DEVELOPMENT OF A FLOW CYTOMETRIC ASSAY OF PLATELET ACTIVATION AND PLATELET FUNCTION

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This research was approved by the Ethics Committee for Research on Human Subjects (Medical), University of the Witwatersrand (Clearance Certificate Number M980551).

DECLARATION:

I, Erin Eleanor Simleit declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Haematology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

.....

on the 10th day of November, 2003.

DEDICATION

To my special husband, Rob and "a-boyz", our beautiful Luke and James.
Also to my wonderful parents.

ABSTRACT

Platelets play a central role in haemostasis, and their malfunction may manifest as haemorrhage, or in certain settings, possibly as thrombosis. Several platelet function tests are available, but those that are feasible to perform as routine diagnostic tests, are in many instances neither sufficiently sensitive, nor specific to be of value.

The aim of this study was to develop testing of platelet activation and platelet function by means of flow cytometry. The bulk of the study involved the optimisation of the assays, focusing on any technical difficulties that might interfere with the standardisation and reproducibility. Normal ranges for circulating levels of endogenously activated platelets in normal, non-smoking, young adults were determined, and an assay for delta storage pool disease was developed.

Significant technical difficulties were encountered in establishing such testing and each laboratory must assess the validity, precision and accuracy of all methodology used, particularly since there is little consensus in the literature.

Whilst the method offers exciting new ways of studying platelets, it cannot be considered to be revolutionary in terms of avoiding the disadvantages that previous methods of assessing platelet activation were subject to. The fragility of platelets and ease of artefactual activation remain problematic.

Once these tests are established and if found to be accurate and reproducible, they may be useful in a variety of settings, such as in assessing the quality of platelet concentrates (since platelet activation has been shown by some groups to correlate with poor platelet survival in vivo), in evaluating platelet function in a different dimension in patients with bleeding and thrombosis, and possibly in the prediction of thrombotic conditions e.g. pre-eclampsia, and in estimating the risk of thrombotic events such as myocardial infarction or cerebrovascular accidents in patients with established coronary artery or cerebrovascular disease respectively (although prospective studies addressing these questions are ongoing.)

In this regard, a further requirement is that researchers in the field come to a consensus on optimal methodology and establish guidelines for use of this technique. This will hopefully encourage researchers to generate studies that are more easily comparable.

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INDEX

ETHICS CLEARANCE
DECLARATION
DEDICATION
ABSTRACT
ACKNOWLEDGEMENTS
INDEX
LIST OF FIGURES
LIST OF TABLES
ABBREVIATIONS
GLOSSARY

INTRODUCTION 1

STUDY OBJECTIVES 19

MATERIALS AND METHODS 21

A) OPTIMISING THE ANALYSIS OF PLATELET ACTIVATION 21
A.1.)Venesection 21
A.2.)Controls 23
A.3.)Interference of monoclonal antibodies used in the combinations chosen 24
A.4.)Titrations 25
A.5.)Optimisation of settings 27
A.5.1.)Light scatter analysis 27
A.5.2.)Expression of activation markers 33
a)CD42a-PerCP/CD62-P-PE/PAC1-FITC 33
b)CD61-PerCP/CD63-PE/Annexin V-FITC 34
A.6.)Gating strategy 38
A.7.)Fixation 50
A.7.1.)Formation of aggregates 50
A.7.2.)Preservation of antigen positivity/negativity 66
PAC1 66
Annexin V 66
CD62-P and CD63 67
A.8.)Time dependant activation of platelets 68
A.8.1.)Varying the period after incubation with antibody and dilution with buffer 68
Platelet Activation Markers 69
Aggregates 70
Microparticles 71
A.8.2.)Varying the period prior to incubation with antibodies 71

B) FINAL PROTOCOL FOR ANALYSIS OF LEVELS OF IN VIVO CIRCULATING ACTIVATED PLATELETS IN NORMAL DONORS 77

Pre-cytometric preparation 77
Venesection 77
Incubation with antibodies 78
Cytometric acquisition of events 78

C) THE ANALYSIS OF THE PRESENCE OF DENSE GRANULES AND THEIR RELEASE 81

Pre-cytometric preparation 81
Venesection 81
Incubation 82
Cytometric acquisition of events 82

D) CORRELATION OF BLEEDING TIME WITH PLATELET ACTIVATION AS ASSESSED BY FLOW CYTOMETRY 85

RESULTS 87

DISCUSSION 94

A) RESULTS OF NORMAL CONTROLS IN THIS STUDY – WHAT ARE THEIR SIGNIFICANCE? 94

1) PLATELET ACTIVATION MARKERS 94
1.1.)CD63 94
1.2.)Annexin V 95
1.3.)CD62-P 97
1.4.)PAC1 100
1.5.)CD42a 100
1.6.)Microparticles 101
1.7.)Aggregates 101

2) MEPACRINE UPTAKE AND RELEASE 103

3) CORRELATION OF BLEEDING TIME WITH PLATELET ACTIVATION AS ASSESSED BY FLOW CYTOMETRY 104

B) EMERGING PRINCIPLES 105

Specific methodology incompatible/compatible with the antibodies used 108
Fixation 108
CD62-P 108

CD63 109
PAC1 and Annexin V 109
Choice of platelet specific identifying monoclonal antibody 109
Aggregates 110
Microparticles 110

CONCLUSION 116

REFERENCES 117

APPENDICES 135

- Method for the isolation and washing of platelets from whole blood
- Composition of HEPES buffer
- Composition of Hank's Balanced Salt Solution(without calcium chloride, magnesium sulfate, phenol red and sodium bicarbonate)
- Mepacrine stock solution
- Solution of fixative

LIST OF FIGURES

Figure 1	Annexin V-FITC Titration	28
Figure 2	Setting the discriminant on CD61-PerCP	30
Figure 3	Comparison of platelet identifying antibodies labeled with different fluorochromes (PerCP and PE)	32
Figure 4	The effects of centrifuging a sample of activated platelets	36
Figure 5	Dot plots of antibodies and buffer, with no cellular component	
	a) Logarithmic settings for forward and side scatter	41
	b) Linear settings for forward and side scatter	42
Figure 6	Dot plots of separated, washed platelets labeled with antibodies to CD42a-PerCP, CD62-P-PE and PAC1-FITC	
	a) Logarithmic settings for forward and side scatter	43
	b) Linear settings for forward and side scatter	44
Figure 7	Dot plots of activated platelets labeled with antibodies to CD42a-PerCP, CD62-P-PE and PAC1-FITC	
	a) Logarithmic settings for forward and side scatter	45
	b) Linear settings for forward and side scatter	46
Figure 8	Dot plots of microparticles labeled with antibodies to CD42a-PerCP, CD62-P-PE and PAC1-FITC	
	a) Logarithmic settings for forward and side scatter	47
	b) Linear settings for forward and side scatter	48
Figure 9	Dot plots depicting the appearance of platelets, leucocytes and erythrocytes	49
Figure 10	Example of a normal donor to illustrate the gating procedure followed	52
Figure 11	Illustration of how the criteria for defining microparticles would affect results	55
Figure 12	Dot plots illustrating the effects of fixing a sample before and after incubating with antibodies	63
Figure 13	Graphs depicting changes in activation status of platelets over time	72
Figure 14	Graphs illustrating mepacrine uptake and release	93

LIST OF TABLES

Table 1	Comparison of venesection with and without a tourniquet	21
Table 2	Comparison of venesection using a Vacutainer, and the double syringe technique with a butterfly	22
Table 3	Comparison of venesection using a Vacutainer, and the double syringe technique with prostaglandin E1	23
Table 4	Final settings for the antibody combination PAC1-FITC, CD62-P-PE and CD42a-PerCP	79
Table 5	Final settings for the antibody combination Annexin V-FITC, CD63-PE and CD61-PerCP	80
Table 6	Final settings for the combination CD42b-PE and mepacrine	83
Table 7	Percentages of platelets expressing activation markers as tested by Annexin V-FITC /CD63-PE/CD61-PerCP	87
Table 8	Percentages of platelets expressing activation markers as tested by PAC1-FITC /CD62-P-PE/CD42a-PerCP	88
Table 9	Percentages of platelets recorded as microparticles or aggregates	89
Table 10	Mean channel number of fluorescence with markers in the Annexin V-FITC /CD63-PE/CD61-PerCP analysis	90
Table 11	Mean channel number of fluorescence with markers in the PAC1-FITC /CD62-P-PE/CD42a-PerCP analysis	91
Table 12	Mepacrine uptake and release	92

ABBREVIATIONS

AA	Arachadonic acid
ACD	Acid Citrate Dextrose
ADP	Adenosine diphosphate
BTG	Beta thromboglobulin
EDTA	Ethylenediamenetetraacetic Acid
-FITC	Fluorescein isothiocyanate
GP	Glycoprotein
Hank's BSS	Hank's Balanced Salt Solution
HEPES	N-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
MCN	Mean channel number
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
-PE	Phycoerythrin
-PerCP	Peridinin chlorophyll protein
PF4	Platelet factor 4
PG	Prostaglandin
PMT	Photomultiplier tube
PS	Phosphatidylserine
RT-PCR	Reverse transcriptase – polymerase chain reaction
TRAP	Thrombin Receptor Activating Peptide
VWF	Von Willebrand factor

GLOSSARY

voltage	By adjusting the voltage signal one can increase the "positivity" of a particular signal. However, this does not necessarily separate negative from positive.
amplifier	Increases or decreases signals from cells so as to put them "on scale".
colour compensation	There is significant overlap of fluorescence spectra between many of the dyes, especially FITC/PE and PE/PerCP. Whilst optical filters minimize the effect of this it is still necessary to reduce unwanted overlap by subtracting from the measured signal the expected proportion which is accounted for by overlap of another fluorochrome. This can be most easily illustrated with two populations of cells expressing only one of the two markers used per cell population. If compensation is correct the population should be recorded in the negative region for the marker not expressed, but not right up against the negative axis, since this is what occurs with overcompensation.
discriminant	Many of the parameters measured can be used to distinguish or "gate out" unwanted events. The value of this chosen discriminant can be sequentially raised, removing at first events which are low on the scale (e.g. very small events) and then later sequentially more positive (e.g. larger) events.
mean channel number	The average channel number of a sample from a population of cells.
gate	A numerical or graphical boundary (region) that defines a subset of data.
acquisition document	A file tailored by the user to acquire the data pertaining to specific events recorded by the detectors in a specific way. Events can be included or excluded by using a discriminant or a gate. The settings are optimized to collect the data in as accurate and clear a way as possible. Such settings include colour compensation, voltage, amplifier gains etc.
analysis document	A file tailored by the analyzer to analyze the parameters required in a specific way. Populations can be focused on or gated out, depending on the user's requirements.

INTRODUCTION

Platelets play a central role in haemostasis, and their inadequacy, either numerically or functionally, may result in haemorrhage. On the other end of the spectrum, the role of platelets in thrombosis, particularly arterial, is becoming increasingly a topic of interest.

Because of the potentially devastating consequences of both haemorrhage and thrombosis, several techniques for evaluating platelet function have been developed. Of the more traditional techniques, only the bleeding time and platelet aggregometry have been utilized as routine clinical tests of platelet function. It should be noted that these tests are not without limitations (George and Shattil, 1991; Miletich, 1995). They are neither sufficiently sensitive for screening purposes, nor sufficiently specific for diagnostic purposes.

Apart from these two tests, which are accessible to the clinician for routine diagnostic purposes, there are other more specialised investigations used predominantly as research tools, which evaluate platelet function or activation in various ways. These include lumiaggregometry, uptake and release of radiolabelled serotonin and electron microscopy used in diagnosis of delta storage pool disease (Laffan and Bradshaw, 1995; Gordon et al, 1995; Wall et al, 1995). Endogenous platelet activation has been assessed by measuring plasma levels of platelet specific proteins such as beta thromboglobulin (βTG), glycocalicin, (Rinder, 1998) platelet factor 4 (PF4) or soluble P-selectin levels (although the latter may be also of endothelial origin) (Michelson, 1996). A major limitation of these techniques

is the lack of reproducibility, and interpretation of results (Laffan and Bradshaw, 1995; Michelson, 1996; Belluci et al, 1993). Metabolites of thromboxane breakdown may also be measured in the urine, reflecting endogenous platelet activation. Tests of platelet adhesion include the Baumgartner technique of passing whole blood over everted rabbit aorta denuded of endothelial cells, and platelet retention in a glass bead column which is rarely done today because it lacks specificity. The ability of activated platelets to support coagulation, specifically the tenase and prothrombinase complex formation has been measured using the prothrombin consumption index. Regarding suspected defects in the signal transduction pathways, highly specific assays for various steps in arachadonic acid metabolism are available (Laffan and Bradshaw, 1995).

The above tests have all been used in research but the vast majority are inappropriate for routine diagnostic purposes.

More recent developments in assessing platelet function include the clot signature analyser, the thrombotic status analyser, the platelet function analyser (PFA-100), the cone and plate analyser and high shear filterometer (Harrison, 2000). These methods assess predominantly mass platelet function rather than individualised platelet activation and function.

Fairly recently a new technique has emerged for testing certain aspects of platelet function and platelet activation - flow cytometry. The basic principle of flow cytometry is that cells (including platelets in this instance) are chanelled in single file

past a laser light source, making use of an appropriate sheath fluid. These cells have been labeled in some fashion, either with fluorescent dyes having a particular affinity for a certain chemical compound within the cells or with fluorescently labeled monoclonal (or polyclonal) antibodies directed at particular antigens on the surface of, or within the cells. As the cells pass the laser beam, they interact with the light source in a number of ways. Light scatter is measured at a position 180° from the direction of the light source. This "forward scatter" gives an indication of cell size. The amount of light scattered towards a detector placed at 90° (side scatter) gives an indication of complexity of the cell. If the cell has bound fluorescently labeled antibody, the fluorochrome is excited by the laser and emits energy of a particular wavelength depending on the fluorochrome chosen. Two or three different fluorochromes may be used to label different antibodies which may then label the same cell simultaneously. Due to physical constraints, this fluorescently generated light is also detected at 90° to the laser beam. However, being of longer wavelength than the laser light, it may be separated by the use of a dichroic mirror. Once the fluorescent light has then passed through the mirror, it may be separated into the various wavelengths generated by the different fluorochromes with the use of further dichroic filters and band pass filters, so that ultimately each wavelength of light is referred its own detector or photomultiplier tube (PMT). The PMT then converts the light signal into an electrical signal with the size of the electrical signal being proportional to the intensity of the light signal at that wavelength. The signals are integrated by computers, stored and displayed graphically in the form of histograms or dot plots etc.

Several aspects of platelet function may be assessed using this technique. Some of these will be considered in the context of the following brief description of the process of platelet recruitment to a site of bleeding and subsequent activation (George and Shattil, 1991; Ware and Coller, 1995; Blockmans et al, 1995).

When a blood vessel is damaged, the subendothelial connective tissue is exposed, becoming accessible to the blood components. By interaction between the subendothelium and certain glycoprotein receptors on the platelet surface, platelets adhere to this connective tissue matrix, spread and become activated. The result of activation is competence to aggregate with other activated platelets, the ability to recruit more platelets to the area in need of repair and the provision of a surface which supports the coagulation cascade.

Multiple surface receptors on the platelet are capable of adhering to components of the subendothelium. These include GPIb/IX/V, a receptor for von Willebrand factor (VWF), and several integrins including GPIa/IIa, a collagen receptor, GPIc/IIa, a fibronectin receptor, GPIc*/IIa, a laminin receptor and $\alpha v\beta 3$, a vitronectin receptor. In addition, many matrix components can interact with each other eg. VWF, fibronectin, thrombospondin and collagen. The relative contributions of each of the receptors to adhesion is not entirely certain, and there appears to be profound complexity and redundancy. From observing the clinical manifestations of patients with inherited deficiencies of GPIb/IX, GPIa/IIa or of VWF, it is clear that the

platelet's interaction with VWF and collagen is essential for normal haemostasis and the GPIb/IX-VWF-matrix interaction is probably the most crucial in the process of adhesion. Some of the other receptors require non-physiological conditions for optimal adhesive function, and may therefore be less significant in vivo (Ware and Coller, 1995).

This process of adhesion does not require metabolic activity of the platelet, but once it occurs, may trigger activation of the platelet. The platelet undergoes a shape change from discoid to spherical with several spiny projections. During activation, thromboxane A2 is generated from arachadonic acid (AA) released from the membrane phospholipids. This diffuses from the platelet and binds to membrane receptors, amplifying activation reactions. Another change that occurs on activation is release of the granules including alpha granules, dense granules and lysosomal granules. Dense granular secretion is most easily triggered, followed by alpha granules and then lysosomal granules, the secretion of which requires strong agonists such as thrombin or collagen at high concentrations (Blockmans et al, 1995). This degranulation results in the release of several agonists which, by binding to their specific receptors on the platelet surface, augment recruitment and activation of new circulating platelets. In addition some of the alpha granule products released mediate and reinforce platelet adhesion and aggregation including thrombospondin, VWF and fibrinogen.

Another very important change that occurs on activation is the conversion of GPIIb/IIIa to an active form, competent to bind fibrinogen and other adhesive

proteins containing the RGDS sequence such as VWF, fibronectin and vitronectin (Abrams and Shattil, 1991), although the GPIIbIIIa receptor in the unactivated state may also bind VWF (George and Shattil, 1991). Fibrinogen, being a dimeric molecule, can form a bridge between two adjacent activated platelets and so enable aggregation.

Central to the coagulation cascade is the necessity for a negatively charged phospholipid surface on which formation of the tenase and prothrombinase complex can occur. Such a surface is provided by activated platelets. In its unactivated state, the platelet has membrane transporter systems which result in the negatively charged phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) being sequestered on the inner leaflet of the membrane. On activation of the platelet when intracellular calcium levels become raised, the transporters responsible for maintaining this balance are inactivated and a transporter with "scrambling" activity is activated resulting in random movement of the phospholipids, and as a result, exposure of the aminophospholipids PE and PS on the outer leaflet of the membrane. These lipids are required for assembly of the tenase and prothrombinase complexes and also for the activation of protein C. In addition, when calcium influx into the cytosol occurs, this may result in calpain activation which facilitates membrane blebbing and the release of microvesicles or microparticles which express PS and are therefore procoagulant. (Zwaal and Schroiy, 1997). This process is also associated with a decrease in the tyrosine phosphorylation of proteins (Pasquet et al, 1997) and is independent of and preceded by the membrane flip-flop. (Dachary-Prigent et al,1995).

The flip-flop of the membrane and shedding of microvesicles is analogous to the processes occurring during apoptosis of a nucleated cell. In fact it was established by RT-PCR that resting platelets were positive for both Bcl-2 and Bax. However after stimulation with ionomycin, platelet microparticle formation was documented at the same time as the Bcl-2/Bax ratio decreasing to ~60% of the levels of unactivated platelets. These changes are similar to those seen in nucleated cells undergoing apoptosis, once again strengthening the case for end stage platelet activation representing platelet apoptosis (Rinder, 1998).

Thus platelet activation and aggregation may be triggered by adhesion to proteins such as collagen, by soluble agonists such as adrenaline, ADP, serotonin, thrombin, vasopressin and the precursor of thromboxane A2, arachadonic acid and possibly by cell to cell contact during aggregation (George and Shattil, 1991). It is by using these agonists, that platelet aggregation may be measured in vitro, utilising the principles of light transmission or impedance to detect the aggregation of platelets.

Some of the aspects of platelet activation that are measurable by flow cytometry include:

1) The presence or absence of surface glycoproteins crucial for normal platelet function. Commonly the antibodies used here include those against CD42a which is GPIX, noncovalently bound in a 1:1 ratio to GPIb, or against CD42b which is GPIb. These antigens are thus typically absent in Bernard-Soulier Syndrome. Antibodies to CD61 (GPIIIa) are used to diagnose Glanzmann's

thrombasthenia. Rarer forms of Glanzmann's disease result in the GPIIb/IIIa complex being present but not functional. Antibodies detecting the activated form of GPIIb/IIIa on platelets activated artificially with agonists may be useful in making such a diagnosis (Ginsberg et al, 1990).

Other platelet membrane receptors may also be assayed, using an appropriate monoclonal antibody directed against that particular protein. By characterising platelets as fully as possible using flow cytometric methods, new insight may be gained as to what the platelet defect is, in conditions which previously have not been fully elucidated (Semple et al, 1997).

2) The presence of dense granules containing ADP and the ability to release their contents can be measured using mepacrine dye which is a compound emitting green fluorescent light when activated by an appropriate light source. This dye has a particular affinity for adenine nucleotides and therefore concentrates in the dense granules, and can be measured there by flow cytometry. Although mepacrine itself inhibits phopholipases, this effect can be overcome using a strong agonist such as thrombin, which then demonstrates the platelets' ability to release dense granular contents (Gordon et al, 1995; Wall et al, 1995; Michelson, 1996).

3) The release of alpha granules can be detected using an antibody to CD62-P, a selectin protein (GMP140 or PADGEM) expressed on the inner membrane of alpha granules which is exposed on the platelet membrane on fusion of these

granules with the cell membrane as the platelet discharges its granular contents. The protein is thought to mediate adhesion of activated platelets to monocytes and neutrophils by interacting predominantly with P-selectin glycoprotein ligand 1 on the white cells (Michelson, 1996). It has been shown, however, that platelets which express P-selectin continue to circulate, and lose their P-selectin expression, continuing to function (Michelson et al, 1996; Berger et al, 1998). This suggests that this test may not be useful under all conditions for the detection of circulating activated platelets but rather under conditions where there is either continual activation, or the blood sample is taken immediately after the stimulus for activation, or the sample is taken from a position anatomically downstream of the source of activation. (Michelson, 1996).

The binding to the platelet surface of certain proteins released in the alpha granules, such as thrombospondin can also be used to measure activation (Abrams and Shattil, 1991).

4) The release of lysosomal contents may also be assessed using an antibody to CD63 which is a protein expressed on the inner membrane of lysosomes, again, exposed on secretion of lysosomal contents (Nieuwenhuis et al, 1987).

5) The conformational change of GPIIb/IIIa required for fibrinogen binding may be measured in a variety of ways. An antibody (PAC1) has been raised against the conformationally altered receptor, which competes with

fibrinogen for binding. The affinity of this IgM antibody for the fibrinogen receptor is actually much greater than that of fibrinogen (Shattil et al, 1987) (although the Fab fragment alone has a somewhat lesser affinity) (Abrams et al, 1994). Thus the plasma level of fibrinogen is not practically a significant variable affecting the binding of PAC1. The specificity of PAC1 may be due to one of its hypervariable regions containing an arginine-tyrosine-aspartic acid sequence which may structurally mimic the arginine-glycine-aspartic acid (RGD) containing site in fibrinogen (Abrams and Shattil, 1991).

Other methods of detecting GPIIb/IIIa activation include fluorescently labelled fibrinogen (Xia et al, 1996), antibodies to a receptor induced binding site (RIBS) (a neoantigen created on fibrinogen by its binding to activated GPIIb/IIIa (Lindahl et al, 1992), or antibodies to a ligand induced binding site (LIBS) (a neoantigen on the receptor created by the binding of fibrinogen) (Michelson, 1996). Thus these three methods can be used to demonstrate the sequential steps involved in activation of IIb/IIIa and binding of its ligand fibrinogen.

This has been useful in specifying the exact point of abnormality of the GPIIb/IIIa receptor in patients with functional variants of Glanzmann's thrombasthenia.

6) Formation of microparticles can be detected as particles much smaller than platelets but expressing the platelet antigens (Abrams et al, 1990). These particles are also characterised by their procoagulant nature and therefore will be expected to bind annexin V. Since microparticles are free to diffuse away from the site of adhesion and activation of platelets (Zwaal and Schroiy, 1997) and be removed from the site of vascular damage, it stands to reason that their detection may overcome the one limitation quoted as a disadvantage of flow cytometry in comparison to the older techniques for measuring platelet activation, such as βTG and PF4. This is that the platelets which are adhering or have been recently consumed in the process of haemostasis or thrombosis would go undetected since they are predominantly not free to circulate (Michelson, 1996). (This would also obviously depend on the half-life of microparticles.) The significance of these circulating microparticles and their function is not certain. It is under debate whether they are present as a result of thrombosis or may themselves cause thrombosis. The anticoagulant role of microparticles has also been documented, and the exact role that that microparticles play in vivo remains under discussion (Nomura et al, 2000).

7) The development of a procoagulant surface. The exposure of the aminophospholipids PS and PE which occurs on flip-flopping of the membrane, as is seen in apoptosis, would result in the binding of annexin V to the membrane (Zwaal and Schroiy, 1997). Incubation with fluorescently labeled annexin V would therefore enable detection of this procoagulant

surface.

Surface bound factor V or VIII can indicate a similar state.

8) On activation of the platelet GPIb/IX is redistributed to the open cannalicular system. This redistribution is reversible with time (~1 hour), but, according to the literature, does not result in recovery of the original density of expression. On repeated stimulation, the GPIb/IX can again be redistributed, but the reduction is smaller and recovery less (Michelson, 1992; Michelson et al, 1994). If this is so, circulating activated platelets would presumably then be a heterogeneous population with expression of GPIb/IX varying in intensity from significantly dimmer than inactive platelets, if they are very recently activated or have been repeatedly activated, to ~80% of the inactive platelet's intensity. There is some debate in the literature regarding the decreased intensity of expression of GPIb/IX after activation. To some extent these findings may also vary with the anticoagulant used e.g. use of EDTA is reported to result in a significant time related down regulation of this receptor after thrombin stimulation which is apparently reversible with restoration of calcium levels. The down regulation is much less dramatic with thrombin activation in the presence of calcium. (Rinder, 1998).

This illustrates a well known problem in the study of coagulation, namely that of in vitro findings not necessarily representing in vivo events.

9) CD36, the thrombospondin receptor is expressed on resting platelets but the density of expression of this receptor increases with activation (Michelson, 1996; Briere et al, 1997).

10) An increase in cytosolic free calcium can be measured with Fluo-3 or Indo-1, but the fluxes are rapidly reversed, making quantitative analysis difficult (Schmitz et al, 1998).

11) An increase in F-actin content of platelets (polymerisation) can be detected with fluorescent phallacidin or phalloidin derivatives (Schmitz et al, 1998).

12) Although the platelet shape change may be associated with a change in light scatter characteristics, this is highly variable depending on instrument optics and the type of data analysis used. Standardisation of such a measurement is therefore difficult (Schmitz et al, 1998).

13) The formation of circulating platelet-leucocyte and particularly platelet-monocyte aggregates was discovered a little later to be a marker of activation which is regarded by many authors to be a more sensitive and stable (Harrison, 2000; Rinder, 1998), clinically relevant test. It is reported to be sometimes significantly increased when other markers of activation may not be (Peyton et al, 1998; Gawaz et al, 1995; Ott et al, 1996). Explanations offered include the possibility that activated platelets are removed from circulation whilst neutrophil-platelet or monocyte-platelet aggregates may be more stable. Since the half life of activated platelets may be shorter, this might result in the

brief period of circulating activated platelets related to an event such as ischaemia, being missed (Ott et al, 1996). In other circumstances the presence or absence of aggregates may be related to the pathogenesis and course of the disease. In sepsis, for example, there are increased percentages of aggregates detectable, whereas in patients with multiple organ failure and in those who demise from the latter, the aggregates are not detected. This could be due to the enhanced peripheral sequestration of leucocyte-platelet aggregates which might account for the development of multiple organ failure in the course of sepsis (Gawaz et al, 1995).

The platelets interact with leucocytes by their expression of P-selectin (CD62-P) which binds to P-selectin glycoprotein ligand-1 on the leucocytes. Other potential links are P-selectin-CD15 (Jy et al, 1995), fibrinogen bridges between IIb/IIIa and CD11b/CD18 and thrombospondin bridges between GP IV receptors on both cells. (Ott et al, 1996; Li et al, 1997). The predominant receptor-ligand interaction may depend on the agonist used for activation (Li et al, 1997). This leucocyte-platelet adhesion has been shown to result in leucocyte activation documented as cytokine production, production of reactive oxidant species, upregulation of cellular adhesion molecules, expression of tissue factor in monocytes and morphological changes (Hagberg and Lyberg, 2000 b).

Advantages of flow cytometry

An advantage that flow cytometry has over other tests of platelet activation such as the measurement of plasma levels of secreted platelet specific proteins such as βTG and PF4 is the fact that flow cytometry can be performed on whole blood, thus avoiding plasma separation procedures which may cause in vitro platelet activation or loss of certain platelet subpopulations. Whole blood is also a more physiological milieu. Like conventional assays for platelet activation, flow cytometry may be used to measure the degree of endogenous platelet activation, but in addition to this it may be used to measure individual platelet reactivity after stimulation with agonists. Platelet aggregation studies may measure reactivity, but utilises averaging techniques, disallowing individualisation. Multiple parameters that characterise activation may be measured on each individual platelet simultaneously, thus allowing for the sensitive detection of platelet subpopulations. Since very small quantities of blood and platelets are necessary, this procedure is useful in individuals from whom one cannot remove large volumes of blood such as neonates as well as in thrombocytopenic individuals. An added advantage is that no radioactivity is required (Michelson, 1996).

Disadvantages of flow cytometry

The recognised disadvantages include expensive capital equipment. In addition, some of the techniques of sample preparation are fairly complicated and it may be necessary to process the sample within a relatively short period of having drawn the blood or to fix the blood where possible (Michelson, 1996). Despite these

disadvantages, this technique appears to have much potential both as a tool for research, enabling understanding of platelet function in a new dimension, as well as in more completely assessing bleeding and thrombotic disorders.

An application of particular interest is the ability of flow cytometry to detect and quantify circulating activated platelets and platelet-leucocyte or platelet-monocyte aggregates in various clinical situations associated with platelet mediated thrombosis. These include pre-eclampsia (Janes et al, 1995; Konijnenberg et al, 1997) (as opposed to normal pregnancy) (Star et al, 1997), coronary artery disease (Furman et al, 1998), including unstable angina (Ott et al, 1996) and myocardial infarction (Michelson, 1996; Katopodis et al, 1997), after cardiopulmonary bypass (Shigeta et al, 1997; Basora et al, 1999), coronary stenting (Gawaz et al, 1996), or angioplasty (Mickelson et al, 1996), in atrial fibrillation when associated with atrial thrombus (Pongratz et al, 1997), in patients with mechanical heart valve replacements (Maugeri et al, 2000), in hypertension (Andrioli et al, 1996) and in venous stasis ulceration (Peyton et al, 1998). The technique has also been employed to attempt to distinguish between vascular and embolic stroke, the former showing an increase in platelet activation (Zeller et al,1999).

Haematological conditions in which this test have been used include sickle cell anaemia (Wun et al, 1997), beta thalassaemia (Del Principe et al, 1993), Essential Thrombocythaemia (Griesshammer et al, 1999), heparin induced thrombocytopenia (Lee et al,1996; Jy et al, 1999; Tomer et al, 1999) and Paroxysmal Nocturnal Haemoglobinuria (Wiedmer et al, 1993) where thrombotic complications are not

uncommon. This technique has also been applied in the detection of activated platelets in platelet concentrates after different collection procedures or methods of filtering, processing and storage (Holme et al, 1997; Grijzenhout et al, 1993; Scharf et al, 1993; Devine et al, 1999). This may give an indication of the quality of the blood product, since the level of certain platelet activation markers has been shown to correlate with the survival of platelets in vivo (Michelson, 1996), although other studies have shown conflicting results. (Lazarus et al, 1995). Flow cytometric analysis of platelets has even been attempted in predicting which of a group of equally thrombocytopenic patients are likely to bleed (Hoffman et al, 1996), and in prediction of development of transplant vasculopathy after heart transplantation (Fateh-Moghadam et al, 2000). Increased levels of circulating activated platelets and microparticles have been detected in HIV infection in association with altered levels of RANTES release from platelets (Holme et al, 1998 (a)).

The sensitivity and specificity of the flow cytometric studies in the clinical setting are continuously being assessed, since there is no accepted gold standard with which to compare the data. The ideal way of determining positive and negative predictive values of the flow cytometry assay is to correlate the data with clinical outcome in a large number of patients. Studies of this nature are currently in progress (Michelson, 1996). Many groups have already shown the usefulness of these assays in a variety of clinical settings. However it is noteworthy that studies often show conflicting results and sometimes contradict each other (Lazarus et al, 1995; Star et al, 1997; Metzelaar et al, 1993). In addition, values for a population of normal controls as

compared to a population of affected individuals may show significant differences, but the ranges for normality versus abnormality, may actually overlap, that is to say that the significance of a result for an individual may not be clear. Whilst the detection of activated platelets may aid in management decisions under these circumstances, the prediction of outcome, based only on the test results, would perhaps be inaccurate.

With regard to studies yielding conflicting results, it is likely, after discovering the tremendous variation in methodology still employed by different groups, that this is one factor which is confusing the issue. This will be referred to again throughout the body of this report, as well as in the discussion.

STUDY OBJECTIVES:

- To investigate the possible use of flow cytometry for assessment of the following aspects of platelet function and to assess the implications for routine clinical implementation:

  1)Detection of activation of platelets measured in terms of :

  - alpha granule secretion (CD62-P)
  - lysosomal granule secretion (CD63)
  - activation of GPIIbIIIa to its ligand binding form (PAC1)
  - changes in density of certain receptors associated with activation (GPIb)
  - development of a procoagulant surface (annexin V)

  These markers may be detected on samples of peripheral blood without the addition of agonists, reflecting the level of endogenous platelet activation, or may be tested for after incubation with an agonist, reflecting the responsiveness of the platelet.

  2) To develop an assay for detection of the presence of, and release of dense granules in platelets, by measuring mepacrine uptake and release.

  3) To determine the levels of circulating activated platelets in normal individuals and to determine normal values for mepacrine uptake and release.

- In order to deepen appreciation of the significance of each of these activation markers, this study will attempt to correlate the sequence of activation marker expression in the physiological milieu of blood emerging from a bleeding time wound.

MATERIALS AND METHODS

A) OPTIMISING THE ANALYSIS OF PLATELET ACTIVATION

All data for this research report was recorded by the use of a FacsCalibur flow cytometer, using Cellquest software. Some of the figures made use of Paint-a-Gate software where the objective was qualitative not quantitative.

A.1.)Venesection

In order to optimize the procedure of venous blood sampling such that platelet activation would be minimal, various methods of blood sampling were compared. The method for blood sampling used most often in the literature is to take venous blood from an antecubital vein using a 21gauge needle, into a Vacutainer tube containing 0,5ml sodium citrate, with the use of, at most, a light tourniquet. A 2ml discard should precede actual sampling. Variations on this method were compared:

1)Blood was sampled twice with a Vacutainer tube on the same patient using the antecubital vein bilaterally. A light tourniquet was used on one side, and released whilst the sample for discarding was being taken. No tourniquet was used on the other side. Although there were too few samples tested in this way for any meaningful statistical analysis, from the author's experience, the difference in activation marker expression between the two methods would appear to be minimal.

Table 1 Comparison of venesection with and without a tourniquet
(Units are activated platelets expressed as a % of total platelets analysed)

	Tourniquet	No Tourniquet
CD63	1,72	1,97
Annexin V	0,80	0,71
CD62-P	0,66	0,91
PAC1	0,73	0,55

2)Sampling with a Vacutainer tube from one antecubital vein was compared to sampling with a 21 gauge butterfly, also into citrate, using the double syringe technique, again with a 2ml discard. A light tourniquet was used in both instances. The double syringe technique showed no advantage.

Table 2 Comparison of venesection using a Vacutainer, and the double syringe technique with a butterfly
(Units are activated platelets expressed as a % of total platelets analysed)

	Vacutainer	Citrated syringe
CD63	2,17	1,89
Annexin V	2,78	2,66
CD62-P	0,47	0,60
PAC1	0,56	0,66

In fact, even taking the blood sample into ACD containing prostaglandin E1 dissolved in ethanol at a final concentration of 3μM in ACD (adapted from Files et al, 1981) using the double syringe technique showed no advantage. Platelet activation (CD62-P expression) was actually noted to continue in vitro. The prolonged contact with the long tubing of the butterfly probably results in platelet activation. Rinder et al (1991) also state that experiments in which prostaglandin E1 (10nmol/l) was included in the platelet preparation had no effect on GMP-140 expression. The samples used in this study were platelet rich plasma prepared from citrated whole blood, then gel filtered and fixed with paraformaldehyde, in some instances after incubation with agonists. Notwithstanding these references, prostaglandin E1 is notoriously unstable, and the possibility of experimental error due to deterioration of the prostaglandins could not be excluded in the absence of repeating the experiment with a new batch.

Table 3 Comparison of venesection using a Vacutainer, and the double syringe technique with prostaglandin E1
(Units are activated platelets expressed as a % of total platelets analysed)

	Vacutainer	Syringe with PGE1
CD63	3,54	11,05
Annexin V	9,44	10,05
CD62-P	3,91	7,76
PAC1	0,89	0,62

* Note that the figures in this table differ from those in the previous tables since different batches of antibodies were used and settings were therefore different.

A.2.) Controls

With regard to controls, the reference method for a negative control for this test would be the blocking of specific antibody binding by an excess of soluble antigen in the tube. This is not practical for routine work. The next alternative is to block specific binding by an excess of unstained F(ab)2- fragments of the specific antibody. This was also not available practically, due to issues of cost and also reagent availability. In the literature, if controls are to be used at all, isotypic control antibodies are generally chosen for negative controls (Levin et al, 2000; Maugeri et al, 2000; Golanski et al, 1996; Schmitz et al, 1998). Occasionally a different approach to negative controls is used, such as using a calcium chelator (EDTA) for annexin V binding (a reaction that is calcium dependant) or a GPIIb/IIIa binding peptide for PAC1 (Tait et al, 1999). One group subtracted any positive fluorescence seen with P-selectin after taking the sample into prostaglandin E1 (1μmol/l), using this as a negative control (Kennedy et al, 1997). (This is in contrast to the findings of this study, with limited use of PGE1.)

In the study presented, controls were not used, since to have an optimal control

antibody one needs to match, not only the isotype, but the fluorochrome and the number of fluorochrome molecules attached to each antibody. Since this would necessitate the purchase of specific antibodies to match each used in the study, the financial cost was considered too great for the potential contribution the controls might add. These issues are discussed in the article European Working Group on Clinical Cell Analysis: Consensus Protocol for the Flow Cytometric Characterisation of Platelet Function (Schmitz et al, 1998).

A.3.) Interference of monoclonal antibodies used in the combinations chosen

There was no obvious interference of antibodies in the combinations chosen as compared to running them alone with another platelet identifier, as done during the titrations of antibody. It was therefore not considered necessary to run each antibody alone to compare to the triple combinations chosen. (In fact as can be seen later, it is best to use one antibody to identify platelets in conjunction with one to measure activation.) The combinations were specifically chosen to avoid possible interference or competitive binding such as may be possible with CD61 and PAC1 antibodies, which bind to different activation states and different sites of the same molecular complex (GPIIb/IIIa).

Other issues taken into consideration include choosing the PerCP conjugated antibody to be that directed to the antigen with the highest density (i.e. GPIIb/IIIa and GPIb/IX) since the PerCP signal tends to be dimmer than that of the PE and FITC fluorochromes. In addition, the spectral overlap between PerCP and the other fluorochromes is

minimal, so the relatively high intensity PerCP signal has minimal overlap that could be interpreted as a weak positive signal by the other photomultiplier tubes and would require careful colour compensation.

A.4.) Titrations

To determine the optimal concentration of antibody to be used, and specifically to determine whether less antibody could be used than is recommended by the manufacturer in order to minimize costs, titrations of antibody were set up over the recommended range and less. The annexin V-FITC (PharMingen, San Diego, California) preparations used were not specifically for the identification of activated platelets, rather for determination of cellular apoptosis versus necrosis. Therefore this titration was in fact necessary to ascertain an optimal concentration. CD42b-PE (Dako, Glostrup, Denmark), CD61-FITC (Dako,Glostrup, Denmark), CD61-PerCP (Becton Dickinson, San Jose, California) and CD42a-PerCP (Becton Dickinson, San Jose, California) were titrated on citrated blood samples using freshly drawn blood without addition of agonists. The identifying antibody in the tubes were CD61-FITC, CD42b-PE, CD42b-PE and CD61-FITC respectively. The optimal (brightest) fluorescence in all cases was the highest concentration of antibody, corresponding to the recommended concentration for the PerCP markers (20µl per 5µl whole blood). If the antibody were being used for simply identifying the presence of the antigen on the platelet such as one does when assessing for a Bernard-Soulier Syndrome or Glanzmann's thrombasthenia, or for identifying the platelet population and not necessarily microparticles (e.g. for analysis of mepacrine uptake and release), it may

suffice to use a lesser concentration. However because of the weaker fluorescence of microparticles and the problem distinguishing them by size, from electronic noise and antibody complexes, (which will be discussed later), the brighter one can make the identifying signal, the better.

To titrate the CD62-P-PE (Becton Dickinson, San Jose, California) and PAC1-FITC (Becton Dickinson, San Jose, California) antibodies, expression of these markers could be achieved by the addition of ADP (final concentration 1 x 10^-4M) and adrenaline (final concentration 1μg/ml). These antibodies were then titrated against activated platelets, using an identifier of another wavelength of fluorescence (CD42b-PE for PAC1-FITC and CD61-FITC for CD62-P-PE). Again the optimal fluorescence was that of the maximum and recommended concentration (20μl per 5μl whole blood).

Since PAC1 is a relatively weakly expressed antigen even at maximal activation, any dilution of the maximal concentration would not suffice. With regard to CD62-P, it is fairly brightly expressed after maximal activation, but in the samples analysed in this study, namely normal controls, the CD62-P expression is initially dim. Therefore it would again be an unacceptable compromise to dilute this antibody.

To titrate the annexin V-FITC (PharMingen, San Diego, California) and CD63-PE (Beckman Coulter, Hialeah, Florida) maximal activation of the platelets was achieved by incubation of washed platelets (see Appendices) with collagen and thrombin at final concentrations of 10μg/ml and 1U/ml respectively. Since ADP and adrenaline could not induce maximal expression, the platelets had to be separated and washed to remove

fibrinogen prior to activation. The platelet count in the sample was adjusted to well within the normal range. Annexin V was titrated using CD42b-PE as the identifying antibody, and the optimal concentration was this time the 1/2 dilution. By looking at the titration curves (Figure 1) one can see that the neat annexin V has a dimmer fluorescence than the 1/2 dilution. This indicates that beyond the optimal concentration of the one in two dilution, steric hindrance is starting to occur.

CD63 –PE was titrated against CD61-FITC on the same platelet preparation and was again found to be optimal as per supplier's recommendation. CD63 expression is particularly weak, even after maximal platelet activation, and a dilution would therefore certainly be sub-optimal.

A.5.) Optimisation of settings

A.5.1.) Light scatter analysis

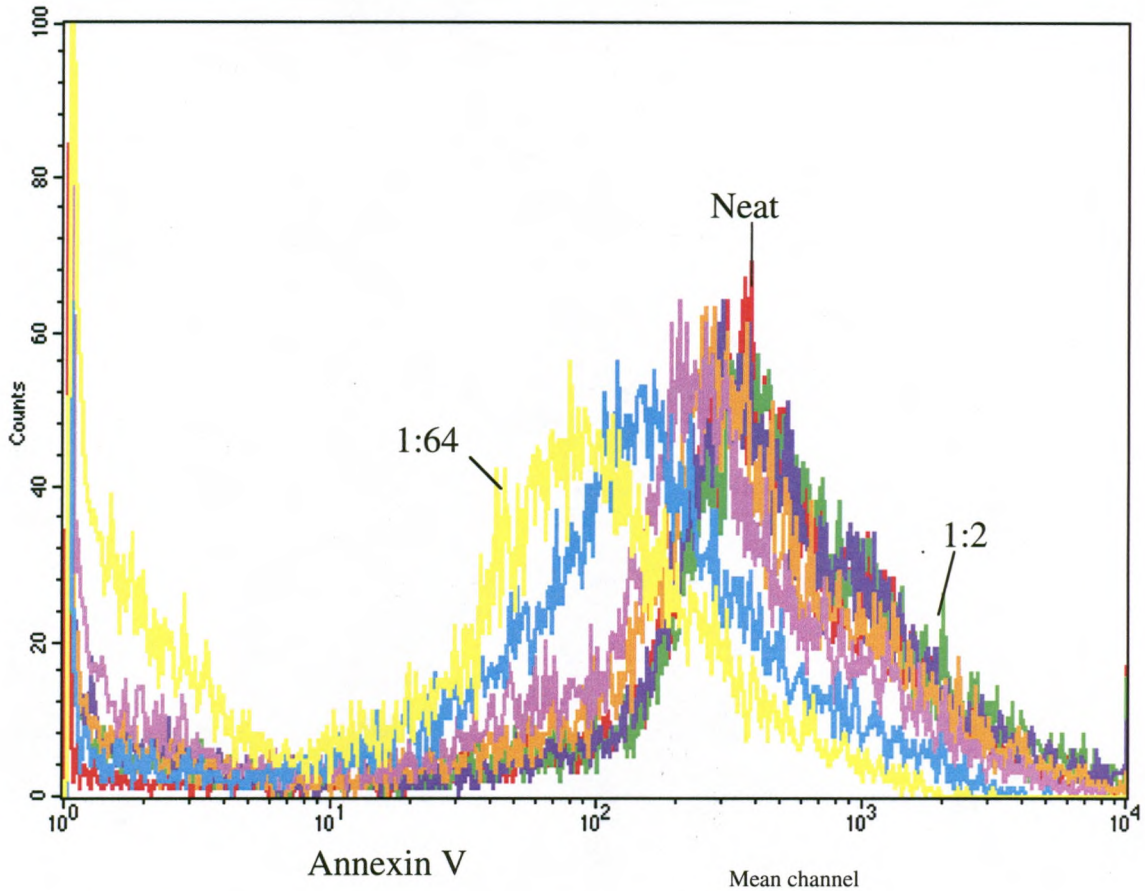
Initially, the platelet assay was set up using linear settings for the forward and side scatter parameters, with the objective of visualizing small differences in size and complexity more easily than might be possible using logarithmic settings. A sample of peripheral blood was incubated with the following antibodies and buffer for 15 minutes in the dark, then diluted with an excess of buffer (500µl – 1ml):

5µl citrated whole blood

40µl HEPES buffer

10µl FITC labeled anti-glycophorin antibody (to visualise red cells)

10µl PE labeled anti-CD42b antibody (to visualise the platelets).



Key	Name	Parameter	Gate	Mean channel fluorescence
	ANN TITR NEAT (20) (NEW).007	FL1-H	No Gate	806,52
	ANN TITR 1/2.009	FL1-H	No Gate	931,62
	ANN TITR 1/4.010	FL1-H	No Gate	862,98
	ANN TITR 1/8.011	FL1-H	No Gate	695,92
	ANN TITR 1/16.012	FL1-H	No Gate	516,42
	ANN TITR 1/32.013	FL1-H	No Gate	282,45
	ANN TITR 1/64.014	FL1-H	No Gate	164,58

Figure 1: Mean channel fluorescence of platelets after incubation with various dilutions of Annexin V-FITC. The neat sample was incubated with 20µl of Annexin V (Pharmingen).

In comparison to the settings that were used for analysis of white cells, the amplifier gains and voltage on forward and side scatter were set much higher, since platelets are far smaller than white cells. It was also necessary to remove the discriminant function delineating the lower limit of events to be recorded based on forward scatter, again because of the platelets and especially microparticles being particularly small. (Having the discriminant on forward scatter would serve to exclude unwanted events such as electronic noise, antibody or protein complexes etc.) The discriminant function was placed on PE fluorescence instead (since the platelet identifying antibody was conjugated to PE in this instance), so that the electronic noise and red cells (which would be in the region of twenty times more frequently recorded events than platelets) could be excluded from analysis as much as possible.

It is important to note at this point, that the higher one sets the discriminant function, the more red cells and electronic noise can be excluded, but the easier it is to exclude events with dim fluorescence such as microparticles (which by virtue of their smaller surface area must bind less "platelet identifying antibody", usually to GPIIb/IIIa or GPIb/IX, as here CD42b). (See Figure 2.) In the literature, this is sometimes recognized to be problematic, but solutions to this problem are not always forthcoming, and certainly it is rare for this to even be discussed in most papers in the literature. (See discussion). It will be demonstrated later that one can calculate a very different percentage of microparticles for example, depending on these very variables. The use of PerCP labeled "platelet identifying antibodies" was also of concern since the fluorescence of the PerCP molecule is dimmer than that of FITC and PE, the latter

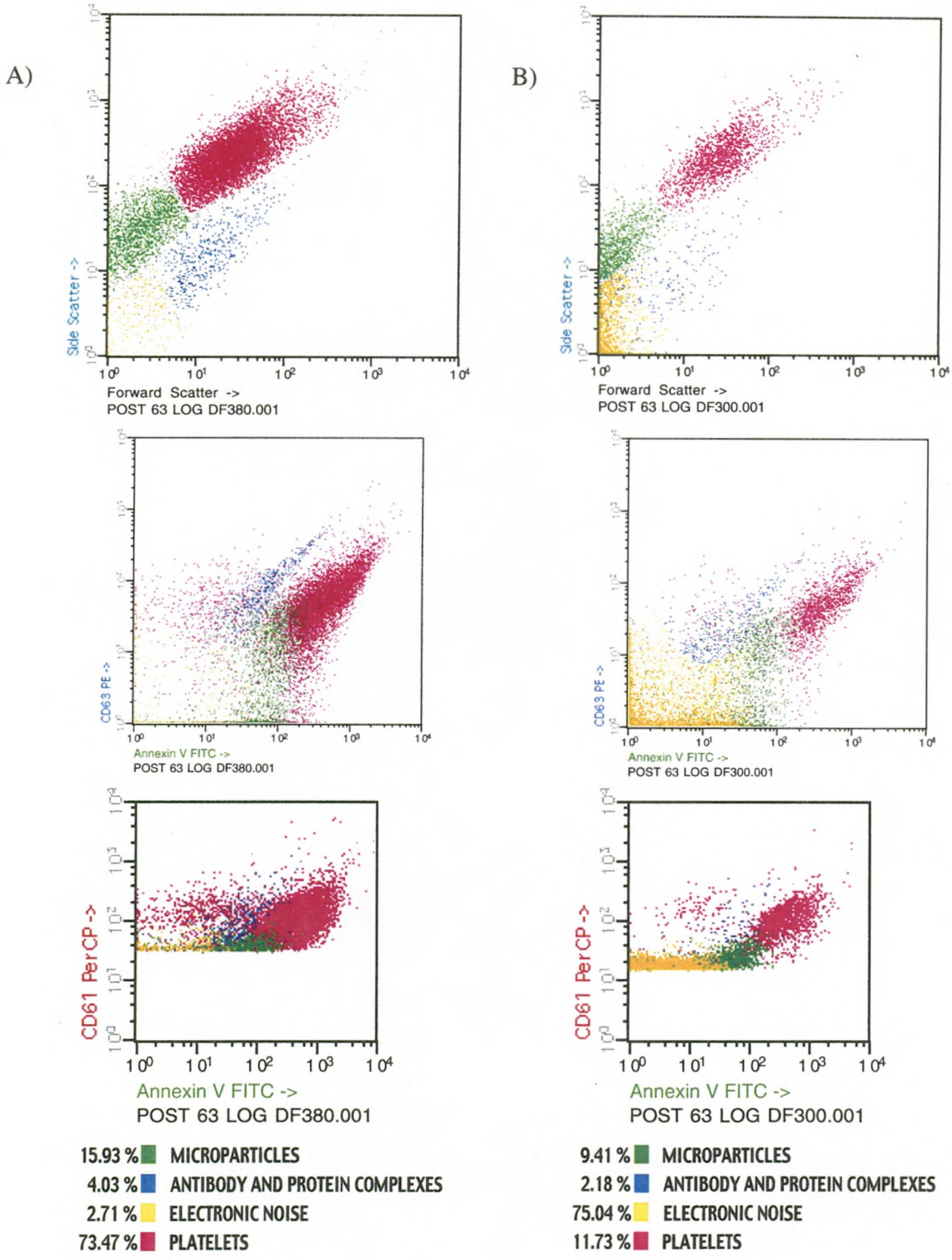


Figure 2:
Dot plots of the same activated sample run with the discriminant function (DF) set on FL3 (PerCP fluorescence) i.e. CD61, at different levels:

A) DF 380 (Units are logarithmic units of fluorescence.)
B) DF 300. (Refer to glossary for definition of discriminant.)

Note the expression of annexin V on the platelets, and more dimly, on the microparticles. Note that the point of distinction between electronic noise and microparticles is somewhat blurred on CD61-PerCP fluorescence, as well as on the plot of forward versus side scatter.

having the brightest fluorescence. One might expect that as a result of this the overlap in fluorescence intensity of platelet microparticles, antibody complexes and electronic noise, and even probably the red cell populations with greatest PerCP fluorescence, would in fact be even greater. Interestingly, this does not seem to be the case. The distinction between background fluorescence of red cells, electronic noise and antibody complexes remains problematic even with the use of a "platelet identifying antibody" conjugated to PE. (See figure 3.)

With the red cell population visualized as FITC positive events much larger than the PE positive platelets, the voltages and amplifier gains were manipulated to optimize the light scatter pattern. Turning up amplifier gains was favoured over maximizing voltages in order to place least stress on the laser. (The amplifier gains and voltage settings serve a similar purpose, however voltage increases are subtler than the large increases one achieves with increasing the amplifier gains.)

It became evident after preliminary investigation, that the distinction between the "small events" i.e. microparticles, electronic noise and antibody/protein complexes, was easier to make with logarithmic settings on forward and side scatter. In addition the distribution of size of normal platelets, microparticles and aggregates is so wide that it required logarithmic settings in order to visualize the populations on the same dot plot of forward versus side scatter. The logarithmic settings were therefore favoured thereafter.

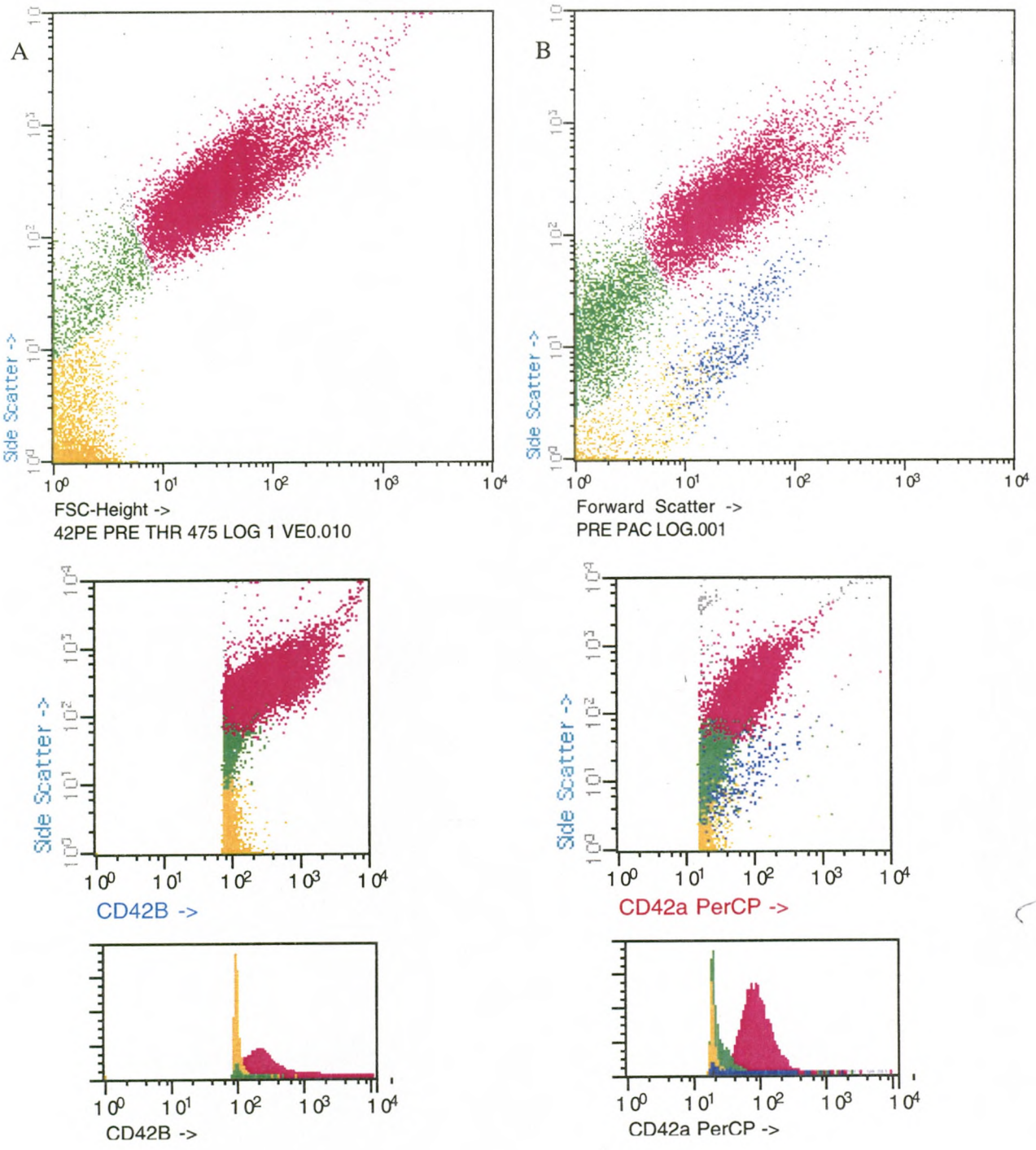


Figure 3:
Comparison of the same sample of separated, washed platelets (which in the process have become activated, including the production of "microparticles") with labeling by antibodies with different fluorochromes attached.

A) CD42b-PE
B) CD42a-PerCP

(Strictly speaking the comparison cannot be made directly between fluorochromes because the antibodies are from different clones and different manufacturers, and are in fact directed at different antigens on proteins which are coupled in a 1:1 ratio). Notwithstanding, note:

1) The overlap region between microparticles and electronic noise, based on forward scatter as well as fluorescence intensity remains problematic with the use of both PerCP and PE labeled antibodies. Even using a combination of side scatter and fluorescence leaves the point of distinction unclear.
2) The absence of significant antibody/protein complexes in Sample A. (It should be noted that the discriminant function in A has been set much lower than it should be specifically to illustrate the overlap problem.)

(Key: yellow = electronic noise; blue = antibody and protein complexes;
green = microparticles; pink = platelets)

The amplifier gains and voltages were manipulated in such a way as to place microparticles predominantly in the first log on forward scatter and second log on side scatter, with electronic noise then falling in the first log for both forward and side scatter. Individual platelets were roughly in the second to third log on forward scatter and third log side scatter while aggregates (platelet-platelet or platelet-leucocyte), as well as red cells, fell in the third to fourth for forward scatter and fourth for side scatter.

A.5.2.) Expression of activation markers

In order to activate the platelets to express the activation markers so that the voltage and amplifier gains settings as well as colour compensation could be optimized, different procedures had to be followed again for different markers.

A.5.2.a.) CD42a-PerCP/CD62-P-PE/PAC-1-FITC

To achieve release of alpha granules (CD62-P expression) and the conformational change of the GPIIb/IIIa receptor (PAC1 expression) it was sufficient to incubate citrated whole blood with ADP and adrenaline at concentrations of 1 x 10^-4M and 1μg/ml respectively. To minimize consumption of antibody, the same sample could be used for CD62-P/PAC1 negative and dual positive platelets. The sample could initially be incubated with antibodies and freshly venesected whole blood, then diluted with buffer and run, optimizing the settings on negative platelets. Adding the ADP and adrenaline to the diluted sample after this also activated the platelets, and the same sample could then be used as dual positive for optimizing colour compensation. (It may be necessary to rerun the dual negative sample after adjustments have been made on the dual positive analysis, in which case one would have to set up another dual

negative sample.) (Note that for the CD61/CD63/annexin V combination, the addition of agonists to a sample already containing antibodies and annexin V should not be performed if formation of microparticles is desirable, since the presence of annexin V is described to inhibit their formation) (Dachary-Prigent et al, 1995).

The voltages and amplifier gains were optimized to show the dual positive population, whilst keeping the dual negative population as close as possible to within the first log (negative region.) The colour compensation settings were not very different from those recommended based on the quality control fluorescent beads run daily.

A.5.2.b.) CD61-PerCP/CD63-PE/Annexin V-FITC

To achieve expression of CD63 and particularly annexin V, and also specifically to obtain microparticle generation required incubation with stronger agonists. Since a combination of thrombin and collagen were to be used, and thrombin would generally cause clotting of the sample by conversion of fibrinogen to fibrin, the platelets were first separated and washed using a method followed in our department for the separation of platelets from plasma prior to performing platelet aggregation studies. (See Appendices). It interesting to observe that this separation procedure alone was in fact capable of generating platelet activation and formation of particles with the size and immunophenotypic characteristics of microparticles. "Microparticles" could in fact be generated by centrifuging a sample of activated platelets at 800g for ten minutes (a method used for separating microparticles from platelets). It was considered whether the buffer used for washing the

platelets in the separation procedure could have caused some artefactual platelet activation since it contained heparin (Schmitz et al, 1998). However the fact that centrifuging and resuspending the sample alone also resulted in production of microparticles (or possibly simply platelet fragmentation resembling microparticles immunophenotypically) implies that the mechanical effects of centrifuging and manipulation generally plays a more dominant part in this phenomenon.
(Figure 4 illustrates this point, showing activation of the platelets after separation and washing, including the production of "microparticles", or fragments of platelets resembling microparticles immunophenotypically. It can be seen from Figure 4 that the step of centrifuging a sample to pellet the platelets and separate microparticles into the supernatant (which would also be used if a fixed sample were to be washed) actually may create microparticles or fragments resembling microparticles immunophenotypically.)

It is noteworthy that the markers which are noted to be expressed on the microparticles in these experiments are those which could conceivably be exposed on the membrane after fragmentation of platelets and their granules, namely CD62-P and annexin V. The antigen which requires an active process to be exposed, PAC1, is negative on the microparticles as well as the platelets in these experiments, even after incubation with agonists. These findings could also be, and in fact are likely to be, compatible with some part of the separation and washing procedure damaging the antigen, such as a component of the buffer etc. Microparticles have been previously described to be largely negative for PAC1 expression (Sims et al, 1988), but certainly after incubation

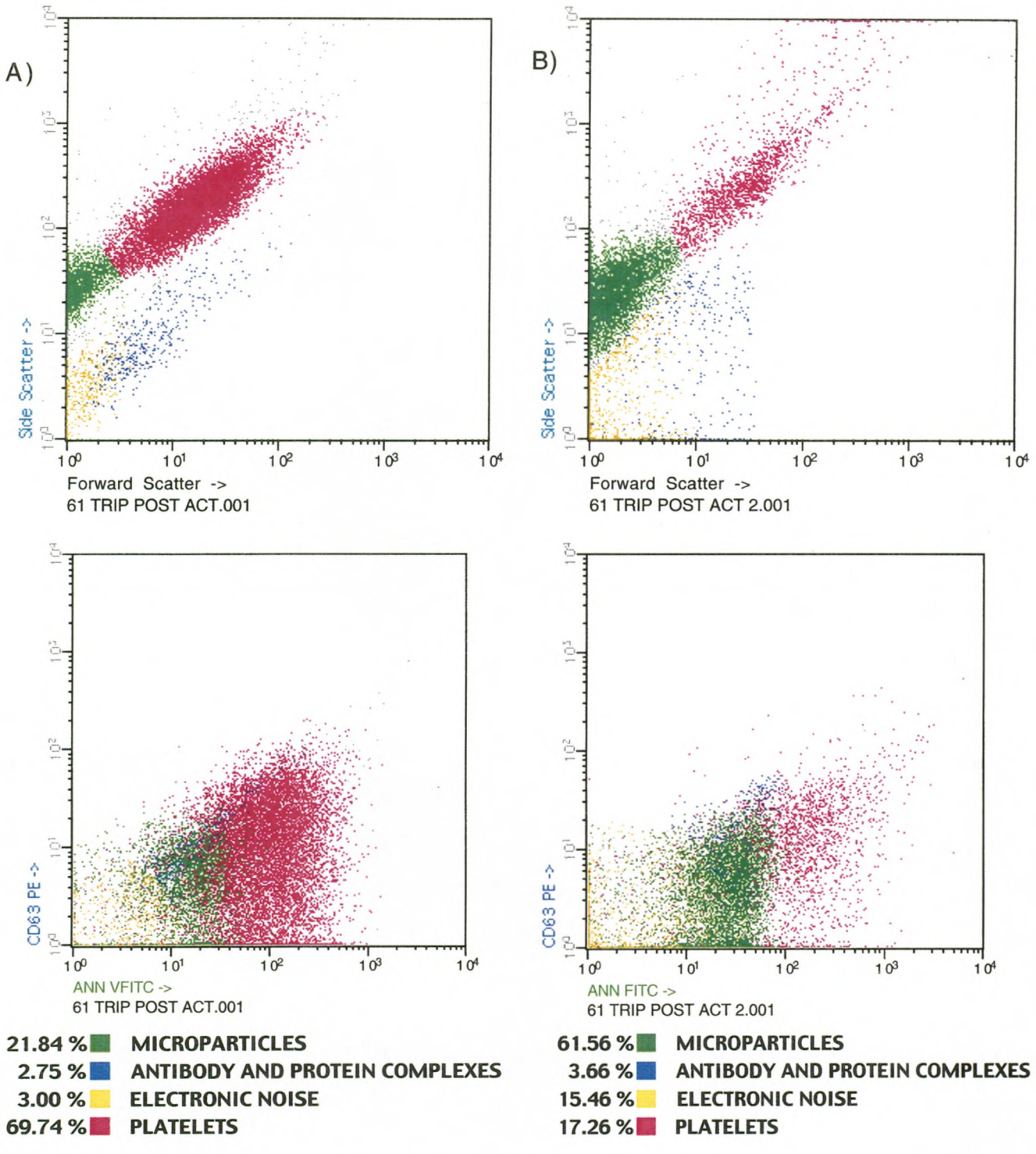


Figure 4:
This figure illustrates the effect of centrifuging a sample. A) and B) were derived from the same donor sample. Platelets were separated from a single whole blood sample and washed as described in the Materials and Methods addendum. The platelets were then activated with thrombin and collagen. Sample A) was then incubated with antibodies and analysed on the cytometer, while sample B) was first centrifuged for 10 minutes at 800g, a procedure used to separate microparticles into the supernatant whilst pelleting the platelets. The sample was then agitated to resuspend the pellet, incubated with antibodies and run on the cytometer.
Note the marked increase in the concentration of "microparticles" relative to platelets which has resulted from centrifuging then resuspending the platelets. Whether the results indicates platelet activation and shedding of microparticles due to sample manipulation, or simple mechanical fragmentation of the platelets, would have to be assessed by other methods such as electron microscopy. (In addition it is possible that the concentration of whole platelets in the sample could be lower, if not all the platelets were resuspended, i.e. some remained in clumps too big to pass through the cytometer tubing, whilst the microparticles clumped to a lesser extent and are falsely elevated as a result.)

with agonists one would expect the platelets to express PAC1 unless they were irreparably damaged during the separation and washing procedures, in which case the light scatter might have alluded to this.

The response to the separation procedure as well as the response of the donor platelets to agonists was noted to be variable. This was not entirely surprising since there are reports in the literature where variability of normal donor platelets has been described in certain settings (Dachary-Prigent et al, 1995). This is noted also with responses to agonists as measured by aggregometry.

The sample of separated, washed platelets was then incubated with collagen (10μg/ml) and thrombin (1U/ml) for at least 10 minutes to allow maximal activation.
It was difficult to optimize settings on this sample since the expression of CD63 on microparticles has not been widely documented. However with the settings similar to those recommended by the quality control fluorescent beads, the CD63 expression appeared to be dim to negative. The annexin V expression on microparticles has been previously described to be positive (Dachary-Prigent et al, 1993 and 1995), and the colour compensation was optimized as such. (It is noted that annexin V negative microparticles have been described, but as it will be seen later in the study, microparticles are not easily distinguished from events such as background noise and antibody complexes, particularly when one does not make use of as many measurable characteristics as possible to delineate this population.)

A.6) Gating strategy

Gates were set up to enumerate platelets, aggregates and platelet microparticles. Gates were also set up to exclude from analysis antibody complexes and electronic background noise, both of which overlap to some extent in terms of forward scatter (size), and to a lesser extent, side scatter (complexity) with microparticles when both logarithmic and linear settings are used. As illustrated, the overall pattern of microparticles is separable from electronic noise and antibody complexes on the logarithmic plot of forward scatter versus side scatter, with a degree of overlap where the groups meet. The characteristic appearance of each of these groups was demonstrated by attempting to isolate and analyse each group alone where necessary.

Buffer and monoclonal antibodies were analysed alone with no cellular component. Their appearance is depicted in Figure 5A) and B).

To produce and enrich for microparticles, the following was performed:

Firstly platelets were separated from plasma following a method used in our department for isolating platelets without activating them in order to perform platelet aggregation studies. (See Appendices.) Once this platelet suspension was available in the absence of fibrinogen, the sample could be maximally activated without interference by fibrin clot formation. (Thrombin receptor activating peptides (TRAP) have been used to achieve this objective more commonly in the literature. The technique described here was more time consuming but less costly.)

To maximally activate the platelets and generate microparticles, the washed platelets

were incubated in HEPES buffer (with calcium) for at least 10 minutes with thrombin (1U/ml) and collagen (10μg/ml) (suggested by SJ Shattil via personal communication).

To enrich for microparticles, the sample was then centrifuged at 800g for 10 minutes and the supernatant used. It was noted in many instances that the percentage of microparticles generated during the separation of platelets from whole blood and washing thereof was similar to that after stimulation with thrombin and collagen. (See Figures 5A) and B) to 8A) and B) for the characteristic appearances of the samples as described above.)

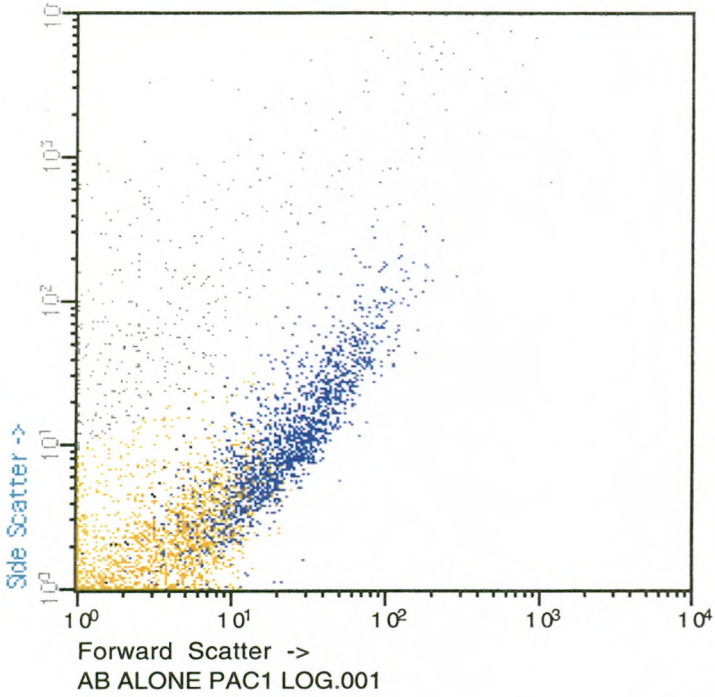
To determine the light scatter pattern of platelet aggregates, platelet-white cell and platelet red cell aggregates, an aliquot of whole blood taken from a citrated tube of blood, was incubated with antibodies to CD61-PerCP, CD45-PE and anti-glycophorin–FITC. The same settings and thresholds as are used normally for the platelet activation assay were used, which is why the colour compensation is not optimal (e.g. the red cells are not confined to the first log (negative on the axis for CD61 PerCP.) (See Figure 9.)

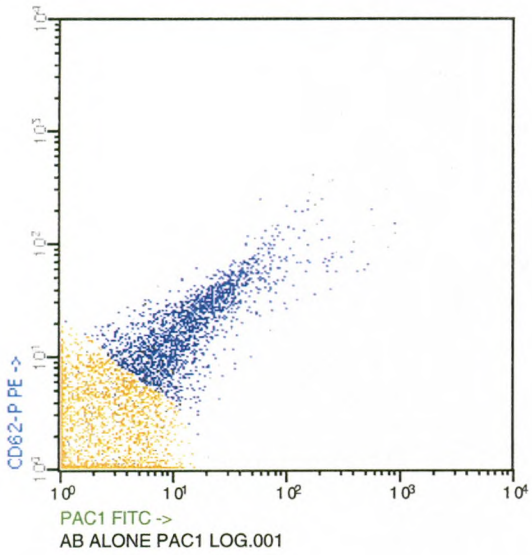
Recognising these typical appearances of the various populations, documented repeatedly during various experiments, antibody complexes were removed from analysis based on their presence in two gates:

1)Events with the same size as microparticles or platelets, i.e. similar forward scatter, but less side scatter for the same amount of forward scatter.

2)Events occurring on the plot of PE versus FITC fluorescence in a

non-specific band of dual positivity. (These events were thought to be unlikely to be electronic noise since they occurred in a region of positive fluorescence for the activation markers, as opposed to true electronic noise described below.)





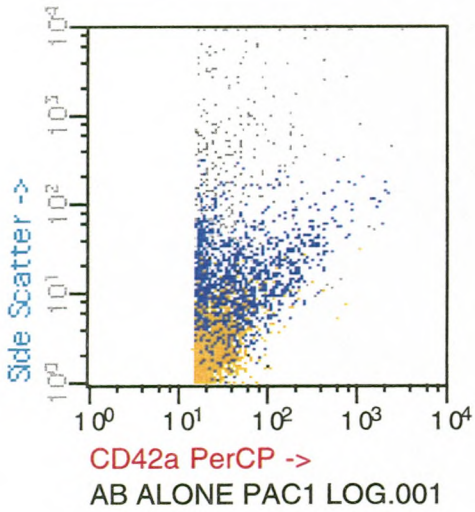
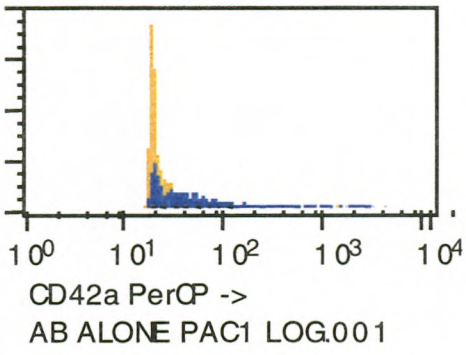


Figure 5:
The appearance of buffer and antibodies alone, with no cellular component.
The sample comprised 50µl HEPES buffer, 20µl CD42a-PerCP, 20µl CD62-P-PE, 20µl PAC1-FITC. After 15 minutes incubation, 1ml HEPES buffer was added and the sample run on the FacsCalibur.

A)Logarithmic settings for forward and side scatter.
B)Linear settings for forward and side scatter.

The population coloured blue is thought to comprise complexes of protein and antibodies because these events appear to have fluorescence for all fluorochromes, in contrast to the yellow events, thought to be electronic noise, which are negative for fluorescence.



36.86 % ANTIBODY AND PROTEIN COMPLEXES
54.26 % ELECTRONIC NOISE

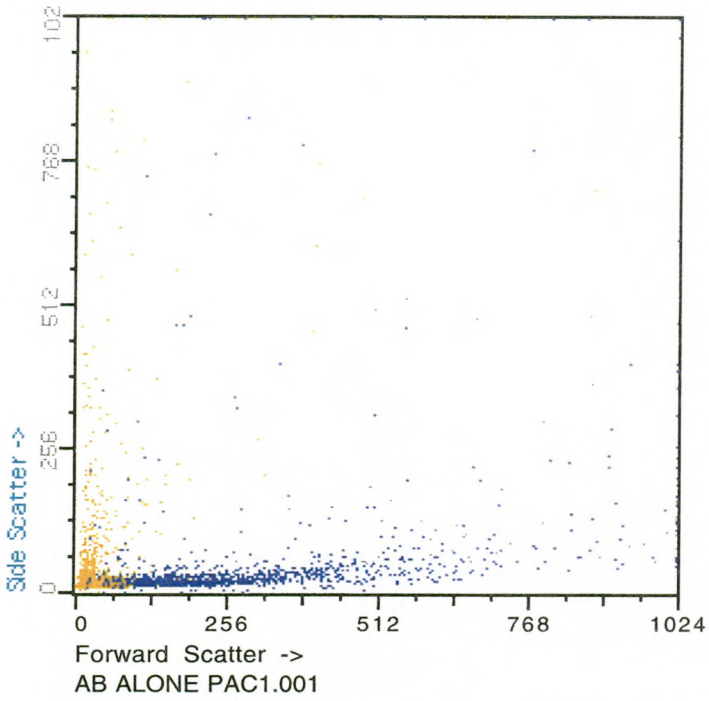
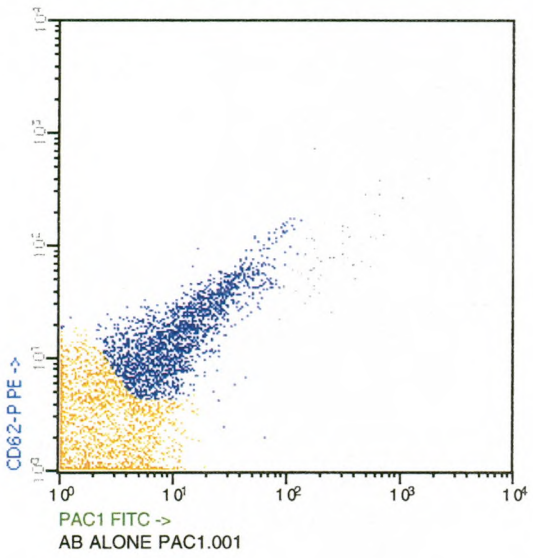
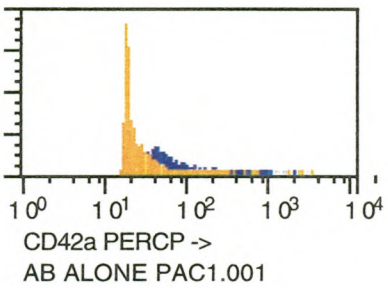


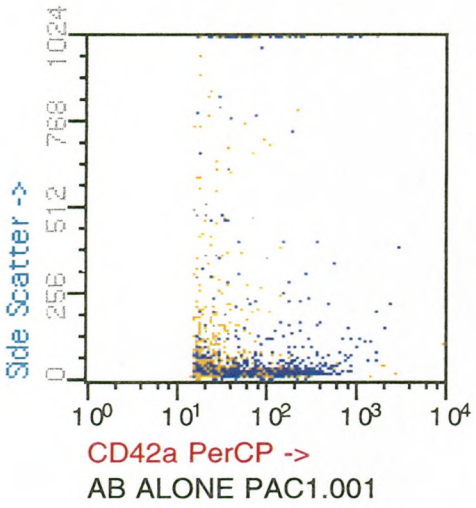
Figure 5:
The appearance of buffer and antibodies alone, with no cellular component.
The sample comprised 50µl HEPES buffer, 20µl CD42a-PerCP, 20µl CD62-P PE, 20µl PAC1FITC. After 15 minutes incubation, 1ml HEPES buffer was added and the sample run on the FacsCalibur.

A)Logarithmic settings for forward and side scatter.
B)Linear settings for forward and side scatter.

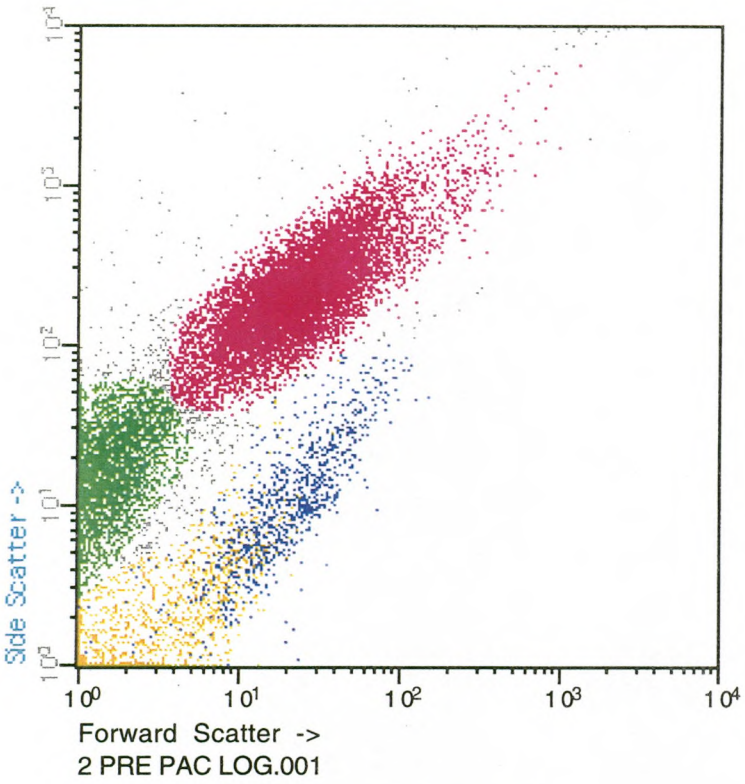
The population coloured blue is thought to comprise complexes of protein and antibodies because these events appear to have fluorescence for all fluorochromes, in contrast to the yellow events, thought to be electronic noise, which are negative for fluorescence.

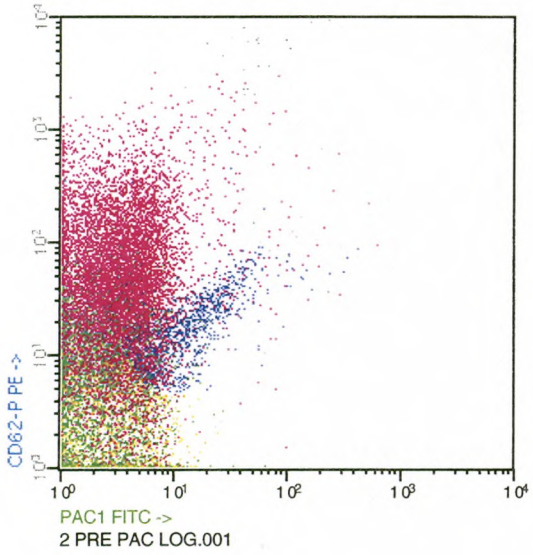






43.36 % ANTIBODY AND PROTEIN COMPLEXES
55.27 % ELECTRONIC NOISE





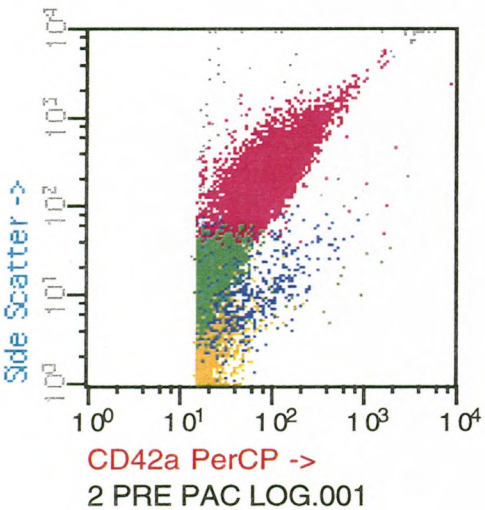
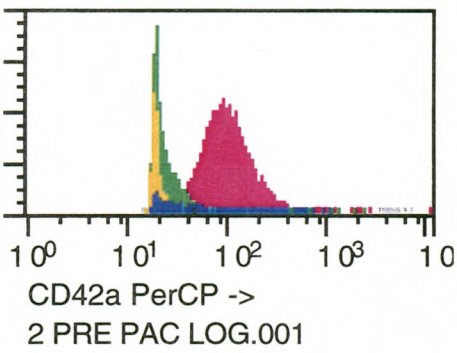
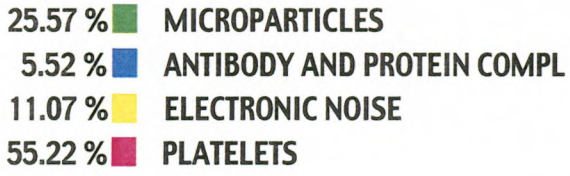


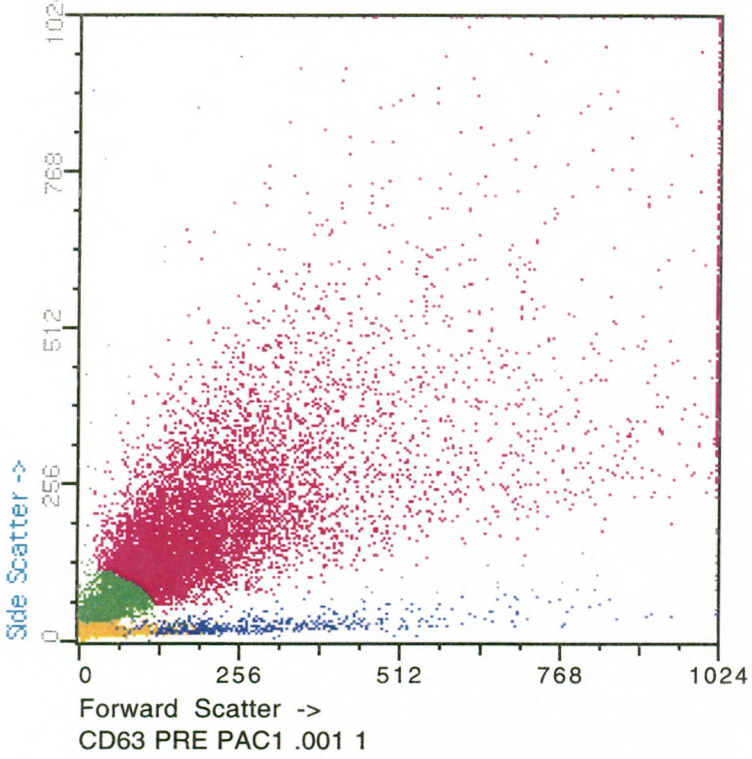
Figure 6:
The appearance of a sample of blood taken into acid-citrate-dextrose (ACD) after the platelets have been separated out and washed (see Materials and Methods Appendix), then incubated with antibodies and buffer as in Fig. 5. "Microparticles" are present already, prior to the addition of agonists.

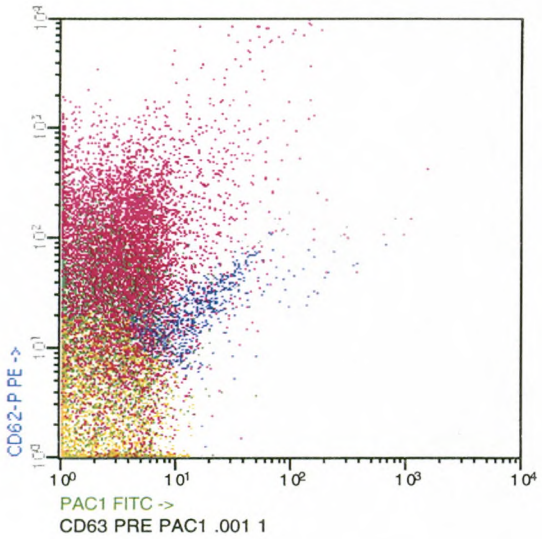
A)Logarithmic settings for forward scatter and side scatter.
B)Linear settings for forward scatter and side scatter.

Note:
1)In this instance microparticles tend to show separation from platelets fairly distinctly on forward versus side scatter as well as CD42a-PerCP fluorescence, and side scatter versus PerCP fluorescence, but this is not always the case.
2)To distinguish the various populations from each other on light scatter alone is very difficult using the linear settings, as is illustrated by the failure of microparticles to comprise a single population on the histogram of CD42a-PerCP fluorescence.









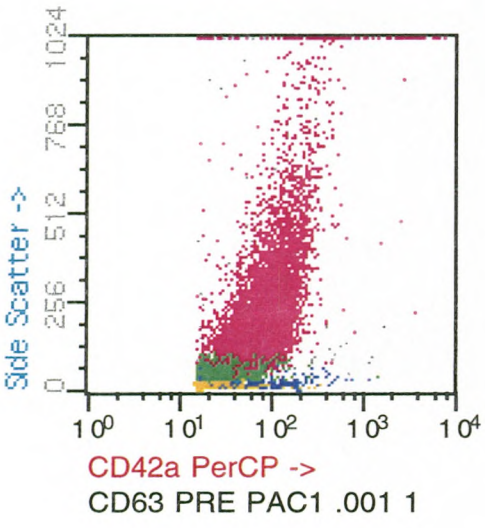
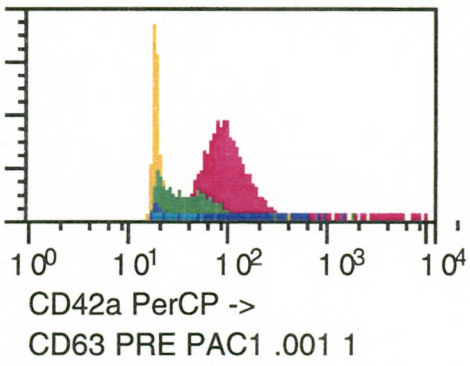


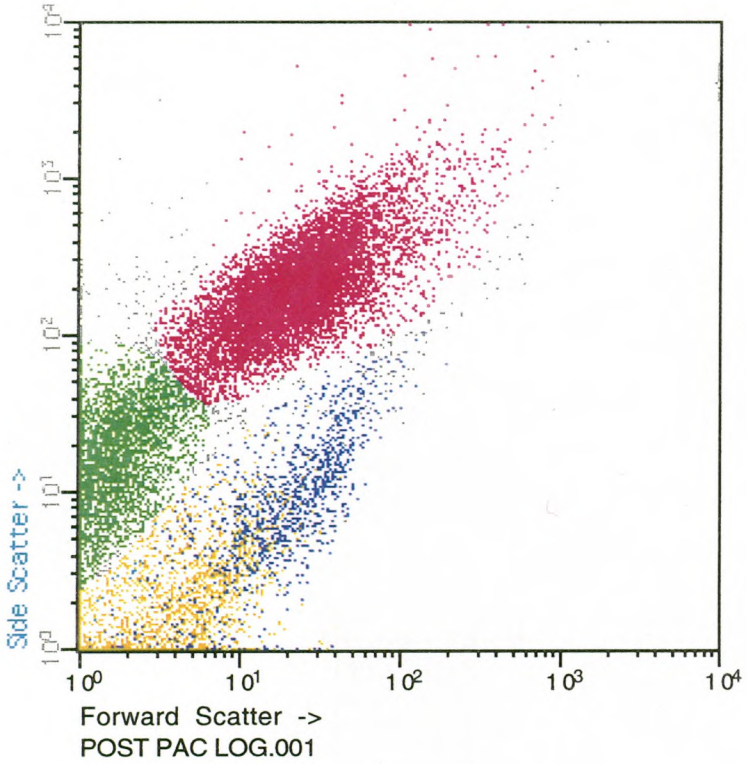
Figure 6:
The appearance of a sample of blood taken into acid-citrate-dextrose (ACD) after the platelets have been separated out and washed (see Materials and Methods Appendix) , then incubated with antibodies and buffer as in Figure 5. "Microparticles" are present already, prior to the addition of agonists.

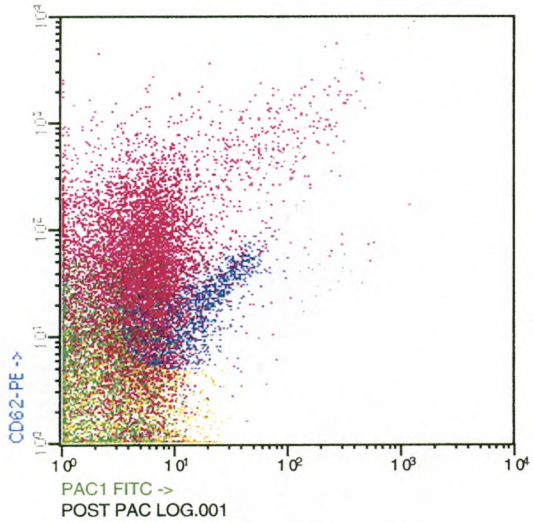
A)Logarithmic settings for forward scatter and side scatter.
B)Linear settings for forward scatter and side scatter.

Note:
1)In this instance microparticles tend to show separation from platelets fairly distinctly on forward versus side scatter as well as CD42a-PerCP fluorescence, or side scatter versus PerCP fluorescence, but this is not always the case.
2)To distinguish the various populations from each other on light scatter alone is very difficult using the linear settings, as is illustrated by the failure of microparticles to comprise a single population on the histogram of CD42a-PerCP fluorescence.



19.63 % MICROPARTICLES
4.26 % ANTIBODY AND PROTEIN COMPLEXES
21.54 % ELECTRONIC NOISE
53.37 % PLATELETS





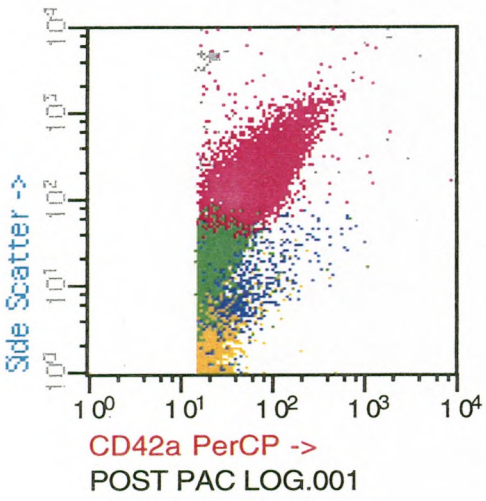
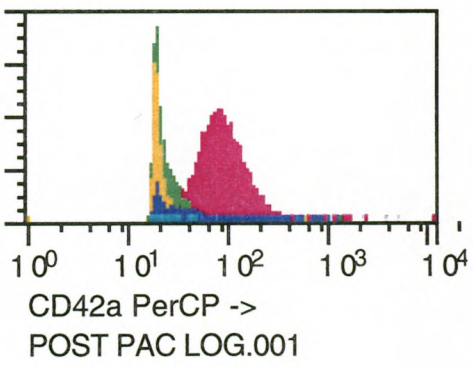
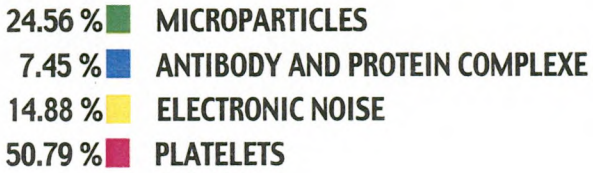


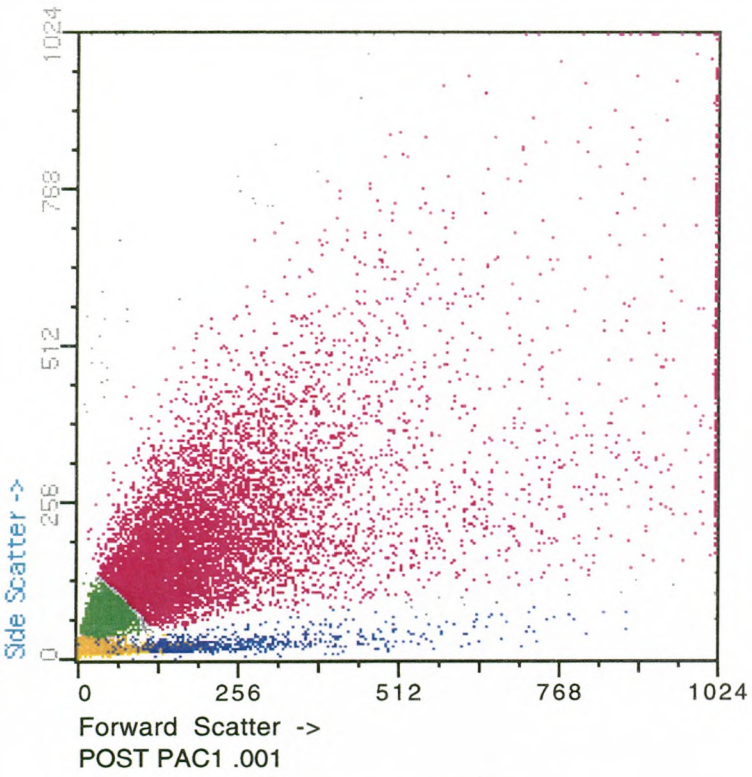
Figure 7:
After incubation of the same separated, washed platelet sample with thrombin (1U/ml) and collagen (10µg/ml), then incubation with antibodies and buffer as described for Fig.5, it can be seen that the sample had a similar appearance to that prior to incubation with agonists in this instance, illustrating again that the separation and washing procedures cause significant activation (and / or possibly mechanical disruption of the platelets.)

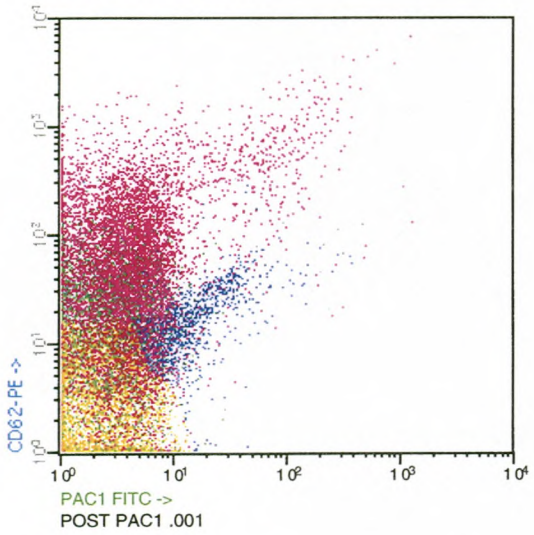
A)Logarithmic settings for forward scatter and side scatter.
B)Linear settings for forward scatter and side scatter.

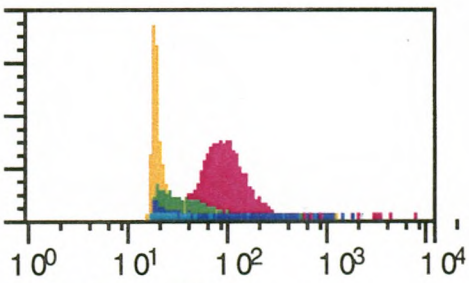
(Refer to Fig.2 for the appearance of the same sample described here, but incubated with a different antibody combination (CD61-PerCP, CD63-PE and annexin V-FITC.)











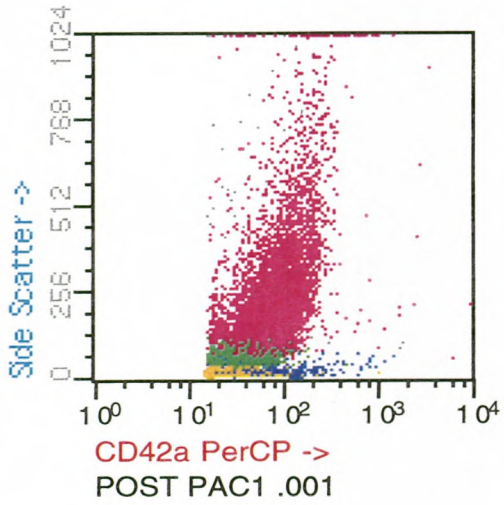
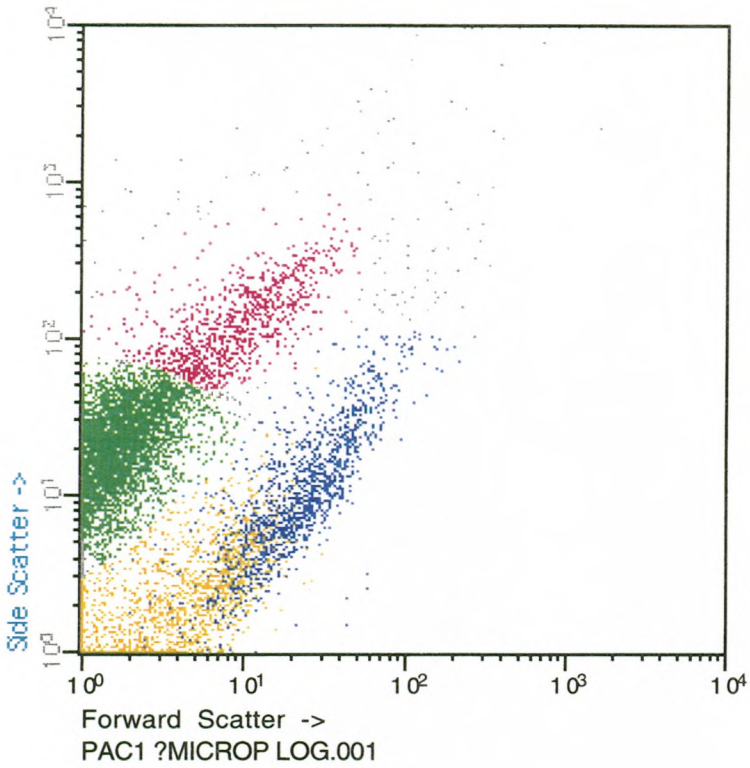
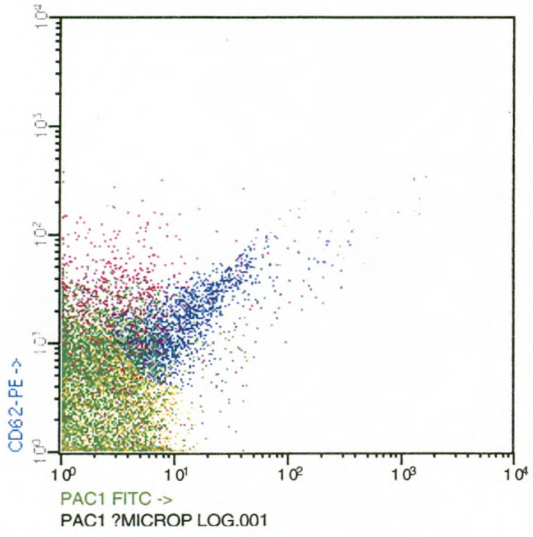


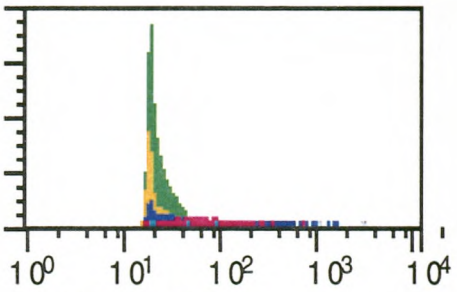
Figure 7:
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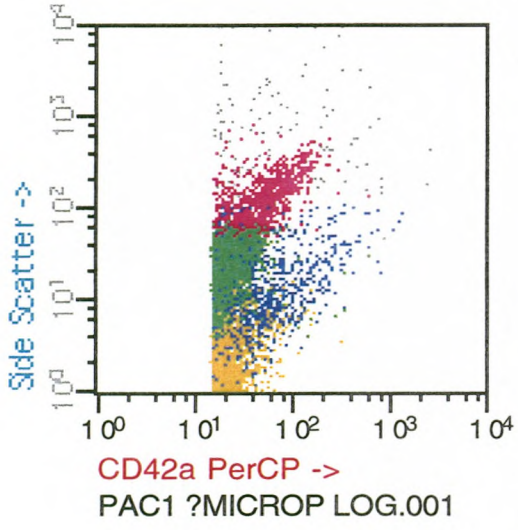
A)Logarithmic settings for forward scatter and side scatter.
B)Linear settings for forward scatter and side scatter.

15.17 % MICROPARTICLES
6.04 % ANTIBODY AND PROTEIN COMPLEXES
26.93 % ELECTRONIC NOISE
48.39 % PLATELETS





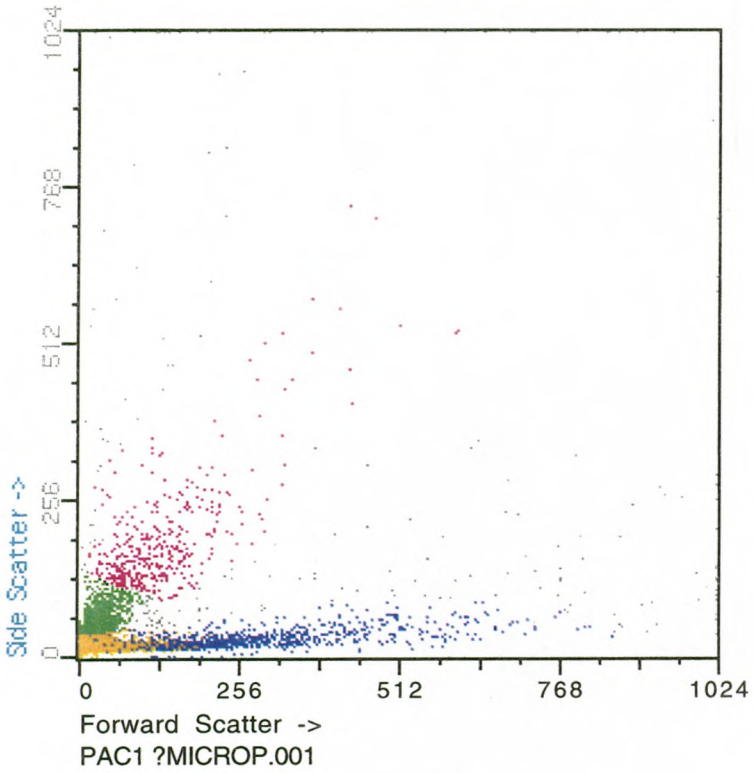


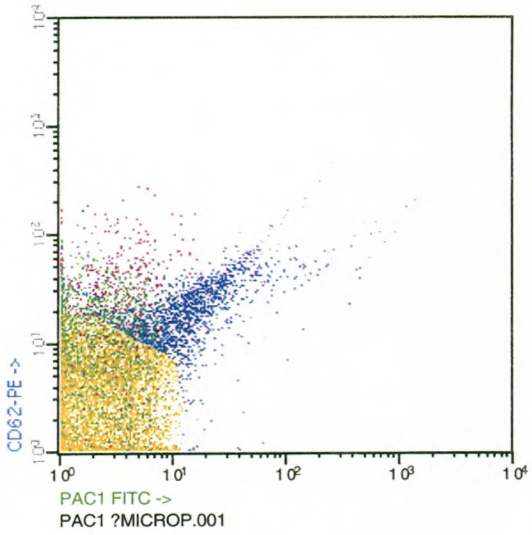


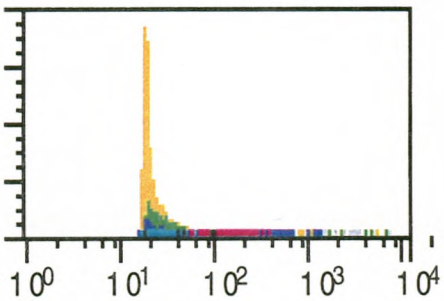
55.26 % MICROPARTICLES
13.28 % ANTIBODY AND PROTEIN COMPLEXES
20.39 % ELECTRONIC NOISE
8.21 % PLATELETS

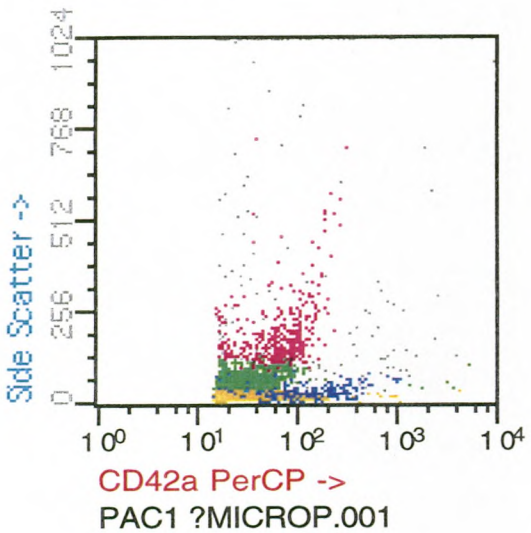
Figure 8:
After activation, the sample was then centrifuged at 800g for 10 minutes to pellet the platelets, the microparticles remaining in the supernatant. The supernatant was then incubated with antibodies and buffer as described in Figure 5. Note the increased proportion of electronic noise and antibody/protein complexes with the lower platelet count and excess of antibody.

A)Logarithmic settings for forward scatter and side scatter.
B)Linear settings for forward scatter and side scatter.





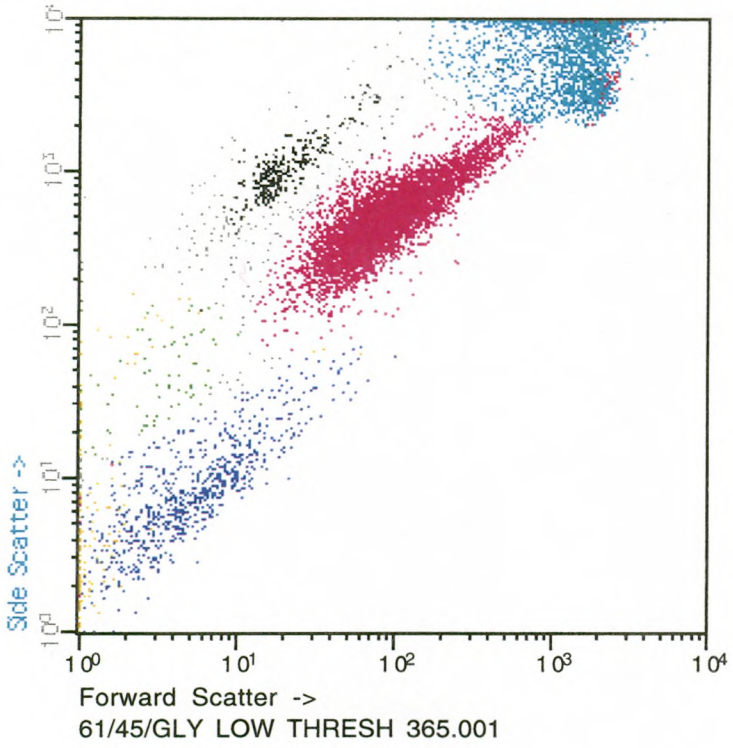


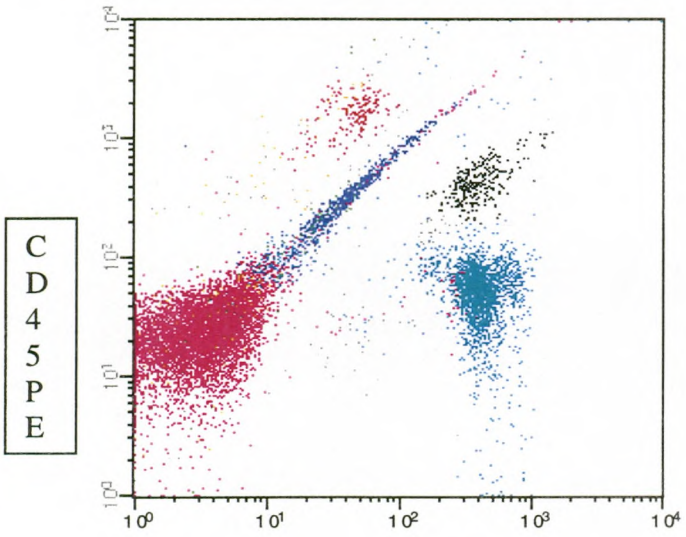


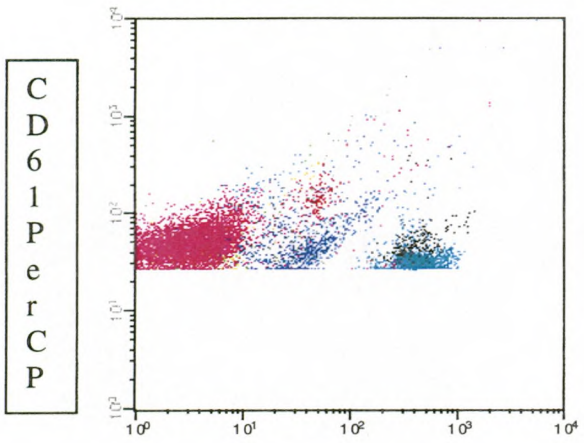
55.26 % MICROPARTICLES
13.28 % ANTIBODY AND PROTEIN COMPLEXES
20.39 % ELECTRONIC NOISE
8.21 % PLATELETS

Figure 8:
After activation, the sample was then centrifuged at 800g for 10 minutes to pellet the platelets, the microparticles remaining in the supernatant. The supernatant was then incubated with antibodies and buffer as described in Figure 5. Note the increased proportion of electronic noise and antibody/protein complexes with the lower platelet count and excess of antibody.

A)Logarithmic settings for forward scatter and side scatter.
B)Linear settings for forward scatter and side scatter.







1.58 % LEUCOCYTE–PLATELET AGGREGATES
0.66 % MICROPARTICLES
6.96 % ANTIBODY AND PROTEIN COMPLEXES
1.71 % ELECTRONIC NOISE
61.84 % PLATELETS
22.45 % RED CELLS
2.64 % ?RBC, WC & PLT MICROPARTICLES

Figure 9:
A citrated whole blood sample labeled with antibodies to identify on light scatter, the position of leucocytes (CD45-PE+), red cells (Glycophorin-FITC+) and platelets (CD61-PerCP+), as well as any aggregates thereof. The discriminant function is set on PerCP fluorescence.

Note that the minority of the red cell population overlaps in intensity of PerCP fluorescence with the platelet population.

The significance of the population coloured black is uncertain. Since these events are smaller than platelets, but positive for all of CD61, Glycophorin and CD45 fluorescence, one has to consider the possibility of these being associations of microparticles derived from platelets, red cells and leucocytes. (The other consideration would be antibody complexes, but their light scatter is not consistent with this.)

Background electronic noise was removed based on its' occurrence in two gates:

1)Events with similar size to microparticles (occurring in the first log on the forward scatter axis) but with lower side scatter than microparticles.

2)Events which are negative for expression of activation markers, specifically annexinV, i.e. occurring in the first log on the annexinV axis.

(Since microparticles should be budded from maximally activated "apoptotic" platelets, they should express negatively charged phospholipids and therefore bind annexin V, and have been previously described to do so (Dachary-Prigent et al, 1995). (See Figure 10 for an illustration of the gating strategy. See Figure 11 for an illustration of how variable the microparticle percentage can be depending on the gating strategy used.)

A.7.) Fixation

For the reasons discussed below, the process of fixation was considered after preliminary investigation, to overall have significant disadvantages, and was not therefore further optimized. Unfortunately this made determination of precision and reproducibility etc. difficult as this would require venesection of the same normal volunteer repeatedly (since as will be seen later, some of the activation markers necessitate immediate processing in the absence of fixation.)

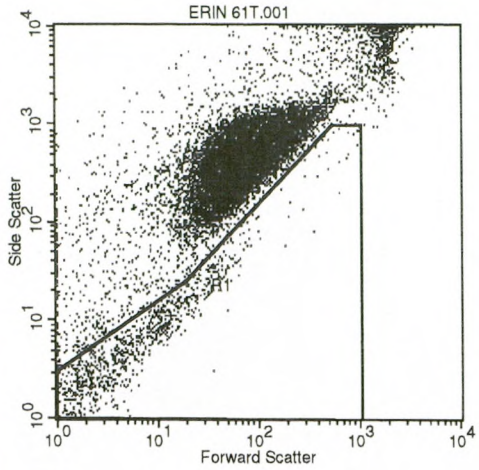
A.7.1.) Formation of aggregates

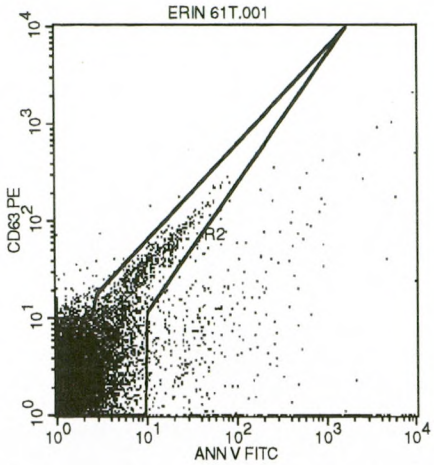
Attempts were made at fixing the platelets, in order to facilitate this assay becoming part of the routine workload. Paraformaldehyde in phosphate buffered saline (PBS)

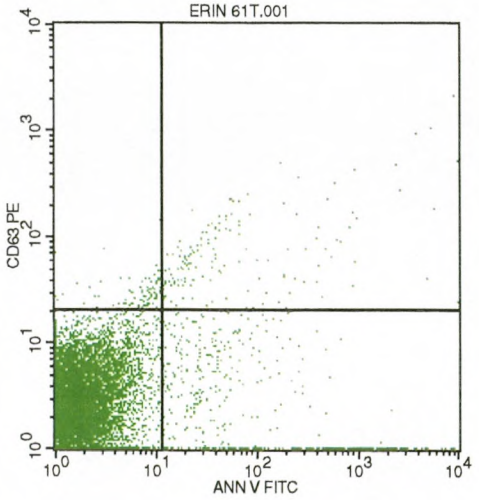
was used at a concentration of 0,5%. Fixing of the sample prior to addition of antibodies was compared to fixing the sample after incubation with antibodies. Some of the problems encountered with fixation follow.

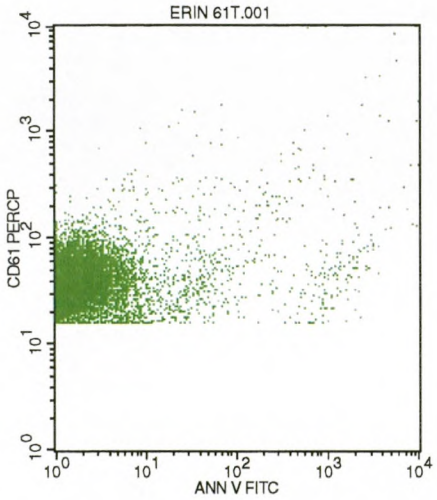
Figure 10:
The dot plots following illustrate the gating procedure followed for platelets and microparticles using the combination of antibodies to CD61 PerCP, CD63-PE and Annexin V-FITC. A similar procedure is followed with the CD42a-PerCP, CD62-P-PE and PAC1-FITC combination to exclude electronic noise and antibody/protein complexes, but microparticles are not quantitated in that instance since expression of Annexin-V cannot be gated for. The example shown is of a normal donor.

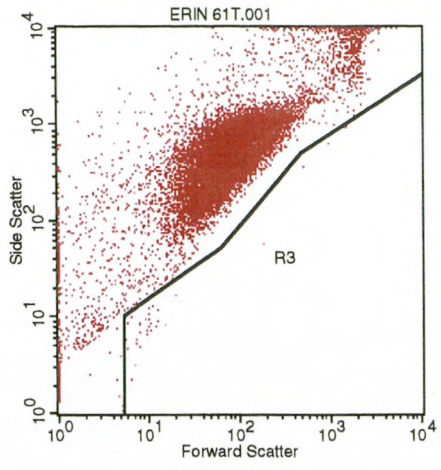
- Events occurring in R1 as well as R2 are excluded since this would be typical of electronic noise or antibody and protein complexes. The remaining events are then plotted in the third dot plot.
- Events in R3 are also excluded since they would again be likely to represent electronic noise or antibodies. The remaining events are plotted in the fourth, fifth and sixth dot plots, and the quantitation of activation marker expression is calculated from these.
- R4 delineates an area on light scatter where microparticles would be likely to fall (as well as any electronic noise or antibody complexes which have escaped the gating procedure thus far).
- The eighth dot plot depicts events falling in R4 alone, and microparticles then comprise the events falling in R6 (Annexin-V positive and CD63 negative).
- R5 delineates an area where platelet-white cell aggregates, platelet-red cell aggregates, platelets co-incident with white cells or red cells, or even red cells alone may fall. These events are plotted in the seventh dot plot. In the absence of an assessment of CD45 expression and a more specific light scatter analysis, this gate is too broad for accurate quantitation of platelet-leucocyte aggregates.









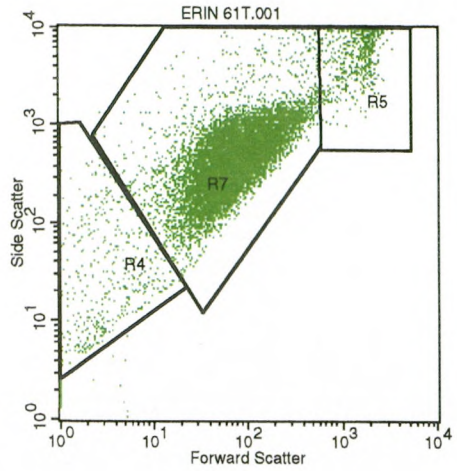


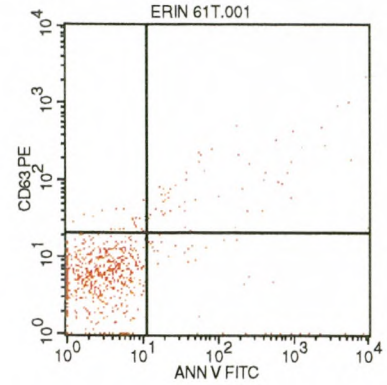
Quadrant Statistics

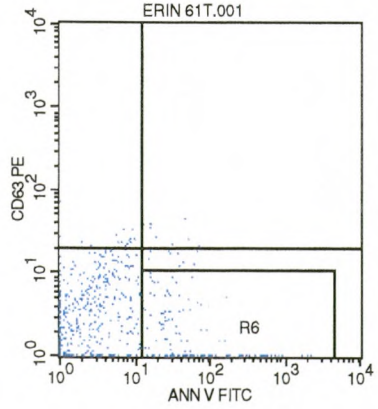
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Tube:
Acquisition Date: 13-Dec-00
Gated Events: 14265
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G2
Total Events: 15338
Y Parameter: FL2-H CD63 PE (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	87	0.61	0.57	7.47	6.57	32.06	30.15
UR	152	1.07	0.99	473.89	67.08	115.07	70.19
LL	13546	94.96	88.32	1.81	1.55	3.23	2.58
LR	480	3.36	3.13	604.53	129.34	4.02	2.31







Region Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 14265
X Parameter: FSC-H Forward Scatter (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G2
Total Events: 15338
Y Parameter: SSC-H Side Scatter (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	11	0.08	0.07	43.56	6.23	65.68	4.87	1, 2
R2	13509	94.70	88.08	151.37	72.02	756.45	413.07	3, 4
R3	0	0.00	0.00	***	***	***	***	1, 2
R4	567	3.97	3.70	3.34	2.35	63.74	29.44	1, 2
R5	692	4.85	4.51	1547.92	1428.51	6243.91	5446.81	1, 2
R6	417	2.92	2.72	103.22	12.95	1613.62	466.15	3, 4
R7	12913	90.52	84.19	93.00	70.59	590.02	430.48	1, 2

Quadrant Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 692
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G5
Total Events: 15338
Y Parameter: FL2-H CD63 PE (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	25	3.61	0.16	6.53	5.51	30.34	29.27
UR	54	7.80	0.35	893.32	123.80	183.15	93.37
LL	575	83.09	3.75	3.34	2.69	6.44	5.26
LR	38	5.49	0.25	658.51	112.83	8.25	4.75

Region Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 567
X Parameter: FL1-H ANN V FITC (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G4
Total Events: 15338
Y Parameter: FL2-H CD63 PE (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Ge
R1	2	0.35	0.01	6.14	4.07	10.39	
R2	382	67.37	2.49	3.21	2.42	5.51	
R3	0	0.00	0.00	***	***	***	
R4	567	100.00	3.70	43.34	6.06	5.37	
R5	0	0.00	0.00	***	***	***	
R6	145	25.57	0.95	155.80	61.09	2.29	
R7	0	0.00	0.00	***	***	***	

s

: (Log)

o Mean	Px,Py
10.27	1, 2
3.48	3, 4
***	1, 2
3.13	1, 2
***	1, 2
1.70	3, 4
***	1, 2

Quadrant Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 567
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G4
Total Events: 15338
Y Parameter: FL2-H CD63 PE (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	16	2.82	0.10	6.43	5.19	28.09	26.66
UR	8	1.41	0.05	33.05	27.57	31.12	30.03
LL	390	68.78	2.54	3.21	2.40	4.78	3.29
LR	153	26.98	1.00	150.01	60.12	3.18	1.95

S4

Figure 11:
Two samples, A) and B), are shown here to illustrate the difficulty of standardizing the quantitation of microparticles.

A) An example of a normal donor.
B) A sample of separated, washed and activated platelets.

i) The dot plots and histograms depict various methods used in the literature, or potentially useful methods, to calculate microparticle percentages.
R1 shows an area identifiable as predominantly electronic noise and antibody complexes, which essentially should be removed from the analysis, and would be by the method described in our study.
R2 shows a gate of events with low side scatter (complexity), but positive for PerCP fluorescence (expected to be microparticles.)
R3 shows a gate of events with low forward scatter (size), but positive for PerCP fluorescence (expected to be microparticles).
The two histograms are gating for microparticles by their separation from the "normal distribution" of platelets as measured by CD61PerCP fluorescence and size respectively.

ii) Samples A) and B) are analysed by the gating procedure described in our study, for comparison.

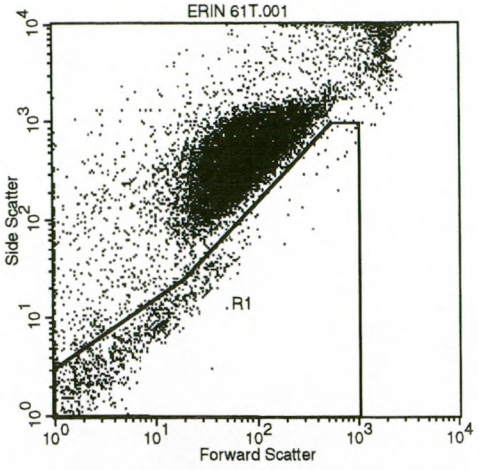
Microparticle percentages according to:

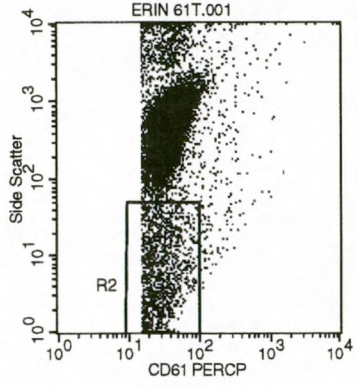
	A	B
CD61 PerCP vs side scatter	9,21	36,81
CD61 PerCP vs forward scatter	6,09	22,97
CD61 PerCP	2,73	5,16
Forward scatter	10,31	34,13
Gating including annexin V binding	1,02	18,61

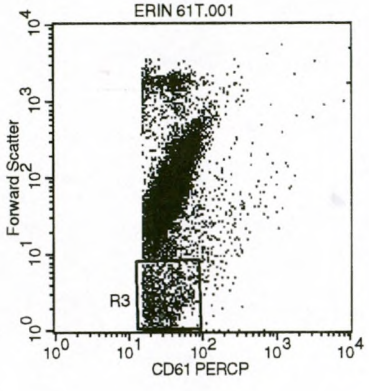
*Note that the discriminant was set low on PerCP fluorescence since the overlapping electronic noise and antibody complexes would be removed by the gating procedure. Other researchers would most likely have set the discriminant higher, and by doing so, could have excluded some of the microparticles.

**On the dot plot of forward versus side scatter, the gate for microparticles has been set at the border of the typical platelet populations seen in normal donors. This is used commonly in the literature, but it should be noted that some researchers object to this on the grounds that a sample of platelets activated in vitro seems to include a range of platelets from the size of microparticles to that of resting platelets. One author claims to have identified a population amongst this range of platelets which is clearly separable, on light scatter and fluorescence, from intact platelets (Bode et al, 2000). It is suggested that one includes fluorescent beads at a known concentration to assess whether there is a true increase in counts as the microparticles are shed. Others include beads of a known size to better discriminate between microparticles and small, activated platelets, as well as electronic noise etc. (Jy et al, 1992). Certainly it is important to describe one's methodology in detail in order to make the findings of the studies easier to compare.

A.i)





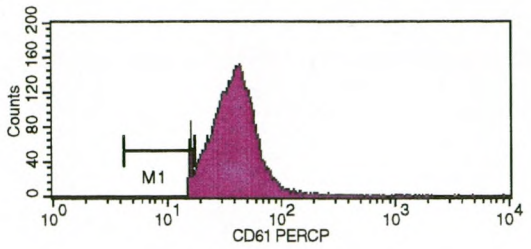


Region Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 15338
X Parameter: FL3-H CD61 PERCP (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 15338
Y Parameter: SSC-H Side Scatter (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	1081	7.05	7.05	44.45	33.09	26.79	5.86	1, 2
R2	1412	9.21	9.21	32.47	28.82	10.44	5.77	5, 2
R3	934	6.09	6.09	33.51	30.00	51.06	8.42	5, 1

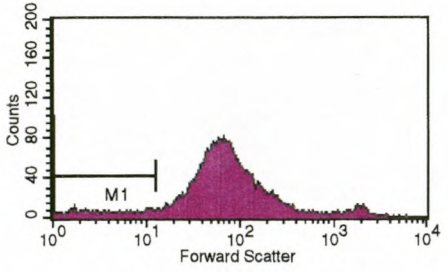


Histogram Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 15338
X Parameter: FL3-H CD61 PERCP (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 15338

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	15338	100.00	100.00	48.40	38.62	352.56	38.20	39
M1	4, 17	418	2.73	2.73	16.03	16.03	2.79	15.82	15



Histogram Statistics

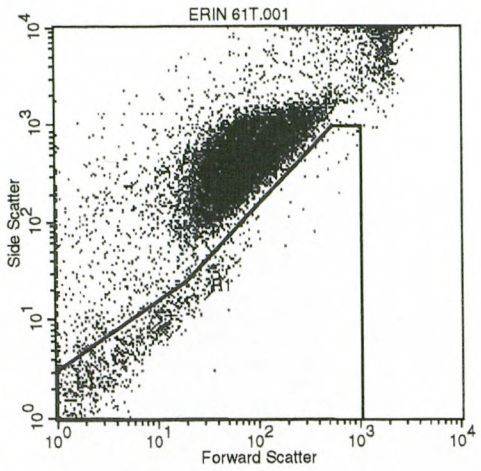
File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 15338
X Parameter: FSC-H Forward Scatter (Log)

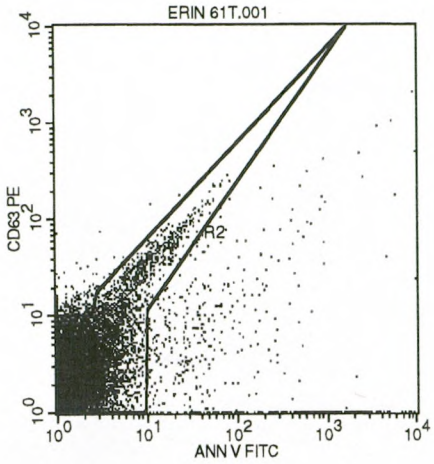
Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 15338

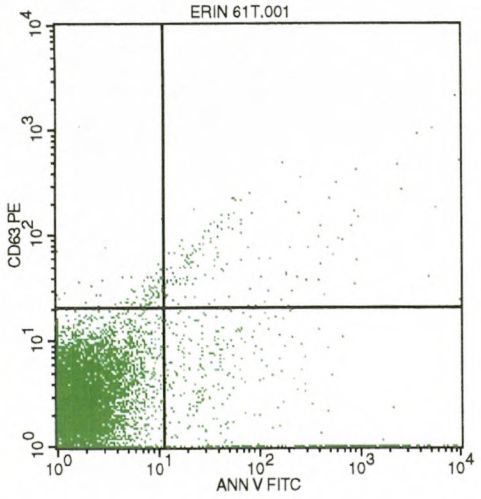
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	15338	100.00	100.00	150.62	58.63	227.39	63.78	1
M1	1, 13	1582	10.31	10.31	4.08	2.88	83.60	2.64	1

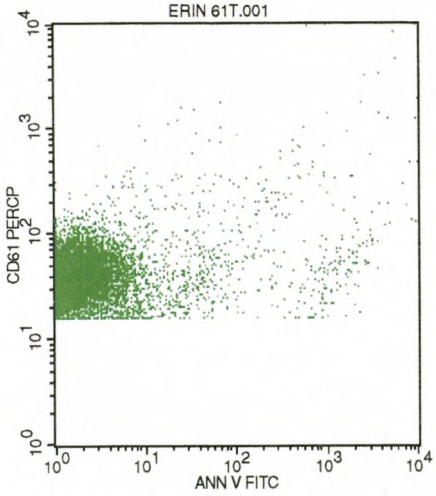
95

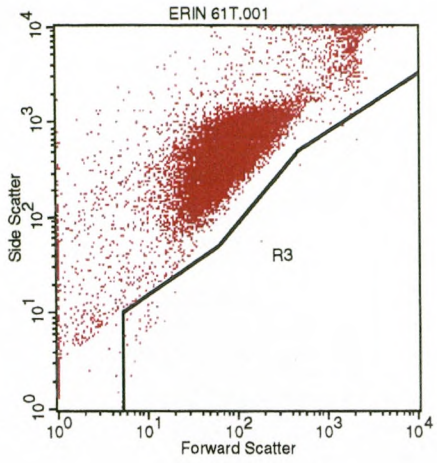
A.ii)











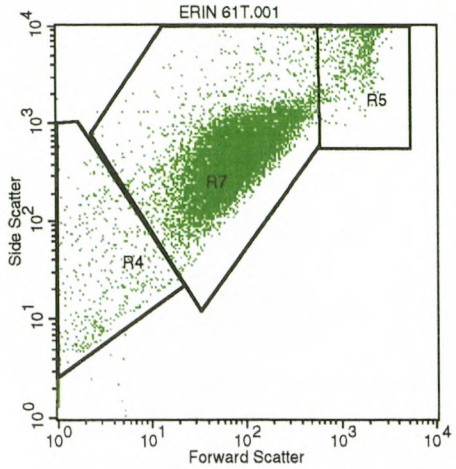
Quadrant Statistics

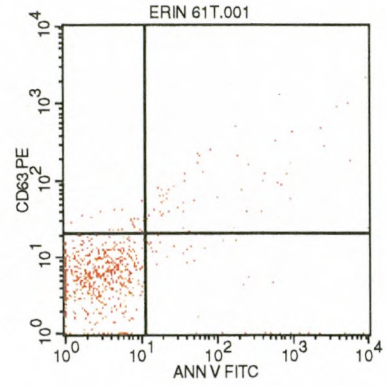
File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 14265
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

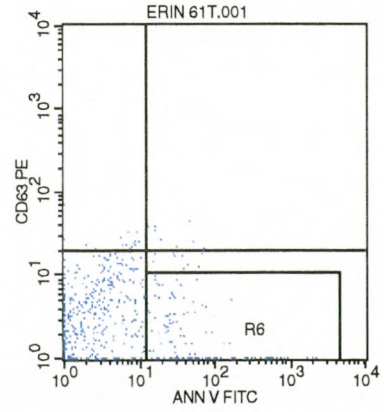
Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G2
Total Events: 15338
Y Parameter: FL2-H CD63 PE (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	87	0.61	0.57	7.47	6.57	32.06	30.15
UR	152	1.07	0.99	473.89	67.08	115.07	70.19
LL	13546	94.96	88.32	1.81	1.55	3.23	2.58
LR	480	3.36	3.13	604.53	129.34	4.02	2.31

LS







Region Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 14265
X Parameter: FSC-H Forward Scatter (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G2
Total Events: 15338
Y Parameter: SSC-H Side Scatter (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	11	0.08	0.07	43.56	6.23	65.68	4.87	1, 2
R2	13509	94.70	88.08	151.37	72.02	756.45	413.07	3, 4
R3	0	0.00	0.00	***	***	***	***	1, 2
R4	567	3.97	3.70	3.34	2.35	63.74	29.44	1, 2
R5	692	4.85	4.51	1547.92	1428.51	6243.91	5446.81	1, 2
R6	417	2.92	2.72	103.22	12.95	1613.62	466.15	3, 4
R7	12913	90.52	84.19	93.00	70.59	590.02	430.48	1, 2

Quadrant Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 692
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G5
Total Events: 15338
Y Parameter: FL2-H CD63 PE (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	25	3.61	0.16	6.53	5.51	30.34	29.27
UR	54	7.80	0.35	893.32	123.80	183.15	93.37
LL	575	83.09	3.75	3.34	2.69	6.44	5.26
LR	38	5.49	0.25	658.51	112.83	8.25	4.75

Region Statistics

File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 567
X Parameter: FL1-H ANN V FITC (Log)

Log Data Units: Linear Value
Patient ID:
Panel:
Gate: G4
Total Events: 15338
Y Parameter: FL2-H CD63 PE

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Ge
R1	2	0.35	0.01	6.14	4.07	10.39	
R2	382	67.37	2.49	3.21	2.42	5.51	
R3	0	0.00	0.00	***	***	***	
R4	567	100.00	3.70	43.34	6.06	5.37	
R5	0	0.00	0.00	***	***	***	
R6	145	25.57	0.95	155.80	61.09	2.29	
R7	0	0.00	0.00	***	***	***	

: (Log)

eo Mean	Px,Py
10.27	1, 2
3.48	3, 4
***	1, 2
3.13	1, 2
***	1, 2
1.70	3, 4
***	1, 2

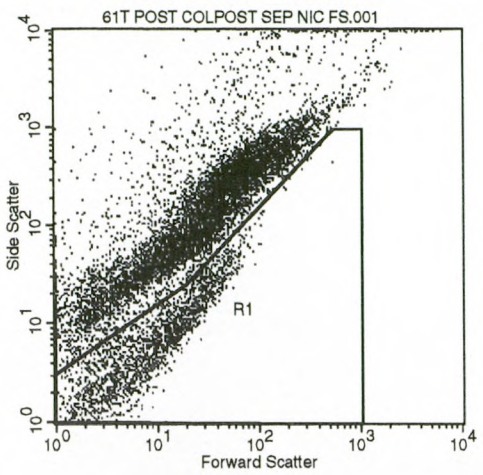
Quadrant Statistics

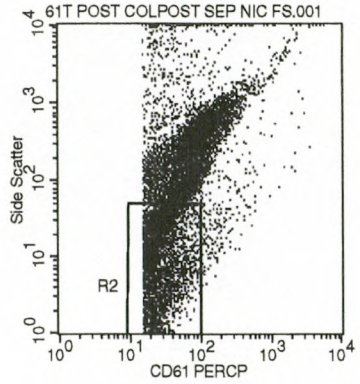
File: ERIN 61T.001
Sample ID: ERIN 61T
Tube:
Acquisition Date: 13-Dec-00
Gated Events: 567
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

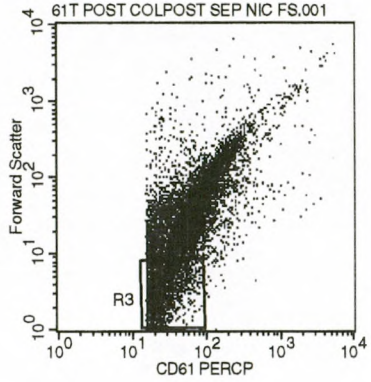
Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G4
Total Events: 15338
Y Parameter: FL2-H CD63 PE (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	16	2.82	0.10	6.43	5.19	28.09	26.66
UR	8	1.41	0.05	33.05	27.57	31.12	30.03
LL	390	68.78	2.54	3.21	2.40	4.78	3.29
LR	153	26.98	1.00	150.01	60.12	3.18	1.95

B.i)





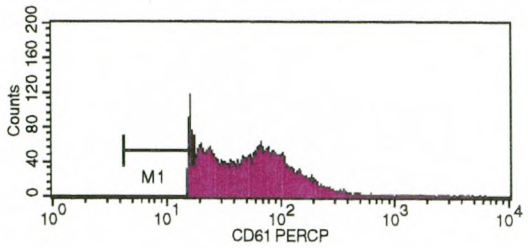


Region Statistics

File: 61T POST COLPOST SEP NIC FS.001
Sample ID: 61T POST COLPOST SEP NIC FS
Tube:
Acquisition Date: 08-Dec-00
Gated Events: 11308
X Parameter: FL3-H CD61 PERCP (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 11308
Y Parameter: SSC-H Side Scatter (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	2226	19.69	19.69	48.25	33.31	13.72	5.87	1, 2
R2	4163	36.81	36.81	30.41	27.46	16.08	9.86	5, 2
R3	2598	22.97	22.97	28.07	25.87	30.44	9.90	5, 1

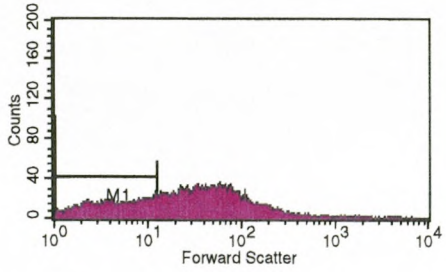


Histogram Statistics

File: 61T POST COLPOST SEP NIC FS.001
Sample ID: 61T POST COLPOST SEP NIC FS
Tube:
Acquisition Date: 08-Dec-00
Gated Events: 11308
X Parameter: FL3-H CD61 PERCP (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 11308

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	11308	100.00	100.00	87.50	54.63	218.46	54.25	15
M1	4, 17	583	5.16	5.16	16.02	16.01	2.76	15.82	15



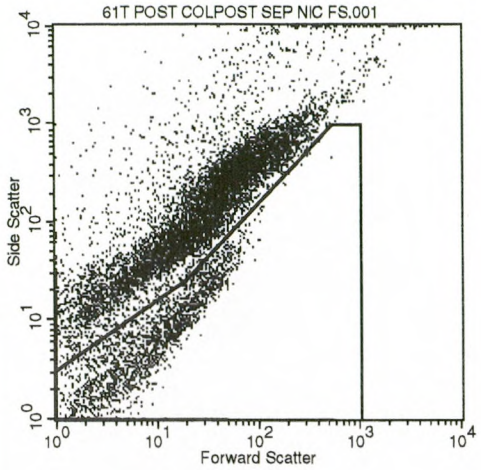
Histogram Statistics

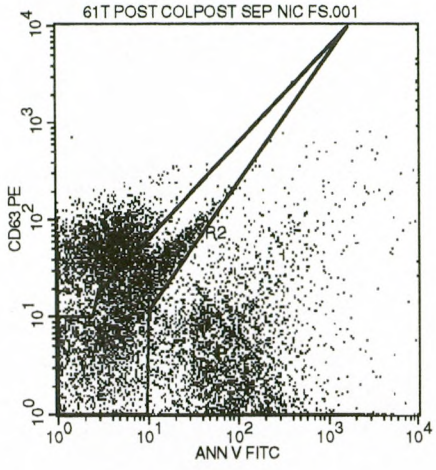
File: 61T POST COLPOST SEP NIC FS.001
Sample ID: 61T POST COLPOST SEP NIC FS
Tube:
Acquisition Date: 08-Dec-00
Gated Events: 11308
X Parameter: FSC-H Forward Scatter (Log)

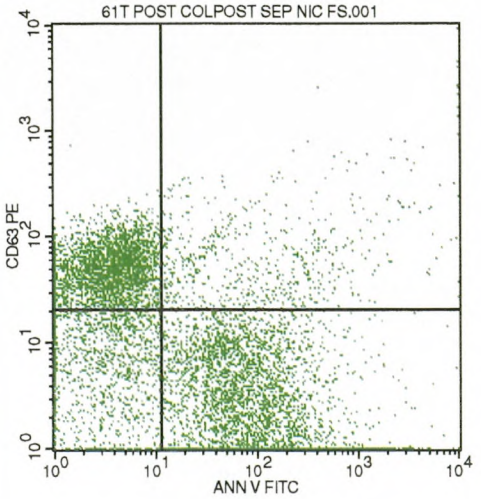
Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 11308

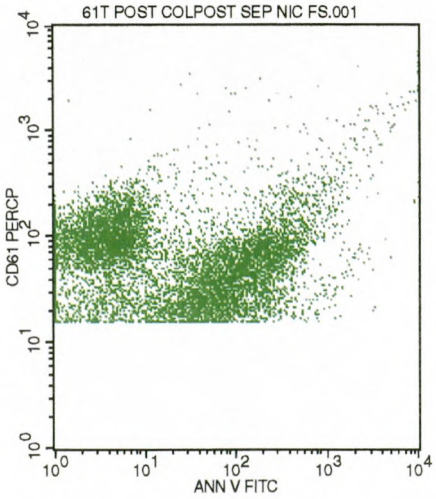
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	11308	100.00	100.00	66.27	21.75	311.83	25.71	1
M1	1, 13	3859	34.13	34.13	5.08	3.86	68.50	4.03	1

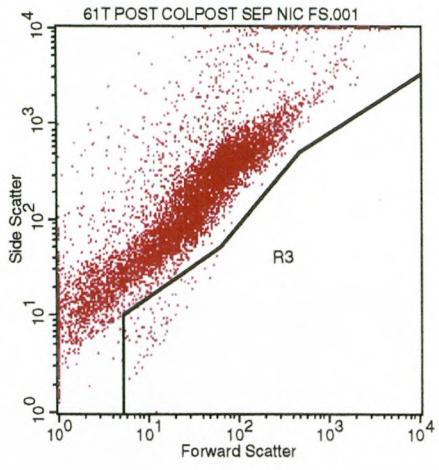
B.ii)









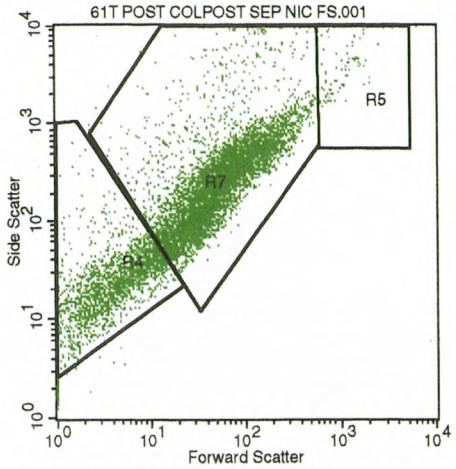


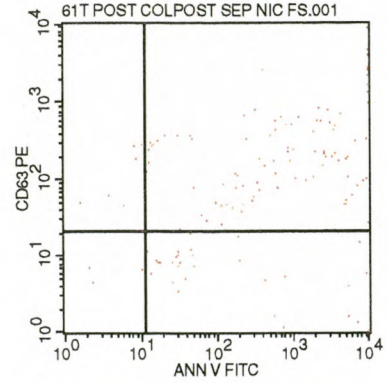
Quadrant Statistics

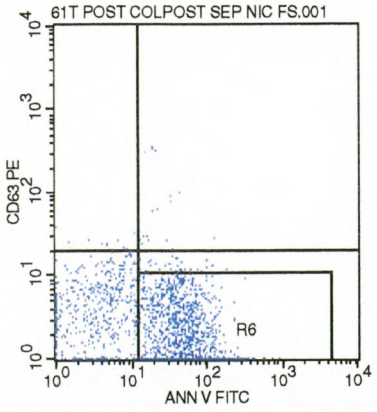
File: 61T POST COLPOST SEP NIC FS.001
Sample ID: 61T POST COLPOST SEP NIC FS
Tube:
Acquisition Date: 08-Dec-00
Gated Events: 9110
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G2
Total Events: 11308
Y Parameter: FL2-H CD63 PE (

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	2707	29.71	23.94	4.13	3.41	58.00	51.42
UR	640	7.03	5.66	599.81	115.44	141.76	67.70
LL	1238	13.59	10.95	4.00	3.06	7.76	5.33
LR	4525	49.67	40.02	211.05	103.27	3.43	2.13







Region Statistics

File: 61T POST COLPOST SEP NIC FS.001
Sample ID: 61T POST COLPOST SEP NIC FS
Tube:
Acquisition Date: 08-Dec-00
Gated Events: 2256
X Parameter: FL1-H ANN V FITC (Log)

Log Data Units: Linea
Patient ID:
Panel:
Gate: G4
Total Events: 11308
Y Parameter: FL2-H C

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Ge
R1	3	0.13	0.03	20.74	20.17	2.41	
R2	463	20.52	4.09	4.35	3.26	6.19	
R3	0	0.00	0.00	***	***	***	
R4	2256	100.00	19.95	55.25	29.01	3.95	
R5	0	0.00	0.00	***	***	***	
R6	1696	75.18	15.00	70.84	54.71	2.24	
R7	0	0.00	0.00	***	***	***	

Region Statistics

File: 61T POST COLPOST SEP NIC FS.001
Sample ID: 61T POST COLPOST SEP NIC FS
Tube:
Acquisition Date: 08-Dec-00
Gated Events: 9110
X Parameter: FSC-H Forward Scatter (Log)

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G2
Total Events: 11308
Y Parameter: SSC-H Side Scatter (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	29	0.32	0.26	101.51	24.38	153.92	34.79	1, 2
R2	1491	16.37	13.19	45.31	17.74	354.60	103.76	3, 4
R3	0	0.00	0.00	***	***	***	***	1, 2
R4	2256	24.76	19.95	5.07	3.78	31.10	22.84	1, 2
R5	135	1.48	1.19	1442.19	1206.61	6259.65	5112.03	1, 2
R6	4180	45.88	36.96	30.94	13.42	312.78	71.17	3, 4
R7	6645	72.94	58.76	76.13	52.46	503.47	259.37	1, 2

Quadrant Statistics

File: 61T POST COLPOST SEP NIC FS.001
Sample ID: 61T POST COLPOST SEP NIC FS
Tube:
Acquisition Date: 08-Dec-00
Gated Events: 135
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G5
Total Events: 11308
Y Parameter: FL2-H CD63 PE (L

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	7	5.19	0.06	7.01	5.94	155.59	119.71
UR	95	70.37	0.84	2658.07	751.81	542.01	211.97
LL	3	2.22	0.03	4.78	3.61	5.84	5.74
LR	30	22.22	0.27	1617.46	143.82	7.31	5.74

r Values

:D63 PE (Log)

ɔo Mean	Px,Py
1.74	1, 2
3.80	3, 4
***	1, 2
2.12	1, 2
***	1, 2
1.67	3, 4
***	1, 2

Quadrant Statistics

File: 61T POST COLPOST SEP NIC FS.001
Sample ID: 61T POST COLPOST SEP NIC FS
Tube:
Acquisition Date: 08-Dec-00
Gated Events: 2256
X Parameter: FL1-H ANN V FITC (Log)
Quad Location: 12, 20

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: G4
Total Events: 11308
Y Parameter: FL2-H CD63 PE (L

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	12	0.53	0.11	5.75	4.71	25.58	25.16
UR	17	0.75	0.15	28.75	23.63	112.24	65.14
LL	500	22.16	4.42	4.52	3.43	4.88	3.53
LR	1727	76.55	15.27	70.54	54.62	2.46	1.74

A general feature associated with fixation was the tendency to form aggregates. (See Figure 12.) This was variable to some extent but appeared to be more pronounced when antibodies were added prior to fixation than after fixation. It also seemed to be more pronounced in more activated samples, particularly in the presence of a high percentage of microparticles. To some extent one could theoretically standardize this by comparing samples to normal controls, and either reporting always on single platelets alone or on all events including aggregates. (Most groups feel that one cannot determine the number of platelets present in an aggregate accurately, and this is simply regarded as one event, although some have tried, with reservations. One cannot be certain that the antibodies will have adequate access to each cell's surface in an aggregate, and one may be underestimating the number of platelets bound if one assumes the fluorescence signal is proportional to the number of platelets present) (Hagberg and Lyberg, 2000 b).

It is the opinion of the writer that comparing activated samples that have been fixed, to fixed normal control samples need not give an accurate reflection of true activation status, particularly if single platelets alone are being assessed. It appears that the more activated a sample is, the more likely it is to form aggregates on fixation. Thus, by excluding aggregates from the analysis one would probably be biasing towards assessing a sample to have a lower level of activation.

However the quantitation of aggregate formation itself as an indication of the degree of activation, and the significance thereof has not been fully addressed in this study.

Figure 12:
Dot plots of forward scatter versus side scatter.
Each row represents the same sample, unfixed/fixed in different ways.

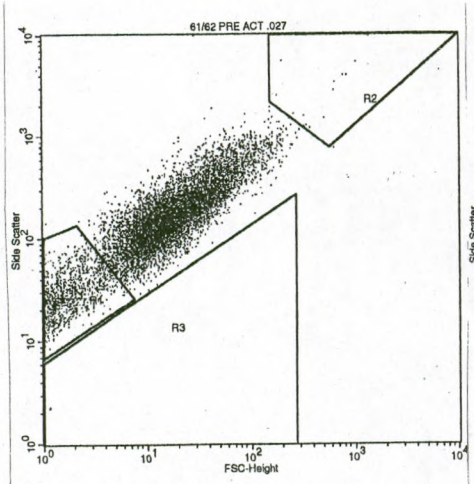
Column A) without fixative
Column B) fixing and washing the cells prior to the addition of antibodies
Column C) fixing and washing the cells after incubation with antibodies

Row 1) Samples of whole blood have been taken into ACD, then the platelets separated and washed (as in Materials and Methods Appendix)
Row 2) Samples have been incubated unstirred with thrombin (1U/ml) and collagen (10µl/ml) for a minimum of 10 minutes.
Row 3) Samples have been activated as above, then centrifuged at 800g for 10 minutes to separate the microparticles (in the supernatant) from the platelets (pelleted), and the microparticles selected for.

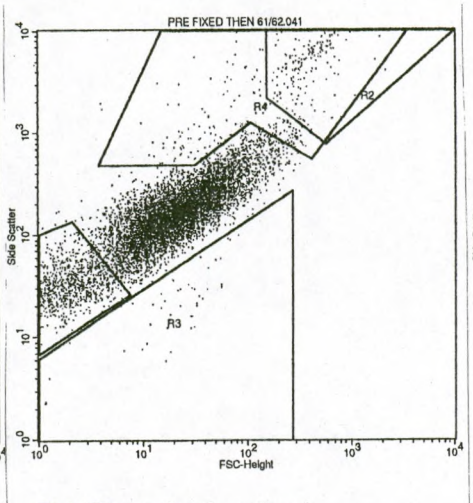
In Column B), fixing the cells was done by the addition of 1ml 0,5% paraformaldehyde to the 5µl whole blood + 10µl CD61-FITC + 20µl CD62-P-PE (or CD63-PE) + 50µl HEPES buffer.
In Column C), 100µl whole blood was fixed in 1ml 0,5% paraformaldehyde.

Samples were fixed at 4°C for longer than 12 hours but less than 24 hours. Subsequently the samples were centrifuged at 1 200g for 5 minutes to pellet the platelets. The supernatant was then centrifuged at 12 000g for 30 minutes to pellet the microparticles and the two pellets resuspended together in 1ml HEPES buffer.
In Column B), this was then analysed on the cytometer (since the antibodies had been added prior to fixation.)
In Column C), 50µl of the suspension of fixed cells was then incubated with antibodies as above for 15 minutes in the dark, then 1ml HEPES buffer added, and the samples run on the cytometer.

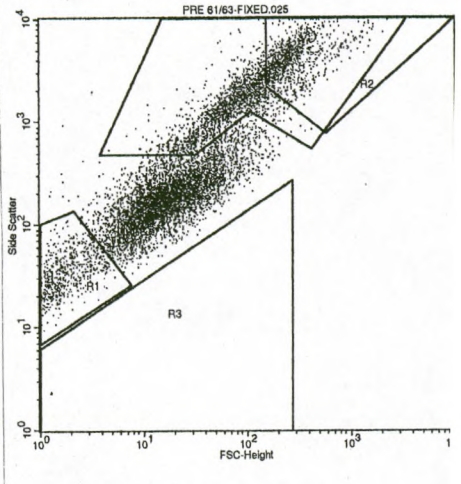
(Note for Row 3), the centrifuging after fixation was done only at 12000g for 30 minutes to pellet the already separated microparticles.)



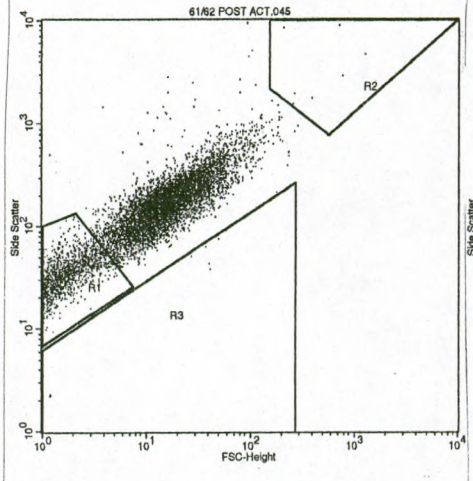
A.1.)Pre-activation, unfixed



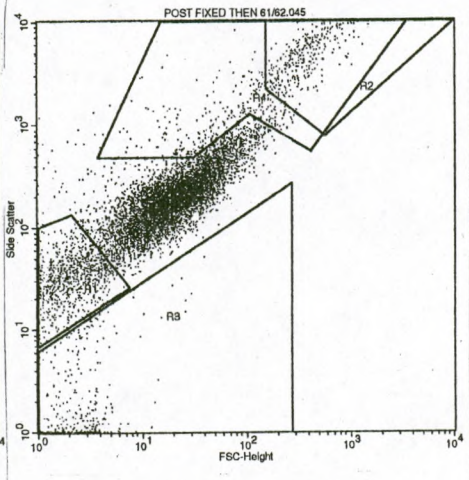
B.1.)Pre-activation, fixed before antibodies



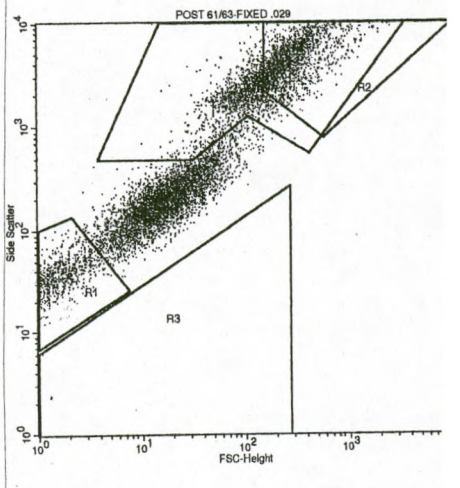
C.1.)Pre-activation, fixed after antibodies



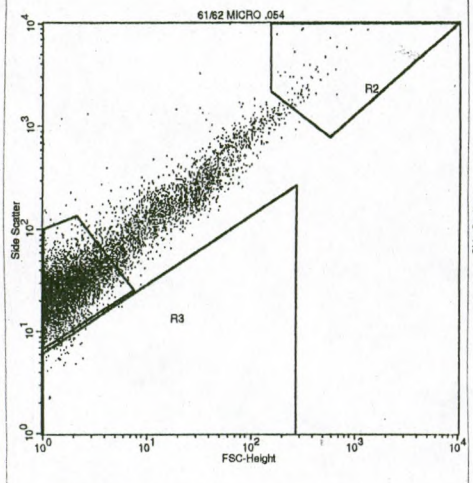
A.2.)Post-activation, unfixed



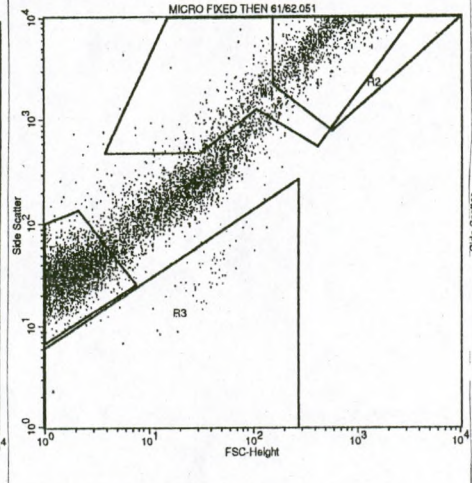
B.2.)Post-activation, fixed before antibodies



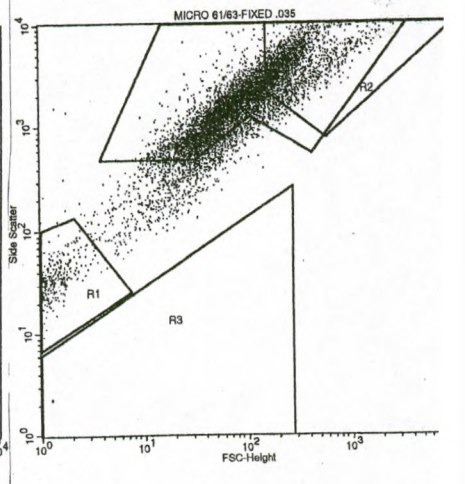
C.2.)Post-activation, fixed after antibodies



A.3.)Microparticles, unfixed



B.3.)Microparticles, fixed before antibodies



C.3.)Microparticles, fixed after antibodies

Figure 12:
Dot plots of forward scatter versus side scatter of a single sample from a donor, processed in different ways. R1 gates microparticles. R2 and R4 gate aggregates.

P.T.O.

Most authors do not discuss the enhanced aggregate formation occurring with fixation of the sample, but this has been previously documented to occur, and was shown to be particularly problematic with increasing centrifuging and washing of a sample. The aggregate formation is also reported to be time related, according to this study, becoming particularly enhanced after 6 hours. (These authors do state that one can still see an increase in aggregates in fixed activated samples as compared to unstimulated fixed samples) (Li et al, 1997).

The reasons suggested to explain this phenomenon include that fixation can reverse binding of fibrinogen to platelets, and may be associated with increased expression of P-selectin both of which could contribute to increased platelet-leucocyte aggregate formation. The cross-linking action of aldehydes on membrane proteins may also aggravate this feature, and the authors felt that the washing and centrifuging of samples may activate and up-regulate selectin and integrin expression on the leucocytes and platelets, allowing for the formation of increased aggregates (Li et al, 1997). In contrast to this study, Hagberg and Lyberg recommend immediate fixation of samples with paraformaldehyde. They did not encounter increased aggregate formation provided the sample was processed within two hours, and suggest that this may be due to the absence of the centrifuging and manipulation involved in washing off the fixative (Hagberg and Lyberg, 2000 b). This is certainly worth investigating further. Centrifuging a sample certainly seems to damage and/or activate the platelets, and it is possible in theory, that forcing the cells into close apposition in this manner encourages more cross linking of proteins on the cell surface. This may be aided by the presence of

monoclonal antibodies in the sample, binding to the cell surface. This could be one explanation why the aggregate formation encountered in our study was seen more prominently when antibodies were added to the sample prior to fixation.

A.7.2.) Preservation of antigen positivity/negativity

PAC1

With regard to the actual preservation of positivity of platelets with the individual antibodies, PAC1 antibody showed diffuse binding of antibodies to the platelets, with fixation both before and after incubation with antibody, and was therefore thought to be incompatible with fixation. It is of interest that certain authors describe successful fixation using PAC1 antibody under certain conditions, notably fixation after incubation with antibodies rather than before (Shattil et al,1987; Peyton et al, 1998). Others do not advocate fixation (Ault, 2001). The different findings are difficult to explain, and one wonders whether differences in the manufacturing process of the antibody, or possibly pH of the buffer or fixative used could be influencing the outcome.

Annexin V

Annexin V is also reported by many authors in the literature to be incompatible with fixation (Schmitz et al, 1998). Our findings were that addition of the fixative after incubation of cells with annexin V resulted in a mean channel shift to the left, i.e. with more negative FITC fluorescence than before fixation. Results would then appear to be difficult to interpret after fixation. Addition of annexin V after the fixation step appeared to yield better results, but was still not optimal. (For an unknown reason, after

fixing the platelets, washing them and then adding the annexin V and CD63, there appeared to be a relative increase in small events negative for the expression of activation markers, relative to true platelet events (resembling electronic background noise or debris). This did not happen with the other combination of antibodies (CD42a, CD62-P and PAC1). No definite reason can be documented for this, but it was found on more than one occasion. A similar finding was encountered by Hagberg and Lyberg, (2000 a). They showed that this phenomenon was dependant on the presence of red cells in the sample, and their degeneration over time. In contrast to this, we encountered this phenomenon after separation and washing of the platelets. Whether the events could represent tiny fragments of red cells could not be confirmed, but this is unlikely since this would have resulted in a similar finding in the tube incubated with CD42a, CD62-P and PAC1. Bacterial contamination of the antibody preparation is also an unlikely explanation since this would presumably have been consistent and ongoing in all experiments.

CD62-P and CD63

Fixation using CD62-P and CD63 antibodies showed more favourable results. There is still aggregate formation, but at least subjectively, there seems to be relative preservation of the antigen positivity on the platelets. (It was also noted however, that there is the possibility of continued alpha degranulation of the sample, and CD62-P expression prior to the cells actually being fixed. Whether this may have been related to the temperature of the fixative not being as low as is optimal (which is unlikely since many studies use fixative at room temperature), or perhaps to the relatively low

concentration of fixative is uncertain, but this was not a consistent feature.)

Many authors are using antibodies to CD62-P in association with fixation (Furman et al, 1998; Zeller et al 1999), but some have noted unsatisfactory differences between fixed samples and unfixed samples. The results described by various authors are not uniform (Kennedy et al, 1997; Shattil et al, 1987; Hu et al, 2000; Cahill et al, 1993). In addition each author describes different effects of fixation on resting samples as compared to activated samples, and also on samples fixed prior to addition of antibodies as opposed to after.

Essentially there is no consensus in the literature as to the effects of fixation. This could be related to the use of different antibodies, buffers, controls, duration of fixation and composition of fixative, or even different flow cytometers and methods of analysis. Each user should investigate the effects of fixation under the laboratory conditions encountered, and details of the method used must be reported, particularly until such time as any consensus has been reached.

Using the methods described in this study, the findings reflect that fixing whole blood samples for the analysis of platelet activation is not satisfactory.

A.8.) Time dependant activation of platelets

A.8.1.) Varying the period after incubation with antibody and dilution with buffer

The period after incubation with antibody and prior to running on the machine was investigated repeatedly on normal blood samples.

Platelet Activation Markers

- Expression of PAC1 appeared not to increase dramatically within the first 30 minutes, but after an hour, gradual increases were documented in some cases.
- Expression of CD63 appeared stable for 30 minutes and probably up to 1,5 hours.
- Annexin V expression appeared to also be stable for 30 minutes and probably up to 1,5 hours.
- CD62-P however showed a rapid time-dependant increase in expression, doubling roughly every 15 minutes. (See Figure 13.)

The findings described in the literature with regard to time dependant in vitro activation are variable. (Some groups state for example, that P-selectin expression is stable for 2 hours in unstimulated unfixed samples (Hu et al, 2000), while others have recorded detectable activation when the sample is processed after 10 minutes, increasing with time (Shattil et al, 1987). However, this same group has stated that the degree of activation at the time point at which antibodies are added to the sample is maintained for up to 2 hours without fixation. Since CD62-P expression represents release of alpha-granules, which occurs more easily than lysosomal granule release, the continuing gradual expression of CD62-P in vitro documented in the study presented here is not surprising. Again the difference in findings is difficult to explain, but perhaps buffer composition and even composition of the tubes and whether they are siliconised or not, could account for some variation.
(The tubes used in the study presented here were not siliconised.)

Aggregates

The apparent variation in number of aggregates over time was interesting. In the tube containing CD61-PerCP, CD63-PE and Annexin V-FITC, the percentage of aggregates was stable over 30 minutes, but tended to increase slightly after that in some experiments. In the tube containing CD42a-PerCP, CD62-P-PE and PAC1-FITC the percentage of aggregates clearly decreased over time in one set of experiments. In another set of experiments, the aggregate percentage clearly increased in the first 40 minutes (from 2,17% to 6,55%), then remained constant. The reason for this difference is uncertain. If any change would be expected with the CD42a/CD62-P/PAC1 combination, it would probably be an increase in aggregate formation, since the continuing CD62-P expression might be expected to result in increasing binding of platelets to the leucocytes, particularly monocytes. One possible mechanism that could perhaps explain our findings is that the PAC1 antibody in this tube is an IgM antibody and one wonders if the aggregates formed between platelets expressing PAC1 and probably CD62-P are perhaps more extensive because of the increased number of binding sites on an IgM molecule. As a result perhaps these complexes settle out of solution more rapidly and since the tubes were not agitated again prior to re-running the sample in which the aggregates were recorded as decreased, perhaps the part of the sample that is aspirated then contains less of these aggregates. (Unfortunately, it was not documented whether the tubes in the other set of experiments were agitated or not, since this was analysed some time after the acquisition, and the proposed explanation remains theoretical.)

Microparticles

Although few samples were analysed for microparticle stability over time, the number of microparticles would appear to be roughly stable over 30 minutes (i.e. this is the subjective opinion of the author, based on previous experience.)

A.8.2.) Varying the period prior to incubation with antibodies

The time prior to pipetting the whole blood into the tube to incubate with antibody was also varied. Again there appeared to be no definite trend to increase the activation markers CD63, annexin V and PAC1. The increase in CD62-P expression over time was similar to that described above. (See Figure 13.)

Figure 13:a)-d)
The following graphs depict the variation in expression of the various activation markers over time. Each series of points on the graph (except the latter two series on each graph) comprises a single sample of citrated whole blood, incubated with antibodies as described in the final methods section of Materials and Methods (page 77). The same tube containing the sample was analysed immediately after incubation with antibodies and dilution with buffer (time 0), and then at various times after that. The latter two series on each graph were samples set up to incubate with antibodies as above then run at time 0. At various times after that a new sample from the same tube of whole blood was prepared again, and incubated again with antibodies, then analysed on the flow cytometer.

(Note that the y-axis on each graph is calibrated differently.)

In Figure 13a), note that although too few samples were available for meaningful statistical analysis, there is a definite trend to increase CD62-P expression with time, both repeatedly running the same sample (Series 1 to 5), as well as after setting up fresh samples from the same whole blood sample at various times after venesection (Series 6 and 7).

In Figures 13b), c) and d), one cannot identify any definite trends to increase expression of these markers with time.

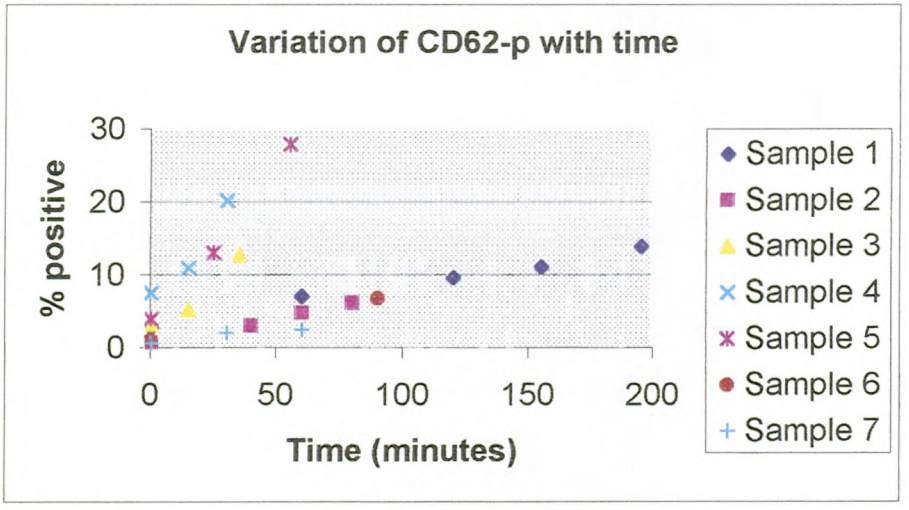

Variation of CD62-p with time
% positive
30
20
10
0
0
50
100
150
200
Time (minutes)
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7

Figure 13a)

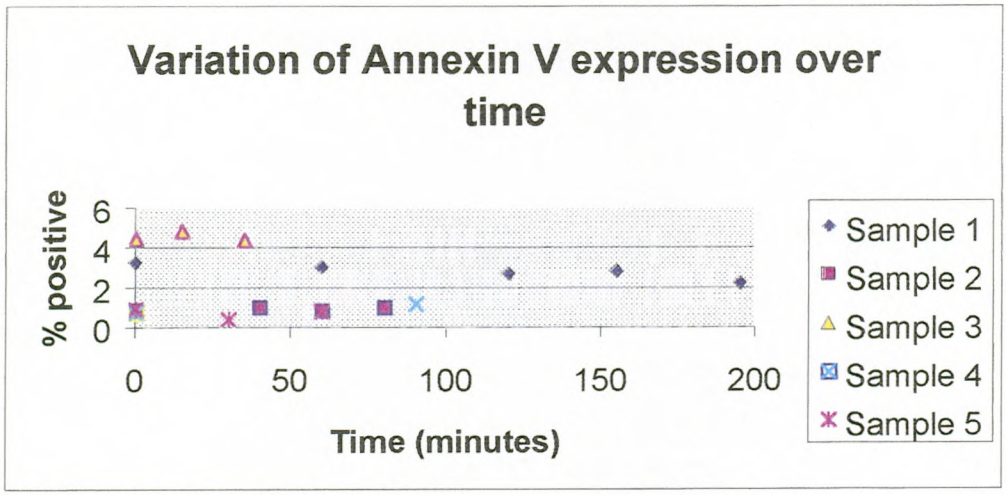

Variation of Annexin V expression over time
% positive
6
4
2
0
0
50
100
150
200
Time (minutes)
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5

Figure 13b)

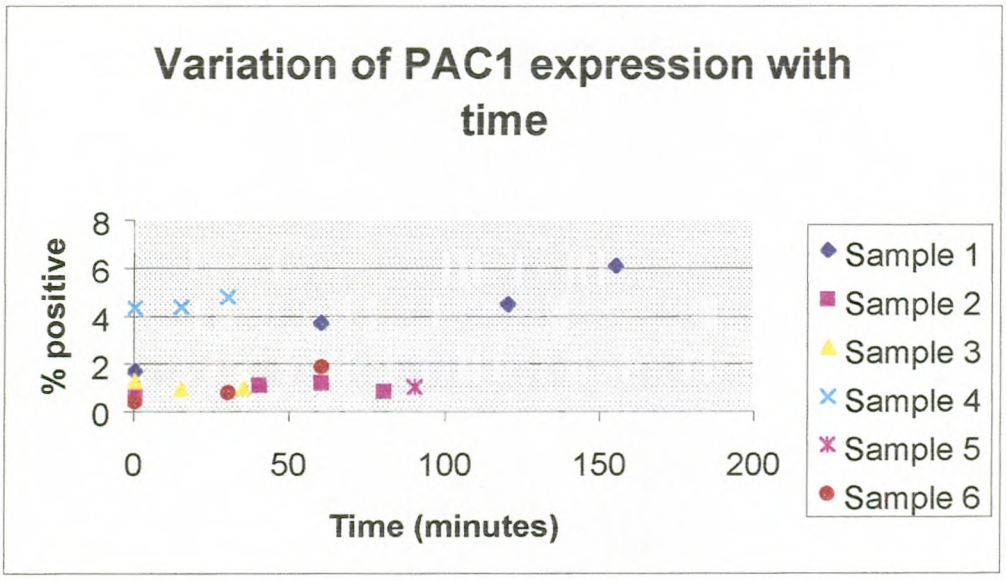

Variation of PAC1 expression with time
% positive
8
6
4
2
0
0
50
100
150
200
Time (minutes)
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6

Figure 13c)

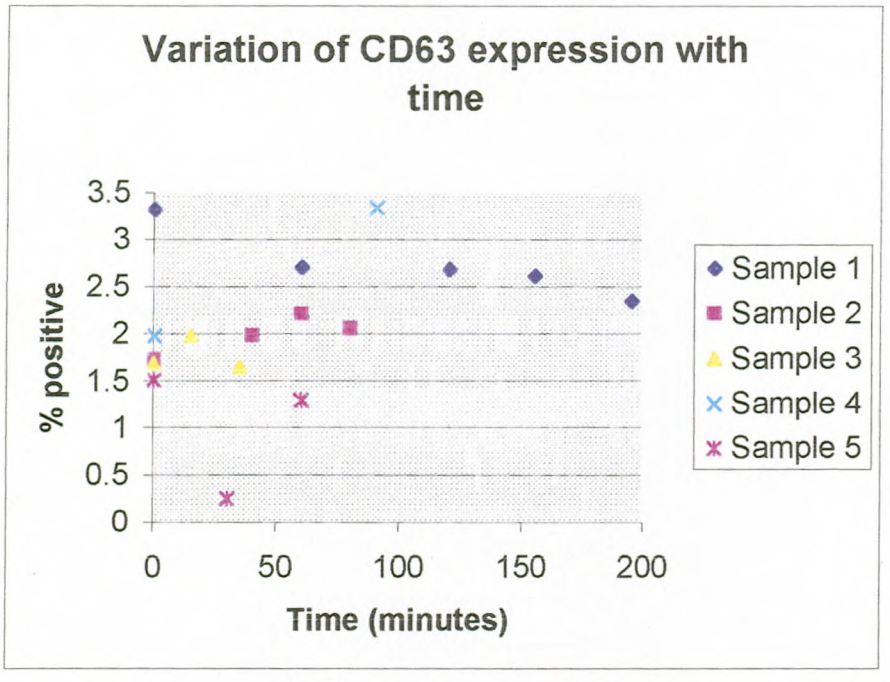

Variation of CD63 expression with time
% positive
3.5
3
2.5
2
1.5
1
0.5
0
0
50
100
150
200
Time (minutes)
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5

Figure 13d)

B) FINAL PROTOCOL FOR ANALYSIS OF LEVELS OF IN VIVO CIRCULATING ACTIVATED PLATLETS IN NORMAL DONORS

Based on the above findings, the optimal and most convenient protocol for the detection of platelet activation was as follows:

Pre-cytometric preparation

Prior to the venesection, polystyrene tubes compatible with the flow cytometer were prepared for the incubation.

Three separate tubes were set up for each donor, containing:

1) 10µl Annexin V-FITC (Pharmingen)
   20µl CD63-PE (Beckman Coulter)
   20µl CD61-PerCP (Becton Dickinson)
   50µl HEPES buffer.

2) 20µl PAC1-FITC (Becton Dickinson)
   20µl CD62-P-PE (Becton Dickinson)
   20µl CD42a-PerCP (Becton Dickinson)
   50µl HEPES buffer.

3) See page 81, C) The Analysis of Presence of Dense Granules and their Release for the mepacrine methodology.

Venesection

After a 2ml discard, 4,5ml of peripheral blood was taken into 0,5ml of 0,105M sodium

citrate in a Vacutainer tube, from an antecubital vein with the donor seated. The needle used was 21G. A light tourniquet was used but removed whilst taking the 2ml discard. The samples were slowly and gently inverted to allow mixing with the anticoagulant, then processed within five minutes.

Incubation with antibodies

Within five minutes of venesection, 5µl of whole blood was pipetted into each of tubes 1) and 2). The tubes were very gently rolled to allow a degree of mixing, then placed in the dark at room temperature for an incubation of 15 minutes. 1ml of HEPES buffer was then added to each tube, gently swirled to resuspend the blood if necessary, and the samples run on the cytometer with tube 2) being run first (since CD62-P expression had been noted to be expressed most readily with time in vitro.)

Cytometric Acquisition of Events

A minimum of 10 000 events positive for the identifying antibody (i.e. CD61 PerCP or CD42a PerCP) were collected on "low flow" settings which resulted in a flow rate of ~100-120 events being accumulated per second.

The cytometer settings for tube 1) and tube 2) are shown in Tables 4 and 5 respectively.

Table 4: Final settings for the antibody combination PAC1-FITC, CD62-P-PE and CD42a-PerCP

Parameter	Detector	Voltage	Amp. Gain*	Mode
P1	FSC	E00	1.30	Log
P2	SSC	540	1.30	Log
P3	FL1**	580	1.00	Log
P4	FL2	610	1.00	Log
P5	FL3	590	1.000	Log
P6	FL2-A		1.00	Linear
P7	FL2-W		1.00	Linear

Threshold:
Primary Parameter: FL3
Value:300 (on a logarithmic scale of size)

Secondary Parameter: None***

Compensation:
FL1 – 1.1% FL2
FL2 – 20.0% FL1
FL2 - 0.0% FL3
FL3 – 12.0% FL2

* Amplifier gains setting
** FL 1 is FITC fluorescence
FL 2 is PE fluorescence
FL 3 is PerCP fluorescence
*** (No other parameter was used to exclude unwanted events.)

Table 5: Final settings for the antibody combination Annexin V-FITC, CD63-PE and CD61-PerCP

Parameter	Detector	Voltage	Amp. Gain*	Mode
P1	FSC	E00	1.30	Log
P2	SSC	540	1.30	Log
P3	FL1**	590	1.00	Log
P4	FL2	560	1.00	Log
P5	FL3	590	1.00	Log
P6	FL2-A		1.00	Linear
P7	FL2-W		1.00	Linear

Threshold:
Primary Parameter: FL3
Value:300 (logarithmic units of fluorescence)

Secondary Parameter: None***

Compensation:
FL1 – 1.4% FL2
FL2 – 20.0% FL1
FL2 – 0.0% FL3
FL3 – 12.0% FL2

* Amplifier gains setting
** FL 1 is FITC fluorescence
FL 2 is PE fluorescence
FL 3 is PerCP fluorescence
*** (No other parameter was used to exclude unwanted events.)

C) THE ANALYSIS OF THE PRESENCE OF DENSE GRANULES AND THEIR RELEASE

The method used here was adapted from one previously described (Wall et al, 1995), where mepacrine is incubated with a whole blood sample in the presence of buffer and a platelet identifying monoclonal antibody. The mepacrine is taken up into dense granules as a result of its affinity for adenine nucleotides. The mepacrine then emits a green fluorescence that is detected in the same way as FITC fluorescence. However, on using the method as described, it was found that the green fluorescence of the platelets was barely detectable. Concentrations of mepacrine were then increased to optimize the green fluorescence up to the point where the light scatter pattern of the platelets was suggestive of their structure having been altered. The concentration of thrombin used to cause degranulation and release of the mepacrine dye was also titrated and optimized. The final method used follows.

Pre-cytometric preparation

A single polystyrene tube was prepared, containing

40µl mepacrine stock solution (5 x 10^{-4}M dissolved in Hank's Balanced Salt Solution without calcium or magnesium)

10µl CD42-PE (Beckman Coulter).

Venesection

The same whole blood sample as was used for the detection of platelet activation, was again used here. This was a 5ml Becton-Dickinson Vacutainer tube containing 0,5ml of 0,105M sodium citrate. This sample was taken with a 21G needle from an antecubital

vein with the use of a light tourniquet, following a 2ml discard during which time the tourniquet was released.

Incubation

Within 5 minutes of venesection, 50µl of whole blood was mixed in 2ml Hank's Balanced salt solution without calcium or magnesium, then 300µl of this solution (A) pipetted into the prepared tube. This tube was incubated in the dark at room temperature for 30 minutes. Subsequently 100µl of the incubated solution was diluted in 2ml Hank's BSS (this dilution being solution B), and an aliquot run on the flow cytometer.

Whilst the above sample was running, 867µl of the solution (B) was incubated with 133µl thrombin in saline, to achieve a final concentration of thrombin of 1U/ml. After 15 minutes to allow full activation and degranulation of the platelets, this was then run on the flow cytometer to demonstrate release of mepacrine as the dense granules are released with activation.

Cytometric Acquisition of events

Ten thousand PE positive events (CD42b+) were acquired on each control sample. There was no apparent disadvantage to running this sample on medium flow settings as compared to low flow, and the medium flow settings were therefore used on the majority of samples.

The final settings used are shown in Table 6.

Table 6: Final settings for the combination CD42b-PE and mepacrine.

Parameter	Detector	Voltage	Amp. Gain	Mode
P1	FSC	E00	1.30	Log
P2	SSC	540	1.30	Log
P3	FL1	580 (610*)	1.00	Log
P4	FL2	700	1.00	Log
P5	FL3	150	1.00	Linear
P6	FL1-A		1.00	Linear
P7	FL1-W		1.00	Linear

Threshold:
Primary Parameter: FL2
Value: 450

Secondary Parameter: None

Compensation:
FL1 – 1.0% FL2
FL2 – 26.0% FL1
FL2 – 0.0% FL3
FL3 – 0.0% FL2

*The voltage on the FL1 detector gives best separation between positive and negative events when set at 610.

Eleven healthy non-smokers under the age of forty were venesected and their results used as normal controls. Ten samples were used for the mepacrine uptake and release. (Note that one sample which had been incubated with CD61, CD63 and annexin V was slightly delayed (10-15 minutes) prior to being run on the cytometer. Since there appeared to be no definite increase in the expression of these markers in that short period, the sample was included. A second sample was incubated with antibodies approximately 15 minutes after venesection rather than within 5 minutes. Since the results, again, were well within the range of normal, if anything CD62-P expression was amongst the lower samples, it was included.)

D) CORRELATION OF BLEEDING TIME WITH PLATELET ACTIVATION AS ASSESSED BY FLOW CYTOMETRY

An attempt was made to correlate the bleeding time on a single normal donor with the sequential expression of platelet activation markers. The method followed was adapted from that used by Abrams et al, (1990), where immediate dilution of the blood sample was used rather than taking the sample into anticoagulant.

A Simplate bleeding time wound was made on the donor's forearm after compressing the upper arm with a blood pressure cuff inflated to 40mmHg. Two 2µl aliquots of blood emerging from the bleeding time wound were taken with a pipette at times 30 seconds, 1 minute and 30 seconds, and 3 minutes and 30 seconds after making the incision.

The blood samples were put into tubes containing:

1) 8µl PAC1-FITC
   8µl CD62-P-PE
   8µl CD42a-PerCP
   20µl HEPES buffer

2) 4µl annexin-V-FITC
   8µl CD63-PE
   8µl CD61-PerCP
   20µl HEPES buffer

These tubes were incubated in the dark for 15 minutes then 1ml HEPES buffer was added to each tube and the samples run on the cytometer as discussed previously. Unfortunately these experiments failed since the blood samples clotted in the test tubes. The platelets had obviously been activated and coagulation triggered and the absence of any anticoagulant, as well as possibly inadequate dilution by buffer, resulted in insufficient single platelets remaining for analysis. (The single platelets remaining would probably also be an inaccurate representation of what was taking place at that stage.)

This experiment would perhaps be better performed using both anticoagulant rather than just dilution of the blood, as well as possibly fixing the blood immediately after sampling. (Fixing a sample does have its own disadvantages, as discussed previously.)

RESULTS:

The tables below show the results obtained on the eleven normal controls tested for circulating activated platelets, and the ten tested for the presence and release of dense granules. In view of the small sample size and the fact that for some of the activation markers there appeared to be one or two skewed results, the median and interquartile range have been given.

Table 7 Percentages of platelets expressing activation markers as tested by Annexin V-FITC /CD63-PE/CD61-PerCP

	AnnexinV total*	AnnexinV aggregates**	CD63 total	CD63 aggregates	CD63/AnnexinV co-expression total	CD63/AnnexinV co-expression aggregates
1	6,77	0,94	1,59	0,60	1,19	0,44
2	3,14	0,73	1,62	0,65	1,08	0,49
3	4,32	0,64	1,51	0,55	1,01	0,38
4	5,18	0,57	1,60	0,47	1,08	0,34
5	2,98	0,48	1,47	0,43	1,09	0,34
6	4,47	1,04	2,04	0,76	1,55	0,58
7	4,14	0,84	1,55	0,52	1,39	0,47
8	3,67	0,49	1,61	0,47	1,23	0,34
9	5,13	0,07	3,39	1,05	2,24	0,82
10	2,28	0,52	1,25	0,48	0,78	0,31
11	4,35	0,68	2,04	0,59	1,54	0,49
Median	4,32	0,64	1,60	0,55	1,19	0,44
Interquartile range	3,14-5,13	0,49-0,84	1,51-2,04	0,47-0,65	1,08-1,54	0,34-0,49

* "Total" refers to all events expressing CD61 also recorded as positive for the activation marker stated, regardless of size (i.e. this includes microparticles and aggregates.)

** "Aggregates" refers to the events expressing CD61 also recorded as positive for the activation marker stated, which fall by light scatter in the "aggregates gate".

Table 8 Percentages of platelets expressing activation markers as tested by PAC1-FITC /CD62-P-PE/CD42a-PerCP

	PAC1 total	PAC1 aggregates	CD62P total	CD62P aggregates	CD62P/PAC1 total	CD62P/PAC1 aggregates
1	4,33	3,53	7,39	3,54	4,01	3,33
2	2,45	1,73	2,68	1,51	1,82	1,41
3	1,26	0,39	3,09	0,48	0,96	0,33
4	4,38	2,50	9,20	2,22	3,16	2,02
5	1,55	0,67	1,80	0,68	1,25	0,61
6	3,08	0,89	4,85	1,03	2,05	0,80
7	2,15	1,36	2,15	1,35	1,76	1,26
8	1,25	0,50	1,69	0,45	0,90	0,37
9	2,40	1,35	5,58	1,56	1,98	1,18
10	1,91	0,95	2,49	1,05	1,57	0,89
11	2,35	0,88	2,72	0,85	1,91	0,75
Median	2,35	0,95	2,72	1,05	1,82	0,89
Interquartile range	1,55-3,08	0,67-1,73	2,15-5,58	0,68-1,56	1,25-2,05	0,61-1,41

Table 9 Percentages of platelets recorded as microparticles or aggregates

	Microparticles (total events)* %	Microparticles Refined** %	Aggregates (total events) %	Aggregates Refined*** %
1	5,70	1,65	5,81	3,76
2	4,21	0,40	5,25	4,11
3	4,18	1,02	4,64	3,56
4	6,58	1,13	5,77	3,11
5	6,18	0,38	7,06	3,78
6	4,27	0,51	6,00	3,97
7	3,09	0,37	5,41	4,14
8	4,56	0,56	5,06	3,12
9	6,37	0,65	6,07	3,77
10	3,06	0,13	6,53	4,65
11	5,26	0,40	5,52	2,80
Median	3,69	0,51	5,53	3,77
Interquartile range	2,80-4,08	0,38-1,02	5,25-6,00	3,12-4,11

*The micropaticle percentages quoted are derived from the AnnexinV/CD63/CD61 analysis only since the refined percentage pertains also to this analysis. However, the aggregate percentages quoted are the mean of the two figures obtained from the two analyses AnnexinV/CD63/CD61 and PAC1/CD62-P/CD42a.

** The microparticle % is calculated as described in the Materials and Methods section. See page ...

*** The aggregate % is calculated after removing events with CD61 or CD42a expression of lower intensity than normal platelets.

Table 10 Mean channel number of fluorescence with markers in the Annexin V-FITC /CD63-PE/CD61-PerCP analysis

	MCN Annexin Total platelets	MCN Annexin Positive platelets	MCN CD63 Total platelets	MCN CD63 Positive platelets	MCN CD61 total platelets
1	31,30	649,28	3,47	61,33	46,90
2	10,79	426,96	4,14	71,36	42,33
3	21,83	709,11	3,71	62,05	44,41
4	28,52	709,93	3,78	58,11	41,99
5	10,30	376,97	4,04	61,43	41,67
6	27,43	800,39	4,71	65,89	44,89
7	22,66	678,67	3,49	67,92	41,43
8	22,10	730,28	3,80	63,95	47,14
9	21,28	529,43	5,37	60,80	50,24
10	8,34	362,72	3,53	60,26	50,11
11	20,31	533,41	3,77	57,28	36,08
Median	21,83	649,28	3,78	61,43	44,41
Interquartile range	10,79-27,43	426,96-709,93	3,53-4,14	60,26-65,89	41,67-47,14

Table 11 Mean channel number of fluorescence with markers in the PAC1-FITC /CD62-P-PE/CD42a-PerCP analysis

	MCN PAC1 Total platelets	MCN PAC1 Positive platelets	MCN CD62P Total platelets	MCN CD62P Positive platelets	MCN CD42a Total platelets
1	1,76	39,26	3,23	22,90	70,33
2	2,41	106,37	2,36	25,38	71,19
3	3,05	160,28	2,87	28,19	62,17
4	4,61	144,66	4,37	26,11	61,35
5	2,87	137,33	2,72	57,84	61,85
6	2,36	40,68	4,67	59,47	69,27
7	2,26	57,47	2,18	23,82	60,30
8	2,38	108,56	2,53	44,69	57,61
9	2,96	122,02	4,19	47,15	62,03
10	2,71	99,80	2,49	26,56	59,23
11	3,49	127,73	2,62	36,59	36,98
Median	2,71	108,56	2,72	28,19	61,85
Interquartile range	2,36-3,05	57,47-137,33	2,49-4,19	25,38-47,15	59,23-69,27

Table 12 Mepacrine uptake and release

	A Platelets neg for mepacrine uptake pre-thrombin %	B Platelets neg for mepacrine uptake post-thrombin %	C B-A	D MCN for mepacrine fluorescence pre-thrombin	E MCN for mepacrine fluorescence post-thrombin	F D-E	G D/E
1	52,85	95,62	42,77	3,00	1,15	1,85	2,61
2	58,67	95,06	36,39	2,85	1,25	1,60	2,28
3	52,87	92,11	39,24	3,68	1,79	1,89	2,06
4	64,96	85,76	20,80	2,74	1,72	1,02	1,59
5	74,27	96,36	22,09	2,18	1,27	0,91	1,72
6	60,01	85,23	25,22	2,56	1,24	1,32	2,06
7	56,12	93,91	37,79	2,74	1,26	1,48	2,17
8	60,24	89,17	28,93	2,52	1,39	1,13	1,81
9	55,96	93,22	37,26	3,02	1,32	1,70	2,29
10	58,98	94,43	35,45	2,78	1,22	1,58	2,28
Median	58,83	93,57	35,92	2,76	1,27	1,53	2,12
Interquartile range	55,96-60,24	89,17-95,06	25,22-37,79	2,56-3,00	1,24-1,39	1,13-1,70	1,81-2,28

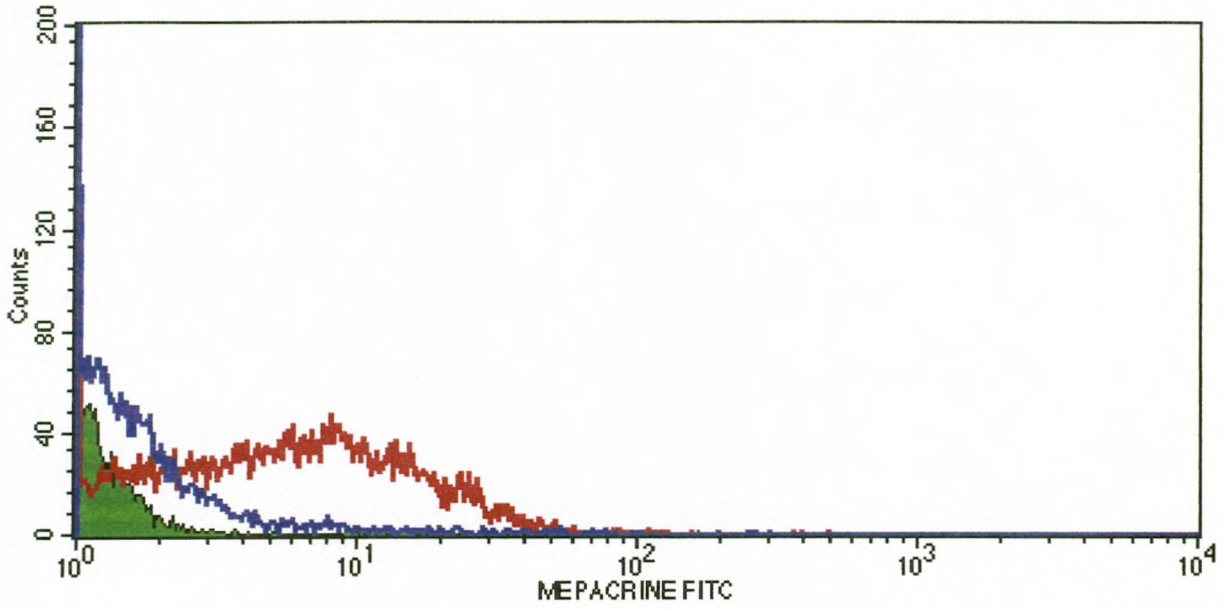


Figure 14:
Mepacrine uptake and release depicted graphically.
Green - a normal donor's platelets labeled with CD42b-PE but no mepacrine.
Red - the same donor's platelets incubated with CD42b-PE and mepacrine.
Blue - the same donor's platelets after subsequent incubation with thrombin (to achieve release of dense granules.)

(Settings used are those recommended based on better separation achieved on the last two samples.)

DISCUSSION

RESULTS OF NORMAL CONTROLS IN THIS STUDY – WHAT ARE THEIR SIGNIFICANCE?

1) PLATELET ACTIVATION MARKERS

The expression of each activation marker was analysed on platelets in total, as well as in two specific gates. (See Figure 10.) The two gates delineated

1) the area (on a plot of forward versus side scatter), in which one would expect to find microparticles,
2) the area in which one would expect to find platelet aggregates, and platelets bound to white cells, or coincident with white cells or red cells.

Where ranges are given, they consist of the median and interquartile range unless otherwise stated.

1.1.) CD63

Since the settings were optimized in such a way that microparticles were shown as CD63 negative, any events occurring in the microparticle gate which were shown to be CD63 positive were subtracted from the total figure. These events were likely to represent antibody complexes or electronic background noise which had slipped through the gating procedure set up to remove as much of this as possible without removing microparticles. In most cases the light scatter pattern in this gate, of the events removed from the analysis was similar to that seen repeatedly with background noise and antibody complexes.

CD63 expression on platelets in total was 1,60% (1,51-2,04). A significant proportion

of this expression localized to the "aggregates" gate, 0,55% (0,47-0,65). The majority of expression also occurred on platelets co-expressing annexin V, 1,19% (1,08-1,54).

It is noteworthy that CD63 expression on activated platelets is somewhat dim, with the fluorescence mean channel number shifting from 3,89 on a negative population of platelets to 58 after this individual's platelets had been maximally activated with collagen (10μg/ml) and thrombin (1U/ml). It is thus easier to document a population shift than to separate positive from negative events with this marker. Amongst normal controls, the range for mean fluorescence channel number for CD63 expression was 3,78 (3,53-4,14).

1.2.) Annexin V

As discussed for CD63, the total number of platelets expressing annexin V was modified by subtracting events in the microparticle gate that were unlikely to be microparticles. (This figure was not substantial, being at most 0,14%.)
The percentage of platelets expressing annexin V was 4,32 % (3,14-5,13). Of this, the positive population localizing to the clumps gate was small, i.e. 0,64 % (0,49-0,84) and the positive population that were considered to be microparticles comprised 0,51% (0,38-1,02). (Microparticles were defined as being annexin V positive.)

Unlike CD63 expression, the majority of annexin V platelet expression was in the absence of CD63 expression, with the annexin V single positive population comprising 2,81% (2,06-3,31). The latter was somewhat surprising since annexin V expression indicates the membrane flip-flop analogous to, and possibly even indicative of here,

apoptosis. One would presume that this represents end stage platelet activation. Certainly under experimental conditions performed in this study, annexin V expression required incubation of platelets with the strongest agonists thrombin and collagen. The possibility exists that these platelets could represent actual senescent, apoptotic platelets rather than activated platelets.

In contrast to the findings of our study, Tait et al, (1999) describe results more in keeping with what one would expect, namely that levels of annexin V expression were far lower in normal controls than levels of CD62-P and PAC1 expression. One of several differences in methodology may account for this finding. Whilst no negative controls were used in this study, Tait et al used negative controls which were different for each activation marker: addition of EDTA was used for annexin V (since calcium is required in detecting annexin V expression), an RGD inhibitor was used for the PAC1 control and an isotypic control monoclonal antibody was used for CD62-P. The annexin V used was different in that the investigators conjugated the protein themselves. (Two different products manufactured by different companies were used at different points of investigation in our study, and these products did appear to reflect different levels of activation in unactivated samples, or perhaps different levels of non-specific binding). On the other hand, despite Tait's samples being taken into prostaglandin E1 and sodium citrate, it was centrifuged and washed into buffer fairly similar in composition to the buffer used in this study. One might expect a greater level of activation here because of this, although it is quite possible that the other activation markers would be even more readily expressed after such manipulation of the sample.

It was also considered whether the events in the "aggregates" gate could actually be red cells which are apoptosed, their PerCP autofluorescence being great enough for them to overlap with the specific PerCP fluorescence of microparticles and the smaller end of the platelet range. However the minority of annexin V positive events localize to the "aggregates" gate and this could not therefore explain this phenomenon entirely.

The mean fluorescence channel number of negative events on a single sample was 2,64 and that of total positive events after maximal activation with thrombin and collagen was 211,05. Positive events are thus much easier to separate from negative than with CD63. The mean fluorescence channel number of microparticles alone after addition of collagen and thrombin was 70,84 and that of activated platelets alone was 286,66. This is similar to what has been described in the literature, i.e. the fluorescence of microparticles should be approximately one third of that of platelets. If one takes their respective volumes into consideration (microparticles, being about 0,2μm in diameter and platelets 2-4μm), per surface area, microparticles are enriched for negatively charged phospholipids in comparison to activated platelets (Dachary-Prigent et al, 1995).

Using final settings, with regard to controls, the mean channel number for annexin V expression on all platelets was 21,83 (10,79-27,43).

1.3.) CD62-P

The gates delineating microparticles and clumps by forward and side scatter were once

again used. However positive events in the microparticle area were not subtracted from analysis since microparticles were not defined as positive or negative at the outset.

CD62-P was expressed on 2,72 % (2,15-5,58) of platelets and 1,05% (0,68-1,56) localised to the "aggregates" gate. Very few events were recorded as positive in the microparticle gate, at most 0.07%, despite the fact that microparticles produced after separation of platelets from whole blood and incubation with collagen and thrombin did appear to express dim CD62-P. They are also documented to express dim CD62-P by other groups (Wiedmer et al, 1990; Jy et al, 1995; Dachary-Prigent et al, 1995). (Perhaps expression of activation markers depends on the agonists encountered, or activating conditions encountered e.g. shear stress. This has been suggested by other authors (Lazarus et al, 1995). The platelet population co-expressing CD62-P and PAC1 comprised 1,82 % (1,25-2,05).

The CD62-P positive population after maximal activation with ADP and adrenaline had a MCN of 93,47, whilst the negative platelet population was at 3,27. The population of positive events in the normal controls was on average recorded at a much lower MCN of 28,19 (25,38-47,15) with total platelets running at 2,72 (2,49-4,19).

The range of CD62-P positive platelets amongst the four male donors from highest to lowest was 2,68% to 9,20%, with a mean of 5,58%. Amongst the seven female donors, the range was 1,69% to 7,39% from highest to lowest, with a mean of 3,05%. The CD62-P expression tended to be higher in males than in females. The control population in the study presented here was very small and results have to be treated

with reserve. However it would not be surprising to find that males have a higher range of P-selectin expression in view of their increased risk of atherosclerosis in general.

The normal control donor with the highest P-selectin expression (9,20%) was venesected again at a later stage, since there was concern that the tourniquet may have been tighter than usual. On the second sampling, the percentage of CD62-P positive platelets was 3,91%. Whether the initial result was in fact higher due to the venesection technique, or whether there was true variability in the percentage of circulating activated platelets over time, this finding is worrying in terms of accuracy and repeatability of the test. (It is quite possible that the activation was artefactual and took place in vitro or during the venesection since we have established that of all the activation markers used in this study, CD62-P expression occurs most readily in vitro, spontaneously over time, and also with manipulation such as separation of platelets from whole blood and even running whole blood down the side of a tube rather than pipetting it neatly into the bottom of the tube. The other platelet activation markers did not appear to be proportionately higher on the first sample, although this could also simply indicate that CD62-P expression is occurring more readily in vivo, than the other markers.)

1.4.) PAC1

The "aggregates" gate and microparticle gates were again delineated. However PAC1 positivity in the microparticle gate was not subtracted, as microparticles were not pre-defined as positive or negative for PAC1.

PAC1 was expressed on 2,35 % (1,55-3,08) of platelets. The percentages falling in the "aggregates" gate were 0,95 % (0,67-1,73). Again, the positivity in the microparticle gate was negligible, at most 0,05%. The majority of PAC1 expression occurred on platelets also expressing CD62-P, with the platelets showing single positivity for PAC1 comprising 0,39 % (0,32-0,63).

The population of platelets negative for PAC1 expression in a single sample were recorded at a MCN of 2,31, at the settings used for the normal donors, whilst the positive population after activation with adrenaline and ADP was at a MCN of 63,10. The platelets recorded as positive in the normal control population were at a MCN of 108,56 (57,47-137,53). It was considered whether the events with the highest fluorescence here could perhaps be complexes of antibodies and platelets which failed to be excluded by the gating procedure, possibly because of the larger size of the complexes of IgM molecules. The total platelet population, i.e. predominantly resting platelets had a MCN of 2,71 (2,36-3,05).

1.5.) CD42a

Expression of CD42a is described by many authors to be weaker after platelet activation (Metzelaar et al, 1993) due to the redistribution of the GPIb/IX complex to

the open cannalicular system. The findings of this study were that on a single sample, the difference in expression between activated platelets (after incubation with ADP and adrenaline) and resting platelets were negligible, the MCN being in fact higher after activation (85,95 as compared to 79,97 before activation.)

Amongst the normal donors, the MCN of platelets for CD42a expression was 61,85 (59,23-69,27). The MCN of platelets for CD61 positivity was 44,41 (41,67-47,14).

1.6.) Microparticles

Following the sequential gating procedure described in Materials and Methods, the percentage of microparticles in the normal controls was 0,51 % (0,38-1,02).

1.7.) Aggregates

The percentage of events falling within the aggregates/clumps gate was recorded. An average was recorded between the two antibody triplet analyses, and this comprised 5,53 % (5,25-6,00). In an attempt to minimize the influence of the contaminating red cell population, the events within the clumps gate which had weaker CD61-PerCP or CD42a-Per-CP expression than an average platelet, were subtracted from the total of the clumps gate. The aggregates then comprised 3,77 % (3,12-4,11).

The setup of an optimal analysis to quantitate platelet-leucocyte aggregates differs from that used in this study in that one would be focusing primarily on the white cells and gating for monocytes and granulocytes by light scatter in association with positivity with a variety of antibodies, possibly the simplest being CD45 alone. The

percentage of monocytes and granulocytes respectively positive for a platelet marker such as CD42a, CD42b or CD61 would then be determined. This would be indicative of platelets bound to white cells, presumably by their expression of CD62-P. (Platelets co-incident with white cells would also have this appearance, but this is apparently a rare occurance in comparison to true platelet-white cell complexes. One study quotes this occurrence at less than 0,05% (Li et al, 1997). In our study, the estimation of aggregates was not the primary focus, and by the cruder way the "aggregate" gate has been set-up, it is not possible to accurately assess such events. Without gating by light scatter and CD45 expression, but gating on forward scatter versus side scatter alone, this area would include platelet-platelet aggregates, platelet-white cell aggregates, platelet-red cell aggregates or platelets co-incident with red cells and even the upper end of the red cell population which overlaps minimally with the range of fluorescence of the microparticles. (See Figure 9.)

A slight improvement is the exclusion of events in the "aggregate" gate which have dimmer PerCP fluorescence than the average platelet. Strictly speaking, this is again suboptimal since this would also exclude from analysis any white cells that had microparticles bound to them. The latter is described in the literature to occur (Jy et al, 1995), and certainly, microparticles were seen to form aggregates on fixation in the study presented here. (Jy et al also used fixation with paraformaldehyde, which could conceivably have increased aggregate formation. However they did not wash off the fixative involving centrifuging steps etc.) Microparticles are also described to form aggregates with intact platelets (Holme et al, 1998 (b)).

2) MEPACRINE UPTAKE AND RELEASE

The results of mepacrine uptake and release were recorded in two ways:

1) A single sample of one of the normal donors was run after incubation with CD42b-PE , but with buffer instead of mepacrine. A gate was drawn to include 99% of all events when the sample was run at low speed. The percentage of events occurring within this gate after the addition of mepacrine was then recorded, as well as the percentage after subsequent addition of thrombin.
2) The mean fluorescence channel numbers were recorded pre- and post- thrombin addition, and the ratio of pre-/post-thrombin recorded.

The platelets falling within the "negative gate" after incubation with mepacrine comprised 58,83 % (55,96-60,24). (Negative control was 99%.) The percentage after subsequent incubation with thrombin was 93,57 % (89,17-95,06). More pertinently, the difference between these two figures i.e. (post-thrombin – pre-thrombin) was 35,92 % (25,22-37,79).

The mean fluorescence channel number after mepacrine incubation was 2,76 (2,56-3,00). (Negative control 1,02.) This shifted to 1,27 (1,24-1,39) after addition of thrombin. The difference in the channel shift per patient was 1,53 (1,13-1,70), and the ratio of pre-thrombin mean channel / post-thrombin mean channel was 2,12 (1,81-2,28).

Since there was no patient with Hermansky-Pudlak Syndrome available to donate a blood sample, separated platelets from a normal donor sample were activated with high

dose thrombin and collagen (1U/ml and 10µg/ml respectively) in the hope of inducing maximal degranulation. The platelet population in this sample had a mean channel fluorescence of 1,75, with 77,46% of events falling within the "negative gate".

Two of the control samples were run with the voltage on the FL1 detector set at a slightly higher level (610 instead of 580). This yielded a far better separation between positive and negative events. The populations occurring in the "negative gate" after mepacrine incubation and before addition of thrombin comprised 28,02% and 22.03% respectively, shifting to 89,49% and 88,21% respectively after addition of thrombin. The mean channel numbers shifted from 7,95 and 9,42 respectively to 1,77 and 1,74 respectively. The pre-/post-thrombin mean channel ratios were 4,49 and 5,41 respectively. These settings are thus recommended over the previous ones used. (See Figure 14.)

3) CORRELATION OF BLEEDING TIME WITH PLATELET ACTIVATION AS ASSESSED BY FLOW CYTOMETRY

Unfortunately these experiments failed since the blood samples clotted in the test tubes. This was most likely due to the absence of any anticoagulant, relying on dilution to prevent clotting, which obviously was inadequate. Insufficient single platelets remained for analysis. This experiment would perhaps be better performed using both anticoagulant rather than just dilution of the blood, as well as possibly fixing the blood immediately after sampling. (Fixing a sample does have its own disadvantages, as discussed previously.)

EMERGING PRINCIPLES

One of the primary aims of this study was to assess the viability of performing platelet activation studies by flow cytometry in a routine laboratory, and to establish some of the problems associated with performing this test. It has been discovered that there are significant technical issues that need to be addressed in order to make possible the performance of this test in a routine laboratory, failing which the validity and comparability of the results would be questionable.

Flow cytometric evaluation of platelet activation was to a certain extent heralded as being revolutionary in this field. It was hoped that it would be far more accurate in comparison to other tests such as platelet factor 4 and βTG which were suboptimal in view of the technical issues around manipulation of the sample which made their results questionable. Unfortunately, there are significant technical difficulties using this methodology as well.

In addition, if results of these tests are to be used by clinicians for diagnosis routinely, and for monitoring of disease or therapy, it would obviously be desirable to have a standardized normal range, which could be comparable with that obtained in other laboratories. As things stand currently, the methods used in various studies in the literature are extremely variable. Some groups are using whole blood whilst others use platelet rich plasma, despite documentation in the literature (Abrams and Shattil, 1991) that manipulation of the sample is very likely to cause activation. (See Figure 4.) Notwithstanding, many groups are in fact fixing their samples, while others do not. Fixation of the samples is being performed prior to sample manipulation (Grieshammer

et al, 1999; Wehmeier et al, 1991) or after incubation with antibodies (Katopodis et al, 1997), and the fixatives used as well as their concentrations are variable. The duration of fixation of a sample is variable, (and some have found significant differences with duration of fixation) (Hagberg and Lyberg, 2000 b). The fixative may or may not be washed off.

It has been noted that the choice of anticoagulant for platelet activation studies may yield different results. (EDTA and heparin may result in increased aggregate formation, and increased P-selectin expression and citrate has been reported to result in a slight increase in calculated microparticles (defined on forward scatter) (Golanski et al, 1996). The use of inhibitors of activation, such as prostaglandin E1 (Tait et al, 1999), trimethylsphingosine (Kennedy et al, 1997) or cocktails of antiplatelet agents such as indomethacin, theophylline and adenosine (Gawaz et al, 1995) or theophylline, adenosine and dipyridamole (Diatube-H) would also show different results with regard to activation status of the platelets (Kuhne et al, 1995).

Even the flow cytometer used may alter one's findings, depending on resolution at the lower end of the scale on forward scatter (Serebruany et al, 1999). Certainly the FacsCalibur used in the study presented here does appear to have excellent resolution of small events, and the problem encountered in our study in distinguishing microparticles from background electronic noise and from protein and antibody complexes may not even be noticed by others if the cytometer used has lower resolution capacity. This is to say that in this instance perhaps a cytometer with good

resolution is in fact required for more accurate estimation of microparticles, but one then needs to have a method to distinguish these small events from each other.

The method for estimating percentage of microparticles is extremely variable and will result in very different results being obtained. (See Figure 11.)
Other factors to consider when results differ, might be the clone of antibody and its' conjugation, as well as the choice of negative controls (discussed previously on page 23.) The results of the studies are also being expressed in different ways. Some groups use percentage of platelets positive for a marker, others use mean channel of fluorescence. Others still, recommend the use of formulae relating the positive population to the background resting platelets (Leytin et al, 2000). It would appear in many respects that we are indeed "comparing apples with oranges"!

For all of the above reasons mentioned, it is the opinion of the author that the test remains essentially still within the realms of research for many applications regarding platelet activation, and since there is no reference method for the detection of circulating activated platelets, a consensus on the optimal method to use would be one requirement necessary to take this test closer to routine use. Several attempts have been made to create guidelines and reach consensus (Schmitz et al, 1998), and it seems this is certainly necessary in order to be able to evaluate the literature more objectively.

Specific methodology incompatible/compatible with the antibodies used

Fixation

As has been discussed previously, the methods of fixation (involving washing off of the fixative) that have been used in this study have yielded results that make the validity of the quantitation of platelet activation, using this method, dubious. This is largely related to the fact that fixing the sample causes aggregate formation, which appears to be proportional to the activation status of the sample. However, our objections to this method have not been tested by evaluation of a population of normal individuals in comparison to thrombotic patients and no comment can be made about the ability of this test to separate normal from abnormal, with a standardized method.

Methods involving fixation with paraformaldehyde followed by sample dilution rather than washing off of the fixative have not been assessed, and could possibly yield better results.

CD62-P

For use of antibodies to CD62-P in particular, a method which does not involve fixation would be difficult, but not impossible, to implement in a routine haematology laboratory. This is because in vitro activation is detectable with this antibody within 15 minutes and continues with time. Fixation appears to be compatible with this antibody and antigen as far as preservation of the status of antigen expression is concerned, but results would be subject to the general objection to fixation. In addition the estimation of CD62-P expression would have to be done only in conjunction with other criteria for assessment of platelet activation which are compatible with fixation, e.g. microparticles

and aggregates could not be estimated on the same fixed sample.

CD63

CD63 expression could probably be done in the absence of fixation since expression would appear to be reasonably stable over a minimum of 30 minutes to an hour, but the antigen and antibody also appear to be relatively compatible with fixation (again, given the general objections to fixation.) The main problem with CD63 is rather the weakness of its expression, making evaluation of a population shift more viable than assessment of expression on each platelet individually.

PAC1 and Annexin V

PAC1 and annexin V expression are not assessed optimally after fixation. Fortunately they too are relatively stably expressed over 30 minutes to an hour. Processing of the sample immediately is therefore not critical.

Choice of platelet-specific identifying monoclonal antibody

With regard to the choice of "platelet identifying antibody", both CD61 and CD42a appear to be adequate. The GPIIb/IIIa complex (including CD61) is reported to be more strongly expressed on activated platelets than resting, and the GPIb/IX complex (including CD42a) is reported by some authors to show decreased expression after activation due to redistribution into the open cannalicular system, making platelet and especially microparticle identification more difficult when using this antigen as a platelet identifier (Scharf et al, 1993). This has not been confirmed by others (Ault et al, 1989). The results of the study presented did not reveal any obvious disadvantage to

using either antibody for identification. Even for the identification of microparticles, the markers were equally dimly expressed. It was also established that the weak expression of "platelet identifying antigens" on microparticles remained similarly problematic with any choice of fluorochrome, and was not peculiar to the PerCP fluorochrome. However it was noted that the problem of antibody and protein complex formation was not present with the use of the two colour programme (PE and FITC, without PerCP). This is discussed further under "Microparticles".

Aggregates

This study did not focus on estimation of platelet-leucocyte aggregates. However it was noted that fixation of a sample by the methods used here would result in falsely elevated levels of aggregates. It cannot therefore be recommended.

Microparticles

One of the major stumbling blocks encountered in this study was the estimation of microparticle percentages, and as such, this issue deserves further comment.
This test is being done by many groups as a marker of platelet activation, with some calculating absolute microparticle counts. The methods used vary significantly and most authors do not discuss the problems encountered in our study with the estimation of microparticles, namely the significant overlap between microparticles, antibody complexes and electronic noise with regard to size (forward scatter) and to a lesser extent complexity (side scatter). Other events that can be confused with microparticles include bacterial contamination of a buffer, cellular

fragments from the blood sample or debris from previous samples in the tubing of the cytometer (Matzdorff et al, 1998). A second problem which is not always addressed is the fact that manipulation of the sample such as centrifuging to separate out platelet rich plasma from whole blood could alter the activation status of the sample significantly. This point is illustrated in Figure 4.

Methods commonly used in the literature to quantitate microparticles range from quantitating events which are positive for the platelet identifying antibody but smaller than the distribution of normal platelets (Golanski et al, 1996; Nomura et al, 2000; Devine et al, 1999) to gating on a plot of fluorescence for the platelet identifying antibody versus side scatter (Tait et al, 1999), or both forward and side scatter (Sloand et al, 1995). One group used intensity of fluorescence for an antibody to GPIb in combination with size (forward scatter) (Lee et al, 1996). Figure 14 illustrates how different methods for quantitating microparticles could yield very different results on the same samples.

Some authors discuss the problems associated with the overlap using this type of method, and a few have gone on to address the matter further. Analysis of samples with the inclusion of fluorescent beads of the estimated size range of microparticles appears to yield more objective results (Combes et al, 1997; Tocchetti et al, 2001), since one then has a way of using a more specific characteristic for the exclusion of a greater proportion of "background and electronic noise" events. Other groups have encountered unexpected results and have difficulty explaining their findings. An example is a group attempting to count microparticles and aggregates by the addition

of a precise number of fluorospheres to a sample. On dilution of the blood sample prior to adding antibodies, they describe an increase in the percentage of microparticles and activated platelets. Surely this must be artefactual. The authors attempt to explain their findings as coincidence or binding of antibody complexes to debris because, relative to cell numbers, antibodies are in excess after dilution. They state that they do not expect an increase in the amount of "machine noise" (debris left in the tubing) after diluting a sample (Matzdorff et al, 1998). In fact, some of the unexpected events could be antibody and protein complexes, which would be easily recognised on a plot of forward versus side scatter. However, I believe the author may not have considered that electronic noise would, in fact, increase relative to the frequency of true cellular events and that the dim fluorescence of microparticles for a "platelet identifying antibody" makes distinction on the basis of size and fluorescence alone, very difficult. This problem illustrates a point in that most authors do not discuss any technical problems that they encounter in defining microparticles, and certainly I, like the group above, had significant difficulties optimising the quantitation of microparticles. One wonders how many other groups are experiencing difficulties with their technique, and whether this explains the conflicting results in the literature to some extent.

The method used here in the study presented, to more specifically quantitate microparticles, has not been previously described to my knowledge, although annexin V co-expression with CD41 on particles of low forward scatter (size) has been described previously. The method used in the study presented makes use of the

following criteria:

1) Exclusion of events not showing positive fluorescence for the platelet identifying antibody (CD42a or CD61).
2) Gating on a logarithmic plot of forward scatter versus side scatter to demarcate the areas in which "background noise" and antibody complexes fall,
   followed by exclusion gating of events with this light scatter AND (using Boolean logic) falling on a plot of fluorescence for platelet activation markers CD63 and annexin V in the negative area (where electronic noise would fall), as well as in a non-specific band of dual positivity noted to be associated with the formation of what is thought to represent antibody complexes.
3) Events remaining in the area on light scatter analysis in which microparticles would fall are then analysed for their expression of annexin V. Only those positive for annexin V are regarded as true microparticles.

In summary then, this method makes use of information regarding not only expression of a "platelet identifying antigen", forward and side scatter, but also the expression of activation markers, and finally more specifically, expression of annexin V.

One might question the necessity of going to these extremes to standardize the calculations on the basis that one could simply compare the percentage of microparticles estimated in a sample to that of normal controls. However, the platelet count in the patient samples might very well vary, and it is noted that in samples with a low count presumably (such as in an activated sample where platelets have been aggregated and therefore removed, or in samples where for example microparticles

have been concentrated, and platelets selectively removed), there is a relatively greater proportion of "background noise" as well as an increase in antibody complex formation. This is why it is desirable to more objectively quantitate the microparticles.

An interesting phenomenon encountered in this study was that when using only two colour analysis, with CD61-FITC and either CD62-P-PE or CD63-PE, the problem of presumed antibody complex formation did not seem to be a factor. Whether this was perhaps because of the PerCP labeled antibodies being instrumental in the formation of complexes of antibodies, or whether perhaps due to the actual setup of this analysis differing from the three colour analysis is not certain, but either way it was a useful observation, since the problem of distinguishing antibody complexes from microparticles is seldom mentioned in the literature, and it is possible that many people have simply not encountered these problems because of their choice of antibody combinations, or because of their method of setting up the parameters of the aquisition document.

To elaborate on the latter, the activated sample used to optimize the parameters on the three colour analysis for CD61-PerCP, CD63-PE and annexin V-FITC, was an activated sample of separated, washed platelets. This was done because for the maximal activation of the sample, and in particular for the production of microparticles, thrombin and collagen were added in high concentrations. Without either removing the fibrinogen by separating and washing the platelets, or bypassing the conversion of fibrinogen to fibrin with the use of a peptide such as TRAP, the

sample would have clotted. Most authors seem to use TRAP, but since this was not available, in this study the alternative was performed. The relevant difference to this discussion is that in this study, for the set-up of this particular acquisition document, the red cells were removed from the test sample. Therefore there was no absolute necessity to raise the discriminant function on fluorescence for the platelet identifying antibody (CD61) sufficiently so that the vast majority of red cells would not be recorded by virtue of their relatively high autofluorescence. As a result, the cutoff point for fluorescence could have been slightly lower, including events with low fluorescence such as antibody complexes. This explanation is probably likely, since the activated samples used to set-up the acquisition document for the CD42a-PerCP, CD62-P-PE and PAC1-FITC combination of antibodies were whole blood samples, since adrenaline and ADP were sufficient to induce expression of these antigens. There still appeared to be antibody complex formation in these samples, but to a lesser extent than in the CD61/CD63/annexin V sample. It still cannot be excluded that the phenomenon of antibody complex formation could be contributed to by the use of PerCP antibodies. In addition, the phenomenon may well be more obvious by the use of the B-D FacsCalibur since the resolution capability at the lower range of the forward scatter (size) is evidently very good.

CONCLUSION

The use of flow cytometry methodology for detection of platelet activation is an exciting development both for coagulationists and flow cytometrists. However, the pitfalls that many other methods used for assessing platelet activation are subject to, in view of the fragility of platelets and the ease with which activation may be triggered, remain significant issues here too. It is imperative that methods to work around this phenomenon be employed. Such methodology must be evaluated by each laboratory so that the individual is satisfied with the test's precision and accuracy prior to commencing research and certainly routine implementation of such testing.

With regard to the literature, it is evident that there is a wide range of methodology being used. For the reader without practical experience, this makes interpretation somewhat confusing, particularly since the results generated are consequently often conflicting. There is a move to come to a consensus regarding acceptable methodology and to recommend practical guidelines. Perhaps once this has been accomplished and accepted, the diversity in methodology may be reduced and consequently results of studies evaluating the effectiveness of this test in predicting thrombotic risk of various clinical conditions may be more uniform.

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Method for the isolation and washing of platelets from whole blood

Blood is taken with a 21 gauge butterfly into ACD (1:7) in a polypropylene tube using the double syringe technique with a discard of at least 2ml. After gentle mixing, the sample is centrifuged at 180g for 10 minutes, and the platelet rich plasma suctioned off using silicone coated glass pipettes.

The latter is then centrifuged again at 1800g for 15 minutes to form a platelet pellet. The supernatant is removed and the pellet resuspended in 10 ml Tyrode's buffer with 500µl apyrase and 500U heparin. This is incubated at 37°C for 15 minutes, then centrifuged again at 1800g for 15 minutes at 37°C in a pre-warmed centrifuge.
The supernatant is then discarded and the pellet resuspended in 10ml buffer again with apyrase but not heparin.

An alliquot is counted on the cell counter whilst the sample is being centrifuged for the last time so that the sample can be resuspended in a volume of buffer calculated to give a platelet count of ~250 x 10^9/l, i.e. a physiological platelet count. The last buffer contains apyrase at 2µl/ml, and no heparin.
The platelets are finally incubated for 30 minutes at 37°C.

(Method adapted from :

Mustard, JF, Perry, DW, Ardlie, NG, Packham, MA. Preparation of suspension of washed platelets from humans. British Journal of Haematology 1972;22:193-204)

Composition of HEPES buffer

137mmol/l	NaCl
2,8mmol/l	KCl
1mmol/l	MgCl2
12mmol/l	NaHCO3
0,4mmol/l	Na2HPO4
0,35%	Bovine serum albumen
10mmol/l	HEPES
5,5mmol/l	glucose
2mmol/l	CaCl2

The buffer was made up freshly in distilled water and adjusted to a physiological pH (7,36-7,44), then filtered to sterilize, and kept at 4°C.

Composition of Hank's Balanced Salt Solution (without calcium chloride, magnesium sulfate, phenol red and sodium bicarbonate)

Potassium Chloride	0,4g/l
Potassium Phosphate Monobasic (anhydrous)	0,06g/l
Sodium Chloride	8,0g/l
Sodium Phosphate Dibasic	0,04788g/l
D-Glucose	1,0g/l

The reagent was purchased from Sigma in dry form and made up into distilled water following instructions given. The pH was adjusted to a physiological range (7,36-7,44) and the buffer filtered for sterilization. The liquid was stored at 2-8°C in the dark.

Mepacrine stock solution

Mepacrine was purchased from Sigma in powder form, and made up in a solution of Hank's Balanced Salt solution without calcium or magnesium (described above) to a concentration of 5×10^{-4}M. This was stored in the dark at 2-8°C.

(Method adapted from:

Wall JE, Buijs-Wilts M, Arnold JT, Wang W, White MM, Jennings LK, Jackson CW. A flow cytometric assay using mepacrine for study of uptake and release of dense granule contents. British Journal of Haematology, 1995; 89:380-385)

Solution of fixative

Paraformaldehyde was dissolved in phospate buffered saline to comprise a 0,5% solution. This required continuous stirring and gentle heating in a fume cupboard to remove toxic fumes.