalent to an area to volume ratio of 13 cm^{-1}) and the various complement inhibitors at the same concentrations as those used in the leukocyte experiments. After incubation, EDTA was added (8 mM final concentration), samples were centrifuged and serum aliquots frozen at –70°C. To analyse complement adsorption on beads by flow cytometry[19], beads were washed once and resuspended in RPMI/FBS. They were then incubated with saturating concentration of anti-iC3b or anti-SC5b-9. After a 20-minute incubation at 4°C, they were washed twice and incubated with GAM-FITC for 20 minutes at

4°C. Following two washes, they were then diluted in HTB and fixed with paraformaldehyde. Bead samples were analysed using the same setting as for beads with leukocytes.

Complement activation in the bulk was determined by enzyme immunoassays (ELISA) for iC3b and SC5b-9 (Quidel Corporation). The principle for both complement products was a three-step procedure utilizing: (1) a microassay plate coated with a mouse monoclonal antibody which binds specifically to the complement product; (2) a horseradish peroxidase conjugated antibody to antigens of the component; (3) a chromogenic substrate.

8) Statistical analysis

All results are reported as means ± SD. To evaluate the significance of the differences in cell activation, analysis of variance (ANOVA) was carried out followed by the Student-Newman-Keuls test, which is a protected test for multiple comparisons[20]. A *p*-value of less than 0.05 was required for statistical significance. The number of repeat experiments was equal to or greater than three with three different donors.

III. Results

1) Complement activation by PS and PS-PEG

PS and PS-PEG generated the same amount of iC3b, but SC5b-9 was almost three times higher with PS-PEG than with PS beads (Table 1). As expected, sCR1, pentamidine and NAAGA blocked complement activation. P5P did not have any significant effect on complement activation as measured by iC3b and SC5b-9 in the bulk.

Complement adsorption as measured by flow cytometry was observed on both types of beads. PS-PEG adsorbed less iC3b than did PS, but similar fluorescence of SC5b-9 was observed (Table 1). Adsorption levels were reduced to those similar to EDTA with sCR1, NAAGA and pentamidine. As it had in the bulk, P5P had no measurable effect on complement adsorption.

Table 1: Complement activation by PS and PS-PEG beads and the effect of various complement inhibitors in pooled human serum.

	Fluid phase		Adsorbed	
	iC3b (μg/mL)	**SC5b-9** (μg/mL)	**iC3b** (%)[a]	**SC5b-9** (%)[a]
No beads	208 ± 38 *	4 ± 1 *	N/A	N/A
Beads + EDTA	N/D	N/D	100 ± 0 *	100 ± 0 *
PS	365 ± 81	8 ± 2	582 ± 65	658 ± 92
+ sCR1	27 ± 1 *	1 ± 0 *	118 ± 13 *	103 ± 18 *
+ P5P	363 ± 23	14 ± 3	465 ± 78	740 ± 102
+ Pentamidine [b]	80 ± 3	0 ± 0	N/D	N/D
+ NAAGA [b]	31 ± 2	1 ± 0	N/D	N/D
PS-PEG	326 ± 52	24 ± 2 #	338 ± 85 #	664 ± 96
+ sCR1	27 ± 1 *	1 ± 0 *	97 ± 19 *	98 ± 10 *
+ P5P	458 ± 27	29 ± 2	322 ± 55	656 ± 96
+ Pentamidine [b]	80 ± 7	1 ± 0	86 ± 5	100 ± 0
+ NAAGA [b]	35 ± 3	2 ± 0	112 ± 33	114 ± 40

N = 3 to 6 ± S.D., [b] N = 2 .

For the ELISA All samples were run in duplicate.

[a] iC3b and SC5b-9 signals on beads represented as a percentage relative to beads+EDTA.

* Significantly different from PS or PS-PEG beads as appropriate ($p < 0.002$)

Significantly different from PS ($p < 0.0003$)

N/A: not applicable. N/D: not determined

2) Isolated leukocytes (autologous heparinized plasma): leukocyte adhesion and activation on beads

Table 2 shows the effect of the various complement inhibitors on leukocyte adhesion and activation on beads, while the level of CD11b activation on adherent leukocytes is shown in Figure 2.

As shown in the first three columns of Table 2. leukocytes adhered to PS and PS-PEG to similar extents. This is based on leukocyte count differences and on the fluorescent intensity of the CD45 and CD14 signals. sCR1 and P5P blocked monocyte and neutrophil adhesion on PS-PEG beads, but were ineffective with PS beads. On the other hand,

pentamidine significantly reduced leukocyte adhesion on PS beads. With PS-PEG beads, pentamidine brought conflicting results: flow cytometry data showed pentamidine to block only monocyte adhesion (i.e., reduced CD14 but not CD45 signals on beads compared to PS-PEG alone), while the relative leukocyte count (neutrophils and monocytes) in the presence of pentamidine was significantly higher than the one observed with PS-PEG beads alone, suggesting that pentamidine was indeed blocking both neutrophil and monocyte adhesion to PS-PEG beads. As for NAAGA, it reduced adhesion with both PS and PS-PEG beads.

Signals for TF, annexin V binding, and CD11b on beads (measuring adherent leukocyte activation) are reported in Table 2 (last three columns). No significant TF expression was seen on adherent monocytes with or without inhibitors. On the other hand, significant CD11b signals were present on both PS and PS-PEG beads. Even in the presence of sCR1 and P5P, leukocytes on PS beads were very activated, as shown by the high CD11b signals. However, CD11b values on PS beads with pentamidine and NAAGA were low since few adherent leukocytes were present. CD11b values were also low on PS-PEG for all complement inhibitors with the exception of with pentamidine. As for annexin V, significant binding was observed on PS beads only, with or without inhibitor (Table 2).

When the numbers of adherent leukocytes are not similar (for example PS-PEG with and without sCR1), comparing CD11b signals may lead to erroneous conclusions. The CD11b signal on beads depends on the level of activation of the adherent leukocytes but also on the number of leukocytes present on the beads: the fewer the cells, the smaller the CD11b signal. Thus, to determine if complement was indeed reducing leukocyte activation and not only adhesion, we defined an index of leukocyte activation on beads as the (% of CD11b on beads) divided by (% of CD45 on beads). Results are reported in Figure 2. As shown in Figure 2, sCR1 and P5P significantly reduced activation of adherent leukocytes on PS-PEG, but not on PS beads. Also, NAAGA reduced activation of adherent leukocytes for PS and PS-PEG beads, while pentamidine was only effective with activation on PS beads.

Table 2: Leukocyte adhesion and activation on PS and PS-PEG beads in the presence of various complement inhibitors (isolated leukocytes in heparinized plasma).

	Leukocyte adhesion			Leukocyte activation on beads		
	Relative leukocyte count (%)[a]	**Leukocytes on beads (CD45, %)[b]**	**Monocytes on beads (CD14, %)[b]**	**TF (%)[b]**	**CD11b (%)[b]**	**Annexin V (%)[b,c]**
Beads + EDTA	100 ± 0 *	100 ± 0 *	100 ± 0 *	100 ± 0	100 ± 0 #*	100 ± 0 #
PS	86 ± 2	140 ± 33	144 ± 32	110 ± 7	248 ± 41	284 ± 69
+ sCR1	92 ± 12	147 ± 26	172 ± 36	108 ± 4	404 ± 49 #	397 ± 105
+ P5P	79 ± 7	163 ± 26	167 ± 53	101 ± 9	483 ± 163 #	492 ± 181 [c]
+ Pentamidine	95 ± 12	81 ± 31 #	72 ± 30 #	89 ± 14	76 ± 19 #	N/D
+ NAAGA	92 ± 10	111 ± 36	103 ± 36	126 ± 25	92 ± 30 #	N/D
PS-PEG	75 ± 13	168 ± 33	145 ± 32	107 ± 9	246 ± 85	132 ± 30 #
+ sCR1	103 ± 13 *	99 ± 10 *	94 ± 4 *	98 ± 7	103 ± 8 *	109 ± 32
+ P5P	82 ± 17	100 ± 14 *	100 ± 6 *	109 ± 15	118 ± 30 *	123 ± 37 [c]
+ Pentamidine	102 ± 9 *	147 ± 19	100 ± 7 *	107 ± 13	246 ± 125	N/D
+ NAAGA [c]	93 ± 0	116 ± 14	107 ± 10	103 ± 4	115 ± 12	N/D

N = 3 to 4 ± SD, [c] N=2

[a] Leukocyte counts are expressed as percent of leukocytes in blood relative to the number of leukocytes in blood with beads and EDTA.

[b] CD45, CD14, TF, CD11b and annexin V signals on beads represented as a percentage relative to beads+EDTA.

Significantly different from PS beads ($p \leq 0.05$)

* Significantly different from PS-PEG ($p < 0.03$)

N/D: not determined

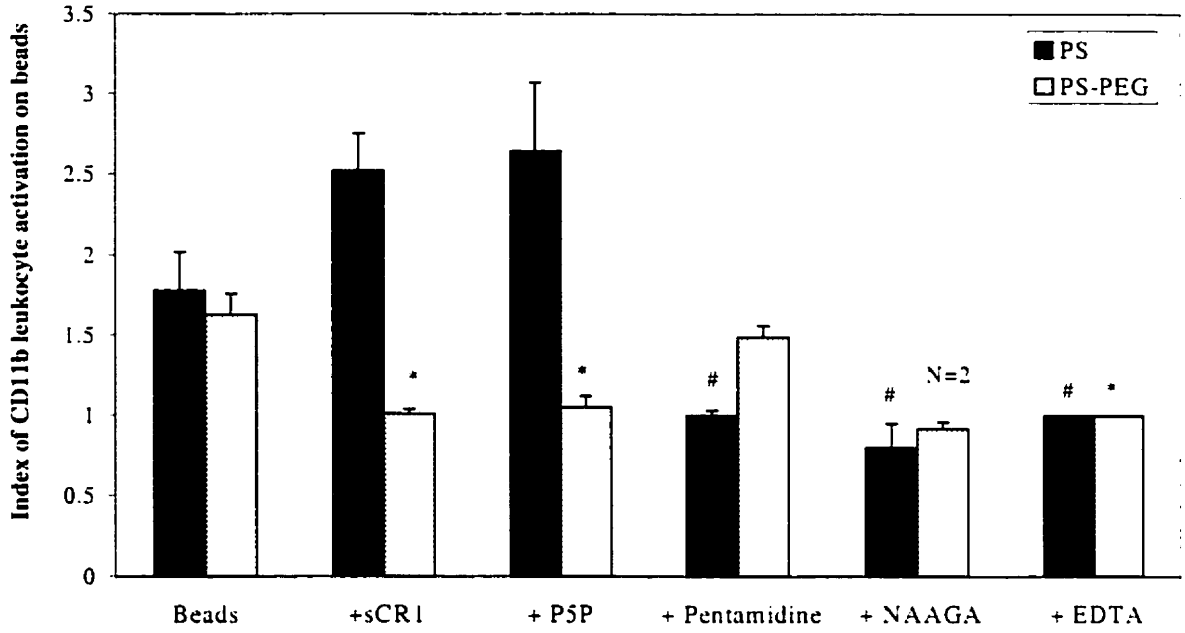

Figure 2: Effect of various complement inhibitors on the index of CD11b activation of leukocytes adherent to PS and PS-PEG beads (autologous heparinized plasma). sCR1 and P5P significantly reduced CD11b upregulation per adherent leukocyte on PS-PEG, while they had no effect on leukocytes adherent to PS beads. The index of CD11b leukocyte activation is calculated as the (% of CD11b on beads)/(% of CD45 on beads). The % of CD11b and CD45 on beads are defined in Table 2. # Significantly different from PS beads ($p \leq 0.01$). * Significantly different from PS-PEG beads ($p \leq 0.02$). N = 3 to 4 ± SD

3) Isolated leukocytes (autologous heparinized plasma): bulk activation with beads

Tables 3-5, and Figure 3 show the effect of the various complement inhibitors on leukocytes in the bulk: TF expression (Table 3), CD11b upregulation (Figure 3 and Table 4) and annexin V (Table 5).

Table 3 shows that TF expression was not significantly higher than background levels on bulk monocytes (with "no" platelets present), except for samples incubated with pentamidine and NAAGA where abnormally high values were observed. This is different than what was seen in whole blood (i.e. with platelets) in Chapter 3 and it agrees with our platelet-poor plasma results from Chapter 4.

Table 3: Effect of various complement inhibitors on tissue factor expression induced by PS and PS-PEG beads (isolated leukocytes in heparinized plasma).

	TF expression (relative fluorescent intensity in %)[a]	**TF expression (% monos expressing TF) [b]**
No beads	47 ± 9 [N.S.]	4 ± 2 [N.S.]
PS	65 ± 9	11 ± 3
PS + sCR1	64 ± 13	11 ± 6
PS + P5P	64 ± 11	9 ± 5
PS + Pentamidine	108 ± 29	54 ± 6
PS + NAAGA	143 ± 49	55 ± 29
PS-PEG [c]	68 ± 10	16 ± 6

N = 3 to 5 ± SD

[a] TF expression is represented as a percent relative to the maximum where maximum expression is determined by the fluorescent intensity of leukocytes incubated with LPS for 2 hours at 37°C

[b] TF expression is represented as the percentage of monocytes staining positive for tissue factor using the dot plot of TF versus CD14.

[c] The complement inhibitors had the same effect on TF expression induced by PS-PEG beads as they had for PS.

[N.S.] Not statistically different from PS or PS-PEG beads.

On the other hand, Figure 3 shows that significant CD11b upregulation occurred on leukocytes in the bulk in the presence of beads, even in the absence of platelets. sCR1 and P5P significantly reduced CD11b upregulation induced by PS-PEG beads but had no effect on PS-induced activation. Also, pentamidine significantly reduced CD11b upregulation for both PS and PS-PEG beads, while NAAGA totally inhibited CD11b upregulation. Note also that CD11b upregulation on leukocytes without beads was high due to the longer preparation and incubation time of these experiments relative to those done previously[21]: plasma was left to sit for a few hours in a polypropylene tube during leukocyte preparation and the presence of activated complement products likely contributed to this "background" leukocyte activation over the 2-hour incubation.

Also, using the differences in size scatter and intensity for CD45 between neutrophils and monocytes, we were able to distinguish the inhibitor effects on the two populations.

Except for P5P with PS beads, the effects of the various complement inhibitors on CD11b were similar for both neutrophils and monocytes (data not shown). However, P5P was only able to significantly reduce monocyte (but not neutrophil) CD11b upregulation induced by PS beads (Table 4).

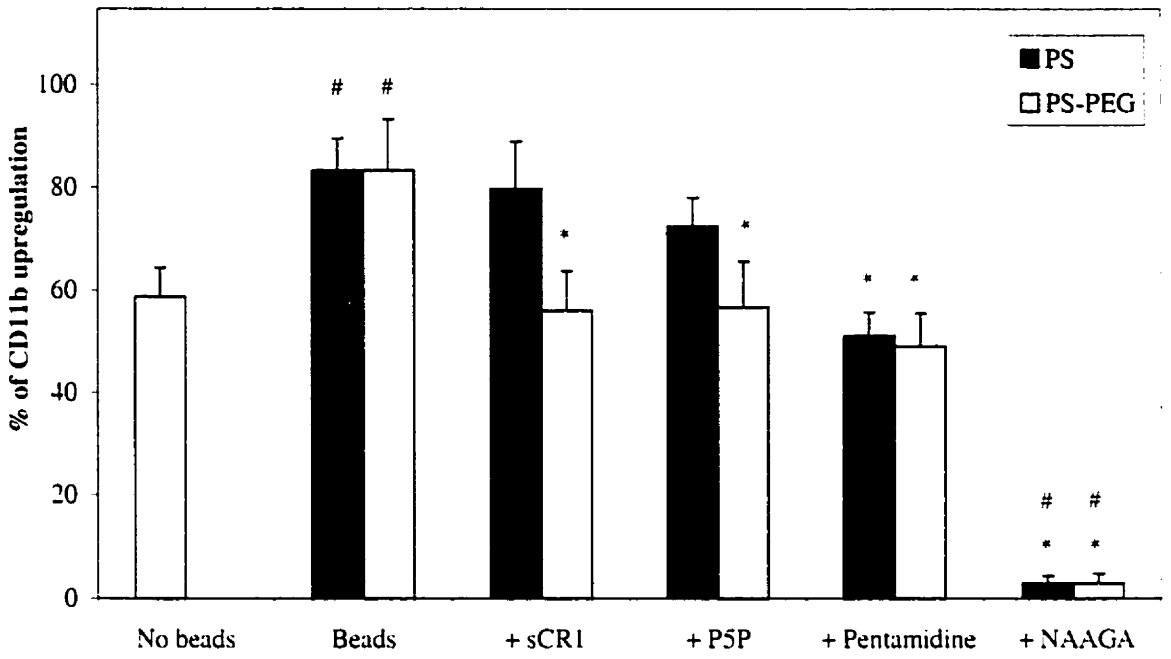

Figure 3: Effect of various complement inhibitors on CD11b upregulation on isolated leukocytes (neutrophils and monocytes) in autologous heparinized plasma. sCR1 and P5P partially reduced CD11b upregulation with PS-PEG beads, but not with PS. CD11b upregulation is represented as a percent relative to the maximum (determined by the fluorescent intensity of leukocytes incubated with LPS) . # Significantly different from no beads, * Significantly different from beads without inhibitor ($p < 0.0002$). N = 3 to 5 ± SD

As shown in Table 5, following contact with beads, annexin V binding on monocytes and neutrophils was not significantly higher than background levels without beads. Annexin V binding on bulk monocytes was reduced with sCR1 and P5P for both PS and PS-PEG beads, but only P5P lead to a reduction that was significant. On the other hand, sCR1 and P5P had no effect on neutrophils, and in some cases, P5P even increased phosphatidylserine exposure on neutrophils, as illustrated by the high annexin V binding levels with PS.

Table 4: Effect of P5P on neutrophil and monocyte activation induced by PS and PS-PEG beads (isolated leukocytes in heparinized plasma).

	% CD11b upregulation on **neutrophils** [a]	% CD11b upregulation on **monocytes** [a]
No bead	60 ± 8 *	67 ± 5 *
PS	83 ± 6	89 ± 11
+ P5P	72 ± 5	67 ± 11 *
PS-PEG	83 ± 10	83 ± 5
+ P5P	57 ± 9 *	60 ± 10 *

N = 3 to 5 ± SD

[a] CD11b upregulation is represented as a percent relative to the maximum determined by the fluorescent intensity of leukocytes incubated with LPS for 2 hours at 37°C.

Monocytes and neutrophils were gated by flow cytometry using their size scatter and CD45 signals.

* Significantly different from PS and PS-PEG beads ($p < 0.02$)

Table 5: Effect of sCR1 and P5P on annexin V binding to isolated leukocytes incubated with beads in heparinized plasma.

	Annexin V on Monos (%)[a]	Annexin V on PMNs (%)[a]
No beads	384 ± 142	272 ± 41 N.S.
PS	638 ± 165	385 ± 76
+ sCR1	461 ± 150	363 ± 79
+ P5P	348 ± 140 #	830 ± 184 *
PS-PEG	578 ± 162	558 ± 296
+ sCR1	370 ± 190	209 ± 110
+ P5P	162 ± 36 *	548 ± 248

N = 3 or 4 ± SD

[a] Annexin V binding is represented as a percent relative to the minimum determined by the fluorescent intensity of leukocytes incubated with beads and EDTA for 2 hours at 37°C.

Significantly different from PS beads ($p \leq 0.05$)

* Significantly different from PS-PEG beads ($p < 0.04$)

N.S. Not statistically different from PS or PS-PEG beads.

4) PMA-stimulated leukocyte CD11b upregulation

After the 2-hour incubation with the various complement inhibitors and beads, leukocytes were stimulated with PMA to verify that they were still able to respond to a strong agonist. PMA is a direct activator of the protein kinase C (intracellular signalling mechanism of activation) that induces strong CD11b upregulation[22]. Table 6 shows how leukocytes, incubated with beads and the complement inhibitors NAAGA or pentamidine for 2 hours, were unable to upregulate CD11b in the presence of PMA. These results suggest that these two agents significantly affected leukocyte intracellular signalling mechanisms. Hence in addition to complement inhibitory effect, NAAGA and pentamidine appeared to inhibit directly leukocytes.

Table 6: PMA-induced activation of leukocytes after a 2-hour incubation with beads and complement inhibitors (isolated leukocytes in heparinized plasma).

	CD11b upregulation (%)
No beads	60 ± 8
No beads + PMA	86 ± 8
PS + PMA	97 ± 11
PS + sCR1 + PMA	101 ± 13
PS + P5P + PMA	97 ± 13
PS + Pentamidine + PMA	55 ± 8 *
PS + NAAGA + PMA	3 ± 3 *
PS-PEG + PMA	101 ± 8

N = 4 ± S.D.

[a] CD11b upregulation is represented as a percent relative to the maximum (determined by the fluorescent intensity of leukocytes incubated with LPS for 2 hours at 37°C).

* Significantly different from No beads + PMA ($p < 0.004$)

5) Isolated leukocytes: pooled serum and pooled-heparinized plasma

To verify that the previous results were independent of the presence of heparin (known to have some complement inhibitory effect), experiments were repeated with

leukocytes resuspended in serum. Only sCR1 and P5P were used with serum as NAAGA and pentamidine were found to affect leukocyte-signalling mechanisms (see above).

Table 7 shows the level of leukocyte adhesion and activation on PS and PS-PEG beads in pooled serum or pooled heparinized plasma conditions. Levels of leukocyte adhesion on beads in serum were lower than those observed in plasma. As observed previously in plasma, neither sCR1 nor P5P blocked adhesion or activation on PS beads, while both were effective with PS-PEG beads.

Table 7: Leukocyte adhesion and activation on beads (isolated leukocytes in serum or heparinized plasma)

	Serum		Heparinized plasma	
	CD45 (%)[a]	CD11b (%)[a]	CD45 (%)[a]	CD11b (%)[a]
Beads + EDTA	100 ± 0	100 ± 0 [#,••]	100 ± 0	100 ± 0
PS	113± 11	155 ± 23	141 ± 1 [b]	397 ± 111 [b]
+ sCR1	114 ± 14	138 ± 12		
+ P5P	133 ± 22	146 ± 26		
PS-PEG	117 ± 14	156 ± 38	143 ± 24 [b]	250 ± 194 [b]
+ sCR1	97 ± 6	96 ± 12 [•]		
+ P5P [b]	105 ± 2	114 ± 8		

N = 3 ± S.D., [b] N = 2

[#] Significantly different from PS beads ($p < 0.03$)

[••] Significantly different from PS-PEG ($p \leq 0.05$)

Figure 4 shows CD11b upregulation on leukocytes in the bulk in the presence of pooled serum or pooled heparinized plasma. Contrary to experiments in heparinized plasma (Figure 4 or Figure 2), no difference in CD11b upregulation was observed with or without beads for leukocytes incubated in serum. Moreover, in the absence of beads, leukocytes in serum were significantly more activated than leukocytes in plasma.

Figure 4 also shows the effect of P5P and sCR1 on material-induced CD11b upregulation in serum. While P5P in heparinized plasma had been previously shown to reduce CD11b upregulation only with PS-PEG, P5P in serum was effective in significantly

reducing leukocyte activation with both PS and PS-PEG beads. As seen in heparinized plasma (Figure 2), sCR1 in serum was only able to partially reduce leukocyte activation induced by PS-PEG, but had no effect with PS.

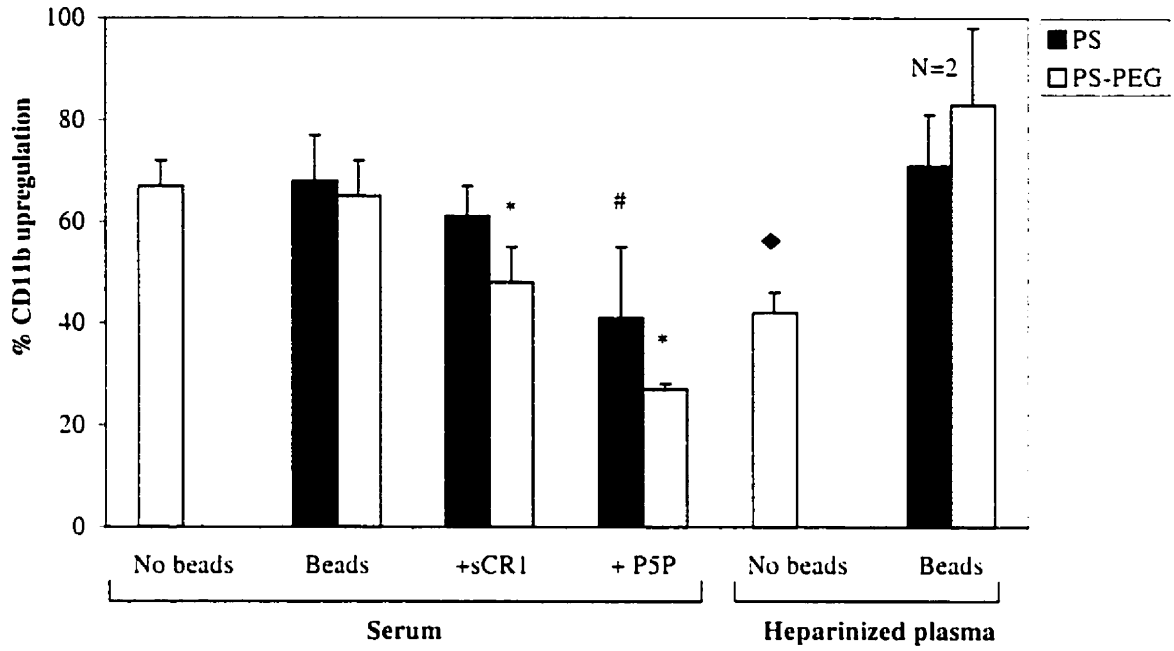

Figure 4: Effect of serum (versus pooled plasma) and various complement inhibitors on CD11b upregulation of isolated leukocytes (bulk) with PS and PS-PEG beads. As seen previously with autologous plasma, sCR1 reduced CD11b upregulation only with PS-PEG. A high background level of CD11b upregulation was observed in serum. * Significantly different from no bead in heparinized plasma ($p = 0.01$). # Significantly different from PS beads ($p \leq 0.03$). ▪ Significantly different from PS-PEG ($p \leq 0.05$). N = 3 ± S.D

IV. Discussion

1) Leukocyte activation with isolated leukocytes

Following contact with PS and PS-PEG beads, significant CD11b upregulation in the bulk was observed on isolated leukocytes in plasma, but TF expression was not much different from that observed without beads. These results confirmed our previous findings (Chapter 4) that platelets were necessary for increases in TF expression on monocytes. P5P, sCR1, pentamidine and NAAGA were unable to block the observed background TF expression, suggesting that the TF expression present in these experiments may be an inevitable consequence of the isolation procedure.

Annexin V was used as a probe to measure the exposure of negatively charged phospholipids following contact with beads. Annexin V is a phospholipid-binding protein, which in the presence of calcium ions exhibits high and selective affinity for phosphatidylserine. Phosphatidylserine, a negatively charged membrane phospholipid (required for the assembly of the clotting enzyme complexes such as the prothrombinase complex), may become exposed upon cell activation. While activated platelets[23,24], platelets adherent to a biomaterial[25] and platelet microparticles[26] are recognized to express phosphatidylserine, very little has been reported about material-activated leukocytes and phosphatidylserine. In our experiments, after contact with PS and PS-PEG beads, both monocytes and neutrophils exposed phosphatidylserine, but to levels that were not significantly different from those observed without beads. This is consistent with a study by Satta et al[27] showing small differences of phosphatidylserine exposure between control and LPS-stimulated monocytes. Strong exposure of phosphatidylserine has been observed previously principally on monocyte microparticles[27]. Our finding showing phosphatidylserine present on neutrophils and monocytes, even without beads, also agrees with earlier studies by Mann et al[28,29] indicating prothrombinase activity on leukocytes. As to whether this observed potential procoagulant activity is a consequence of the isolation procedure is unclear, since sCR1 and P5P had many different effects. sCR1 and P5P were unable to block background exposure of phosphatidylserine on neutrophils, suggesting then that it may be a consequence of isolation. On the other hand, P5P significantly reduced exposure on monocytes with PS and PS-PEG beads, but to different extents, suggesting that exposure of phosphatidylserine on monocytes was a consequence of activation induced by the beads. Further work is needed to fully characterize phosphatidylserine exposure on leukocytes.

In the presence of plasma (pooled or autologous), PS beads induced a significant CD11b upregulation on leukocytes compared to the no beads sample. CD11b upregulation was also observed with isolated leukocytes in serum, however no significant differences were present between the beads and no-bead samples, because of the high background activation. CD11b upregulation on leukocytes without beads in serum was significantly higher than in heparinized plasma. Both serum and plasma came from the same pool of donors, dismissing the possibility of an inter-individual variation. Compared to plasma, serum is more likely to contain activated complement products and other inflammatory mediators generated by

activation of the coagulation cascade (e.g; platelet release, kallikrein). Indeed, recent work by Karlsson et al[30;31] showed that serum from blood (clotted for 10 minutes) induced CD11b upregulation, leukocyte priming, increased respiratory burst and intracellular calcium levels on neutrophils. Different phospholipids were also found in serum, and the authors believed that they may potentially be important intercellular signalling molecules[31] that are responsible for the observed leukocyte activation. In addition, significant complement activation was generated by the glass tube used to prepare serum[30]; and it likely contributed to the observed serum-induced leukocyte activation. Thus, the inflammatory products generated during serum preparation may have primed leukocytes to respond strongly to the complement activation induced by the polypropylene tube during our 2-hour incubation period. This hypothesis is further supported by previous results showing that adding sCR1 to the rest sample was able to significantly reduce leukocyte activation, even in the absence of beads (data not shown). When studying mechanisms of leukocyte activation, serum is usually thought to represent a better system since no anticoagulant is added. However, it appears that the presence of inflammatory mediators generated during blood clotting primes leukocytes and results in unwanted leukocyte activation.

On the other hand, the disadvantage of using heparinized plasma over serum is that heparin has recognized inhibitory effects on complement activation[32]. However, the inhibitory effect of heparin on complement activation varies with the type of heparin, concentration, incubation time and material[33-35]. In fact, we (see Chapter 6) and others[35;36] have shown that 5 U/mL of heparin did not have a significant inhibitory effect on complement activation. It is then advised that heparinized plasma, at 5 units/mL or lower concentration, be used when studying leukocyte/material interactions.

2) Complement activation with PS and PS-PEG beads

PEG or PEO grafting is often thought of as a solution to blood compatibility as it prevents protein adsorption and platelet adhesion[37;38]. Although blood compatibility is not limited to platelet-material interactions, few studies have actually studied complement activation by PEO or PEG-grafted surfaces. In our *in vitro* system, PS-PEG was significantly more complement activating than PS as it generated more SC5b-9. PEO grafting on silane glass has also been shown to result in a higher degree of complement activation than silane glass[39]. One hypothesis is that the presence of hydroxyl groups at the end of the PEG chain

is responsible for this increased complement activity. It is important to note that for the PS-PEG beads, the PEG chains were immobilized on the PS matrix by anionic graft copolymerisation to ensure a free functional hydroxyl group[40]. Hydroxyl and amino groups are recognized to allow covalent binding of C3b[10]. Attachment of C3b to the surface would result in the formation of the alternative pathway C3 convertase. The clustering of C3b around the C3 convertase, conferred by the presence of numerous hydroxyl groups on the surface, may then lead to the formation of the C5 convertase with generation of C5a and C5b-9[10;41]. The activating effect of PEG on complement may also be explained by the low molecular weight of the chain used in our study (3000 kd) and it might not be as effective in repelling protein. Also the PS surface may also not be entirely grafted with PEG and then the PEG would not be able to effectively prevent protein adsorption. Since we did not characterize protein adsorption directly, we cannot explain the increased complement activation observed with PS-PEG beads. However, these results underscore that when using PEG coating, conclusions on the biocompatibility of this surface should be drawn with caution. While PEG treatment of surfaces prevents platelet adhesion, it may not be the solution to blood-compatibility since, in some cases, the presence of PEG strongly activates complement.

3) Leukocyte activation and complement inhibition in the isolated leukocyte system

Table 8 summarizes the effects of the various complement inhibitors on leukocyte activation and adhesion.

a) Pentamidine

Pentamidine significantly reduced complement activation and CD11b upregulation in bulk with both PS and PS-PEG. However, leukocytes incubated in the presence of pentamidine were unable to further upregulate CD11b in the presence of PMA. PMA is a direct protein kinase C activator and bypasses receptor-mediated pathways of activation. Pentamidine thus appeared to be interfering with leukocyte intracellular signalling mechanisms, suggesting that pentamidine's effect on leukocytes was not related to the associated complement inhibition. Its inhibitory effects on platelet activation[17;42] and complement had made pentamidine an interesting prospect to resolve material incompatibility. However, pentamidine's side effects on leukocytes will likely limit the pharmaceutical potential of this drug. Indeed, neutropenia in AIDS patients is a frequently

Table 8: Summary table on the effect of various complement inhibitors on material-induced activation of isolated leukocytes in heparinized plasma.

Isolated leukocytes		sCR1	P5P	Pentamidine	NAAGA
Bulk	TF	-	-	↑	↑
	CD11b	Material-dependent (↓ with PS-PEG)	Material dependent (↓ with PS-PEG)	↓	↓↓↓
	Annexin V	↓	↓↓	N/D	N/D
Adhesion	CD45 CD14	Material-dependent (↓ with PS-PEG)	Material-dependent (↓ with PS-PEG)	↓	↓
Adherent	TF	-	-	-	-
	CD11b	Material-dependent (↓ with PS-PEG)	Material-dependent (↓ with PS-PEG)	Material-dependent (↓ with PS)	↓
	Annexin V	-	-	N/D	N/D

- No effect

N/D: Not determined

occurring side effect of pentamidine's use[43]. Pentamidine is an antimicrobial agent and these agents have also been recognized to compromise phagocytic cell functions[44]. Pentamidine has actually been found in vitro to interfere with neutrophil degranulation[45], and inhibit superoxide radical formation[46] and chemotactic cytokine release[47]. The inhibitory effect of pentamidine on PMA-induced CD11b upregulation was in the range of therapeutic concentrations. While others have not previously reported pentamidine's effect on CD11b upregulation, our results agree with previous reports[45-47] showing that the inhibitory effect of pentamidine on leukocytes is at the intracellular level and not via inhibition of inflammatory mediators such as complement activation. The mechanism, by which pentamidine acts on leukocyte activation, remains unknown. Thus despite interesting platelet and complement inhibitory properties, pentamidine, given as a drug, does not represent a viable solution to the bioincompatibility problem since it significantly impairs leukocyte signalling functions.

b) NAAGA

NAAGA totally inhibited CD11b upregulation on bulk isolated leukocytes with PS and PS-PEG beads. Complement activation was also inhibited by NAAGA. However, the effect of NAAGA on leukocytes is unlikely to be related to complement inhibition since, like pentamidine, PMA was unable to induce CD11b upregulation. Moreover, all other inhibitors, when effective, were seen to prevent biomaterial associated CD11b upregulation (i.e.: CD11b upregulation with beads and inhibitors was similar to the no-bead level) while NAAGA totally abrogated CD11b expression on leukocytes to a level lower than the one observed with EDTA. Washing leukocytes, that had been first in contact with beads and NAAGA, prior to incubation with anti-CD11b did not change CD11b expression, suggesting that NAAGA had either altered the CD11b binding epitope in an irreversible manner or that NAAGA, by interacting with calcium pools or channels, interfered with CD11b expression. The latter hypothesis seems to agree with other experiments where the treatment of isolated leukocytes with NAAGA was shown to inhibit release of histamine[48] and leukotriene B_4[49], in a mechanism thought to involve selective inhibition of calcium influx from the extracellular cytoplasm.

c) sCR1

In accordance with Cheung et al.[50], complement activation was inhibited by more than 90% by sCR1 with both PS and PS-PEG beads. On the other hand, CD11b upregulation was only partially reduced by complement inhibition, and only with PS-PEG beads. The

effect of sCR1 on isolated leukocytes was not an effect of heparin since similar results were found with serum. The inadvertent presence of a few platelets during incubation with isolated leukocytes was unlikely to have contributed to PS-induced leukocyte activation since adding anti-IIb/IIIa during the experiments was without any effect (data not shown). PS-PEG generated high levels of SC5b-9 and thus can be characterized as a strong complement activating material, when compared to PS. Considering the complement activating property of our materials, the fact that sCR1 was only effective in reducing CD11b upregulation with a high complement activating material (PS-PEG) but not with a moderate activator is then not as surprising. The results with sCR1 also tend to suggest that the terminal pathway of complement (C5a, C5b-9) is more important in leukocyte activation (CD11b upregulation) than the common pathway (C3a, C3b, iC3b). Moreover, the absence of an effect of sCR1 for PS-induced leukocyte activation implies that with some materials, other mechanisms of activation (independent of complement) exist, at least for isolated cells.

The material-dependent effect of sCR1 on activation of isolated leukocytes agrees with our previous work with discs of biomaterials, showing that sCR1 was only effective in reducing leukocyte activation with high complement activating materials such as cellophane[21]. Rozenkranz et al. also showed CD11b upregulation induced by cuprophan on isolated neutrophils to be only partially inhibited by sCR1, leaving neutrophils still significantly activated when compared to the control[51]. In their study, they also demonstrated that complement-dependent neutrophil activation was dependent on a C5-mechanism, which supports our observation. Cheung et al.[50] also noted that the inhibitory effect of sCR1 on leukocyte release was only partial (25 to 70%) and material-dependent, suggesting that material-induced neutrophil activation was mediated in part by pathways other than complement. Other pathways of activation, such as cytokines[52], have been shown to upregulate CD11b, even in the presence of sCR1. Indeed, in the absence of complement, interleukin-1 and arachidonic acid metabolites, two inflammatory mediators able to support leukocyte activation, have been observed with some hemodialysis membranes[53]. With some materials, such as PS, several pathways of the inflammatory response may be activated (complement being only one part of it), while with others, such as PS-PEG, the inflammatory response to this foreign material may be centered on complement activation. Thus, generalization on the mechanisms of material-induced leukocyte activation should be done

with caution: while materials induce similar level of leukocyte activation, the mechanisms, by which activation is regulated, differ and depend on the material's properties.

d) Pyridoxal 5-phosphate

P5P, a compound related to vitamin B6, significantly reduced CD11b upregulation induced by PS-PEG beads while it had only a small effect with PS-beads. We were unable to measure any significant complement inhibition by P5P, even though P5P is believed to be a C1 inhibitor. P5P, at 2mM (the same concentration that was used in our study), totally inhibited C1 activation, by reacting with lysyl residues on C1q and inhibiting its ability to associate with C1s-C1r-C1r-C1s[54]. It must be noted that inhibition at the C1 level may not result in a significant inhibition of complement activation, as other pathways are able to bypass this component to still activate complement. P5P, at low concentrations and for short incubation times (up to 10 minutes), is a leukocyte anti-aggregant[55] without any effect on other leukocyte functional parameters (adhesion, respiratory burst)[56]. How P5P acts on leukocytes at higher concentration and for longer period of times such as our 2-hour incubation has not been reported. On the other hand, we know that, due to its negative charge and hydrophilic nature, P5P does not cross the cell membrane[57]. In the concentration range used here, P5P has also been reported to inhibit platelet aggregation[58;59] and clotting[60], most likely due to a modification of the fibrinogen binding site on thrombin. The mechanism by which P5P inhibits platelet aggregation may be similar to how P5P inhibit leukocyte activation. With platelets, P5P affected neither c-AMP levels [61] nor Ca^{2+} mobilization induced by calcium ionophore[58]. On the other hand, P5P inhibited platelet aggregation induced by ADP and thrombin[58;62]. Taken together, these studies suggest that P5P has a direct effect on receptor-mediated pathways of platelet activation and that it is interacting with specific glycoproteins on the platelet membranes. It is then conceivable that P5P also interacts with a leukocyte receptor to inhibit CD11b upregulation induced by PS-PEG beads: however, our results do not allow us to speculate which membrane activation pathway P5P may inhibit. Harrington et al[58] also speculated that P5P has a direct effect on Na^+/H^+ exchange or on its mechanisms of platelet activation. This might also be true for its effect on leukocyte activation. On the other hand, another hypothesis is that the P5P effect on leukocyte activation is directly linked to its inhibition of C1q. C1q has been demonstrated to have inflammatory functions with leukocytes, enhancing phagocytosis[63;64] and oxidative burst[64;65]. Also, stimulated but not resting neutrophils selectively respond to elevated levels

of free C1q, resulting in enhanced CD11b expression[66]. Our present data does not allow us to conclude if P5P affects leukocyte activation via C1q inhibition or via its interaction with receptor-mediated leukocyte activation. Further experiments with C1-INH will help in understanding the mechanisms of action of P5P.

The fact that P5P had a significant effect on monocyte activation induced by PS beads but not on neutrophil activation underscores the existence of activation mechanisms that are different for neutrophils and monocytes. The different effect of a stimulus on monocytes and neutrophils is not uncommon[67;68].

P5P was found to significantly reduce CD11b upregulation and exposure of phosphatidylserine associated with PS-PEG beads, while it had only a minor effect with PS beads. As for sCR1, the material-dependent effect of P5P on leukocyte activation is likely related to the fact that PS and PS-PEG activate different mechanisms of leukocyte activation. PS-PEG may be a material activating the classical pathway of complement more than PS does. Or, PS and PS-PEG may have similar classical complement activity but PS may activate other pathways of leukocyte activation that can bypass a C1q-dependent mechanism. The latter hypothesis appears more reasonable, since, from our results, leukocyte activation induced by PS-beads occurs via complement-dependent and independent mechanisms, while for PS-PEG, complement seems to be playing a more central role. On the other hand, upon contact with PS-PEG but not PS, leukocytes may release an inflammatory mediator (such as a cytokine or platelet activating factor) with which P5P interacts to block the signal for leukocyte activation. However, in the absence of any data on classical complement activation by PS and PS-PEG, and not knowing the specific mechanisms of actions of P5P, only suggestions can be made. Further studies are warranted to fully elucidate the properties of P5P.

4) Leukocyte adhesion and complement inhibition in the isolated leukocyte system

Since NAAGA and pentamidine affected leukocyte-signalling mechanisms, results on adhesion with these two inhibitors will not be discussed.

Significant leukocyte adhesion and activation of adherent cells was observed on both PS and PS-PEG and to similar levels, despite the presence of PEG on the latter beads. Since complement inhibition, by means of sCR1, inhibited leukocyte adhesion on PS-PEG but not

PS beads, it appears that leukocyte adhesion at least on this material is complement dependent.

On PS-PEG beads, both sCR1 and P5P were effective in blocking adhesion and activation of adherent leukocytes. P5P blocked leukocyte adhesion on PS-PEG beads, just as well as sCR1, even though iC3b levels remained high with P5P. This suggests that C1q may be an important mediator in adhesion to PS-PEG beads or that P5P interferes directly with leukocyte adhesion on PS-PEG. The latter hypothesis is less likely as, if P5P interfered directly with leukocyte adhesion on PS-PEG, it should also have had some effect on leukocyte adhesion on PS. Further experiments with C1-INH or measurement of C1q on PS-PEG will help in understanding the mechanisms by which P5P acts on adhesion with PS-PEG beads.

Despite the absence of iC3b on PS beads with sCR1, leukocyte adhesion still occurred in heparinized plasma. The fact that isolated leukocytes are able to adhere to surfaces in the absence of complement activation and platelets is not in itself surprising since leukocyte adhesion has been reported even in the absence of plasma proteins[69]. The leukocyte adhesion observed here with sCR1 is more likely mediated by fibrinogen adsorbed on PS beads than by the few platelets that might be present. Tang et al.[70;71] also found similar level of leukocyte adhesion to Mylar® films adsorbed with normal or decomplemented plasma. Fibrinogen and IgG were shown to play an important role in leukocyte adhesion on PET rather than complement products[70]. Our serum results tend to reinforce the fact that fibrinogen is an important ligand in leukocyte adhesion to PS beads, as less leukocyte adhesion was observed on PS beads in serum (where no fibrinogen is left) than in plasma. [71].

The fact that leukocytes adherent to PS beads were more activated with sCR1 than without sCR1 remains a mystery. This is not due to endotoxin contamination of sCR1 since it tested negative for endotoxin. A similar increase in activation on adherent leukocytes was also observed with P5P. In both cases, this enhanced activation on adherent leukocytes was observed in plasma only, but not in serum, which tends to suggest that adsorbed fibrinogen may play a role. In the absence of complement products adsorbed on the surface, adhesion through fibrinogen may be favoured and trigger signalling mechanisms that lead to higher levels of activation. Indeed, Anderson et al[72-74] have found that fibrinogen on polystyrene activated monocytes to produce certain cytokines but not others.

The fact that sCR1 was effective in blocking adhesion to PS-PEG also suggests that fibrinogen adsorption was minimal on these beads (as expected, since PEG is recognized to prevent protein adsorption). Thus, in the absence of both complement products and fibrinogen on the surface, the PS-PEG beads were not able to support leukocyte adhesion. Our results on the effects of sCR1 on leukocyte adhesion all point toward fibrinogen as being an important ligand for adherent leukocytes. With isolated leukocytes, fibrinogen may thus be more important than complement products in mediating adhesion to materials and subsequent activation. These results also underscore how material properties, such as their ability to selectively adsorb proteins, are an important parameter in adhesion and activation of adherent leukocytes.

The presence of annexin V binding on leukocytes adherent to PS and not PS-PEG suggest that exposure of phosphatidylserine is governed by a different mechanism of activation than CD11b upregulation, since both PS and PS-PEG induced CD11b on adherent leukocytes. The elevated annexin V signal on PS beads cannot be explained by the few platelets present on the surface since both PS and PS-PEG had similar background signals of CD41. Again, difference in material properties, such as difference of protein adsorption or material chemistry, may be linked to difference in exposure of phosphatidylserine.

V. Conclusion

Following contact with PS and PS-PEG beads, isolated leukocytes in plasma in the absence of platelets were found to express less procoagulant activity than in whole blood: only a significant CD11b upregulation was observed, while TF expression and exposure of phosphatidylserine remained at background levels. None of the complement inhibitors used were able to effectively reduce the background fluorescence of TF expression or phosphatidylserine exposure, suggesting that the cells were primed during the isolation procedure and inflammatory inhibitors other than complement were involved in the background expression. Pentamidine and NAAGA inhibited complement and were effective in reducing CD11b upregulation for PS and PS-PEG. However, they also affected directly leukocyte signalling mechanisms, which make them unsuitable to improve blood-material compatibility.

Complement inhibition via sCR1 significantly reduced CD11b upregulation only in the presence of a high complement activating surface such as PS-PEG. The lack of effect of

sCR1 on leukocyte activation and adhesion induced by PS beads but not on PS-PEG suggests that with isolated leukocytes, despite similar levels of leukocyte activation, (1) mechanisms of activation are material-dependent, and that (2) complement-independent mechanisms of leukocyte activation exist. The CD11b inhibition by P5P was also material-dependent and further showed that generalization on the mechanisms of material-induced leukocyte has to be done with caution, as no one material activates leukocytes via the same mechanisms than another.

Our results with isolated leukocytes and sCR1 also suggest that adsorbed proteins other than complement, such as fibrinogen, play an important role in mediating leukocyte adhesion and activation on the surface. This further underscores that the low complement activation of a material does not preclude low leukocyte activation and adhesion. Material chemistry and properties can also affect leukocyte activation even in the absence of complement activation.

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Chapter 6

Role of complement and platelets in leukocyte activation induced by polystyrene and PEG-immobilized polystyrene beads in whole blood

Abstract

In whole blood, inhibiting both complement and platelets significantly reduced material-induced leukocyte activation. In the presence of sCR1 (a specific complement inhibitor), material-induced CD11b upregulation in bulk was significantly reduced only with a high complement-activating surface (such as PS-PEG) but not with the moderate activating PS. sCR1 had no significant effect on material-induced TF expression or platelet-leukocyte aggregates. Anti-GPIIb/IIIa (a GPIIb/IIIa platelet receptor antagonist) only partially reduced material-induced TF expression and platelet-leukocyte aggregates, while it had no effect on CD11b upregulation. Both adherent platelets and adsorbed complement products mediated leukocyte adhesion to materials. Complement played a role in CD11b upregulation of adherent leukocytes, while TF expression on adherent monocytes was dependent on adherent platelets. Combining both sCR1 and anti-GPIIb/IIIa resulted in a significant reduction (but not total) of material-induced TF expression and platelet-leukocyte aggregates, while CD11b upregulation and leukocyte adhesion were blocked. Pyridoxal-5-phosphate (P5P) alone significantly reduced both material-induced TF expression and CD11b upregulation, in a way we believed to be associated with its C1q and platelets inhibitory activities.

Material-induced leukocyte activation, associated with expression of procoagulant activities, appeared to be secondary to both platelet and complement activation. This suggests that blood-material compatibility will be greatly improve by the presence of biomaterial that minimally activates both platelet and complement or by the use of therapeutic agents, such as maybe P5P, that simultaneously affects platelets and complement.

I. Introduction

With the use of cardiovascular devices, thrombotic complications continue to occur despite anti-platelet and anticoagulant therapies. Leukocyte activation, leading to the expression of procoagulant activities, may contribute to thrombotic failure of cardiovascular devices. In Chapter 3, monocytes were found to express functional TF, and CD11b upregulation and platelet-leukocyte aggregates were observed following material contact. In Chapter 5, phosphatidylserine exposure was also demonstrated on isolated leukocytes. TF expression, by activating the extrinsic coagulation cascade; CD11b, by binding Factor X or fibrinogen; phosphatidylserine by promoting coagulation complexes binding; and platelet-leukocyte aggregates, by creating a microenvironment for activation; are all potential ways by which leukocytes may participate in thrombin formation. TF expression on monocytes following blood-material contact has been reported by others *in vitro*[1,2] and *in vivo*[3-5] but the mechanisms of its expression remains unknown. A role for complement has been suggested in CD11b upregulation of bulk leukocytes but it is unclear as to whether it is exclusively dependent on complement or not. As for adherent leukocytes in whole blood, very little is known, apart from the fact that complement (and other proteins such as fibrinogen) are likely playing a role in adhesion. Understanding the mechanisms leading to the expression of procoagulant activities on leukocytes, both in the bulk and adherent to surfaces, will offer valuable means to improve blood-compatibility.

Blood-material interactions are complex since many protein systems (complement, coagulation) and blood cells are activated. We have hypothesized that both platelets and complement play a role in leukocyte activation and that they affect TF and CD11b in different ways. Using isolated leukocytes, a role for complement in CD11b upregulation was shown to be dependent on the material properties (Chapter 5), while platelets were shown to play a role in TF expression (Chapter 4). Since the isolation procedure may create artefacts, whole blood experiments were undertaken to determine the role of complement and platelet activation in whole blood. We hypothesized that inhibiting complement activation and platelets in whole blood reduced material-induced leukocyte activation. To isolate platelet effects, a complement inhibitor, a recombinant soluble form of the CR1 receptor (sCR1), was used: sCR1 is a specific complement inhibitor blocking the classical and alternative convertases by binding to C3b and C4b and promoting their inactivation by Factor I[6]. Platelet activation due to fibrinogen binding was blocked using Reopro, a platelet antagonist

for GPIIb/IIIa. Promising results obtained with Pyridoxal-5-phosphate (P5P) (Chapter 5 and Gemmell's work[7]), the biologically active coenzyme form of vitamin B6 compounds which inhibits the classical pathway by modifying C1q[8], led us to also test this compound in whole blood.

To be consistent with the isolated leukocyte experiments (Chapter 5), whole blood experiments were first run in heparinized whole blood. For the isolated leukocyte experiments, heparin had been chosen over PPACK, due to PPACK's instability and the long process required for leukocyte isolation.

II. Materials and methods

1) Reagents and antibodies

The same reagents and antibodies as in Chapter 5 were used. Reopro® Abciximab (anti-IIb/IIIa) was from Eli Lily and Company (Indianapolis, IN). The saturating concentration of Reopro had been previously determined at 25 μg/mL by Gemmell (personal communication, 2000). The same antibodies for flow cytometry and reagents were used as in Chapter 5.

2) Beads

The same 45μm beads were used as in previous chapters: polystyrene (PS) and TentaGel beads, polystyrene beads immobilized with polyethylene glycol (PS-PEG). They were cleaned from endotoxin contamination as described earlier and resuspended in endotoxin-free PBS. In all experiments, the residual endotoxin on the beads was equivalent to a concentration of endotoxin that was less than 0.1 EU/ml, which is below the recommended maximum FDA level[9] of 0.5 EU/mL.

3) Blood-material contact

Whole blood from normal human volunteers was drawn into syringes preloaded with anticoagulant (5 Units/mL heparin or 120μM PPACK) after discarding the first millilitre. Blood was incubated with only one concentration of beads ($2x10^5$ beads/mL – equivalent to a surface area to volume ratio of $13cm^{-1}$) for 2 hours at 37°C on a haematology mixer. The inhibitors were added one at a time or in combination with anti-IIb/IIIa to the blood before the beads at the following concentrations: sCR1 (200 μg/mL), P5P (500 μg/mL), and anti-IIb/IIIa (25μg/mL). For the negative control, EDTA (8 mM EDTA final concentration) was

added to blood and beads while the positive control was blood incubated with endotoxin (5 μg/mL) without beads.

As previously described, following incubation, beads were allowed to settle and small blood aliquots were taken for flow cytometry analysis of non-adherent cells. To analyse adherent cells on beads, red blood cells were lysed for 10 minutes with FACSlyse (Becton-Dickinson). Beads were washed with RPMI/FBS and then incubated with the appropriate antibodies. The introduction of FACSLyse as a lysing buffer represents a modification from the protocol used in Chapter Three. FACSlyse is a prepared solution that lyses RBC more effectively than the NH_4Cl lysing buffer. FACSlyse also contains fixative and this ensures that cell activation does not occur during the lysing process[10]. The disadvantage of the fixation step introduced with FACSlyse is that fixation before staining is recognized to increase expression of some receptors[11,12] (namely CD45, CD14 and CD41 in our case). Thus, comparisons of the flow cytometry adhesion results between Chapter 3 and 6 should be avoided.

In some experiments, SEM (Hitachi, S-520) was performed on beads following fixation in 2.5% glutaraldehyde and dehydration in a graded series of ethanol solutions.

4) Flow cytometry

Flow cytometric analysis was carried out on blood and beads as described before in Chapter 3. Briefly, small aliquots (5 to 30 μL) of blood or beads, diluted in 50μL of HTB were incubated with saturating concentrations of fluorescently labelled monoclonal antibodies for 30 minutes at 4°C. For leukocyte activation in the bulk, following incubation with antibodies, erythrocytes were lysed for 10 minutes in the dark with FACSlyse. They were then washed, diluted in HTB and fixed with paraformaldehyde (1% final concentration). All other samples were directly diluted in HTB and fixed. Samples were analysed on a Becton Dickinson FACScan flow cytometer (Mountain View, CA), using CELLQuest software.

5) Complement activation in the presence of anticoagulants

Serum, from at least four individuals, was prepared following standard procedures. Pooled aliquots were stored at –70°C. They were thawed and ultracentrifuged for 3 minutes prior to experiments.

To isolate the effect of anticoagulants, heparin (5 U/mL final concentration) or PPACK (final concentration 120 μM) was added to serum and the samples with beads were incubated for 2 hours at 37°C on the haematology mixer. After a 2 hour-incubation, EDTA was added (8 mM final concentration), samples were centrifuged and serum aliquots frozen at –70°C. Complement activation in the bulk and adsorption on beads were assessed as previously described in Chapter 5 by flow cytometry and ELISA respectively.

6) Statistical analysis

All results are reported as means ± SD. To evaluate the significance of the differences in cell activation, analysis of variance (ANOVA) was carried out followed by the Student-Newman-Keuls test, which is a protected test for multiple comparisons[13]. A *p*-value of less than 0.05 was required for statistical significance. The number of repeat experiments was equal to or greater than three with three different donors.

III. Results

1) Effect of sCR1, P5P and anti-IIb/IIIa on leukocyte adhesion.

Table 1 summarizes flow cytometry results on leukocyte and platelet adhesion on PS and PS-PEG beads. Figures 1 to 4 show the effects of complement inhibition and platelets on leukocyte adhesion (Figures 1 and 2) and the level of activation on adherent leukocytes (Figure 3, TF and Figure 4, CD11b).

Table 1 shows that similar levels of leukocyte adhesion (CD45 on beads: neutrophils and monocytes) were observed on PS and PS-PEG beads. Monocyte adhesion (CD14 signal on beads, Table 1) was significantly less on PS-PEG than on PS, suggesting that while fewer monocytes adhere to PS-PEG, neutrophils strongly adhered to this surface. These observations are in accordance with our previous results in PPACK whole blood (Chapter 3).

Both platelets and complement played a role in leukocyte adhesion to PS beads (Table 1). On the other hand, for PS-PEG beads, which did not support platelet adhesion, leukocyte adhesion was reduced to background levels with complement inhibition (sCR1) alone. P5P alone had no effect on leukocyte adhesion to PS beads. However, P5P in combination with anti-IIb/IIIa reduced leukocyte adhesion to background levels. All inhibitors had an effect on CD14 and CD45 signals, suggesting that they acted indiscriminately on monocyte and neutrophil adhesion (Table 1).

Table 1: Effect of complement inhibition and anti-IIb/IIIa on leukocyte adhesion and activation and platelet adhesion on PS and PS-PEG beads in heparinized whole blood.

	Leukocyte adhesion (CD45, %)[a]	Monocyte adhesion (CD14, %)[a]	TF on beads (%)[a]	CD11b on beads (%)[a]	Platelet adhesion (CD41, %)[a]
Beads + EDTA	100 ± 0 [#,*]	100 ± 0 [#,*]	100 ± 0 [#,*]	100 ± 0 [#,*]	100 ± 0 [#,*]
PS	272 ± 121	217 ± 45	263 ± 45	891 ± 286	1479 ± 368
+ sCR1	165 ± 41 [#]	176 ± 32 [#]	188 ± 28 [#]	368 ± 122 [#]	1514 ± 525
+ P5P	243 ± 85	183 ± 39	197 ± 36 [#]	525 ± 241	1041 ± 336 [#]
+ anti-IIb/IIIa	164 ± 45 [#]	167 ± 30 [#]	158 ± 27 [#]	1108 ± 300	165 ± 16 [#]
+ sCR1 + anti-IIb/IIIa	112 ± 36 [#]	109 ± 19 [#]	124 ± 18 [#]	185 ± 127 [#]	109 ± 30 [#]
+ P5P + anti-IIb/IIIa	121 ± 31 [#]	113 ± 14 [#]	130 ± 18 [#]	129 ± 32 [#]	120 ± 15 [#]
PS-PEG	232 ± 21	124 ± 13 [#]	146 ± 10 [#]	1006 ± 163	186 ± 5 [#]
+ sCR1	125 ± 19 [*]	107 ± 4	115 ± 10 [*]	166 ± 64 [*]	114 ± 10 [*]
+ anti-IIb/IIIa	238 ± 21	122 ± 6	156 ± 13	1071 ± 259	192 ± 17
+ sCR1 + anti-IIb/IIIa	128 ± 21 [*]	108 ± 6	110 ± 11 [*]	158 ± 55 [*]	113 ± 12 [*]

N = 3 to 6 ± S.D.

[a] TF, CD14, CD45, CD11b and CD41 signals on beads are represented as a percentage relative to beads+EDTA.

[#] Statistically significant from PS beads ($p \leq 0.02$)

[*] Statistically significant from PS-PEG beads ($p \leq 0.02$)

Scanning electron microscopy, as illustrated in Figures 1 and 2, confirmed the leukocyte adhesion results obtained with flow cytometry.

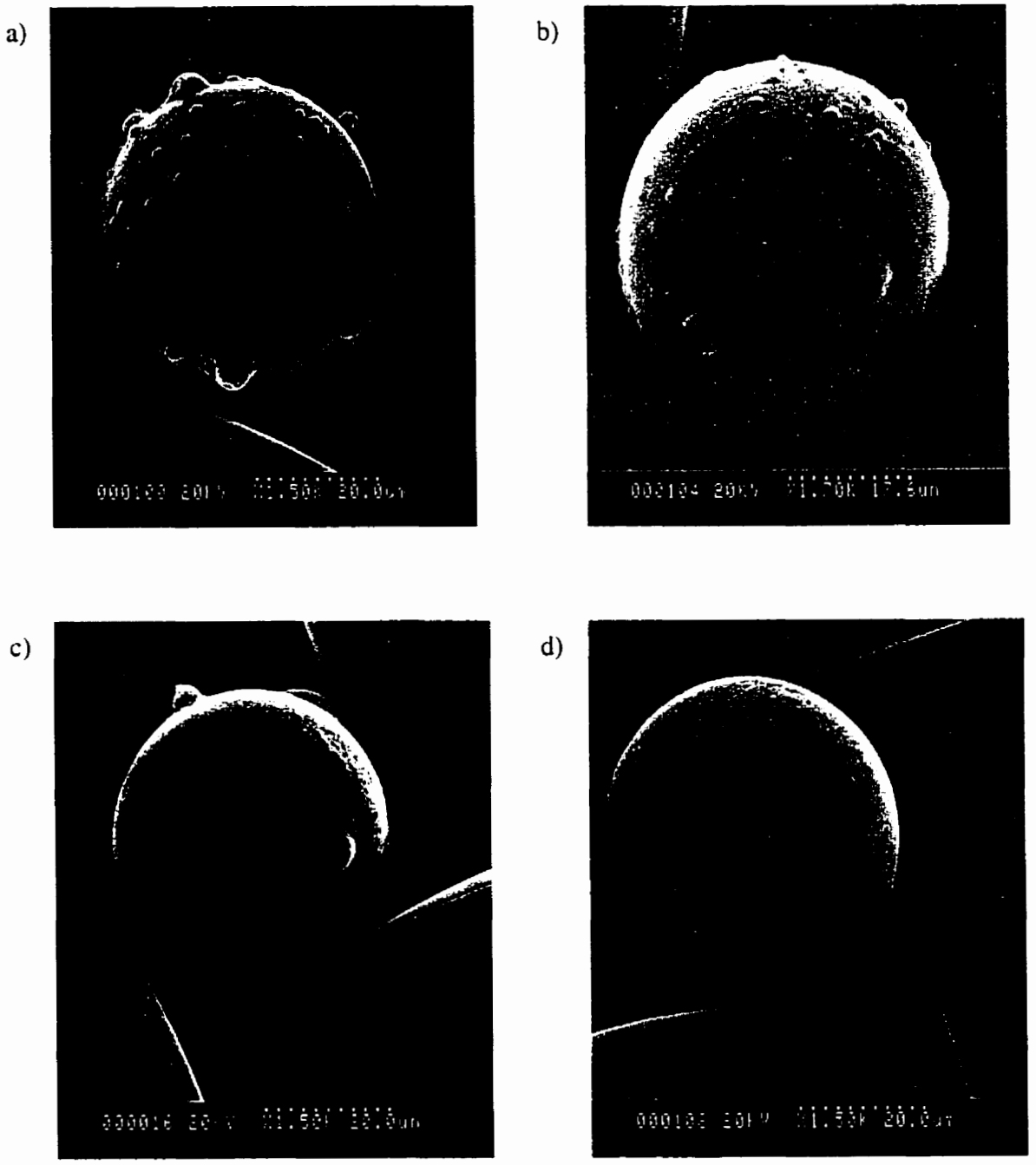

Figure 1: Scanning electron micrographs of PS beads with and without inhibition of complement and platelet activation in heparinized whole blood: (a) no inhibitor, (b) with sCR1, (c) with anti-IIb/IIIa, (d) with sCR1 and anti-IIb/IIIa.

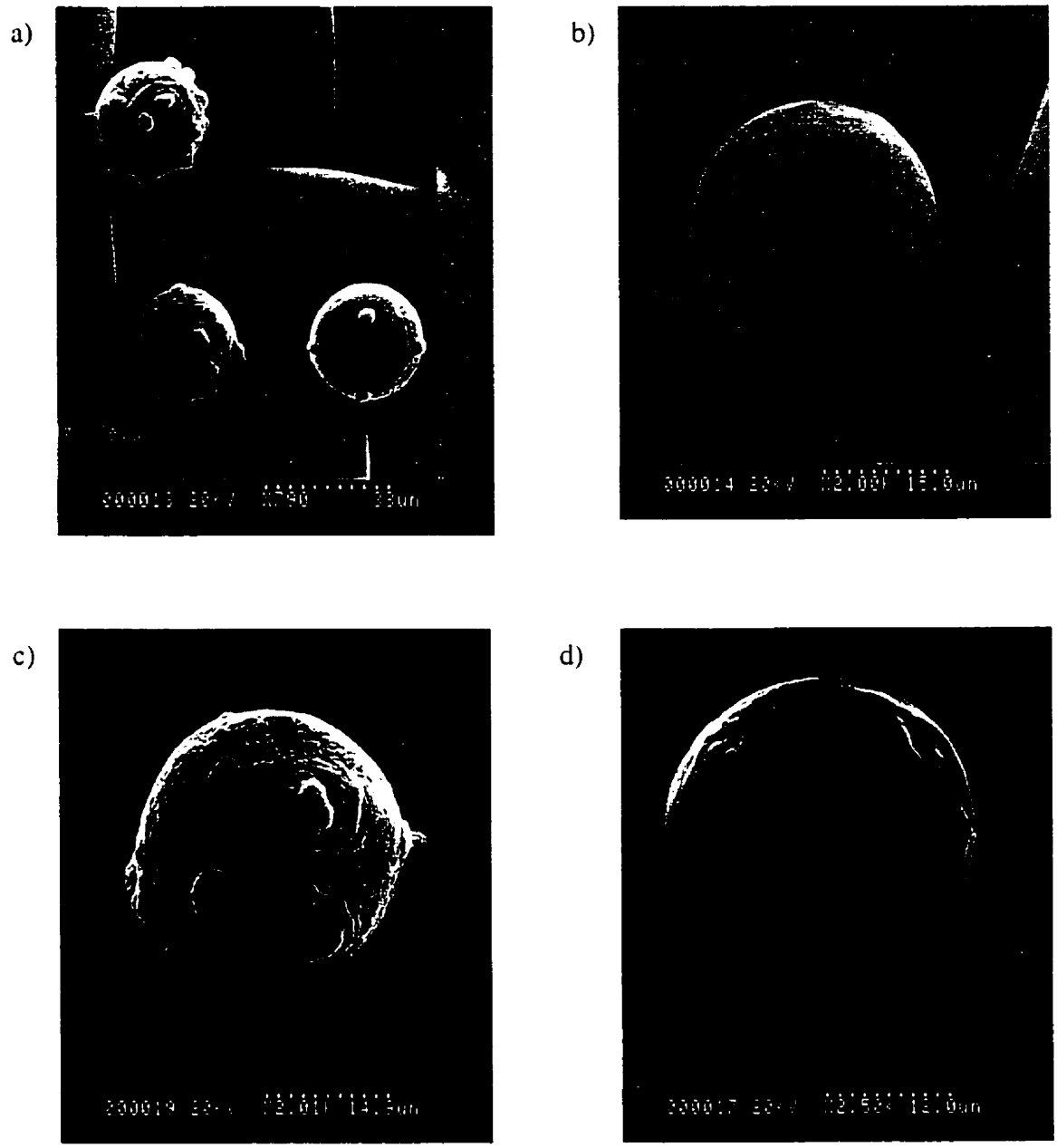

Figure 2: Scanning electron micrographs of PS-PEG beads with and without inhibition of complement and platelet activation in heparinized whole blood: (a) no inhibitor, (b) with sCR1, (c) with anti-IIb/IIIa, (d) with sCR1 and anti-IIb/IIIa. The creases observed in the micrographs of PS-PEG are artefacts generated during the fixation and dehydration process.

As shown by the TF signal on beads (Table 1), significant levels of TF were observed on monocytes adherent to PS and PS-PEG beads. All inhibitors alone or in combination significantly reduced TF signal on PS beads, while for PS-PEG only sCR1 had an effect. The reduction of TF signal on beads was usually associated with a reduction in monocyte adhesion (i.e., reduced CD14 signal). To ensure that the reduced TF signal on beads was a result of inhibition of activation rather than a consequence of a reduced number of cells, the index of activation was calculated: (% of TF expression on beads) divided by (% of CD14 on beads). As illustrated in Figure 3, the index of activation for TF indicates that only anti-IIb/IIIa and P5P significantly reduced TF expression per adherent monocyte on PS beads, suggesting that the other significant differences observed with TF signal on beads (Table 1) were most likely due to the associated reduced monocyte adhesion. The lack of significant reduction for the TF index of activation with sCR1 further suggests that complement activation plays a minimal role on the expression of TF on monocytes.

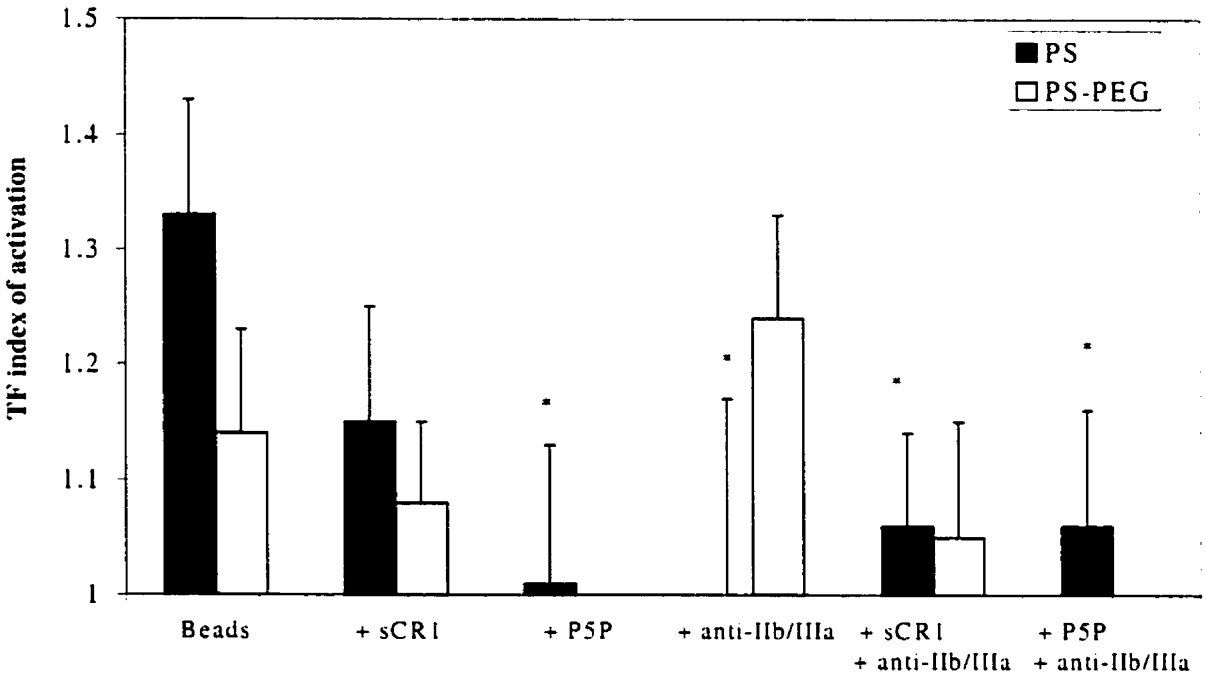

Figure 3: Effect of complement and platelets on the index of activation for TF expression on adherent monocytes in heparinized whole blood. P5P and anti-IIb/IIIa alone significantly reduced TF expression per adherent monocyte on PS beads. TF index of activation is calculated as the (% of TF on beads)/(% of CD14 on beads), whereby the % of TF and CD14 on beads are expressed relative to the signal on beads+EDTA. * Significantly different from PS beads without inhibitor ($p<0.04$). N = 3 to 6 ± S.D.

Similar levels of CD11b signals were observed on PS and PS-PEG beads (Table 1). Only sCR1 and P5P significantly reduced CD11b signal on cells on beads (Table 1). In Figure 4, the index of activation for CD11b shows that, for both PS and PS-PEG beads, complement inhibition with sCR1 significantly reduced CD11b upregulation per adherent leukocyte. On the other hand, the reduction observed when combining sCR1+anti IIb/IIIa or P5P+anti-IIb/IIIa was not significant when compared to inhibitor alone, suggesting that the small CD11b signals (Table 1) were more a consequence of reduced leukocyte adhesion than a reduced activation.

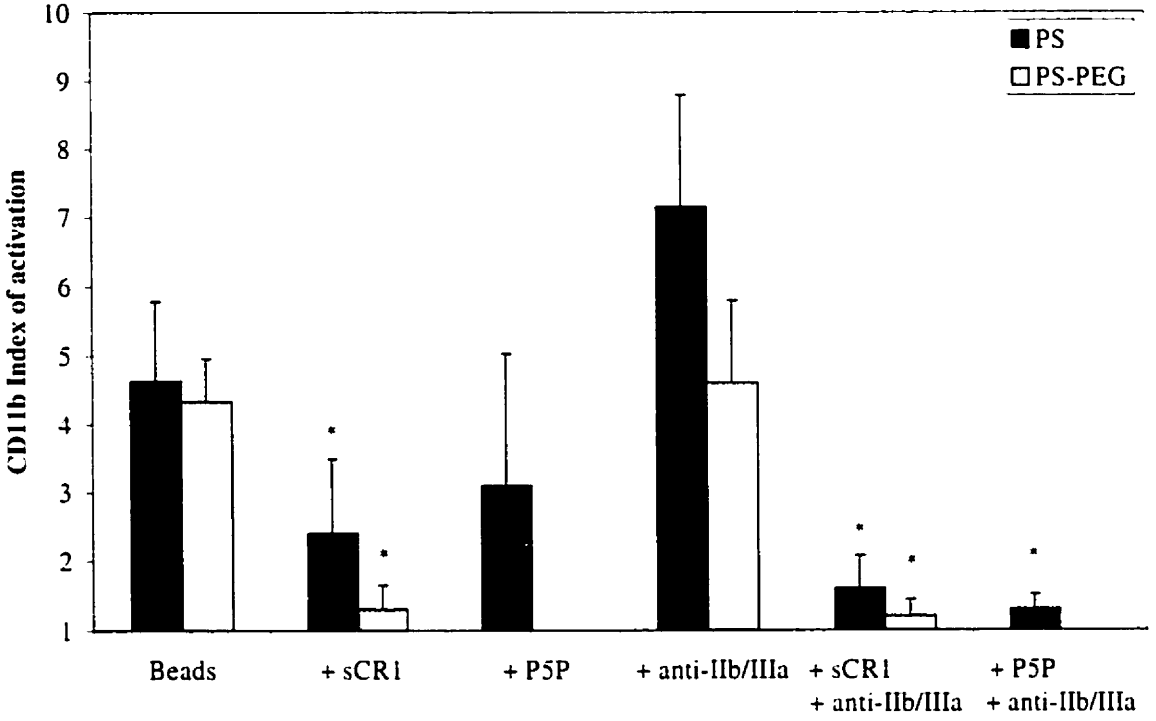

Figure 4: Effect of complement and platelets on the index of activation for CD11b upregulation on adherent leukocytes in heparinized whole blood. Complement inhibition via sCR1 significantly reduced CD11b upregulation on adherent leukocytes to PS and PS-PEG beads. CD11b index of activation is calculated as the (% of CD11b on beads)/(% of CD45 on beads), whereby the % of CD11b and CD45 are expressed relative to the signal on beads+EDTA.
* Significantly different from respective beads without inhibitor ($p < 0.05$). N = 3 to 6 ± S.D

2) Effect of sCR1, P5P and anti-IIb/IIIa on leukocyte activation in the bulk.

Table 2 and Figures 5, 6 and 7 show the effect of complement and platelets on TF expression (Table 2) and CD11b upregulation (Figure 6) on leukocytes in the bulk incubated

with PS beads. Table 3 and Figure 7 show the effect of complement and platelets on TF expression and CD11b upregulation with PS-PEG beads.

As shown in Table 2, only P5P alone was able to significantly reduce TF expression on monocytes (in the bulk) induced by PS beads. Anti-IIb/IIIa alone only reduced the number of monocytes expressing TF, but not the level of TF expression. Adding anti-IIb/IIIa to P5P further reduced TF expression. Combining anti-IIb/IIIa and sCR1 also resulted in a significant reduction of TF expression to the no beads level. TF expression in the no bead sample may appear relatively high compared to the initial TF expression. This may be explained by the presence of some platelet-monocyte aggregates that formed even in the absence of beads (due most likely to low levels of platelet and leukocyte activation). Interestingly, for a similar level of TF expression between sCR1+anti-IIb/IIIa and the no bead samples, similar low levels of monocyte-platelet aggregates were observed (Table 2). Indeed, Figure 5 shows a positive correlation (Pearson R = 0.75; $p < 0.001$) found between TF expression and monocyte-platelet aggregates, the correlation study was performed using data from all the experiments.

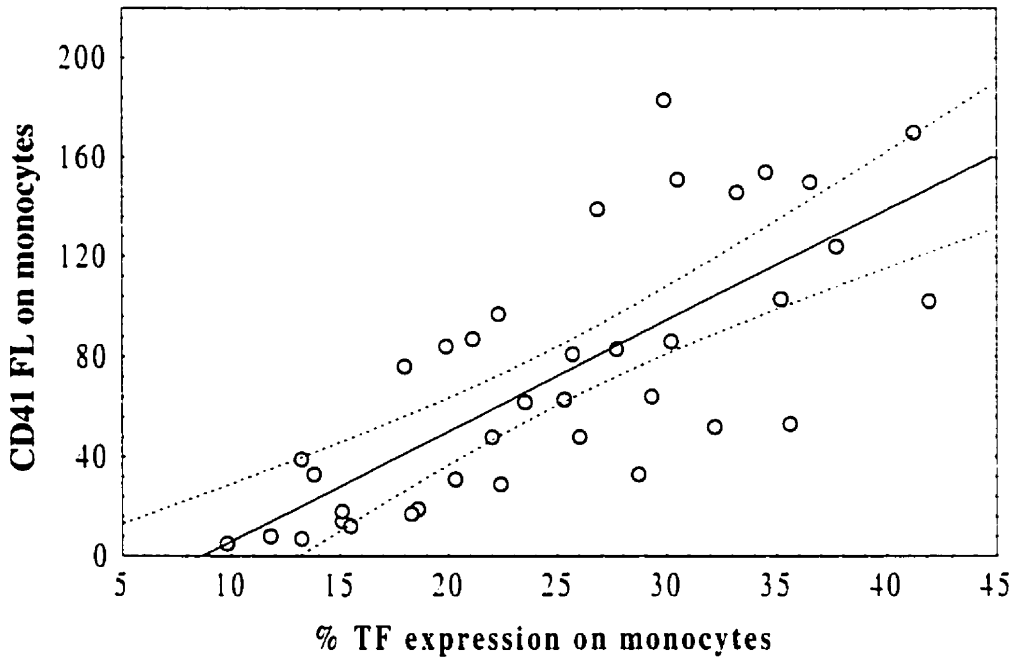

Figure 5: Correlation graph between TF expression on monocytes and platelet-monocyte aggregates (CD41 signal on monocytes) induced by PS beads in heparinized whole blood. (Pearson R = 0.75; $p < 0.001$; N = 37)

Table 2: Effect of complement inhibition and anti-IIb/IIIa on TF expression, platelet-leukocyte aggregates and platelet activation induced by PS beads in heparinized whole blood.

	Leukocyte activation			Platelet activation	
	TF expression (%)[a]	Monocytes expressing TF (%)[b]	Platelet-leukocyte aggregates (FL units)[c]	Platelet microparticles (%)	P-selectin expression (%)
t = 0	13 ± 2 *	0 ± 0 *	7 ± 3 *	6 ± 2 *	100 ± 0 *
No beads	22 ± 3 *	4 ± 3 *	34 ± 17 *	7 ± 3 *	100 ± 0 *
PS	35 ± 6	26 ± 10	88 ± 26	40 ± 10	256 ± 55
+ sCR1	31 ± 5	18 ± 11	95 ± 18	41 ± 6	240 ± 40
+ P5P	22 ± 6 *	6 ± 5 *	16 ± 7 *	14 ± 4 *	102 ± 4 *
+ anti-IIb/IIIa	30 ± 4	14 ± 6 *	25 ± 5 *	4 ± 1 *	100 ± 2 *
+ sCR1 + anti-IIb/IIIa	22 ± 4 *	4 ± 3 *	21 ± 10 *	4 ± 1 *	100 ± 0 *
+ P5P + anti-IIb/IIIa	16 ± 2 *	3 ± 3 *	7 ± 2 *	4 ± 1 *	100 ± 3 *

N = 4 to 6 ± S.D.

[a] TF expression is represented as a percent relative to the maximum where maximum expression is determined by the fluorescent intensity of leukocytes incubated with LPS for 2 hours at 37°C

[b] Monocytes staining positive for TF expression.

[c] CD41arbitrary fluorescence on leukocytes.

* Significantly different from PS beads ($p \leq 0.03$)

Figure 6 shows that, as expected from the results with isolated leukocytes, CD11b upregulation induced by PS beads in heparinized whole blood was unaffected by sCR1 or anti-IIb/IIIa. However, as opposed to results with isolated leukocytes, P5P significantly reduced CD11b upregulation induced by PS beads in blood. Adding anti-IIb/IIIa to sCR1 or P5P totally inhibited CD11b upregulation induced by PS beads (levels similar to t=0).

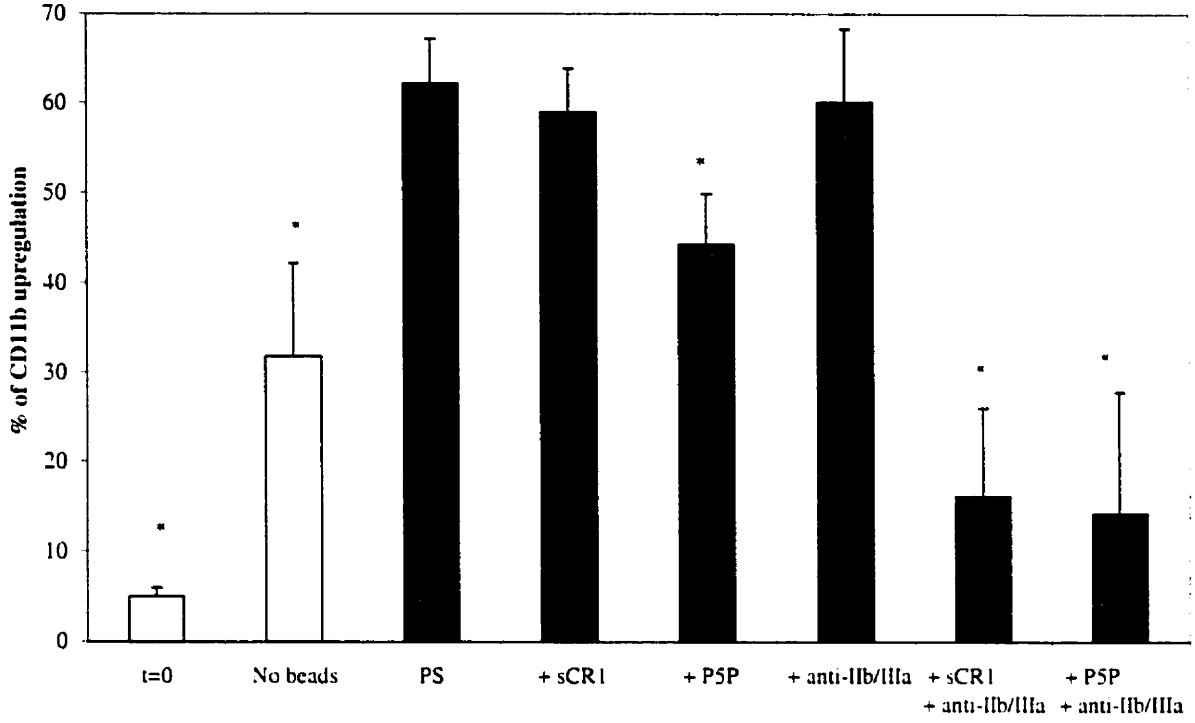

Figure 6: Effect of complement and platelets on CD11b upregulation on leukocytes induced by PS beads in heparinized whole blood. Combining sCR1 and anti-IIb/IIIa or P5P and anti-IIb/IIIa inhibited CD11b upregulation induced by PS beads. CD11b upregulation is represented as a percent relative to the maximum (determined by the fluorescent intensity of leukocytes incubated with LPS for 2 hours at 37°C). * Significantly different from PS beads ($p \leq 0.0001$). N = 4 to 6 ± S.D.

Table 2 also shows the effect of sCR1, P5P and anti-IIb/IIIa on the formation of platelet-leukocyte aggregates and platelet activation. In accordance with results from Chapter 3, significant association between platelets and leukocytes was observed in the presence of PS beads. These platelet-leukocyte aggregates were unaffected by sCR1 while anti-IIb/IIIa and P5P alone significantly reduced platelet-leukocyte association to the no-

bead level (anti-IIb/IIIa) or lower (P5P). In the presence of both P5P and anti-IIb/IIIa, platelet-leukocyte aggregates were similar to t=0. Platelet microparticle formation and P-selectin expression were reduced by P5P but not sCR1 (Table 2), as expected based on the results of Gemmell[7]. Not surprisingly and in accordance with other studies[14;15], anti-II/IIIa totally blocked platelet MP formation and P-selectin expression.

For PS-PEG beads in heparinized whole blood, high leukocyte adhesion was observed with PS-PEG alone and with anti-IIb/IIIa, leaving too few leukocytes in the bulk to perform a proper analysis of leukocyte activation in the bulk[i]. However, we were able to obtain some values for PS-PEG: values for PS-PEG alone are either from the same inhibition experiments (enabled by higher collection time, n=2) or from others experiments. Table 3 shows that TF expression on bulk leukocytes, similar to the one induced by PS, was observed with PS-PEG beads. As for PS beads, sCR1 alone had no effect on TF expression, while combining sCR1 and anti-IIb/IIIa reduced TF expression. As illustrated in Figure 7, sCR1 significantly reduced CD11b upregulation induced by PS-PEG beads in heparinized whole blood, which is in accordance with our results from isolated leukocyte experiments. Adding anti-IIb/IIIa to sCR1 further reduced CD11b upregulation, in a way that was significantly different from the effect of sCR1 alone.

Table 3: Effect of complement inhibition and anti-IIb/IIIa on TF expression induced by PS-PEG beads in heparinized whole blood.

	TF expression (%)[a]	**Monocytes expressing TF** (%)
t = 0	15 ± 2 *	1 ± 1 *
No beads	23 ± 3 *	4 ± 3 *
PS-PEG	44 ± 6	25 ± 8
+ sCR1	39 ± 12	18 ± 6
+ sCR1 + anti-IIb/IIIa	31 ± 5	8 ± 3 *

N = 3 to 4 ± S.D.

[a] TF expression is represented as a percent relative to the maximum where maximum expression is determined by the fluorescent intensity of leukocytes incubated with LPS for 2 hours at 37°C.

* Statistically significant from PS-PEG beads ($p \leq 0.02$)

[i] Such a problem had not been encountered before (Chapter 3) when PPACK-anticoagulated blood was used.

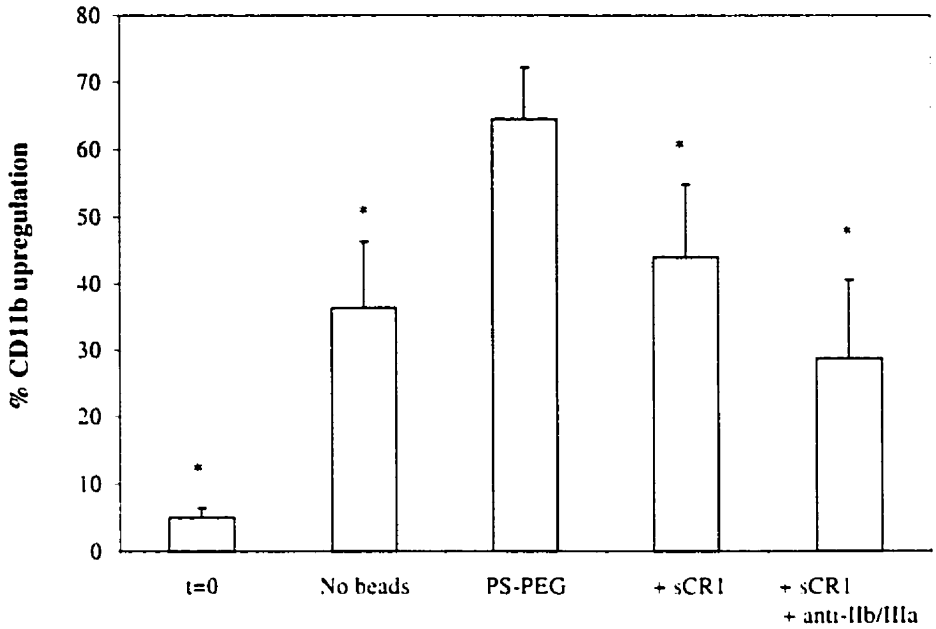

Figure 7: Effect of complement and platelets on CD11b upregulation on leukocytes induced by PS-PEG beads in heparinized whole blood. Complement inhibition significantly reduced (but not entirely) CD11b upregulation induced by PS-PEG beads. Ordinate is explained in Figure 8. * Significantly different from PS-PEG beads ($p \leq 0.0008$). N = 3 to 4 ± S.D

3) Effect of sCR1 and anti-IIb/IIIa on bulk leukocyte activation and leukocyte adhesion in PPACK whole blood.

Experiments with sCR1 and anti-IIb/IIIa were also performed in PPACK blood with PS and PS-PEG beads to verify results obtained with PS-PEG beads in heparinized blood, since too few leukocytes remained in the bulk in these conditions for PS-PEG and PS-PEG with anti-IIb/IIIa. We were also interested in investigating the effect of anticoagulant on material-induced leukocyte activation in the presence of complement and platelet inhibitors. Figure 8 and tables 4 and 5 show the effect of complement and platelets in PPACK whole blood on leukocyte adhesion on PS and PS-PEG (Table 4) and leukocyte activation in the bulk (Table 5, TF and Figure 8, CD11b).

As shown in Table 4, contrary to heparinized blood, sCR1 had no significant effect on leukocyte adhesion or activation of adherent leukocytes (as measured by TF and CD11b signals) on either PS or PS-PEG beads. Furthermore, as opposed to previous results in PPACK blood (Chapter 3) and in heparinized blood (above), significantly lower adhesion occurred on PS-PEG compared to PS beads. On the other hand, similarly to its effects in

heparinized whole blood, anti-IIb/IIIa reduced leukocyte adhesion on PS beads and subsequent TF expression on adherent monocytes.

Table 4: Effect of complement inhibition and anti-IIb/IIIa on platelet and leukocyte adhesion and activation of adherent leukocytes on PS and PS-PEG beads in PPACK whole blood.

	Leukocyte adhesion (CD45, %)[a]	**TF on beads (%)[a]**	**CD11b on beads (%)[a]**	**Platelet adhesion (CD41, %)**
Beads + EDTA	100 ± 0 #•	100 ± 0 #	100 ± 0 #•	100 ± 0 •
PS	251 ± 20	200 ± 38	264 ± 55	4494 ± 1577
+ sCR1	262 ± 37	210 ± 51	306 ± 82	4227 ± 1584
+ anti-IIb/IIIa [b]	143 ± 17	132 ± 6	213 ± 10	266 ± 137
+ sCR1 + anti-IIb/IIIa [b]	121 ± 12	115 ± 3	148 ± 15	268 ± 196
PS-PEG	167 ± 29 #	114 ± 12 #	166 ± 35 #	118 ± 16 #
+ sCR1	161 ± 26	112 ± 9	116 ± 34	118 ± 12
+ anti-IIb/IIIa	174 ± 20	111 ± 6	178 ± 51	119 ± 26
+ sCR1 + anti-IIb/IIIa	153 ± 24	119 ± 11	119 ± 13	106 ± 10

N = 4 ± S.D. [b] N = 2

[a] CD45, TF, CD11b and CD41 signals on beads are represented as a percentage relative to beads+EDTA.

Statistically significant from PS beads ($p < 0.05$)

• Statistically significant from PS-PEG beads ($p < 0.05$)

TF expression in the bulk is shown in Table 5. With PS-PEG beads in PPACK blood, the expected inhibitory effect of anti-IIb/IIIa on TF expression in the bulk was observed, further confirming our hypothesis that platelets played a role in TF expression independently of the material. Complement inhibition alone had no effect on TF expression. As for PS beads in PPACK blood, results similar to heparinized blood were obtained for TF expression in the presence of the various inhibitors (Table 5).

As found previously with PS beads in heparinized conditions, the platelet antagonist anti-IIb/IIIa alone was without effect on CD11b upregulation in the bulk induced by PS-PEG (Figure 8). On the other hand, Figure 8 shows that, while sCR1 was found to be ineffective

in reducing CD11b upregulation induced by PS beads in heparinized blood, sCR1 in PPACK blood significantly reduced CD11b upregulation in bulk with PS.

Table 5: Effect of complement inhibition and anti-IIb/IIIa on TF expression and platelet activation induced by PS and PS-PEG beads in PPACK whole blood.

	TF expression (%)[a]	**Monocytes expressing TF (%)**	**Platelet microparticles (%)**
t = 0	13 ± 3 *#	1 ± 1 *#	
No beads	26 ± 5 #	4 ± 3 *#	8 ± 2 *
PS	44 ± 8	14 ± 4	25 ± 6
+ sCR1	35 ± 4	8 ± 3	27 ± 9
+ anti-IIb/IIIa [b]	25	4	9 ± 3
+ sCR1 + anti-IIb/IIIa [b]	25	3	10 ± 2
PS-PEG	35 ± 8	16 ± 11	10 ± 1
+ sCR1	35 ± 8	16 ± 9	10 ± 3
+ anti-IIb/IIIa	22 ± 5 *	4 ± 2 *	5 ± 1
+ sCR1 + anti-IIb/IIIa	18 ± 2 *	2 ± 1 *	6 ± 2

N = 3 to 4 ± S.D. [b] N = 1 for TF, N = 2 for platelets

[a] TF expression is represented as a percent relative to the maximum where maximum expression is determined by the fluorescent intensity of leukocytes incubated with LPS for 2 hours at 37°C

Statistically significant from PS beads ($p \leq 0.02$)

* Statistically significant from PS-PEG beads ($p \leq 0.01$)

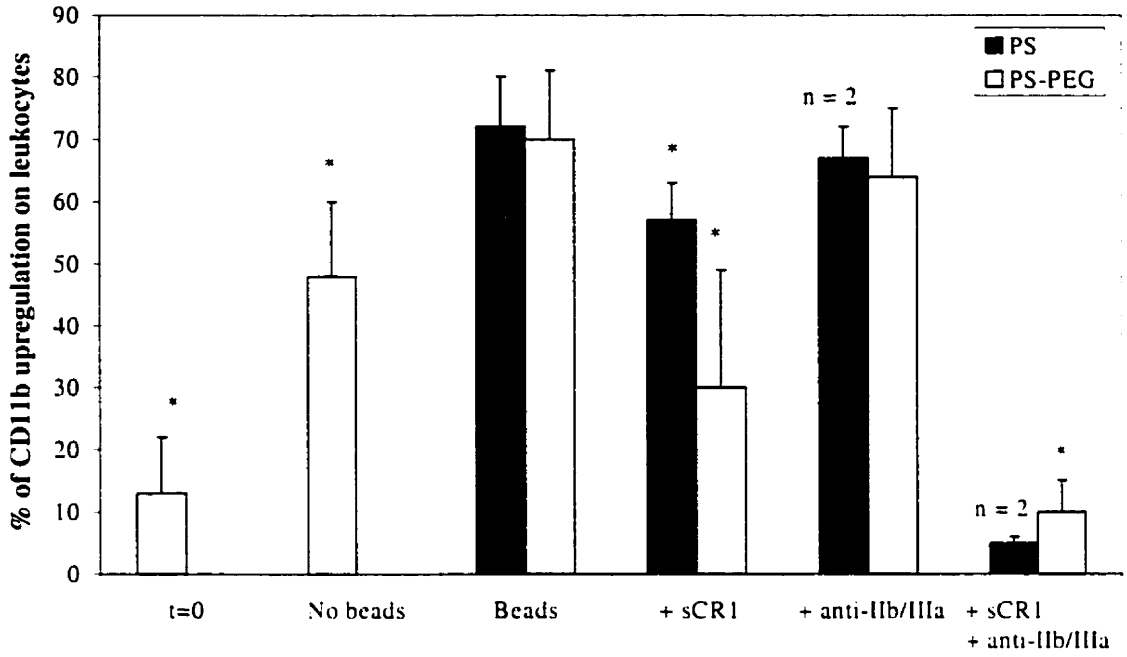

Figure 8: Effect of complement and platelets on CD11b upregulation on leukocytes by beads in PPACK whole blood. As opposed to results in blood, sCR1 significantly reduced (but not entirely) CD11b upregulation by both PS and PS-PEG beads. Ordinate is explained in figure.7. * Significantly different from beads without inhibitor ($p < 0.05$). N = 3 to 4 ± S.D

4) Effect of anticoagulant on complement activation

sCR1 was able to reduce leukocyte activation in the bulk induced by PS beads in PPACK blood but not in heparinized blood. On the other hand, sCR1 had a significant effect on adhesion and activation of adherent leukocytes in heparinized blood but not in PPACK blood. To further understand these differences, complement activation was measured in the presence of heparin and PPACK. To isolate the effect of just the anticoagulants, they were added to serum and samples with beads were incubated for 2 hours at 37°C.

As shown in Table 6, heparin at 5 units/mL reduced (but not significantly) complement activation in the bulk induced by both PS and PS-PEG beads. On the other hand, PPACK at 120μM significantly increased complement generated with PS beads in the bulk by a factor of 4. Similar results were obtained with PS-PEG beads. The addition of PPACK to serum resulted in increased complement activation so that iC3b levels generated with both the beads and the no bead samples were then identical, while SC5b-9 levels were identical for PS and PS-PEG but still greater than the no bead sample. The addition of

heparin had no such effect for SC5b-9, although iC3b levels were the same for both PS and PS-PEG beads (but higher than the no bead sample). Plasma from blood experiments was also analysed and similar effects of PPACK were found when compared to heparinized blood (data not shown).

Table 6 also presents results on complement adsorption on beads in the presence of PPACK or heparin. To our surprise, while the presence of PPACK resulted in an increase in complement products in bulk, very little complement was actually adsorbed on the beads.

Table 6: Effect of heparin and PPACK on complement activation induced by beads in serum

	Fluid Phase		Adsorbed
	iC3b (μg/mL)	SC5b-9 (μg/mL)	iC3b (%)[a]
No beads	175	6	N/A
+ Heparin (5 units/mL)	126	5	N/A
+ PPACK (120 μM)	1619	15	N/A
PS	397 ± 85	10 ± 1 [#]	585 ± 65
+ Heparin (5 units/mL)	192 ± 37	7 ± 1 [#]	612 ± 101
+ PPACK (120 μM)	1597 ± 233 [•]	45 ± 5 [•]	125 ± 17 [•]
PS-PEG	311 ± 36	25 ± 2	374 ± 85 [b]
+ Heparin (5 units/mL)	206 ± 30	21 ± 3	345 ± 18 [b]
+ PPACK (120 μM)	1461 ± 337 [•]	43 ± 4 [•]	101 ± 20 [b]

N = 3 ± S.D, [b] N = 2

[a] iC3b signal on beads is represented as a percentage relative to beads+EDTA (100%).

[•] Significantly different from PS and PS-PEG beads with or without heparin ($p < 0.0005$)

[#] Significantly different from PS-PEG beads with or without heparin ($p < 0.0002$)

To ensure that the effect of PPACK was not due to an interaction with antibodies during the ELISA or flow cytometry procedures, several controls were run. To verify that the unexpectedly high values observed with the ELISA were not due to non-specific binding, PPACK was added to EDTA-serum or PBS, incubated for 2 hours at 37°C, frozen at –70°C and then analysed for SC5b-9. Only background values of SC5b-9 were observed with or without PPACK. Adding PPACK to bead samples before incubation with the antibody for flow cytometry did not have any effect. PPACK was also not contaminated by endotoxin.

Also, the acidified buffer in which PPACK was dissolved was not acidic enough to significantly affect physiological pH: the acidified buffer was then not responsible for the enhanced complement activation observed with PPACK. Taken all together, these control experiments suggested that a specific interaction occurred between PPACK and complement, which resulted in an enhanced complement activation.

IV. Discussion

1) Effect of complement and platelets on leukocyte adhesion in heparinized whole blood

Following a two hour-incubation in heparinized whole blood, leukocyte adhesion occurred on PS and PS-PEG beads to the same extent. Our results demonstrated that complement activation products and platelet presence on the material surface mediate leukocyte adhesion. As a consequence of complement inhibition by sCR1, no iC3b was adsorbed on the beads (see Chapter 5). The reduction in leukocyte adhesion may be explained by the absence of iC3b on beads, as iC3b is a recognized ligand in leukocyte adhesion to biomaterials[16]. However, our results show that it is not the only ligand to mediate leukocyte adhesion: in the absence of adsorbed iC3b, adherent platelets (when present) were able to support leukocyte adhesion. Under low shear conditions, leukocyte adhesion to surface-adherent platelets may be mediated by interaction of CD11b with fibrinogen bound to the IIb/IIIa receptor on platelets[17;18] or via binding of platelet P-selectin to its leukocyte counter-receptor PSGL-1[19;20]. The presence of platelets on the leukocyte membrane may also represent a means of adhesion to surfaces, whereby the leukocyte attach to the biomaterial via its adherent platelets.

Anti-IIb/IIIa reduced leukocyte adhesion on PS beads, in a way we believe to be associated with its inhibition of platelet adhesion. There have been some reports on anti-IIb/IIIa interacting directly with leukocyte CD11b and blocking leukocyte adhesion to fibrinogen and ICAM-1[21,22]. While our results with PS-PEG showed no effect of anti-IIb/IIIa on leukocyte adhesion, this does not provide more insight on how anti-IIb/IIIa may affect leukocyte adhesion (i.e; via its inhibition of platelet adhesion or via an interaction with CD11b) since the presence of PEG on the PS-PEG beads most likely prevented fibrinogen adsorption. However, our previous results from isolated leukocytes with sCR1 showing no effect of anti-IIb/IIIa on leukocyte adhesion suggested that anti-IIb/IIIa did not interact with

CD11b and supports our hypothesis that anti-IIb/IIIa effect on leukocyte adhesion is via its inhibition of platelet adhesion. Mickelson et al[15] also found that Reopro did not bind to neutrophils; while Makkar et al[23] observed leukocyte adhesion on stents implanted in dogs in the presence of 7E3. These two reports further suggest that an interaction between anti-IIb/IIIa and leukocytes is unlikely. The type of antibody used as well as the cells (cell lines versus fresh blood cells) may explain the interaction found in the earlier studies by Plescia[22] and Simon[21]. Coller[24] also recently reported that 7E3 appears to bind only to an activated conformation of CD11b, conformation that may not be adopted during physiologic activation. Furthermore, 7E3 binds to CD11b with a much lower affinity (K_p~160nM) than it binds to GPIIb/IIIa (K_p~4nM)[24]. This confirms our hypothesis that, in our experiments, 7E3 did not interact with leukocytes.

Complement inhibition via sCR1 had a small (but not significant) effect on TF expression on adherent monocytes on PS and PS-PEG beads, as shown by the TF index of activation. On the other hand, sCR1 significantly reduced CD11b upregulation on leukocytes adherent to PS and PS-PEG beads. The fact that TF expression and CD11b upregulation are not regulated by the same mechanisms of activation is not surprising, as TF needs to be synthesized while CD11b is translocated from intracellular granules. The effect of complement activation on CD11b may occur either through binding of leukocytes to adsorbed complement products (such as iC3b) or complement products in the bulk (such as the anaphylatoxins C3a and C5a) may activate the adherent leukocytes. Both mechanisms may be relevant in this situation, since opsonized surfaces with iC3b tend to enhance leukocyte activation[25-27] and presence of C3a and C5a in the fluid phase have been shown to activate adherent leukocytes[25,27-29].

Anti-IIb/IIIa did not reduce CD11b upregulation on adherent leukocytes. However, adding anti-IIb/IIIa to sCR1 further reduced CD11b expression induced by adhesion on PS beads relative to sCR1 alone, suggesting that platelets may also contribute to CD11b upregulation of adherent leukocytes. The lower CD11b index of activation observed with PS-PEG beads (which did not support platelet adhesion) in the presence of sCR1, compared to PS, further supports the hypothesis that platelets play a role in CD11b upregulation of adherent leukocytes. On the other hand, the fact that in the presence of both sCR1 and anti-IIb/IIIa, leukocyte activation in the bulk was inhibited may also have affected activation of adherent leukocytes.

Anti-IIb/IIIa significantly reduced TF expression per adherent monocyte on PS beads. Anti-IIb/IIIa also blocked platelet adhesion and activation. This suggests that the presence of platelets on the surface or of platelet activation products (such as thromboglobulin or platelet activating factor) plays an important role in the expression of TF on adherent monocytes. The significant difference in TF expression of monocytes adherent to PS versus PS-PEG beads supports our hypothesis that platelet adhesion and/or platelet activation participate in TF expression of adherent monocytes. While our experiments do not allow us to distinguish between the effect of platelet activation versus platelet adhesion, our previous experiments with isolated leukocytes and platelets suggested that platelet presence on the surface may be an important factor in TF expression on adherent monocytes. Further experiments with anti-P-selectin, which would block platelet-leukocyte interactions on the surface but not platelet activation, would provide valuable information on the mechanisms of TF expression on adherent monocytes.

P5P significantly reduced TF expression per adherent monocytes, suggesting that C1q may be an important mediator in TF expression. A role for C1q in TF expression on monocytes appeared earlier in a study by Osterud and Eskeland[30], where monocytes resuspended in plasma free of Clq and factor D failed to synthesize tissue factor in the presence of LPS. They later showed that adding back C1q partially restored LPS-induced TF expression[31]. That sCR1 alone, while inhibiting complement activation, was not able to reduce TF expression does not contradict the hypothesis that C1q may play an important role in TF expression, since sCR1 does not act on complement at the level of C1 but at the C3, C4 and C5 levels. P5P reduced but not significantly CD11b upregulation on adherent leukocytes. On the other hand, combining P5P and anti-IIb/IIIa surprisingly inhibited CD11b upregulation on adherent leukocytes. Since neither reagent was effective alone, this inhibition may be due to the fact that in the presence of both P5P and anti-IIb/IIIa, leukocyte activation in the bulk was inhibited and this may have prevented CD11b upregulation of adherent leukocytes.

2) Effect of complement inhibition on leukocyte adhesion in PPACK whole blood.

In PPACK-anticoagulated whole blood, significant less leukocytes adhered to PS-PEG compared to PS beads, which disagrees with our earlier results showing similar levels of leukocyte adhesion on both beads (Chapter 3). This may be explained by the fact that a

new batch of PS-PEG was used. Upon XPS analysis, differences in C1s spectra were observed, suggesting that the PS-PEG used herein had more PEG immobilized on its surface (see Appendix E). Any comparison with leukocyte adhesion results in Chapter 3 should then be done with caution, since different PS-PEG beads were actually used. The fact that a difference appeared in leukocyte adhesion between the different batches of PS-PEG but not in leukocyte activation in the bulk agrees with our hypothesis that material surface chemistry affects leukocyte adhesion but not leukocyte activation in the bulk (Chapter 3).

The lack of effect of sCR1 on leukocyte adhesion to PS and PS-PEG beads in PPACK blood is not surprising, since in the presence of PPACK, little iC3b was adsorbed on the beads. The platelet presence on PS beads was almost entirely responsible for leukocyte adhesion on PS beads in PPACK blood, since anti-IIb/IIIa (which blocked platelet adhesion) significantly reduced leukocyte adhesion to EDTA levels. This further confirms our results in heparinized blood, showing that in the absence of iC3b, platelets (when present) mediate leukocyte adhesion. The fact that leukocyte adhesion to PS-PEG in PPACK blood was not affected by sCR1 (contrary to our heparinized results) implies that proteins other than iC3b mediate leukocyte adhesion on PS-PEG in PPACK blood.

Despite the absence of iC3b on the surface (due to the effect of PPACK) and the high level of complement activation in the bulk, CD11b upregulation occurred on adherent leukocytes, but at a much lower level than the one observed with heparinized blood where iC3b was present on the beads. Moreover, in PPACK blood, sCR1 was without significant effect on CD11b upregulation of adherent leukocytes, suggesting complement activation in the bulk has no effect on adherent CD11b upregulation. This implies that the complement effect on CD11b upregulation on adherent leukocytes observed previously in heparinized blood is due to the adsorbed complement products and not complement activation in the bulk.

3) Effect of sCR1 and anti-IIb/IIIa on platelet adhesion in whole blood

Significant platelet adhesion occurred on PS beads, while, as expected, few platelets were present on PS-PEG beads. Complement inhibition via sCR1 had no effect on platelet adhesion on PS beads, while P5P had a small but significant effect. The lack of effect of sCR1 on platelet adhesion in whole blood is in accordance with Gemmell's study, where sCR1 did not block platelet adhesion on PS beads in platelet-rich plasma[7]. In that study[7],

P5P was also shown to reduce platelet adhesion, but the degree of inhibition was much higher than the one we observed (65% reduction in CD41 signal on PS beads versus 30%). The difference in incubation times (20 minutes versus 2 hours), of *in vitro* models (static versus low shear) and mediums (platelet-rich plasma versus whole blood) may account for the variation in P5P efficacy. Indeed, in a 1-hour rocking experiments in whole blood, P5P was not as effective as in platelet-rich plasma in inhibiting platelet adhesion to polyethylene tubes[7].

As expected, anti-IIb/IIIa blocked platelet adhesion to PS beads. On the other hand, anti-IIb/IIIa failed to inhibit the small platelet adhesion occurring on PS-PEG, while sCR1 prevented it. This is most likely due to the fact that platelets present on PS-PEG beads are actually associated with leukocytes via P-selectin and not directly adhering to PS-PEG. SEM further confirmed that direct platelet adhesion on PS-PEG beads was extremely rare. The effect of sCR1 on platelet signal on PS-PEG beads is thus likely a consequence of its inhibition of leukocyte (associated with platelets) adhesion.

4) Effect of complement and platelets on leukocyte activation in the bulk in whole blood

Leukocyte activation, as measured by TF expression, CD11b upregulation and association with platelets, was observed with both PS and PS-PEG beads in PPACK and heparin anticoagulated blood. In accordance with our earlier study in PPACK whole blood (Chapter 3), both materials activated leukocytes to the same extent. The effect of complement and platelets on leukocyte activation in the bulk in PPACK and heparinized blood will be discussed together, since a difference between the two anticoagulants only appeared in the case of sCR1 and PS-induced CD11b upregulation. In the discussion, the type of anticoagulant will not be mentioned unless significant difference in the inhibitor effect was observed depending on the anticoagulant used.

a) Anti-IIb/IIIa

Adding anti-IIb/IIIa to whole blood prior to incubation with beads significantly reduced the number of monocytes expressing TF (but not to the no-beads level). This follows the trend of our previous results with isolated leukocytes where platelets were found to play a significant role in material-induced TF expression. In the absence of a material, a

role for platelets in TF expression has been shown before, whereby the presence of platelets enhanced LPS-induced TF expression[32;33].

On the other hand, the presence of the platelet IIb/IIIa antagonist during blood incubation with either type of beads was totally ineffective in reducing CD11b upregulation on leukocytes. This further confirmed our results with isolated leukocytes showing the lack of effect of platelets alone on material-induced CD11b upregulation, suggesting that platelet activation is not a primary mechanism for CD11b upregulation and other mechanisms, such as complement activation, played a role. However, there have been some reports of anti-IIb/IIIa[15;34] reducing CD11b upregulation in myocardial infarction and during angioplasty: these effects were obtained after 12 and 24 hours, respectively. The longer time periods or the fact that it might be a situation of very low complement activation with high platelet activation (a case that we did not study here) may account for the difference in the effect of anti-IIb/IIIa. Time may actually be an important factor for platelets to have a significant effect on leukocyte CD11b upregulation. May et al showed that, after coronary stent implantation, a significant reduction in CD11b upregulation appeared only after the 4th day of antiplatelet therapy (ticlopidine) [35].

As expected, blocking fibrinogen binding to platelets with anti-IIb/IIIa significantly inhibited both material-induced platelet activation and formation of platelet-leukocyte aggregates. Reduced platelet-leukocyte aggregates have also been observed *in vivo* following administration of a GPIIb/IIIa antagonist[15;34;36;37]. The lack of P-selectin expression on platelets, a primary ligand for the association between platelets and leukocytes (Chapter 4 and de Gaetano[38]), explains the minimal presence of platelet-leukocyte aggregates. Anti-IIb/IIIa may also have blocked bridging between platelets and leukocytes via fibrinogen, a common ligand for platelet GPIIb/IIIa and leukocyte CD11b, further inhibiting any possible association between platelets and leukocytes.

b) sCR1

Complement inhibition (with sCR1) alone did not significantly reduced TF expression, the number of monocytes expressing TF, and platelet-leukocyte aggregates induced by PS and PS-PEG beads in whole blood. This suggested that complement activation did not play a primary role in material-induced TF expression and formation of platelet-leukocyte aggregates, and that other mechanisms were involved.

In heparinized blood, sCR1 significantly reduced CD11b upregulation induced by PS-PEG beads but not by PS. These results agree with our isolated leukocyte experiments that demonstrated the primary role of complement activation in PS-PEG induced CD11b upregulation while pointing to other complement-independent mechanisms of activation for CD11b upregulation induced by PS beads. To further discuss the effect of complement inhibition on CD11b, the platelet and complement activating properties of each material should be considered. As shown previously in Chapter 5, PS-PEG is a highly complement activating material (high levels of [SC5b-9]), but induces minimal platelet activation as measured by formation of platelet microparticles and P-selectin expression. PS is a mild complement activating material (low [SC5b-9]), but a strong platelet activator. Considering these differences, the fact that sCR1 in whole blood is effective with one material but not the other may then not appear as surprising; i.e, if the material is not a strong complement activator, then the effect of sCR1 should be small.

Our results on the lack of effect of sCR1 on PS-induced CD11b upregulation may appear to disagree with the many reports on the importance of complement activation in material-induced leukocyte activation *in vitro*. However, it is necessary to look closely at the activating properties of the material tested[39].[40-45] Himmelfarb et al.[43] and Rinder et al.[40] showed a significant reduction in CD11b upregulation with sCR1 with a material that was highly complement activating but minimally activated platelets, which agrees with our results with a material with similar properties, PS-PEG. With a strong platelet and complement activating material such as PVA[45], PVC[39,44] or extracorporeal circuits[41,42], sCR1 or inhibition of complement at C5 or C9 levels was found effective in blocking leukocyte activation. While we did not study such a type of material in our study with heparinized blood, a parallel may be drawn to the case of PS in PPACK blood characterized by high [SC5b-9] complement generation (to be explained later) and platelet activation. In this case, we also showed an effect of sCR1. Taken all together, our results and others on the effect of complement inhibition on material-induced CD11b upregulation tend to suggest that a primary role for complement in CD11b upregulation is only observed when high terminal pathway activation is present.

While our experiments do not directly assess how the complement pathways C3 and C5 are involved in CD11b upregulation, our results with sCR1, showing its effect primarily when high SC5b-9 levels are observed, tend to suggest that the C5 complement pathway may

be important in the mechanisms of material-induced CD11b upregulation. Gillinov et al[46] showed that the terminal pathway of complement activation was most important in material-induced CD11b upregulation: blocking the C3 pathway but not the terminal pathway resulted in similar leukocyte activation relative to the absence of inhibition. Other studies[41;47] also demonstrated that the terminal pathway, but not C3a, contributed to leukocyte activation. Thus the material-dependent effect of complement inhibition appears to be directly linked to the complement activating properties of the material: only when high terminal complement activation is present is complement inhibition effective. This further supports the notion that activation of the terminal pathway of complement may be a more relevant index of blood compatibility. Videm et al[48] also proposed that measuring the terminal complement activation may be the best index of complement activation during cardiopulmonary bypass.

When a role for complement was shown in our study, a reduction in CD11b upregulation to level similar to the no beads sample was usually observed. But the no bead level was greater than the EDTA or initial levels, suggesting that there was some material-independent activation occurring. Anti-IIb/IIIa had to be present with sCR1 to totally inhibit CD11b upregulation, suggesting that platelet activation, even when minimal, contributed CD11b upregulation. This partial inhibition in CD11b upregulation upon complement inhibition seems to contradict other *in vitro* studies in blood, which observed a total abrogation of CD11b upregulation (level with complement inhibition similar to initial level). These discrepancies may be the result of the differences in experimental protocol or in the *in vitro* model used. In the *in vitro* studies by Rinder et al.[40-42], the priming solution used to dilute blood contains mannitol, a hydroxyl scavenger. Hydroxyl radicals are known to activate leukocytes[49] and platelets[50-52]. Thus the presence of mannitol most likely inhibited other pathways of leukocyte activation, making complement activation the exclusive mechanism. Difference in temperatures of incubation (120 min at 37°C versus 27°C for 60 min, and 37°C for 30 min)[40-42] may also account for the difference as lower incubation temperature have been reported to induce lower leukocyte activation[53;54]. The 25% blood dilution and higher heparin concentration (10 U/mL versus 5U/mL) in the study of Himmelfarb[43] may also have an impact on the extent of leukocyte activation. Finally the *in vitro* model used, such as the one developed with PVC by Larsson et al[39;44], may affect to which degree the different mechanisms of activation are involved. Their model used a smaller surface area to blood volume ratio (6 cm^{-1}) with a large volume of air (4 mL of gas

for 5 mL of blood). In such a system, the effect of complement activation induced by the air bubble rather than by the material may be measured. The presence of gas bubbles has been previously reported to significantly activate complement[55-57].

In PPACK blood, sCR1 significantly inhibited (but not entirely) CD11b upregulation induced by PS beads, while it had been ineffective in heparin. Our complement results have shown that in PPACK blood, high complement activation is observed, changing PS from a moderate complement activator to a high complement activator with concentrations of [SC5b-9] similar to PS-PEG. Thus, the mechanism of leukocyte activation in PPACK blood appears to be driven in part by the high complement activation for PS, hence the effect of sCR1. The role of platelet activation as a secondary mechanism for leukocyte activation is also observed whereby the reduction in CD11b upregulation in the presence of sCR1 is stronger with PS-PEG beads (minimal platelet activation) than with PS beads. This is further verified by adding anti-IIb/IIIa with sCR1, which combination totally blocked CD11b upregulation induced by incubation with beads. The partial role of complement in leukocyte activation induced by a strong platelet and complement activating material was further demonstrated *in vivo* by Fitch et al[47], where a recombinant C5 complement inhibitor was shown to reduce but not entirely CD11b upregulation on leukocytes in patients undergoing bypass graft surgery with cardiopulmonary bypass. Their results further confirm our hypothesis that in a case of a high complement and platelet activating material, both complement and platelet activation have to be inhibited to totally block material-induced CD11b upregulation.

c) sCR1 and anti-IIb/IIIa

Inhibiting both complement activation and platelet activation via fibrinogen binding (with anti-IIb/IIIa) reduced TF expression and the percentage of monocytes staining positive for TF to the no-beads level. Complement and platelet activation appear then to act together in material-induced TF expression in the bulk. Further reduction might have been achieved if fewer platelet-monocytes aggregates were present. While we showed a positive correlation between platelet-monocyte aggregates and TF expression on monocytes, we were unable to demonstrate directly the effect of platelet-monocyte association on TF expression in whole blood. Our commercial source of anti-P-selectin was contaminated with endotoxin and another source could not be found in the appropriate time frame. Warnes et al[58] have actually shown that anti-P-selectin reduced LPS-induced monocyte TF expression and from

our isolated leukocyte experiments with platelets (Chapter 4), a similar trend would be expected for material-induced TF expression in whole blood.

Combining both sCR1 and anti-IIb/IIIa also resulted in a significant inhibition of CD11b upregulation with PS beads, while none of the reagents were effective alone. This suggests that both complement and platelet activation were playing a role in PS-induced CD11b upregulation: inhibiting one pathway of activation was ineffective as the other one was still present to activate leukocytes. Adding anti-IIb/IIIa to sCR1 with PS-PEG also further reduced CD11b upregulation observed with sCR1 alone, suggesting that the minimal platelet activation present with PS-PEG beads was responsible for some of the remaining leukocyte activation in the presence of sCR1.

These results further emphasize the existence of more than one pathway for leukocyte activation and show that depending on the properties of the material, inhibiting one pathway of leukocyte activation, such as complement activation, may not have a significant effect. *In vivo* studies by Rousseau et al[59] and Videm et al[60] also point toward a material effect on leukocyte activation that is not always related to complement activation but also to platelet activation. As discussed previously, generalization on material-induced leukocyte activation from one material to another should be done with caution unless they appear to have similar complement and platelet activating properties.

d) Pyridoxal-5 phosphate

The presence of P5P alone in whole blood with beads reduced TF expression to the no-beads level. P5P also significantly reduced the association between platelets and monocytes. The effect of P5P on TF is unlikely due solely to the reduction in platelet-monocyte aggregates since, with anti-IIb/IIIa, similar levels of platelet-leukocyte aggregates did not result in such a significant reduction of TF expression on monocytes. The C1q inhibitory effect of P5P might be responsible for its effectiveness in inhibiting TF expression induced by PS-beads. As mentioned above, C1q has been shown to play a role in LPS-induced TF expression[30]. Adding anti-IIb/IIIa to P5P totally abrogated TF expression on monocytes (level similar to initial TF expression), suggesting that the low TF expression induced by PS beads in the presence of P5P was due to the remaining platelet activation.

P5P, alone, significantly reduced CD11b upregulation induced by PS beads. These results can also not be explained by reduced platelet activation and leukocyte-platelet aggregates alone since anti-IIb/IIIa failed to have such an effect on CD11b. P5P effect may

suggest C1q participation in CD11b upregulation. Presence of C1q has been reported to increase PMA-induced CD11b upregulation[61]. The inhibition of C1q by P5P may reduce CD11b upregulation by blocking C1q stimulative effect on activated leukocytes. Adding anti-IIb/IIIa to P5P blocked CD11b upregulation induced by PS-beads, further confirming our hypothesis that complement and platelet activation, both, play a role in leukocyte activation.

Material-induced formation of platelet-leukocyte aggregates was blocked by P5P. The low P-selectin expression on platelets may not entirely explain this observation; despite similar levels of P-selectin present with PS-PEG, platelet-leukocyte aggregates still formed. The combined effect of P5P on platelet and leukocyte activation may prevent the association between platelets and leukocytes.

While we discussed the effect of P5P focussing on its reported C1q inhibition, it is also important to remember that P5P may have other activities (previously discussed in chapter 5) that may be linked to its effect on leukocyte activation: these potential activities for P5P were studied with platelets[62-65]. Briefly, P5P may be able to interact with a glycoprotein receptor on leukocyte and may also have an effect on Na^+/H^+ exchange. Further studies with C1-INH are warranted to characterize the mechanisms by which P5P inhibited leukocyte activation.

5) Effect of complement inhibition on platelet activation in whole blood

In our *in vitro* system, complement inhibition via sCR1 did not affect platelet activation. This agrees with previous works by Gemmell[66] and Larsson et al[39]. On the other hand, in Rinder's studies, complement inhibition with antibodies against C5[41] or C8[42] significantly reduced platelet activation. The contradiction on the effect of complement on platelets may arise from the presence of mannitol, a hydroxyl scavenger, in Rinder's studies. Hydroxyl radicals have been reported for their capacity to activate platelets[50-52]: their neutralization by mannitol may suppress an effective mechanism of platelet activation and explain Rinder's finding for a primary role of complement in platelet activation in these studies.

In the presence of P5P, material-induced platelet microparticle formation was reduced, which is in accordance with previous results from Gemmell[66]. P5P has also been previously reported to inhibit platelet aggregation[62;67] and platelet release[65;68] *in vitro*. While

not directly demonstrated yet, P5P effect on material-induced platelet activation is thought to be through its inhibition of C1q[66].

6) Effect of anticoagulants on complement activation

The presence of heparin in serum reduced, but not significantly, complement activation induced by beads. Heparin is an anticoagulant that also has anti-complement properties[69]. However, the inhibitory effect of heparin on complement activation appears to vary with the type of heparin[70-73] and concentration[73-76]. The reported effects of complement inhibition by heparin have usually been observed at concentration of 10U/mL and higher[73;74;76]. During surgery and haemodialysis, the therapeutic concentration of heparin is usually 5U/mL[77]. At this concentration, our results and others[73;78;79] suggest that heparin at 5U/mL has a minimal effect of complement activation.

From earlier work by Kettner and Shaw[80], PPACK is believed to be a very selective thrombin inhibitor[81;82]. However, our results points to an interaction between PPACK and complement, since in the presence of PPACK, high complement activation was observed even in the absence of beads. The high specificity of PPACK has been reported at low concentrations of PPACK (between 10^{-8} and 10^{-9} M)[80], while we used a fairly high concentration of anticoagulant (120μM) to compensate for the instability of PPACK and our long incubation time. At high concentration, PPACK may not be as specific as expected. Others have found that PPACK at concentration in the micromolar ranges inhibited plasmin and tPA[83;84]. Gilboa et al[84] actually showed that PPACK inhibition of plasmin and tPA was concentration dependent.

In our experiments, PPACK, at 120μM, led to a significant level of complement activation in the fluid phase while little iC3b was observed on the beads. The very high activation in the fluid phase suggests that PPACK may be interacting with some of the regulatory proteins of complement. The fact that little iC3b was observed on the beads (a monoclonal antibody to iC3b was used) suggests that Factor I may be that protein. By interacting with Factor I, PPACK would prevent the inactivation of the C3b and thus favour formation of the C3 and C5 convertases, amplifying complement activation. Knabb et al[85] have reported that PPACK at 200nM inhibited complement Factor I. While no other study has been reported yet with PPACK and complement, an interaction of PPACK with Factor I cannot be dismissed (personal communications, Jeffrey Weitz, Robert Knabb, 2000). A

derivative of PPACK, DuP714 has been reported to directly act on complement proteins. DuP714 is a boroarginine derivative of PPACK, which was shown to be a highly effective, slow binding inhibitor of thrombin[86]. DuP714 was also found to activate complement through its potent inhibition of Factor I[87:88]. Factor I and thrombin are both trypsin-like serine proteases and have a very similar amino acid sequences, especially in the areas of the ligand binding region for DuP714 or PPACK[87]. In our study, the interaction of PPACK with the complement regulatory protein Factor I is likely due to the high concentration of PPACK used and may explain the increase in complement activation..

It then appears that there is no "inert" anticoagulant for *in vitro* use; while heparin is known to bind to cells and plasma proteins[89], PPACK specificity for thrombin at high concentrations is questionable. The properties of the anticoagulants also have to be weighed against their ease of use, price and availability. Thus heparin, at concentration of 5U/mL (or lower) may be a better choice over PPACK for long time experiments.

V. Conclusion

Following contact with beads, significant TF expression, CD11b upregulation and leukocyte association with platelets were observed. All these markers of leukocyte activation have been recognized to have potential procoagulant activities and their expression suggests that material-induced leukocyte activation may play a role in thrombosis associated with cardiovascular devices. Table 7 summarizes the results obtained with sCR1, P5P and anti-IIb/IIIa. Platelet activation plays a significant role in TF expression on monocytes, both in the bulk and adherent to beads. A role for platelet-leukocyte aggregates was also suggested in material-induced TF expression, and further studies with anti-P-selectin are warranted to better characterize the role of aggregates in material-induced TF expression.

sCR1 was only effective in partially reducing CD11b upregulation in the bulk when high complement activation had been present (i.e., high levels of [SC5b-9], suggesting that complement-independent mechanisms of activation participated in material-induced CD11b upregulation. Both complement and platelet activation had to be blocked to totally inhibit CD11b upregulation induced by PS and PS-PEG beads, confirming that complement is not the exclusive mechanism of activation: platelet activation appears to play a secondary role in material-induced CD11b upregulation.

Both adherent platelets (when present) and adsorbed/bound complement products were found to mediate leukocyte adhesion on beads. However, others proteins, yet unidentified, appear to participate in leukocyte adhesion on PS-PEG in the absence of iC3b on the surface. Upregulation of CD11b on adherent leukocytes was primarily due to complement activation.

This study shows that material-induced leukocyte activation is mediated by both platelet and complement activation. Depending on the properties of the material, inhibiting one pathway of leukocyte activation may not have any significant effect on leukocyte activation, and hence on leukocyte expression of procoagulant activities. The expression of procoagulant activities by leukocytes is also regulated differently by complement and platelets, and thus by inhibiting one pathway will only affect one aspect of leukocyte procoagulant activities. A combination of anti-IIb/IIIa and P5P was also very effective in inhibiting material-induced leukocyte and platelet activation. While the mechanisms by which P5P inhibits leukocyte activation are not yet well understood, its potential, as a biocompatible agent, should be seriously considered. Furthermore, it appears that blocking both platelet and complement activation may significantly improve blood compatibility with cardiovascular devices. When trying to design better biocompatible materials or to improve existing materials via surface modification, one should remember that the ideal biomaterial seems be a material that activates neither platelets nor complement. Meeting only one of these conditions will likely not be enough. Both surface adhesion and bulk activation are also important parameters to keep in mind.

Table 7: Summary table on the effect of complement inhibition and platelets on material-induced leukocyte activation in whole blood.

Leukocytes in whole blood		sCR1	Anti-IIb/IIIa	sCR1 + anti-IIb/IIIa	P5P	P5P + anti-IIb/IIIa
Bulk	TF	-	↓	↓	↓	↓↓
	CD11b	↓ if high [SC5b-9] generating material	-	↓↓↓	↓	↓↓↓
	Association with platelets	-	↓↓	↓↓	↓↓	↓↓↓
Adhesion	CD45 CD14	↓	Material-dependent (↓ with PS)	↓↓↓	-	↓↓↓
Adherent	TF	-	↓	↓↓↓	↓↓↓	↓↓↓
	CD11b	↓	-	↓↓↓	-	↓↓↓

- No effect

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Chapter 7

Summary and perspectives

Biocompatibility of blood contacting material is a complex problem where interactions between inflammatory, haemostatic and cellular systems are present. Despite over 25 years of material research, the ideal blood-compatible surface has not been found and surface coatings have failed to provide significant improvement. Thrombotic complications remain a recurrent problem in the use of cardiovascular devices, even in the presence of anticoagulant and anti-platelet therapy. This thesis took a more fundamental approach to the problem by trying to understand how leukocytes were activated by material contact and how they can contribute to thrombogenesis. The previous chapters investigated how materials, platelets and complement are involved in leukocyte activation that can be linked to procoagulant activity: TF expression, CD11b upregulation, and formation of platelet-leukocyte aggregates were studied. An *in vitro* assay using small beads (45μm) was developed to assess leukocyte activation both in the bulk and adherent to beads by flow cytometry. Polystyrene beads and PEG-immobilized polystyrene beads were used as models of blood-activating surfaces.

1) Materials activate leukocytes and induce the expression of procoagulant activity.

The presence of a material, regardless of its surface area, was sufficient to stimulate monocytes to express tissue factor (Chapter 3). TF expression was first assessed by flow cytometry. We also demonstrated using a clotting test (PTT) and anti-TF that the measured TF expression was indeed functional (i.e, initiated the extrinsic pathway) and resulted in fibrin formation (Chapter 3).

On the other hand, CD11b upregulation in the bulk and formation of platelet-leukocyte aggregates were dependent on surface area: significant CD11b upregulation and aggregate formation were observed when the surface area to volume ratio reached 13 cm^{-1} (Chapter 3). CD11b may be associated with thrombin generation as it may bind fibrinogen or Factor X. In some experiments, we were able to show that Factor X was directly activated to Factor Xa in the presence of material-activated leukocytes.

The presence of PEG on PS beads did not result in a reduced level of leukocyte activation in the bulk when compared to unmodified PS beads (Chapters 3, 4, 5 and 6).

These results further confirmed our understanding that material chemistry does not play a significant role in leukocyte activation, at least in the bulk.

We also demonstrated that leukocyte adhesion increased as the surface area increased, regardless of the type of beads studied (Chapter 3). The presence of PEG alone on PS beads did not significantly reduce leukocyte adhesion. However, activation of adherent leukocytes (TF, CD11b) was dependent on material chemistry, suggesting that specific interactions with adsorbed proteins, platelets or chemical groups at the interface regulated the activation of adherent leukocytes.

Our studies on material-induced leukocyte activation and procoagulant activities were limited to the membrane-associated receptors TF and CD11b. Release of procoagulant mediators, such as elastase and Cathepsin G, also occurs during material-induced leukocyte activation (see Chapter 2-section 4). Future work on leukocyte release would add another dimension to the characterization of material-induced leukocyte activation associated with procoagulant activities. Furthermore, studying leukocyte release would also provide evidence that leukocyte activation contributes to the formation of platelet-leukocyte aggregates. The procoagulant activity of *in vitro* material-induced leukocyte activation also needs further characterization, such as the contribution to thrombin generation of adherent versus bulk leukocytes, and the relative contribution of neutrophils and monocytes. However, at this time, we believe, it is more relevant to assess directly the contribution of material-induced leukocyte activation to fibrin formation *in vivo*.

2) Mechanisms of material-induced TF expression

Platelets were found to have a significant role in TF expression on monocytes in both isolated leukocytes resuspended with platelets (Chapter 4) and whole blood (Chapter 6). We demonstrated that the presence of platelets was a necessary condition for monocytes to significantly express TF in the presence of a foreign surface (Chapter 4). Under extremely low platelet conditions ($\leq 1x10^6$ platelets/mL), only background levels of TF expression were observed on monocytes (Chapters 4 and 5). In Chapter 4, a role for platelet-monocyte aggregates in material-induced TF expression was also shown using an anti-P-selectin antibody, which blocked the formation of leukocyte-platelet aggregates. A positive correlation found between TF expression in whole blood and monocyte-platelet aggregates (Chapter 6) further confirmed that monocyte association with platelets played a significant

role in TF expression. Platelet activation per se did not appear to play a direct role in TF expression, since similar TF expression occurred with PS, a strong platelet activator, and PS-PEG, which minimally activated platelets.

Complement activation alone was not responsible for material-induced TF expression, as sCR1 failed to have any significant effect on TF expression (Chapters 5 and 6). However, sCR1 in whole blood in combination with anti-IIb/IIIa (which alone reduced partially TF expression) resulted in a significant reduction of TF expression (Chapter 6). This showed that both complement activation and platelets (via association with monocytes) played a role in material-induced TF expression. The synergistic effect of complement activation and platelets in material-induced TF expression was further confirmed with P5P, a C1q complement inhibitor that also reduced platelet-leukocyte aggregates. P5P was the only inhibitor that when used alone was able to significantly reduced material-induced TF expression (Chapter 6).

3) Mechanisms of material-induced CD11b upregulation

Using the specific complement inhibitor sCR1, a role for complement activation in material-induced CD11b upregulation on bulk leukocytes was demonstrated only in the presence of a material that significantly activated the terminal pathway of complement, such as PS-PEG. This was seen with isolated leukocytes in the absence of platelets (Chapter 5) and with leukocytes in whole blood (Chapter 6).

When effective, inhibition of complement activation was also shown to only partially reduce CD11b upregulation, suggesting that other mechanisms of activation existed (Chapters 5 and 6). Indeed, platelet activation was identified as another mechanism that contributed to CD11b upregulation (Chapter 6). Material-induced CD11b upregulation was blocked, only when both complement and platelet activation were inhibited.

Pentamidine and NAAGA, two other complement inhibitors tested, were shown to significantly inhibit CD11b upregulation with all beads (Chapter 5). However, despite their effect on CD11b upregulation, pentamidine and NAAGA do not represent valuable means to improve blood-material compatibility as they were found to significantly affect leukocyte intracellular mechanisms. On the other hand, the presence of P5P in whole blood resulted in a significant reduction in material-induced CD11b upregulation (Chapter 6).

While we only measured material-induced CD11b upregulation (i.e., increase in CD11b surface expression), it is important to remember that functional/conformational changes of CD11b also occur upon leukocyte activation. These changes result in increased avidity of CD11b for certain ligands (see Chapter 2 – Section 4) and promote leukocyte adhesion and aggregation. During our experiments, CD11b increased avidity for fibrinogen and iC3b was likely an important factor in leukocyte adhesion to the beads. CD11b conformational changes are also relevant for activated leukocytes in the bulk, as these can enhance the avidity of CD11b for Factor X, and hence increase leukocyte contribution to thrombin formation.

4) Mechanisms of formation of platelet-leukocyte aggregates

In Chapter 3, we showed that despite minimal platelet activation with PEG-immobilized PS beads, many platelet-leukocyte aggregates were present (similar in number to PS beads, which substantially activated platelets). These results suggest that leukocyte activation plays a role in the association between platelets and leukocytes. As TF expression appears to correlate with platelet-monocyte aggregates, understanding the mechanisms by which platelets associate with leukocytes also becomes an issue.

Complement activation did not appear to be directly involved in the formation of platelet-leukocyte aggregates, since sCR1 had no effect (Chapter 6). On the other hand, unsurprisingly, platelet activation played a role in the formation of platelet-leukocyte aggregates, as shown by the effect of the platelet antagonist anti-IIb/IIIa (Chapters 4 and 6). The formation of platelet-leukocyte aggregates was primarily mediated by interaction with P-selectin: in Chapter 4, we showed that an antibody against P-selectin inhibited their formation.

5) Mechanisms of leukocyte adhesion to materials

In whole blood, both the presence of adherent platelets and adsorbed complement products were demonstrated to mediate leukocyte adhesion (Chapter 6). Our results also suggested that adsorbed or bound iC3b was one ligand responsible for leukocyte adhesion (Chapter 6). However, monocyte adhesion was also strongly dependent on the presence of platelets on the surface. Even in the presence of adsorbed complement products, minimal monocyte adhesion was observed on PEG-immobilized PS beads, which did not support

platelet adhesion (Chapters 4 and 6). Monocyte adhesion to adherent platelets on surfaces also occurred primarily via interaction with P-selectin (Chapter 4).

There were some contradictions on the role of platelets and complement in neutrophil adhesion between whole blood and isolated leukocytes experiments. With isolated leukocytes, neither complement activation nor adherent platelets were found to significantly affect neutrophil adhesion to PS beads while they both did in whole blood. This is most likely explained by the difference in experimental conditions. As a consequence of the separation procedure, isolated neutrophils may be more adhesive than neutrophils in whole blood and thus less sensitive to changes in the cell population and proteins at the material interface. Adhesion of isolated leukocytes may also be regulated by different mechanisms than leukocyte adhesion in whole blood. Thus, while working with separated cells helps in isolating mechanisms of interactions, it is important to verify results in the more physiological system that whole blood represents.

Complement activation also played a role in CD11b upregulation on adherent leukocytes, regardless of the capacity of the material to activate the terminal pathway of complement (Chapters 5 and 6). Adsorbed complement products, and most likely iC3b, appeared to be responsible for CD11b upregulation (Chapter 6). On the other hand, interactions with adherent platelets but not complement products played a role in TF expression on adherent monocytes (Chapters 4 and 6).

Our *in vitro* experiments were performed under low shear conditions. Contradictory reports exist on the role of shear in leukocyte adhesion (Chapter 2, section II-6) and more research is required in this area. Future investigations should assess the effect of shear on leukocyte adhesion to materials in the presence or absence of platelets. As experimental conditions appear to affect the outcomes of the shear studies, we recommend that if isolated leukocytes have to be used, both plasma and red blood cells should be present.

6) Role of platelets and complement activation in material-induced leukocyte activation

Using anti-IIb/IIIa and sCR1 in whole blood, a role for both platelets and complement activation was demonstrated in material-induced leukocyte activation, as measured by TF expression, CD11b upregulation and platelet-leukocyte aggregates (Chapter 6). Figures 1 and 2 illustrate the mechanisms involved in material-induced TF expression

and CD11b upregulation identified by our experiments. Inhibiting either one pathway alone was only effective with some materials (even though they induced similar levels of leukocyte activation) or with some markers of activation. This showed that (1) the mechanisms of material-induced leukocyte activation were material-dependent and (2) that markers of leukocyte activation, such as TF and CD11b, were not regulated by the same mechanisms. We demonstrated that blocking both platelet and complement activation significantly inhibited material-induced leukocyte activation and leukocyte adhesion, suggesting that leukocyte activation was secondary to platelet and complement activation.

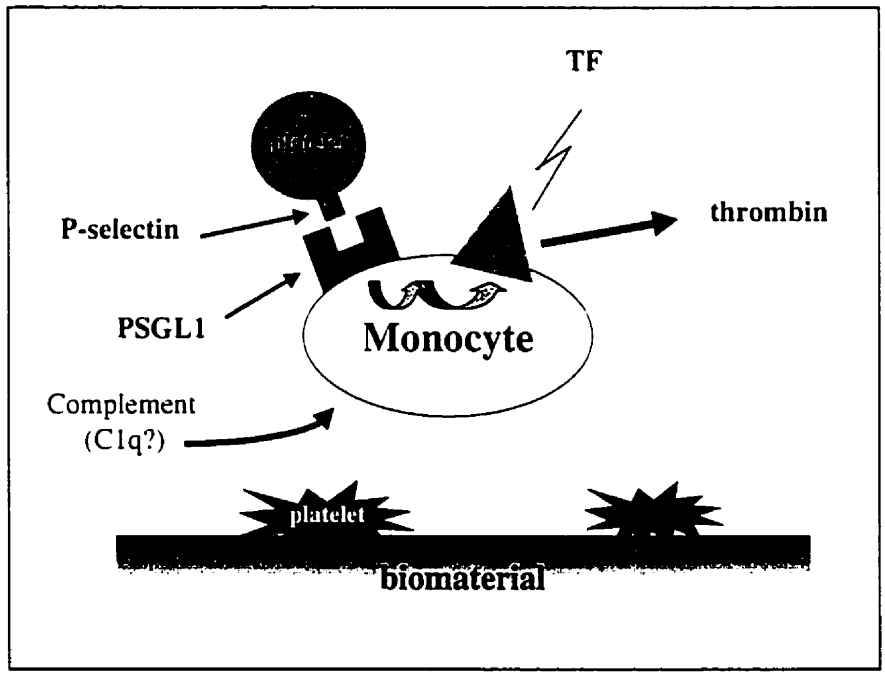

Figure 1: Mechanisms of material-induced TF expression. Note that the presence of activated platelets on the biomaterial underlines the role of platelets in monocyte adhesion and subsequent TF expression. The arrow inside the monocyte indicates intracellular signalling triggering protein synthesis.

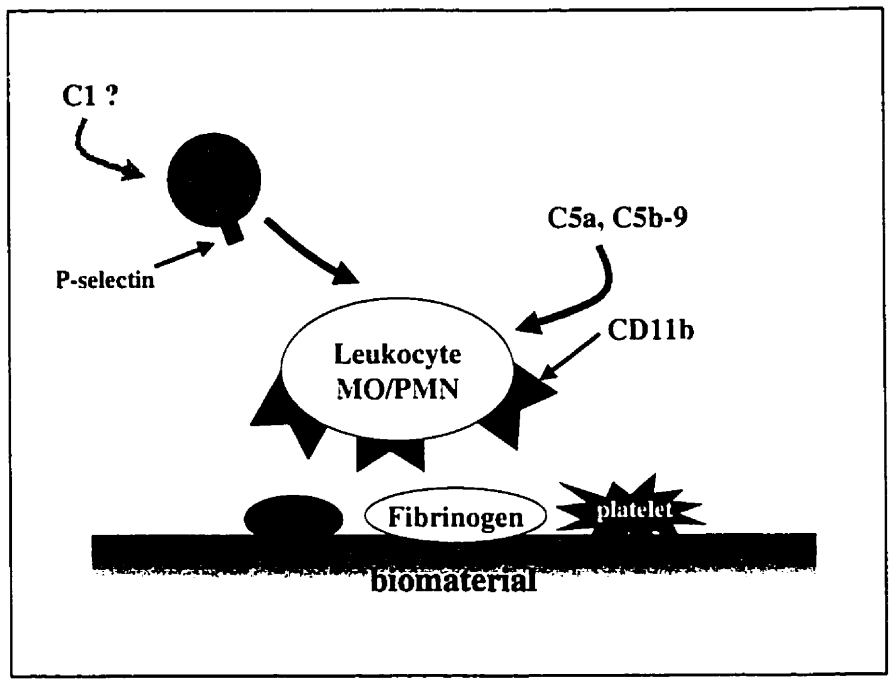

Figure 2: Mechanisms of material-induced CD11b upregulation. The presence of platelets iC3b, and fibrinogen on the biomaterial underlines their role in leukocyte adhesion. The two latter ones are also believed to play a role in CD11b upregulation on adherent leukocytes. MO: monocyte, PMN: neutrophil

The fact that the regulatory mechanisms for TF expression and CD11b upregulation appear to differ is not as surprising when considering that TF needs synthesis while CD11b is translocated from intracellular granules. The requirement for platelet-monocyte interaction in material-induced TF expression suggests that the binding of P-selectin to its ligand on leukocytes PSGL-1 triggers the intracellular mechanism to synthesize TF. In the absence of such a signal, no significant material-induced TF expression will occur. Complement activation products, such as C1q binding to its receptor, act as a cooperative stimulator. On the other hand, for CD11b, binding of a complement product alone, such as C5a, to its receptor is sufficient to trigger the mechanism for CD11b translocation. Platelet release products, such as PAF or β thromboglobulin, appear as another alternative stimulator. The fact that platelet activation contributed minimally to CD11b upregulation with PS-PEG beads, suggest that either high concentrations or a combination of platelet mediators are required.

7) Perspective on blood-material compatibility

While we have shown that *in vitro* contact with a material resulted in leukocyte activation with expression of procoagulant activities such as TF and CD11b, the role of leukocyte activation in thrombogenesis associated with cardiovascular devices has not yet been proven. Our *in vitro* experiments provide information on the early molecular and cellular events. A relationship between these events and formation of a thrombus still has to be established. Future investigation should then focus on an *in vivo* model whereby the role of leukocytes on thrombin or fibrin formation can clearly be demonstrated. An in vivo model would also help in characterizing the timing of the different factors (Factor XII, platelet activation, leukocyte activation) that contribute to material thrombogenecity. Since TF requires synthesis, it is likely that TF contribution to thrombin generation would appear at a later time than the platelet contribution ing a material. The effect of neutrophil release Using the ex vivo dog shunt in our laboratory, and antibodies against P-selectin, GPII/IIIa, TF and CD11b, we should be able to demonstrate how the presence of leukocytes on a material significantly affects fibrin deposition. The role of leukocytes in thrombin generation in the bulk may be more difficult to elucidate due to dilution effects.

Our *in vitro* results, showing that material-induced leukocyte activation is secondary to platelet and complement activation, suggest that the ideal biomaterial should be a material that minimally activates both complement and platelets. Such conditions have to be verified in the bulk and on the surface, implying:

- minimal platelet adhesion
- minimal bulk platelet activation (such as platelet microparticle formation and P-selectin expression)
- minimal bulk complement activation
- minimal adsorption of complement products.

It may be difficult to create such a material, and inhibitors of activation, such as the ones used in this thesis, may represent a better solution. It also appears that C1q mediates platelet activation. Thus inhibiting both classical and alternate pathways of complement activation may be a useful approach to improve blood-material compatibility. While we do not know yet the mechanisms of P5P on leukocytes and platelets, the pharmaceutical potential of P5P as blood-compatible therapeutic agent should also be seriously considered.

To date, both anti-IIb/IIIa and sCR1 are undergoing separate clinical trials. Should it be shown *in vivo* that leukocytes play a significant role in thrombogenesis, the combined use of anti-IIb/IIIa and sCR1 may one day prove to be useful in eliminating the thrombotic complications observed with cardiovascular devices.

8) Contributions

Most studies on the problems related to the failure of cardiovascular devices due to thrombotic complications have focussed on the role of platelets and the extrinsic pathway of coagulation. In this thesis, the mechanisms involved in material-induced leukocyte activation with expression of leukocyte procoagulant activities were identified. Our *in vitro* results showing significant expression of leukocyte procoagulant activities suggest that leukocytes are likely to play role in cardiovascular device thrombotic failure. We developed an in vitro bead assay to assess material-induced leukocyte activation both in the bulk and adherent to the beads. Using this in vitro model, the principal contributions of this research are summarized below.

Mechanisms of material-induced leukocyte activation

- Characterization of the effect of material surface area on leukocyte activation and adhesion (TF expression, CD11b upregulation, formation of platelet-leukocyte aggregates).
- Demonstration that blood contact with a material results in increased leukocyte procoagulant activity.
- Identification of the role of platelets in material-induced leukocyte activation.
- Demonstration that material-induced leukocyte activation is secondary to both platelet and complement activation. This provides a new avenue towards the development of therapies and/or biomaterials that will effectively reduce cardiovascular device failures.

Biocompatible Materials/Therapies

- PEG immobilization may not be the ideal solution to blood-material compatibility as it has the potential to activate complement and leukocytes.

- Characterization of the efficacy of combining sCR1 and anti-IIb/IIIa in reducing material-induced leukocyte activation.
- Identification of P5P as a promising agent to improve blood-material compatibility.

Experimental design

- Characterization of the effect of endotoxin contamination on material-induced leukocyte activation.
- Characterization of the effect of anticoagulants (heparin and PPACK) on complement and leukocyte activation in the presence of materials.
- Comparisons of material-induced leukocyte activation in whole blood versus isolated cells systems.

Chapter 8
Conclusions

In whole blood, leukocyte activation in the bulk but not on the beads was independent of material chemistry

- The presence of a material, regardless of its surface area, induced TF expression on monocytes
- CD11b upregulation in the bulk, platelet-leukocytes aggregates and leukocyte adhesion were dependent on surface area.
- Material-induced leukocyte activation in the bulk was independent of material-induced platelet activation.
- Monocyte adhesion and subsequent TF expression were related to the presence of adherent platelets.
- Material-induced TF expression was procoagulant.

With isolated leukocytes, material-induced TF expression but not CD11b upregulation was dependent on the presence of platelets

- Material-induced TF expression in the bulk was related to platelet-monocyte aggregates.
- P-selectin mediated the formation of platelet-leukocytes aggregates during material interaction.
- Monocyte adhesion but not neutrophil adhesion was dependent on the presence of platelets on beads.

With isolated leukocytes in the absence of platelets, complement inhibition, via sCR1, was only effective in reducing CD11b upregulation with a highly complement activating material (PEG-immobilized PS).

- Despite similar levels of material-induced CD11b upregulation, the mechanisms regulating leukocyte activation were different for PS and PS-PEG.
- The effect of P5P, a C1q inhibitor, on CD11b upregulation induced by PS-PEG beads suggested a role for classical complement in leukocyte activation.

- Other mechanisms of activation (independent of complement) were responsible for leukocyte activation induced by PS beads.
- In the absence of platelets, plasma proteins and/or adsorbed complement products mediated leukocyte adhesion.

Material-induced leukocyte activation in whole blood is secondary to both platelet and complement activation.

- Material-induced leukocyte activation was inhibited when both complement activation and platelets were blocked with sCR1 and anti-IIb/IIIa, respectively.
- P5P inhibitory effects on PS-induced leukocyte activation suggested a role for C1q in TF expression and CD11b upregulation.
- Complement inhibition with sCR1 was effective in reducing CD11b upregulation in the bulk only with a high complement-activating surface (high [SC5b-9]).
- Platelet activation contributed to CD11b upregulation in the bulk.
- TF expression in the bulk correlated with platelet-monocyte aggregate formation..
- Both adherent platelets (when present) and adsorbed complement products mediated leukocyte adhesion to beads.
- TF expression on adherent monocytes was regulated primarily by platelets, while adsorbed complement products regulated CD11b upregulation on adherent leukocytes.

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General Conclusion

Material-induced leukocyte activation, resulting in the expression of membrane associated procoagulant activity, depends on both platelet and complement activation.

Appendix A

Flow cytometric study of *in vitro* neutrophil activation by biomaterials

By M.B Gorbet, E.L. Yeo, and M.V. Sefton.

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Flow cytometric study of *in vitro* neutrophil activation by biomaterials

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Abstract: Neutrophil activation for adherent and nonadherent cells, as measured by flow cytometry, was not strongly dependent on material surface chemistry. We had hypothesized that material-induced neutrophil activation was an important parameter associated with material failure. All materials tested [cellophane, an acrylonitrile copolymer (AN69), Pellethane®, nylon, polyethylene terephthalate, low density polyethylene, and polydimethylsiloxane] activated isolated human neutrophils, which were resuspended in plasma or serum, to similar extents based on L-selectin shedding, CD11b upregulation, and stimulation of the oxidative burst after 30-min exposure. Inhibition of complement activation by sCR1 unexpectedly had little effect if any on nonadherent neutrophils. However, neutrophil adhesion, but not the level of activation of the adherent cells, was strongly dependent on complement activation. Pretreatment with albumin did not inhibit adhesion or reduce neutrophil activation, but plasma pretreatment resulted in increased activation for nonadherent and adherent cells. More adhesion and a higher level of activation of adherent cells was observed following pretreatment with fibrinogen, a ligand of CD11b. Taken together these results suggest that upon contact with a material, neutrophil activation may occur though mechanisms that are not mediated by complement. For example, the presence of plasma proteins such as fibrinogen at the interface may trigger activation and the release of other activating agents. Although the material differences are small, the extent of activation may be significant and warrant further study of the mechanism and consequences of that activation. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res, 44, 289–297, 1999

Key words: flow cytometry; *in vitro* neutrophil activation; biomaterials

INTRODUCTION

Blood–material contact triggers a complex series of events including protein adsorption, platelet and leukocyte adhesion and activation, complement activation, and coagulation. No material has been found yet to be truly compatible with blood. Despite anticoagulant and antiplatelet activation therapy, thrombosis and thromboembolism continue to occur in cardiovascular devices. Although platelets and activation of the coagulation cascade play a central role in thrombosis, material-induced leukocyte activation may also play a role in material failure. *In vivo* leukocyte activation has been observed during cardiopulmonary bypass[1] and hemodialysis.[2] The roles of adsorbed proteins, complement, and material chemistry in leukocyte activation are unclear.

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Neutrophils (or polymorphonuclear leukocytes, PMNs) are the predominant subpopulation of leukocytes. Although short lived, they possess potent proinflammatory properties with the secretion of lysosomal enzymes, cytokines, reactive oxygen species, and lipid mediators.[3,4] Neutrophil activation results in alterations in surface membrane receptors, such as L-selectin (L-sel) shedding and CD11/CD18 upregulation,[5] leading to an increased adhesiveness to artificial and biological surfaces and in the release of reactive oxygen products ("oxidative burst")[6] and proteases (degranulation).[7] Stimulation of neutrophils with inflammatory mediators (e.g., C3a, C5a, or cytokines) activates the oxidative burst, resulting in the generation and release of tissue damaging reactive oxygen products. These may be especially important in those devices (e.g., stents) for which blood–material contact may influence other tissues located nearby. Repetitive oxidative bursts by leukocytes may enhance endothelial cell membrane lipid peroxidation and lead to endothelial dysfunction[7] and may also alter the neutrophil response to infection due to exhaustion.[8,9]

The multiple responses possible during leukocyte activation and an incomplete understanding of their interactions necessitate measurement of more than one response to fully characterize the extent of activation. Here we use fluorescent activated flow cytometry (FAFC) to assess leukocyte activation with monoclonal antibodies and fluorescent dyes. Upon activation, neutrophils increase their cell-surface expression of the CD11b/CD18 (Mac-1) integrin receptor via translocation and fusion of intracellular granules with the plasma membrane.[10] Another marker of cell activation is the shedding of L-sel (CD62L); L-sel is a lectin glycoprotein that is cleaved at a membrane proximal site, resulting in its rapid shedding from the cell surface.[11] The oxidative burst begins with the production of superoxide by a reaction catalyzed by NADPH oxidase, and then H_2O_2 and chlorinated oxidants are generated.[4] FAFC analysis of the regulation of the adhesion molecules L-sel and CD11b requires monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), while the production of intracellular reactive oxygen species can be detected using the fluorescent probe dihydrorhodamine 123 (DHR). These markers of activation have been used in *in vitro* and *in vivo* studies to document leukocyte activation in cardiovascular devices.[1,2,12]

The specific objectives of this study were to characterize neutrophil activation on a range of clinically relevant cardiovascular biomaterials and determine the effects of complement inhibition (sCR1) and protein precoating (albumin, fibrinogen, and plasma). *In vitro* experiments were performed on isolated neutrophils to distinguish the direct effect of the material on neutrophil activation from indirect effects mediated, for example, through platelets. Nonadherent and adherent neutrophils were both assessed; adherent neutrophils were tested after EDTA treatment to resuspend them.

MATERIALS AND METHODS

Reagents, antibodies, and biomaterials

Endotoxin-free bovine serum albumin (BSA), human serum albumin (HSA, high purity), and phorbol myristate acetate (PMA) were purchased from Calbiochem (San Diego, CA). Lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5), EDTA, and paraformaldehyde (Sigma, St. Louis, MO) were used. Monoclonal antibodies to CD11b and L-sel (CD62L), both fluorescein isothiocyanate conjugates, were from Immunotech (Marseilles, France). Soluble human recombinant complement receptor type 1 (sCR1), a generous gift from T Cell Sciences (Dr. James Levin, Needham, MA), was used in some experiments. All other chemicals were of analytical or reagent grade.

A range of clinically relevant biomaterials were tested including the two NIH reference materials low density polyethylene (LDPE) and polydimethyl siloxane (PDMS) (Abiomed, Danvers, MA), which were used as our reference materials. Pellethane® and nylon (courtesy of Ms. A. Sawyer, Cordis, Miami Beach, FL), polyethylene terephthalate (PET, in the form of Mylar film, Advanced Polymers Inc., Salem, NH), a cellophane dialysis membrane (Germany/IUPAC Working group, courtesy of Dr. M. Josefowicz, Villetaneuse, France), and sulfonated polyacrylonitrile (AN69, Hospal, Meyzieu, France; sample provided by Dr. J. Breilatt, Baxter Health, Round Lake, IL) were tested. Disks (15-mm diameter) were cut and washed as described previously.[13] X-ray photoelectron spectroscopy (XPS) analysis was performed on the washed surfaces (Tables I, II) and confirmed the expected surface chemistries and the expected presence of small amounts of silicon.

Endotoxin levels

Various buffers and reagents (distilled water, albumin), as well as the materials, were routinely tested for endotoxin contamination using a chromogenic substrate assay[14] because of the known effect of endotoxin on leukocytes (Table III). Saline-rinsed disks of materials were incubated in sterile polystyrene tubes with endotoxin free water for 30 min at 37°C. Limulus amebocyte lysate was then added, and the samples were incubated for 45 min at 37°C. Following addition of the chromogenic substrate (Boc-Leu-Gly-Arg-pNa, Novabiochem, San Diego, CA) and a 5-min incubation, samples were transferred to a 96-well plate in triplicate and glacial acetic acid was used to stop the reaction. The plate was read at 410 nm on an ELISA reader (model MR_{700}, Dynatech Lab Inc., Chantilly, VA). The concentration of endotoxin was determined using a standard curve from serial dilutions of LPS 055:B5. Results were obtained in endotoxin units per milliliter (EU/mL) and converted to nanograms per milliliter (ng/mL; 5 EU/ng LPS 055:B5; G. Wall, personal communication, 1996).

Leukocyte and plasma/serum preparation

Whole blood (from donors who did not take medication within 72 h of the phlebotomy) was collected without a tour-

TABLE I
Composition (atom %) of Biomaterials from Low Resolution XPS Analysis

	O1*s*	N1*s*	C1*s*	Na	S2*p*	Si2*p*
PET	23.5	0.3	75.3			0.5
Pellethane	19.2	2.6	76.8			1.0
Nylon	7.9	6.3	85.1			0.6
LDPE	0.7		99.0			0.3
PDMS	26.7		51.1			22.2
AN69	4.0	17.4	76.1	1.3 (A)	1.0	
Cellophane	34.0		62.2	0.3 (KLL)		3.2

TABLE II
Composition (atom %) of Biomaterials from High Resolution C1s Spectra

	C-C or C-Si (%) (285 eV)	C-O and/or C-N (%) (286.5 eV)	C=O (%) (288 eV)
PET	55.9	25.1	19
Pellethane	87.1	6.5	6.4
Nylon	79.8	11.9	8.3
LDPE	100		
PDMS	100		
AN69	36.8	57.5	5.7
Cellophane	38.4	46.3	15.3

niquet into a heparinized syringe (5 U/mL) after discarding the first milliliter to minimize blood activation. Neutrophils (PMNs) were isolated by one-step density gradient centrifugation on Polymorph Prep (Nycomed, The Netherlands), and washed twice in PBS without Ca^{2+} and Mg^{2+} supplemented with glucose and albumin (137 mM NaCl, 2.7 mM KCl, 6.45 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM KH_2PO_4, 1 g/L glucose, 3.5 g/L BSA, pH 7.4). They were then resuspended in Tyrode-albumin (TA; 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2 \cdot 6H_2O$, 0.36 mM $NaH_2PO_4 \cdot H_2O$, 11.9 mM $NaHCO_3$, 1 g/L glucose, 3.5 g/L HSA, pH 7.4) to a concentration of 4–5 × 10^6 PMNs/mL. Cell counts (white blood cell count and differential) were performed using a multiparameter, automated hematology analyzer (Sysmex® E-2500, TOA Medical Electronics, Japan). All cells were >99% viable by Trypan blue. The initial status of the prepared leukocytes was determined by flow cytometry: after isolation, neutrophils were in a resting state; less than 2% had shed L-sel, and the mean fluorescence for CD11b and DHR was minimal (<200 and <50, respectively).

Serum and plasma were prepared following standard procedures. Plasma or serum from at least five individuals was pooled to limit interindividual data variation. Pooled aliquots were stored at −70°C.

Disk experiments

Neutrophils were incubated with 15-mm diameter disks of the test biomaterials fixed to the bottom of a 24-well culture plate by pieces of Silastic® tubing (1/2-in. id × 5/8-in. od × 1/16-in. wall × 1/5-in. length, Dow Corning).[13] Prior to the experiment the biomaterials were rinsed with 0.9% NaCl. Albumin [3% (w/v) HSA in PBS], fibrinogen [1% (w/v) in PBS], or plasma were preadsorbed to the materials at 37°C for 30 min on a rotary shaker for some experiments. Biomaterials (with or without preadsorbed proteins) were rinsed twice with Tyrode's buffer and kept in Tyrode's buffer until incubation with neutrophils. Preadsorption of proteins was scheduled so that no more than 10 min would elapse before neutrophil incubation. The neutrophil suspension (200 μL) with 25% (v/v) plasma or serum in Tyrode-HSA was incubated on a rotating table (120 rpm, American Rotator V, R4140) at 37°C for 30 min, at which time EDTA (5 mM final concentration) was added. Nonadherent cells were removed and prepared for flow cytometric analysis. Biomaterials were then washed with TA and Tyrode. Remaining adherent neutrophils were either fixed, stained with Giemsa, and assessed microscopically or removed by a 10-min incubation with cold EDTA (5 mM in HEPES Tyrode buffer, HTB) for subsequent flow cytometric analysis. Incubation with 5 mM EDTA has been shown to be satisfactory in removing cells from most materials (95% recovery).[15] The detached neutrophils were transferred into microtubes containing 1% paraformaldehyde, washed, and resuspended in TA.

For each experiment a resting neutrophil sample (250 μL) and one containing EDTA (final concentration 5 mM) at 37°C were used as controls. At the end of the experiment the resting control was stimulated with the strong agonist PMA (10 μM) to ensure full reactivity of the neutrophils.

Flow cytometry

Flow cytometric analysis was carried out on leukocyte samples before and after exposure to test materials. Small aliquots (10–20 μL) of cell suspensions, diluted in 50 μL of HTB (137 mM NaCl, 2.7 mM KCl, 16 mM $NaHCO_3$, 5 mM $MgCl_2$, 3.5 mM HEPES, 1 g/L glucose, 2 g/L BSA, pH 7.4), were incubated with saturating concentrations of FITC-labeled monoclonal antibodies (anti-CD11b and anti-CD62L) for 20 min at room temperature. Samples were then diluted, fixed with paraformaldehyde (1% final concentration), and assessed by one color flow cytometry on a Becton-Dickinson FACScan flow cytometer using CELLQuest software. The degree of leukocyte CD11b upregulation was determined by gating on positive CD11b events and recording the average fluorescent intensity of the signal (FL1). This was compared to that obtained for the fully stimulated sample. To assess the degree of L-sel shedding, the fluorescent intensity of the L-sel signal was recorded, as well as the percentage of cells negative for L-sel. The fully stimulated control was used to define the negative FL1 peak for L-sel: those neutrophils having totally shed L-sel. For the fully stimulated control, 95–100% of neutrophils had shed L-sel. The L-sel fluorescent intensity was compared to that of the EDTA and fully

TABLE III
Endotoxin Contamination of Biomaterials

	Mean (ng/mL)	SD
PET	0.70	0.06
Pellethane	0.05	0.02
Nylon	0.54	0.07
LDPE	0.13	0.1
PDMS	0.04	0.02
AN69	0.78	0.03
Cellophane	2.96	0.4

The concentration given indicates the concentration of pure endotoxin (reference LPS 055:B5) having the same color yield chromogenic substrate (Boc-Leu-Gly-Arg-pNa) as the biomaterial surface (1.76 cm^2). Prior to endotoxin testing, materials were prepared and washed as for neutrophil experiments. $n = 2$.

stimulated controls. Irrevelant antibodies of the same isotype were used as controls.

The production of intracellular reactive oxygen species by activated leukocytes was detected using the fluorescent probe dihydrorhodamine 123 (DHR, Molecular Probes, Eugene, OR). DHR is freely permeable and localizes in the mitochondria.[16] After oxidation by H_2O_2 and O_2^- to rhodamine 123, it emits a green fluorescent signal upon excitation. DHR has been shown to be one of the most sensitive indicators of oxidative burst activation.[16] Neutrophils were loaded before exposure with biomaterials with 10 μM of DHR for 5×10^6 cells/mL at 37°C for 5 min under slight agitation. After incubation, they were fixed, stored at 4°C, and analyzed within the next 2 h.

The mean fluorescence of the samples (FL1) was normalized with that obtained with EDTA neutrophils and/or fully stimulated neutrophils for the same experiment. EDTA fluorescent intensities were generally between 120 and 150 for L-sel, 150 and 200 for CD11b, and 15 and 20 for DHR. Stimulated values were between 5 and 20 for L-sel, 800 and 1000 for CD11b, and 1000 and 1200 for DHR.

For each marker of activation, a minimum of 5000 neutrophil events were analyzed for nonadherent cells; data was collected for adherent cells for a set time (70 s).

Quantitative assessment of cell adhesion was performed with the flow cytometer using a modified bead method from Yano et al.[17] Ten-micron calibration beads were added to the fixed samples before FACScan analysis. For analysis of samples containing the nonadherent cells, a set number of neutrophil events (5000 minimum) was acquired; thus, the number of bead events collected allowed for calculation of the decrease in neutrophil count due to adhesion or other processes. These results were verified with the analysis of the adherent cell samples containing added beads for a set acquisition time (70 s). For these samples the collected number of beads was constant while the number of cell events varied, allowing for the estimation of the numbers of detached cells.

Statistical analysis

Analysis of variance was carried out to evaluate the significance of differences between neutrophil adhesion or activation. Comparisons were made using Fisher's least significant difference test. A p value of less than 0.05 and corrected for the number of comparisons was required for statistical significance. The number of repeat experiments was three or more.

RESULTS

Nonadherent neutrophils

In the presence of normal human plasma, alterations in the expression of adhesion molecules (L-sel shedding, CD11b upregulation) and generation of superoxide were observed for all surfaces as shown in Table IV. All materials activated neutrophils at least to a limited extent. However, there were few material differences among materials. Only cellophane pretreated or not with albumin induced significantly more L-sel shedding than nonpretreated LDPE and PDMS ($p < 0.005$). No significant difference among biomaterials was observed with either plasma or serum (as the suspending medium for the neutrophils) for CD11b upregulation. As for stimulation of the oxidative burst, only neutrophils that had contacted cellophane were strongly activated. In the presence of serum (Table V), neutrophil activation was also observed for all biomaterials, and slightly higher activation levels were usually observed in the presence of serum when compared to plasma. Regardless of the materials, pretreatment with albumin had no passivating effect on neutrophil activation (data not shown).

PET and LDPE were then selected to further examine the effect of protein preadsorption and complement. These two surfaces were chosen because of their differences in inducing cell adhesion (see below) and their low level of endotoxin contamination. In the complement study, some experiments were also performed with cellophane, despite its higher level of endotoxin contamination, because of its high complement reactivity. Pretreatment with albumin and fibrinogen had little effect on neutrophil activation (Fig. 1). However, pretreatment of PET with plasma had a significant effect: CD11b upregulation by plasma pretreated PET was found to be significantly higher than all LDPE surfaces, untreated PET, and the other pretreated PET surfaces ($p < 0.002$). The L-sel shedding with plasma pretreated PET was also significantly different from LDPE again (except that treated with al-

TABLE IV
Effect of Biomaterials on Leukocyte Activation: Nonadherent Neutrophils in Plasma

Material	L-sel Shedding ± SD (%)	CD11b Upregulation ± SD (%)	Oxidative Burst ± SD (%)
PET	37.1 ± 13.4	43.5 ± 6.5	129 ± 8
Pellethane	29.3 ± 3.1	43.5 ± 10.6	129 ± 15
Nylon	25.7 ± 1.3	41.3 ± 10.6	147 ± 13
LDPE	12.4 ± 9.7	39.0 ± 13.1	136 ± 19
PDMS	16.1 ± 11.4	38.8 ± 13.0	152 ± 30
AN69	31.3 ± 5.9	42.5 ± 9.5	169 ± 9
Cellophane	47.0 ± 7.2*	47.6 ± 10.1	567 ± 351†

L-sel shedding is represented as percent relative to the maximum, where the maximum shedding is determined as the difference in fluorescent intensity of unstimulated EDTA control and fully stimulated (PMA) sample. CD11b upregulation is represented as percent relative to the maximum, where the maximal upregulation is determined by the fluorescent intensity of a fully stimulated (PMA) sample. Oxidative burst is represented as percent relative to the fluorescent intensity of unstimulated EDTA control. $n = 3 \pm$ SD.

*Significantly different from LDPE and PDMS.

†Significantly different from all ($p < 0.003$).

TABLE V
Effect of Biomaterials on Leukocyte Activation: Nonadherent Neutrophils in Serum

Material	L-sel Shedding ± SD (%)	CD11b Upregulation ± SD (%)
PET	40 ± 21	53 ± 5
Pellethane	39 ± 23	54 ± 4
Nylon	44 ± 19	56 ± 5
LDPE	37 ± 23	62 ± 10
PDMS	43 ± 18	61 ± 5
AN69	42 ± 19	54 ± 7
Cellophane	69 ± 12	68 ± 5

See Table IV footnote. $n = 3 \pm$ SD.

bumin, data not shown). No significant change was observed in the oxidative burst measurements (data not shown).

The effect of plasma could be accounted for by adsorbed complement products, because neither albumin nor fibrinogen pretreated samples lead to such a difference. This hypothesis was supported by performing these experiments in the presence of sCR1 (a complement inhibitor). In this case, the levels of activation were identical to the samples that were not pretreated, regardless of whether sCR1 was added during the pretreatment with plasma or added to the neutrophil suspension (Fig. 2). Adding sCR1 to the neutrophil suspension had no effect on the degree of neutrophil activation for untreated LDPE and PET, but

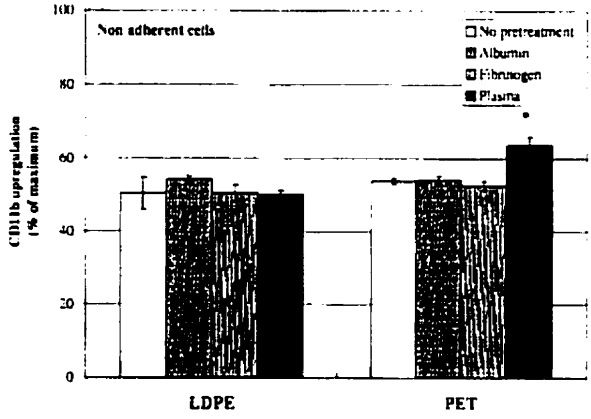

Figure 1. Effect of pretreatment with plasma proteins on CD11b upregulation by nonadherent neutrophils in serum after a 30-min incubation at 37°C. Note the significantly higher degree of CD11b upregulation with plasma pretreatment of PET but not LDPE, and the absence of an effect of albumin and fibrinogen on either surface. Upregulation is represented as percent relative to the maximum where the maximal upregulation is determined by the fluorescence intensity of the fully stimulated sample (PMA). All pretreatments were performed at 37°C for 30 min. HSA, human serum albumin; Fbg, fibrinogen; LDPE, low density polyethylene; PET, polyethylene terephthalate. *Significantly different from all others ($p \leq 0.001$). $n = 3 \pm$ SD.

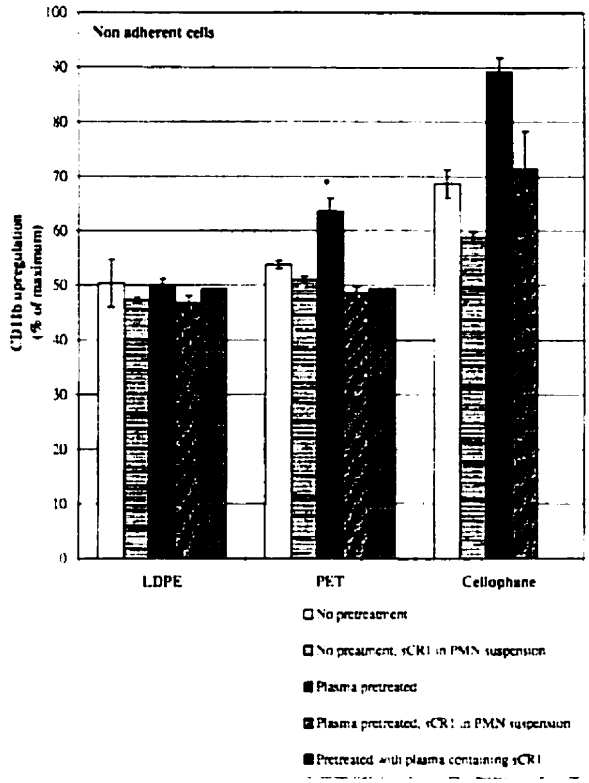

Figure 2. Effect of complement inhibition by sCR1 on CD11b upregulation by nonadherent neutrophils in serum. Upregulation as in Figure 1. sCR1 added to neutrophil suspension or added to the plasma for plasma pretreatment inhibited the increase in activation seen with plasma pretreated PET. $n = 3 \pm$ SD, except for plasma + sCR1 pretreated, $n = 1$. *Significantly different from LDPE, untreated PET, and plasma pretreated PET/sCR1 (in bulk) ($p \leq 0.001$). No statistical analysis was performed with cellophane because only two experiments were performed.

did result in a 10% reduction for cellophane (compare to the first two columns of Fig. 2). However, in all the experiments with sCR1, its presence did not reduce the extent of neutrophil activation to zero. Superoxide generation was also not significantly affected by sCR1 (data not shown).

Adherent neutrophils

Neutrophil adhesion was observed on all surfaces but to different degrees: LPDE and PDMS had the fewest adherent cells while PET, Pellethane, and cellophane had the most (Fig. 3). In the absence of serum or plasma, few cells adhered to any surface (data not shown). Albumin pretreatment did not inhibit cell adhesion: for all biomaterials, similar levels of adhesion were observed for albumin treated samples and samples without pretreatment (data not shown). Pre-

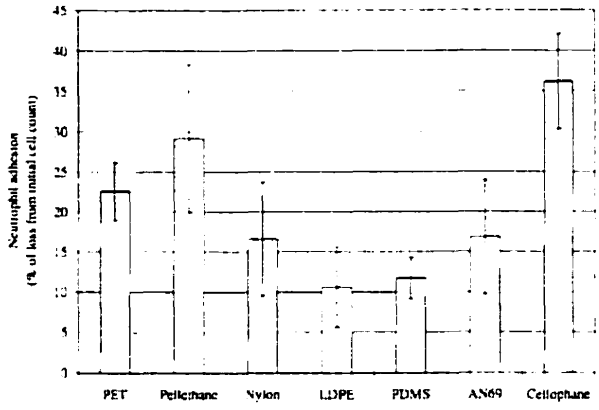

Figure 3. Neutrophil adhesion (expressed as a percentage of cell loss from the initial cell count) after a 30-min incubation with neutrophils in plasma. $n = 3 \pm SD$.

treatment with fibrinogen and plasma led to significant increases in cell adhesion, however. When complement activation was inhibited, a significant decrease in cell adhesion was observed on PET (Fig. 4) and other materials (data not shown), regardless of the protein pretreatment.

The L-sel shedding for adherent cells was almost complete, regardless of the conditions or the biomaterial: mean values were all above 80% loss of L-sel, which was expected because L-sel shedding is one of the first events in neutrophil adhesion (Table VI). On the other hand, as shown in Table VI, the degree of CD11b upregulation was only slightly material dependent.

Pretreatment with plasma had the most effect on

TABLE VI
Effect of Biomaterials on Leukocyte Activation: Adherent Neutrophils in Plasma

Material	L-Selectin Shedding ± SD (%)	CD11b Upregulation ± SD (%)
PET	89.9 ± 1.3	58.3 ± 4.5
Pellethane	88.7 ± 1.6	54.6 ± 8.1
Nylon	88.2 ± 2.6	57.1 ± 8.2
LDPE	90.4 ± 1.2	43.1 ± 8.5
PDMS	88.9 ± 2.2	38.7 ± 8.3
AN69	87.3 × 2.9	59 ± 12.1
Cellophane	87.5 ± 3.4	71 ± 2.1*

See Table IV footnote. $n = 3 \pm SD$.
*Significantly different from LDPE and PDMS.

CD11b upregulation on PET (Fig. 5). For PET, only slight increases in CD11b upregulation were observed with albumin and fibrinogen pretreatments. Except for albumin, pretreatment with different proteins had no significant effect on the degree of adherent neutrophil activation for LDPE. With sCR1 to inhibit complement activation in the bulk (i.e., neutrophils were resuspended in serum and sCR1), fewer adherent cells were observed (Fig. 4), but the degree of CD11b upregulation was similar, regardless of the protein pretreatment (Fig. 5). When the plasma pretreatment was performed in the presence of sCR1 and followed by incubation with neutrophils in serum, the level of CD11b upregulation of the few adherent neutrophils was 70.5% of maximum. This was a reduction compared to plasma treated PET, and a level similar to that observed on untreated PET.

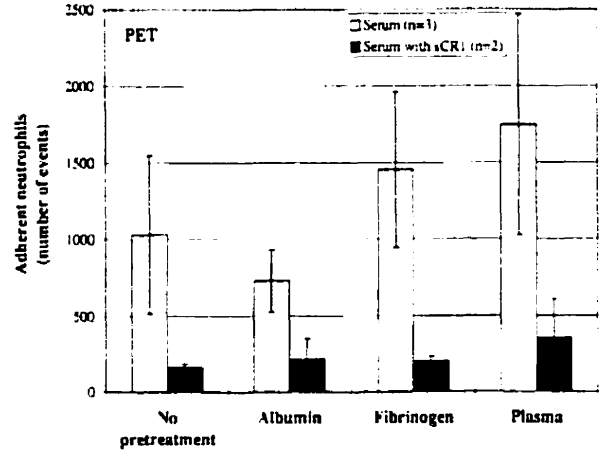

Figure 4. Effect of complement inhibition and/or pretreatment with plasma proteins on neutrophil adhesion on PET after a 30-min incubation with neutrophils in serum. All pretreatments were performed at 37°C for 30 min. Adherent neutrophils are represented by the number of neutrophil events acquired by flow cytometry in a set time (and specific volume). $n = 3 \pm SD$.

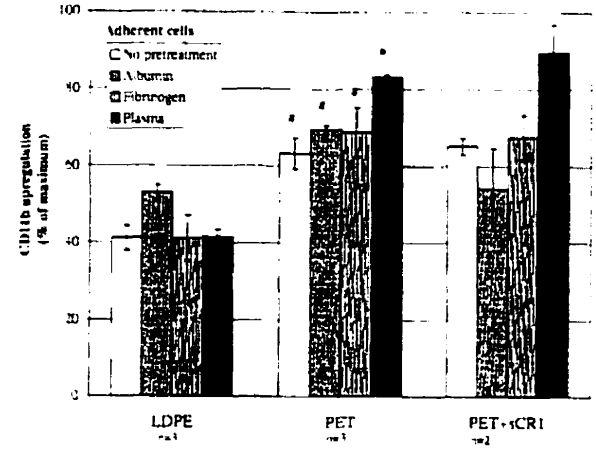

Figure 5. Effect of pretreatment with plasma proteins (37°C, 30 min) on CD11b upregulation by adherent neutrophils in serum. Upregulation is represented as in Figure 1. *Significantly different from all LDPE ($p \leq 0.002$). #Significantly different from all LDPE except HSA ($p \leq 0.004$). $n = 2$ or $3 \pm SD$.

DISCUSSION

Assay system

An *in vitro* assay using isolated neutrophils was used to assess the effect of material chemistry on neutrophil activation. Although a whole blood assay would be closer to the *in vivo* situation, it was thought that whole blood would make it more difficult to isolate the mechanism of material-induced activation. The presence of platelets, which would be activated,[18] would influence leukocyte activation substantially, making result interpretation more difficult. In this assay activation was not an artifact of agitation because similar results were observed under static conditions.

While low levels of endotoxin were detected, it did not appear to have a significant effect on neutrophil activation. Experiments were performed with pure endotoxin (using LPS 055:B5); only slight cell activation could be observed at 30 min at 1 ng/mL, and a more pronounced activation occurred at 5 ng/mL. It is also important to note that such experiments were performed with purified endotoxins that are known to be more potent that "environmental" endotoxins.[19,20] At the concentration of our contaminated biomaterials, environmental endotoxins (i.e., samples of endotoxin contaminated water) did not activate leukocytes (data not shown).

Slight differences could be observed in material-induced neutrophil activation between plasma and serum. In the presence of plasma nonadherent cells were usually less activated, and more adherent cells with higher activation levels were observed. The presence of heparin (5 U/mL) and fibrinogen in the plasma may explain these observations. Heparin at this concentration[21] is known to have some complement inhibitory effects. A slight reduction in complement activation in the bulk could be responsible for the observed decrease in neutrophil activation in the bulk. This is consistent with our complement inhibition results with sCR1 that showed a slight (but not significant) decrease in nonadherent cell activation.

Protein pretreatment

Knowing the passivating effect of albumin on platelets,[22,23] it was presumed that this effect would also be true for PMNs. However, consistent with other studies, albumin did not significantly attenuate neutrophil adhesion.[24–26] Albumin coating also did not have a passivation effect on activation: slightly (but not significant) higher PMN activation was usually present with all albumin coated surfaces except for AN69 and cellophane. A recent study of protein adsorption on hemodialysers also showed that pretreatment with albumin does not reduce leukopenia, suggesting that leukocyte activation still occurs.[24]

Increased adhesion was observed following pretreatment with fibrinogen or plasma. It is likely that the increase in cell adhesion observed with plasma, relative to serum, was due to the presence of fibrinogen. Fibrinogen adsorbed to the surfaces could be an additional ligand for neutrophil adhesion.[25]

Effect of complement on cell adhesion

Although albumin failed to reduce adhesion, adhesion was greatly reduced in the presence of sCR1, regardless of the surface studied. Various researchers[13,27] demonstrated previously that C3b and C3b degradation products are present on some of these surfaces in a significant amount. Among other proteins, iC3b was found on AN69, PDMS, and cellophane. Neutrophils possess several receptors for C3b, iC3b, and C3d.[28] *Only* C3b (but not iC3b) was adsorbed on LDPE.[13] Thus, the low PMN adhesion observed on LDPE and the reduced adhesion obtained when inhibiting complement activation suggests, in accordance with the work of Cheung et al.,[29] that C3b degradation products (but not intact C3b) have an important role in PMN adhesion.

On the other hand, low adhesion was observed on AN69. Although AN69 is not usually considered to be an activator of complement, it is recognized to specifically adsorb complement activation products such as C3a[30,31] and other C3b products[13,31] (C3b, iC3b, C3c, and C3d). The presence of complement adsorbed products on AN69 did not result in "massive" neutrophil adhesion. This may indicate that the adsorbed complement proteins on AN69 are sterically hindered from interaction with their counterreceptor on leukocytes. Further work is needed to clarify these observations.

Effect of complement on PMN activation

Because cellophane and to a lesser extent PET were strong complement activators, we expected that there would be substantial differences in leukocyte activation among at least these materials. The previously observed differences in complement activation did not translate into differences in neutrophil activation. Perhaps this is because neutrophils are reported to be more prone to activation by C5a than by C3a, which would have required longer incubation times.[13] On

the other hand, increasing the PMN incubation time to 60 min did not lead to any significant differences among biomaterials (results not shown).

Complement inhibition, by means of sCR1, also did not fully block the observed activation, although it did block the effect of plasma treatment. Furthermore, neutrophil activation could also be observed when cells were resuspended in TA buffer. Both of these results suggest that complement is only partly responsible for neutrophil activation and other factors such as released products[32,33] and direct contact activation may play a role in activation.

CONCLUSIONS

All biomaterials measurably activated neutrophils. Despite a wide range of material chemistries, however, few differences were observed, suggesting that material chemistry may not be as important as one would expect. The presence of the material, per se, may be sufficient to trigger the nonspecific activation observed here, at least for the nonadherent cells. Complement seemed to have a small effect, if any, on nonadherent neutrophil activation, leading to the conclusion that other mechanisms of activation exist. Significant differences in activation could only be observed with adherent cells: the number of neutrophils and their degree of activation were both material dependent. Neutrophil adhesion was dependent on complement activation, presumably because of the interaction with adsorbed C3b degradation products (iC3b and C3d). However, the degree of activation of the adherent cells appeared to be independent of complement, similar to what was observed for nonadherent neutrophils.

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Appendix B

Effect of flow on neutrophil activation

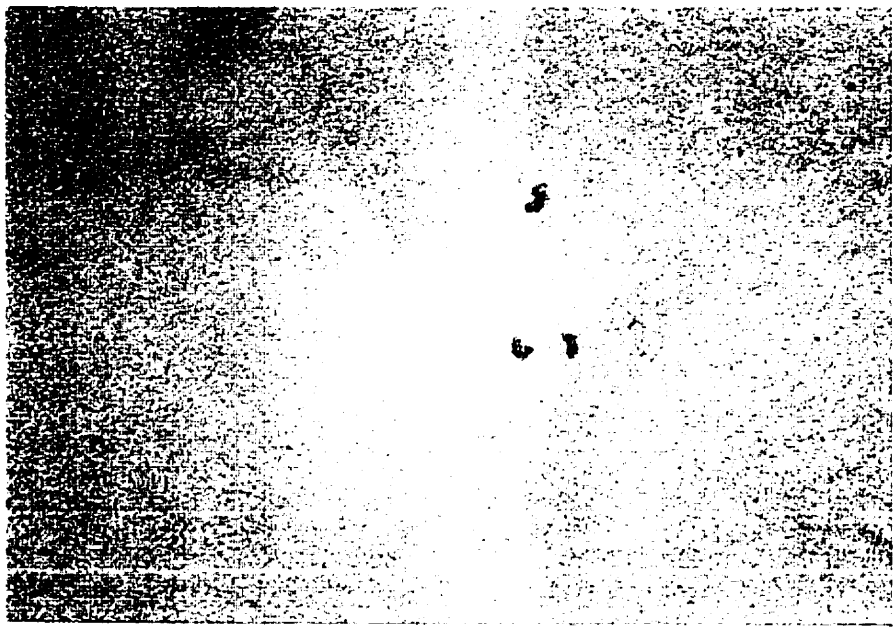
Figure 1: Light micrographs of human neutrophils adherent to fibrinogen-coated polyethylene surfaces after a 30-min contact with neutrophils/red blood cells (RBC) suspension with and without plasma, flow of 165 s-1 using a Grabowski type flow model. Page 226

Figure 2: Light micrographs of human neutrophils adherent to polyethylene (PE) and polyethylene terephtalate (PET) surfaces after a 30-min contact with neutrophils/ RBC/plasma suspension, flow of 100 s-1 using a Grabowski type flow model. Page 227

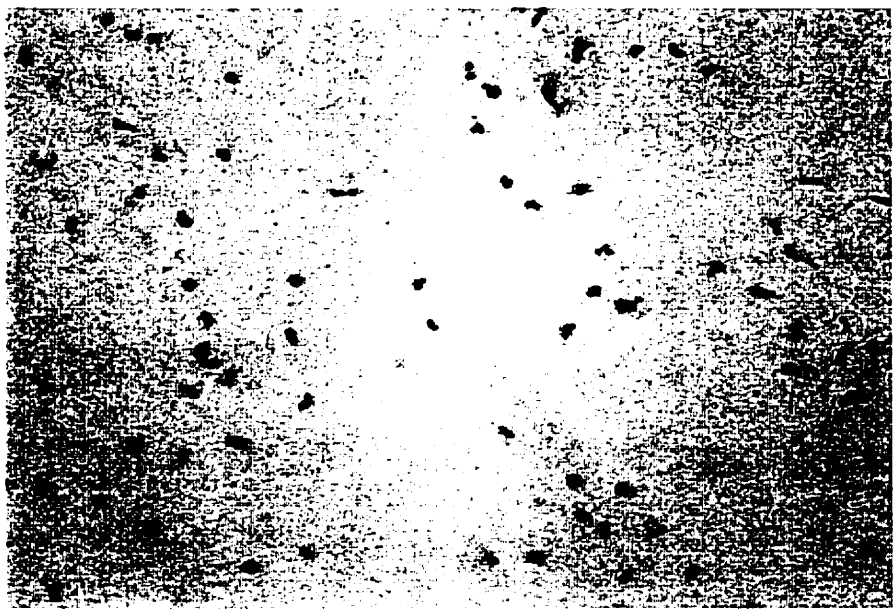
Figure 3: Light micrographs of human neutrophils adherent to polyethylene (PE) and polyethylene terephtalate (PET) surfaces after a 30-min contact with neutrophils/ RBC/plasma suspension, flow of 250 s-1 using a Grabowski type flow model.**Figure 4:** Effect of flow on CD11b upregulation (relatively to EDTA) of non-adherent neutrophils after a 30-min contact with PE and PET surfaces, using a Grabowski type flow model. Neutrophils resuspended with red blood cells (35% hematocrit) and plasma (25%). Page 228

Page 229

Figure 1: Light micrographs of human neutrophils adherent to fibrinogen-coated polyethylene surfaces after a 30-min contact with neutrophils/red blood cells (RBC) suspension with and without plasma, flow of 165 s^{-1} using a Grabowski type flow model.

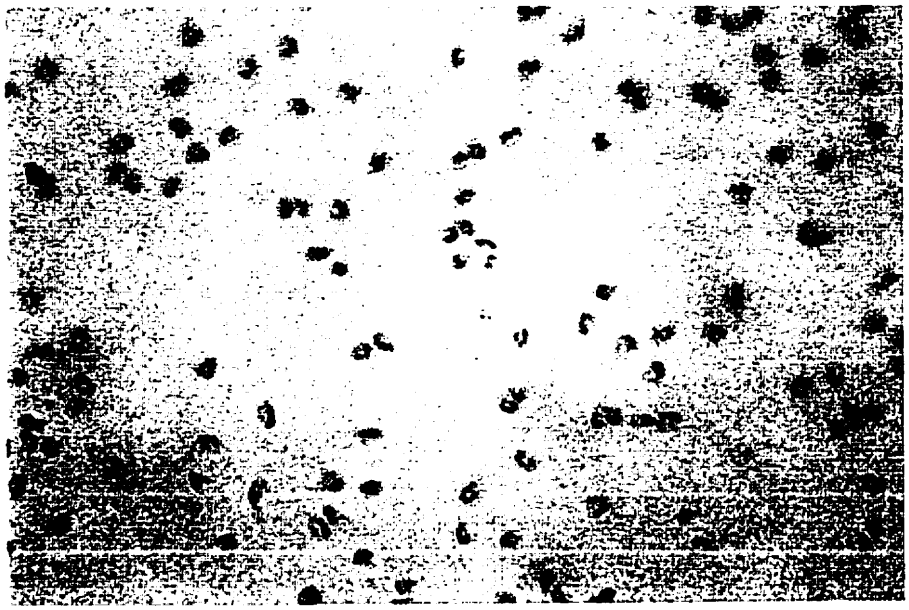


PE (x100), neutrophils/RBC suspension in absence of plasma

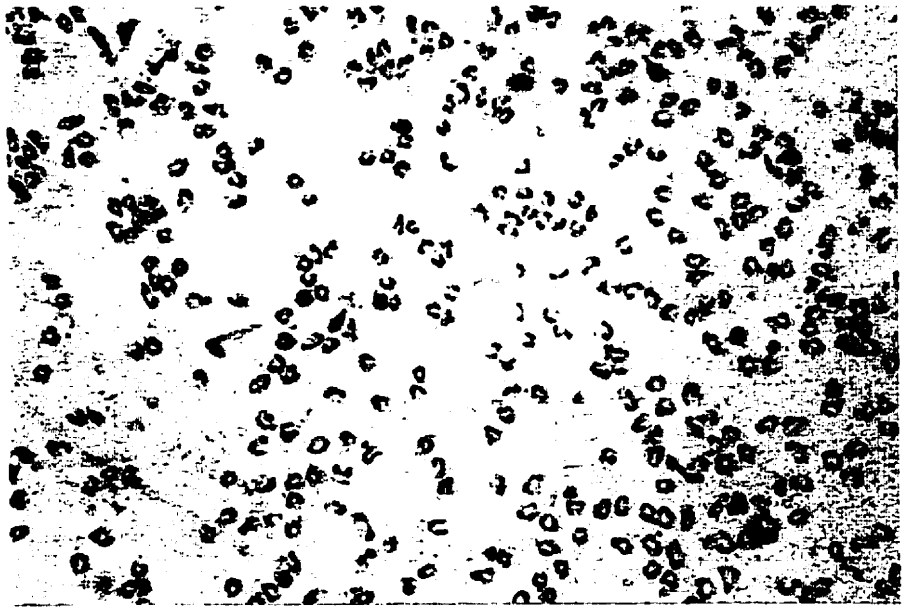


PE (x100), neutrophils/RBC suspension in 25% plasma

Figure 2: Light micrographs of human neutrophils adherent to polyethylene (PE) and polyethylene terephtalate (PET) surfaces after a 30-min contact with neutrophils/ RBC/plasma suspension, flow of 100 s^{-1} using a Grabowski type flow model.

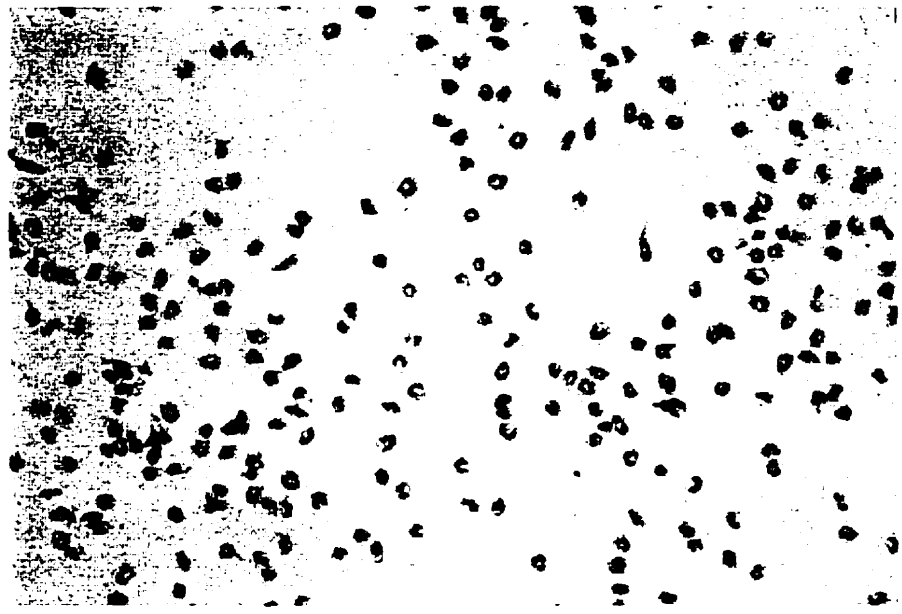


PE (x100)



PET (x100)

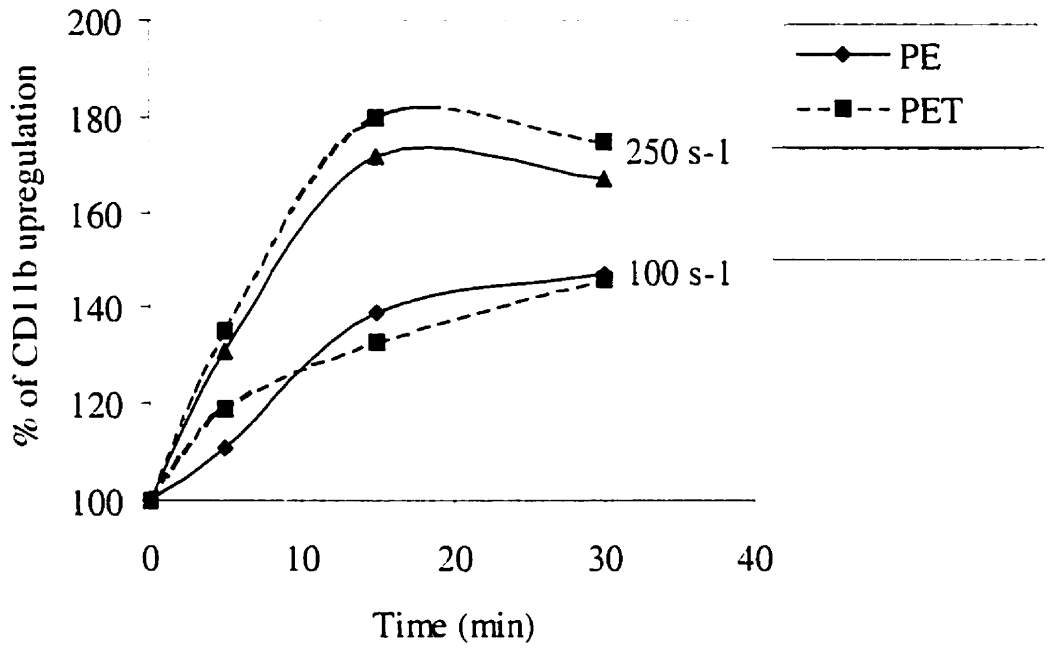
Figure 3: Light micrographs of human neutrophils adherent to polyethylene (PE) and polyethylene terephtalate (PET) surfaces after a 30-min contact with neutrophils/ RBC/plasma suspension, flow of 250 s^{-1} using a Grabowski type flow model.



PE (x100)

PET (x100)

Figure 4: Effect of flow on CD11b upregulation (relatively to EDTA) of non-adherent neutrophils after a 30-min contact with PE and PET surfaces, using a Grabowski type flow model. Neutrophils resuspended with red blood cells (35% hematocrit) and plasma (25%).



Appendix C

Surface characterization of washed versus unwashed beads

Table 1: Elemental composition from XPS low resolution analysis of beads before and after acid/base/ethanol wash to remove endotoxin contamination. Page 231

Figure 1: C1s spectrum of PS beads before and after acid/base/ethanol wash to remove endotoxin contamination. Page 232

Figure 2: C1s spectrum of PS-PEG beads before and after acid/base/ethanol wash to remove endotoxin contamination. Page 232

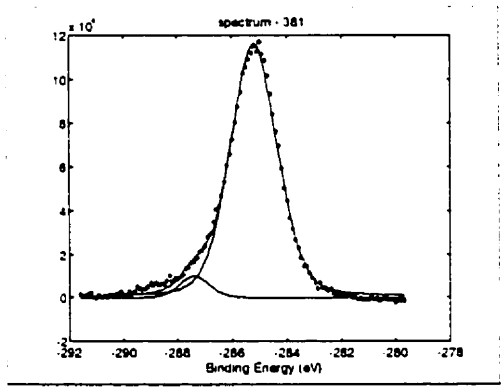
Figure 3: C1s spectrum of PS-PEG-NH_2 beads before and after acid/base/ethanol wash to remove endotoxin contamination. Page 233

Figure 4: Surface roughness of beads, as seen by SEM. Page 233

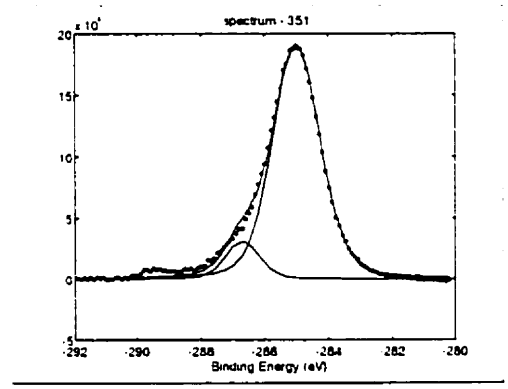
Table 1: Elemental composition from XPS low resolution analysis of beads before and after acid/base/ethanol wash to remove endotoxin contamination.

	Before wash				**After wash**			
	O	N	C	Si	O	N	C	Si
PS	30.3	0	56.5	13.2	13	0	85.8	1.2
PS-PEG	18.4	0	81	0.6	20.5	0	77.8	1.8
PS-PEG-NH_2	18.7	0.4	80.3	0.6	18.8	0.3	79.9	1

Figure 1: C1s spectrum of PS beads before and after acid/base/ethanol wash to remove endotoxin contamination

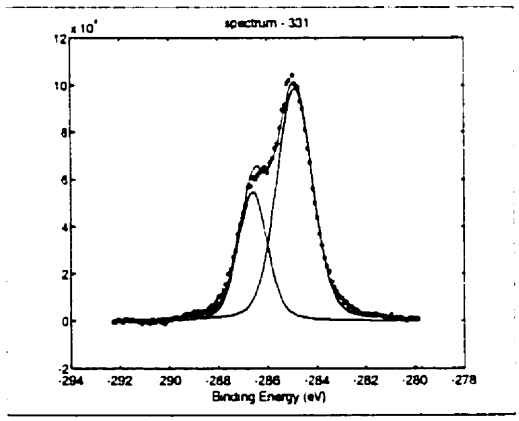


Before wash

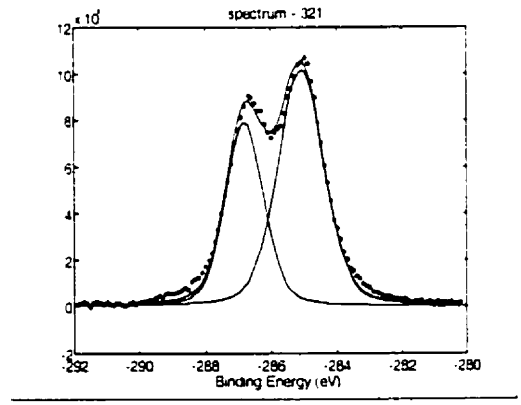


After wash

Figure 2: C1s spectrum of PS-PEG beads before and after acid/base/ethanol wash to remove endotoxin contamination

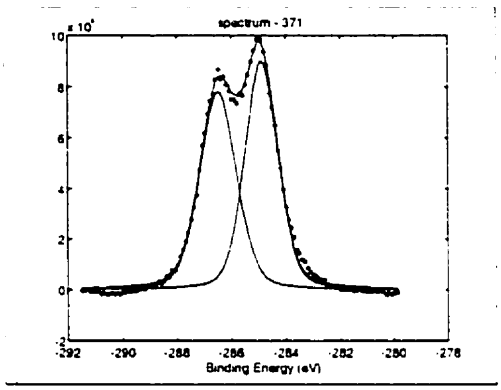


Before wash

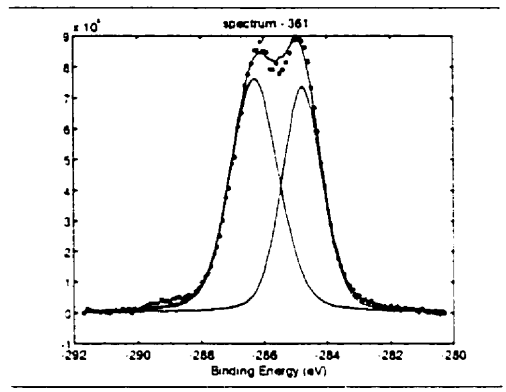


After wash

Figure 3: C1s spectrum of PS-PEG-NH_2 beads before and after acid/base/ethanol wash to remove endotoxin contamination

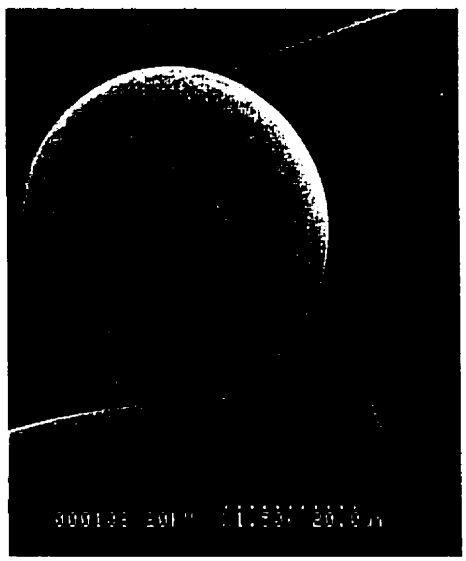


Before wash

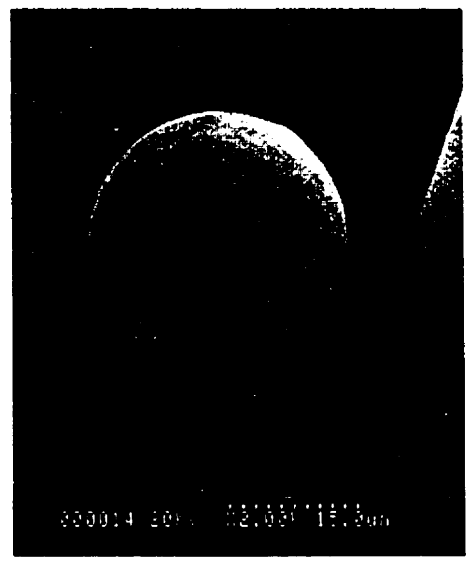


After wash

Figure 4: Surface roughness of beads, as seen by SEM



PS



PS-PEG[a]

[a] The creases observed on PS-PEG are an artifact of the dehydration and drying process

Appendix D

Scanning electron micrographs of leukocytes adherent to beads

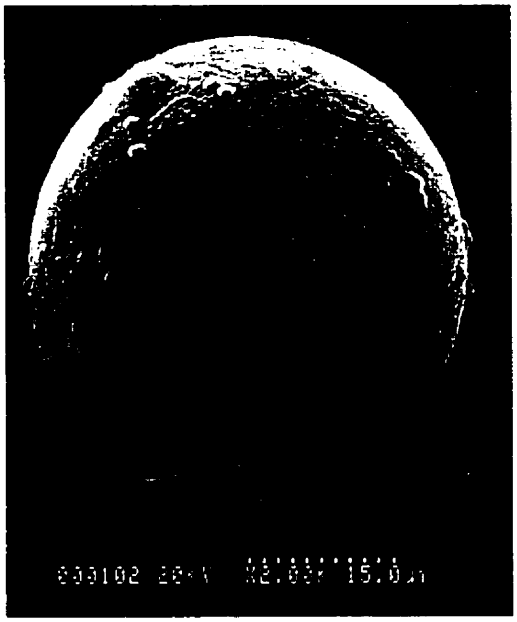
Figure 1: Scanning electron micrographs of leukocyte adhesion on PS after contact with blood for 2 hours. Page 235

Figure 2: Scanning electron micrographs of leukocyte adhesion on PS after contact with blood for 1 hour (PPACK blood – 20x104 beads/mL). Page 236

Figure 3: Scanning electron micrographs of leukocyte adhesion on PS-PEG after contact with blood for 2 hours. Page 237

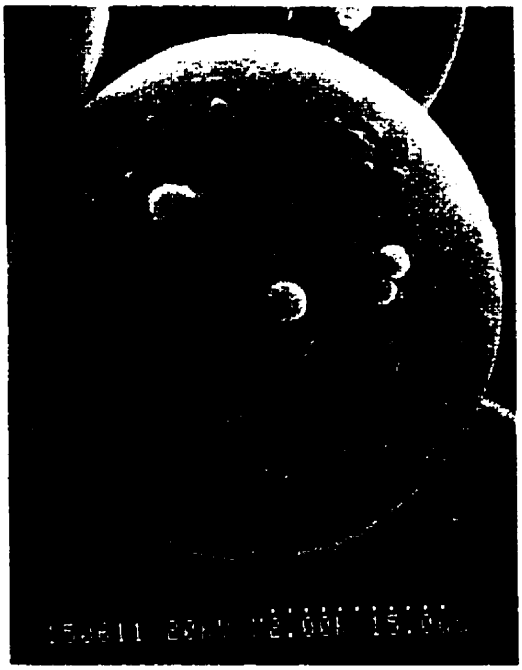
Figure 1: Scanning electron micrographs of leukocyte adhesion on PS after contact with blood for 2 hours.

PPACK blood – $20x10^4$ beads/mL



PPACK blood – $20x10^4$ beads/mL

PPACK blood – $20x10^4$ beads/mL

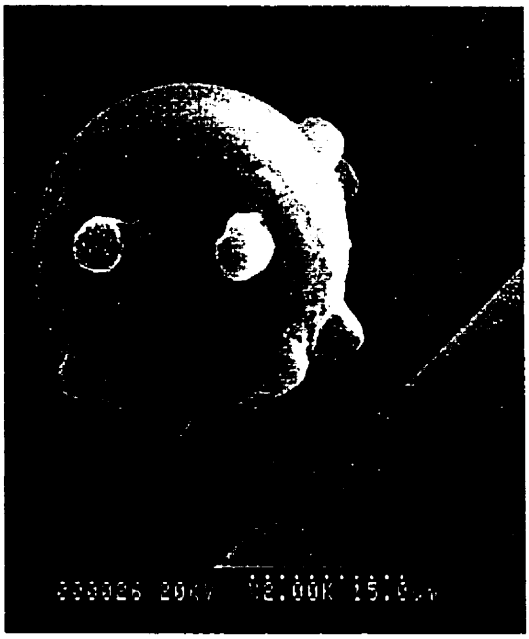


PPACK blood – $20x10^4$ beads/mL

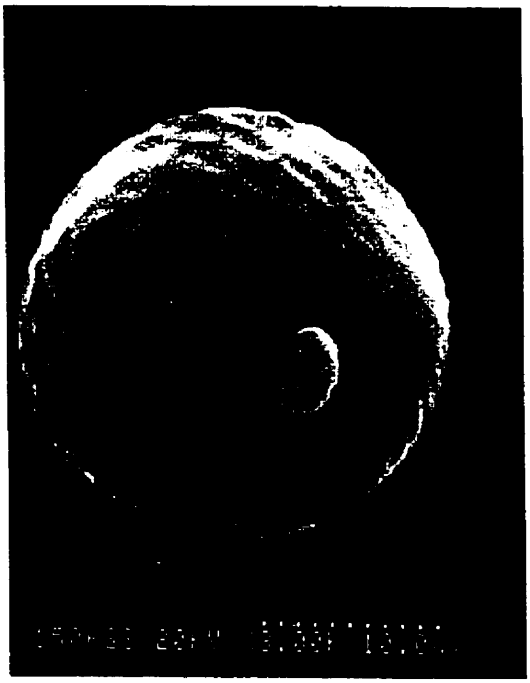
Figure 2: Scanning electron micrographs of leukocyte adhesion on PS after contact with blood for 1 hour (PPACK blood – $20x10^4$ beads/mL)

Figure 3: Scanning electron micrographs of leukocyte adhesion on PS-PEG after contact with blood for 2 hours

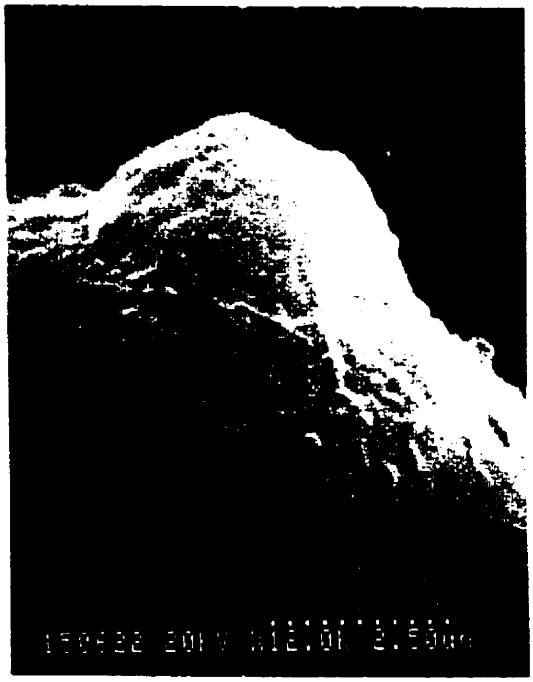
Heparinized blood – $20x10^4$ beads/mL



PPACK blood – $20x10^4$ beads/mL



PPACK blood – $54x10^4$ beads/mL



PPACK blood – $0.55x10^4$ beads/mL

Appendix E

XPS analysis of the new batch of PS-PEG beads

Table 1: Comparisons of elemental composition of PS-PEG beads (dry form) between two batches. Page 239

Table 2: Comparisons of the high resolution C1s composition of PS-PEG beads (dry form) between two batches. Page 239

Figure 1: High resolution C1s spectra of the two different batches of PS-PEG beads (dry form): (a) old batch (Chapter 3), (b) new batch (Chapter 6) Page 239

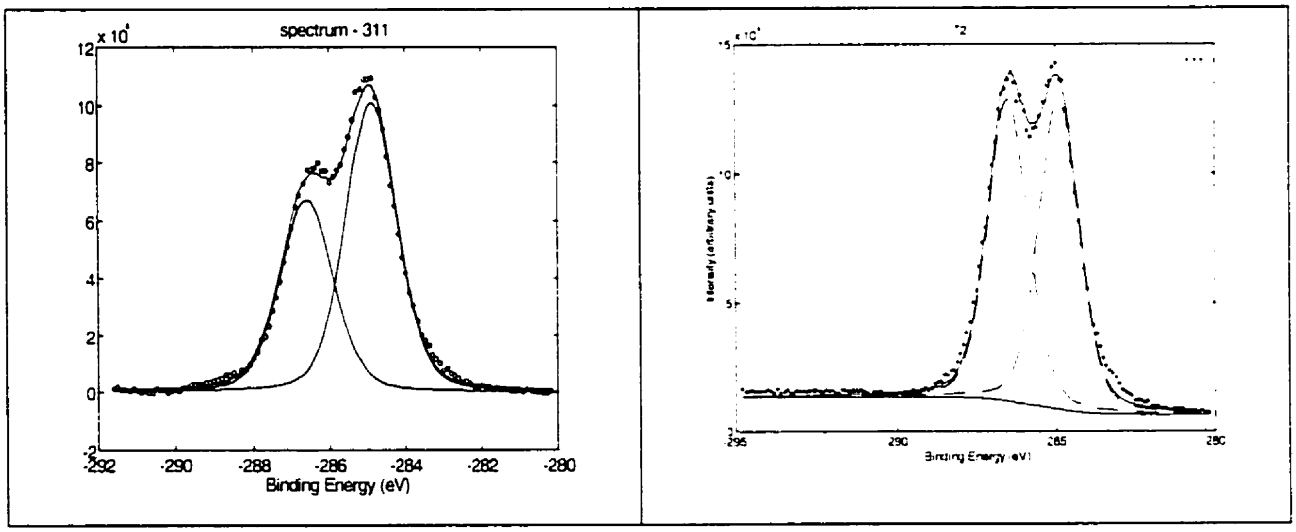
Table 1: Comparisons of elemental composition of PS-PEG beads (dry form) between two batches.

PS-PEG	O1s	C1s	Si2p
Old batch (Chapter 3)	20.3	79.2	0.5
New batch (Chapter 6)	21.9	77.8	0.3

Table 2: Comparisons of the high resolution C1s composition of PS-PEG beads (dry form) between two batches.

PS-PEG	285 eV (C-C bonds)	286.5 eV (C-O bonds)
Old batch (Chapter 3)	53.8	**46.2**
New batch (Chapter 6)	47.5	**52.5**

Figure 1: High resolution C1s spectra of the two different batches of PS-PEG beads (dry form): (a) old batch (Chapter 3), (b) new batch (Chapter 6)



a) b)