

**FLOW CYTOMETRIC OBSERVATIONS IN QUANTITATIVE
AND QUALITATIVE PLATELET DISORDERS AND IN
HUMANS RECEIVING INFUSIONS OF A CHIMAERIC
MONOCLONAL ANTIBODY TO GLYCOPROTEIN IIb-IIIa**

A thesis submitted to the University of London
for the Degree of M.D. in the Faculty of Medicine.

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ABSTRACT

A flow cytometric method was developed for quantitation of platelet surface-bound immunoglobulin G (IgG). A novel approach was used to calibrate the assay; this was based on the creation of reference curves by measuring PSiGg in platelets coated with known amounts of a chimaeric construct (supplied by Centocor Inc.) consisting of human IgG whose hypervariable region (HVR) had been replaced by the HVR of the murine monoclonal antibody (MoAb) 7E3, which binds with high affinity to human platelet glycoprotein (gp) IIb-IIIa. By using a fluorescein-conjugated polyclonal anti-IgG it was calculated that platelets of healthy subjects (n=71) bear 1463 (SD=927) IgG molecules, whereas PSiGg elevations of up to 39,000 molecules/platelet were often detected in a cohort (n=87) of thrombocytopenic individuals.

FC was also used to monitor platelet-bound 7E3, residual 7E3 binding sites and PSiGg in stable angina patients receiving infusions of Fab fragments of chimaeric MoAb 7E3 (c7E3-Fab), which is currently under evaluation as an anti-platelet agent. The distribution of c7E3-Fab in the platelet population was unimodal at all time points following the infusion, demonstrating *in vivo* transfer of antibody to newly released platelets; clearance of platelet-bound antibody followed an exponential model but all circulating platelets were bearing c7E3-Fab at time points beyond the lifespan of the platelets exposed to c7E3-Fab during the infusion. *Ex vivo* and *in vitro* mixing experiments suggested that c7E3-Fab transfer between platelets was possible.

Turbidometrically measured platelet aggregation response to ADP was shown to be a linear function of the logarithm of residual binding sites for 7E3.

Infusion of c7E3-Fab was frequently associated with recruitment of IgG onto the platelet surface; in vitro evidence suggested that this was caused by naturally occurring IgG bindable to proteolytically derived Fab fragments of chimaeric but not murine 7E3-IgG.

A new type of pseudothrombocytopenia, seen upon EDTA exposure of c7E3-Fab-bearing platelets was observed; this also appeared to be immune-mediated.

The physiological, pathophysiological and therapeutic implications of these findings are discussed.

To Berry and George

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LIST OF ABBREVIATIONS

ABP	actin-binding protein
ADP	adenosine di-phosphate
ATP	adenosine tri-phosphate
BSS	Bernard-Soulier syndrome
β -TG	beta-thromboglobulin
BT	bleeding time
cAMP	cyclic adenosine monophosphate
c7E3	chimaeric monoclonal antibody 7E3
c7E3-Fab	Fab fragment of chimaeric monoclonal antibody 7E3
c7E3-IgG	whole IgG molecule of chimaeric monoclonal antibody 7E3
CD	cluster of differentiation
CHO	chinese hamster ovary
CNS	central neural system
CPD-A	citrate-phosphate-dextrose with adenine
CV	coefficient of variation
DTS	dense tubular system
DG	diacyl-glycerol
EDTA	ethylene-diamino-tetracetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FAB	proteolytically derived Fab or F(ab') ₂ fragments of IgG
Fc	the constant region of the IgG molecule
FC	flow cytometry
Fgn	fibrinogen
FITC	fluorescein isothiocyanate
FM	fluorescence microscopy
Fn	fibronectin
FS	forward light scatter
GMP-140	granule membrane protein 140
Gp	blood group
gp	glycoprotein
GT	Glanzmann's thrombasthenia
GTP	guanine tri-phosphate
HACA	human anti-chimaeric antibodies
HAMA	human anti-murine antibodies
HEL	human erythroleukemia
IP3	inositol tri-phosphate
ITP	idiopathic thrombocytopenic purpura
IV	intravenous
KD	affinity constant
LEU-CAM	leucocyte cell adhesion molecules
LRG	leucine-rich glycoprotein
LFS	logarithm of forward light scatter

LSS	logarithm of side light scatter
m7E3	murine monoclonal antibody 7E3
m7E3-Fab	Fab fragment of murine monoclonal antibody 7E3
MFC	mean fluorescence channel
MoAb	monoclonal antibody
MW	molecular weight
NSF	non-specific fluorescence
n	number of observations or patients
PADGEM	platelet activation-dependent granule externalized membrane protein
PAGE	polyacrylamide gel electrophoresis
PAF	platelet activating factor
PAIgG	platelet-associated IgG
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PG	prostaglandin
PF4	platelet factor 4
PMT	photomultiplier tube
PRP	platelet-rich plasma
PPP	platelet-poor plasma
PSC3	platelet surface complement (3rd component)
PSIg	platelet surface immunoglobulin
PSIgG	platelet surface immunoglobulin G
r	Pearson's coefficient of correlation
RAF	Royal Air Force
RBC	red blood cells
RGD	the tripeptide Arg-Gly-Asp
R-TPA	recombinant tissue-type plasminogen activator
RES	reticulo-endothelial system
RFI	relative fluorescence intensity
RIA	radioimmunoassay
RT	room temperature
SDS	sodium dodecyl-sulphate
SCCS	surface-connected cannalicular system
SD	standard deviation
SE	standard error
SF	specific fluorescence
SLE	systemic lupus erythematosus
SS	side light scatter
TSP	thrombospondin
TxA ₂	thromboxane-A ₂
TX-100	triton X-100
v	versus
VLA	Very Late Antigen
VnR	vitronectin receptor
vWF	von Willebrand factor

PREFACE - AIMS AND STRUCTURE OF THESIS

*CHORUS: ...We know about the sudden thrombosis
And the slowly hardening artery...*

T.S.ELIOT, The Family Reunion

These lines, written more than half a century ago, were placed by the poet in the mouth of a chorus enumerating some of the great anxieties of Western Man. They highlight the public appreciation of occlusive arterial disease as a major cause of death in the developed world.

Recognition of the prominent role played by the platelet in the pathogenesis of complications and, probably, also in the initiation of the atherosclerotic process, is the main reason for the present rapid expansion in platelet-related research. Encouraging results obtained with aspirin in the setting of coronary artery disease signalled the start of a race for development of new, more effective antiplatelet agents. At the same time, the increasing dependence on platelet transfusions to support thrombocytopenia resulting from widely used myelotoxic chemotherapy, has added another source of interest in understanding platelets and particularly their immunology.

The general aim of the work presented in this thesis was to develop new investigational approaches, based mainly on the use of fluorescence flow cytometry, and explore their application in the diagnosis of platelet disorders and the monitoring of the effects of novel antiplatelet therapies. The thesis has been organized in seven chapters:

Chapter 1 presents relevant background information. Chapter 2 contains a description of general laboratory methods employed in the present studies, with the emphasis on platelet flow cytometry. Chapter 3 presents results of immunoenzymatic staining of platelets, as a contribution to understanding the distribution of some platelet glycoproteins; one of them (glycoprotein IIb-IIIa) is the target of monoclonal antibody 7E3, which has been used extensively throughout the studies included in this thesis. Chapter 4 describes the development and assessment of a flow cytometric method for quantitative detection of platelet-bound IgG. Chapter 5 examines the application of flow cytometry to monitor platelet-bound antibody in patients receiving trial infusions of Fab fragments of chimaeric anti-glycoprotein IIb-IIIa monoclonal antibody 7E3. Chapter 6 presents observations on the recruitment of IgG onto platelets treated *in vivo* or *in vitro* with Fab fragments of 7E3. Finally, Chapter 7 is a general discussion of the main findings.

Apart from some preliminary results from earlier stages of these studies (Christopoulos *et al* 1990 & 1991, Haga *et al* 1990, Christopoulos and Mattock 1991), the material presented in this thesis is previously unpublished.

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Chapter 1

INTRODUCTION

1.1 PLATELETS IN HAEMOSTASIS AND THROMBOSIS AND AS MEMBERS OF A PRIMITIVE DEFENCE SYSTEM

Platelets were first identified as a distinct class of blood cells more than a hundred years ago and at the same time their important role in haemostasis and thrombosis was recognized (Bizzozero 1882). As occlusive arterial disease constitutes the major cause of death in the western world, it is not surprising that the protagonistic role of platelets in the pathogenesis of arterial thrombosis has made these cells an object of intensive investigation. The study of platelet structure and function has revealed a complicated network of activating mechanisms enabling the explosive transformation of the circulating inert "smooth discs" into adhesive "spiny spheres" forming plugs in areas of disrupted vascular integrity. These activating mechanisms are finely balanced by equally complicated inhibitory ones, so that platelet activation and thrombus formation are limited to the area of damaged vascular endothelium.

Beside the specialised functions of haemostasis, platelets are also involved in more "primitive" activities as part of an unspecific defence system (Schmid-Schönbein 1979) operating in parallel with the sophisticated specific humoral (antibodies) and cellular (immunocompetent cells) defence system. As members of this system, platelets can react indiscriminately to various noxious agents as exemplified by their ability to phagocytose foreign material (Lewis *et al* 1976) or become activated by immune complexes binding to the Fc receptors present on their surface (Rosenfeld *et al* 1985).

This introduction includes a brief description of platelet structure and a discussion of platelet function with the emphasis on the aspects most relevant to the studies presented in the chapters that follow.

1.2 PLATELET STRUCTURE

Platelets are anuclear cells originating from fragmentation of the cytoplasm of megakaryocytes. Both the mechanism of cytoplasmic fragmentation and the site of platelet release into the circulation are debated issues. Although the latter event has traditionally been considered to take place in the bone marrow sinusoids, a number of authors have produced evidence suggesting the pulmonary capillaries as a main site of platelet release (for review see Eldor *et al* 1989). Resting platelets circulate in the blood as smooth biconvex discs $1\mu\text{m}$ thick with a diameter of $2\text{-}4\mu\text{m}$ and a volume of $7\text{-}8\text{fl}$. It should be noted that exposure of platelets to various anticoagulants can result in structural alterations with the best known example being the variable swelling caused by EDTA (Zucker and Borelli 1954).

The main structural elements of a platelet are: (a) plasma membrane, (b) cytoskeleton, (c) internal membrane systems and (d) organelles. Fig 1.1 is a diagrammatic depiction of platelet ultrastructure.

1.2.1 The plasma membrane

The platelet plasma membrane is similar in its basic structure to that of other cells. A phospholipid bilayer with integrated glycoproteins constitutes the *unit membrane*. The long glycoprotein (gp) tails are rich in carbohydrates and are the main component of the *exterior coat* or *glycocalyx* which is thicker (50nm)

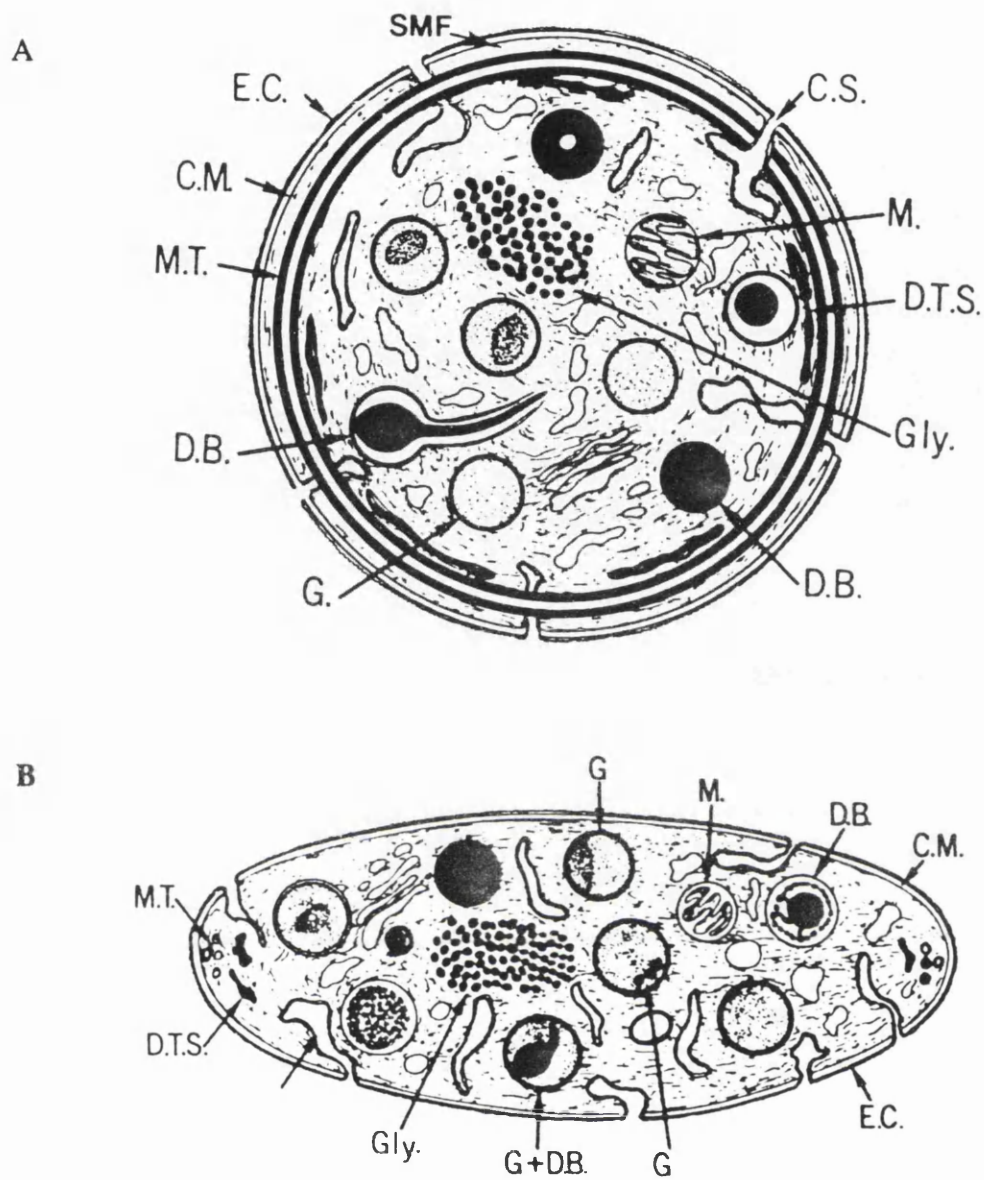


FIGURE 1.1 Diagrams of main ultrastructural features as seen in equatorial plane section (A) and cross-section (B) of a resting discoid platelet (reproduced from White *et al* 1981): **EC** exterior coat (glycocalyx), **CM** unit membrane, **MT** circumferential bundle of microtubules, **DB** dense bodies, **G** granules (α -granules and lysosomes), **DB** dense bodies, **Gly** glycogen, **DTS** dense tubular system, **M** mitochondria, **CS** surface-connected (open) canalicular system, **SMF** submembrane filaments (membrane skeleton).

and denser in platelets compared to other blood cells. In contrast to the multiple villous projections present on the surface of leucocytes, the surface of the resting platelet is smooth. A unique feature of the platelet plasma membrane is its multiple invaginations which form an extensive intracellular canalicular system communicating with the extracellular milieu through small openings of the surface. The main functions of the plasma membrane, apart, of course, from maintaining platelet integrity are:

(a) It houses a variety of proteins, some of them acting as transmitters of molecular signals to the cell interior, some functioning as ion pumps and others acting as physical links for cell-cell and cell-matrix interactions which, beside their known importance in platelet adhesion and aggregation, could be playing a major role in platelet activities as diverse as the IgE-mediated killing of parasites (Ameisen *et al* 1986) or the propagation of tumour metastasis (Karpatkin *et al* 1986, Nierodzik *et al* 1991). A list of platelet surface receptors and their ligands is given in Table 1.1.

(b) It serves as a source of fatty acids which are essential for the metabolic processes of the cyclo-oxygenase pathway.

(c) It offers, through its phospholipid bilayer, a catalytic surface essential for the assembly of enzyme-cofactor-substrate complexes, which is the central event in the plasmatic coagulation cascade (Walsh 1985).

Constituents of the plasma membrane, especially glycoproteins, are responsible for platelet immunological specificity. A selection of surface antigens specific for platelets is presented in Table 1.2.

1.2.2 Cytoskeleton

Actin represents the major structural protein accounting for up to 20% of total platelet protein. Actin filaments existing in equilibrium with free cytoplasmic

actin are arranged in a three-dimensional fashion to form a cytoskeleton traversing the cytoplasm (Fox 1987). Their organization is regulated by their interaction with proteins such as *actin-binding protein (ABP)*, α -*actinin* and *tropomyosin*. Shorter actin filaments form a distinct two-dimensional structure, the *membrane skeleton*, lining the inner aspect of the plasma membrane. The membrane skeleton is linked to surface glycoproteins, mainly gpIb-IX (Fox 1985) via proteins such as ABP and appears to be the main structure responsible for maintaining the discoid shape of resting platelets (Fox *et al* 1988). Another structure contributing to shape maintenance is the circumferential bundle of *microtubules* which is linked to both actin networks (White *et al* 1986, Fox *et al* 1988). During platelet activation, changes in the membrane skeleton and polymerization of cytoplasmic actin with association of the resulting filaments with contractile proteins and microtubules are involved in pseudopod extension, secretion and clot retraction.

1.2.3 Internal membrane systems

1.2.3.1 Dense tubular system (DTS)

This is a peripherally located system of branched membranes forming 30-40nm wide channels which enclose an area of electron density similar to that of the cytoplasm. Its two main functions appear to be: (a) regulation of cytoplasmic levels of ionized calcium through sequestration of calcium ions using a Ca^{2+} -activated ATPase (Cutler *et al* 1978, Kaeser-Glanzmann *et al* 1978) and (b) supply of enzymes involved in arachidonate metabolism (Gerrard *et al* 1976).

1.2.3.2 *Surface-connected canalicular system (SCCS)*

This system appears as a peripheral network of ducts of varying width, communicating with each other and with the platelet surface. It is generally thought to represent multiple invaginations of the plasma membrane as evidenced by the fact that its walls stain with the same cationic dyes that bind to the glycocalyx and it is estimated that the walls of the SCCS account for 50-60% of total platelet plasma membrane (Morgenstern and Kho 1977, Wurzinger *et al* 1987). It has been suggested that the SCCS serves as a route for secretion (Ginsberg *et al* 1980, White and Clawson 1981) and endocytosis of particulate foreign material (White and Clawson 1981, Zucker-Franklin 1981). Two fundamental structural issues are still disputed:

(a) Is the SCCS communicating freely with the extracellular milieu in resting platelets? This would result in a dramatic increase in surface available for molecular interactions and would reduce the distance of any intracellular point from the environment. Freeze-fracture studies by Sixma (1986) have indicated that the SCCS orifices are closed by a non-bilayer lipid structure. Breton-Gorius *et al* (1983) found that anti-gp antibodies stained the SCCS only during activation when the system was dilated although others (Woods *et al* 1986) noticed that Fab fragments but not whole molecules of certain antibodies could enter the system, suggesting that access to the SCCS depends on molecular size.

(b) Can the SCCS be evaginated - resulting in a dramatic increase in platelet surface/volume ratio - without structural damage? It has been suggested (Milton and Frojmovic, 1979) that the system can be evaginated following activation and shape change. On the other hand, White and Clawson (1980) have suggested that the SCCS would have to suffer extensive structural damage before it could be evaginated, although in more recent work the same group

(Escobar *et al* 1989) have shown that the SCCS can be evaginated in response to surface activation.

1.2.4 Organelles

Apart from the storage granules discussed below, platelets also contain *mitochondria*, *glycogen particles*, *microperoxisomes* containing catalase and other oxidases and occasional *lipid droplets*.

1.2.4.1 α -granules

These are spheroid, oval or elongated membrane-coated bodies numbering an average of 36 per platelet and occupying up to 15% of total platelet volume. Their contents can generally be divided into three groups:

- (a) Platelet-specific proteins like β -thromboglobulin (β -TG), platelet factor 4 (PF4), platelet-derived growth factor (PDGF).
- (b) Coagulation proteins like von-Willebrand factor (vWF), fibrinogen, factor V, high molecular weight kininogen.
- (c) Other proteins: Thrombospondin (the endogenous platelet lectin - a multi-functional trimeric molecule secreted upon activation and mediating cell-cell and cell-matrix interactions [Lawler 1986]), fibronectin, histidine-rich glycoprotein, α_1 -antitrypsin, chemotactic factor, vascular permeability factor, albumin, immunoglobulins (George 1990).

Some of these proteins are synthesized wholly or partly by the megakaryocyte, while others are taken up from the plasma. α -granules show significant structural variability and the observation that all of them contain lysosomal enzymes (Morgenstern and Janzarik 1985) suggests that the previously separately classified platelet lysosomes belong to the same organelle type.

The α -granule membrane attracts increasing attention because it has been identified as a possible intraplatelet pool of gpIIb-IIIa (Wenzel-Drake *et al* 1986, Cramer *et al* 1990). It has also been shown to house the Platelet Activation-dependent Granule Externalised Membrane (PADGEM) protein (Hsu-Lin *et al* 1984), which is identical to the Granule Membrane Protein (GMP)-140 (McEver and Martin 1984), the most extensively studied platelet membrane activation marker.

Grey platelet syndrome (Raccuglia 1971) is a rare familial bleeding disorder characterized by large, hypogranular platelets and variable - usually moderate - thrombocytopenia. The abnormal appearances are due to a specific deficiency of α -granules and the disorder has become a popular model for investigation of the physiological role of these organelles. Diagnosis is confirmed by electron microscopy and measurement of α -granule proteins (e.g. β -TG, PF4, TSP); impaired aggregation and secretion responses to thrombin and collagen also occur (for review see Hardisty 1989)

1.2.4.2 Dense bodies

These electron dense structures, formerly called δ -granules, number an average of 5 per platelet. They contain the secretable "storage pool" of densely packed adenine nucleotides with an ATP:ADP ratio of 2:3, while the ratio in the "metabolic pool" in the cytoplasm is 8-10:1. Dense bodies also contain high concentrations of calcium (ca. 50% of total platelet calcium) and phosphorus and substantial concentrations of serotonin (5-HT). Other biogenic amines are also present in lower concentrations.

1.3 PLATELET SURFACE RECEPTORS

The major platelet surface glycoprotein complexes, gpIb-IX and gp IIb-IIIa, are discussed separately in sections 1.4 and 1.5. Many of the other platelet membrane glycoproteins are confirmed or putative receptors for a variety of ligands and those that have been characterised so far are shown in Table 1.1.

1.3.1 The VLA family

VLA-2, VLA-5 and VLA-6, corresponding to the gpIa-IIa, gpIc-IIa and gp Ic'-IIa complexes (Table 1.1) are members of the family of VLA (very late antigen) adhesion molecules present on many different cell types. They are calcium dependent heterodimers (Parmentier *et al* 1991) consisting of a common β_1 chain (gpIIa) and variable α chain (α_1 to α_6), a structure characteristic of the members of the broader *integrin supergene family* of adhesion molecules (Hynes 1987). These proteins are receiving increasing attention because of accumulating evidence for their involvement in a multitude of interactions as diverse as adherence of cells to each other and to subendothelium, lymphocyte function, embryogenesis and tumour metastasis.

1.3.2 The Fc receptor

Another recently characterized platelet surface protein is the Fc γ RII receptor for the Fc region of the IgG molecule (Rosenfeld *et al* 1985, King *et al* 1990). It has been known for a long time (Humphrey and Jaques 1955) that washed platelets can be activated in vitro by immune complexes and the presence of a platelet Fc receptor analogous to that of white cells was postulated (Israels *et*

TABLE 1.1

PLATELET MEMBRANE RECEPTORS

Name	CD No	MW(kD)	Heterodimer with	Receptor for	Reference
gpIa	CDw49b	166	gpII	collagen	1,2
gpIb	CD42b	170	gpIX	vWF, thrombin	3,4
gpIc	CDw49e	160	gpIIa	fibronectin	1,5
gpIc'	CDw49f	160	gpIIa	laminin	1,5
gpIIa	CD29	130	gpIa,Ic,Ic'*	collagen, fibronectin, laminin	1,2,5
gpIIb	CD41	140	gpIIIa	fibrinogen, vWF, fibronectin, vitronectin	6,7
gpIIIa	CD61	110	gpIIb	as for gpIIIa	
gpIV	CD36	90		thrombospondin, collagen	8,9
gpV		82		thrombin **	10,3
gpVI		62		? collagen	11
gpIX	CD42a	22	gpIb	as for gpIb	
FcγRII	CDw32	40		complexed IgG	12,13,14

* gpIa-IIa, Ic-IIa and Ic'-IIa complexes correspond to the VLA-2, VLA-5 and VLA-6 integrins respectively.

** gpV acts as a substrate rather than a receptor for thrombin.

gp: glycoprotein, MW: approximate molecular weight (reduced) CD: cluster of differentiation as defined at the four International Workshops on Human Leucocyte Differentiation Antigens (Knapp *et al* 1989). The "w" means that the cluster needs re-evaluation in future workshops.

Key to references: 1.Hemler *et al* 1988, 2.Coller *et al* 1989a, 3.Berndt *et al*,1985, 4.Berndt *et al* 1989, 5.Kawai *et al* 1989, 6.Phillips *et al* 1988, 7.Newman 1991, 8.Tandon *et al* 1989, 9.Silverstein *et al* 1989, 10.Berndt & Phillips 1981, 11.Moroi *et al* 1989, 12.Karas *et al* 1982, 13.Rosenfeld *et al* 1985, 14.King *et al* 1990

al 1973) long before it was characterized. Platelet activation by aggregated IgG was shown to be inhibited by monomeric IgG, normal plasma and even by IgG deficient plasma, although binding of IgG aggregates to platelets continued to occur at physiological platelet concentrations (Pfueller *et al* 1977). This raised the possibility that immune complex binding to platelets might not directly activate/damage them *in vivo* but could, of course, result in their removal by the reticuloendothelial system. The demonstration (Spycher and Nydegger 1986) of internalization by human platelets of complexed IgG bound to their surface suggested that platelet Fc receptors might be contributing to the clearance from the circulation of immune complexes and opsonized microorganisms. Karas *et al* (1982), using radiolabelled IgG dimers, estimated the average number of Fc receptors per platelet at 400-2,000. King *et al* (1990), using trimeric IgG estimated *ca.* 8500 sites per platelet, while McCrae *et al* (1990), using radiolabelled IV3 (a MoAb to the Fc γ RII) estimated 891 receptors per platelet increasing by 55% upon thrombin activation. The platelet Fc γ RII appears to be identical to the 40kD low affinity Fc receptor of monocytes, whereas the high affinity (72kD) monocyte Fc receptor could not be identified on platelets (Rosenfeld *et al* 1985). A monoclonal antibody (IV3) to the Fc γ RII abolished platelet aggregation by heat-aggregated IgG, while the responses to thrombin, collagen and ADP were not affected (Rosenfeld *et al* 1985). It has been shown that the Fc γ RII is involved in the pathogenesis of heparin induced thrombocytopenia through binding of heparin-IgG complexes (Kelton *et al* 1988) and in platelet activation induced by certain MoAbs to platelet membrane glycoproteins (Horsewood *et al* 1991, Anderson *et al* 1991).

There is evidence that platelets also possess a receptor for IgE and participate in IgE-dependent cytotoxic functions (Ameisen *et al* 1986) and the presence of binding sites for the third component of complement has also been proposed (Yu *et al* 1986).

1.4 GLYCOPROTEIN Ib

1.4.1 Historical background - Bernard-Soulier Syndrome (BSS)

This rare autosomal recessive platelet disorder was first described in 1948 by two French physicians, Drs Bernard and Soulier, who reported the case of a young patient with an inherited haemorrhagic diathesis characterised by thrombocytopenia and large platelets. It was subsequently recognized that these platelets failed to adhere to subendothelium (Weiss *et al* 1974) and, while maintaining normal aggregation responses to ADP, collagen and adrenaline, they did not respond to ristocetin - an antibiotic that agglutinates platelets by inducing binding of vWF (Howard and Firkin 1971, Caen and Levy-Toledano 1972). This defect was not correctable by addition of normal plasma as a source of vWF. A platelet membrane abnormality was suspected when it was shown that BSS platelets lacked surface sialic acid (Grottum and Solum 1969). In 1975, Nurden and Caen, by means of electrophoretic studies, demonstrated specific deficiency of a glycoprotein (gpIb) and subsequently other workers (Clemetson *et al* 1982, Berndt *et al* 1983) showed that two other gps (gpIX and gpV) are also deficient in BSS. There is evidence (Finch *et al* 1990,) that classical BSS represents a genetically heterogenous disorder.

The clinical characteristics of BSS have been reviewed by George and Reimann (1982), Bernard (1983) and Coller (1984). The spectrum of clinical severity is very wide. Serious spontaneous bleeding is unusual in adult BSS patients, the commonest manifestations being skin bruising and mucosal haemorrhages but bleeding secondary to trauma can be life-threatening. Most haemorrhagic deaths have occurred during early childhood (Bernard 1983). The diagnosis is suspected in the presence of prolonged bleeding time, thrombocytopenia with very large platelets and absent agglutination response to ristocetin, not correctable by addition of normal plasma. Confirmation of the diagnosis

requires demonstration of gpIb deficiency, usually by polyacrylamide gel electrophoresis of platelet lysates or, more conveniently, by labelling of intact platelets with radioactive or fluorescent anti-Ib probes. In diagnosing BSS one should be aware of the existence of "BSS-like" or "pseudo-BSS" abnormalities (Berndt *et al* 1988, Stricker *et al* 1985, Devine *et al* 1987). Management of serious bleeding is with blood and platelet (preferably HLA matched) transfusions, always keeping in mind the possibility of developing anti-gpIb antibodies, which can inhibit the adhesive function of normal platelets (Degos *et al* 1977).

1.4.2. Structure and function of gpIb.

gpIb has an apparent molecular weight (MW) of *ca.* 170,000 on SDS-polyacrylamide gels and exists as a heterodimeric complex with gpIX, MW 22,000 (Du *et al* 1987). Most workers estimate *ca.* 25,000 copies of gpIb-IX complex per platelet (Berndt *et al* 1985). The gpIb molecule consists of a large (MW=135,000) α chain and a smaller (MW \approx 25,000) disulphide-linked β chain. There is significant polymorphism of the Ib_{α} chain (Moroi *et al* 1984). This subunit alone appears to be sufficient for expression of the adhesive function of gpIb (Wicki & Clemetson 1985). gpIb is a transmembrane protein; its intracellular portion is thought to be linked to the platelet cytoskeleton through its association with actin binding protein (ABP, Fox 1985). The extracellular part of the Ib_{α} subunit (MW \approx 120,000), called glyocalycin for its presence in the glycocalyx (see 1.2.1) has two distinct structural domains: a proximal (COOH-terminal) macroglycopeptide chain containing most of the carbohydrate of gpIb and a distal (NH₂-terminal, MW \approx 45,000) domain that carries the binding activity for vWF and one of the two distinct thrombin receptors present on platelets (Wicki & Clemetson 1985, McGowan & Detwiler 1986, Titani 1987).

The primary structure of gpIb_α, gpIb_β and gpIX, which has become known following cloning and sequencing of the respective cDNAs, has revealed that all three of them contain the unique 24-amino acid sequence that distinguishes the LRGs (leucine-rich glycoproteins), a family of widely distributed proteins with no clear functional relationship (Roth 1991).

The main function of gpIb is as a major mediator of platelet adhesion to exposed subendothelium in the presence of vWf and blood flow conditions characterized by high shear rate, like those encountered in arteries and arterioles. The exact molecular mechanisms leading to adhesion are not known; the available evidence suggests that, under appropriate flow conditions, conformational changes occur in the vWF and/or gpIb molecules resulting in their interaction. The vWF side of the complex binds to subendothelium exposed following endothelial injury/disease. The sequence of events, i.e. whether vWF binding to subendothelium precedes or follows its interaction with gpIb, is not known (for review of possible mechanisms see Roth 1991). Although the initial (contact) step of adhesion is a passive process not requiring platelet energy expenditure, once it has occurred it initiates the active processes of spreading and aggregation which are mediated by the other major platelet surface gp, the IIb-IIIa complex (Sakariassen *et al* 1986). It should, of course, be noted that the described adhesion mechanisms have been based on *in vitro* perfusion experiments and might not accurately reflect the situation in various clinical settings, most importantly the setting of platelet adhesion to damaged endothelium during atherogenesis. In surfaces that differ fundamentally from the vascular endothelium (e.g. synthetic grafts), there is evidence that the initial platelet deposition does not involve gpIb (Sheppeck *et al* 1991).

1.5 GLYCOPROTEIN IIB-IIIa

1.5.1 Historical background - Glanzmann's Thrombasthenia (GT)

Like BSS, GT is another typical example of a rare hereditary (autosomal recessive) bleeding disorder, the study of which directly led to the discovery and understanding of function of a major platelet membrane gp. It was first described by Glanzmann in 1918 as "hereditary haemorrhagic *thrombasthenia*" (Gk. for "weakness of clot") characterized by absent or subnormal clot retraction. It was further defined by Caen *et al* (1966) as a disorder presenting with prolonged bleeding time and absent aggregation responses to all physiological agonists combined with normal platelet count and morphology. In 1974, Nurden and Caen performed SDS-PAGE of platelet membrane gps and demonstrated that one of the three then known gps (later named IIB-IIIa) was missing from the platelets of patients with GT. More detailed electrophoretic studies that followed confirmed that a severe gpIIB-IIIa deficiency was the underlying molecular abnormality in GT (Phillips & Agin 1977). Later studies showed that qualitative defects of quantitatively normal gpIIB-IIIa could also present with the clinical and aggregometric picture of GT (Ginsberg *et al* 1986, Nurden *et al* 1987). GT heterozygotes are asymptomatic and their platelets show normal responses in standard aggregometry despite bearing only *ca.* 50% of the normal level of gpIIB-IIIa (Coller *et al* 1986c). Recent molecular studies suggest that classical GT represents a genetically heterogenous entity (Bray & Shuman 1990, Newman *et al* 1991). As with BSS, acquired forms of GT, usually caused by autoantibodies against gpIIB-IIIa have been described (DiMinno *et al* 1986, Niessner *et al* 1986, Kubota *et al* 1989).

The clinical presentation of GT appears to be almost identical with that of BSS and has been extensively reviewed by George *et al* (1990). As in BSS,

spontaneous bleeding is uncommon and most haemorrhagic episodes are due to trauma or exaggeration of physiological bleeding. Petechiae, ecchymoses, epistaxes, gingival bleeding and menorrhagia are the commonest manifestations. Gastrointestinal blood loss is not uncommon and can lead to iron deficiency anaemia. The clinical severity seems to decrease with age and most of the few reported haemorrhagic deaths have occurred during childhood. As with BSS, management is supportive, based on local measures (e.g. pressure, topical thrombin) to control minor superficial bleeding in accessible sites, and administration of blood and platelet transfusions for more serious haemorrhages. Drugs and foodstuffs interfering with haemostasis and especially with platelet function (George & Shattil 1989) should be avoided. It should be noted that the severity of bleeding is unpredictable and does not correlate with the degree of gpIIb-IIIa abnormality; this necessitates prophylactic administration of (ideally HLA-matched) platelet concentrates prior to any invasive procedure. The probability of clinically significant isoimmunization to IIb-IIIa is reported to be low (George *et al* 1990) but should be kept in mind (Levy-Toledano *et al* 1978). In patients refractory to platelet transfusions bone marrow transplantation could be considered; it has been performed successfully in a case of GT (Bellucci *et al* 1985).

1.5.2 Structure of the gpIIb-IIIa complex.

The gpIIb-IIIa complex represents the major platelet membrane glycoprotein, numbering, according to most estimates, 40-50,000 copies on the surface of normal circulating platelets although recent work (Jordan *et al* 1991) suggests that the number might be significantly higher (*ca.* 80,000). The complex exists as a Ca^{2+} dependent heterodimer (Fig 1.2) which can be split into its two components (gpIIb and gpIIIa) by treatment with calcium chelators, e.g. EDTA

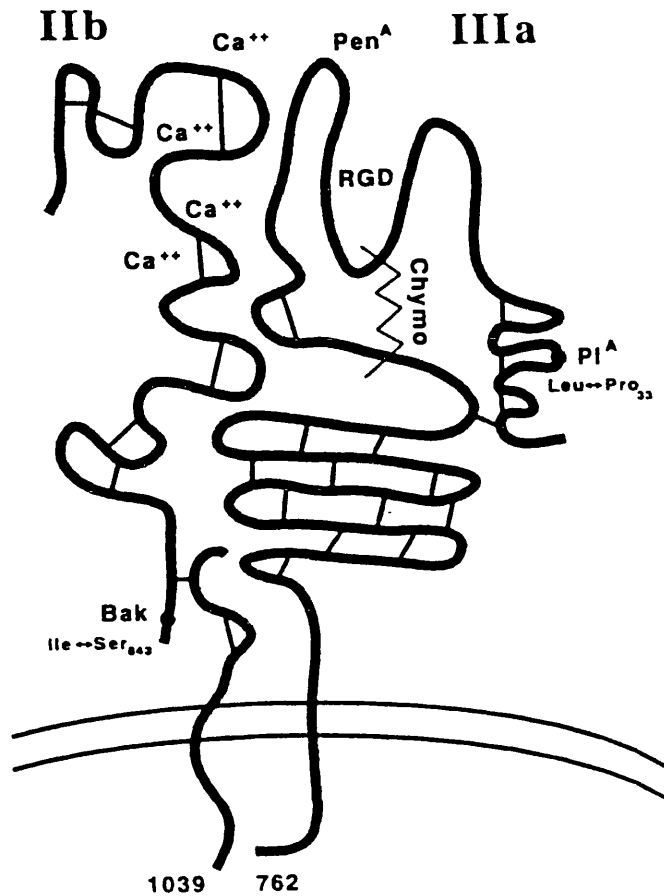


FIGURE 1.2 Schematic representation of the gpIIb-IIIa complex (Newman 1991). Disulphide bonds are shown as thin intra-chain and inter-chain lines.

Jagged line: Chymotrypsin cleavage sites; treatment of platelets with this enzyme results in loss of ligand-binding capacity.

RGD: The presumed site of binding of adhesive ligands and RGD-containing peptides (see section 1.5.3).

The sites of polymorphism responsible for the PL^A and Bak epitopes do not necessarily contain the respective epitopes (see section 1.6.1).

(Pidard *et al* 1986). Beside gpIIb-IIIa that is evenly distributed on the surface of resting platelets (Wencel-Drake 1986) and appears to constitute *ca.* 70% of total platelet IIB-IIIa (Isenberg 1987), intraplatelet pools of the complex exist, mainly in the membranes of the SCCS (Woods *et al* 1986, Wencel-Drake 1986) but also in the α -granules (Wencel-Drake 1986, Cramer *et al* 1990). The complete primary structure of both gpIIb and gpIIIa has been established by sequencing cDNAs cloned from HEL (human erythroleukaemia) cells which are known to carry IIB-IIIa (Bray *et al* 1987, Rosa *et al* 1988, Sosnoski *et al* 1988, Zimrin *et al* 1988). The two separate genes encoding gpIIb and gpIIIa reside in the proximal long arm of chromosome 17. It is noteworthy that preliminary studies of gpIIb-IIIa biosynthesis have indicated that the assembly of the complex precedes its insertion into the plasma membrane (Rosa *et al* 1986) and therefore, theoretically, a defect in any of the two gp components could prevent the formation of the complex and its insertion into the membrane.

gpIIb (MW=145,000) consists of a large α (MW=125,000) and a small β (MW=25,000) subunit linked by a disulphide bridge. gpIIb is the subunit of the complex that binds calcium (Charo *et al* 1986) and the dissociation of the complex following Ca^{2+} chelation is probably a result of conformational changes in the IIB molecule.

gpIIIa (MW=105,000) is a single polypeptide chain crosslinked with multiple intrachain disulphide bonds. gpIIIa represents the common β_3 chain shared with the other member of the *cytoadhesin* family of adhesion molecules, the *vitronectin receptor* (VnR) which is present on endothelial cells, fibroblasts, smooth muscle and osteoclasts and shows crossreactivity with complex-specific anti-IIB-IIIa MoAbs (Charo *et al* 1987). The α chain of the VnR is structurally similar although not identical with gpIIb and the receptor has functional similarities with the platelet IIB-IIIa complex, as evidenced by fibrinogen and vWF binding (Cheresh 1987).

The cytoadhesins are members of the broader *integrin supergene family* (Hynes 1987) which also includes the VLA molecules ($\alpha\beta_1$, see 1.3.1) and the LEU-CAM ("leucocyte cell adhesion molecules") sharing the structure $\alpha\beta_2$ (Anderson & Springer 1987). All integrins consist of non-covalently associated α and β subunits existing as Ca^{2+} dependent heterodimers. Each subfamily contains the same β subunit complexed, in individual members, with different α subunits.

1.5.3 The role of gpIIb-IIIa in the haemostatic function of platelets.

Evidence of the central role of gpIIb-IIIa in platelet aggregation has been accumulating since the demonstration (Nurden & Caen 1974) that this gp is missing from GT platelets, which fail to aggregate in response to agonist stimulation. In a recent report, Frojmovic and co-workers (1991) provide evidence that this gp is the only unique platelet surface component required for aggregation, as shown by its ability to confer aggregability on heterologous cells: In a series of elegant transfection experiments they expressed recombinant normal human gpIIb-IIIa onto the surface of CHO (chinese hamster ovary) cells and demonstrated that these cells acquired the ability to aggregate. This was not the case when the same CHO cells were transfected with gpIIb-IIIa containing a point mutation abrogating fibrinogen binding to the complex. Aggregation was dependent of the presence of extracellular fibrinogen and Ca^{2+} and required prior activation of gpIIb-IIIa (which they achieved by using an activating anti-IIIa MoAb). These results also highlight the fundamental role of two events necessary for aggregation: "activation" of the gpIIb-IIIa complex and binding of fibrinogen to the complex.

gpIIb-IIIa is a receptor for at least 3 large adhesion molecules: Fibrinogen (Fgn, MW=340kDa), vWF (MW 1-20x10³kDa) and fibronectin (Fn, MW=440kDa). The key role of Fgn in the plasmatic coagulation cascade is well known; it has

also been known for a long time that platelets suspended in a medium devoid of Fgn and Ca^{2+} do not aggregate (Mustard *et al* 1972). Since the concentration of Fgn in the plasma is much higher than that of the other I Ib-IIIa-bindable adhesive molecules, it would appear that fibrinogen is the preferred ligand *in vivo*, while vWF might support platelet aggregation in cases of afibrinogen-aemia (De Marco 1986). It is now established that, following platelet activation, the gpIIb-IIIa complex transforms into a Fgn receptor, the number of Fgn molecules binding per platelet being comparable to the number of gpIIb-IIIa copies on the platelet surface (Coller 1983). The exact mechanism by which the Fgn receptor is exposed upon activation is not known but the latest studies (Frojmovic *et al* 1991) support the theory proposed by Shattil *et al* (1985) that involves conformational changes in the I Ib-IIIa molecule itself rather than alterations in its microenvironment (Coller 1985).

The existence of a common recognition mechanism for the binding of adhesive ligands to gpIIb-IIIa is suggested by the fact that all three of them (and other adhesive molecules like TSP, collagen, laminin and vitronectin) contain the tripeptide Arg-Gly-Asp (RGD). RGD-containing synthetic peptides bind to resting and activated platelets and inhibit ligand binding to activated platelets (Plow *et al* 1985, Ruggeri *et al* 1986). The RGD sequence occurs twice in the α chain of Fgn (Hawiger *et al* 1989). A dodecapeptide contained in the γ chain of Fgn has also been shown to bind to gpIIb-IIIa and inhibit Fgn binding to activated platelets (Kloczewiak *et al* 1989). Synthetic "hybrid" peptides containing combinations of the RGD sequence and the dodecapeptide have been constructed and shown to have enhanced potency of inhibiting Fgn binding to platelets (Timmons *et al* 1989). It is supposed that bound Fgn mediates aggregation by acting as a bridge between platelets (Fig. 1.3). Ligand binding may initiate secondary events like clustering of gpIIb-IIIa molecules on the platelet surface (Isenberg *et al* 1987) and association of gpIIb-IIIa with the

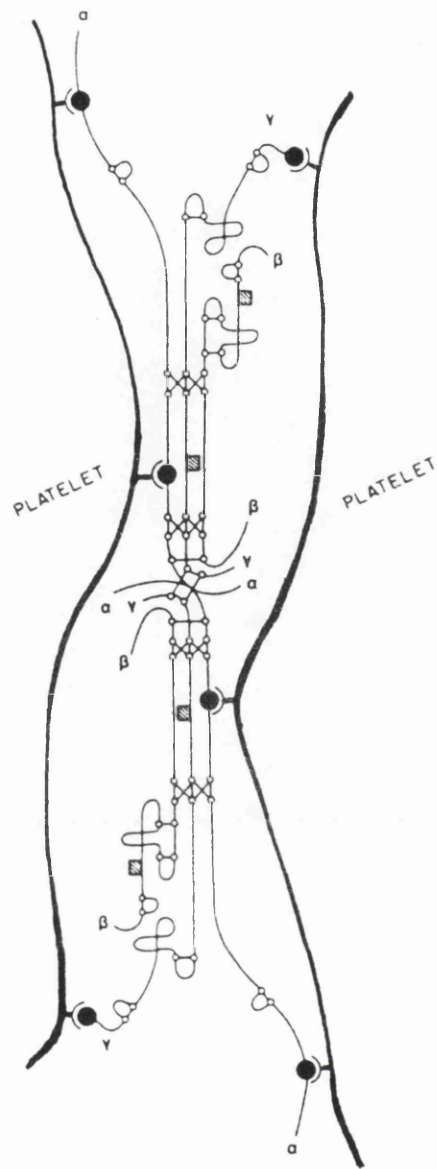


FIGURE 1.3 Proposed model of interaction of fibrinogen (composed of 3 pairs of non-identical chains: α , β and γ) with platelets (Hawiger *et al* 1989). The domains recognizing the platelet fibrinogen receptor (on gpIIb-IIIa) are marked as black dots (two in the α chain, residues 95-98 and 572-575 and one in the γ chain, residues 400-411). The hatched boxes represent carbohydrates on β and γ chains.

platelet cytoskeleton (Phillips *et al* 1980) which might be part of the process of clot retraction.

1.5.4 A brief overview of platelet activation responses.

Platelet activation responses are discussed briefly at this point in order to place the - already discussed - contribution of the major platelet membrane glycoproteins in the general context of platelet function. As with most other cells, many biochemical aspects of the complicated network of stimulatory and inhibitory pathways of platelet activation are incompletely understood. Detailed accounts of the current knowledge about these mechanisms have been published recently (Kroll & Schafer 1989, Siess 1989, Brass *et al* 1990).

Disruption of the continuity of vascular endothelium triggers an immediate recruitment of platelets from the circulating blood to the damaged area. The interaction between platelets and exposed subendothelium (adhesion) involves binding of large "adhesive" molecules present in the subendothelial matrix (collagen, vWF, fibronectin, laminin, vitronectin) to appropriate platelet receptors (see Table 1.1). Adhesion leads to activation manifested morphologically as spreading onto subendothelial matrix and recruitment of additional platelets through platelet-platelet interactions (aggregation) to form a hemostatic plug. Apart from adhesion to subendothelium, platelet activation can also be induced by soluble agonists such as ADP, epinephrine, serotonin, vasopressin, thrombin, thromboxane A_2 , PAF (platelet activating factor) and possibly by platelet-platelet contact alone during aggregation, while other molecules (e.g. PGI₂, PGD₂, endothelium-derived relaxing factor) act as activation antagonists upon binding to their membrane receptors. Platelet surface receptor occupancy causes changes in a family of GTP-binding regulatory proteins (G-proteins) which activate membrane-linked enzymes (e.g.

phospholipases, adenyl-cyclase) acting on highly phosphorylated substrates (e.g. phosphatidylinositol, ATP) to convert them into intracellular second messengers like inositol triphosphate (IP_3), diacyl-glycerol (DG) and cAMP. The second messengers induce conformational changes of target proteins either directly or through activation of protein kinases (PKs); IP_3 releases calcium, which binds to calmodulin and activates Ca^{2+} /calmodulin-dependent PKs, whereas DG activates PKC and cAMP activates cAMP-dependent PKs. As in other cells, platelet activation responses are heralded by an increase in Ca^{2+} content of the cytosol and are abolished by an increase in intracellular cAMP. Figure 1.4 is a diagrammatic summary of the current platelet activation concepts.

The first morphological sign of platelet activation is shape change from a smooth disk to a sphere with long pseudopods produced through reorganization of cytoskeletal proteins. Once activation has occurred, gpIIb-IIIa becomes bindable to subendothelial components and fibrinogen, thus mediating platelet spreading and aggregation respectively. It has been proposed that aggregation of activated platelets in the presence of adhesive ligands could be considered as a passive process, since there appears to be no further consumption of platelet energy after the initial shape change (Steen & Holmsen 1987).

Secretion of platelet granule contents is a process involving contraction of reorganized cytoskeletal elements and exocytosis via an as yet not clearly defined route. The release of dense body contents (e.g. ADP, serotonin) amplifies the aggregatory response, while the many α -granule proteins (1.2.4.1) contribute to a variety of activities. Secretion in response to weak agonists like ADP and epinephrine will only occur after aggregation, whereas with strong agonists like thrombin and collagen secretion can run virtually in parallel with shape change (Gear & Burke 1982).

Although platelet adhesion and aggregation appear to be sufficient to temporarily control haemorrhage from damaged vessels, deposition of fibrin is necessary for permanent haemostasis. The surface of activated platelets plays a key

catalytic role (Walsh 1985) that enables the reactions of the plasmatic coagulation cascade to proceed, resulting in fibrin formation.

1.5.5 Platelets in atherogenesis and thrombogenesis: Rationale for the evaluation of anti-gpIIb-IIIa MoAbs as therapeutic agents.

For obvious reasons, ischaemic coronary syndromes are the best studied example of occlusive arterial disease. While the antagonistic role of platelets in thrombus formation - a frequent complication of atheromatous arterial lesions - is undisputed, their involvement in early atherogenesis, although strongly suspected, is not clearly defined. Evidence for such involvement has been present for a long time: Organized mural microthrombi were demonstrated by Duguid as early as 1948; platelet antigens have been detected within atheromatous plaques (Carstairs 1965); platelet derived growth factor (PDGF) - an α -granule constituent secreted upon activation following adhesion to the vessel wall - has been shown to stimulate proliferation and migration of smooth muscle cells into the intima, thus generating a substrate for the formation of atheromatous plaques (Ross *et al* 1974). It is not known what the initiating event of the atherosclerotic process is: Endothelial damage related to haemorrhological factors appears to play a major role (Reidy & Boyer 1977), although the endothelial cell-damaging effects of cells of the monocyte-macrophage system and platelets are receiving increasing attention (Ross 1984). The prevailing current concepts on the issue have been reviewed by Ross (1986) and Fuster *et al* (1988).

Once superficial endothelial damage has occurred, platelet adhesion and activation mechanisms come into play. Besides, secretion of α -granule growth factors contributing to the progression of the atherogenic process (PDGF) and other proteins (PF4, β -TG) which are chemotactic for neutrophils and

monocytes also takes place. Progression of the lesion will cause coronary stenosis which may result in the clinical syndrome of angina pectoris. Evidence in support of the concept of continuous platelet activation and consumption in vascular disease has been produced by Fuster *et al* (1981) who demonstrated decreased platelet survival in patients with coronary heart disease and smokers. The potency of the platelet activating stimulus is increased when the growing atherotic plaque undergoes deeper injury (involving the tunica media) which can expose highly thrombogenic type I and III collagen fibres and release tissue thromboplastins which will amplify the - already activated by subendothelial collagen - plasmatic coagulation cascade resulting in thrombin generation. The combination of thrombin and collagen is the strongest stimulus for platelet aggregation. Thrombi thus generated can become organized and incorporated into the plaque, resulting in stenosis progression. Plaque disruption and/or the superimposed thrombus may result in critical lumen obstruction locally or - following detachment and embolization - distally; this is the pathophysiological background of acute coronary syndromes such as unstable angina, myocardial infarction and sudden ischaemic death (Davies & Thomas 1985, Fuster *et al* 1988). Additionally, the release by activated platelets of vasoconstrictors (thromboxane- A_2 , serotonin, histamine) can aggravate myocardial ischaemia.

Clinical studies with aspirin - the most widely used anti-platelet agent - have shown beneficial effects in the setting of ischaemic heart disease, the most characteristic being protection against acute infarction and death in unstable angina (Lewis *et al* 1983) and efficacy in the secondary prevention of myocardial infarction (ISIS-2 1988). These results support the experimental evidence for the importance of platelet involvement in the pathogenesis of acute coronary syndromes. Aspirin inhibits the formation of thromboxane- A_2 (TxA_2), which is a metabolic product of arachidonic acid liberated from membrane phospholipids through the action of phospholipase- A_2 , activated following

binding to membrane receptors. TxA_2 is a potent platelet activator that diffuses extracellularly and amplifies the response to weak agonists like ADP. As can be seen in Fig. 1.4, other strong agonists are still able to bring about platelet aggregation - through activation of the phospholipase-C pathway - even when the amplifying effect of TxA_2 is abolished. As mentioned above, strong agonists often operate in the setting of arterial thrombosis and, therefore, the need for anti-platelet treatment effective against the "aspirin resistant thrombus" is obvious. This can theoretically be achieved by inhibiting multiple pathways of platelet activation /aggregation or, alternatively, by blocking a common final step like the binding of fibrinogen to its platelet receptor, gpIIb-IIIa. The latter approach is currently being explored in studies involving in vivo administration of MoAbs known to be potent in vitro inhibitors of Fgn binding to gpIIb-IIIa (Coller *et al* 1986b, Takami *et al* 1987, Hanson *et al* 1988).

1.5.5.1 General characteristics of MoAb 7E3

(Coller 1985, Coller *et al* 1986a).

7E3 is the most extensively tested anti-human gpIIb-IIIa MoAb. It was launched in 1985 by B.S.Coller, the product of a hybridoma cell line derived from the same fusion as MoAb 10E5 (Coller *et al* 1983, Coller 1985). It belongs to the IgG1 subclass. At concentrations $\geq 5\mu\text{g/ml}$ it completely blocks ADP-induced in vitro platelet aggregation through high affinity ($K_D=3.4\text{nmol/L}$) binding to the gpIIb-IIIa complex, which prevents binding of Fgn to its receptor. Although the rate of binding is faster in activated platelets (Coller 1985), saturation of IIb-IIIa receptors of resting platelets occurs in 30-40 minutes at RT (personal observations). The antibody is complex dependent: binding is prevented by dissociation of the complex through calcium chelation. Bound 7E3 can be displaced by EDTA treatment. An important characteristic of 7E3 is its ability

to bind with high affinity to canine platelets and platelets of primates; this allows study of its in vivo effects on artificial animal models of thrombosis.

1.5.5.2 Clinical experience with 7E3.

Animal studies

In 1985, Collier and Scudder injected 3 dogs with F(ab')₂ fragments of 7E3 (up to 0.81mg/kg) and demonstrated a dose-dependent inhibition of aggregation responses to ADP ex vivo, with the highest dose producing nearly total inhibition, which correlated with nearly quantitative blockade of IIB-IIIa receptors. There was no evidence of spontaneous bleeding. With the higher doses, impaired aggregation responses were still present 6 days post injection, when the number of free 7E3-binding sites on the platelet surface had returned to baseline levels. Interestingly, although there were no instances of thrombocytopenia, one of the animals had a considerably elevated platelet count (142% of baseline) one week post injection.

When 7E3-F(ab')₂ (0.7-0.8mg/kg) was injected I.V. into 4 dogs and 4 monkeys with experimentally induced arterial thrombosis in the coronary and carotid arteries respectively (Collier *et al* 1986b), the antibody completely blocked new thrombus formation despite multiple provocations, including intimal damage combined with epinephrine infusion - a thrombogenic setting in which aspirin had been proven ineffective (Folts & Rowe 1988) and only very potent inhibitors like prostacyclin had shown antithrombotic effect (Bertha & Folts 1984).

Further studies in primates (Collier *et al* 1989b) showed that 0.2mg/kg 7E3-F(ab')₂ could prevent thrombus formation in the carotid artery experimental thrombosis model. This dose did not always abolish ex vivo responses to ADP

and caused only moderate prolongation of the bleeding time (BT). The BT became more significantly prolonged when the 7E3 dose was increased to levels that completely abolished *ex vivo* ADP-induced aggregation. The authors postulated that it might be possible to separate the antithrombotic from the antihaemostatic effect of gpIIb-IIIa receptor blockade. This was in agreement with previously reported preliminary results by Takami *et al* (1987), who had observed that infusion into pigs of a MoAb to porcine gpIIb-IIIa had a minimal effect on BT combined with complete abolition of *ex vivo* responses to ADP. Qualitatively similar results using two different anti-IIb-IIIa MoAbs (AP-2 and LJ-CP8) have been reported in baboons by Hanson *et al* (1988). They assessed antithrombotic effect by means of measuring platelet deposition onto synthetic (DACRON) vascular grafts; there is now strong evidence that the mechanisms of platelet deposition on artificial surfaces can be fundamentally different from those on damaged endothelium (Sheppeck *et al* 1991). Moreover, the antibodies used by Hanson and co-workers appeared to have significantly lower affinities for primate platelets and the infusions were associated with a high incidence of thrombocytopenia.

The suggestion of dissociation between antithrombotic and antihaemostatic effect is at variance with the results of other studies employing 7E3 (Gold *et al* 1988) in a dog model of coronary artery reocclusion after thrombolysis with recombinant tissue-type plasminogen activator (R-TPA), showing that virtually complete inhibition of *ex vivo* aggregation was required to prevent reocclusion. The efficacy of 7E3-F(ab')₂ in preventing rethrombosis in canine models of coronary artery thrombolysis with R-TPA is well proven (Mickelson *et al* 1990, Yasuda *et al* 1990a,b). In this setting (Mickelson *et al* 1990), aggregation responses to ADP and arachidonic acid were <10% of baseline 3 hours after bolus injection of 7E3-F(ab')₂ at doses (0.8mg/kg) preventing reocclusion post R-TPA in 7 of 9 animals. In a canine model of platelet-rich occlusive coronary thrombus resistant to thrombolysis, the combined administration of 7E3-F(ab')₂

and R-TPA achieved stable reperfusion (Yasuda *et al* 1990a). The same group (Yasuda *et al* 1990b) compared the effects of aspirin, a synthetic thrombin inhibitor (Argatroban) and 7E3-F(ab')₂ on coronary reperfusion with R-TPA in a canine preparation: 7E3 was statistically as effective as the combination of aspirin and Argatroban (significantly more effective than either of them used alone) but caused a much more pronounced prolongation of the BT compared with that caused by the combination of the other two agents (27±8 mins versus 12±11 mins; baseline 4.2±1.2 mins, n=5).

Studies in humans

Coller *et al* (1988) administered 7E3-F(ab')₂ intravenously to a person who had just died and was being maintained on a respirator. Boluses of 0.1 and 0.2 mg/ml resulted in respective blockade of 74% and 92% of the gpIIb-IIIa receptors and 84% and 100% inhibition of ADP-induced ex vivo platelet aggregation. No acute toxicity was noted.

A pharmacodynamic study of 7E3-F(ab')₂ was performed by Gold *et al* (1990) in 16 patients with unstable angina pectoris. Following a bolus of 0.2 mg/ml (the highest dose used), the BT prolonged to >30 mins (baseline 6.3±1.9) after 1 hour and decreased to 13±7.8 after 12 hours and 8.3±1.5 in 24 hours. The number of unblocked IIb-IIIa receptors (baseline 32±3 x10⁹/platelet) decreased to 13±7% of baseline at 1 hour and returned to 67±7% of baseline at 72 hours post injection. The BT was inversely proportional to the logarithm of the residual IIb-IIIa receptors. Ex vivo aggregation response to ADP (using the lowest ADP concentration that gave maximal aggregation at baseline) was practically absent at 1 hour and returned to ~65% of baseline within 72 hours. The response to collagen was also abolished 1 hour post injection. Angina was not observed in any of the patients during the first 12 hours (while the BT was

significantly prolonged) but occurred in 6 of the 16 patients within 72 hours post injection. A significant fall in the platelet count to $94 \times 10^9/l$ (baseline $240 \times 10^9/l$) was seen in one of the 16 patients at 12 hours; this gradually returned to normal over the following two days. Two of the 16 patients developed low titres of IgG antibodies specific for 7E3-F(ab')₂.

Similar results were reported by Machin *et al* (1990) using Fab fragments of 7E3 in 71 patients with stable angina. In addition they showed that the profound anti-platelet effect could be sustained by continuous infusion of 7E3-Fab for up to 36 hours. They reported a 25% incidence of human-anti-murine antibody development and one case of significant thrombocytopenia (platelet count $62 \times 10^9/l$ at 4 hours, recovering spontaneously by 48 hours). The same workers (Machin *et al* 1991) have reported that Fab fragments of chimaeric (human/murine, see following section) 7E3 given as bolus or constant infusion for up to 72 hours had effects comparable to those of the murine Fab in respect of inhibition of aggregation, prolongation of BT and blockade of IIb-IIIa receptors. Three of the 50 patients who received the chimaeric 7E3-Fab developed transient asymptomatic thrombocytopenia.

1.5.6 General issues related to the therapeutic use of anti-platelet monoclonal antibodies.

In general, the purpose of systemic administration of anti-platelet MoAbs (and, indeed, any MoAb) can be diagnostic or therapeutic. Diagnostic use aims at cell labelling, usually employing radioactive MoAbs; a typical example is thrombus imaging (Ezekowitz *et al* 1986, Peters *et al* 1986, Palabrica *et al* 1989). Therapeutically, the aim can be lysis or modification of function; only the latter has been explored so far in platelets. The epitope on the platelet surface and the nature of the MoAb molecule are the main factors determining if the antibody

will have an effect on platelet function and whether the effect will be stimulatory or inhibitory. A number of MoAbs have been described, which can activate platelets (Boucheix *et al* 1983, Higashihara *et al* 1985, Chen *et al* 1987, Moddermann *et al* 1988, Morel *et al* 1989); it appears that in many cases activation is mediated through binding of the Fc region of the MoAb to the platelet Fc receptor (Horsewood *et al* 1991, Anderson *et al* 1991).

Research interest has recently focused on the potential therapeutic application of MoAbs which inhibit platelet aggregation, usually by preventing gpIIb-IIIa binding of fibrinogen or other adhesive ligands (1.5.5.2). Platelets have certain characteristics that facilitate their targeting by MoAbs:

- They exist in a natural suspension and can therefore be easily accessed by the MoAb; accessibility is a major problem with MoAb use in other settings (e.g. solid tumours). Nonetheless, little is known about the degree of accessibility of platelets in the splenic pool or megakaryocytes (sharing platelet antigens) in the bone marrow.
- They possess unique antigens so that they do not have to compete for antibody binding with plasma proteins or other cells.
- They do not undergo antigenic modulation.

F(ab')₂ fragments of anti-platelet antibodies are usually employed for the following reasons: a. To limit the possibility of increased platelet clearance mediated by the Fc region of the MoAb (through recognition by the recipient's RES or via complement recruitment) and b. To prevent RES blockade that might be caused by binding of the Fc region of circulating antibody to the macrophage Fc receptors. Monovalent Fab fragments might have the additional advantages of better access to the target molecule due to their smaller size and of being unable to agglutinate platelets through crosslinking of gp molecules on neighbouring cells.

In view of the small cell size and the density of the major glycoproteins populating the surface of platelets, steric factors are likely to play a significant role in the binding of antibodies to the platelet surface.

The most formidable problem associated with the *in vivo* use of MoAbs is immunogenicity. Murine Igs are immunogenic in humans and although the Fc region accounts for most of the immunogenicity of the Ig molecule, neutralizing immune responses triggered by the unique idiotypic determinants of any MoAb represent a frequently encountered major obstacle. The most extensive clinical experience with *in vivo* administration of a MoAb has been obtained with OKT3 (anti-CD3) in the setting of acute rejection following cadaveric renal transplant. Antibody responses to the infused MoAb have been reported in up to 100% of patients with a high frequency (60%) of anti-idiotypic responses (Ortho Multicenter Transplant Study Group 1985, Jaffers *et al* 1986). In the case of monoclonals aiming at modification of platelet function, development of anti-murine constant region antibodies would result in recruitment of Igs onto the platelet surface upon re-exposure to the MoAb, which could eventually cause increased platelet clearance and thrombocytopenia, whereas development of anti-idiotypic antibodies might prevent binding of the MoAb to its epitope. Immunogenicity could therefore limit such applications to a once only use in acute vascular complications (e.g. unstable angina, acute occlusion of coronary arteries after thrombolysis or angioplasty, acute occlusion of vascular grafts, cerebrovascular transient ischaemic attacks) which would not be satisfactory, in view of the chronic nature of most vascular disorders.

Recent advances in recombinant DNA technology have allowed the re-design of therapeutic antibodies. An interesting example is refashioned antibody molecules that have been produced by the introduction into cells of genetic constructs consisting of combinations of cloned genes encoding parts of the

murine MoAb and of normal human IgG. The final product consists of the variable (or just the hypervariable) region of murine IgG while the rest of the molecule is "human"; in this way up to 90-95% of the aminoacids of murine IgG can be substituted with human sequences (Morrison *et al* 1987, Morrison & Schlom 1990). The so produced "chimaeric" (mouse/human) MoAbs permit selection not only of the specificity determining variable region but also of a human constant region with desirable biological behaviour (Steplewski *et al* 1988). Chimeric MoAbs have the same specificity and comparable binding affinity with their murine parent molecules, combined with longer circulation times and have been tested in early-phase clinical trials (Hale *et al* 1988, LoBuglio *et al* 1989, Knox *et al* 1991). Although the presence of the immune component could still elicit an anti-idiotypic immune response in humans, there is evidence (LoBuglio *et al* 1989, Knox *et al* 1991) that the administration of chimaeric MoAbs is associated with substantially lower immunogenicity than that seen with their murine counterparts.

1.6 THE IMMUNOLOGICAL SPECIFICITY OF PLATELETS

Platelet surface alloantigens have been traditionally divided into "platelet specific" and "common" (i.e. shared with other cells). This classification is not theoretically justified since antigens previously thought to exist only on platelets are now known to be also present on other cells (endothelia, smooth muscle, fibroblasts, white cells). Platelet antigens are aetiologically involved in serious clinical disorders through allo-immunization. Table 1.2 summarises platelet "specific" and "common" antigens of proven or suspected clinical importance.

1.6.1 Platelet "specific" alloantigens

These are located on glycoproteins and, apart from PL^T, represent diallelic systems with a high and a low frequency allele inherited in a autosomal co-dominant fashion. These polymorphisms appear to result from single amino-acid substitutions in gp molecules which, in turn, correspond to single base changes in the respective mRNAs (Newman *et al* 1989, Lyman *et al*, 1990). It should be noted that, although these polymorphisms are necessary for the formation of the alloepitopes (Goldberger *et al* 1991), the substituted amino-acids are not necessarily included in the epitopes, which may be formed by minor changes in the conformation of the gp molecule (Flug *et al* 1991).

The frequency and clinical importance of platelet alloantigens varies significantly among different ethnic groups. In caucasians, the PL^A (formerly Zw) system is the one most commonly implicated in clinical disorders. It consists of two alleles, PL^{A1} and PL^{A2} resulting from a leucine³³/proline³³ polymorphism in the molecule of gpIIIa (Newman *et al* 1989). The respective allele frequencies in the population are 0.85 and 0.15, giving approximate phenotypic

TABLE 1.2 PLATELET SPECIFIC ALLOANTIGENS

System name (other names)*	Alleles	Glycoprotein localization	Reference
PL^A (Zw, HPA-1)	PL ^{A1} /PL ^{A2} (Zw ^a /Zw ^b , HPA-1a/HPA-1b)	gpIIIa	Kunicki and Aster 1979 Newman 1991
Pen (Yuk, HPA-4)	Pen ^a /Pen ^b (Yuk ^b /Yuk ^a , HPA-4a/HPA-4b)	gpIIIa	Furihata <i>et al</i> 1987 Newman 1991
Bak (Lek, HPA-3)	Bak ^a /Bak ^b (Lek ^a /Lek ^b , HPA-3a/HPA-3b)	gpIIb	von dem Borne <i>et al</i> 1980 Lyman <i>et al</i> 1990
Br (HPA-5)	Br ^a /Br ^b (HPA-5b/HPA-5a)	gpIa	Santoso <i>et al</i> 1989
Ko (HPA-2)	Ko ^a /Ko ^b (HPA-2a/HPA-2b)	gpIb	Kuijpers <i>et al</i> 1989
PL^E	PL ^{E1} /PL ^{E2}	gpIb	Furihata <i>et al</i> 1988
PL^T	-	gpV	Beardsley <i>et al</i> 1987

* Nomenclature of platelet specific alloantigens based on the designation HPA (human platelet antigen) has been proposed recently (von dem Borne *et al* 1990). The systems are numbered in chronological order of first description. The high frequency allele is indicated with the letter "a" and the low frequency one with the letter "b".

frequencies of 72% for A1/A1, 26% for A1/A2 and 2% for A2/A2. The usual ways of allo-immunization are through pregnancy or transfusion as discussed below.

1.6.1.1 Neonatal alloimmune thrombocytopenia (NAIT)

This disorder is usually seen in A1/A2 (PL^{A1} positive) newborns of A2/A2 (PL^{A1} negative) mothers who have developed antibodies against the A1 antigen. Fetal platelet destruction is caused by maternal IgG antibody crossing the placenta. NAIT, in contrast to Rhesus incompatibility, is characterized by its failure to spare the first incompatible offspring. Only about 1-2% of PL^{A1} negative mothers will have affected babies as can be inferred by the incidence of NAIT which is 1 for every 2-5,000 live births (Muller 1987, McFarland & Aster 1991). There is a strong association between allo-immunization and the HLA antigen DR3 (Reznikoff-Etiévant *et al* 1983) present in 90% of alloimmunized mothers but only 25% of controls. The commonest manifestations in the newborn are cutaneous and mucosal haemorrhages but intracranial bleeding is not uncommon and is responsible for the significant mortality of the condition. The thrombocytopenia resolves spontaneously over a period of a few weeks but initial support with transfusions of PL^{A1} negative platelets is usually given. Fetal thrombocytopenia leading to intrauterine death or permanent neurological impairment can occur. It is therefore important to establish a diagnosis by cordocentesis early (21-22 weeks) in the pregnancy of women with a history of previously affected babies and offer treatment with frequent intrauterine transfusions of PL^{A1} negative platelets, which appears to be the only effective management available at present (Kaplan *et al* 1988, Kelsey *et al* 1991).

1.6.1.2 Post-transfusion purpura (PTP)

This is a rare syndrome of obscure pathogenesis presenting with severe thrombocytopenia 7 to 10 days following transfusion of blood products containing platelets. The patients are usually PL^{A1} negative elderly women sensitized to the PL^{A1} antigen through previous pregnancies but nulliparous women and men with a history of multiple previous transfusions can also be involved. The thrombocytopenia usually resolves spontaneously within a period that can extend to several weeks. Treatment with high doses of intravenous immunoglobulin appears to be effective (Mueller-Eckhardt 1986), whereas transfusions of PL^{A1} (-) platelets are of little value. The question of why PTP patients destroy their own platelets that lack the antigen to which the alloantibody is directed has not found a definitive answer so far. Kickler *et al* (1986) have shown that PL^{A1}(-) platelets can be converted to weak PL^{A1} positivity by incubation with PL^{A1}(+) plasma, presumably through adsorption of soluble PL^{A1} antigen. The latter has been detected in stored blood from PL^{A1}(+) donors and in the plasma of PTP patients (Kickler *et al* 1986, Shulman *et al* 1990). It is therefore possible that the destruction of autologous cells is mediated by alloantibody binding to platelets bearing adsorbed soluble antigen or by antigen-antibody complexes binding to platelet Fc receptors.

1.6.2 Common alloantigens

1.6.2.1 Blood group antigens

These glycoconjugate antigens (ABH, Le, P, I) are present on platelet membranes but there is marked heterogeneity in the density of antigen sites per platelet (Dunstan and Simpson 1985), which is also present in cultured

megakaryocytes (Dunstan 1986). Although these antigens are synthesized by megakaryocytes, there is evidence that they are also partly adsorbed from the plasma (Kelton *et al* 1982a, Lewis *et al* 1977). It is possible that ABO incompatibility could be a contributing factor in some cases of refractoriness to platelet transfusions (Lee and Schiffer 1989).

1.6.2.2 HLA antigens

Only class I HLA antigens (HLA-A, -B and -C) are present on platelets. Their density distribution shows less variability compared to that of blood group antigens (Dunstan and Simpson 1985) but, as with the latter, there is evidence of antigen adsorption from the plasma beside endogenous synthesis (Lalezari and Driscoll 1982, Santoso *et al* 1986a). There is no agreement regarding the number of antigenic sites per platelet but this appears to differ widely between individuals and types of antigen. Janson *et al* (1986) incubated platelets with human anti-HLA sera and, using a radiolabelled MoAb to human IgG, estimated binding of 4,400-10,000 anti-HLA-A2, 870-8,400 anti-Bw4 and 1,300-5,800 anti-Bw6 molecules per platelet. HLA-C expression on platelets is generally considered to be very weak. Alloimmunization against HLA class I antigens is the main cause of immune refractoriness to platelet transfusions (Hogge *et al* 1983).

1.7 PLATELET IgG

The concepts concerning the presence, amount and biological role of immunoglobulins and in particular IgG associated with the platelets of healthy individuals have evolved in parallel with the methodology for its measurement, which is discussed in more detail in Chapter 4. Some early methods (e.g. Dixon *et al* 1975) estimated unrealistically high numbers (many hundreds of thousands) of IgG molecules per platelet but, even when later modifications or new methods brought this estimate down to several thousands (reviewed by Schwartz, 1988), it was still difficult to find a physiological role for platelet-associated IgG (PAIgG). Moreover, methods using lysed platelets to measure PAIgG consistently yielded significantly higher estimates compared to those using intact platelets to measure mainly surface IgG (PSIgG) and it is now established that the main pool of PAIgG (up to *ca.* 20,000 molecules) is intracellular, located mainly in the α -granules, where its concentration reflects that of the plasma (George 1990). The exact mechanism of acquisition of IgG (and of other plasma proteins) by the platelets remains poorly understood, although platelet endocytotic activities have been known to exist for many years (Zucker-Franklin 1981). The parallel acquisition of apparently irrelevant (e.g. albumin) proteins by the α -granules implies that the intraplatelet presence of IgG is, at least in healthy individuals, a non-specific phenomenon probably resulting from indiscriminate fluid-phase endocytosis of plasma proteins, which is reminiscent of the traditional perception of the platelet as a "passive sponge". The explanation proposed for the often dramatically raised levels of total PAIgG in patients with immune and, occasionally, presumably non-immune consumptive thrombocytopenias is that, due to increased thrombopoietic stimulation, large "stress" platelets with increased endocytotic capacity are being produced (George 1990). Although firm experimental evidence to confirm this hypothesis is lacking, the current tendency is to focus on platelet surface

IgG, assuming that elevation of its level is more likely to reflect specific platelet antibody activity.

The current concepts about PSiGg in normal platelets have been profoundly influenced by the work of LoBuglio and co-workers (1983), who were the first to use a radiolabelled monoclonal antibody to the Fc region of human IgG in order to measure PSiGg. Their estimate (*ca.* 170 molecules of surface IgG per platelet) was 10-100 times lower than that of most contemporary methods employing polyclonal antibodies. This amount of PSiGg was considered more "realistic", one reason being that it was of the same order with the amount present on the surface of red cells. This argument should be treated with scepticism, in view of the profound differences in surface structure between platelets and all other blood cells.

Whatever the number of IgG molecules on the surface of normal platelets is, their spatial orientation and biological role remain unknown. Generally, there are two recognized ways in which IgG may be bound to the platelet surface:

- Specifically, through the antigen-binding Fab region. The nature of the antigen that might attract IgG to the platelet surface in healthy individuals can only be a matter of speculation. The possibility of naturally occurring antibodies binding to altered membrane antigens of senescent platelets has been suggested (George 1990).

- Non-specifically, through binding of the Fc region of monomeric or complexed IgG to the platelet Fc γ RII receptor (see section 1.3.2).

In contrast to the situation in normal platelets, there is ample evidence that, in the setting of immune-mediated thrombocytopenia, at least a proportion of the frequently elevated PSiGg represents specific antibody directed against components of the platelet membrane, usually glycoproteins (Van Leeuwen *et al* 1982, Beardsley *et al* 1984, Woods *et al* 1984a,b, McMillan *et al* 1987, Szatkowski *et al* 1986, Berchtold *et al* 1989). Platelet membrane phospholipids

have also been implicated as potential antigens (Harris *et al* 1985) although the issue of specific binding of antiphospholipid antibodies to intact platelets is still debated (Out *et al* 1991, Haga *et al* 1990). Binding of IgG to the platelet surface can have one or more of the following effects:

- Reticuloendothelial system sequestration of antibody-coated platelets (Karpatkin 1980, McMillan 1981, Saleh *et al* 1989).
- Direct lysis through complement fixation (Tsubakio *et al* 1986, Lehman *et al* 1987).
- Alteration of platelet function (Clancy *et al* 1972, Zahavi and Marder 1974, Stricker *et al* 1985, Niessner *et al* 1986, Devine *et al* 1987, Pfueller *et al* 1989).

It should be noted that elevated PSiGg is not incompatible with normal platelet count, lifespan or function, as has been reported in cases of PSiGg elevation associated with SLE (Mueller-Eckhardt *et al* 1980, Endresen and Foerre 1982), liver disease (Landolfi *et al* 1979) and hypergammaglobulinaemic states other than myeloma (McGrath *et al* 1979).

It appears that the end result of IgG binding to the platelet is determined by many factors, which might include the amount, subclass and spatial orientation of PSiGg, as well as the nature of the glycoprotein or other antigen involved and in particular the functional and structural (maintenance of membrane integrity) role of the epitope to which the platelet antibody is binding. Clearly, more research is needed in order to clarify these issues.

Chapter 2

GENERAL METHODS

This chapter contains details of general laboratory methods employed in the investigations described in this thesis. Modifications of some of these methods as well as specialised techniques that are only relevant to particular experiments will be cited separately in the appropriate results chapters.

An effort has been made to focus on methodology and avoid exhaustive descriptions of materials. Formulations of buffers, anticoagulants, fixatives and other reagents mentioned in this chapter are given separately in Appendix I. Unless stated otherwise, all the experiments were performed at room temperature.

2.1 PLATELET PREPARATION

2.1.1 Samples from normal controls. The following sources of presumably normal platelets were used:

- a. Donations by members of laboratory staff. Small volumes of blood, usually 10 to 20ml, were collected via a 21-gauge Butterfly needle into a 20ml polypropylene syringe and 9ml were transferred quickly to 10ml polypropylene tubes containing 1ml of either trisodium citrate (105mM) or disodium EDTA (50mM) in isotonic saline.
- b. Surplus blood from full blood counts performed on healthy RAF personnel as part of routine medicals. The blood samples were taken in 4.5ml VACUTAINER tubes (Becton-Dickinson, Meylan, France) containing tri-potassium-EDTA at a final concentration of 4mM.
- c. In some experiments requiring comparatively large numbers of normal control platelets, newly expired platelet concentrates that had been separated from whole

blood anticoagulated with CPD-A1 (63ml CPD-A1 in 450ml blood; see Appendix I for formulation of CPD-A1) were obtained from the Blood Bank of University College Hospital.

2.1.2 Blood grouping. In some experiments involving incubation of control platelets with under investigation sera, the use of platelets from blood Group O individuals was required. Rapid ABO grouping was performed as follows: One volume (1-2 drops) of a 10% (v/v) whole blood dilution in normal saline was mixed in a 10x75mm polypropylene tube with the same volume of anti-A or anti-B serum (Lorne Laboratories Ltd., Reading) and, after centrifugation at 400g for one minute, red cell agglutination was looked for macroscopically and under a magnifying lens.

2.1.3 Samples from patients. These were taken in citrate or EDTA anticoagulant as described in 2.1.1 above. Some citrated samples were taken directly into 4.5ml siliconized VACUTAINER tubes (Becton-Dickinson, Meylan, France) which contained sodium citrate giving a final concentration of 10.5mM.

2.1.4 Preparation of platelet-rich plasma (PRP). Platelet-rich plasma was prepared from anticoagulated whole blood in 4.5ml VACUTAINER tubes or 10x75mm polypropylene tubes. The tubes were centrifuged at 200g for 10 minutes in a bench-top centrifuge. When large platelets were present, lower centrifugal forces and shorter centrifugation times were used (e.g. 170g for 8 minutes) to limit the loss of large platelets co-sedimenting with the red cells. The upper two thirds of the supernatant were removed with a plastic transfer pipette without disturbing the buffy coat and were transferred to a 10x75mm polypropylene tube. In order to isolate platelets from

small (e.g. fetal) samples, PRP was prepared in 8x60mm polypropylene tubes after diluting the sample with an equal volume of wash buffer (see 2.1.6).

2.1.5 Preparation of platelet-poor plasma (PPP). Anticoagulated whole blood or PRP prepared as above were centrifuged at 2000g for 15 minutes. Without disturbing the pelleted cells, the supernatant was transferred to another tube and centrifuged at 2000g for 10 minutes. When the PPP was not used immediately, it was stored at -20°C or -70°C.

2.1.6 Platelet washing method. Two types of platelet wash buffer were used (see Appendix I for formulations): Phosphate buffered saline containing EDTA (PBS-EDTA) and a ACD-acidified buffer containing PGE₁ (citrate-PGE₁). For washing in PBS-EDTA, PRP in a 10x75mm polypropylene tube was diluted with an equal volume of the wash buffer and centrifuged at 2000g for 5 minutes. Following complete removal of the supernatant using a fine tipped plastic transfer pipette, the platelet pellet was gently resuspended in the wash buffer and the platelets pelleted by centrifugation as above. One washing cycle or "wash" consisted of suspending the platelets in wash buffer and pelleting by centrifugation. For washing in citrate-PGE₁, the initial platelet pellet was prepared by centrifugation of PRP which had been acidified to pH 6.4 by adding an appropriate volume of ACD (see appendix I).

2.1.7 Lysing red cells. When avoidance of contamination of the platelet suspension by red blood cells was indicated, the red cells were lysed by suspending the platelet pellet in 1ml of 1% (w/v) ammonium oxalate for 10 minutes.

2.1.8 Platelet fixation. In studies employing fixed platelets, fixation was achieved by mixing the platelet suspension with an equal volume of 2% (w/v) paraformaldehyde (see Appendix I) for 15 minutes.

2.1.9 Platelet counting. Platelet counts were determined in whole blood, PRP or washed platelet suspensions using a Coulter STKR analyzer (Coulter electronics, Luton, UK). Histograms of platelet volume distribution were obtained (Fig 2.1) and, where indicated, manual platelet counts were performed using a Neubauer chamber (Dacie and Lewis 1991).

2.2 ANTIBODIES

The various antibodies used in the flow cytometric and immunoenzymatic studies were either purchased or donated by their producers as indicated in Table 2.1. Stock antibodies were aliquoted and kept frozen at -70 or -20°C until used. When dilution of the stock antibody was indicated, this was done with PBS pH 7.2 containing 0.1% sodium azide. The chimaeric 7E3 antibody provided by Centocor Inc. will be described in more detail in Chapter 5. The FITC labelling of c7E3-Fab and rabbit-anti-mouse IgG was performed by Dr Carrie Wagner using modifications of published methods (Goding 1976).

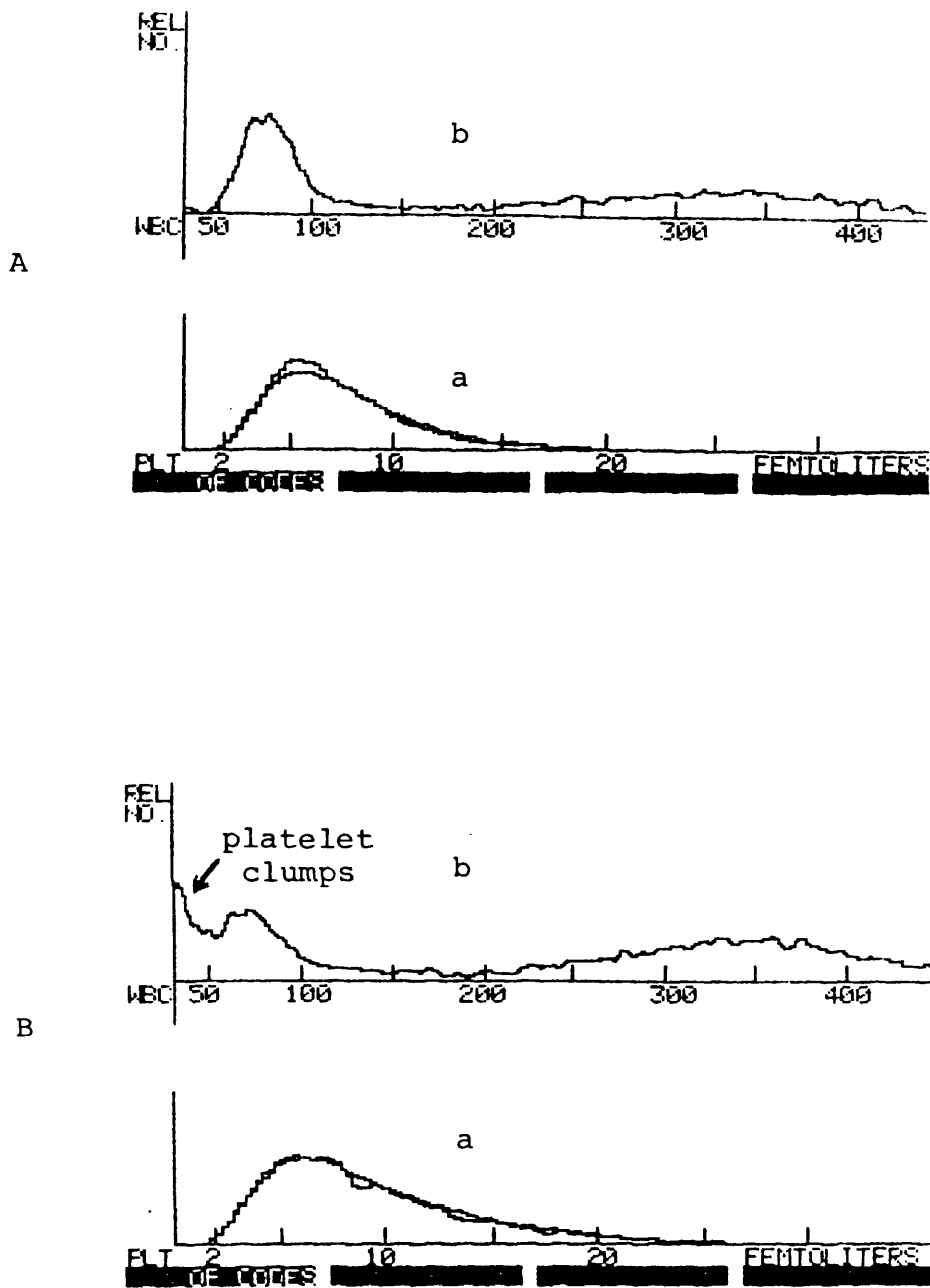


FIGURE 2.1 Coulter printout of platelet (a) and white cell (b) volume distribution histograms in a normal blood sample (A) and a sample containing platelet clumps (B).

TABLE 2.1 SOURCES OF ANTIBODIES

TYPE OF ANTIBODY	ANIMAL SOURCE	SUPPLIER
A. FITC conjugates of antibodies to human immunoglobulins		
Human IgG (γ -chain specific) affinity isolated, antigen specific	goat	Sigma No F-0132
Human IgG,IgM,IgA (polyvalent) IgG fraction of antiserum	goat	Sigma No F-6506
Human IgG (Fc-specific) affinity isolated, antigen specific	goat	Sigma No F-9512
Human IgG (Fc-specific) nonoclonal	mouse	Sigma No F-5016
B. Antibodies to platelet proteins		
gpIb (AN51) monoclonal	mouse	DAKO No M-719
gpIb (MM/174) monoclonal	mouse	Dr M Wilkinson, London
gpIIIa (Y2/51) monoclonal	mouse	DAKO No M-753
gpIIb-IIIa (J15) monoclonal	mouse	Dr D Mason, Oxford
gpIIb-IIIa (7E3) monoclonal fab fragments	mouse	Centocor Inc.
7E3 chimaeric monoclonal IgG, fab and FITC-fab	mouse/human	Centocor Inc.
Thrombospondin monoclonals (FT1/7, FT1/10, FT1/52, FT1/85)	mouse	Dr C Mattock, London
Thrombospondin monoclonal (P12)	mouse	The Binding Site, Birmingham
GMP-140 (S12) monoclonal	mouse	Dr R McEver, Texas
Thrombospondin (S12) monoclonal	mouse	The Binding Site, Birmingham

TABLE 2.1 (cont.)**C. Other antibodies and controls**

FITC-IgG (non-immune)	goat	Sigma No F-7381
FITC-IgG1 monoclonal non-human reactive	mouse	Coulter No 6602928
FITC-IgG2a monoclonal non-human reactive	mouse	Coulter No 6602906
IgG (non-immune)	mouse	Sigma No I-5381
FITC-anti-mouse IgG	goat	Sigma No F-2012
FITC-anti-mouse IgG	goat	Coulter No 6602159
FITC-anti-mouse IgG	rabbit	Centocor Inc.
Anti-HLA-DR monoclonal	mouse	DAKO No M-775

2.3 FLOW CYTOMETRY

As the development of flow cytometric methods for the study of platelets was one of the objectives of the present studies, certain points related to methodology will be discussed critically in the results' chapters. In this section, the methods are described in their "final" form, i.e. as they are currently being used in our laboratory. Because flow cytometry is not yet established as a routine method in the investigation of platelet disorders, the section that follows gives an outline of the principles of fluorescence flow cytometry, with the emphasis on its application for studying platelets.

2.3.1 Principles of fluorescence flow cytometry (FC)

The general definition of flow cytometry (Shapiro 1988, modified) is "measurement of selected physical and/or chemical characteristics of a cell (or particle) while the cells pass, preferably in a single file, in a fluid stream through a suitable detection apparatus". The best known application of this system in haematology is the Coulter blood cell analyzer where the physical characteristic measured is the change in electrical impedance across a small orifice, caused by cells passing in a single file through that orifice. The last decade has witnessed an increasing interest in the development of flow cytometry systems based on the measurement of fluorescence emitted by cells that have been exposed to fluorescent probes binding to cellular elements like nucleic acid or protein macromolecules (for a historical review see Shapiro 1988). The emission of fluorescence by the probe requires excitation (absorption of photon energy of the appropriate wave length) which is usually provided by a focused laser light beam, through which the cells are forced one by one. The main components of a fluorescence flow cytometry (FC) system are: (a) the flow system, (b) the optical system, (c) the electronic signal generation system and (d) the

electronic data analysis system. Figure 2.2 is a diagrammatic representation of the function of a flow cytometer.

2.3.1.1 *The flow system*

The function of this system is to present the cells under analysis in a single file to the *flow chamber (flow cell)*, where those cells will be "interrogated" by a beam of laser light. This is achieved by automatic *hydrodynamic focusing*, which involves guiding the cell sample into a stream of pressurised *sheath fluid* and regulating the pressures of sample and sheath fluid through various pinch valves, so that a single file of cells in the middle of the stream of sheath fluid in laminar flow is delivered to the flow chamber at a constant rate.

2.3.1.2 *The optical system*

This system includes three main components: (a) The light source, usually a laser (e.g. argon ion, krypton ion) emitting light of a fixed intensity and wave length. (b) An assembly of lenses to shape the laser beam and focus it on the observation point in the flow chamber. (c) An assembly of mirrors, lenses and optical filters to collect the light after its passage through the flow chamber, separate the various parts of interest (e.g. specified wavelengths) and present them to the appropriate sensors.

When the laser beam "hits" a cell at the observation point (the intersection of laser beam and column of cell sample in the flow chamber) the laser light is scattered by the cell at various angles. Light scattering in a forward direction (FS) i.e. 0.5-10 degrees to the path of the laser beam is roughly proportional to the size of the cell, whereas light scattering at a right angle (side scatter - SS) relates to cell shape, surface irregularity and intracellular structure. Measurement of FS and SS can be used to differentiate various cell populations. Laser light of appropriate wavelength can also

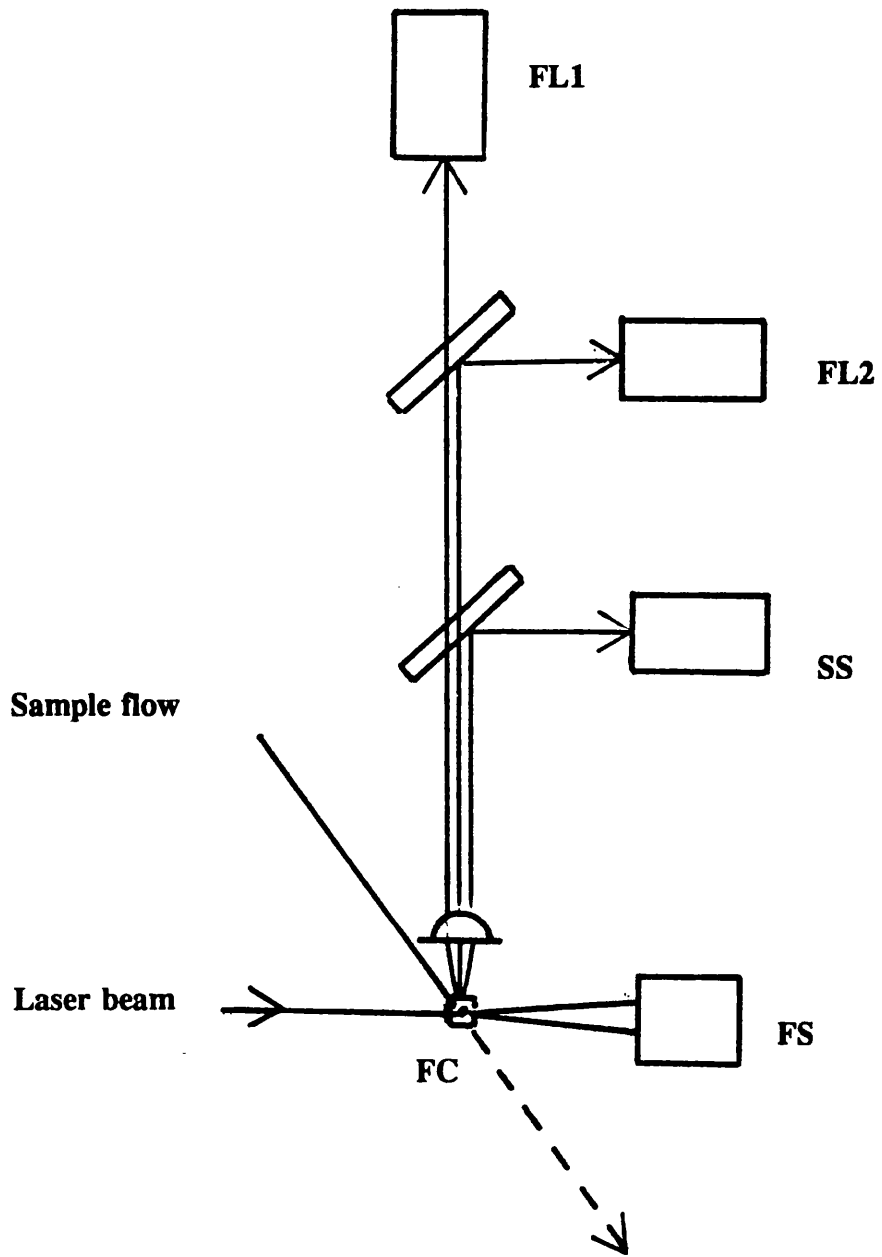


FIGURE 2.2 The principle of fluorescence flow cytometry: A cell (dot) in the flow chamber (FC) is being "interrogated" by the laser beam. Scattered light is directed to the appropriate photodetectors for measurement of FS (forward light scatter), SS (side light scatter), FL1 ("green" fluorescence - 525nm), FL2 ("red" fluorescence - 575nm).

excite fluorescence emission by molecules that are intrinsic to the cell (autofluorescence) or that have been attached to it as fluorescent probes.

2.3.1.3 Electronic signal generation system

Photodetectors convert the light signals into pulses of electric current with voltage accurately proportional to the intensity of light they receive. The photodetector for FS light is usually a simple silicon photodiode, whereas SS and fluorescence photodetectors (i.e. the sensors for relatively low intensity light) are photomultiplier tubes (PMT). The pulses of different voltage generated by the photodetectors are amplified and converted into digital signals by an Analog-to-Digital converter (ADC). The digitized signals are finally stored in a computer.

Amplification

Before being digitally converted, the signals generated by the photodetectors pass through voltage amplifying electronic circuits (or "amplifiers"). *Linear amplifiers* will amplify the signal by a selected factor (e.g. 1, 2, 5, 10, 20, 50, or 100). It follows that the voltage ratio of two signals post amplification will be the same as it was pre amplification (if the linear amplifier is accurate). *Logarithmic amplifiers* will amplify the signal logarithmically. Figure 2.3 shows the transfer function of an "ideal" three-decade log amplifier. As can be seen in the figure, logarithmic amplification expands the scale for weak signals and compresses the scale for strong signals. This permits the inclusion of a greater range of measurements in the final histogram. Log amplifiers have become popular, mainly because they allow a more "biologically relevant" presentation of data, as biological parameters often appear as log-normal distribution. The main problem related to the use of log amplifiers is the difficulty in making direct comparisons between signal intensities of different samples. This could be theoretically overcome by mathematically converting the log values into a linear scale. Unfortunately, in practice, log amplifiers are not perfectly logarithmic and the use of

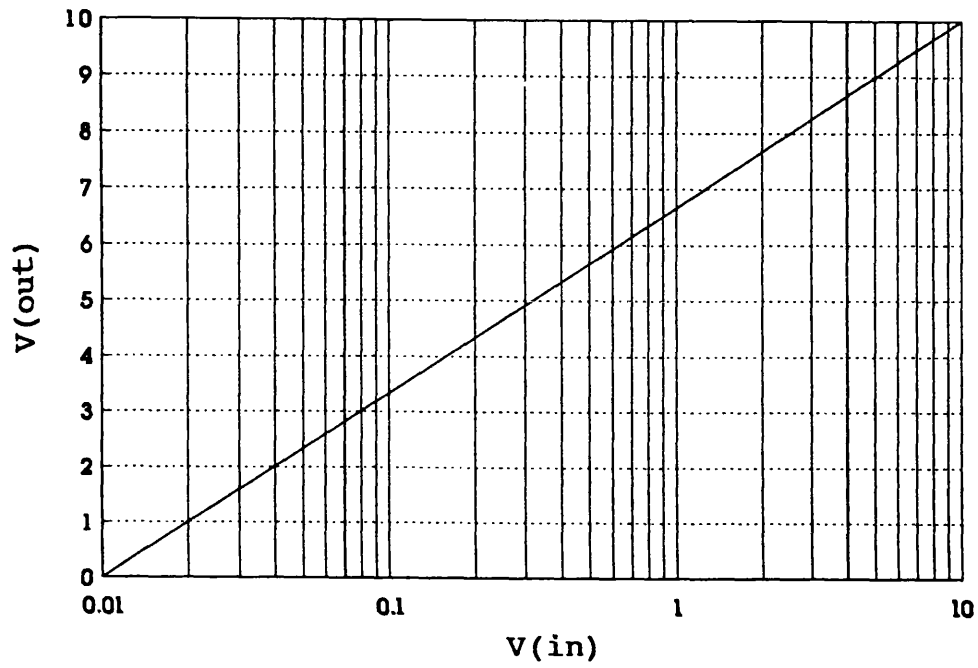


FIGURE 2.3 Signal amplification by a "perfect" three-decade log amplifier.
 $V(\text{in})$: voltage of signal before amplification
 $V(\text{out})$: voltage of signal after amplification

calibration methods is necessary before any comparisons between samples are possible (Schmid *et al* 1988).

Digital conversion

Using a computer, the signal voltages obtained after linear or log amplification are expressed as "channel numbers", which are a linear function of signal voltage. Channel numbers are stored in the computer's memory and are subsequently processed and presented, usually in the form of histograms.

2.3.1.4 Data analysis system

Most modern flow cytometers contain appropriate software for the presentation of the digitized signals as dot plots (Fig 2.4) or histograms (Fig 2.5). They are also able to perform simple statistical tests (min, max, %, mean, coefficient of variation) either on the entire population of events included in a histogram or on subpopulations selected by the operator (Fig 2.6).

Gating

In most flow cytometry systems, the operator has the facility of setting "gates" i.e. defining the range of measurements that are allowed to be included in a histogram. Single parameter (e.g. FS or fluorescence) gates usually set a threshold of intensity which must be exceeded by a signal in order for it to be eligible for inclusion in the histogram. A two parameter or *bitmap* gate is an area defined by any two parameters (e.g. FS and SS) and can have any shape (Fig 2.4). The computer will compare the coordinate of the two parameter measurements obtained from a cell/particle to the bitmap coordinates and only if it falls inside the bitmap will the cell be included in the histogram analysis.

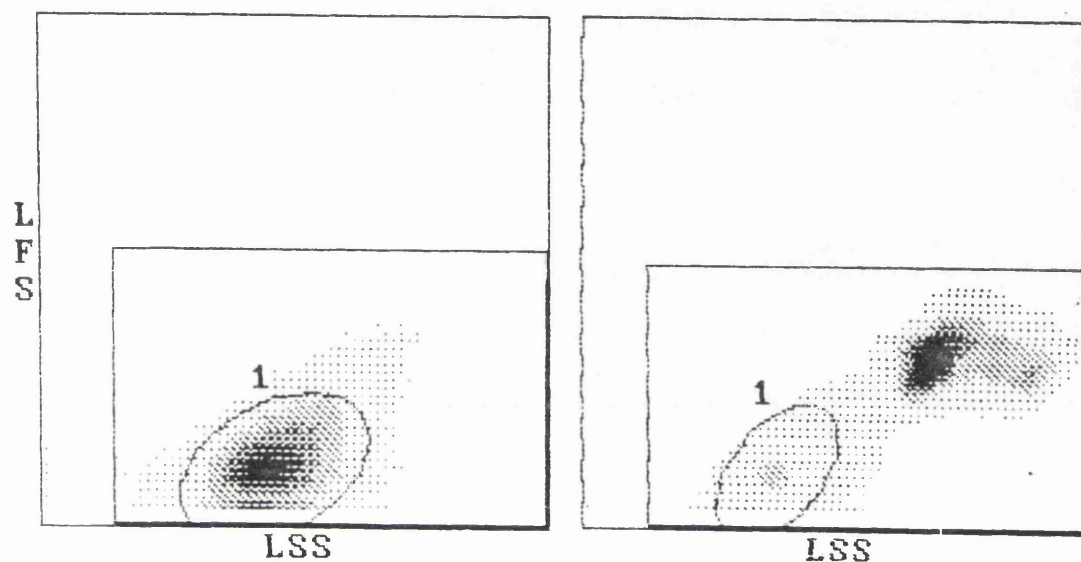


FIGURE 2.4 **A.** Light scattering profile of a sample of dilute PRP (2×10^9 platelets/l). Dot plot of log forward scatter (LFS) plotted against log side scatter (LSS). The circled area ("bitmap" 1) defines the cell population for analysis. **B.** LFS v LSS of a sample of dilute whole blood ($1 \mu\text{l}$ blood in 1ml PBS) to show the differences in light scattering behaviour between platelets (bitmap 1) and red (R) cells.

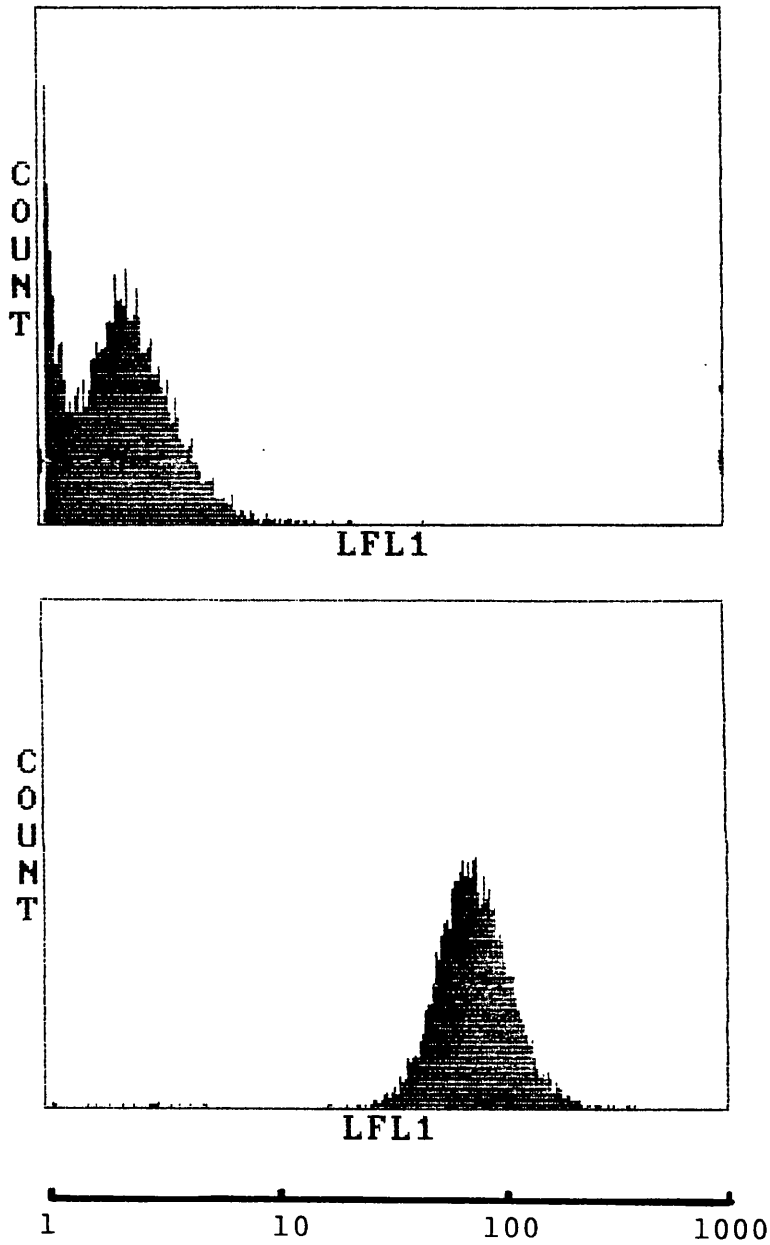


FIGURE 2.5 Single parameter histograms. Histogram of log amplified "green" (525nm) fluorescence of platelets incubated with (a) FITC-labelled mouse IgG1 (non-human reactive) and (b) FITC-labelled murine MoAb to gp IIb-IIIa. Horizontal axis: channel number (obtained by digital conversion of the log amplified voltage of the fluorescent signal). Vertical axis: cell count.

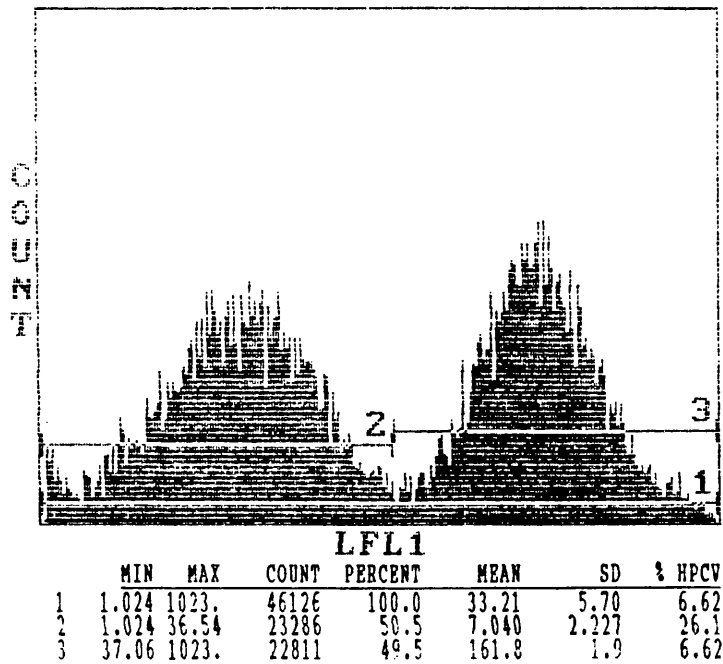


FIGURE 2.6 Analysis of fluorescence histogram showing the presence of two cell populations bearing different amounts of the fluorescent probe. The areas being analysed are indicated with numbered horizontal bars. The statistics of each area are printed below the histogram. MIN, MAX: minimum and maximum channels of area; COUNT: absolute cell count in area; PERCENT: cell count as % of total count in histogram; MEAN: mean fluorescence channel in area; SD: standard deviation and %HPCV: half-peak coefficient of variation (i.e. the CV of the upper half of the frequency distribution histogram).

2.3.2 Preparation of flow cytometer

All flow cytometric studies described here were performed using a Coulter EPICS-Profile (Coulter Electronics, Luton, UK) flow cytometer (Figure 2.7) equipped with a air-cooled 488nm argon laser. The instrument was used as supplied by the manufacturer, without modifications in the optical filters, which included a 525nm band pass filter in front of the green (FITC) fluorescence detector. According to the manufacturer's specification, the system was able to measure light scatter of particles ranging from 0.3 to 40 μ in diameter with a capacity of analyzing up to 10,000 cells per second. Isoton-II (Coulter) was used as sheath fluid. Instrument settings were as follows:

Sample volume: 100 μ l

Sample flow rate: 20-100 μ l/min

Laser power: 15mW

Sheath pressure: 7.20 psi

Side scatter PMT voltage: 400v

Green fluorescence PMT voltage: adjustable (around 1300v)

Before running samples, the laser beam was aligned and the photomultiplier tube voltage calibrated using standard fluorescent beads (Immuno-Check and Standard-Brite respectively, Coulter Electronics, Hialeah, FL). Cell concentration in the samples to be analysed on the cytometer did not exceed 5×10^6 per ml of suspending liquid. Gating on the cell population to be analysed was performed using a log forward scatter (LFS) versus log side scatter (LSS) histogram (Fig 2.4). Forward light scatter (FS) and green fluorescence (FL) signals were amplified through the instrument's three-decade log amplifier and, after digital conversion, were automatically plotted as frequency distribution histograms on a 1024-channel scale (Fig 2.5).



FIGURE 2.7 The Coulter EPICS-Profile flow cytometer.

2.3.3 Quantitation of platelet surface glycoproteins

2.3.3.1 Direct method using PRP

PRP prepared as described in 2.1.4 was diluted (usually 1:10) with PBS pH7.2 so that the concentration of platelets was approximately $30 \times 10^9/l$ and 100 μ l of the diluted PRP were incubated with the appropriate volume of FITC-labelled anti-gp MoAb (usually 5 μ l for most of the MoAbs used in these studies) for 1 hour at RT in the dark. FITC-labelled irrelevant MoAb or FITC-labelled non-immune mouse IgG of the same subclass were used as negative controls. Following incubation, the platelets were washed once in 3 ml of buffer (2.1.6), resuspended in 200 μ l PBS and analysed immediately in the flow cytometer.

2.3.3.2 Direct method using whole blood

Anticoagulated whole blood was diluted 1:10 with PBS pH7.2 and incubated with FITC-labelled MoAb or control Ab as described in 2.3.3.1. Following incubation, 3 ml of wash buffer were added and the red and white cells were sedimented by centrifugation at 400g for 1 min. The supernatant containing the platelets was transferred to another tube and analysed in the flow cytometer either directly or after a final wash.

2.3.3.3 Indirect method

150 μ l of PRP diluted 1:5 with PBS were incubated with anti-gp MoAb or control Ab as described in 2.3.3.1. Incubation was followed by one wash in 3 ml of wash buffer, with resuspension of the platelet pellet in 100 μ l PBS. This suspension was incubated with 10 μ l of the appropriate dilution (usually 1:20) of a polyclonal FITC-anti-mouse IgG (usually Sigma or Coulter goat-anti-mouse, see Table 2.1) for 40 minutes in the

dark. Finally, the platelets were washed once in 3 ml of wash buffer and resuspended in 200µl PBS for analysis in the flow cytometer.

Where indicated, the above labelling methods were used with washed or fixed washed platelets instead of dilute PRP.

2.3.4 Measurement of platelet surface-bound immunoglobulins

Platelets were isolated from EDTA anticoagulated blood samples which were kept at RT until the platelets were harvested, usually within 24 hours of blood collection. PRP was prepared as described in 2.1.4 and the platelets were pelleted and washed three times as described in 2.1.6 and they were finally resuspended in an appropriate volume of PBS pH7.2 to prepare a working suspension of $30 \pm 10 \times 10^9/l$. 150µl of the working suspension were incubated with 20µl of the appropriate dilution (see 2.4.6) of FITC-labelled goat-anti-human Ig polyclonal antibody for 1 hour at RT in the dark. Incubation with FITC-labelled non-immune goat IgG of the same IgG concentration as the anti-human antibody served as negative control. Positive controls consisted of platelets coated with IgG through preincubation (see 2.3.5) with anti-PL^{A1} serum. Following incubation, the platelets were washed in 3ml of wash buffer and the pellet was resuspended in 250µl PBS for analysis in the flow cytometer.

In cases where significant numbers of red blood cells (RBCs) were present in the PRP (e.g. in cases of severe thrombocytopenia or microcytic RBCs and large platelets) the RBCs were lysed using 1% ammonium oxalate (see 2.1.7) after the first washing cycle.

2.3.5 Detection of platelet bindable immunoglobulins in serum or plasma

Control platelets were prepared from PRP of healthy GpO individuals by pelleting (2.1.5), washing three times (2.1.6) and resuspending in PBS pH 7.2 at a concentration of $150 \times 10^9/l$. $100 \mu l$ of this suspension were mixed with $50 \mu l$ of serum or plasma under investigation and incubated for 1 hour at RT. Following this, the platelets were washed twice in 3ml of wash buffer, resuspended in PBS at a concentration of $40-50 \times 10^9/l$ and incubated with FITC-labelled anti-immunoglobulin for measurement of surface Ig as described in 2.3.4. Sera from patients with high titres of anti-PL^{A1} and/or anti-HLA antibodies were used as positive controls, whereas sera from young, untransfused, male, GpAB donors served as negative controls.

2.3.6 Determination of optimal antibody concentrations

In order to determine the optimal concentration of FITC-labelled anti-gp or anti-Ig antibody for direct platelet labelling, a range of concentrations obtained by serial dilutions of both specific and control antibody were incubated with the platelets as described in 2.3.3.1. The ratio of *signal* (mean fluorescence channel [MFC] of platelets incubated with specific antibody) over *noise* (MFC of platelets incubated with control) was calculated and the antibody concentration giving the highest signal-to-noise ratio was selected.

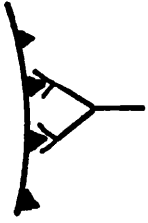
For determination of the optimal concentration of secondary FITC-labelled antibody in the indirect labelling methods, platelets were first sensitised with an excess of a . a . unlabelled anti-gp monoclonal, washed and then incubated with serial dilutions of the secondary antibody and control for calculation of signal-to-noise ratios.

2.4 PEROXIDASE-ANTI-PEROXIDASE (PAP) IMMUNOENZYMATIC STAINING OF BLOOD SMEARS

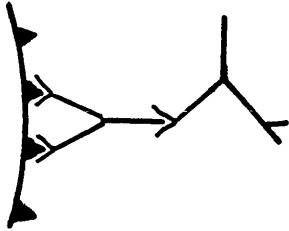
PAP and other immunoenzymatic methods have been used extensively for the study of surface and intracellular white cell markers (Mason *et al* 1975 and 1982, Moir *et al* 1983). The principle of the method is described in Figure 2.8

Method description

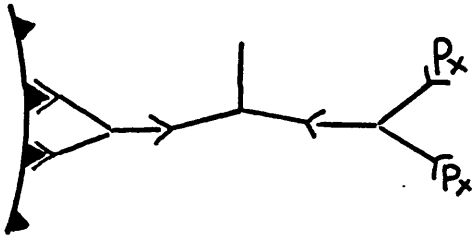
EDTA anticoagulated peripheral blood samples were routinely used. The epitopes of some antibodies were EDTA sensitive and in those cases citrated samples were used instead. Air-dried blood smears on glass slides were kept at room temperature for up to 5 days before staining. Some smears were stored wrapped in parafilm at -20°C for up to 3 months. Before staining, the smears were fixed for 10 minutes in methanol containing H₂O₂ (see appendix I) to block endogenous peroxidase activity and air dried. Thirty µl of the appropriate dilution in TBS (as determined by serial dilution experiments of the primary murine MoAb) were applied to a coverslip and the fixed smear was applied to this carefully, avoiding inclusion of air bubbles. After 40 min incubation at RT in a moist chamber, the coverslip was removed and unbound (or loosely bound) antibody was washed off by three brief (10 seconds) immersions in TBS. A 1 in 20 dilution (in PBS) of polyclonal rabbit-anti-mouse antibody (DAKO, Copenhagen) was applied as described for the primary antibody, followed by 40 min incubation and 3 washes as above. A third 40 min incubation was performed with monoclonal PAP complexes (DAKO) diluted 1 in 50 in PBS and followed by 3 washes and a final rinse in Tris-HCl. Then, the slides were flooded for 15 minutes with diamino-benzidine/H₂O₂ substrate (see appendix I), washed in tap water and air dried. Finally, they were counterstained for 5 minutes in Mayer's haematoxylin, washed for 5 minutes in tap water, air dried and mounted.



1st layer: binding of primary MoAb



2nd layer: excess of rabbit-anti-mouse antibody (RAM) results in monovalent binding of RAM to primary MoAb



3rd layer: the free arm of RAM binds to the Fc region of the murine PAP complex

For colour development:

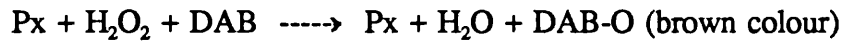


FIGURE 2.8 PRINCIPLE OF THE PEROXIDASE-ANTIPEROXIDASE IMMUNOENZYMATIC STAINING METHOD

Px peroxidase **PAP** peroxidase-antiperoxidase complex (immune complex between Px and a murine monoclonal antibody raised against Px)

2.5. PLATELET AGGREGOMETRY

Platelet aggregation studies were performed in an AGGRECORDER II four channel automatic aggregometer (Clandon, Kyoto, Japan) using PRP prepared from citrate anticoagulated blood as described in 2.1.4.

A platelet count was performed on the PRP and the platelet concentration was adjusted to $300 \times 10^9/l$ using platelet poor plasma (PPP) prepared from the same sample after removal of PRP. 270 μ l of this diluted PRP were pipetted into each of four cuvettes containing magnetic stir bars and were warmed to 37°C without stirring in the aggregometer heating block. After calibration of baseline light absorbance using a cuvette containing PPP, the pre-warmed PRP cuvettes were placed into each channel and stirring at 850rpm with parallel recording of light absorbance was started. When a stable baseline was obtained in the monitor, 30 μ l of the appropriate dilution of platelet agonist were quickly pipetted into each cuvette. Monitoring was stopped when the aggregation trace reached a steady plateau, with no further change in transmittance. Representative aggregation responses are shown in Figure 2.9. Preparation of reagents is described in Appendix I.

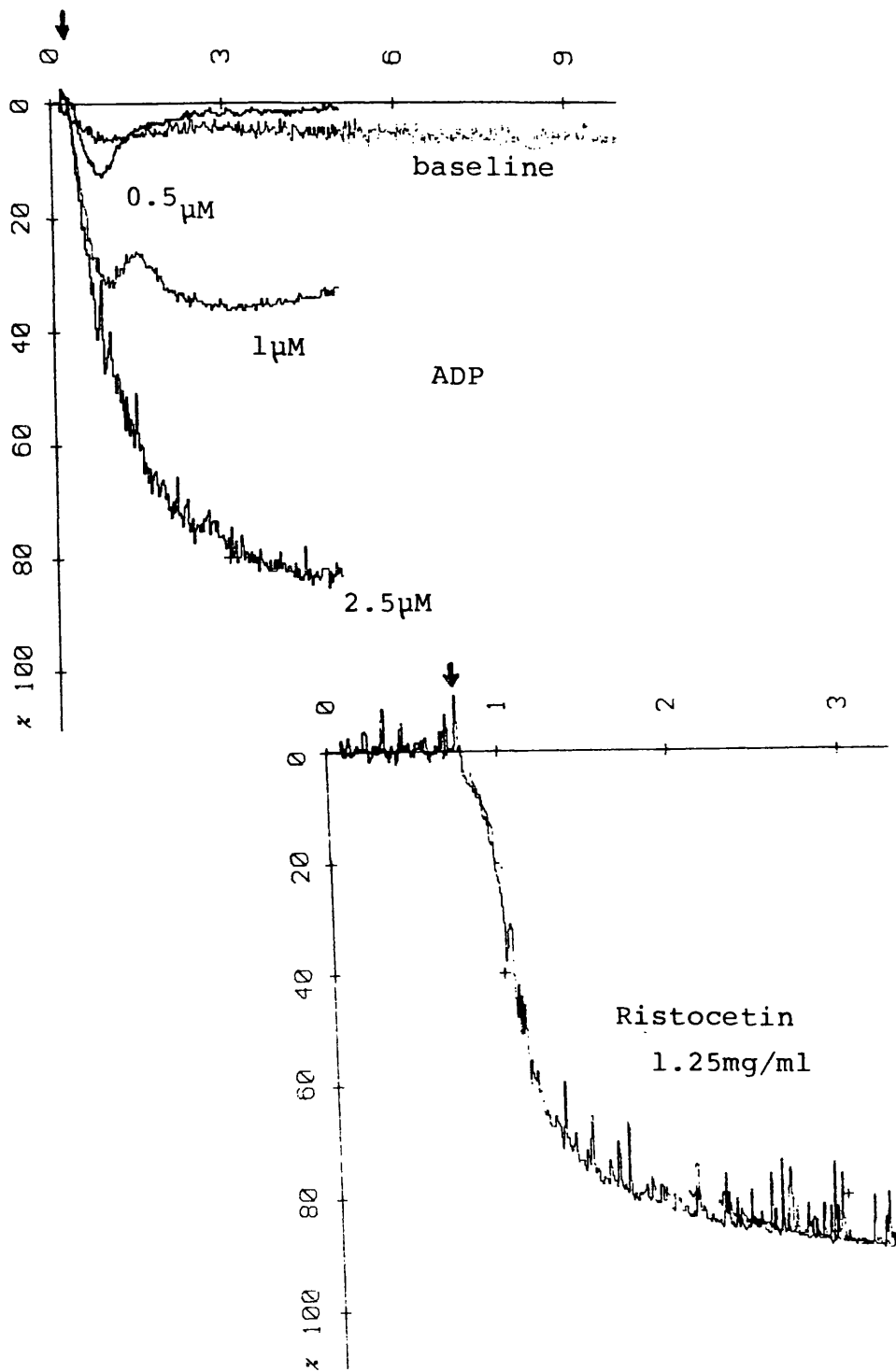


FIGURE 2.9 Normal platelet aggregation responses to ADP and ristocetin (turbidometric technique). The vertical axis indicates % change in light transmission through the PRP (increasing when platelets aggregate). Horizontal axis: Time in minutes (arrows indicate point of agonist addition).

2.6 RADIOIMMUNOMETRIC ASSAY (RIA) FOR QUANTITATION OF c7E3 BINDING SITES

The number of platelet surface sites available for binding chimaeric 7E3-IgG (c7E3-IgG) was measured by the following assay employing ^{125}I -labelled-c7E3-IgG. All the RIA measurements were performed by the staff of the Haemostasis Research Laboratory at UCH, under the direction of Professor S.J. Machin.

PRP was prepared as described in 2.1.4. and the PRP platelet count was measured. 350 μl of PRP were incubated in a 1.5ml microfuge tube with a near saturating amount of ^{125}I -labelled c7E3-IgG (final concentration of antibody in incubation mixture 0.018 mg/ml) for 30 minutes at room temperature. After the incubation, 100 μl of the mixture was carefully layered on top of 200 μl of 30% sucrose in each of three 0.4ml microfuge tubes and the platelets were sedimented through the sucrose by microcentrifugation at 12,000g for 4 minutes. Following this, the platelet pellet was separated from the supernatant by cutting off the bottom of the tube and radioactivities of pellet and supernatant (triplicates) were counted in a gamma counter. The fraction of radio-labelled antibody that became platelet-bound was calculated from the formula:

$$Y (\mu\text{g}) = [P/(P+S)] \times 1.8$$

where P = radioactivity of pellet measured as counts per minute (cpm)

S = radioactivity of supernatant (cpm) and

1.8 = amount of antibody (in μg) added to PRP.

The number of IgG molecules in 1 μg of antibody can be calculated from Avogadro's Number (6.022×10^{23} = number of molecules contained in 1 Mol) and the molecular weight of IgG (approximately 150,000) and is approximately 4×10^{12} . Therefore, the number of ^{125}I -7E3-IgG molecules bound per platelet was:

$$X (\text{molecules/platelet}) = [Y \times 4 \times 10^{12}] / C$$

where C = the number of platelets in 100 μl of PRP (i.e. the volume of PRP incubated with the radiolabelled antibody).

2.7 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR HUMAN ANTI-CHIMAERIC ANTIBODIES

Assays for human IgG antibodies to the chimaeric 7E3-fab were performed at Centocor Inc. using a standard ELISA method. Briefly, various dilutions of sera under investigation were incubated for 1 hour at 45°C in microtiter wells that had been coated with c7E3-fab and blocked with bovine serum albumin. After washing, the wells were incubated with a horseradish peroxidase-conjugated goat anti-human IgG followed by treatment with substrate (*O*-phenylenediamine and hydrogen peroxide for colour development. Colour intensity was reported as the difference in absorbance at 490 and 650nm on an automatic microplate reader. A normal range was created by analysing sera from people who had never received murine or chimaeric monoclonals. Patient samples showing reactivity above the normal range, were subsequently retested to demonstrate inhibition of binding to the plate in the presence of fluid-phase c7E3-fab, which was a requirement for classification of the response as positive. Serial dilutions of samples with positive responses were subsequently tested to establish the maximum serum dilution giving a positive response.

Appendix to Chapter 2

REAGENTS

All reagents were supplied by the Sigma Chemical Co., Poole, Dorset or by British Drug Houses (BDH), Poole, Dorset and were made up in distilled tap water (DW).

1. Formulation of phosphate-buffered saline (PBS) pH 7.2

NaCl	4.5g/l
NaH ₂ PO ₄ ·2H ₂ O	2.8g/l
Na ₂ HPO ₄	8.1g/l

2. Formulation of CPD-A1

Trisodium citrate dihydrate	26.30g/l
Citric acid monohydrate	3.27g/l
H ₂ PO ₄ monohydrate	2.22g/l
Dextrose	31.85g/l
Adenine	0.28g/l
pH adjusted to 5.6	

3. Formulation of PBS-EDTA platelet wash buffer

Disodium EDTA	3.35g/l
NaCl	4.10g/l
Na ₂ HPO ₄ (anhydrous)	3.75g/l
Bovine Serum Albumin	0.1% (w/v)
pH 7.0	

4. Formulation of ACD pH 4.9

Trisodium citrate dihydrate	22.0g/l
Citric acid monohydrate	8.0g/l
Dextrose	24.5g/l

5. Formulation of citrate-PGE₁ acidified platelet wash buffer

Hepes (free acid)	4.76g/l
NaCl	8.77g/l
Dextrose	0.90g/l
PGE ₁	0.05mg/l
ACD (see 4 above) 10% (v/v)	
pH adjusted to 6.5 with 1M HCl	

6. Preparation of platelet fixative

A 4% (w/v) solution of paraformaldehyde in PBS was heated to 70°C with occasional stirring and when this temperature was reached, 1M NaOH was added dropwise under continuous stirring until the solution was clear. An equal volume of PBS was added so that the final concentration of paraformaldehyde was 2% (w/v), the pH adjusted to 7.2 with 1N HCl and the solution stored at 4°C in the dark and used within 6 weeks.

7. Platelet aggregation reagents

ADP (Sigma): 10mM aliquots made up in isotonic saline were kept at -70°C. Shortly before use, an aliquot was thawed and 200, 100, 50, 25, 10 and 5 µM dilutions were made and placed on ice.

Ristocetin (Lundbeck Ltd, Luton): Aliquots of 12.5mg/ml in isotonic saline were kept frozen at -70°C and thawed shortly before use.

8. Reagents for PAP method:

i. Tris-HCl buffer:

250ml Tris(hydroxymethyl) aminomethane (24.23g/l)

39ml 1N HCl

q DW to 1L

adjust pH to 7.6

ii. Tris buffered saline (TBS):

Tris-HCl (above) diluted 1 in 10 in Normal Saline

iii. Fixative:

1ml 10vol.(30%) H₂O₂ + 99ml methanol

iv. Diaminobenzidine tetrahydrochloride (DAB) stock:

50mg DAB (Sigma) is dissolved in 10ml Tris-HCl and

1ml aliquots are frozen at -20°C until required.

v. Substrate for colour development (to be made up immediately before use):

(a) 1ml of stock DAB is made up to 10ml with Tris-HCl

(b) 0.1ml of a 1 in 15 dilution of 10vol H₂O₂ in Tris-

HCl is added to (a).

Chapter 3

IMMUNOENZYMATIC LABELLING OF SURFACE AND INTRACELLULAR PLATELET GLYCOPROTEINS

IN WHOLE BLOOD SMEARS:

Its use in combination with flow cytometry

3.1 BACKGROUND

A significant part of our understanding of platelet function originates from the study of clinical disorders caused by deficiency of platelet glycoproteins, like Glanzmann's thrombasthenia (GT) and Bernard-Soulier syndrome (BSS). The molecular study of glycoprotein (gp) abnormalities was facilitated by the development of polyacrylamide gel electrophoresis techniques enabling high resolution separation of glycoproteins (gps) from platelet lysates (Laemmli 1970). Topographic distribution of the various gps has traditionally been studied with immuno-electron microscopy, whereas the binding to platelets of radiolabelled anti-gp MoAbs has been the most popular method for gp quantitation. Despite the progress that has been made in the study of platelet gps, a number of important questions remain unanswered; one of them concerns the intracellular localization of the major platelet membrane gps Ib and IIb-IIIa. The small cell size with overlapping intracellular structures prohibits accurate localization and the presence of the surface-connected canicular system (SCCS), which is thought to represent multiple invaginations of the plasma membrane makes the distinction between "surface" and "intracellular" localization particularly

difficult, even when EM is employed. Moreover, activation or damage during processing may result in redistribution of many glycoproteins.

3.1.1 Flow cytometry and its limitations in the study of platelet proteins.

Most of the investigations described in subsequent chapters of this thesis are based on the use of fluorescence flow cytometry (FC) to study platelet surface antigens. In the study of platelet surface gps, FC has certain advantages over methods based on measurements of the average of a cell population, the most important being that it provides quantitative information on the distribution of antigens, thus enabling detection of phenotypically (and possibly also functionally) distinct subpopulations (Fig.3.1, Fig.3.2 and see Jennings *et al* 1986, Michelson 1987, Marti *et al* 1988, George *et al* 1986, Shattil *et al* 1987, Ginsberg *et al* 1990, Abrams *et al* 1990,). A disadvantage of FC is that, although measurements are made on individual cells, the cells themselves are not visualised and little information is obtained about cell-cell interactions, which can be related to the expression of specific antigens. Another limitation of FC is related to the difficulty in staining intracellular antigens of cells in suspension without significant distortion of cell structure. Although flow cytometric detection of intracellular gps after permeabilization of the plasma membrane has been reported (Hourdillé *et al* 1990, Michelson and Barnard 1990 and see 5.4.7), the author's experience using a number of methods is that permeabilization generally results in significant structural damage and increases non-specific antibody uptake, which limits the sensitivity of detecting low density antigens (Fig.3.3a).

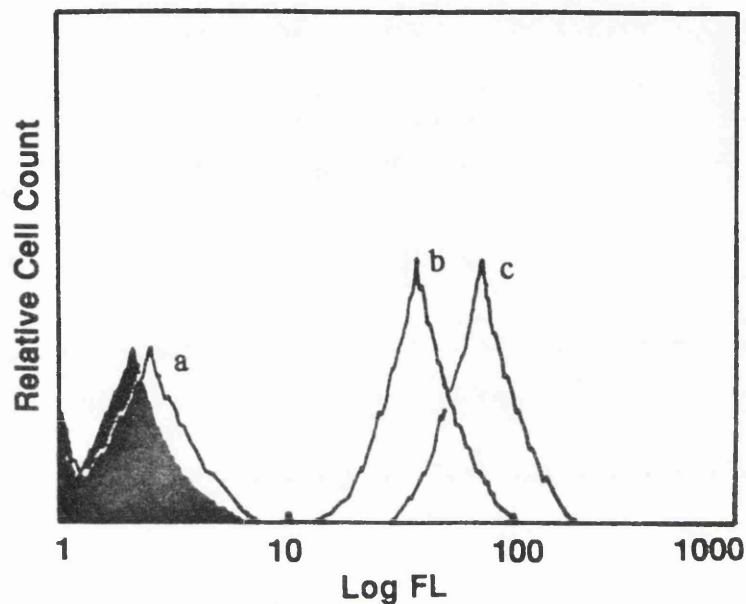


FIGURE 3.1 Example of quantitative detection of platelet surface glycoproteins by flow cytometry. Platelets labelled with a FITC-conjugated anti-IIb-IIIa MoAb (7E3-Fab) as described in 2.3.3.1. Over this range of fluorescence intensity, the Mean Fluorescence Channel number (MFC) on the 1024-channel log fluorescence scale of the instrument used can, for practical purposes, be considered as a linear function of the number of gp molecules on the platelet surface.

- a: GT homozygote, MFC=2.9
- b: GT heterozygote, MFC=41
- c: Normal control (brother of b), MFC=80

The shaded histogram represents background fluorescence (platelets incubated with FITC-conjugated, non-immune mouse IgG).

Flow cytometric measurement of platelet surface gps enables easy detection of the heterozygous state. Platelets of heterozygotes have approximately 50% of the IIb-IIIa content of matched normal controls (see also Wautier & Gruel 1989).

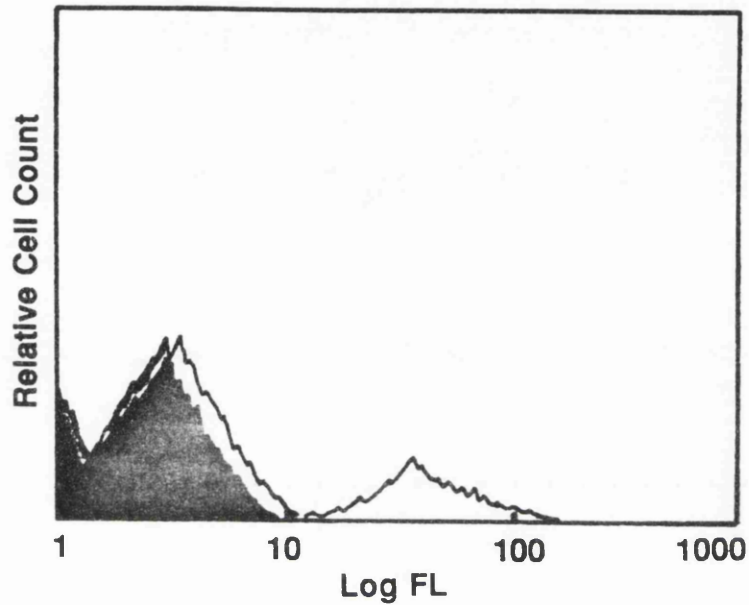


FIGURE 3.2 The ability of FC to detect phenotypically distinct subpopulations of platelets: Patient with GT and gastrointestinal haemorrhage treated with platelet transfusions. Platelets in PRP have been labelled with FITC-7E3-Fab as described in 2.3.3.1. The transfused platelets are easily distinguishable and constitute approximately 20% of the circulating platelet population. The shaded histogram represents background fluorescence (platelets incubated with FITC-conjugated, non-immune mouse IgG).

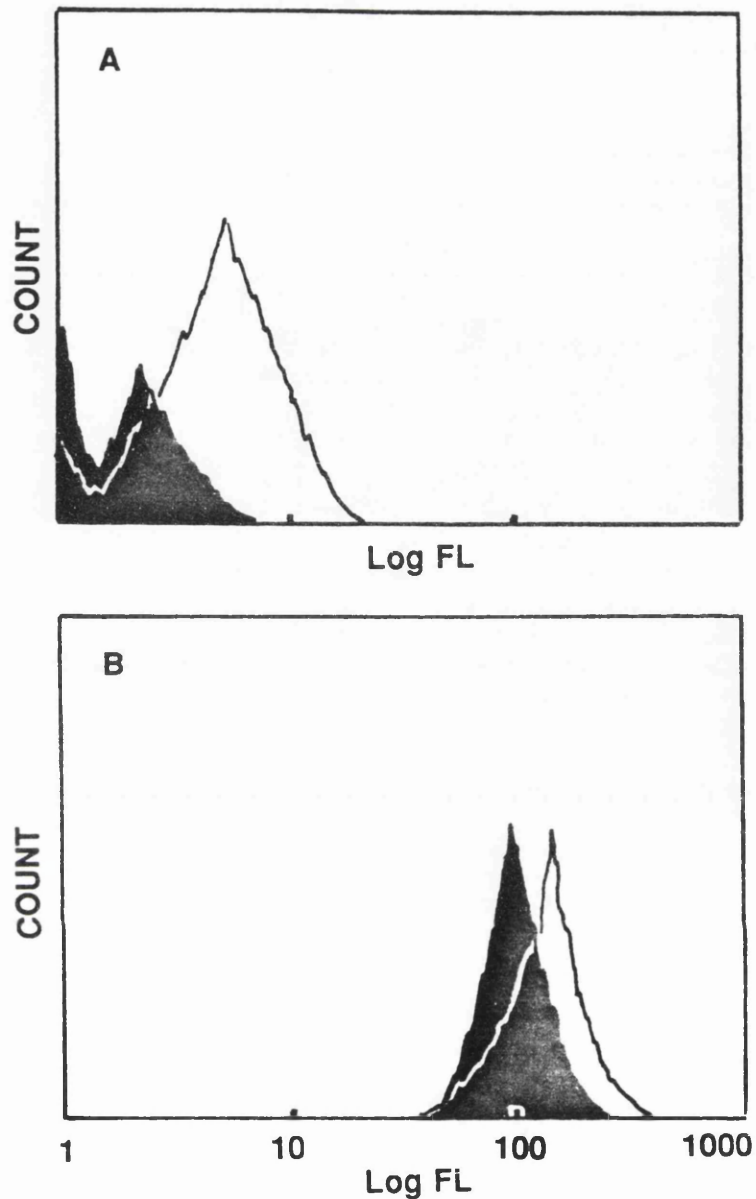


FIGURE 3.3 A Fluorescence histogram of paraformaldehyde-fixed normal platelets incubated with FITC-conjugated, non-immune mouse IgG before (shaded histogram, MFC=2.2) and after (open histogram, MFC=5.4) treatment with 0.1% (v/v in PBS) of the non-ionic detergent Triton X-100 (TX-100) for 10 minutes. TX-100 permeabilization resulted in significant increase of non-specific fluorescence.

FIGURE 3.3 B Flow cytometric measurement of gpIIb-IIIa in fixed normal platelets incubated with FITC-7E3-Fab before (shaded histogram, MFC=87) and after (open histogram, MFC=127) TX-100 permeabilization. Even after subtraction of the increased non-specific fluorescence (shown in Fig.3.3a) the IIb-IIIa amount detectable after permeabilization is significantly increased (by 43.5%). It should be noted, however, that the method provides little information regarding the possible location of this additional pool of IIb-IIIa (see discussion of this chapter).

3.1.2 Rationale and aims of immunoenzymatic labelling of platelets.

The limitations of flow cytometric measurement of platelet glycoproteins could be partly compensated for if a microscopy-based method were used in parallel with FC. Electron microscopy (EM) is not practical for routine screening of large numbers of samples. Fluorescent microscopy (FM) is limited by the necessity to label the cells as soon as possible after sampling (to avoid the non-specific fluorescence of dead cells) and the rapid fading of fluorescence intensity, which makes long-term storage of labelled preparations impossible. Added to these limitations is the poor visualization of cell morphology, especially of negative cells, by FM.

Immunoenzymatic staining of haematological samples, usually employing peroxidase or alkaline phosphatase conjugates of antibodies, has been extensively used since the early seventies, mainly in the study of white cell markers (Mason *et al* 1975, Moir *et al* 1983). Blood smears can be stored for long periods before and after staining and surface as well as intracellular antigens can be detected using simple light microscopy. It is more sensitive than FM and counterstaining with conventional reagents allows morphological assessment of the entire cell population, while sites of antibody binding are identified by insoluble colour products generated by the action of the antibody-bound enzyme on an appropriate chromogenic substrate.

In order to explore the potential usefulness of immunoenzymatic staining, platelets of healthy individuals and patients with various platelet disorders were examined with both FC and a peroxidase-

antiperoxidase (PAP) blood smear staining technique for detection of membrane and intracellular platelet antigens. There were two main aims:

-To assess the performance of PAP in detecting intraplatelet antigens and compare their staining patterns with those of platelet surface antigens.

-To obtain information about the distribution of the major platelet membrane gps Ib and IIb-IIIa. The latter contains the epitope of the MoAb 7E3, the therapeutic use of which is the subject of the studies described in chapters 5 and 6.

3.2 MATERIALS AND METHODS

The PAP staining technique and flow cytometric measurement of platelet gps have been described in chapter 2.

Platelets studied were from normal controls (section 2.1.1) and patients with BSS (n=3), GT (n=2), possible heterozygotes for GT (n=4), myeloproliferative thrombocythaemia (n=3), α -granule deficiency ("grey platelet syndrome", n=1), generalised arteriopathy with subacute synthetic arterial graft occlusion (n=2) and recent (<24 hours) cardiopulmonary by-pass (n=2). The diagnosis of BSS and GT had been made by aggregometry and flow cytometry and that of grey platelet syndrome by electron microscopy, measurement of β -TG and PF4 and aggregometry (see 1.2.4.1).

The sources of the various MoAbs are given in Table 2.1. MoAbs used were against GP-IIIa (Y2/51), GP-Ib (MM2/174 and AN/51), GP-IIb-IIIa (J15), thrombospondin (FT1/7,10,52,85 and P12) and GMP-140 (S12). Anti-HLA-DR served as non-platelet-bindable control MoAb.

3.3 PAP STAINING PATTERNS OF SURFACE AND INTRACELLULAR PLATELET GLYCOPROTEINS

3.3.1 Specificity.

No staining of platelets was observed when an irrelevant (anti-HLA-DR) first layer MoAb was used (Fig.3.4) or when the first layer was omitted. The MoAbs raised against platelet gps did not cross-react with other blood cells. The only exemption was S12 (anti-GMP-140, McEver and Martin 1984), which showed a strong reaction with neutrophils and a weak one with the majority of lymphocytes (Fig.3.11). This was not due to non-specific uptake because eosinophils and monocytes were negative.

3.3.2 Membrane antigens.

Staining with MoAbs directed against the major platelet surface gps (Ib, IIb-IIIa) resulted in a peripheral staining pattern with a "hairy" outline (Fig.3.5), which was thought to be characteristic of plasma membrane antigens. The centre of the cell was usually clear. Some platelets showed a mixed pattern with peripheral as well as central staining (Fig.3.6). The "membrane" pattern was absent and the platelets were usually completely negative when smears from patients with GT (Fig.3.7) and patients with BSS (Fig.3.8) were stained with anti-gpIIb-IIIa and anti-gpIb respectively. Interestingly, in a case of BSS diagnosed by aggregometry and FC, anti-Ib produced a weakly positive, homogenous staining pattern (Fig.3.9) contrasting with the typical peripheral "membrane" pattern seen in the same platelets when they were stained with anti-IIb-IIIa.

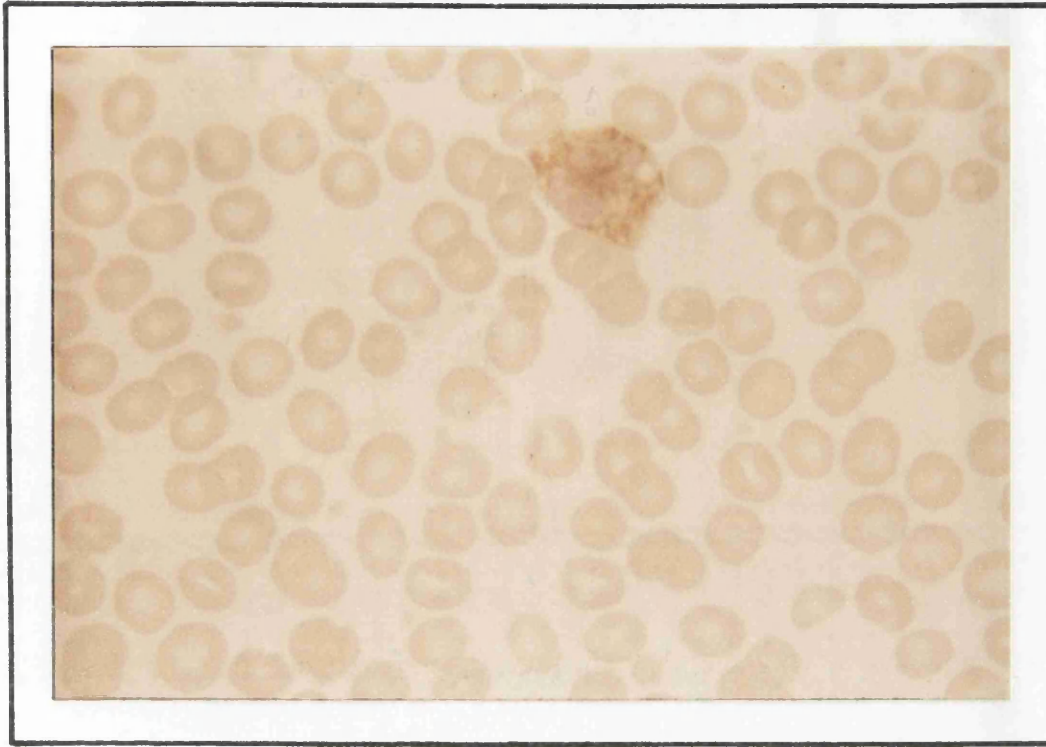


FIGURE 3.4 Use of an irrelevant (anti-HLA-DR) first layer MoAb as control, to assess non-specific staining in the PAP method. The platelets are negative, while a monocyte visible in the same field is -as expected- positive.

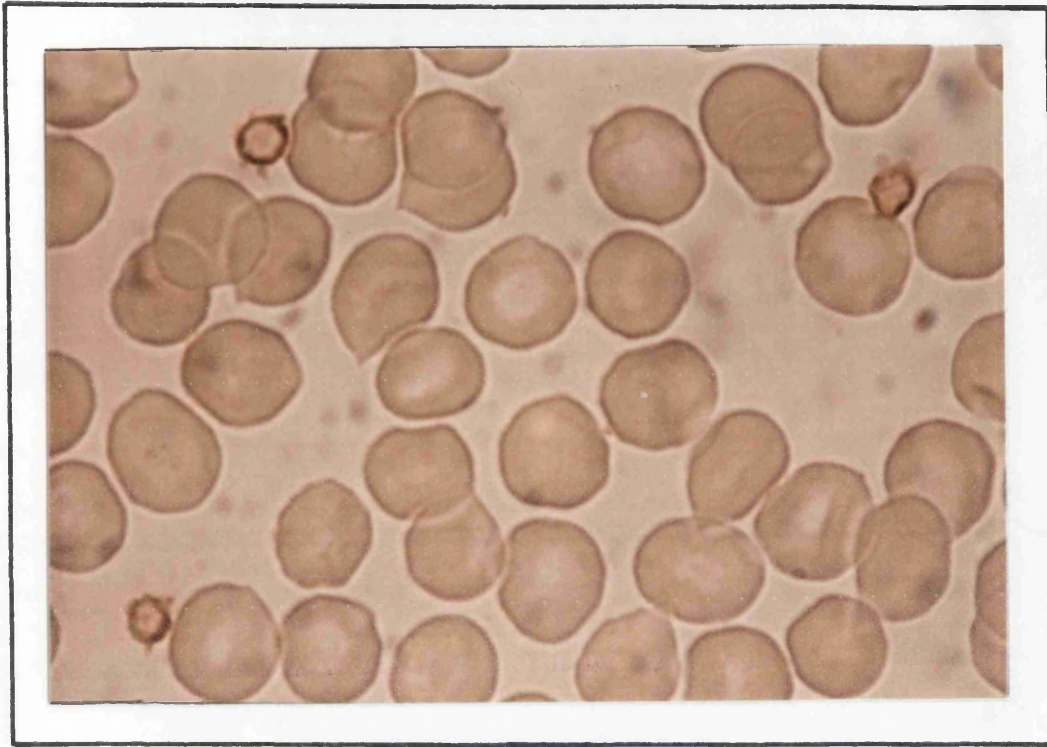


FIGURE 3.5 PAP staining of normal blood smear with an anti-gpIb MoAb (MM2/174). Membrane staining with characteristic "hairy" outline. The cell centre is clear. The same pattern was seen with anti IIb-IIIa and anti-IIIa MoAbs.

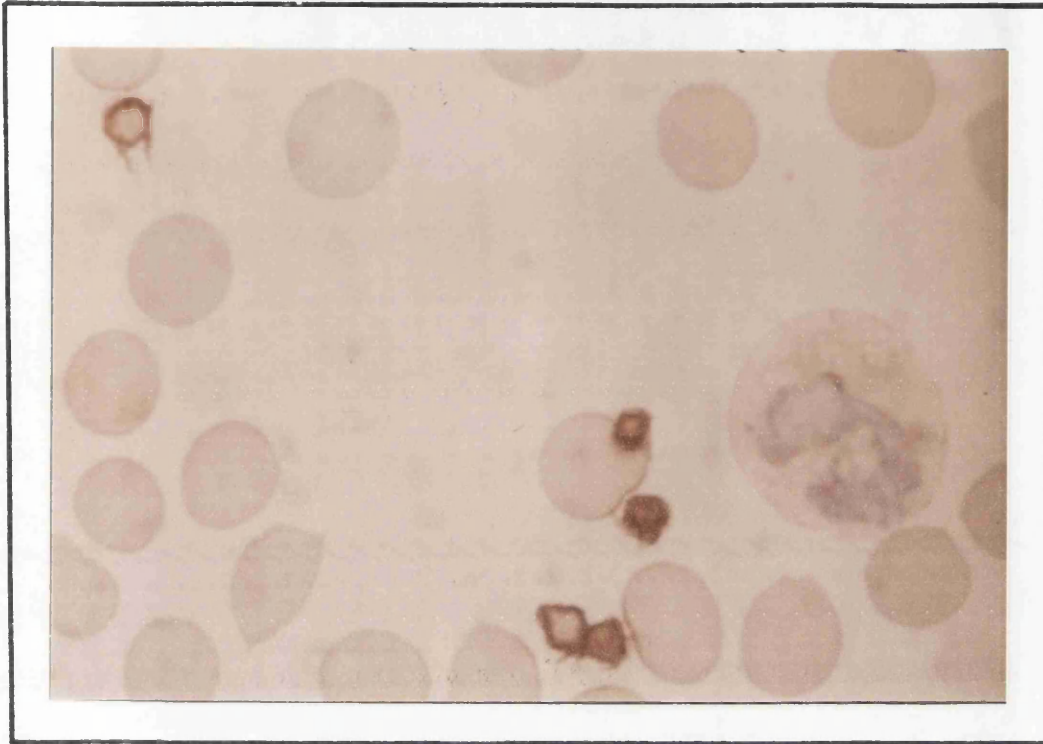


FIGURE 3.6 PAP staining of a normal blood smear with an anti-IIIa MoAb (Y2/51) showing two platelets with "mixed" (membrane and central) staining pattern. The white cell included in the field is negative.

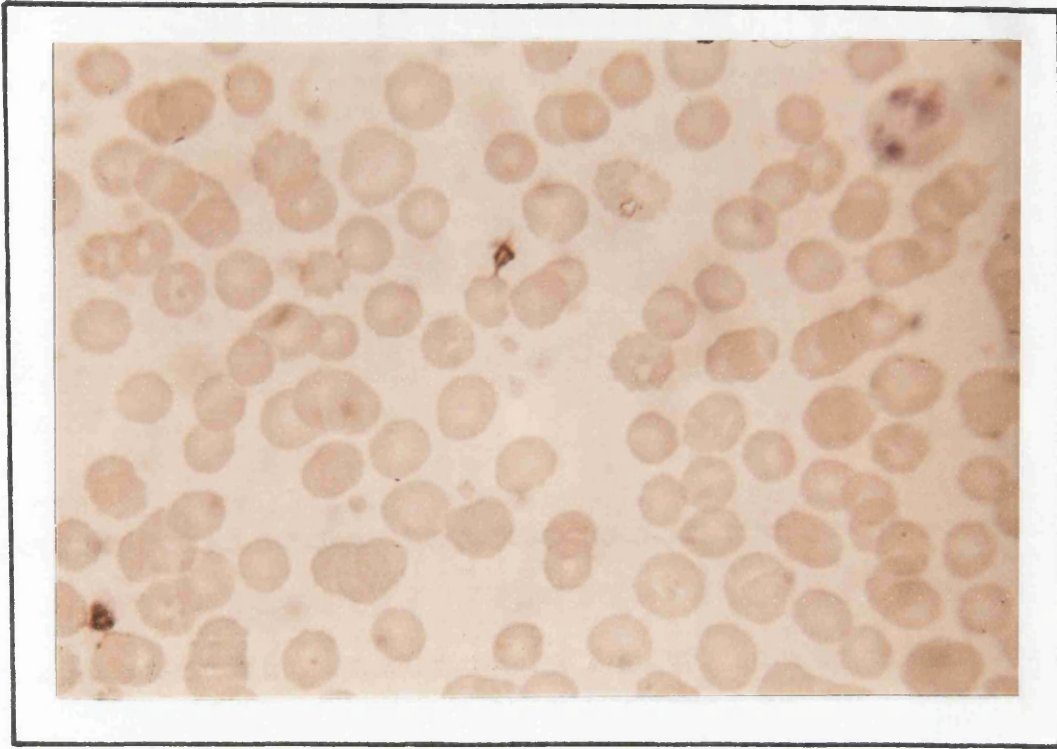


FIGURE 3.7 PAP staining of thrombasthenic platelets using anti-gpIIIa MoAb (Y2/51). Patient with GT receiving platelet transfusions. Many negative platelets are visible. The two positive (transfused) platelets act as positive controls. The percentage of transfused platelets estimated from the PAP stained smear was the same as that measured by FC (Fig.3.2).

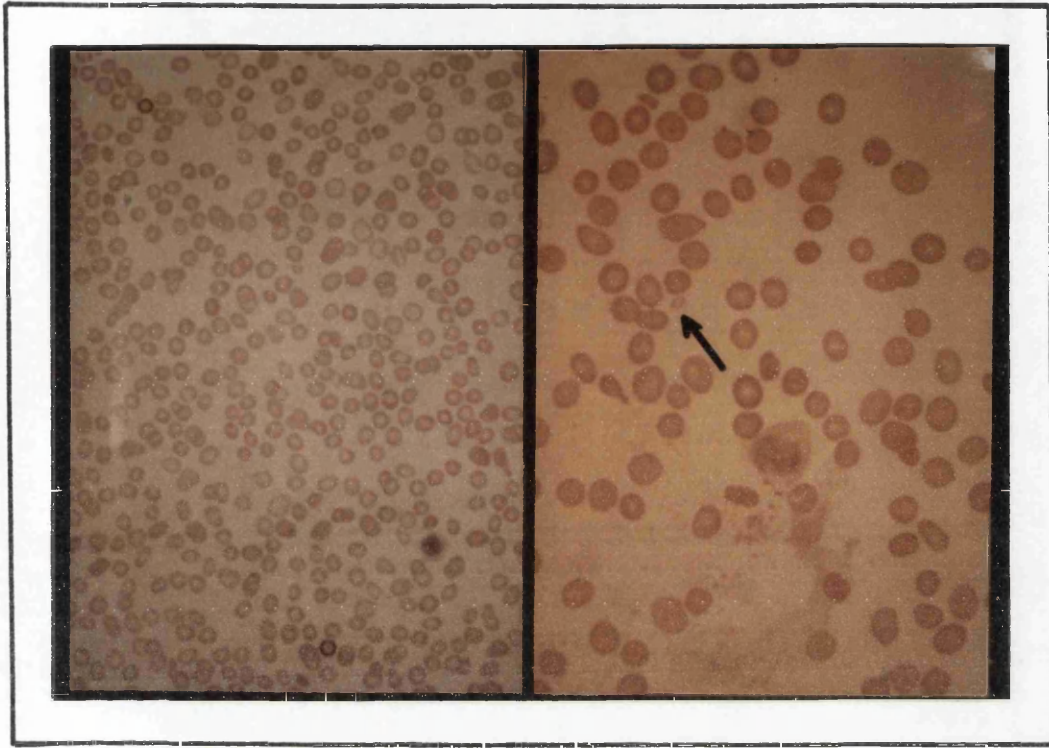


FIGURE 3.8 PAP staining of platelets in a case of BSS with severe thrombocytopenia.

LEFT: Anti-gpIIb-IIIa (J15) gave typical "membrane" staining of the large BSS platelets (two are visible in this field).

RIGHT: Platelets were negative (arrow) when stained with anti-Ib (MM2/174).

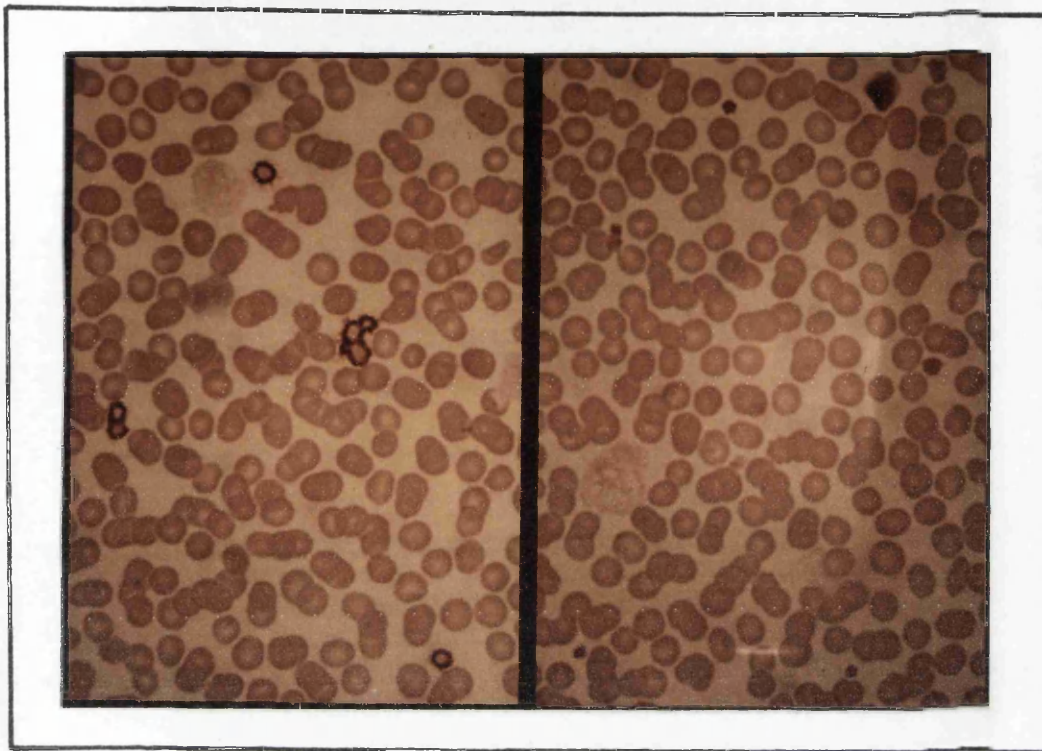


FIGURE 3.9 PAP staining of platelets in a case of BSS diagnosed by aggregometry and FC. The latter had failed to detect any gplb on the platelet surface. The weak, homogenous staining pattern obtained with anti-Ib (right) contrasts with the characteristic "membrane" pattern obtained with anti-IIb-IIIa (left).

In contrast to flow cytometry (Fig. 3.1), PAP staining did not enable reliable discrimination of GT heterozygote from normal platelets.

3.3.3 Intracellular antigens.

MoAbs to thrombospondin (TSP) produced a coarse granular or homogeneous central staining pattern without a "hairy" margin (Fig.3.10). A similar pattern was obtained with MoAbs to GMP-140 (Fig.3.11) but it was noticed that TSP often gave coarse granules or clumps of antigenic activity, a pattern not seen with GMP-140 (Figs.3.12, 3.13). This "intracellular" pattern was easily distinguishable from the "membrane" pattern obtained with anti-gpIb/IIb-IIIa staining (Fig.3.14). TSP staining showed a broad spectrum of intensity with a high percentage of negative platelets in a case of myeloproliferative thrombocythaemia associated with multiple thrombotic phenomena and a case of severe generalized arteriopathy with subacute occlusion of a synthetic femoropopliteal graft. This last patient also showed marked EDTA-dependent platelet satellitism restricted to TSP positive platelets and has been reported elsewhere (Christopoulos and Mattock 1991). In a case of grey platelet syndrome there was decreased but not absent TSP and GMP-140 reactivity. No abnormal staining patterns were seen in two patients post cardiopulmonary by-pass.

3.4 DISCUSSION

Although extensively used in the study of white cell markers, there are few reports describing the use of immunoenzymatic blood smear staining in the investigation of platelet disorders. Published evidence suggests that it can be useful in the diagnosis of gp deficiencies (Tooze *et al* 1990) and the detection of platelet surface immunoglobulins (Schmidt *et al* 1980). So far, the ability of these technique to differentiate between membrane and intracellular platelet antigens has not been studied.

The results presented here demonstrate that with PAP staining of whole blood smears it is possible to make the distinction between intracellular and surface platelet antigens. The staining patterns obtained with anti-Ib and anti-IIb-IIIa MoAbs suggest that the bulk of these glycoproteins is associated with the plasma membrane. To interpret these appearances, one should keep in mind that the MoAbs are applied to platelets adhering to glass. Escolar *et al* (1990) have shown that evagination of the SCCS onto the platelet surface occurs during platelet spreading. It is possible that the long, slender projections that stained with anti-Ib and anti-IIb-IIIa represent exteriorized channels of SCCS. This is further supported by the fact that these projections failed to stain with the MoAbs to α -granule proteins, which gave a smooth, round, cell outline. The "mixed" (membrane and intracellular) pattern seen in some platelets stained with anti-Ib/IIb-IIIa was probably due to staining of gps associated with non-evaginated membranes of the SCCS.

Woods *et al* (1986) using immunofluorescence have demonstrated the presence of a centrally located, surface-connected compartment containing a large pool of gpIIb-IIIa which, in resting platelets, was accessible to some but not other extracellular proteins and could be

exteriorized upon thrombin stimulation. Similar findings were reported by Wencel-Drake *et al* (1986) who employed immunofluorescence and immunoelectron microscopy to show the presence of gp Ib and IIb-IIIa in intracellular membrane-bounded vacuolar structures that were inaccessible to antibody (IgG) or chymotrypsin in fixed resting platelets. The same group (Wencel-Drake 1989) have since provided evidence of cycling between surface and intracellular pools of IIb-IIIa. This gp complex has also been detected in the membrane of α -granules (Gogstad *et al* 1981, Wencel-Drake *et al* 1986, Wencel-Drake 1989, Cramer *et al* 1990). In the present study, staining with monoclonal anti-IIb-IIIa or anti-IIIa antibodies resulted in a "membrane" pattern associated with a clear centre in the majority of platelets, which is against the presence of significant amounts of this gp complex in the α -granules. This was not due to artefactual loss of intracellular organelles because the same platelets gave strong central staining with antibodies to α -granule proteins. The absence of central staining with MoAbs to IIb-IIIa is not surprising. Consistent labelling of this gp in the α -granule membrane has only been achieved with polyclonal antibodies, which presumably recognize multiple epitopes on the IIb-IIIa molecule (Wencel-Drake *et al* 1986, Cramer *et al* 1990); Wencel-Drake *et al* (1986) failed to label the α -granule membrane with six different anti-IIb-IIIa MoAbs. These reports are in agreement with the immunomorphologic appearances seen in the present study and suggest that only a minute proportion of total platelet IIb-IIIa resides in the α -granule membrane. It therefore seems that the significantly increased amounts of IIb-IIIa detectable by FC after permeabilization of the plasma membrane result mainly from labelling of gp in the SCCS, which is inaccessible to the fluorescent probe in intact resting platelets (Fig.3.3b).

Glycoprotein Ib has not been detected in the α -granules but otherwise its distribution is similar to that of IIb-IIIa. Several groups have reported intracellular localization, probably in the SCCS (Wencel-Drake *et al* 1986, Michelson *et al* 1988, Hourdillé *et al* 1990, Cramer *et al* 1991) and in contrast with IIb-IIIa, there was no evidence of cycling between surface and intraplatelet stores (Wencel-Drake 1989). The findings of the present study are compatible with predominantly plasma membrane and SCCS localization for the reasons discussed in the previous paragraph. The finding of gpIb positivity in a case of BSS is not surprising, as it is known that residual amounts of Ib can be detected in the platelets of some of these patients (Finch *et al* 1990); the mainly intracellular staining pattern in the absence of any flow-cytometrically detectable surface Ib raised the question of a defect in the mechanism of surface expression of the glycoprotein, which might be playing a role in the pathogenesis of some cases of BSS.

The frequently observed coarse granular staining pattern obtained with anti-TSP MoAbs (Fig.3.12) was thought to reflect the α -granule localization of this large adhesive molecule. This was supported by the finding of weak, diffuse "intracellular" staining when the same MoAb was used to stain α -granule deficient platelets (grey platelet syndrome). The finding of a high percentage of TSP negative platelets in some myeloproliferative thrombocythaemias could be due to primary TSP deficiency of the abnormal (clonal) platelets or to the presence of "exhausted" platelets, i.e. platelets that are recirculating following in vivo activation and release/aggregation (Boughton *et al* 1977). Although abnormalities of the TSP molecule have been described in these disorders (Booth *et al* 1984) the latter appears to be a more plausible explanation, as similar appearances were seen in a case of severe arteriopathy with strong

clinical evidence of *in vivo* platelet activation. The fact that staining with anti-GMP-140 produced patterns which, while generally similar to those obtained with anti-TSP, showed less coarse granularity, might be related to the different localization of the two proteins within the α -granule (TSP contained in the granule, GMP-140 embedded in its membrane). The specific staining of neutrophils obtained with the S12 MoAb (anti-GMP-140) was an unexpected finding; it was probably due to cross-reaction with an epitope shared with some other, structurally related, cell adhesion molecule that might be present in neutrophils.

It should, of course, be acknowledged that immunolabelling methods based on microscopy are non-quantitative and only a limited number of cells can be examined. Despite these limitations, the use of PAP immunoenzymatic staining of membrane and intracellular platelet glycoproteins in whole blood smears made available new immunomorphologic information, which was useful in the diagnosis of thrombocytopathies and could contribute to a better understanding of platelet pathophysiology. Finally, it is noteworthy that the ability of PAP staining to discriminate between membrane and intracellular antigens could be useful in the screening of new anti-platelet MoAbs.

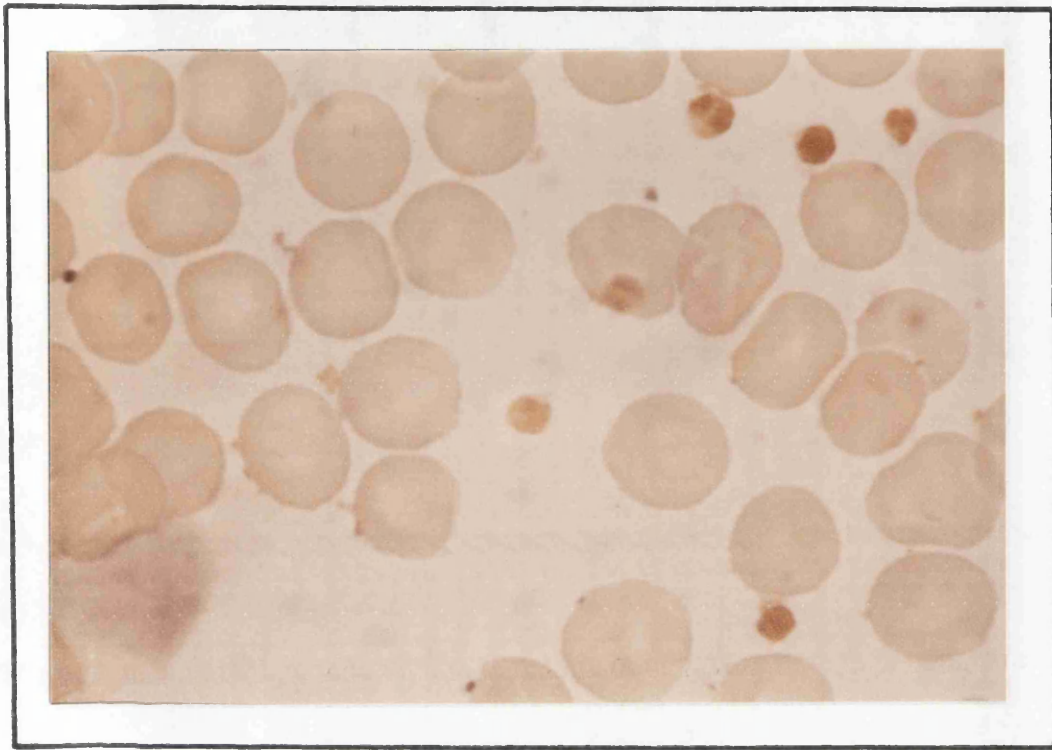


FIGURE 3.10 PAP staining of normal control platelets with anti-TSP MoAb (P12): Central, homogenous or "patchy" staining pattern with smooth edge. A negative white cell is visible in the left lower corner.

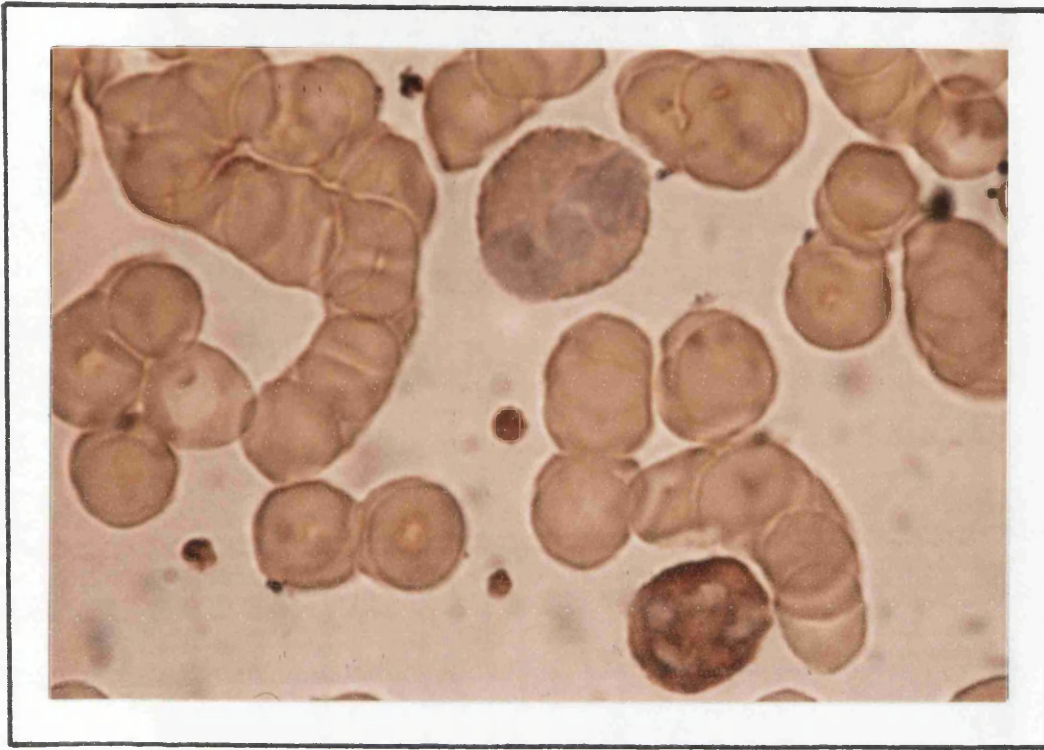


FIGURE 3.11 PAP staining of normal control platelets with anti-GMP-140 MoAb (S12): Staining pattern similar to that obtained with TSP (Fig.3.10). There is a strongly positive neutrophil. The presence of a negative eosinophil in the same field confirms the specificity of the stain (i.e. the positive reaction given by the neutrophils is not due to incomplete blocking of endogenous peroxidase activity).

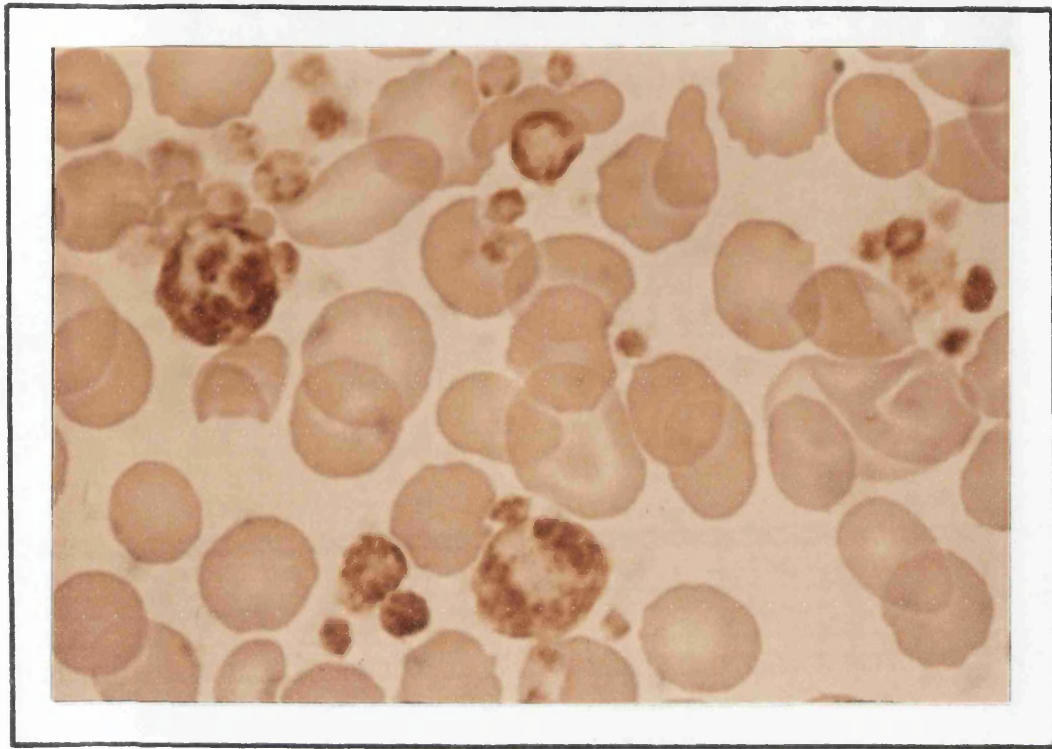


FIGURE 3.12 PAP staining of platelets with an anti-TSP MoAb (FT1/7) in a case of myeloproliferative thrombocythaemia with marked platelet anisocytosis shows clumps of "intracellular" antigenic activity.

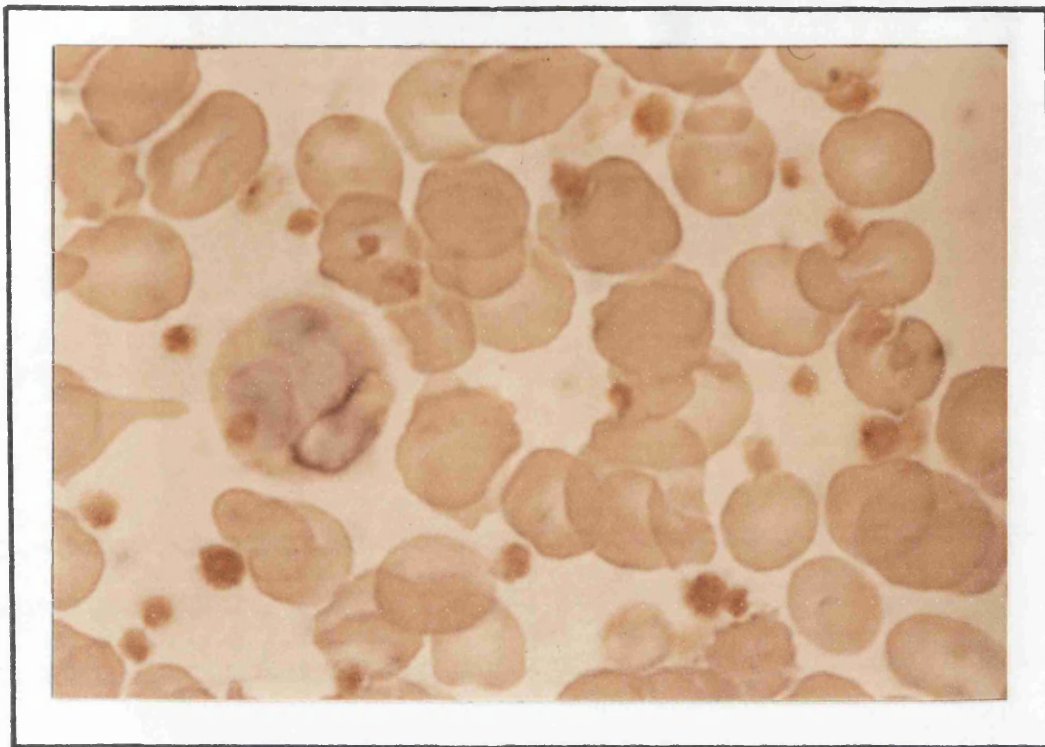


FIGURE 3.13 PAP staining with anti-GMP-140 (S12) in the same case shows a more homogenous pattern.

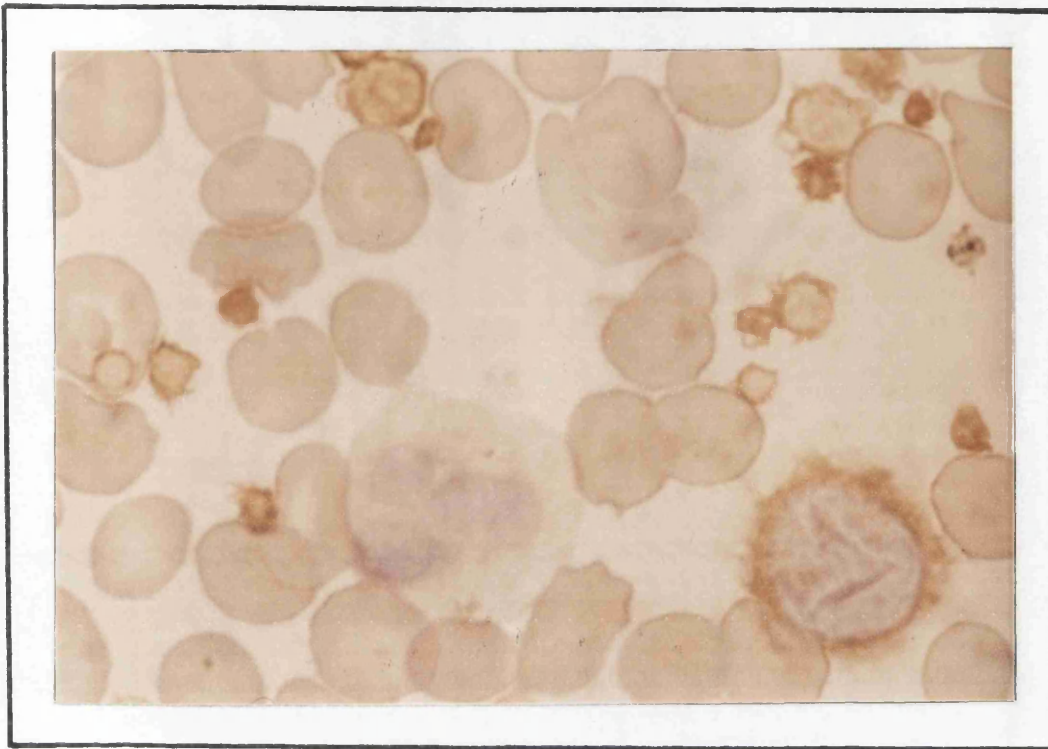


FIGURE 3.14 The same myeloproliferative platelets stained with an anti-gpIb MoAb (MM2/174) give the characteristic "membrane" pattern. Compare with the "intracellular" pattern of Figs 3.12 and 3.13.

Chapter 4

A FLOW CYTOMETRIC APPROACH TO QUANTITATIVE DETECTION OF PLATELET-SURFACE IgG

4.1 INTRODUCTION

Increased platelet associated immunoglobulin (PAIg), most commonly IgG, appears to be a cardinal feature of thrombocytopenic states where immune mechanisms are thought to be operating, like acute and chronic idiopathic thrombocytopenic purpura (ITP), post-transfusion and neonatal alloimmune thrombocytopenia (TCP) and TCP associated with drugs, SLE and other collagen disorders, human immunodeficiency virus, lymphoid neoplasia and certain infections. The nature and clinical significance of elevated PAIgG in ITP are far from well defined (Mueller-Eckhardt *et al* 1980, Kelton *et al* 1982, Shulman *et al* 1982, George 1990). Nevertheless, a test for detection of increased PAIgG is commonly performed as part of the diagnostic work-up of thrombocytopenic states and occasionally when, despite a normal platelet count, the presence of compensated immune destruction or an antibody interfering with platelet function is suspected.

4.1.1 Historical background.

Since the demonstration, in 1951, by Harrington and colleagues of a thrombocytopenic factor in the blood of patients with ITP and the subsequent localization (Shulman *et al* 1965) of this factor in the gamma globulin fraction of serum, a large number of methods for detection of

"platelet antibodies" have been developed (for review see Kelton 1983 and Schwartz 1988). Early methods were based on the measurement of secondary phenomena like aggregation, release and lysis resulting from the binding of immunoglobulin to the platelet surface. Their sensitivity, specificity and reproducibility were very limited and they have largely been abandoned. In 1975, Dixon and colleagues published a method for quantitative determination of platelet bound antibody in ITP and in the following ten years a large number of alternative methods, all based on immunological recognition of the PAIg, were developed. Some of these methods were measuring total (surface plus intracellular) platelet IgG and it is now thought that elevation of intraplatelet IgG, which is mainly located in the alpha-granules and constitutes the majority of total platelet IgG, merely indicates increased platelet turnover, whereas elevation of surface bound IgG (PSIgG) is more likely to reflect the presence of a platelet antibody (George 1990). If a method is to be useful in the investigation of immune-mediated platelet disorders it should be able to measure PSiGg excluding contributions from intracellular IgG but, even when this requirement is fulfilled, there are wide disparities in the estimates of PSiGg of both normal and ITP platelets when different methods are used (Rosse *et al* 1984, Blumberg *et al* 1986).

Fluorescence flow cytometry (FC) has emerged in the last decade as a powerful tool for the study of cell membrane antigens using fluorescently labelled antibodies (Garratty 1990). It is fast, objective, requires minimal sample manipulation and avoids the problems associated with the use of radioisotopes. It has the unique advantage that, although cells are not visualized, measurements are made on individual cells, so that a phenotypic profile based on examination of a large cell population is obtained, enabling reliable detection of subpopulations bearing different

amounts of antigen. The main application of FC in haematology has been the study of white cell markers but platelet FC is being increasingly used for the study of platelet membrane glycoproteins (Jennings *et al* 1986, Michelson 1987, Marti *et al* 1988, Abrams *et al* 1990). Detection of PSiG using FC has been the subject of several reports (Corash and Rhein-schmidt 1986, Lazarchick and Hall 1986, Rosenfeld & Bodensteiner 1986, Rosenfeld *et al* 1987, Heim and Petersen 1988, Ault 1988) and the general impression is that flow-cytometric methods are diagnostically useful in the setting of immune-mediated thrombocytopenia although objective definition of their sensitivity in terms of numbers of IgG molecules detectable on the platelet surface is not available. This is because the results of PSiG estimation by FC are expressed either qualitatively or as arbitrary units based on fluorescence intensity which makes comparisons between samples difficult, especially when, as usual, the fluorescent signals are scaled after passing through logarithmic amplifiers.

In this chapter, a flow cytometric PSiG estimation method designed for use in a clinical service laboratory is presented together with a novel approach to quantitation of PSiG measured by FC. This is based on calibration curves generated by measuring the fluorescence of platelets that have been coated with known amounts of IgG, through incubation with a chimaeric monoclonal antibody, which binds with high affinity to the glycoprotein IIb-IIIa complex. The resulting estimates of IgG amounts on normal platelets and platelets from patients with immune and non-immune thrombocytopenias are in agreement with reported data obtained using different methods and demonstrate the sensitivity of FC in detecting PSiG.

4.2 DESIGN OF THE FLOW CYTOMETRIC METHOD FOR MEASUREMENT OF PSiGg

The following were considered essential features of the method:

- Reproducibility.
- Ability to define its sensitivity objectively, i.e. by detecting known amounts of IgG on the platelet surface rather than by estimating the frequency of abnormal results in patients with clinically diagnosed immune TCP.
- Applicability in the setting of profound TCP (platelet counts $<20 \times 10^9/l$) and small sample volumes (e.g. paediatric and fetal samples).

4.2.1 Preparation of platelets and flow cytometry

The procedure for measurement of PSiGg was as described in section 2.3.4. To increase platelet recovery from profoundly thrombocytopenic samples, centrifugations at 2000g were prolonged to 8 minutes resulting in $\geq 80\%$ recovery after three centrifugations.

Cytometer preparation and gating on the platelet population was performed as described in 2.3.2. Logarithmically amplified fluorescent signals from fifty thousand platelets were usually analysed in each test and the Mean Fluorescence Channel (MFC) number on the 1024-channel scale of the instrument was recorded. The MFC numbers obtained with the FITC-labelled anti-IgG and FITC-labelled non-immune IgG of the same species were used as arbitrary units of specific (SF) and non-specific (NSF) fluorescence respectively.

4.2.2 Feasibility of PSiG detection by FC.

Isolation of adequate numbers of platelets for PSiG quantitation was possible even when the platelet count was as low as $2 \times 10^9/l$ although in cases of such profound thrombocytopenia a comparatively large volume of blood (e.g. 20ml) was used to harvest adequate numbers of platelets. The test was performed successfully on several very small (*ca.* 0.5ml) thrombocytopenic ($14-30 \times 10^9/l$) samples obtained by cordocentesis of pregnant women with a history of alloimmune neonatal thrombocytopenia complicating previous pregnancies. There were no instances of failure to complete the test due to technical problems. A batch of eight tests (including controls) could be completed in 2½ hours. Standing of EDTA samples at RT for up to 24 hours did not affect the results.

Sensitization of platelets to serve as positive controls was achieved as described in 2.3.5, using serum from patients with high titres of anti-PL^{A1} antibody. Figure 4.1 shows typical histograms of NSF (with FITC-non-immune goat IgG) and SF (with a polyclonal FITC-goat-anti-human IgG) of normal control platelets before and after sensitization.

4.2.3 Selection of fluorescent probe.

The following FITC-conjugated anti-immunoglobulins were assessed as probes for detection of PSiG (see Table 2.1):

- Monoclonal anti-human IgG (Fc-specific).
- Polyclonal goat-anti-human IgG (γ -chain specific).
- Polyclonal goat-anti-human IgG (Fc-specific).
- Polyclonal goat-anti-human IgG,IgM,IgA (polyvalent).

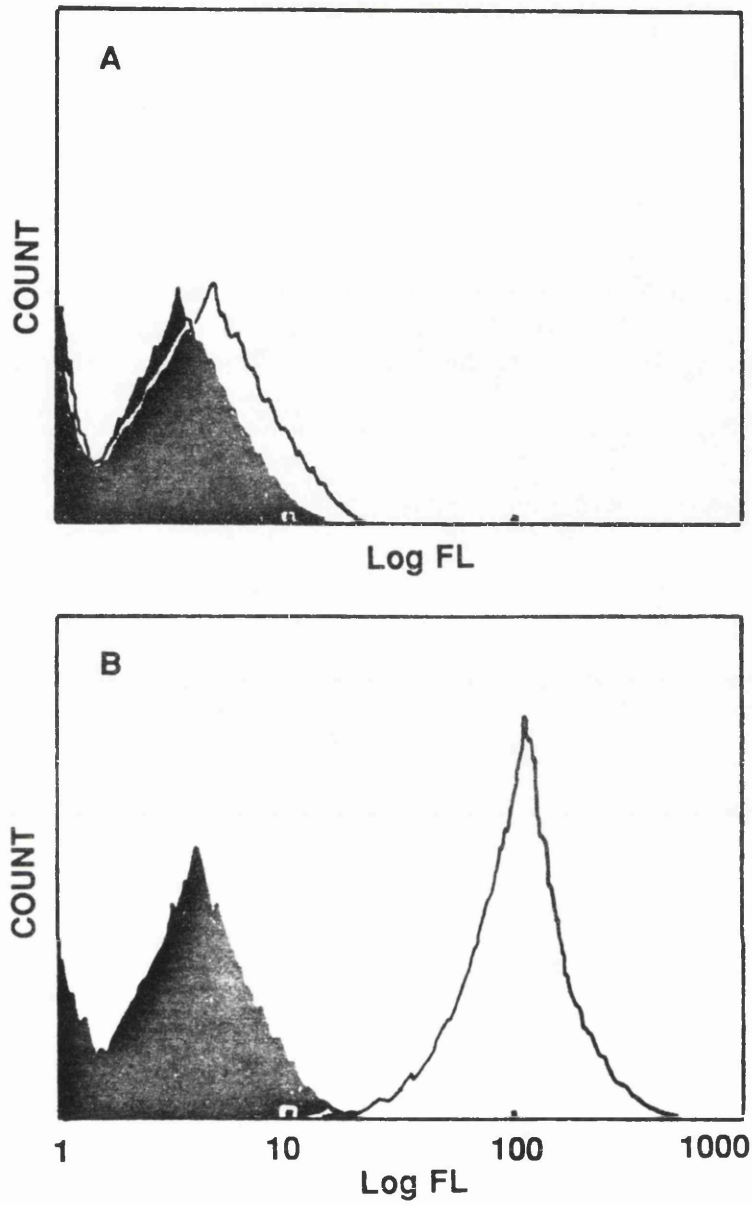


FIGURE 4.1 PSiG detection: Normal control platelets (PLA¹ positive) incubated with FITC-conjugated non-immune goat IgG (shaded histograms) and FITC-conjugated goat-anti-human IgG (open histograms) before (A) and after (B) sensitization with anti-PLA¹ serum.

The optimal concentration of the various FITC-labelled anti-immunoglobulins was determined by incubating control platelets before and after anti-PL^{A1} sensitization with serial dilutions of the anti-immunoglobulins and selecting the concentration that gave the best positive-negative separation (highest signal-to-noise ratio, Fig. 4.2).

As shown in Fig. 4.2, the polyclonal anti-immunoglobulins gave a much higher signal-to-noise ratio than the monoclonal. This was presumably due to the polyclonal recognizing more than one epitope on the IgG molecule and suggested that, as PSiG is normally a low density antigen, its flow cytometric detection using MoAbs might have an unacceptably low sensitivity. This was subsequently confirmed when the monoclonal anti-IgG failed to detect raised PSiG easily detectable by the polyclonal in platelets coated with several thousands IgG molecules during calibration experiments (4.2.4). Furthermore, the polyclonal raised to the whole gamma-chain bound more anti-immunoglobulin molecules per platelet than the one directed against the Fc region of IgG. The polyclonal FITC-conjugated anti-IgG (γ -chain specific) was therefore selected for PSiG quantitation studies. Comparable sensitivity was obtained with the polyclonal polyvalent (anti-IgG,IgM,IgA) which might be preferable for routine diagnostic use because of the theoretically reduced probability of missing cases with isolated elevations of PSiGM or PSiGA in the presence of normal PSiG (Nel *et al* 1983).

4.2.4 PSiG in normal controls.

The method was applied to measure PSiG in EDTA-anticoagulated samples from 71 healthy individuals (2.1.1). There was a comparatively weak ($r=0.46$) but statistically significant ($P<0.001$) correlation between

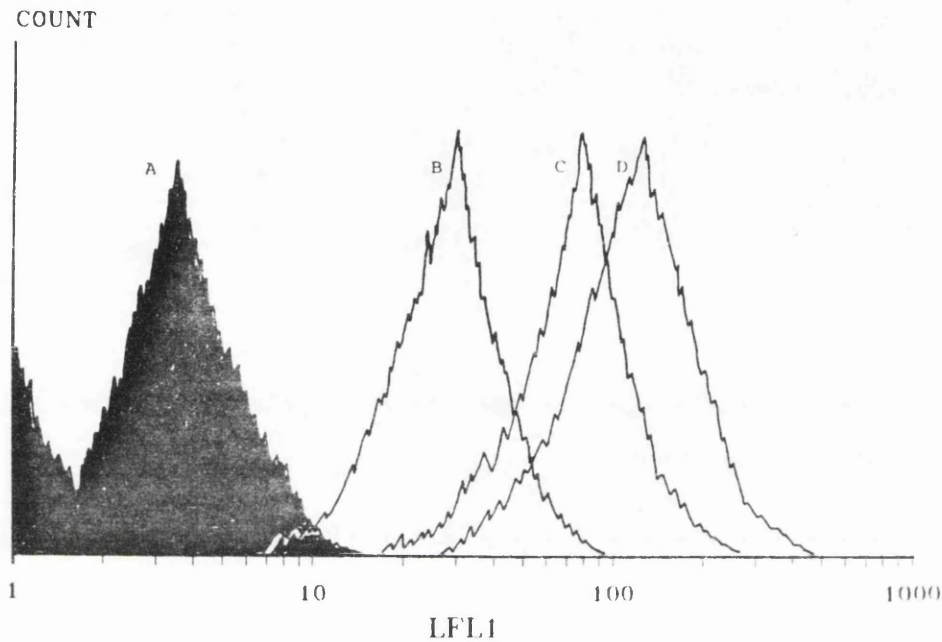


FIGURE 4.2 Fluorescence histograms of anti-PLA¹-sensitised platelets incubated with optimal concentrations of FITC-mono anti-IgG (Fc-specific) (B), FITC polyclonal anti-IgG (Fc-specific) (C) and FITC polyclonal anti-IgG (gamma-chain specific) (D). The respective mean fluorescence channels (MFC) were 28, 80 and 138. Histogram A (shaded) represents the background fluorescence (noise) which was obtained by incubating the same platelets before sensitisation. Background fluorescence was standardised at MFC=4 for all three antibodies by appropriate adjustment of the photo-multiplier tube voltage. The optimal concentration for each antibody was the one that gave the highest signal-to-noise ratio. LFL1: Logarithmic scale of green fluorescence.

SF and NSF in the normal controls. To compensate for this, results of PSiG detection tests were expressed as Relative Fluorescence Intensity (RFI) defined as the ratio SF/NSF. There was no correlation between RFI and NSF ($r=0.08$) whereas there was significant ($P<0.001$) strong correlation ($r=0.92$) between RFI and SF. The individual results of SF, NSF and RFI in the normal controls are given in Table IV.1 (Appendix to Chapter 4); their statistical analysis is included in Table 4.1.

In normal control platelets the SF was always higher than the NSF ($RFI>1$, $P<0.001$, mean $RFI=1.63$) despite using identical concentrations of FITC-anti-IgG and FITC-control IgG with the same F/P (fluorescein to protein) ratios. Moreover, as can be seen in Table 4.1, the coefficient of variation (CV) of the SF was higher than the CV of the NSF. These differences suggest that the signal obtained with the FITC-anti-IgG reflected either specific detection of variable amounts of surface IgG in normal platelets or platelet-bound immune complexes resulting from the reaction of the fluorescent probe with free IgG in the suspension. This free IgG could be the result of release from intraplatelet stores caused by activation or damage during platelet preparation (Pfueller and David 1986, George 1990).

4.2.5 PSiG in thrombocytopenic patients.

The method was applied to measure PSiG in 87 patients with thrombocytopenia (platelet count $<150 \times 10^9/l$). Among them were occasional patients who, despite platelet counts within the normal range, had impaired platelet function of suspected immune aetiology or had shown only partial recovery from an acute episode of thrombocytopenia. The patients were divided in three groups:

"Positive clinical controls": Sixteen patients with a pre-established clinical diagnosis of immune-mediated TCP (7 acute ITP, 6 chronic ITP, 1 SLE, 2 other connective tissue disorders). Diagnoses were based on the history, presence of megakaryocytic hyperplasia and clinical course including response to treatment). This group had a mean platelet count of $36.6 \times 10^9/l$ (range $2-125 \times 10^9/l$).

"Negative clinical controls": Nine patients in whom an immune aetiology of TCP was considered unlikely (4 chemotherapy related, 3 previously untransfused myelodysplastic syndromes, 1 thrombotic thrombocytopenic purpura, 1 microangiopathic haemolytic anaemia). This group had a mean platelet count of $52.1 \times 10^9/l$ (range $9-138 \times 10^9/l$).

"Unknown aetiology": Samples from 62 patients with TCP or a suspicion of immune-mediated disorder of platelet function in whom a clinical diagnosis was difficult to make were tested prospectively (mean platelet count 83.6, range 2 to $190 \times 10^9/l$). In this group, 31% consisted of cases of incidental TCP in otherwise healthy individuals or patients with disorders not known to be associated with TCP (n=19). Other subgroups included pregnancy-associated TCP (n=8), TCP associated with deranged liver function (n=4), lymphoproliferative disorders (n=3), Gaucher's disease (n=3), probable drug-induced TCP (n=3), rheumatoid arthritis (n=2), sarcoid (n=2) and TCP associated with morphological abnormalities of megakaryocytes suggestive of myelodysplasia in the presence of essentially normal myelo- and erythropoiesis (n=6). Patients incidentally discovered to have unexplained low platelet counts and normal bone marrow appearances were not classified as chronic ITP because the latter implies immune aetiology.

The SF, NSF and RFI results of the patients studied (Tables IV.2 and IV.3, Appendix to Chapter 4) are compared with those of normal controls in Table 4.1. The distribution of PSiG (expressed as RFI) in the above patient groups is presented in Fig. 4.3. It can be seen that the RFI of the majority (75%) of the immune thrombocytopenia controls was above the upper limit (mean + 2SD) of the normal range (mean difference 4.74, $P < 0.001$) and this percentage did not change when the upper limit of normal was raised to 3 SDs above the mean. Seven of the 9 non-immune thrombocytopenia controls had RFI within the normal range, while in the remaining two the RFI was between 2 and 3 SD above the mean of the normal controls. From the prospectively studied group of 62 TCP patients with no established aetiology, 22 had increased RFI and their individual results and clinical details are shown in Table 4.2.

4.2.5.1 The distribution of PSiG.

In cases of raised PSiG, the distribution of IgG in the platelet population was usually normal, albeit of varying width. Occasionally, two distinct populations, one with high and one with normal levels of PSiG were present. This was usually but not exclusively seen in the setting of severe thrombocytopenia and has also been reported by other investigators (Ault 1988). Staining of these samples with anti-platelet glycoprotein MoAbs (Fig. 4.4) showed that both populations consisted of platelets, thus excluding the presence of "non-platelet particles" e.g. red cell fragments commonly found in severe ITP (Zucker-Franklin and Karpatkin 1977) co-purifying with the platelets. It appears that, at least in some cases, this phenomenon is artefactual as it was seen in only one of three samples taken from the same patient within 24 hours. What is

		MEAN	SD	CV(%)	RANGE
Normal controls n=71	NSF	3.22	0.36	11.3	2.74-5.05
	SF	5.27	1.47	27.9	3.13-10.5
	RFI	1.63	0.40	24.5	1.04-3.03
Immune thr/penia n=16	NSF	3.44	0.61	17.8	2.65-4.75
	SF	22.6	16.9	75.0	4.07-67.2
	RFI	6.37	3.97	62.4	1.09-14.2
Non-immune thr/penia n=9	NSF	3.19	0.53	16.6	2.51-4.31
	SF	6.62	1.55	23.5	4.07-9.58
	RFI	2.09	0.43	20.4	1.46-2.59
Thr/penia ?cause n=62	NSF	3.44	0.49	14.2	2.46-4.87
	SF	10.8	13.7	127.6	3.16-86.8
	RFI	3.03	3.42	112.9	1.01-19.2

TABLE 4.1 Summary statistics of Non-Specific (NSF) and Specific (SF) fluorescence and Relative Fluorescence Intensity (RFI) of the 158 samples analysed. SD: standard deviation of the mean. CV: coefficient of variation.

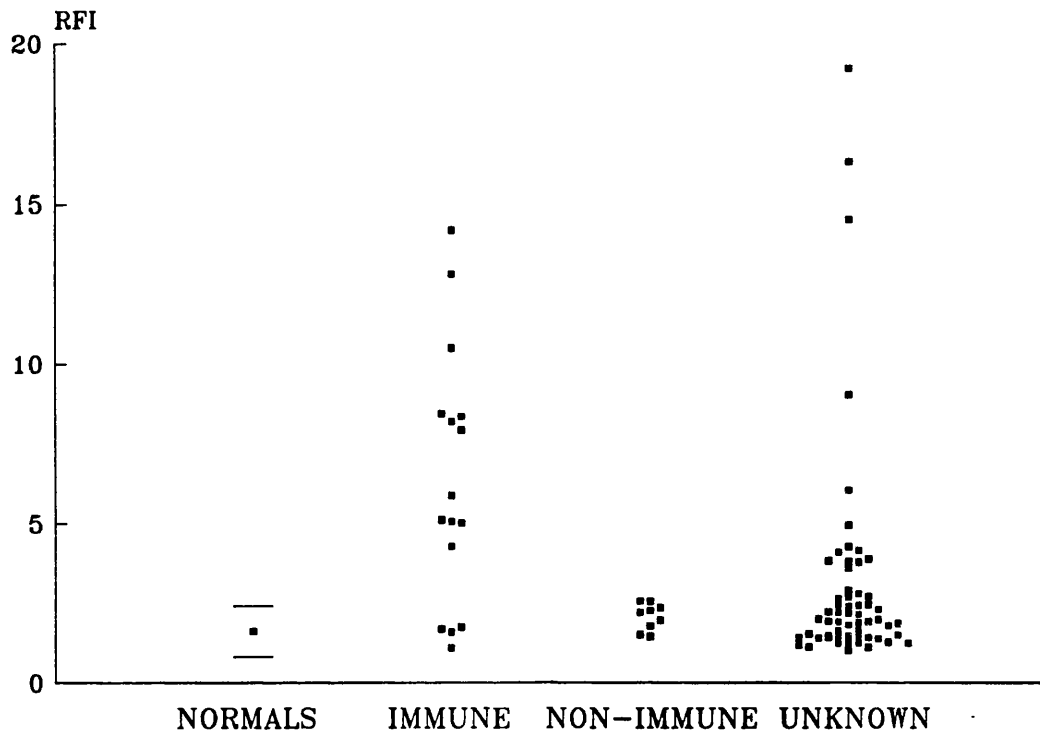


FIGURE 4.3 Results of PSiG detection expressed as Relative Fluorescence Intensity (RFI) in normal controls (n=71), patients with previously diagnosed immune (n=16) or non-immune (n=9) thrombocytopenia and prospectively studied patients with thrombocytopenia or platelet dysfunction of unknown aetiology (n=62). The normal range is depicted as mean \pm 2SD of the group of 71 normal controls.

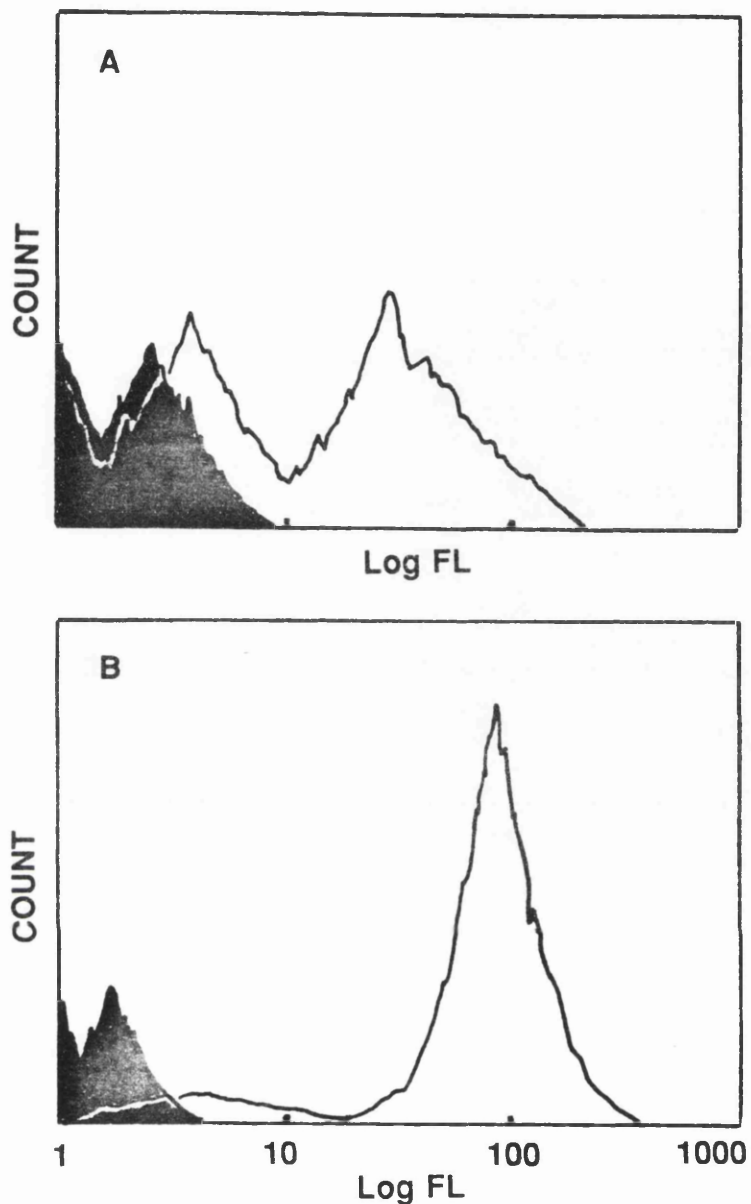


FIGURE 4.4 **A:** Bifid fluorescence histogram suggesting the presence of two platelet populations bearing different amounts of PSiG in a case of acute thrombocytopenia. **B:** Staining of the same sample with an anti-gpIIIa MoAb (section 2.3.3.1) demonstrates that practically all the analyzed cells are platelets.

The shaded histograms represent background fluorescence obtained by incubating the platelets with irrelevant, FITC-conjugated IgG of the same species as the specific fluorescent probe.

the mechanism accounting for the loss of platelet-bound IgG from only a proportion of the platelets in a sample is difficult to postulate.

4.2.6 Detection of platelet-bindable immunoglobulins in serum/plasma.

Throughout this thesis, the term "platelet-bindable immunoglobulin" has been used instead of "serum anti-platelet antibody" because the fact that an Ig binds to platelets does not necessarily mean that this Ig represents a specific antibody to a platelet antigen. Non-specific binding to the platelet Fc receptor or other components of the platelet membrane is always a possibility. Moreover, recent studies have identified intracellular and cytoskeletal platelet proteins that bind normal serum immunoglobulins and naturally occurring antiglobulins (Reid *et al* 1990, Pfueller *et al* 1990).

The reported frequency of demonstrating platelet-bindable Ig in ITP sera is very variable - 26% (Faig and Karpatkin 1982) to 65% (von dem Borne 1980) - and a positive result, especially of low titre, is of limited diagnostic help compared to the finding of raised PSIg. Nevertheless, in certain clinical settings (e.g. alloimmune neonatal TCP, post-transfusion purpura, refractoriness to platelet transfusions, drug-dependent TCP) the diagnosis depends on the ability to demonstrate the presence of serum Ig that can, under certain conditions, bind to intact platelets.

Flow cytometric detection of platelet-bindable Ig was performed by incubating test serum/plasma with control GpO platelets as described in 2.3.5. Figure 4.5 shows an example of results obtained when this method

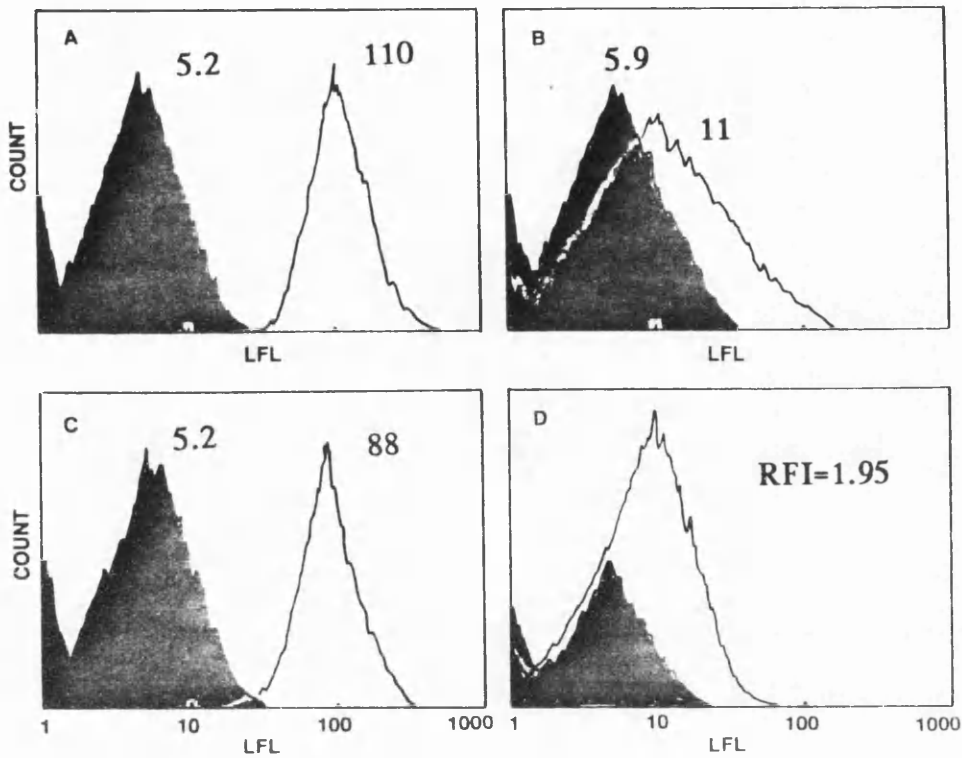


FIGURE 4.5 Neonatal alloimmune thrombocytopenia. On-call flow cytometric investigation of platelets and plasma (from a EDTA sample) of a mother who had just delivered a severely thrombocytopenic baby (platelet count $11 \times 10^9/l$).

A. PSiGg of control GpO, PL^{A1} -positive (A1/A1) platelets incubated with a serum containing high titres of anti- PL^{A1} IgG (open histogram) or serum from a GpAB, previously untransfused male (shaded histogram).

B. PSiGg of mother's platelets incubated with the same sera. The very low level of IgG binding to her platelets indicates that she is PL^{A1} negative. Platelets of PL^{A1} heterozygotes (A1/A2) bind ca. 50% of the amount of IgG bound to platelets of homozygotes (A1/A1) upon incubation with strong anti- PL^{A1} sera and give MFC values approximately 50% of those obtained with A1/A1 platelets (see calibration curve in Fig. 4.9).

C. PSiGg of control PL^{A1} -positive platelets incubated with mother's PPP (open histogram) or control serum (shaded histogram) demonstrates that her plasma contains high titres of IgG bindable to PL^{A1} -positive platelets (compare with A). It was later shown that her husband and the newborn were PL^{A1} -positive.

D. PSiGg (expressed as RFI) of washed mother's platelets. Open and shaded histograms represent fluorescence obtained with FITC-conjugated goat-anti-human IgG and non-immune goat IgG respectively. The absence of elevated PSiGg in the presence of high plasma titres of IgG bindable to heterologous platelets is against autoimmune aetiology of the neonatal TCP.

In panels A to C the mean fluorescence channels (noted by the histograms) of platelets incubated with polyclonal, FITC-conjugated goat-anti-human IgG are used as a measure of PSiGg.

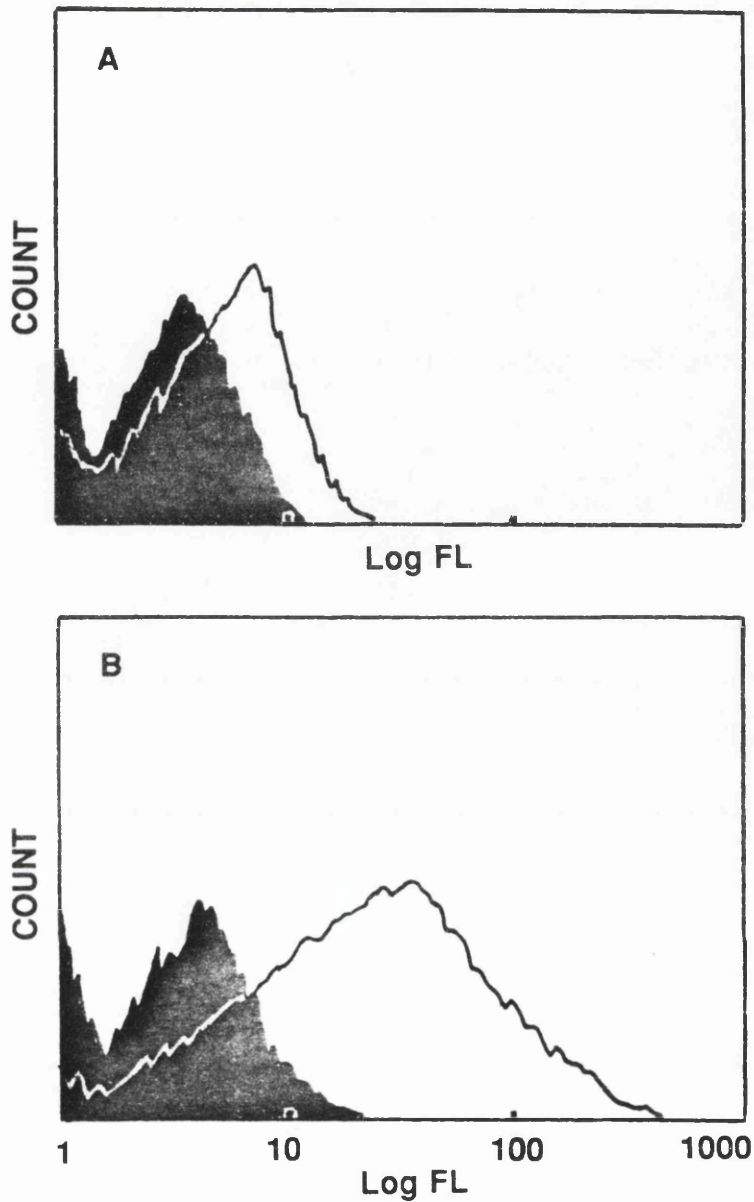


FIGURE 4.6 Detection of PSiGg in platelets from GpO (A) and GpA (B) healthy donors that have been incubated with serum from a GpO individual containing anti-A IgG. The broad-based fluorescence histogram in B indicates heterogenous distribution of the GpA antigen in the platelet population.

Open histogram: Fluorescence obtained with FITC-conjugated goat-anti-human IgG.

Shaded histogram: Fluorescence obtained with FITC-conjugated non-immune goat IgG.

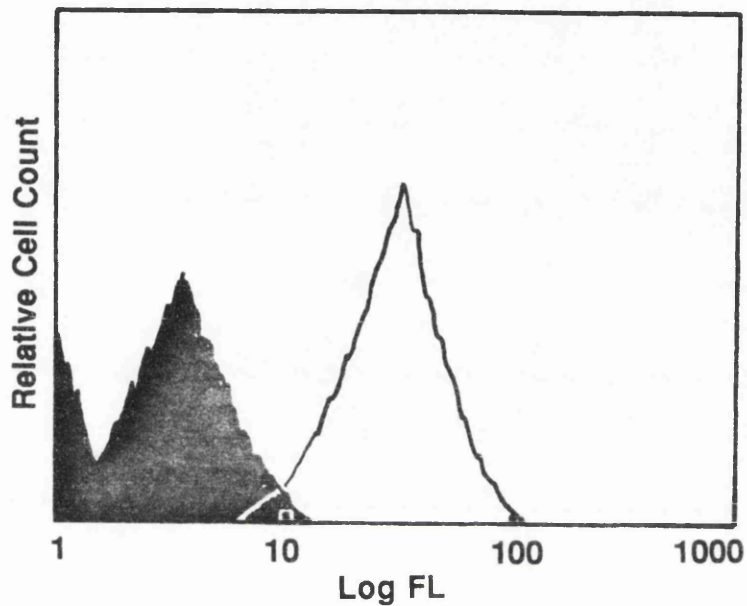


FIGURE 4.7 Detection of PSiGg in control GpO platelets incubated with a serum containing high titres of anti-HLA antibodies. Histogram appearances can be indistinguishable from those given by IgG bound to high density platelet-specific antigens, as platelets can be bearing high numbers of HLA antigens (see section 1.6.6.2). Open histogram: Specific fluorescence obtained with FITC-goat-anti-human IgG. Shaded histogram: Background fluorescence obtained with FITC-goat IgG (non-immune).

was applied to investigate a case of alloimmune neonatal TCP due to PL^A incompatibility. Figures 4.6 and 4.7 show results obtained with Ig bindable to platelet surface antigens less uniformly distributed than those of the PL^A system. Sera giving strong labelling of control platelets could be serially diluted to determine the highest titre giving positive results.

4.2.7 A calibration system for quantitation of PSiGg.

A model of platelets with predetermined amounts of IgG on their surface was created by coating platelets in vitro with a chimeric monoclonal antibody to gpIIb-IIIa. The murine MoAb 7E3 binds with high affinity to the gpIIb-IIIa complex (Coller 1985). A chimaeric construct (c7E3-IgG) consisting of human IgG with murine 7E3 hypervariable region (see 5.1) was generously provided by Centocor Inc., Malvern, PA. The platelet binding characteristics of the chimaeric molecule were very similar to those of the murine antibody (Centocor, data on file). Chimaeric IgG concentration in the stock antibody solution was 1.37g/l as determined by spectrophotometry following ultracentrifugation at 100.000g for 30 minutes.

4.2.7.1 Coating platelets with c7E3-IgG.

Because the epitope of 7E3 is EDTA sensitive (Coller 1985), citrate or CPD-A1 anticoagulated platelets were used for this purpose. Platelets washed as described in 2.1.6 were resuspended in PBS pH7.2 at a concentration of 200-300x10⁹/l and incubated with a range of concentrations of c7E3-IgG at room temperature for one hour, following which they were washed twice in citrate-PGE₁ and resuspended in PBS. As shown in

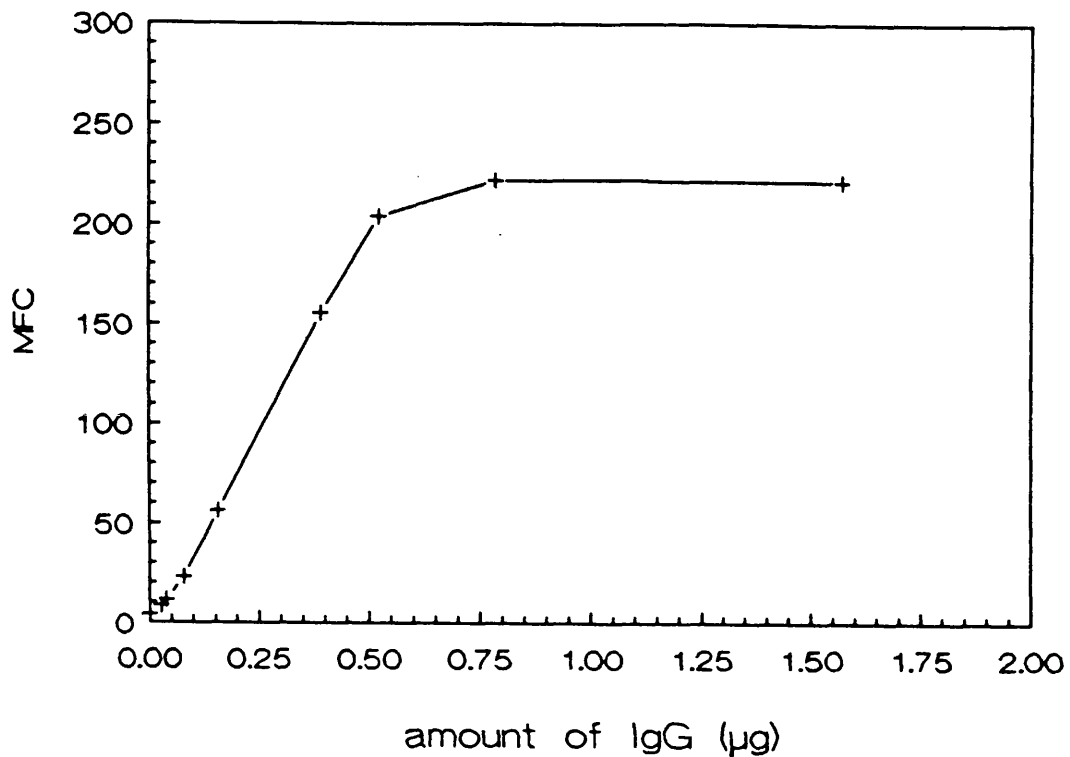


FIGURE 4.8 Mean platelet fluorescence (MFC: mean fluorescence channel) after incubation of 25×10^6 platelets with increasing amounts of c7E3-IgG in a total volume of $100 \mu\text{l}$, followed by washing and labelling with FITC-goat-anti-human IgG. Each point is the mean of duplicate experiments.

Fig. 4.8, flow cytometric measurement of PSiGg in platelets that had been incubated with increasing concentrations of c7E3-IgG gave a dose response curve typical of saturation kinetics. To simplify calculations it was assumed that, at subsaturating concentrations, practically all the c7E3-IgG became platelet-bound. This assumption was based on binding studies with radiolabelled (¹²⁵I) chimaeric IgG (Centocor Inc., method described in 2.6) showing that, at <50% saturating antibody concentrations, 69-75% of added radioactivity became platelet-bound. As radioiodination results in inactivation of a fraction of the antibody, the percentage of unlabelled antibody (used in the calibration studies) that would bind to the platelets under the same conditions should be higher, probably >80%. The number of chimeric IgG molecules bound per platelet at subsaturating concentrations of cIgG was calculated by dividing the total number of molecules added (estimated from the concentration of IgG in the stock antibody solution, the molecular weight of IgG and Avogadro's Number) by the number of platelets in the incubation mixture.

4.2.7.2 Estimation of the number of IgG molecules per platelet.

It was assumed that, in view of the very slow off-rate of platelet-bound 7E3 (Coller 1985), c7E3-IgG levels on the platelet surface remained stable throughout the assay. Calibration curves (Fig. 4.9) were created by measuring PSiGg on platelets coated with various amounts of c7E3-IgG and plotting platelet fluorescence against the estimated number of cIgG molecules per platelet. The best curve fit was given by the following second degree polynomial:

$$\text{MFC} = 3 + 1.534 \times \text{Mols} + 0.014 \times (\text{Mols})^2$$

where MFC is the mean fluorescence channel number and Mols the number of cIgG molecules per platelet ($\times 10^{-3}$). When MFC=3 (i.e. the approximate MFC obtained with the non-immune IgG as measure of

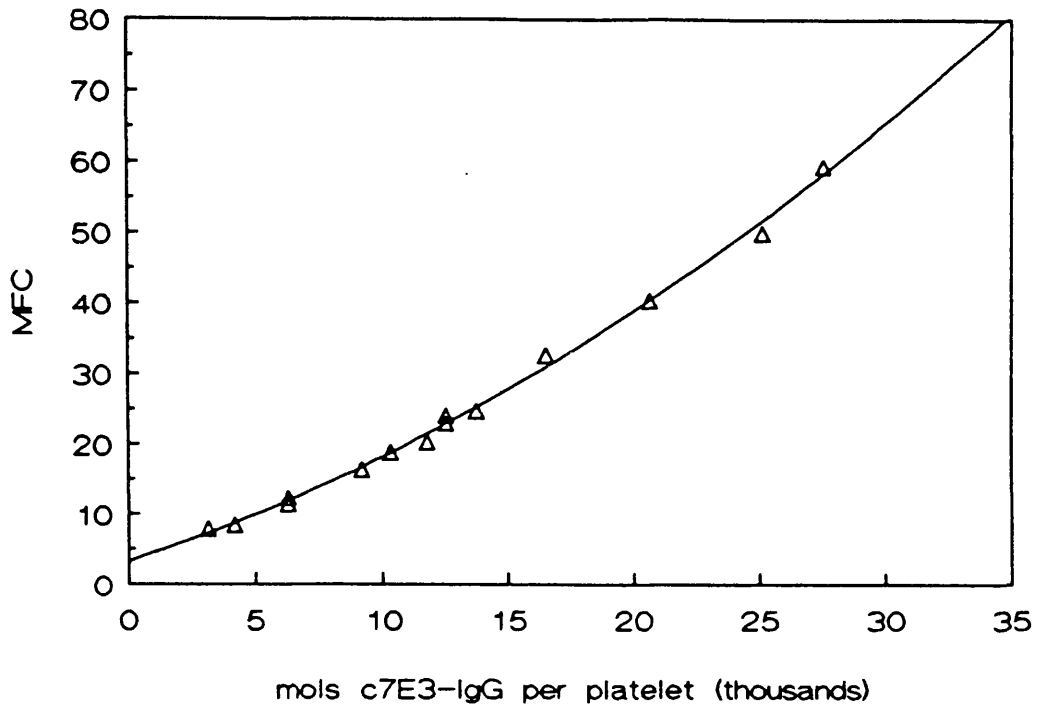


FIGURE 4.9 Reference curve generated by plotting the mean fluorescence channel (MFC) of c7E3-IgG-coated platelets incubated with FITC-anti-IgG against the number of c7E3-IgG molecules per platelet. Each point represents the mean of two independent experiments. To minimize the contribution by native PSiGg, only platelets which in preliminary flow cytometric screening had practically undetectable native PSiGg were used for c7E3-IgG coating.

background fluorescence) then Mols=0 (i.e. no IgG is being detected). When the MFC is known, the number of IgG molecules per platelet can be calculated from the formula:

$$\text{mols/plat (x10}^{-3}\text{)} = (\sqrt{0.056 \times \text{MFC} + 2.185} - 1.534)/0.028$$

As the hypervariable region of cIgG is of murine origin, it is possible that the polyclonal anti-IgG recognizes more epitopes on a human than a chimaeric IgG molecule and therefore, with this calibration method, it is likely to overestimate rather than underestimate the number of IgG molecules per platelet. Such overestimation is unlikely to be significant because (a) the constant region is responsible for most of the antigenicity of the IgG molecule and (b) epitopes in the hypervariable region are likely to be inaccessible when the IgG is specifically bound to its target antigen.

By coating control platelets with known numbers of c7E3-IgG molecules it was shown that, at low levels of PSiG (2-3,000 molecules per platelet), the method was able to discriminate between platelets differing by an estimated 500-600 molecules of PSiG (Fig 4.10). Less marked differences could not be detected reliably with the method and instrument used.

Application of the above formula to the sample of 71 normal controls gave an average of 1,463 (SD=927, range 85 to 4,693) molecules per platelet. The negative control sample of non-immune TCP had a slightly higher mean at 2,309 molecules per platelet (SD=965, range 689 to 4,133) while in the immune TCP group the mean was 11,535 (SD=8,561, range 690 to 32,328). In the more heterogenous group of 62 prospective

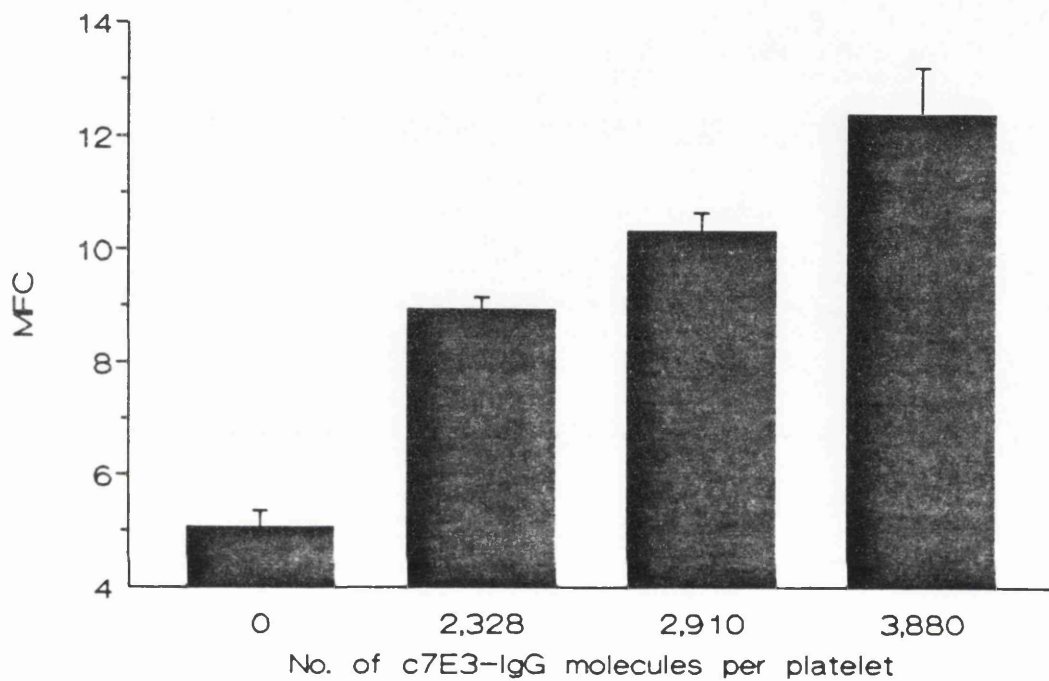


FIGURE 4.10 Platelet fluorescence (MFC) after FITC-anti-IgG incubation of normal platelets (0) and the same platelets coated with increasing amounts of c7E3-IgG (see section 4.2.7.1 for technical details). Means of triplicate tests are shown with standard deviations represented by thin vertical bars. Mean differences were significant by Student's t-test ($P < 0.01$).

ly investigated patients the mean was 4,855 (SD=6.613, range 101 to 40,032) and 20 were above the upper limit (mean+2SD) of normal as opposed to 22 when the results were expressed as RFI (see Table 4.2 for individual details). When the cut-off point was placed at 3SD above the mean only 14 cases of raised PSiG could be identified with either way of results expression, i.e. in 6 out of 20 cases the elevations of PSiG were marginal.

4.2.8 Comments on patients with elevated PSiG.

From the prospectively studied group of patients with TCP or impaired platelet function of unknown cause, those who were found to have elevated PSiG levels are discussed below.

4.2.8.1 Incidental thrombocytopenia.

Patients with incidentally discovered, usually mild TCP (platelet count below $150 \times 10^9/l$ on at least two occasions) in the absence of associated disorders, constituted the main subgroup of cases studied. These patients are likely to represent a heterogenous group. A specific diagnosis was difficult to make, mainly because of the frequent absence of remarkable marrow changes and lack of platelet survival studies. Of 19 such patients (mean platelet count $93.7 \times 10^9/l$, range 35 to $142 \times 10^9/l$), five had raised PSiG but in only three of them was the elevation greater than 3 SDs above the normal mean. This finding was suggestive of chronic immune-mediated platelet destruction and directed the diagnosis away from other possibilities (e.g. early myelodysplastic syndrome which can present as

TABLE 4.2 Clinical presentation and individual results of prospectively investigated patients found to have elevated PSiG. These represent 35.7% of the group of 62 patients with thrombocytopenia (TCP) or platelet dysfunction of unknown aetiology.

NR: normal range (defined as mean±2SD of 71 healthy controls). RFI: Relative Fluorescence Intensity (see text for definition).

CLINICAL FEATURES	PL. COUNT ($\times 10^9/L^{-1}$)	RFI (NR:0.83-2.43)	MOLS IgG/PLATELET (NR:<3,330)
Mild thrombocytopenia during pregnancy persisting 5 months post-delivery.	98	2.71	3,614
Early CLL, normal megakaryocytes in marrow.	111	3.84	5,014
Pregnant thrombocytopenic.	42	2.93	4,073
Incidental thrombocytopenia. Normal megakaryocytes.	32	2.82	3,530
Incidental thrombocytopenia. Normal megakaryocytes.	110	3.63	5,846
Gaucher's disease. Old splenectomy. Mild bleeding tendency; defective platelet response to ristocetin.	140	4.29	7,484
18 months old. Leucopenia-thrombocytopenia. Marrow morphology within normal.	31	3.81	6,025
Sarcoidosis, on prednisolone 5 mg daily. Pregnant.	47	9.04	17,533
Probable heparin induced thrombocytopenia.	53	4.18	7,976
Incidental thrombocytopenia. Normal megakaryocytes.	70	4.96	7,828
Mother of severely thrombocytopenic newborn.	180	2.66	4,107
Incidental thrombocytopenia.	142	2.48	3,450
Incidental thrombocytopenia. Normal megakaryocytes.	80	4.12	4,828
Chronic liver disease. Portal hypertension. Positive antimitochondrial antibodies.	106	19.2	39,050

Chronic thrombocytopenia following glandular fever. Normal marrow morphology.	115	2.74	3,440
Age 72. Acute onset TCP. Absent megakaryocytes; normal erythro- and myelopoiesis.	2	14.52	25,289
Pregnant thrombocytopenic.	40	3.91	4,873
Age 73. Chronic severe TCP. No bleeding. Refused B/Marrow examination.	6	3.85	8,034
Chronic thrombocytopenia refractory to immunosuppression. Plentiful megakaryocytes with dysplastic changes. Normal erythro- and myelopoiesis.	13	16.32	27,814
African. Kaposi's sarcoma. TCP refractory to standard Rx. HIV-negative.	19	2.46	3,320
Incidental thrombocytopenia. Normal B/Marrow.	70	2.48	3,309
Pregnant thrombocytopenic.	51	6.05	8,976

isolated TCP) with different prognosis and management. Patients with chronic stable TCP who are asymptomatic and usually maintain platelet counts $>50 \times 10^9/l$ on no treatment, appear to constitute a good prognosis group and are usually found to have normal levels of PSiG (LoBuglio *et al* 1983).

4.2.8.2 Pregnancy-associated TCP.

Eight patients with incidentally discovered, pregnancy-associated TCP were studied. This sample (median platelet count $78.5 \times 10^9/l$) was not representative of the degree of TCP seen in pregnancy (platelet count usually $>100 \times 10^9/l$), as a result of referral bias. Four of these 8 women were found to have elevated PSiG with the level being higher than 3 SDs above the normal mean in two. The latest evidence suggests that incidentally discovered TCP of

pregnancy is, in the vast majority of cases, a benign condition (Samuels *et al* 1990, Burrows and Kelton 1990), which is best managed conservatively. The apparently higher incidence of TCP in the pregnant population might simply reflect a state of compensated consumptive coagulopathy occurring in pregnancy (Tygart *et al* 1986). The nature of elevated PSiG found in some of these cases is unclear and neither this nor the maternal platelet count and, probably, not even the presence of circulating platelet-bindable IgG appear to predict the development of fetal/neonatal thrombocytopenia (Hart *et al* 1986, Aster 1990)

4.2.8.3 Other disorders.

One of 2 patients with sarcoidosis was found to have markedly raised PSiG (17,500 mols per platelet); elevations of PSiG associated with this disorder have been reported by others (Semple 1975, Lawrence and Greenberg 1985). Autoimmune TCP in lymphoproliferative disorders (One of 3 early chronic lymphocytic leukaemia cases had raised PSiG) is also well recognized (Kaden *et al* 1979).

Very high levels of PSiG (39,000 mols per platelet) were seen in one of the four cases of TCP associated with impaired hepatic function. This patient had cirrhosis with portal hypertension and positive antimitochondrial antibodies. Raised PSiG appears to occur commonly in chronic liver disease and does not correlate with the platelet count (Landolfi *et al* 1979, Barrison *et al* 1981). In Barrison's study (1981), from a total of 25 patients with chronic liver disease, the only one with positive antimitochondrial antibodies had also the highest levels of circulating immune complexes in the series. Platelet-bound immune complexes could be the reason for the unusually high levels of PSiG in the cirrhotic patient reported in the present study.

One case of TCP that had been ascribed to heparin was associated with significantly raised PSiG. Kelton's group (1988) have provided evidence that heparin-induced TCP results from the binding to the platelet Fc receptor of complexes formed between heparin and anti-heparin antibodies.

4.2.8.4 Impaired ristocetin response/associated with raised PSiGg:

A case of "pseudo-pseudo Bernard-Soulier syndrome"?

Qualitative platelet abnormalities are not uncommon in patients with past or present immune-mediated TCP (Clancy *et al* 1972) or simply elevated PSiGg without evidence of an immune disorder involving the platelets (McGrath *et al* 1979). The qualitative defect is usually manifested as hypoactivity but autoantibodies resulting in clinically significant platelet hyperactivity have also been described (Pfueller *et al* 1990). The following is a case of isolated loss of the platelet agglutination response to ristocetin in a patient with raised PSiGg:

A 45-year-old female with Gaucher's disease and previous splenectomy being investigated for a bleeding tendency and a normal platelet count of $140 \times 10^9/l$ was found to have severely impaired platelet agglutination response to ristocetin not correctable by porcine Factor VIII or cryoprecipitate (Fig. 4.11). These abnormalities were unchanged on re-testing 4 months later. Responses to other agonists were normal. She had a recent history of blood transfusions complicated by the development of red cell and HLA antibodies and was also found to have a polyclonal B-cell lymphocytosis. Flow cytometric analysis of her platelets revealed high levels of PSiGg and serum IgG bindable to heterologous GpO platelets and also to platelets from a patient with Bernard-Soulier syndrome (BSS), the level binding to BSS platelets being higher than that to normal platelets (probably as a result of steric factors and the larger size of BSS platelets). On follow-up it was seen that the response to ristocetin returned toward normal in parallel with amelioration of the bleeding tendency and normalization of the PSiGg, while there was no appreciable change in the levels of serum platelet-bindable IgG (presumably anti-HLA). Platelet surface gpIb levels were normal by FC. Normal control platelets incubated with plasma from the patient showed normal response to ristocetin. Interestingly, absent response to ristocetin was seen in a second patient (19-year-old female) with Gaucher's disease, previous splenectomy and recent blood transfusions complicated by development of anti-HLA antibodies, in association with mildly impaired liver function, hypergammaglobulinaemia and raised PSiGg. A third female patient with long standing Gaucher's disease, borderline platelet count and previous splenectomy but without recent transfusion history or anti-HLA antibodies was subsequently investigated and showed normal response to ristocetin, normal levels of PSiGg and no platelet-bindable immunoglobulins in her serum.

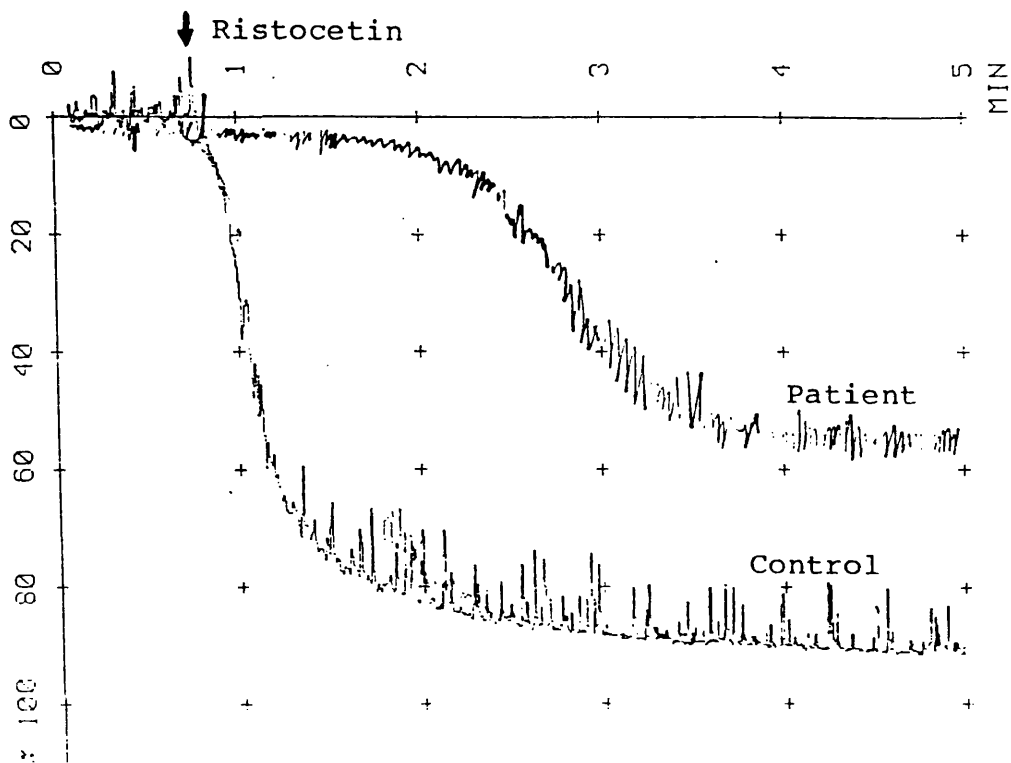


FIGURE 4.11 Absent ristocetin (1.25mg/ml) agglutination response in a patient with Gaucher's disease, excessive operative bleeding, multiple red-cell alloantibodies and anti-HLA antibodies, polyclonal B lymphocytosis and elevated PSiGg. The ristocetin response returned toward normal simultaneously with normalization of PSiGg.

Horizontal axis: Time in minutes. Vertical axis: Light transmission through PRP (as % of transmission in PPP). The only important assessment of "aggregation" to ristocetin (which is really an agglutination of platelets) is the angle of the initial slope and not the height of maximal response (see Yardumian *et al* 1986 for review of platelet aggregometry).

Although the Bernard-Soulier-like abnormalities observed in the above cases appear to be immune-mediated, the available data are not sufficient to determine the underlying pathogenetic mechanism. The fact that the platelet-bindable IgG present in the serum of these patients gave a stronger reaction with BSS than normal platelets questions (but does not exclude) the possibility that the abnormal ristocetin response was due to an antibody binding to gpIb. This mechanism has been suggested in both cases of immune-mediated or "pseudo-" BSS reported so far in the literature (Stricker *et al* 1985, Devine *et al* 1988). It is possible that IgG binding to some other component of the platelet membrane might also interfere with ristocetin response; the term "pseudo-pseudo BSS" was coined to describe this hypothetical situation. The failure to reproduce the abnormality *in vitro* by incubating normal platelets with patient sera suggests that factors intrinsic to the membrane of Gaucher's platelets (probably related to the accumulation of sphingolipid) might also be playing some part. It is conceivable that abnormal platelet function could be contributing to the bleeding tendency which is often disproportional to the TCP in patients with Gaucher's disease. Platelet antibody production could have been the result of an abnormal immune response of these splenectomised patients to the antigenic stimulation by the recent blood transfusions.

4.2.8.5 Amegakaryocytic acute ITP.

A 72-year-old male presented with purpura of sudden onset and was found to have a platelet count of $2 \times 10^9/l$. He had no other illnesses. Bone marrow examination revealed absence of megakaryocytes. Flow cytometric analysis showed markedly raised PSiGg (*ca.* 25,000 IgG mols per platelet). The possibility of an autoimmune mechanism involving immune destruction of megakaryocytes was considered. The patient showed a delayed, partial (platelet count reaching $70 \times 10^9/l$ after 6 weeks) response to steroids. When reduction in the dose of prednisolone was attempted, the platelet count fell rapidly and the patient became anaemic with no evidence of bleeding but with a strongly positive direct Coombs. Thus, the subsequent development of autoimmune haemolytic anaemia provided evidence in support of an autoimmune mechanism for the amegakaryocytic TCP at presentation, as had been suggested by the flow cytometrically

detected elevation in PSiG. In view of the paucity of megakaryocytes, which is very unusual in ITP, this case was labelled as "atypical Evans' syndrome".

4.3 DISCUSSION

Techniques for detection of IgG bound to the platelet surface were published as early as 1961 (Dausset *et al* 1961, Van de Wiel *et al* 1961). The first methods to quantitate PSiG in normal and ITP platelets (McMillan *et al* 1971, Dixon *et al* 1975, Cines and Schreiber 1979) were cumbersome and therefore unsuitable for routine use in a clinical laboratory. Results of PSiG in normal controls were sometimes unrealistically high, e.g. $>10^6$ IgG molecules per platelet (Dixon *et al* 1975). Nevertheless, these quantitative methods were shown to be sensitive and reproducible and contributed significantly to the study of immune thrombocytopenias. Modifications of Dixon's original complement lysis-inhibition assay yielding more realistic PSiG estimates (e.g. Kelton *et al* 1980) became established in many research laboratories.

PSiG measurement was greatly facilitated by the introduction, in the late seventies, of comparatively simple direct tests employing labelled anti-immunoglobulins. Cines and Schreiber (1979) incubated washed platelets with ^{125}I -labelled polyclonal rabbit-anti-human IgG and compared platelet radioactivity with the radioactivity of red cells which had been coated with known amounts of IgG prior to incubation with the radioactive probe. Their estimate was of an average 2,400 IgG molecules per normal platelet whereas 90% of ITP patients showed raised PSiG ranging from 5,000 to 48,000 molecules per platelet. A large number of sensitive quantitative assays employing radiolabelled or enzyme-linked

polyclonal anti-immunoglobulins have since been introduced; they usually detect several thousands of IgG molecules on normal platelets and up to several tens of thousands on platelets of patients with immune-mediated thrombocytopenia (Kelton 1983, Schwartz 1988). Some investigators (Leporrier *et al* 1979, Blumberg *et al* 1986) using peroxidase or alkaline phosphatase labelled polyclonal anti-IgG and making a number of stoichiometric assumptions, have calculated a much lower number (*ca.* 250) of IgG molecules on the surface of normal subjects' platelets, which is at variance with the estimates usually obtained using polyclonal probes. In 1983, LoBuglio and co-workers published a simple PSiG detection method based on the use of an ¹²⁵I-anti-human IgG (Fc-specific) monoclonal antibody. Making stoichiometric assumptions, they estimated the average number of IgG molecules on normal platelets at 169 (SD=79) which was at least 10 times less than the numbers reported by most methods using polyclonal antibodies. Very similar estimates were subsequently published by other groups (George and Saucerman 1988, Kelton *et al* 1989, Tjihuis *et al* 1991) using the same or slightly modified methods. Although these estimates appear to be gaining general acceptance, there is evidence that they might be underestimating PSiG. Using the MoAb employed in LoBuglio's original report, Rosse and colleagues (1984) found that only 60% of the IgG molecules present on the platelet membrane could be detected and that, at any given level of membrane-bound IgG, a ¹²⁵I-labelled polyclonal anti-IgG bound more molecules per platelet than the MoAb. This can be explained by the fact that MoAbs recognize only a single epitope on the IgG molecule, which may not always be accessible in the case of platelet-bound IgG due to steric hindrance. Polyclonal antibodies might be more practical tools in this setting as they have an increased probability of binding one or more

antibody molecules per molecule of platelet-surface IgG through recognition of multiple epitopes.

A number of methods for PSiG detection using fluorescent probes - usually FITC-conjugated antiglobulins - have been developed, the most popular being the platelet suspension immunofluorescence test, a rapid and technically simple method for qualitative assessment of PSiG introduced by Von dem Borne in 1980. Quantitative immunofluorescent techniques based on fluorometry have also been described (Van Boxtel *et al* 1975, Sugiura *et al* 1980) and using such a method Sugiura and co-workers (1980) estimated an average binding of 3,100 (SD=800) anti-IgG molecules per platelet.

Flow cytometry entered the scene of PSiG detection methodology in the mid-eighties with a number of preliminary results presented mainly as symposia abstracts. Several more detailed method reports have been published in the last five years (Corash and Rheinschmidt 1986, Lazarchick and Hall 1986, Rosenfeld *et al* 1987, Heim and Petersen 1988, Ault 1988) and their main technical characteristics are summarized in Table 4.3.

There is no consensus regarding the advantages of platelet fixation prior to PSiG measurement, the only obvious one being that it allows the tests to be done in batches at a convenient time, which is important when flow cytometer availability is limited. The author's opinion and that of others (Lazarchick and Hall 1986) is that fixation should be avoided if possible; it tends to alter significantly the light scattering behaviour of the platelets (personal observations) which reflects structural changes. In view of the very low platelet counts often encountered in these investigations, a single ("one layer") staining step using a FITC

Authors (reference)	Platelet fixation	Antibody used	Irrelevant control	Expression of results	Cytometer used
Corash and Rheinschmidt (13)	PFH 1% x5 minutes	Biotin-F(ab') ₂ -goat-anti-human IgG, FITC-avidin	None	MFC	FACS Becton-Dickinson
Lazarchick and Hall (14)	No	FITC-F(ab') ₂ -goat-anti-human IgG	None	MFC of stained cells over MFC of unstained cells	Ortho Spectrum
Rosenfeld et al (15)	No	FITC-goat-anti-human IgG (whole molecule)	Sheep-anti-mouse	Labelling Index (% cells with fluorescence over a fixed threshold)	Coulter EPICS
Heim and Petersen (16)	No	FITC-F(ab') ₂ -goat-anti-human IgG	Goat-anti-horse	% fluorescence (as labelling index)	Coulter EPICS
Ault (17)	PFH 1% for 2 hours or overnight	FITC-F(ab') ₂ -rabbit or goat-anti-human IgG	None	MFC	FACS Becton-Dickinson

TABLE 4.3 Main technical features of published flow cytometric methods for PSiGG detection.

conjugated anti-immunoglobulin appears to be the only practical approach. Most investigators have used the F(ab')₂ fragment of a goat polyclonal and although the whole molecule goat-anti-human IgG performed well in the present study, it is possible that the use of F(ab')₂ fragments could reduce interference from Fc receptor binding.

Incubation with FITC-labelled non-immune goat IgG in parallel with the specific antibody appeared to offer a good index of non-specific antiglobulin adsorption which tends to be increased in immune TCP, presumably as part of the general tendency of these platelets to non-specifically adsorb irrelevant plasma proteins (Kelton and Steeves 1983, Hotchkiss *et al* 1986, Rosenfeld *et al* 1987).

Corash and Rheinschmidt (1986) studied 171 patients with thrombocytopenia or a clinical suspicion of a qualitative platelet defect and reported that flow cytometric measurement of PSiG had a 93.8% sensitivity and 94.5% specificity in detecting those with a clinical diagnosis of immune-mediated platelet disorder. Rosenfeld and colleagues (1987) in a study of 102 patients reported a 75% sensitivity rising to 91% when PSiGM was measured in addition to PSiG. Ault (1988) reported increased PSiG in 63% of 299 patient samples tested (clinical diagnoses were not stated) and found that separate assessment of PSiG, PSiGM and PSC3 had no practical advantage over a single test using a pan-specific (anti-IgG,IgM,IgA) antibody. He was also the first to report the occasional presence of two platelet populations bearing different levels of PSiG, usually in the setting of recovery from acute ITP. Holme and colleagues (1988) used flow cytometry to demonstrate that raised PSiG in thrombocytopenic patients involved proportionally all size classes of platelets.

In the present study, a method for of flow cytometric PSiG detection was applied to a control group of 25 patients with clinically defined

immune or non-immune thrombocytopenia as well as a group of 62 patients with thrombocytopenia or impaired platelet function in whom it was difficult to make a clinical diagnosis. Retrospectively, the finding of a raised PSiG in 35.5% of the latter group, especially when the rise was considerable (more than 3SD above the mean of the normal controls), would have been of potential help in their clinical management. In many of these cases, an immune aetiology was further supported by the clinical course and/or response to treatment. It should at this point be stressed that, failure to detect raised PSiG, irrespectively of the method used, can not exclude the diagnosis of immune-mediated TCP, one possible reason being the wide distribution of PSiG levels in healthy individuals (Tijhuis *et al* 1991) although other possibilities, e.g "washing-off" of low affinity antibodies during platelet preparation should also be considered. It is, of course, possible that the pathogenesis of some cases of immune TCP does not involve significant increases in platelet surface immunoglobulins and this is why the last few years have witnessed the development of more "dynamic" assays focusing on monocyte-platelet interactions rather than the "static" measurement of PSiG (Saleh *et al* 1989, Hymes *et al* 1990). These comments notwithstanding, simple and reliable methods for PSiG measurement will continue to be essential tools for the investigation of platelet disorders.

It is logical to argue that the sensitivity and specificity of any new method for PSiG detection should refer to its ability to reproducibly detect known amounts of IgG on the platelet surface rather than the compatibility of its results with the subjective clinical diagnosis of ITP which, after all, is a heterogenous entity and a diagnosis of exclusion. This issue was addressed by using the flow cytometric method to

measure PSiGg in platelets bearing known amounts of chimeric IgG bound to the gpIIb-IIIa complex. This glycoprotein complex contains the most frequently identified target for platelet autoantibodies (Van Leeuwen *et al* 1982, Beardsley *et al* 1984, McMillan *et al* 1987, Berchtold *et al* 1986) and it was considered that this model offered an acceptable simulation of the *in vivo* platelet membrane microenvironment in cases of immune-mediated platelet disorders where specific binding of monomeric IgG occurs and less so in disorders associated with the binding of immune complexes to platelets. The data presented here show that FC gives reproducible results over a wide range of PSiGg levels and is sensitive enough to detect differences of a few hundred IgG molecules at low PSiGg levels.

To date, there have been no published flow cytometric estimates of numbers of IgG molecules detectable on the platelet surface of normal and thrombocytopenic subjects. Estimations of the number of binding sites for the major platelet membrane glycoproteins have been attempted using reference curves obtained by measuring the fluorescence of beads coated with known numbers of FITC molecules (Tschoepe *et al* 1990). There are many dissimilarities between synthetic fluorescent beads and live cells bearing fluorescent probes, mainly in terms of size distribution, shape, internal complexity, autofluorescence and signal quenching. The ideal calibration standards should consist of cells or biological material identical to those being investigated by a particular technique and the use of cIgG coated platelets to create calibration curves for PSiGg estimation represents a step in this direction. The estimate of the average number of IgG molecules detectable on the surface of normal platelets (*ca.* 1500 mols per platelet) is in good agreement with published data (Kelton 1983, Schwartz 1988) obtained with entirely

different methods also employing polyclonal antibodies. It should be noted however, that when Kelton and colleagues (1989) compared four methods differing widely in their estimated number of IgG molecules per normal platelet (86 to 10,850 mols) they found that all four of them had comparable sensitivity and specificity in the setting of clinically diagnosed immune or non-immune thrombocytopenia. The results of the present study indicate that, although useful for research purposes, the expression of results as IgG molecules per platelet has no practical clinical advantage over the use of arbitrary units (Table 4.2.).

In conclusion, FC was found to offer an efficient means of quantitative PSiGg estimation for both research and clinical service purposes. Objective comparison of its performance with that of radioisotopic or ELISA methods currently in use should be the subject of future studies.

Appendix to Chapter 4

Case No	NSF	SF	RFI	Case No	NSF	SF	RFI
1.	2.745	4.308	1.56	37.	3.169	6.803	2.15
2.	2.795	4.106	1.47	38.	3.194	5.836	1.83
3.	2.812	4.962	1.76	39.	3.198	6.349	1.98
4.	2.821	3.516	1.25	40.	3.207	3.771	1.17
5.	2.845	3.134	1.10	41.	3.216	3.967	1.23
6.	2.857	4.038	1.41	42.	3.216	5.444	1.69
7.	2.869	3.208	1.12	43.	3.219	6.834	2.12
8.	2.872	4.505	1.56	44.	3.222	5.060	1.57
9.	2.878	4.907	1.70	45.	3.232	6.238	1.93
10.	2.895	4.870	1.68	46.	3.250	5.370	1.65
11.	2.816	3.215	1.14	47.	3.259	5.871	1.80
12.	2.910	8.043	2.76	48.	3.261	5.079	1.56
13.	2.921	4.496	1.53	49.	3.273	4.568	1.35
14.	2.922	5.949	2.03	50.	3.280	3.410	1.04
15.	3.926	3.843	1.31	51.	3.285	9.884	3.01
16.	2.930	4.976	1.70	52.	3.314	4.396	1.33
17.	2.939	4.907	1.67	53.	3.325	4.802	1.44
18.	2.951	3.766	1.27	54.	3.377	6.262	1.85
19.	2.954	4.627	1.57	55.	3.414	4.474	1.31
20.	2.965	5.413	1.82	56.	3.414	5.225	1.53
21.	2.989	3.593	1.20	57.	3.428	6.079	1.77
22.	3.043	4.860	1.59	58.	3.438	4.659	1.36
23.	3.047	4.905	1.61	59.	3.464	10.510	3.03
24.	3.057	4.684	1.53	60.	3.534	7.064	2.00
25.	3.062	3.834	1.25	61.	3.550	5.590	1.57
26.	3.085	4.774	1.55	62.	3.553	8.586	2.42
27.	3.091	4.963	1.61	63.	3.613	4.453	1.23
28.	3.092	5.542	1.79	64.	3.617	4.490	1.24
29.	3.102	5.752	1.85	65.	3.670	5.656	1.54
30.	3.109	4.523	1.45	66.	3.732	8.329	3.23
31.	3.129	3.988	1.27	67.	3.774	5.283	1.40
32.	3.131	6.920	2.20	68.	3.851	6.744	1.75
33.	3.133	4.901	1.56	69.	3.944	5.991	1.52
34.	3.133	5.143	1.73	70.	4.048	6.604	1.63
35.	3.155	3.405	1.08	71.	5.056	7.460	1.47
36.	3.165	4.733	1.49				

TABLE IV.1. Non-specific (NSF) and specific (SF) fluorescence and Relative Fluorescence Intensity ($RFI = SF/NSF$) of platelets from 71 healthy subjects. The Mean Fluorescence Channel (MFC) numbers obtained with FITC-goat IgG (non-immune) and FITC-goat-anti-human IgG were used as arbitrary units of NSF and SF respectively.

A. IMMUNE

Case No	NSF	SF	RFI	Case No	NSF	SF	RFI
1.	3.250	5.730	1.76	9.	3.070	15.440	5.03
2.	3.699	4.068	1.09	10.	4.089	33.510	8.20
3.	4.032	6.066	1.70	11.	3.618	38.030	10.51
4.	3.032	24.620	8.44	12.	2.880	12.390	4.30
5.	3.035	25.380	8.56	13.	3.256	16.660	5.12
6.	2.924	4.688	1.60	14.	4.753	67.510	14.20
7.	2.960	37.930	12.81	15.	3.372	19.820	5.88
8.	2.684	13.450	5.07	16.	4.500	35.700	7.93

B. NON-IMMUNE

Case No	NSF	SF	RFI	Case No	NSF	SF	RFI
1.	3.688	9.582	2.59	6.	2.927	6.529	2.23
2.	3.229	5.797	1.79	7.	2.781	4.067	1.46
3.	3.057	6.053	1.98	8.	3.139	7.960	2.58
4.	2.508	5.738	2.29	9.	4.315	6.556	1.52
5.	3.071	7.300	2.38				

TABLE IV.2 Results of NSF, SF and RFI obtained from 25 patients with pre-established clinical diagnosis of immune-mediated (n=16) or non-immune (n=9) thrombocytopenia (see legend of Table IV.1 for explanation of units and abbreviations).

Case No	NSF	SF	RFI	Case No	NSF	SF	RFI
1.	3.153	4.861	1.54	32.	3.610	7.819	2.17
2.	3.300	3.754	1.13	33.	3.485	13.290	3.81
3.	3.461	56.500	16.32	34.	3.139	4.583	1.46
4.	2.651	3.158	1.19	35.	3.952	6.489	1.64
5.	2.895	5.240	1.81	36.	3.971	10.580	2.66
6.	2.760	10.810	3.91	37.	3.421	8.321	2.43
7.	3.952	7.719	1.95	38.	3.565	4.087	1.15
8.	3.621	5.325	2.02	39.	3.919	35.410	9.04
9.	3.258	3.681	1.12	40.	4.183	17.480	4.18
10.	3.139	4.033	1.28	41.	3.548	7.922	2.23
11.	3.050	4.632	1.51	42.	3.042	4.345	1.43
12.	2.936	6.859	2.33	43.	4.867	8.912	1.83
13.	4.209	16.230	3.85	44.	4.345	6.278	1.44
14.	2.967	17.960	6.05	45.	3.509	4.937	1.41
15.	3.000	5.663	1.88	46.	3.351	8.250	2.46
16.	3.588	4.486	1.25	47.	3.256	16.160	4.96
17.	3.360	6.740	2.00	48.	3.915	3.685	1.26
18.	3.399	4.837	1.42	49.	2.669	4.454	1.67
19.	3.543	7.985	2.25	50.	3.773	4.745	1.26
20.	3.366	9.118	2.71	51.	3.690	7.044	1.90
21.	2.823	10.850	3.84	52.	3.256	6.286	1.93
22.	3.368	9.876	2.93	53.	4.733	6.583	1.39
23.	2.818	3.734	1.32	54.	3.728	9.232	2.48
24.	2.861	4.234	1.48	55.	2.465	10.150	4.12
25.	3.066	8.660	2.82	56.	3.753	7.293	1.94
26.	3.461	5.308	1.53	57.	3.324	8.232	2.48
27.	3.914	5.119	1.31	58.	4.523	86.850	19.20
28.	3.618	13.130	3.63	59.	3.030	4.480	1.48
29.	3.754	16.120	4.29	60.	3.253	4.170	1.28
30.	3.586	7.907	2.20	61.	3.083	8.452	2.74
31.	3.642	3.670	1.01	62.	3.550	51.550	14.52

TABLE IV.3 Results of NSF, SF and RFI obtained from 62 consecutive patients with thrombocytopenia of unknown aetiology (see legend of Table IV.1 for explanation of units and abbreviations).

Chapter 5

FLOW CYTOMETRIC OBSERVATIONS IN HUMANS RECEIVING INFUSIONS OF Fab FRAGMENTS OF A CHIMAERIC MONOCLONAL ANTIBODY TO PLATELET GLYCOPROTEIN IIb-IIIa

5.1 INTRODUCTION - CHIMAERIC 7E3

Monoclonal antibodies to the gpIIb-IIIa complex are the most potent anti-platelet agents ever described (see section 1.5.5.2 and Fitzgerald 1989). Among them, the most extensively tested is 7E3, a murine IgG1 monoclonal produced by B.S.Coller (1985). As discussed in the introduction, murine monoclonals, being foreign proteins, are immunogenic and this limits their potential therapeutic usefulness. Production of human monoclonals is still very impractical and therefore, in an effort to reduce the immunogenicity of murine MoAbs, "chimaeric" (murine/human) MoAbs have been developed. These are constructs in which, through genetic engineering, the antigen binding region of a murine monoclonal has been inserted into the molecule of human IgG (see section 1.5.6). Such a chimaeric construct, consisting of murine 7E3 hypervariable region (the specific complementarity determining region) inserted into the framework of human IgG, has recently been developed by Centocor Inc. and is currently undergoing evaluation in early-phase trials. Figure 5.1 shows a diagrammatic representation of the molecule of chimaeric 7E3.

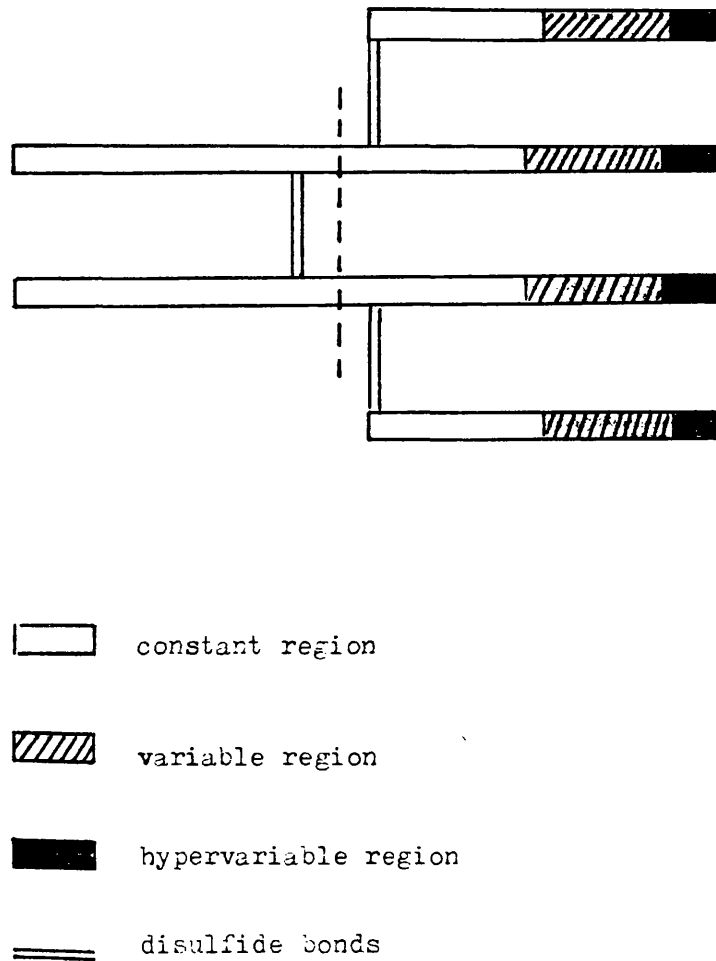


FIGURE 5.1 Schematic representation of chimaeric 7E3-IgG. The specificity-determining hypervariable areas (black) of the variable region are of murine (7E3 MoAb) origin, while the rest of the molecule is normal human IgG. The Fab fragments of this molecule (obtained by papain cleavage at the sites indicated by broken line) were infused into stable angina patients.

Before clinical studies of c7E3-Fab administration were undertaken, in vitro studies (Centocor, data on file) had shown that the binding affinity of the chimaeric antibody and its Fab fragments to isolated human platelets was comparable to that of the murine antibody and that, at saturating antibody concentrations, it produced complete abolition of ADP-induced platelet aggregation.

5.2 PHASE I STUDIES OF CHIMAERIC 7E3-Fab (c7E3-Fab) INFUSIONS IN PATIENTS WITH STABLE ANGINA

The majority of the observations described in this chapter were made on patients with stable angina receiving trial infusions of c7E3-Fab at Northwick Park Hospital, London, U.K.. The main aims of this trial were to determine the safety of c7E3 administration and compare (with ex-vivo studies) its fibrinogen receptor blocking and platelet aggregation inhibiting effect to that of murine 7E3-Fab (m7E3-Fab). The original design of the trial did not include flow cytometric investigations; these were undertaken while the trial was in progress, in an effort to elucidate the pathogenesis of thrombocytopenia occasionally associated with 7E3 administration and obtain more information about the pharmacokinetics of c7E3-Fab. Although a general overview of the trial is given below, the results presented here refer exclusively to the group of trial patients on whom flow cytometric studies were performed.

5.2.1 Brief description of c7E3-Fab trial protocol

5.2.1.1 Study population

The study was approved by the local ethical committee and written informed consent was obtained by all participating patients. Patients between the ages of 18 and 75 years were enrolled, excluding females of child-bearing potential. The diagnosis of ischaemic heart disease was documented by a typical history of angina pectoris and/or arteriographic evidence of >50% stenosis of at least one coronary artery and/or previous myocardial infarction confirmed electrocardiographically and biochemically. Patients with a history of thrombocytopenia, hypertension, recent (8 weeks or less) surgery, CNS diseases, immune disorders, insulin dependent diabetes, significant abnormalities on routine haematological and biochemical screening and a personal or family history of haemorrhagic diathesis were excluded. Patients who had received thrombolytic therapy within 4 weeks or antiplatelet agents/ anticoagulants within 7 days of entry were also excluded, as were patients on medication known to cause thrombocytopenia, people previously treated with murine monoclonal antibodies and unstable cardiac patients with a high likelihood of requiring invasive procedures or emergency anticoagulation/thrombolysis. All standard antianginal treatment (beta-blockers, calcium antagonists, nitrates) was continued during the study.

5.2.1.2 Administration of c7E3-Fab and monitoring of antiplatelet activity

Participating patients were hospitalised during and for 48 hours after the infusion and were carefully monitored for evidence of bleeding. c7E3-Fab fragments were supplied by Centocor Inc. as a sterile, preservative free solution of 2mg/ml of 0.15M NaCl and 0.01M sodium phosphate, pH 7.2. The fragments had been prepared by papain digestion of c7E3-IgG followed by extensive purification to ensure the absence of residual IgG molecules or Fc fragments. All patients received a I.V. loading c7E3-Fab dose of 0.25mg/kg, which was shown in previous studies with m7E3-Fab to completely inhibit platelet response to physiological agonists through near saturation blockade of gpIIb-IIIa receptors (Machin *et al* 1990). This was followed by continuous I.V. infusion of 5µg/min for 24 hours (5 patients), or 10µg/min for 48 hours (7 patients), 72 hours (6 patients) or 96 hours (1 patient). Platelet response to antibody administration was monitored by baseline, during and post-infusion measurements of platelet count, bleeding time (Simplate II), platelet aggregation and number of available 7E3-binding sites on platelets (see section 2.6).

5.3 FLOW CYTOMETRIC STUDIES IN PATIENTS RECEIVING c7E3-Fab INFUSIONS

5.3.1 Rationale for using flow cytometry to monitor platelet-surface c7E3-Fab.

The studies of in vivo administration of anti-gpIIb-IIIa MoAbs published so far have used radioimmunometric assays (RIA) to monitor available

antibody-binding sites; the amount of platelet-bound antibody has been inferred by subtraction of the number of binding sites from that of baseline. The number of available binding sites returns gradually to normal following the infusion. RIA studies give average numbers of free binding sites per platelet but do not give any information about the distribution of these sites in the platelet population. One could assume that, as new platelets, presumably not exposed to 7E3, are released into the blood stream following the infusion, two distinct subpopulations (7E3-positive and 7E3-negative) will coexist until the 7E3-coated platelets are cleared from the circulation. The rate of clearance of these platelets would be expected to correlate with the rate of normalization of platelet function parameters (aggregation, bleeding time) and could be inappropriately increased in patients developing thrombocytopenia during or after the infusion. As shown in Chapter 3 (Fig. 3.2), flow cytometry of platelets labelled with anti-gp antibodies can be used to reveal the presence of platelet subpopulations bearing different amounts of a particular gp. It was therefore thought that, by applying flow cytometric analysis to platelets incubated with a FITC-labelled anti-7E3 antibody at various time points before, during and after c7E3-Fab infusion, useful information might be obtained to improve understanding of the *in vivo* c7E3-Fab kinetics. It should also be noted that, direct quantitation of platelet-bound 7E3 using an anti-7E3 antibody is theoretically preferable to the indirect quantitation by subtracting post-infusion from baseline free 7E3-binding sites, as the latter calculation is based on the assumption that the total number of gpIIb-IIIa molecules on the platelet surface remains constant. This assumption is challenged by evidence for the presence of an intraplatelet pool of gpIIb-IIIa with recycling of gp between this pool and platelet surface (Wencel-Drake 1990).

5.3.2 Monitoring platelet-surface c7E3-Fab

An FITC-conjugated polyclonal rabbit-anti-m7E3 (Centocor Inc.) cross-reacting with the variable region of the chimaeric 7E3 was used to label platelet-bound c7E3-Fab (Fig. 5.2a). Stock antibody concentration was 2.2mg/ml and the fluorescein-to-protein ratio 1.8:1. Labelling was performed using dilute PRP (see 2.3.3.1) prepared from citrate-anti-coagulated whole blood within 3 hours of venesection. In view of the sensitivity of the epitope of 7E3 to EDTA (Coller *et al* 1986), citrate-PGE₁ (Appendix to Chapter 2) was used to wash the platelets. Figure 5.2b shows fluorescent histograms obtained with platelets labelled at baseline and 30 minutes after the onset of a c7E3-Fab infusion, by which time, as evidenced by the fluorescence distribution, the entire circulating platelet population was uniformly coated with antibody. The mean fluorescence channel (MFC) number was considered to be directly proportional to the amount of c7E3 on the platelet surface; this is in agreement with the calibration curve presented in Fig. 4.9, showing that, over the range of fluorescence intensities encountered in these studies, the MFC on the 1024-channel scale of the cytometer is, for practical purposes, a linear function of the number of FITC-labelled antibody molecules bound to the cell surface (see also 5.3.5.1 below). In order to consider the MFC as a linear function of the amount of platelet-bound c7E3, one also needs to assume that the number of fluorescent antibody molecules binding per molecule of c7E3-Fab is the same at high and low levels of platelet-bound c7E3-Fab, i.e. ignore the possible role of steric hindrance. Figure 5.3 shows fluorescence histograms obtained at baseline and at various time points after the end of a 48 hour infusion. It is obvious that, contrary to what was expected, the distribution of platelet-bound 7E3 remained unimodal at all time points post-infusion, i.e. no

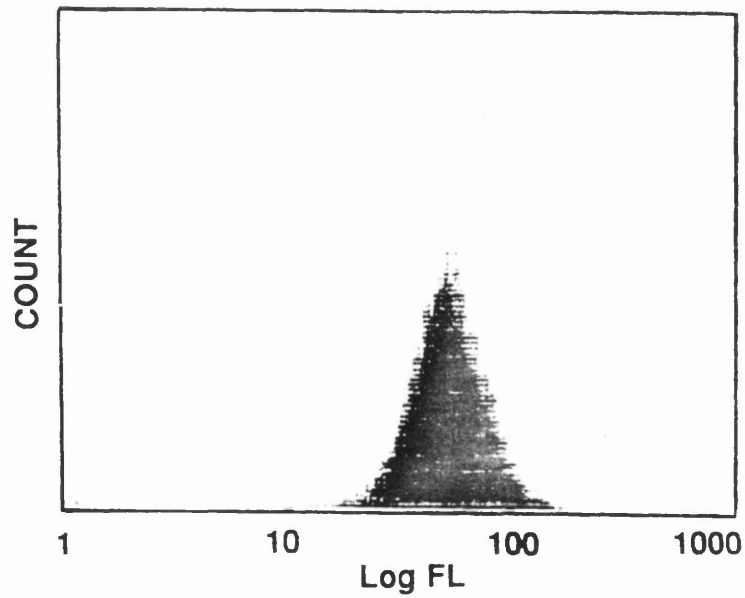
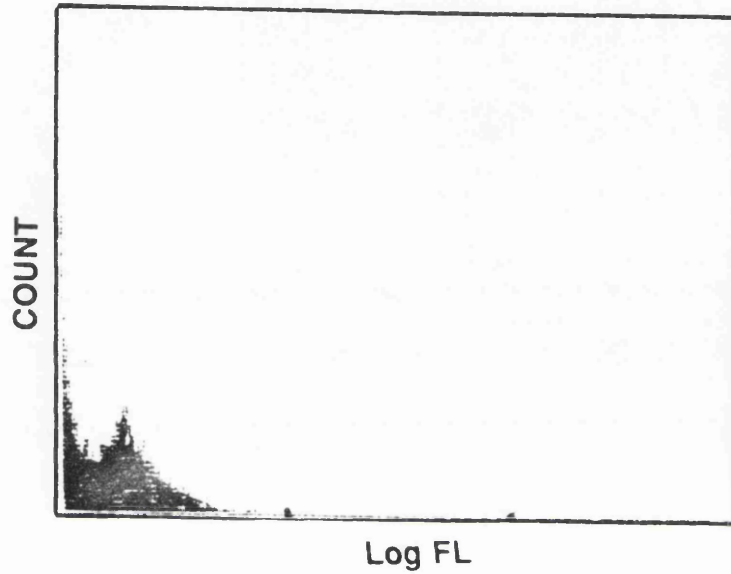
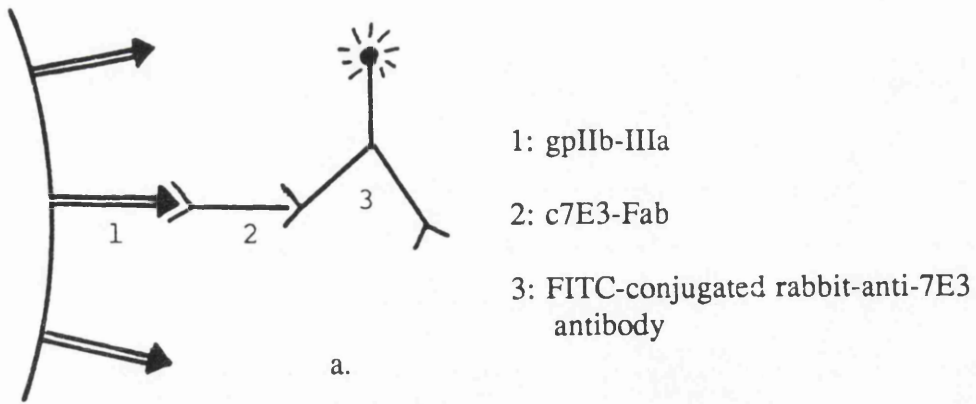


FIGURE 5.2 a. Labelling of platelet-bound c7E3-Fab with fluorescent polyclonal probe; b. Representative fluorescence histograms obtained at baseline (upper panel) and 30 minutes into the infusion of c7E3-Fab (lower panel).

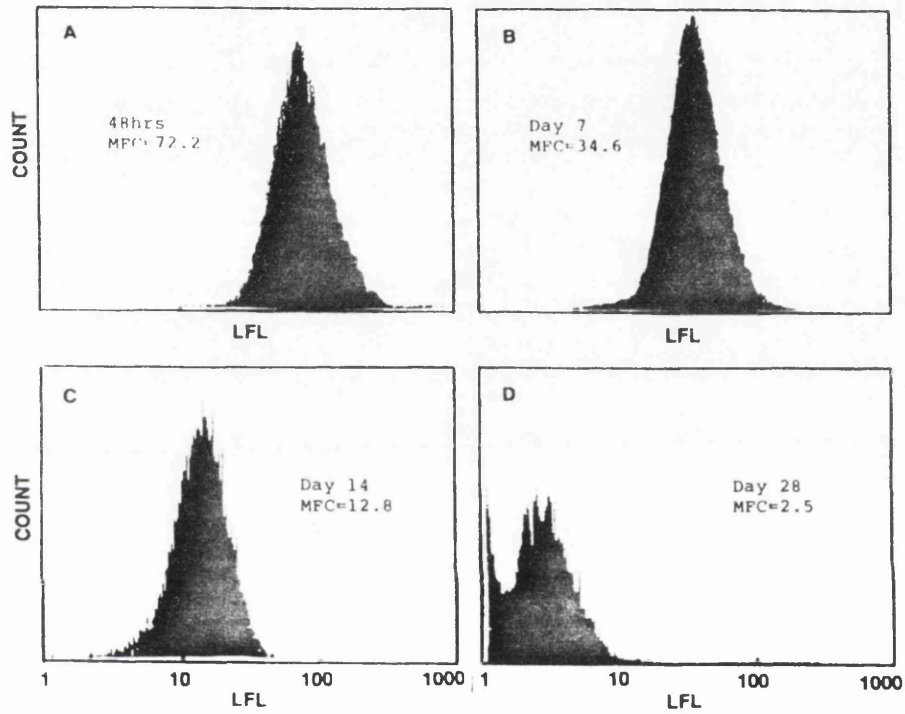


FIGURE 5.3 Fluorescence histograms obtained by incubation of platelets at the end of a 48-hour infusion and various time points thereafter with FITC-conjugated rabbit anti-7E3. MFC: Mean fluorescence channel.

dual (negative-positive) populations were present as a result of release of new, unexposed to 7E3, platelets, in any of the patients investigated. Moreover, the entire platelet population was bearing surface-bound 7E3 at a time (2 weeks) well beyond the lifespan (10 days) of the platelets originally exposed to the antibody. This means that, in some way, transfer of antibody to the newly released platelets was taking place.

5.3.2.1 24-hour infusions

Five patients received a loading dose of 0.25mg/kg followed by a 24-hour infusion of 5µg/min. Table 5.1 shows the levels of platelet-bound c7E3-Fab measured at baseline (just prior to the loading dose), 30 minutes into the infusion, end of infusion and at various post-infusion times. Differences between duplicate measurements on the same sample were consistently <2.5% and therefore single measurements were routinely performed. The fall in absolute levels (expressed as mean fluorescence channel [MFC]) of c7E3-Fab post-infusion is shown graphically in Fig. 5.4, whereas Fig. 5.5 shows c7E3-Fab levels expressed as % of the levels at the end of the infusion. In two patients (nos 1 and 2), no samples were available for flow cytometric analysis before 48 hours. In the remaining three patients, the time needed for platelet-bound antibody to fall to 50% of the level achieved at 24 hours was about 4 days (Fig. 5.5).

TIME	PATIENT NUMBER				
	1	2	3	4	5
Baseline	N/A	N/A	2.0(4.0)	1.9(3.5)	2.0(5.0)
30 minutes	N/A	N/A	70(145.0)	44(76.0)	33(84.5)
24 hours	N/A	N/A	48(100.0)	58(100.0)	39(100.0)
48 hours	41	55	36(75.0)	49(84.5)	36(92.0)
72 hours	32	44	35(73.0)	35(60.5)	25(64.0)
5 days	30	41	24(50.0)	25(43.0)	21(54.0)
7 days	19	25	16(33.5) ⁺	22(38.0)	19(49.0)
14 days	5.5 [*]	2.5 [*]	5.7(13.0)	6.5(11.5)	6.7(18.0)
28 days	2.3	1.9	2.2(4.5)	2.1(3.5)	2.2(5.5)

⁺ 8 days, ^{*} 16 days, [·] N/A: sample not available

TABLE 5.1 24 hour infusions: Mean fluorescence channel (MFC) of platelets incubated with FITC-conjugated rabbit-anti-7E3 at various time points. The numbers in parentheses are MFC numbers expressed as % (0.5% approximation) of the MFC at 24 hours.

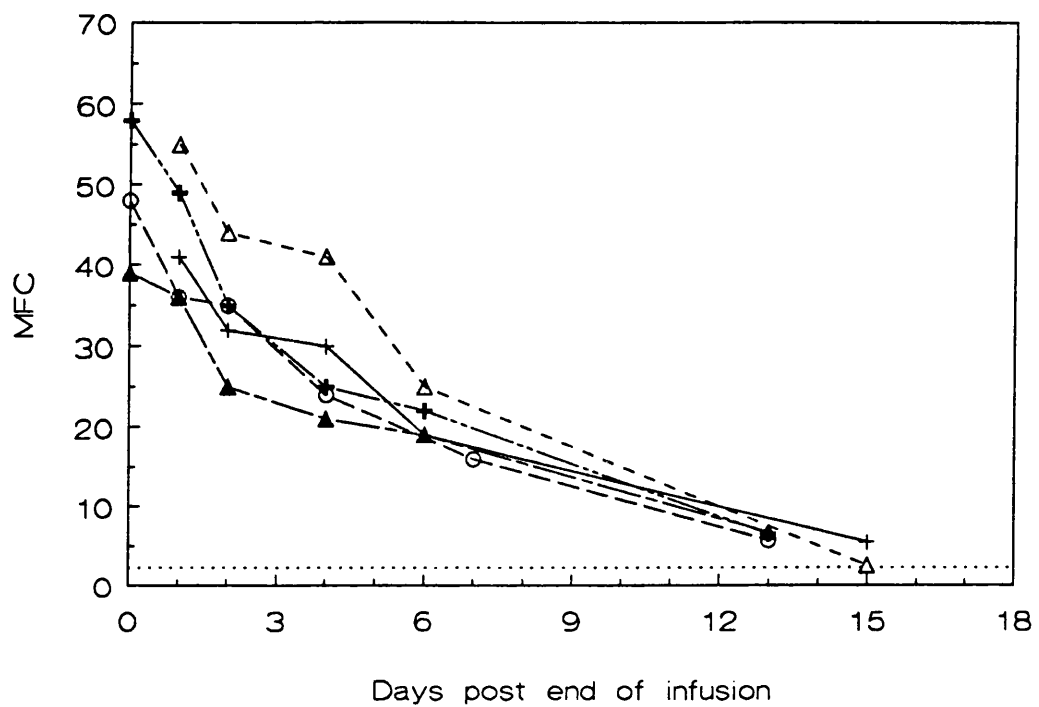


FIGURE 5.4 Post-infusion fall in absolute levels (expressed as MFC) of platelet-bound c7E3-Fab in 5 patients given 24-hour infusions. The horizontal dotted line represents background fluorescence.

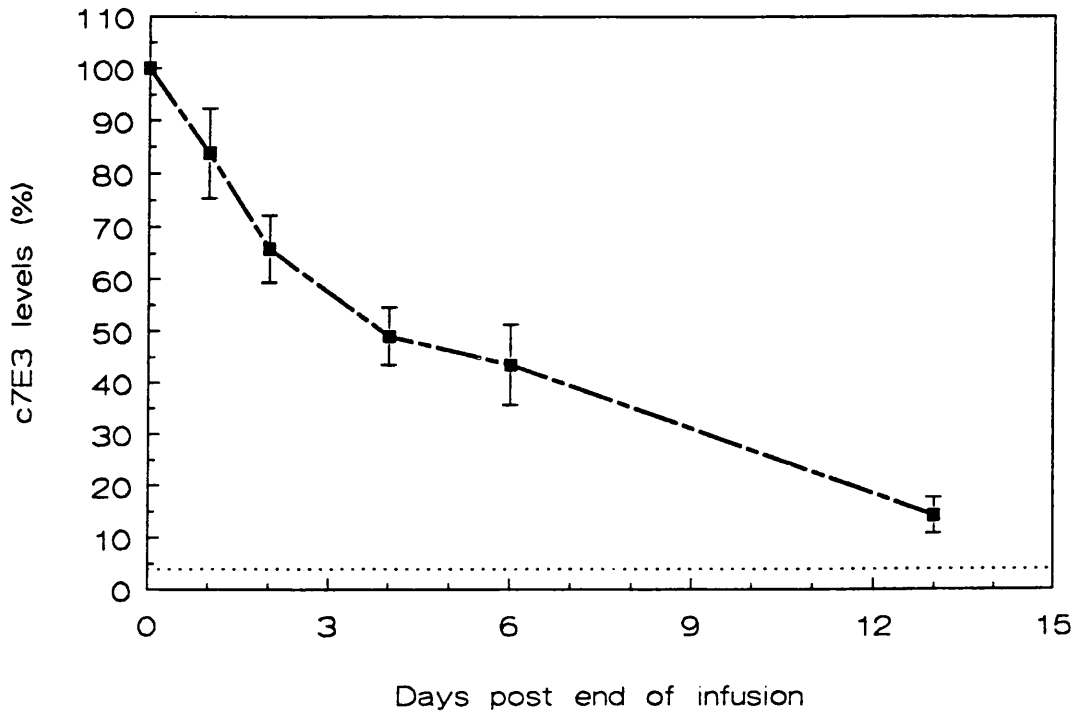


FIGURE 5.5 24-hour infusions (n=5). Mean post-infusion levels of platelet-bound c7E3-Fab expressed as % of the level attained at the end of the infusion. The thin vertical bars represent SD of the mean and the horizontal dotted line mean background fluorescence.

5.3.2.2 48-hour infusions

Seven patients received a 0.25mg/kg loading dose followed by continuous infusion of 10µg/min for 48 hours. The results of platelet-bound c7E3-Fab measurements are summarized in Table 5.2. Figure 5.6 is a graphical representation of the post-infusion fall in absolute levels of platelet-bound antibody (expressed as MFC) in individual patients. Figure 5.7 shows the clearance rate of antibody expressed as % of the level that had been achieved at the end of the infusion. The average "halving-time" of platelet-bound antibody in this group was *ca.* 5 days.

5.3.2.3 72-hour infusions

Six patients received a 0.25mg/kg loading dose followed by continuous infusion of 10µg/min for 72 hours. The results of monitoring c7E3-Fab levels are summarized in Table 5.3 and represented graphically in Figures 5.8 and 5.9. It can be seen from Fig. 5.9 that the halving-time of platelet-bound antibody in this group of patients was about 6 days.

5.3.2.4 96-hour infusion

One patient received a loading dose of 0.25mg/kg followed by continuous infusion of 10µg/min for 96 hours. The measurements obtained in this case are given in Fig. 5.10.

TIME	PATIENT NUMBER						
	6	7	8	9	10	16	18
Baseline	1.7 (3.0)	1.8 (2.5)	1.9 (3.0)	2.0 (3.5)	2.2 (3.5)	2.0 (3.5)	2.1 (4.0)
30 minutes	46 (78)	59 (82)	53 (79)	54 (95)	59 (89.5)	52 (91)	43 (82.5)
24 hours	53 (90)	63 (87.5)	54 (80.5)	61 (107)	71 (107.5)	54 (95)	51 (98)
48 hours	59 (100)	72 (100)	67 (100)	57 (100)	66 (100)	57 (100)	52 (100)
72 hours	52 (88)	62 (86)	60 (89.5)	50** (87.5)	48** (73)	47 (82.5)	46 (88.5)
5 days	35 (59.5)	46 (64)	36 (53.5)	43 (75.5)	48 (73)	30** (52.5)	N/A
7 days	34 (57.5)	35 (48.5)	33 (49.5)	32 (56)	30+ (45.5)	36 (63.0)	26 (50)
14 days	13 (22)	13 (18)	N/A	13 (23)	12 (18)	6 (10.5)	8.2 [§] (16)
28 days	2.3 (4.0)	N/A	3.1* (4.5)	2.3 (4.0)	2.1 (3.0)	2.1 (3.5)	2.1 (4.0)

* 21 days, ** 60 hours, + 8 days, ** 4 days, § 11 days, N/A: sample not available

TABLE 5.2 48 hour infusions: Mean fluorescence channel (MFC) of platelets incubated with FITC-conjugated rabbit-anti-7E3 at various time points. The numbers in parentheses are MFC numbers expressed as % (0.5% approximation) of the MFC at 48 hours.

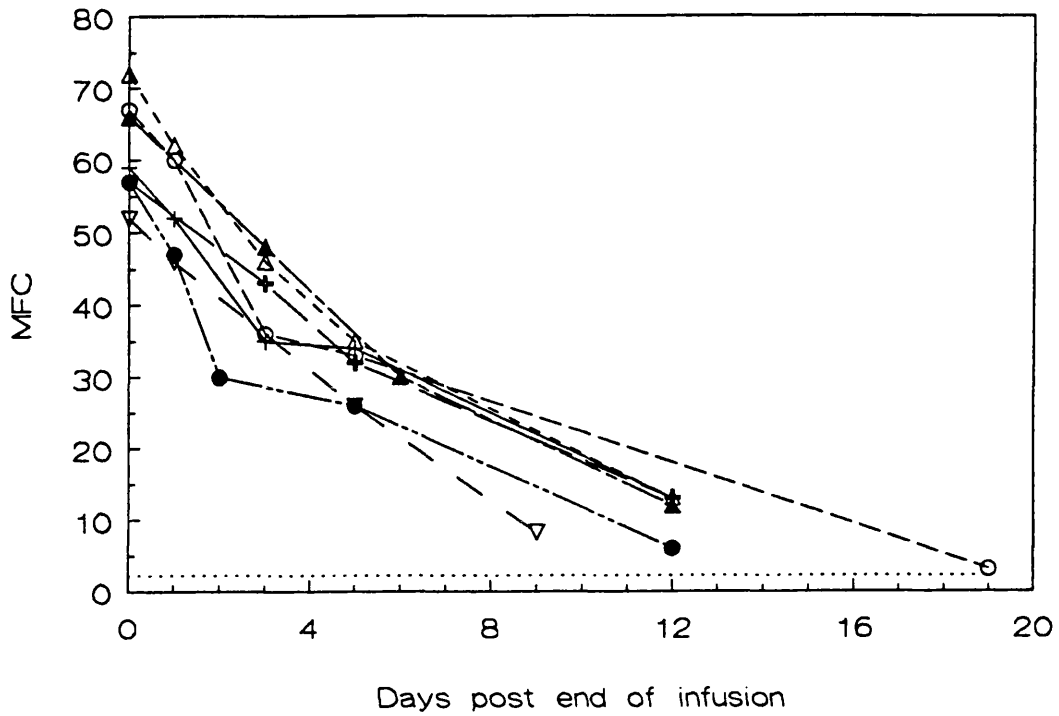


FIGURE 5.6 Post-infusion fall in absolute levels (expressed as MFC) of platelet-bound c7E3-Fab in 7 patients given 48-hour infusions. The horizontal dotted line represents background fluorescence.

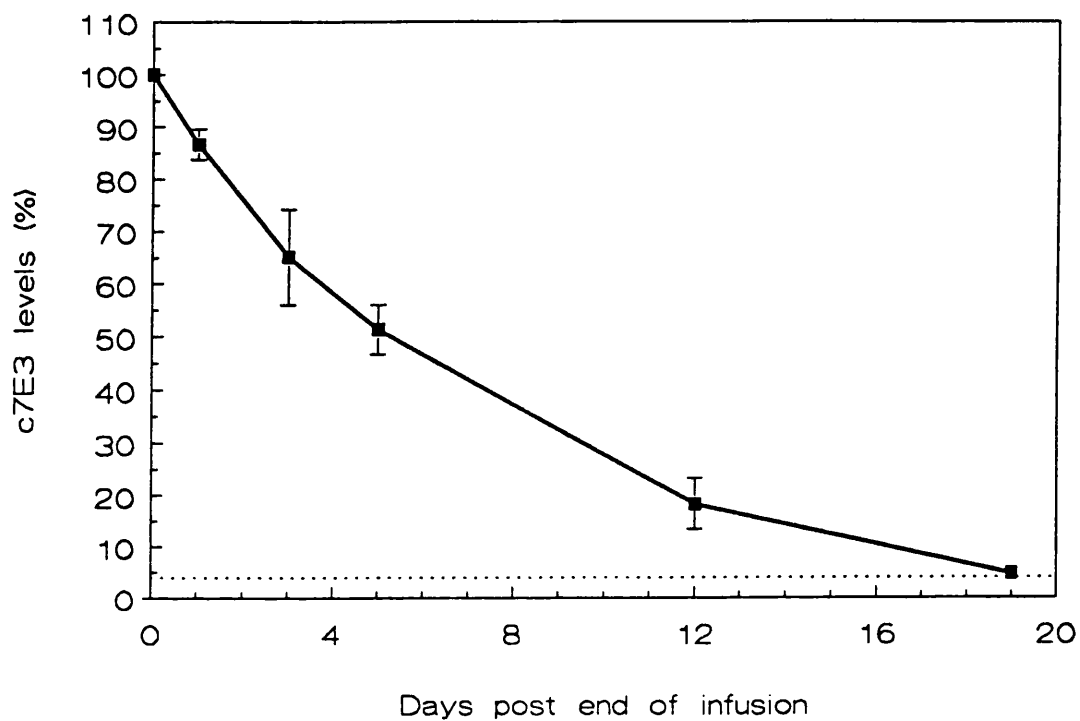


FIGURE 5.7 48-hour infusions (n=7). Mean post-infusion levels of platelet-bound c7E3-Fab expressed as % of the level attained at the end of the infusion. The thin vertical bars represent SD of the mean and the horizontal dotted line mean background fluorescence.

TIME	PATIENT NUMBER					
	11	12	13	14	15	17
Baseline	1.8 (3.5)	2.0 (3.0)	2.1 (3.5)	1.8 (3.5)	1.8 (3.0)	2.0 (3.5)
30 minutes	56 (110)	52 (84)	49 (80.5)	41 (74.5)	50 (77)	41 (69.5)
24 hours	49 (96)	59 (95)	57 (93.5)	45 (82)	56 (86)	51 (86.5)
48 hours	56 (110)	56 (90.5)	49 (80.5)	53 (96.5)	66 (101.5)	59 (100)
72 hours	51 (100)	62 (100)	61 (100)	55 (100)	65 (100)	59 (100)
5 days	37 (72.5)	50 (80.5)	49 (80.5)	48 (87.5)	49 (75.5)	53* (90)
7 days	36 (70.5)	26** (42)	35** (57.5)	38 (69)	42 (64.5)	40 (68)
10 days	N/A	N/A	N/A	25 (45.5)	26 (40)	25 (42.5)
14 days	9.1+ (18)	5.5+ (9.0)	9.3+ (15)	17.1 (31)	13.9 (21.5)	11.6 (19.5)
28 days	2.0 (4.0)	2.1 (4.0)	2.0 (3.5)	2.9 (5.5)	2.4 (4.0)	2.2 (4.0)

* 4 days, ** 8 days, + 15 days, N/A: no sample available

TABLE 5.3 72 hour infusions: Mean fluorescence channel (MFC) of platelets incubated with FITC-conjugated rabbit-anti-7E3 at various time points. The numbers in parentheses are MFC numbers expressed as % (0.5% approximation) of the MFC at 72 hours.

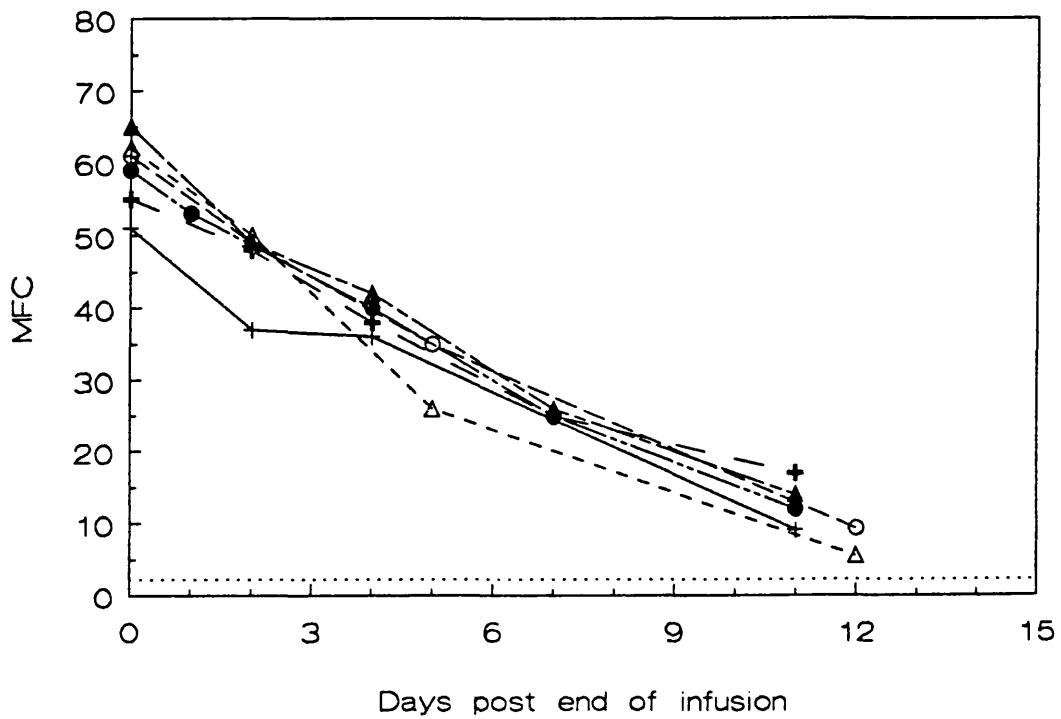


FIGURE 5.8 Post-infusion fall in absolute levels (expressed as MFC) of platelet-bound c7E3-Fab in 6 patients given 72-hour infusions. The horizontal dotted line represents background fluorescence.

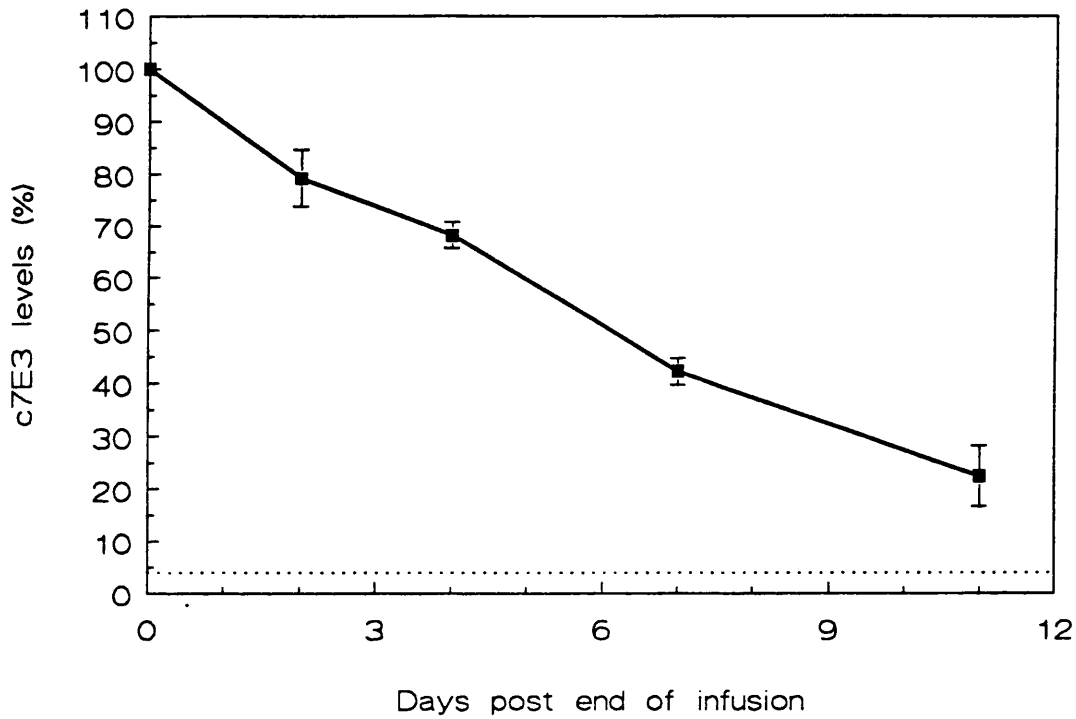


FIGURE 5.9 72-hour infusions (n=6). Mean post-infusion levels of platelet-bound c7E3-Fab expressed as % of the level attained at the end of the infusion. The thin vertical bars represent SD of the mean and the horizontal dotted line mean background fluorescence.

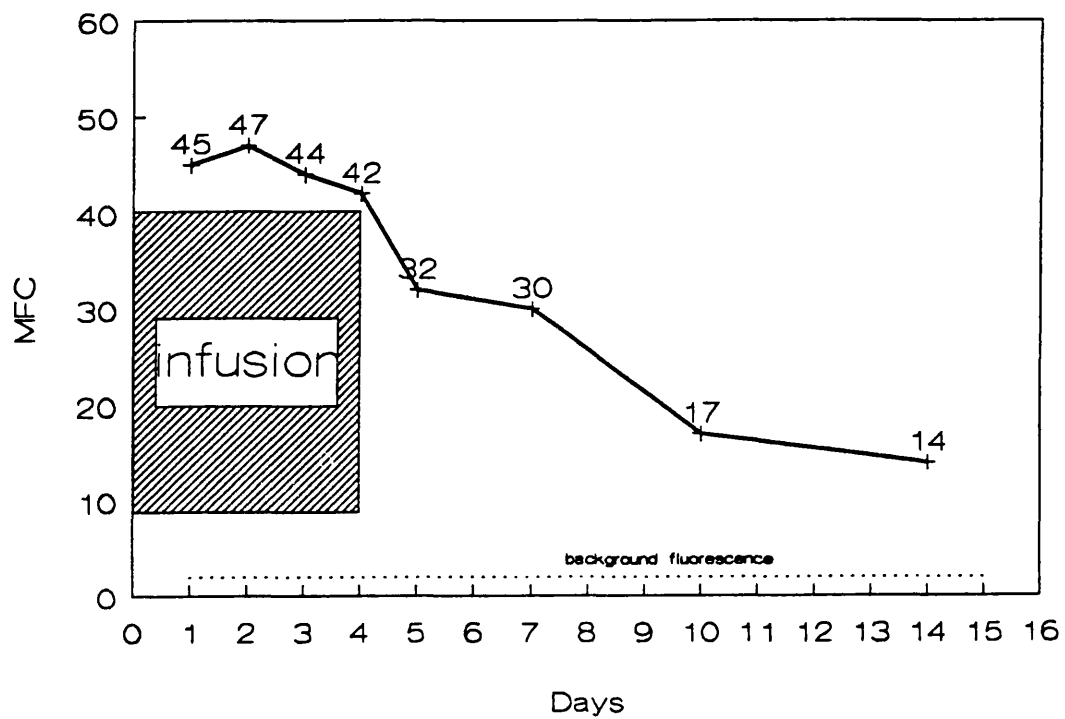


FIGURE 5.10 Platelet-bound c7E3-Fab levels (as MFC numbers) at various time points of a 96-hour infusion.

5.3.3 Platelet-bound c7E3-Fab levels versus number of free binding sites.

The number of available binding sites for 7E3 on the platelet surface was routinely monitored using radiolabelled c7E3-IgG as described in 2.6. Individual patients' measurements performed simultaneously with flow cytometric platelet-bound c7E3-Fab estimations are given in Table V.1 (Appendix to Chapter 5). Figure 5.11 shows that the rate of recovery of free binding sites post infusion was similar (parallel lines) in the three groups of patients. It can also be seen that, the percentage of binding sites blocked at the end of the 24hr infusion was significantly lower compared with that of the 48 and 72hr infusions, due to the lower infusion dose of 7E3 used in the former group (the loading dose was the same in all groups).

There was a generally good negative correlation between absolute levels of platelet-bound c7E3-Fab (PS7E3) measured by FC and free binding sites for c7E3 estimated by RIA (BS-RIA). Figure 5.12 shows PS7E3 plotted against BS-RIA on day 7 of all three infusion groups ($r=-0.666$, $P<0.001$). In Figure 5.13, the post-infusion fall of PS7E3 measured by FC has been plotted against the increase in BS-RIA in individual patients (those on whom at least 4 simultaneous FC/RIA assays were performed). There was generally a very strong correlation between rate of fall of PS7E3 and increase in BS-RIA.

Although the number of free binding sites had returned to baseline (mean=103.7%, SD=16%) on day 7 in the patients who received 24hr infusions, in the same group of patients the levels of flow cytometrically measurable c7E3-Fab on the platelet surface were 40% (SD=8%) of those

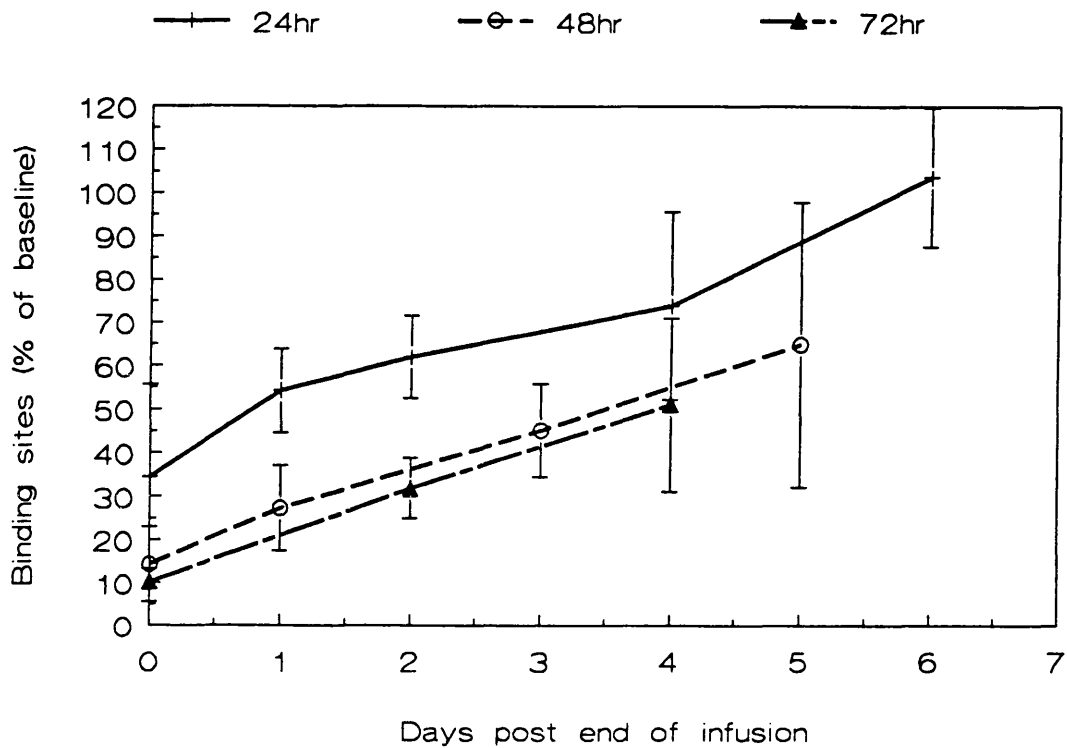


FIGURE 5.11 Recovery of c7E3 binding sites (as % of baseline) estimated by RIA following 24, 48 or 72-hour infusions of c7E3-Fab. The thin vertical bars represent SD of the mean.

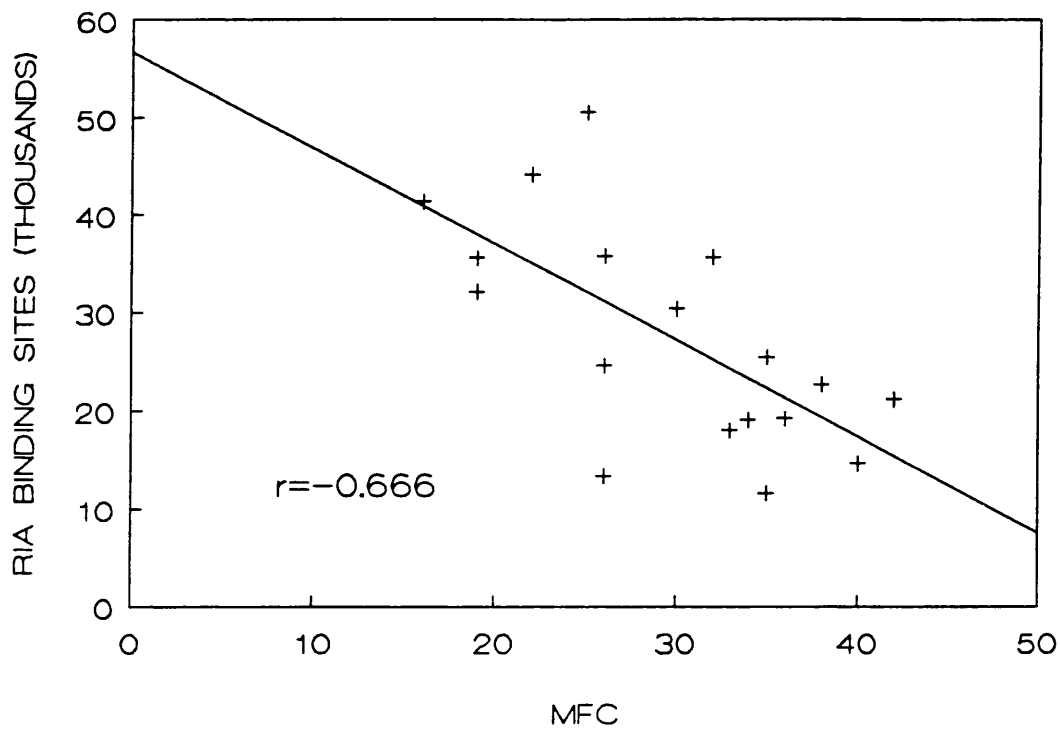
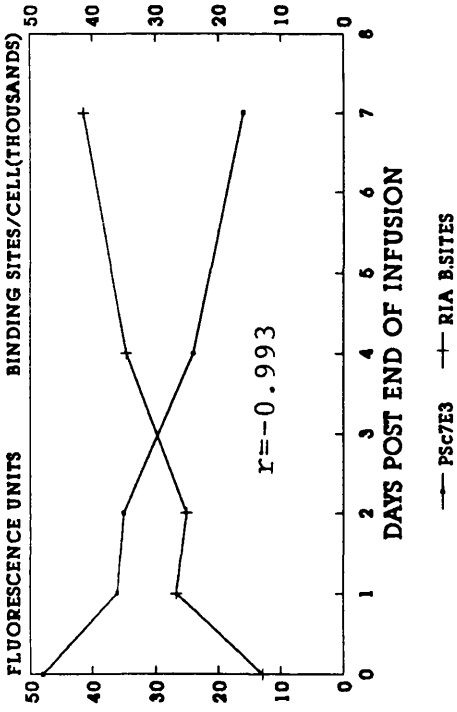
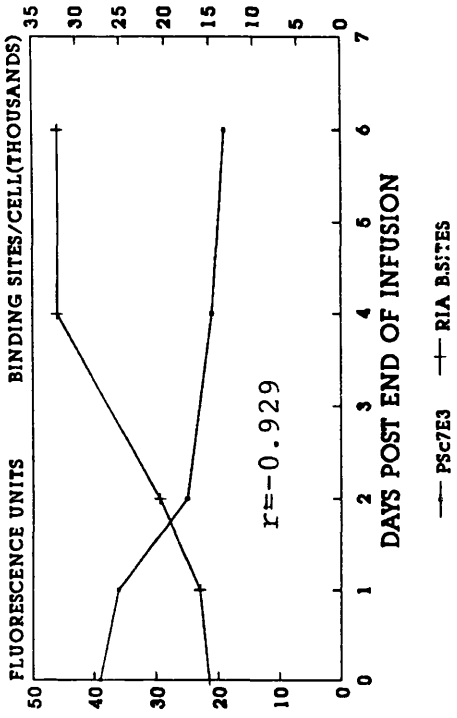


FIGURE 5.12 Number of residual 7E3 binding sites (RIA) plotted against absolute levels of platelet-bound c7E3-Fab (expressed as the MFC obtained using FITC-conjugated rabbit-anti-7E3).

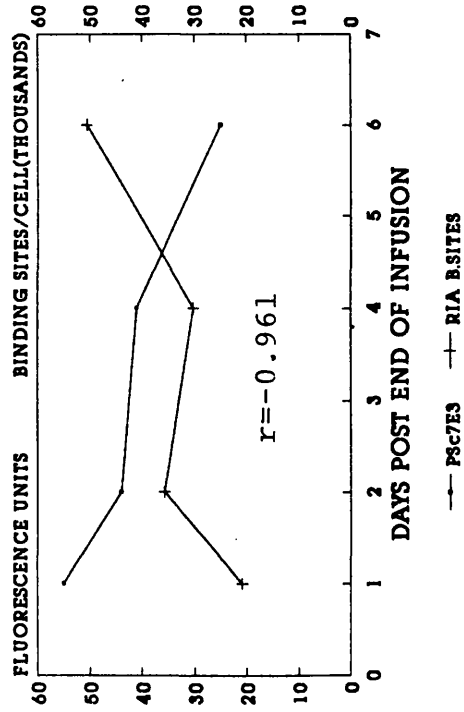
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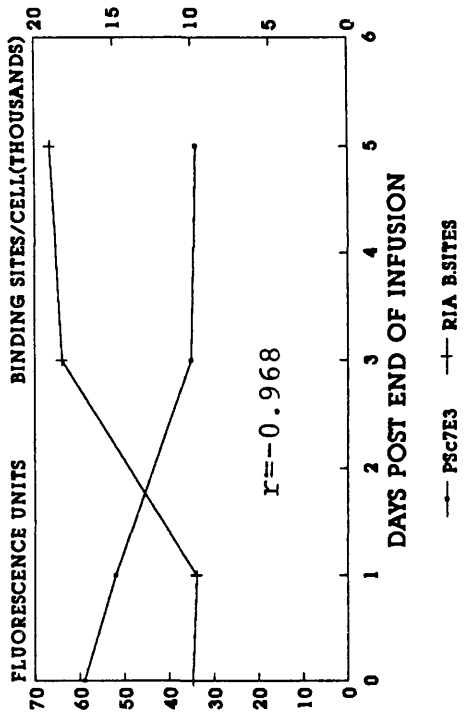
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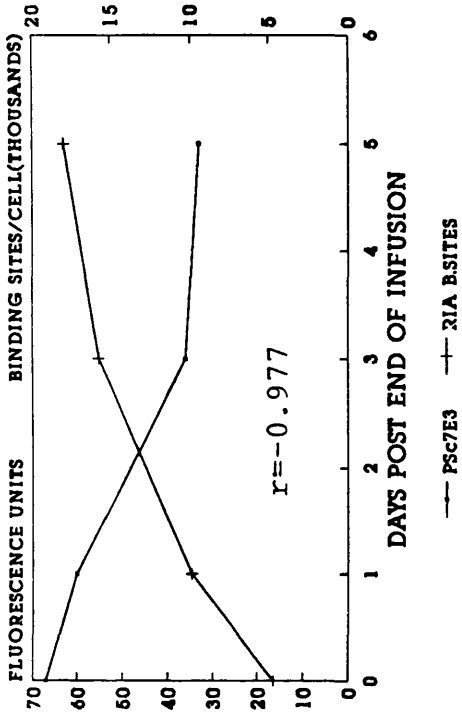
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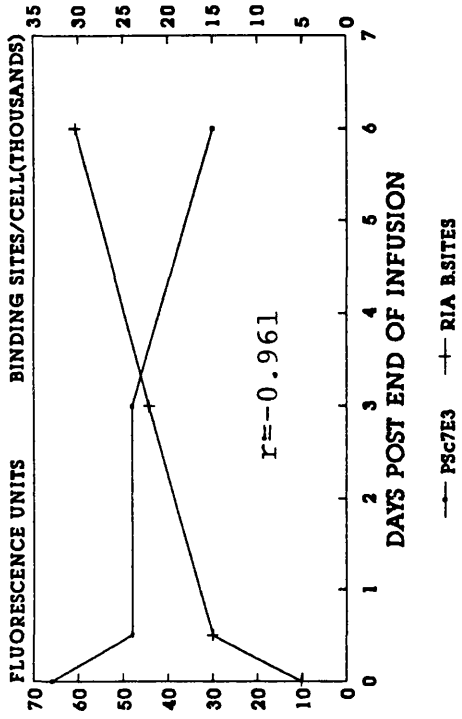
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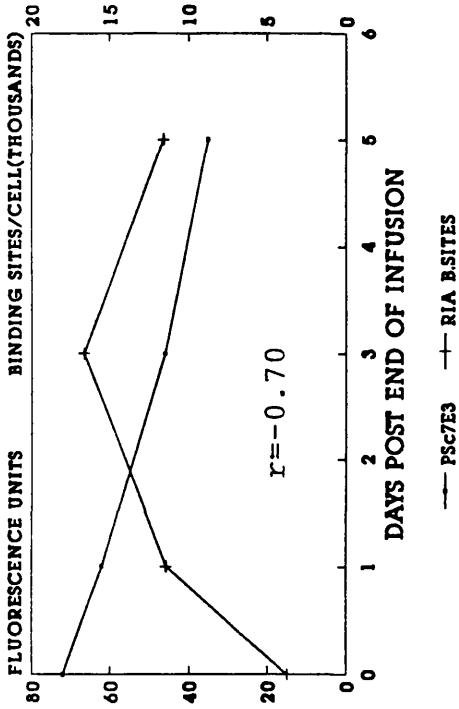
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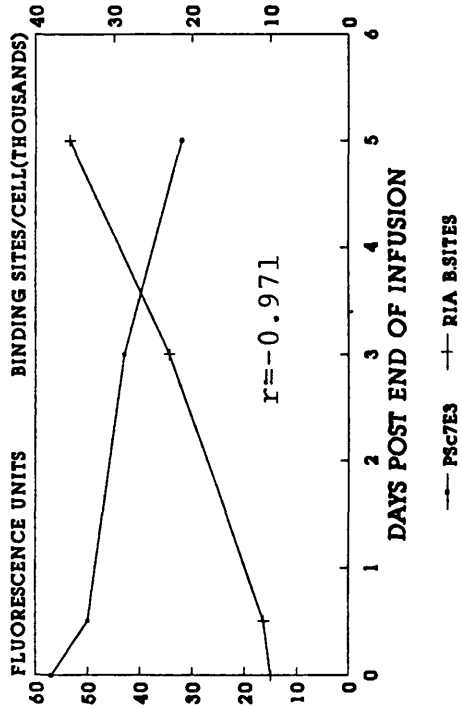
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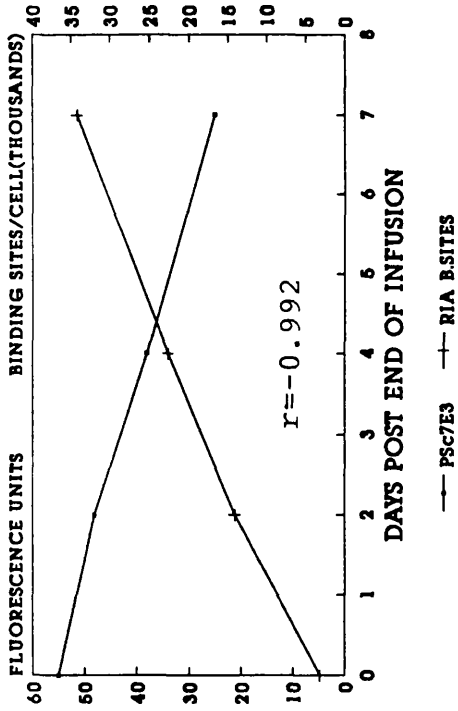
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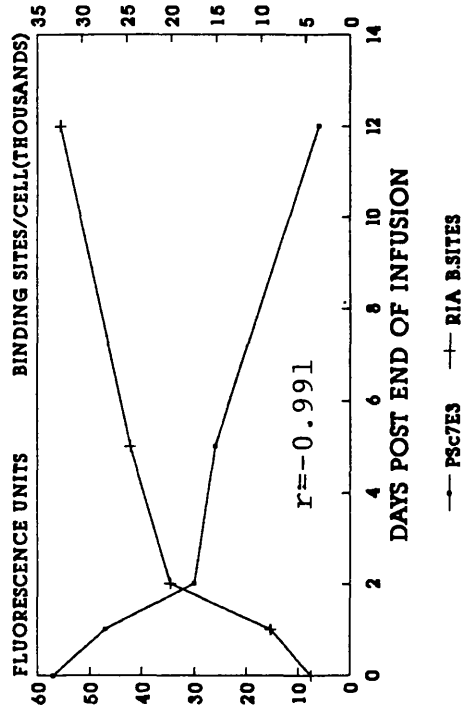
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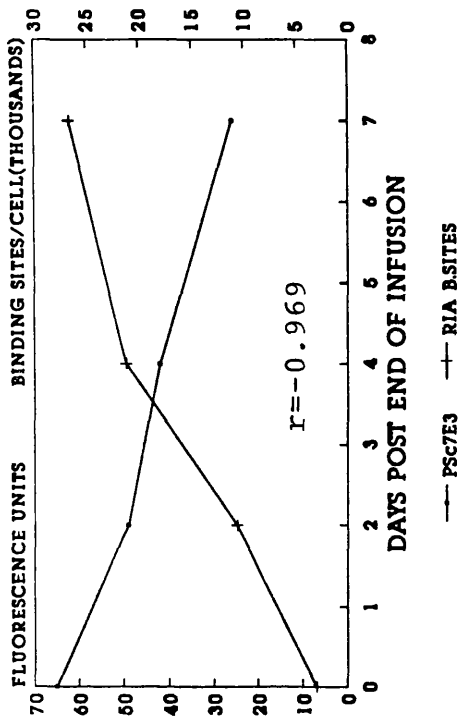
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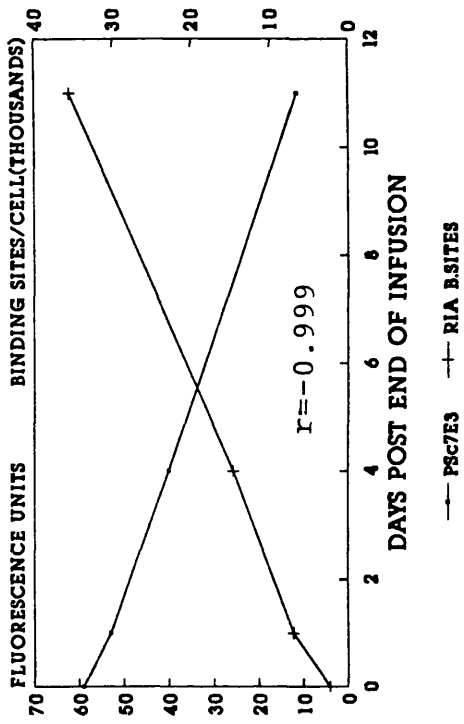
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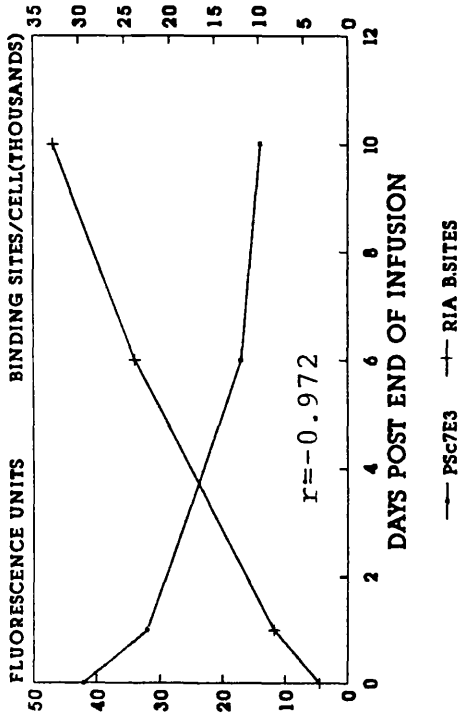
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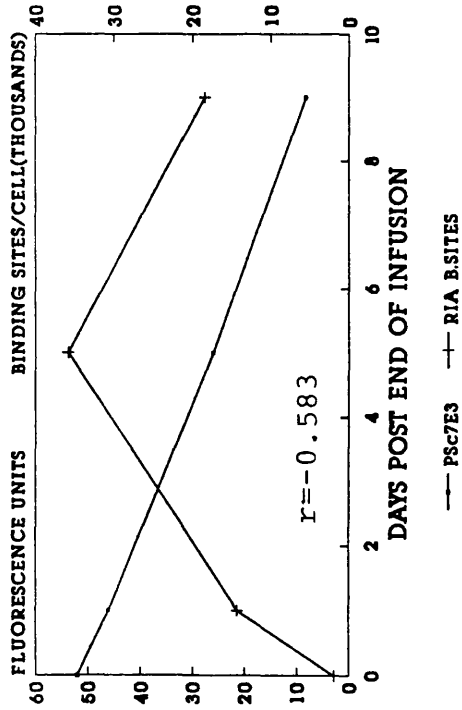


FIGURE 5.13 Rate of post-infusion fall in platelet-bound c7E3-Fab (PS7E3) estimated by FC, plotted against the increase in number of residual 7E3 binding sites measured by RIA.

Patients with at least four simultaneous FC/RIA measurements are included. There is generally a very strong negative correlation.

Fluorescence units = mean fluorescence channel number.

r: Pearson's coefficient of correlation.

at 24 hours. Four days post infusion, free binding sites and 7E3 levels were respectively 73.8% (SD=21.7%) and 49% (SD=5.5%). This suggests that the platelets might be expressing more surface gpIIb-IIIa post infusion compared with baseline. In the groups who received longer and higher dose infusions, this apparent discrepancy between recovery of free binding sites and amount of platelet-bound 7E3 was less marked. In the 48hr infusion group, binding sites on day 7 had returned to 64.9% (SD=32.8%) of baseline, while platelet-bound 7E3 was 52.8% (SD=6.1%) of the level achieved at 48 hours. In the 72hr infusion group, binding sites four days post infusion were 51.1% (SD=19.9%) of baseline and 7E3 levels 61.9% (SD=10.8%) of those at the end of the infusion.

5.3.4 c7E3-Fab binding sites by flow cytometry

Using incubation with c7E3-IgG to estimate available binding sites following administration of c7E3-Fab has the theoretical disadvantage of overestimating the true number of free binding sites due to displacement of platelet-bound cFab during incubation with cIgG. Furthermore, Fab fragments could have better access to binding sites located in the surface-connected canalicular system (Woods *et al* 1986). In order to assess the efficacy of FC in monitoring free c7E3-Fab binding sites in parallel with platelet-bound c7E3-Fab, platelets were labelled with FITC-conjugated c7E3-Fab at baseline and at various time points during and after the infusion. The antibody concentration (stock) was 0.8mg/l with a fluorochrome:protein ratio of 5.1:1. Labelling was performed using dilute PRP as described in 2.3.3.1 and a saturating final FITC-c7E3-Fab concentration of 10µg/ml. The final wash was in citrate-PGE₁ buffer. Representative histograms are shown in Fig. 5.14.

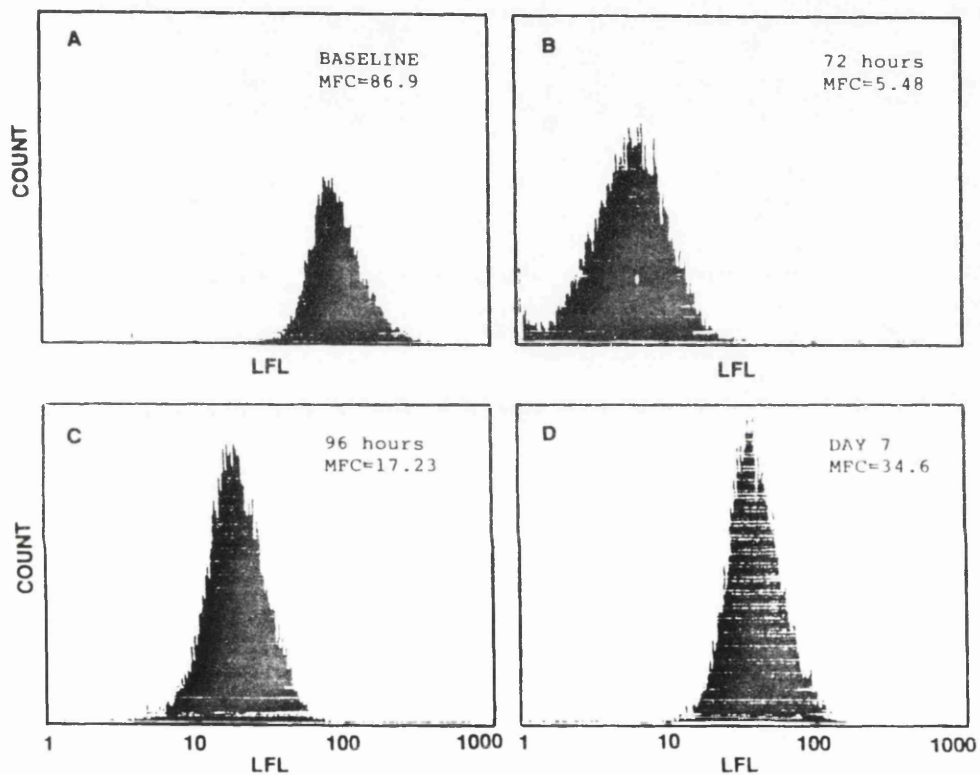


FIGURE 5.14 Flow cytometric estimations of c7E3-Fab binding capacity of platelets at various time points of a 72-hour c7E3-Fab infusion. Fluorescence histograms were obtained by incubation with FITC-conjugated c7E3-Fab.

The results obtained in the six patients who were studied in this way are shown in Table 5.4 both as absolute fluorescence levels (expressed as MFC) and percent of baseline fluorescence. All patients had received a loading dose of 0.25mg/kg at time zero, followed by continuous infusion of 10µg/min for 48 (n=2), 72 (n=3) or 96 (n=1) hours. At 30 minutes, the 7E3-binding capacity had fallen to 6.8% (SD=0.7%) of baseline. At the end of the infusion it was 7.7% (SD=2.6%) compared to 8.7% (SD=2.3%) estimated by RIA. In the three 72hr-infusion patients studied, the free binding sites on day 7 were 50.5% (SD=9.5%) by FC and 61.2% (21.3%) by RIA. As shown in Fig.5.15, there was usually an excellent correlation between the rate of post infusion recovery of binding sites estimated by FC and that estimated by RIA.

Five patients had 7E3-binding capacity measured by FC on day 28. As shown in Fig.5.16, antibody binding at that point was 134.2% (SD=17%) of baseline.

5.3.5 The phenomenon of 7E3-Fab transfer between platelets

As described in 5.3.2, c7E3-Fab was present on the surface of all circulating platelets at time points well beyond the life span of the platelets originally exposed to the antibody. This was not due to prolonged survival of 7E3-coated platelets because antibody distribution in the platelet population was always unimodal. One or more of the following mechanisms could be responsible:

- Transfer of antibody between receptors as platelets come into contact (i.e. "jumping" of 7E3 to unoccupied binding sites; a simple competition mechanism).

TIME	PATIENT NUMBER					
	14*	15*	16**	17*	18**	19*
Baseline	94.6 (100)	81.0 (100)	91.1 (100)	86.9 (100)	84.0 (100)	110.0 (100)
30 mins	6.1 (6.5)	5.8 (7.2)	7.1 (7.8)	6.0 (6.9)	5.8 (6.9)	6.1 (5.5)
24 hours	12.3 (13.0)	11.7 (14.4)	8.7 (9.5)		8.8 (10.5)	5.4 (4.9)
48 hours	12.1 (12.7)	11.0 (13.5)	11.1 (12.2)	7.8 (9.0)	4.8 (5.7)	5.1 (4.6)
72 hours	8.5 (9.0)	6.6 (8.1)	30.1 (33.0)	5.5 (6.3)	26.4 (31.4)	7.6 (6.9)
96 hours			60.6 (66.5)	17.2 (19.8)		5.5 (5.0)
day 5	34.1 (36.0)	32.8 (40.5)				14.0 (12.7)
day 7	50.7 (53.6)	47.1 (58.1)	69.0 (75.7)	34.6 (39.8)	64.3 (76.5)	29.0 (26.4)
day 10	74.2 (78.4)	62.7 (77.4)		54.0 (62.1)	87.3 ^s (103.9)	42.0 (38.2)
day 14	74.8 (79.5)	65.6 (81.0)	84.4 (92.6)	87.1 (100.2)		86.3 (78.5)
day 28	111.0 (117.3)	97.1 (119.9)	118.0 (129.5)	130.0 (149.6)	130.0 (154.7)	

* 72hr infusion, ** 48hr infusion, + 96hr infusion, ^s day 11

TABLE 5.4 Mean fluorescence channels (MFC) of platelets incubated with FITC-conjugated c7E3-Fab before (baseline) and at various time points during and after c7E3-Fab infusion. The numbers in parentheses are MFC expressed as percent of MFC at baseline.

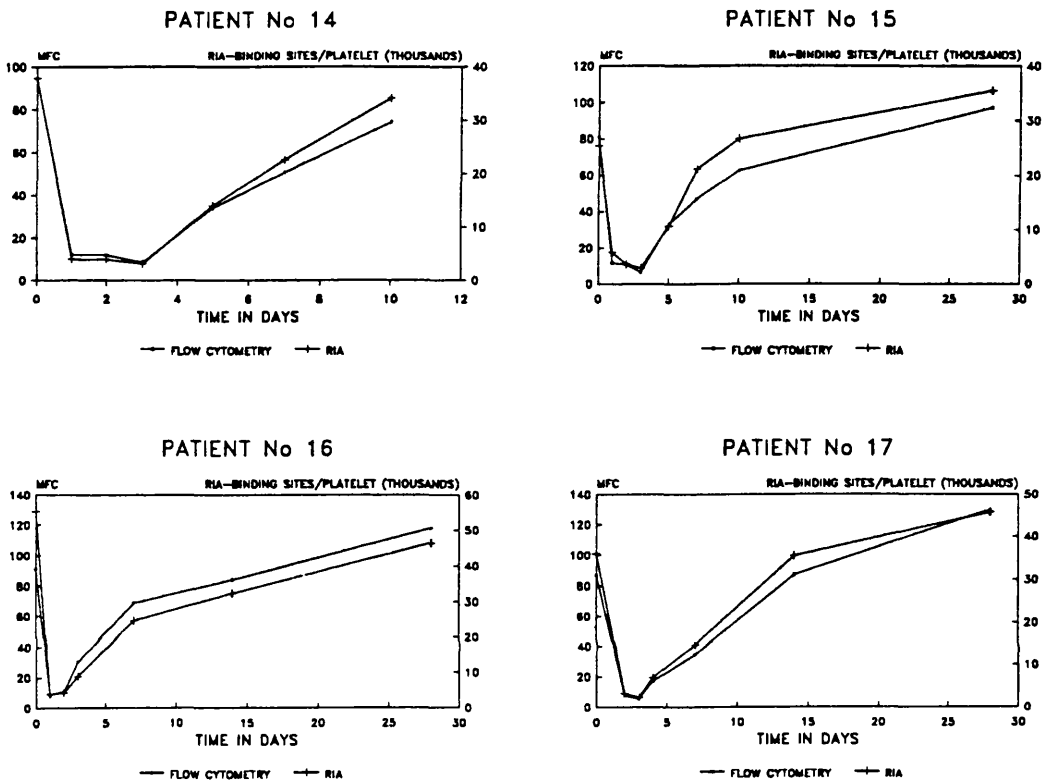


FIGURE 5.15 Parallel flow cytometric and RIA estimation of c7E3 binding capacity of platelets at various time points of three 72-hour and one 48-hour (patient No 16) infusions. There is an excellent correlation between the two methods with an average linear correlation coefficient of $r=0.97$ ($SD=0.028$).

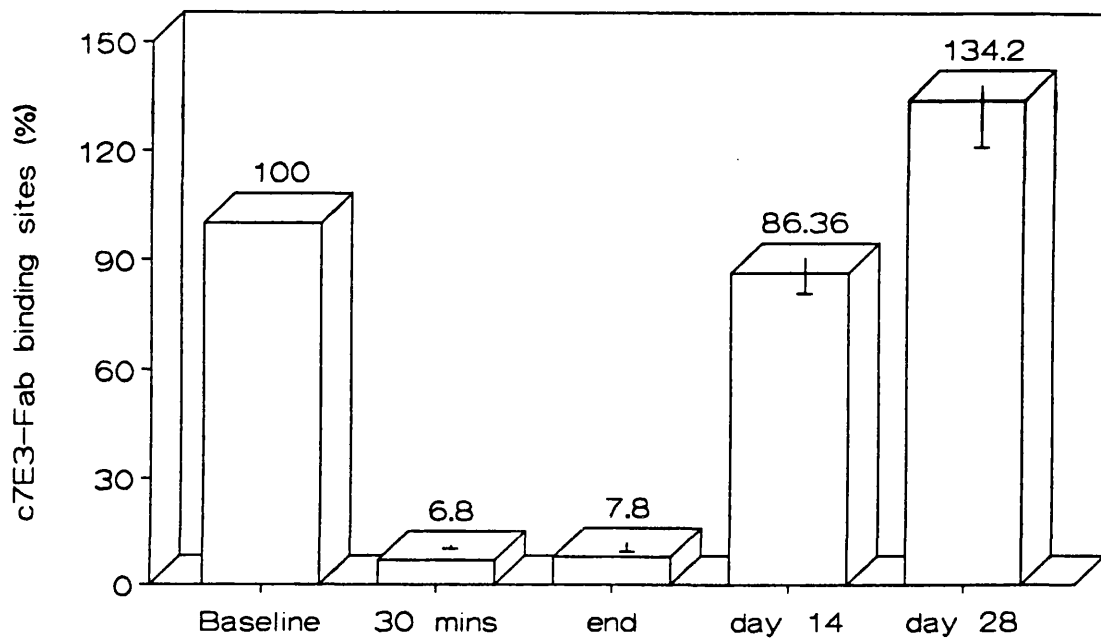


FIGURE 5.16 Comparison of flow cytometrically estimated c7E3-Fab-binding capacity (expressed as % of baseline) at various time points during and after the infusion. The numbers given are means of the 5 patients who had this measurement performed on day 28; the thin vertical bars represent SD of the mean.

- Exchange of antibody-carrying receptors between platelets.
- Antibody binding to megakaryocytes or other sites (e.g. endothelia), with subsequent incorporation/transfer to newly released platelets.

In order to investigate the first two possibilities, the following *ex vivo* and *in vitro* mixing experiments were performed:

(a). PRP was made from citrate-anticoagulated blood samples obtained at baseline and during the c-7E3-Fab infusion and the platelet count adjusted to $300 \times 10^9/l$. Equal volumes of the two PRPs were mixed in polypropylene tubes and incubated at room temperature, standing or on a rotary mixer. Platelet-bound 7E3 was measured by FC (5.3.2) immediately after mixing and at intervals during the incubation. The results of a representative experiment are shown in Fig. 5.17. Two distinct platelet populations, a negative (baseline) and a positive (7E3-coated) were present immediately after mixing. With time, the fluorescence of the positive population decreased and that of the negative increased, eventually resulting in complete merging of the two populations. The mean fluorescence at equilibrium (the point where only a single peak was visible in the fluorescence histogram) was about 50% of the original fluorescence of the 7E3-coated platelets. Equilibrium was usually reached within 18 hours. Continuous mixing had no appreciable effect on the time needed to reach equilibrium.

(b). PRP from normal volunteers was incubated with $5 \mu\text{g/ml}$ c7E3-Fab for 30 minutes and, following 2 washes in citrate- PGE_1 the platelets were resuspended in autologous PPP. Mixing with an equal volume of PRP which had not been exposed to 7E3 and measurements of platelet-bound

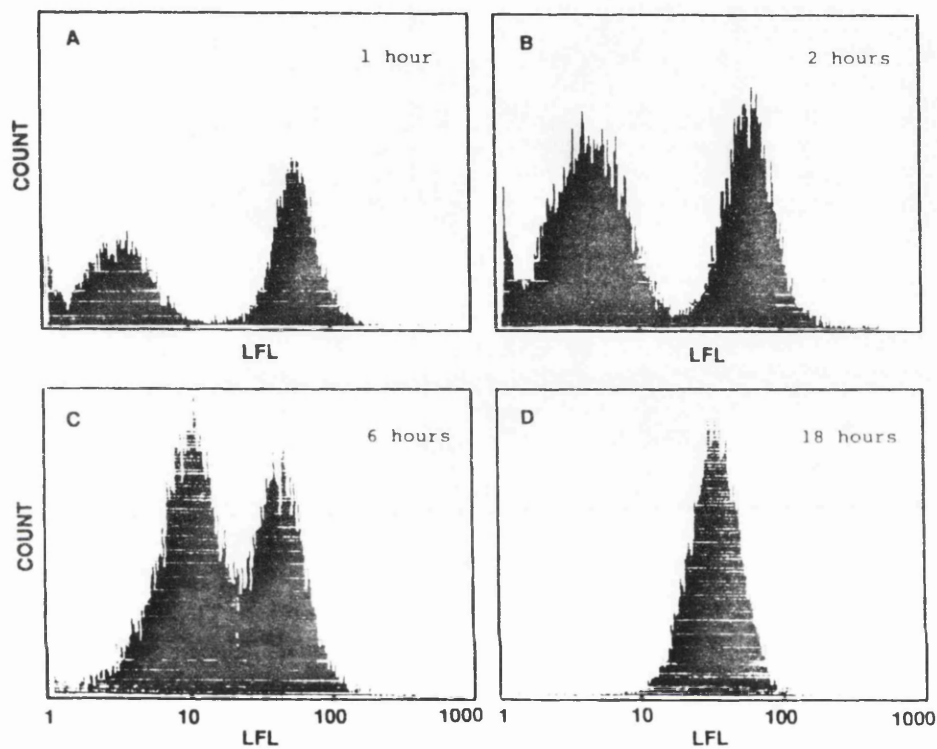


FIGURE 5.17 Fluorescent histograms showing the distribution of platelet-bound c7E3-Fab at various time points following mixing equal numbers of c7E3-Fab-coated and non-coated platelets.

7E3 were performed as in (a). Again, a gradual transfer of antibody from positive to negative cells was observed. The rate of transfer varied between individuals but, as with the ex vivo mixing, equilibrium was usually achieved within 18 hours. The same pattern of antibody transfer was observed when the Fab fragment of murine 7E3 was used instead of chimaeric 7E3-Fab.

(c). Washed platelets (in citrate-PGE₁) were incubated with a saturating concentration of c7E3-Fab and, following a further wash, were resuspended in PBS and mixed with an equal volume of washed, non-7E3 coated platelets from the same donor. There was no difference in the pattern of antibody transfer compared to platelets suspended in autologous plasma.

(d). The above experiment (c) was repeated using washed platelets which were fixed (paraformaldehyde 1%) prior to 7E3 coating. Transfer of antibody to non-coated, non-fixed platelets took place at an increased rate, probably reflecting lower affinity of 7E3 for the fixed receptor.

(e). Experiments (b) and (c) were repeated using an excess of the whole molecule of c7E3-IgG (instead of its Fab fragment) to coat the platelets. In this case no significant antibody transfer from positive to negative platelets took place (Fig. 5.18). This suggests that 7E3-IgG binds to the IIB-IIIa receptor with significantly higher affinity than its Fab fragment. This is most likely due to bivalent binding, i.e. each Fab arm of the 7E3-IgG molecule binding to a different IIB-IIIa molecule, even in conditions of large antibody excess.

(f). c7E3-Fab-coated platelets were mixed and incubated with non-coated platelets whose IIB-IIIa complexes had been dissociated through

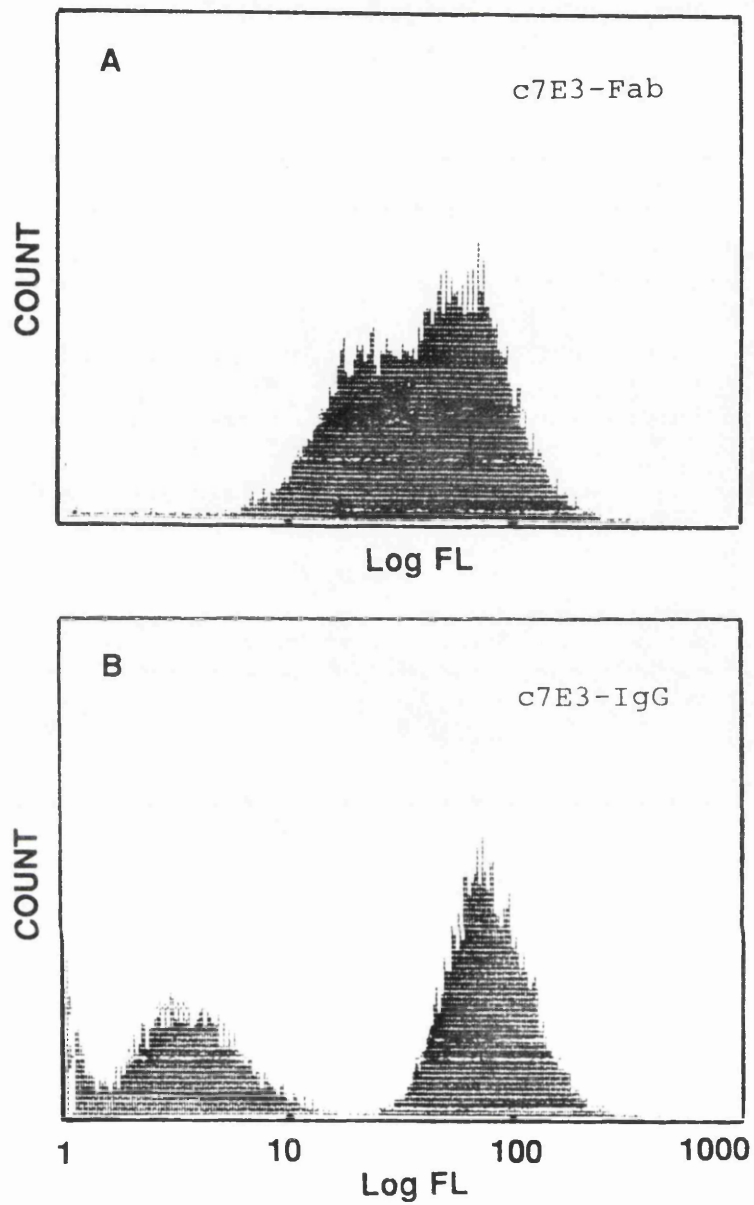


FIGURE 5.18 The distribution of platelet-bound c7E3 five hours after mixing platelets coated with c7E3-Fab (A) or c7E3-IgG (B) with equal numbers of non-coated platelets (see also Fig. 5.17).

treatment with 10mM EDTA at 37°C and pH 8.0 for 1 hour (Pidard *et al* 1986, Zucker & Grant 1978). In this case, antibody transfer after 24 hr incubation was negligible.

5.3.5.1 Use of the 7E3-Fab transfer phenomenon to calibrate the flow cytometer

By mixing known proportions of 7E3-Fab-coated and non-coated platelets and incubating until equilibrium is reached (i.e. all the platelets in the mixture carry the same number of 7E3-Fab molecules), it should be possible to create suspensions of platelets with known ratios of surface-bound 7E3-Fab. Labelling of these platelets with a FITC-conjugated anti-7E3-Fab would give a range of fluorescence intensities which could be plotted against the percentage of 7E3-Fab-coated platelets in the original mixture (directly proportional to platelet-bound 7E3-Fab after equilibrium). In this way, cytometer calibration curves could be generated, enabling quantitative comparisons between different samples.

To confirm this, washed platelets were incubated with saturating concentrations of c7E3-Fab and, after further washing to remove unbound antibody, they were suspended in PBS, counted and mixed with known numbers of identically treated platelets from the same donor, which had not been exposed to c7E3-Fab. When equilibrium was reached in all mixtures as judged by the presence of a single histogram on labelling with FITC-rabbit-anti-7E3, the mean fluorescence channel (MFC) was recorded (results of duplicate experiments shown in Table 5.5) and plotted against the percentage of c7E3-coated platelets in the

Ratio of c7E3-Fab-coated/non-coated platelets in mixture (%)	Mean fluorescence channel	
	experiment 1	experiment 2
0/100	1.77	1.75
25/75	12.81	11.56
50/50	22.59	23.77
75/25	37.66	36.44
100/0	51.39	52.60

TABLE 5.5 Mean fluorescence channels at equilibrium, obtained by FITC-rabbit-anti-7E3 labelling of various mixtures of c7E3-Fab-coated and non-coated platelets.

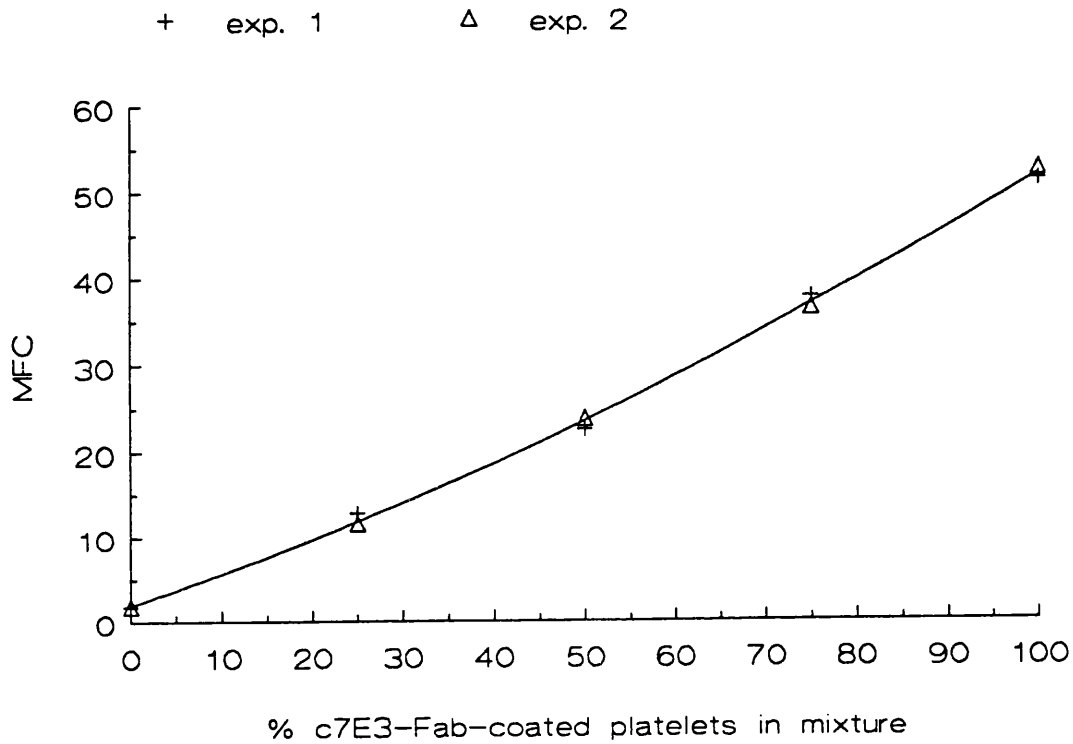


FIGURE 5.19 A five-point calibration curve for the flow cytometer based on the results of the two independent mixing experiments presented in Table 5.5. Each point represents the mean of two independent measurements.

mixture (Fig. 5.19). Although the best curve fit was given by a 2nd degree polynomial, for practical purposes, the MFC on the 1024-channel log fluorescence scale could be considered a linear function of the number of 7E3 molecules on the platelet surface.

5.3.6 Labelling of c7E3-Fab after platelet permeabilization

The fluorescence of intact platelets incubated with FITC-conjugated anti-7E3 reflects the amount of surface-bound antibody. It is possible that some of the platelet-associated c7E3-Fab becomes internalized and therefore inaccessible to the fluorescent probe. To investigate this possibility, platelets from 3 patients who were on day 7 of a 72 (n=2) or 96-hour (n=1) infusion were labelled with FITC-conjugated rabbit anti-7E3 before and after platelet membrane permeabilization with the non-ionic detergent Triton X-100 (TX-100). Permeabilization was achieved by fixing washed platelets with 1% paraformaldehyde (section 2.1.8) and then incubating for 10 minutes with 0.1% (v/v) TX-100. Following two washes, labelling with the fluorescent anti-7E3 was performed as described in 5.3.2 above. Normal platelets, not exposed to 7E3, were used as control to estimate non-specific fluorescence.

There was a significant increase in non-specific fluorescence after permeabilization but, even when this was allowed for, there was a marked increase (55-64%) in the amount of detectable c7E3-Fab in the TX-100 treated platelets compared with that of fixed but not TX-100 treated ones in all three patients. The results of a representative experiment are shown in Fig. 5.20.

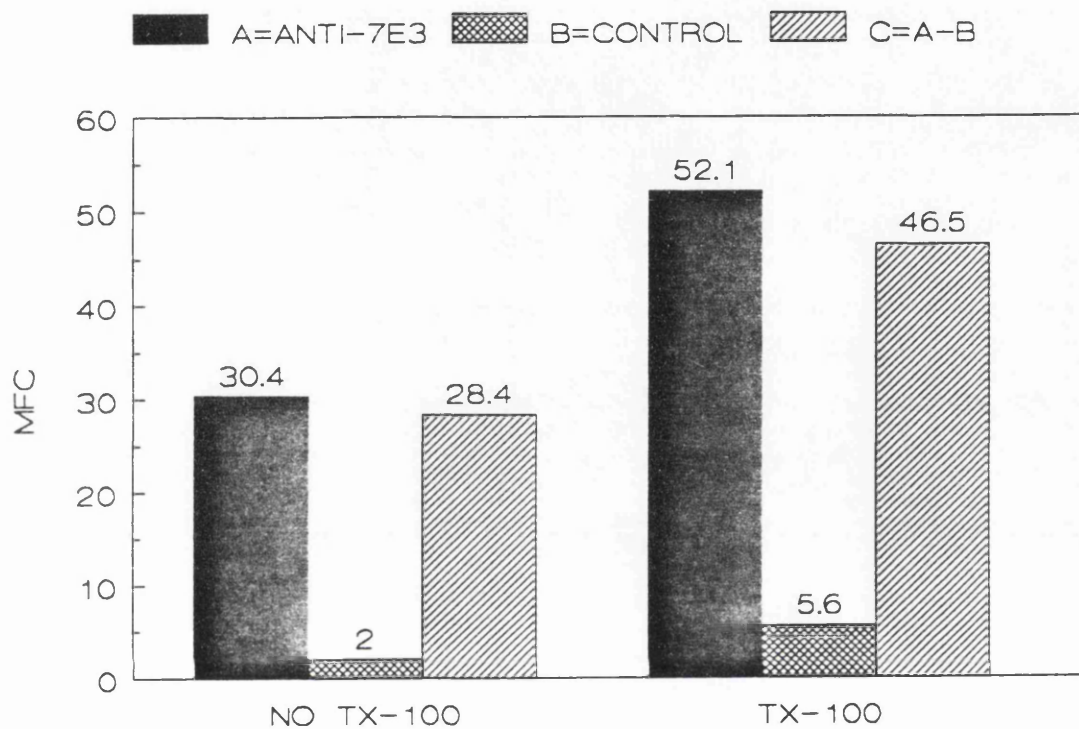


FIGURE 5.20 Fluorescence of platelets isolated from a day 7 sample of a 96-hour c7E3-Fab infusion and incubated with FITC-rabbit-anti-7E3 (A) and of control platelets (B) incubated with the same fluorescent probe. The measurements were performed before and after permeabilization with TX-100. "Specific" fluorescence (C) is calculated by subtraction of B from A. MFC: Mean fluorescence channel.

5.3.7 Correlations between aggregation responses to ADP and 7E3 binding sites or platelet-bound c7E3-Fab.

Table V.2 (Appendix to Chapter 5) shows platelet aggregation responses to 5 and 20 μM ADP at baseline and up to day 7 in the 24, 48 and 72 hour infusion groups. In some patients, aggregation studies were also performed on days 10, 14 and 28 (results not shown). In the 24hr infusion group (5 $\mu\text{g}/\text{min}$), aggregation responses at the end of the infusion were 32.5% (43.8%), SD 5.3% (9.3%) of baseline (the first number refers to 5 μM and the number in parenthesis to 20 μM ADP). These had returned to the pre-infusion levels by 72 hours. In the 48hr infusion group (10 $\mu\text{g}/\text{min}$), the responses to 5 and 20 μM ADP at the end of the infusion were 5% (7.5%), SD 6.6% (8.3%) of baseline and had returned to 89.3% (93.6%), SD 35.8% (28.3%) by day 7. In the patients who received 72hr infusions (10 $\mu\text{g}/\text{min}$), the responses to 5 μM (20 μM) ADP at the end of the infusion were 7.9% (11.1%), SD 7.5% (6.7%) of baseline, with recovery to 86.5% (99.4%) by day 7.

There was a very strong positive correlation (Fig. 5.21 & 5.22) between aggregation response to ADP and the logarithm of the number of free 7E3 binding sites on the platelet surface estimated by FC ($r=0.84$ [0.92] for 5 μM [20 μM] ADP). For practical purposes, the aggregation response to ADP could be considered a linear function (Fig. 5.21) of the number of free 7E3-binding sites from the onset of the infusion until the number of binding sites had returned to about 55% of baseline. From that point until the number of free binding sites returned to pre-infusion levels there was no correlation, suggesting that approximately half of the available gpIIb-IIIa receptors can be blocked without appreciable effect on platelet responsiveness to ADP as assessed by standard turbidometric methods.

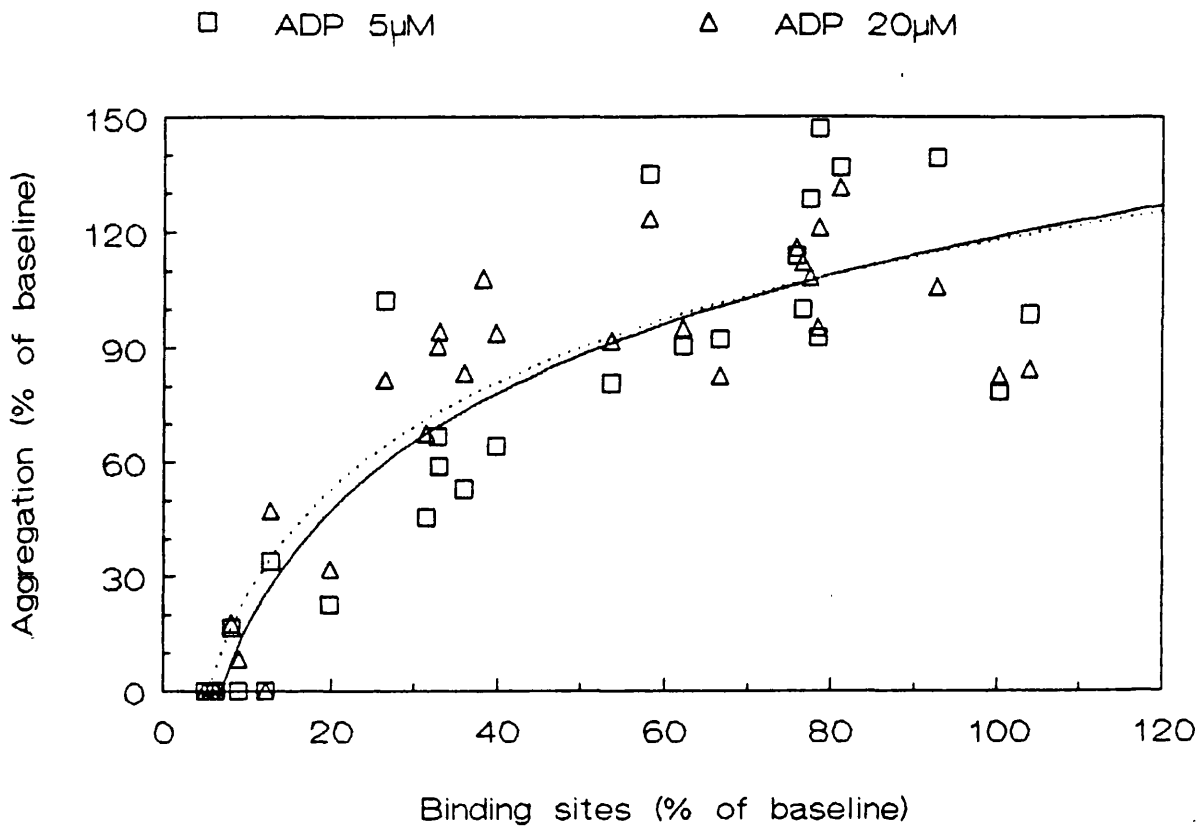


FIGURE 5.21 A logarithmic curve gives the best fit when ex vivo platelet aggregation responses to 5 and 20 μ M ADP are plotted against the amount of residual 7E3-Fab binding sites measured by FC. The continuous and dotted line refer to 5 and 20 μ M ADP respectively.

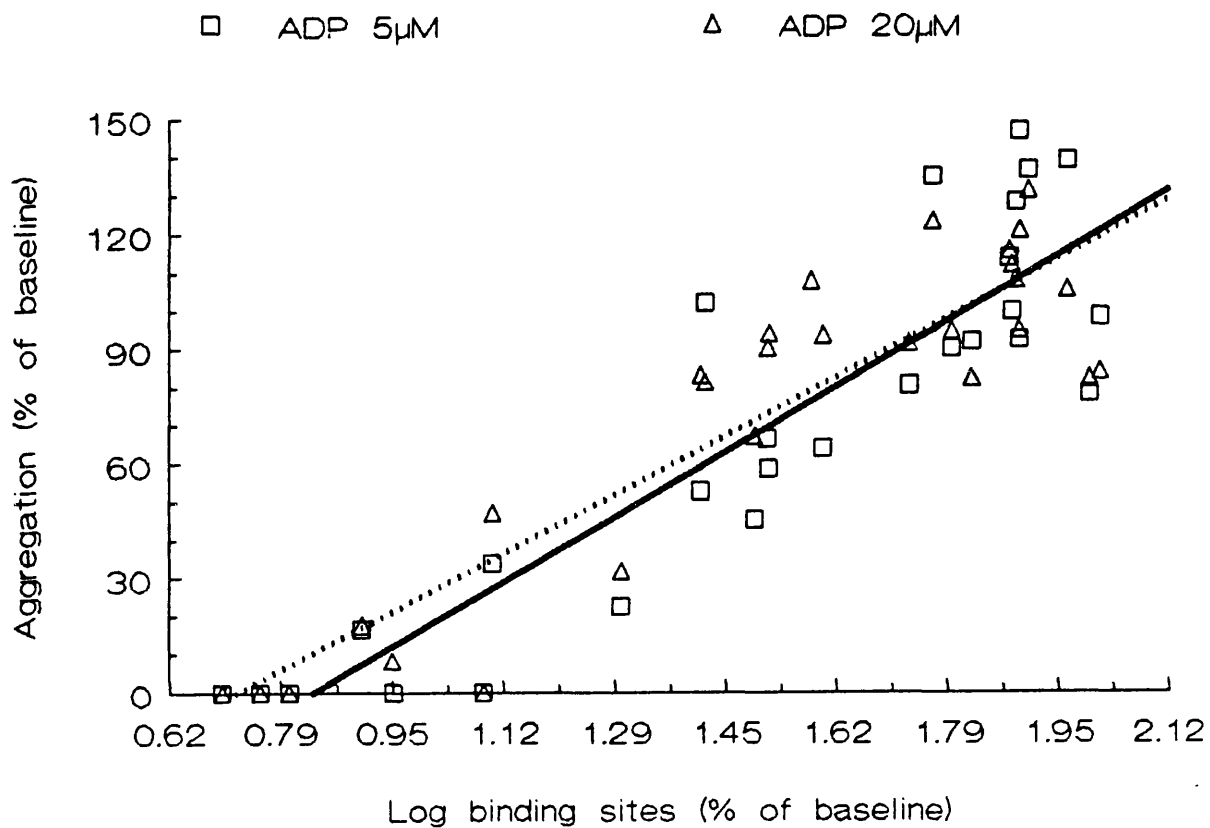


FIGURE 5.22 Same data as in Fig. 5.21 after logarithmic transformation of the amount of residual binding sites. Aggregation to ADP is a linear function ($r=0.9$, $P<0.0001$) of the logarithm of available $7E3$ binding sites (i.e. fibrinogen receptors).

A similar picture was obtained when aggregation responses were plotted against the amount of platelet-bound c7E3-Fab (expressed as percent of the amount bound at the end of the infusion). A strong negative linear correlation ($r=-0.83$ [-0.81] for $5\mu\text{M}$ [$20\mu\text{M}$] ADP) was present only until c7E3-Fab levels fell to 40-50% of those achieved at the end of the infusion (Fig. 5.23). From that point until the point when platelet-bound 7E3 was no longer detectable, there was no correlation between levels of antibody on the platelet surface and aggregation response to ADP.

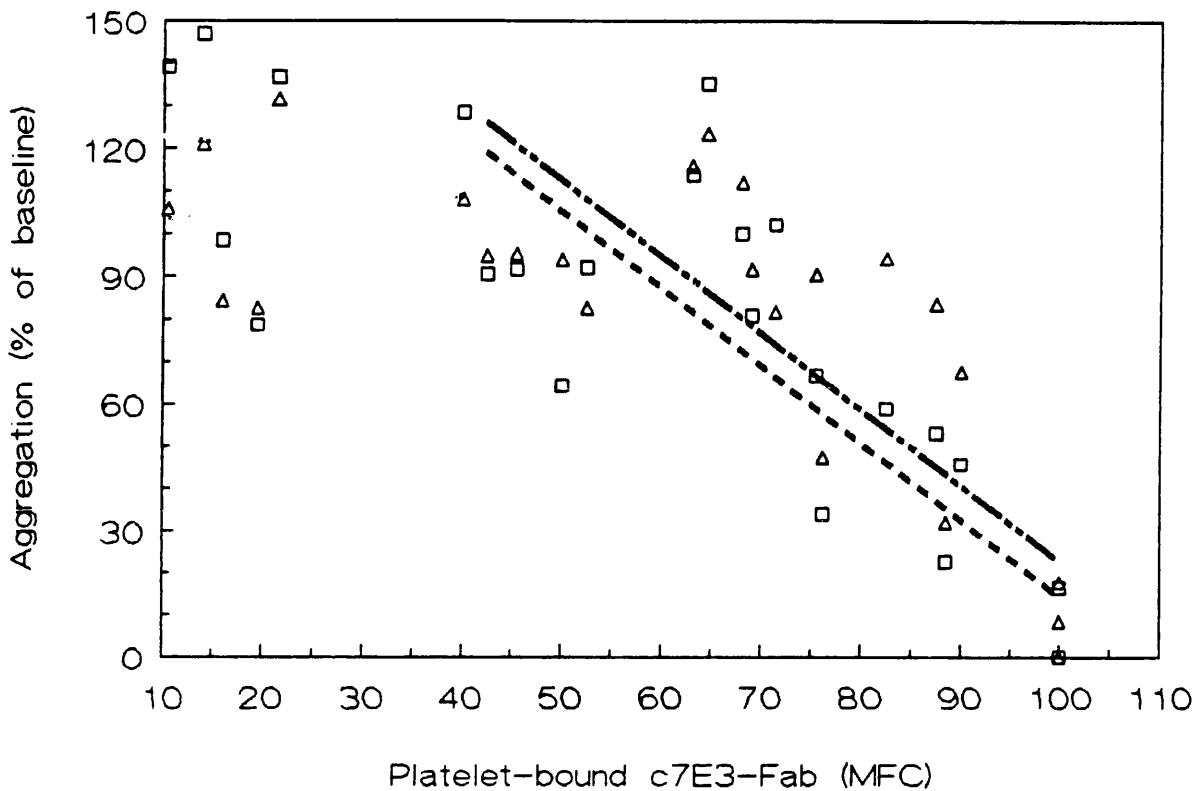


FIGURE 5.23 Ex vivo platelet aggregation responses to 5 (open squares) and 20 μ M (open triangles) ADP are plotted against the amount of platelet surface-bound c7E3-Fab (expressed as % of the level reached at the end of the infusion) measured by FC. There is a strong linear correlation between aggregation response and amount of platelet-bound antibody until the level of the latter falls to 40-50% of that measurable at the end of the infusion.

5.4 CONCLUSIONS

The studies described in this chapter represent the first published application of flow cytometry to monitor the platelet surface in the setting of therapeutic administration of anti-platelet MoAbs. The results of flow cytometric monitoring of platelet-bound c7E3-Fab and c7E3-Fab-binding sites were consistent and correlated well with the results of RIA and aggregometry. Moreover, important new information was obtained.

An unexpected finding was the unimodal distribution of antibody in the platelet population at all time points following the infusion of c7E3-Fab. This, combined with the fact that practically all platelets were bearing antibody at post-infusion times exceeding the lifespan of normal platelets, clearly suggests that, in some way, transfer of antibody to the newly released platelets had occurred. The *ex vivo* and *in vitro* mixing studies described in 5.4.5 showed that transfer of c7E3-Fab or m7E3-Fab could take place from antibody coated platelets to platelets bearing no antibody or, following the direction of the concentration gradient, between two platelet populations bearing different levels of antibody, eventually resulting in normal distribution of platelet-bound 7E3-Fab in the mixture. The occurrence of this phenomenon in platelets which had been fixed prior to being coated with 7E3-Fab and the failure to transfer antibody to platelets whose 7E3 epitopes had been destroyed through treatment with EDTA, suggest that exchange of receptors was not responsible for antibody transfer between platelets. Furthermore, the lack of antibody transfer from platelets coated with c7E3-IgG instead of c7E3-Fab suggests that the transfer is made possible by lower affinity of the Fab fragment for the receptor compared to that of the whole IgG molecule, probably due to bivalent binding of the latter. Bivalent binding

is supported by the findings of Jordan *et al* (1991) who showed that platelets from the same donor bound *ca.* 80,000 molecules/platelet of radiolabelled 7E3-Fab or a heterobifunctional F(ab')₂ fragment (constructed by chemically linking a 7E3-Fab with the Fab of an irrelevant MoAb) but only *ca.* 40,000 molecules/platelet of 7E3-F(ab')₂ or 7E3-IgG. This suggests that the number of Iib-IIIa receptors per platelet is closer to 80,000 rather than the generally quoted 40,000, the discrepancy having presumably arisen from the common use of the whole molecule of anti-glycoprotein antibodies for receptor quantitation studies.

Although simple competition for 7E3-Fab binding between available receptors explains the antibody transfer seen in the *ex vivo* and *in vitro* mixing studies, the contribution of other mechanisms in the antibody transfer observed *in vivo* cannot be excluded. Megakaryocytes express Iib-IIIa on their surface (Berridge and Ralph 1985, Breton-Gorius and Vainchenker 1986) and binding of 7E3 with subsequent incorporation into platelets should be considered. The fact that, Fab fragments, due to their smaller size, might have better access to bone marrow extracellular matrix compared to IgG or F(ab')₂ could also be playing a role. Another theoretically possible scenario might involve binding of 7E3-Fab to endothelia, with subsequent transfer to circulating platelets. Indirect evidence suggests that binding of 7E3 to endothelial cells does occur (Charo *et al* 1987, Martinez *et al* 1989).

As a result of c7E3-Fab transfer to new platelets, a significant amount of antibody (*ca.* 25% of the level reached at the end of the infusion) was still detectable on all circulating platelets (Fig. 5.24) 10 days after the infusion had finished, *i.e.* at a time when very few of the platelets originally exposed to the antibody would be expected to have survived.

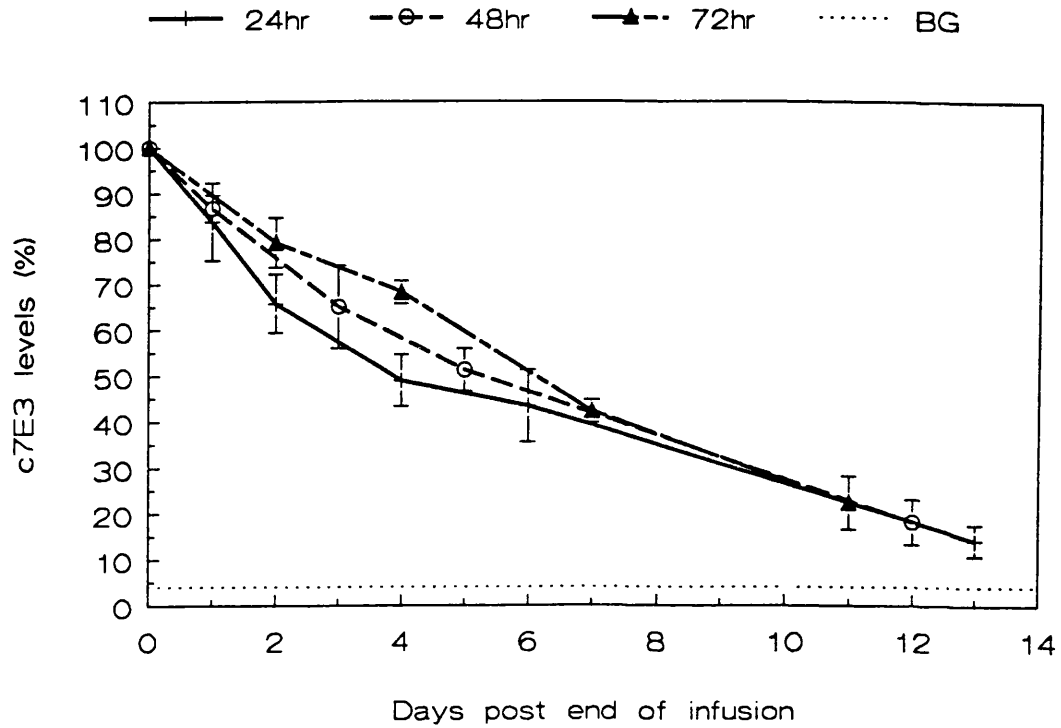


FIGURE 5.24 Comparison of the rate of clearance of platelet-bound c7E3-Fab in the 3 main groups of patients studied (24, 48 and 72-hour infusions).

BG: level of non-specific (background) fluorescence.

The possible implications of this prolonged circulation of the antibody are discussed in Chapter 7.

In general, the clearance of platelet surface-bound c7E3-Fab (PS7E3) post-infusion followed an exponential model (Fig. 5.25). The initial post-infusion elimination rate of PS7E3 (expressed as "halving time") appeared to be fastest in the 24-hour infusion group and slowest in the 72-hour group (Fig. 5.24), although the small number of patients in each of the three main groups did not allow statistical significance to exceed the 5% level. It is possible that, antibody infusion of higher dose or longer duration (or both) results in increased binding of 7E3 to sites other than the platelet surface (e.g. intraplatelet I Ib-IIIa, megakaryocytes, endothelia) with subsequent transfer to the platelet surface, thus slowing down the clearance of platelet-bound antibody. Harrison *et al* (1990), studying patients receiving murine or chimaeric 7E3-Fab, reported detection of antibody intracellularly, probably in the open canalicular system, which suggests that antibody-bearing I Ib-IIIa can shift from the surface to the interior of the cell. Shift in the opposite direction is also conceivable as part of the I Ib-IIIa pool cycling (Wencel-Drake 1990).

Internalisation of other MoAbs bound to the I Ib-IIIa complex has also been described (Santoso *et al* 1986, Isenberg *et al* 1990) and it has been suggested that sequestration of antibody-bearing I Ib-IIIa into the SCCS might be responsible for the resulting refractoriness to aggregation by platelet agonists. The results of flow cytometric monitoring of platelet surface-bound 7E3 presented here show that this mechanism is not playing an important role in 7E3 induced refractoriness, because, although antibody-receptor complex internalization does seem to occur, large amounts of antibody are present on the surface (presumably

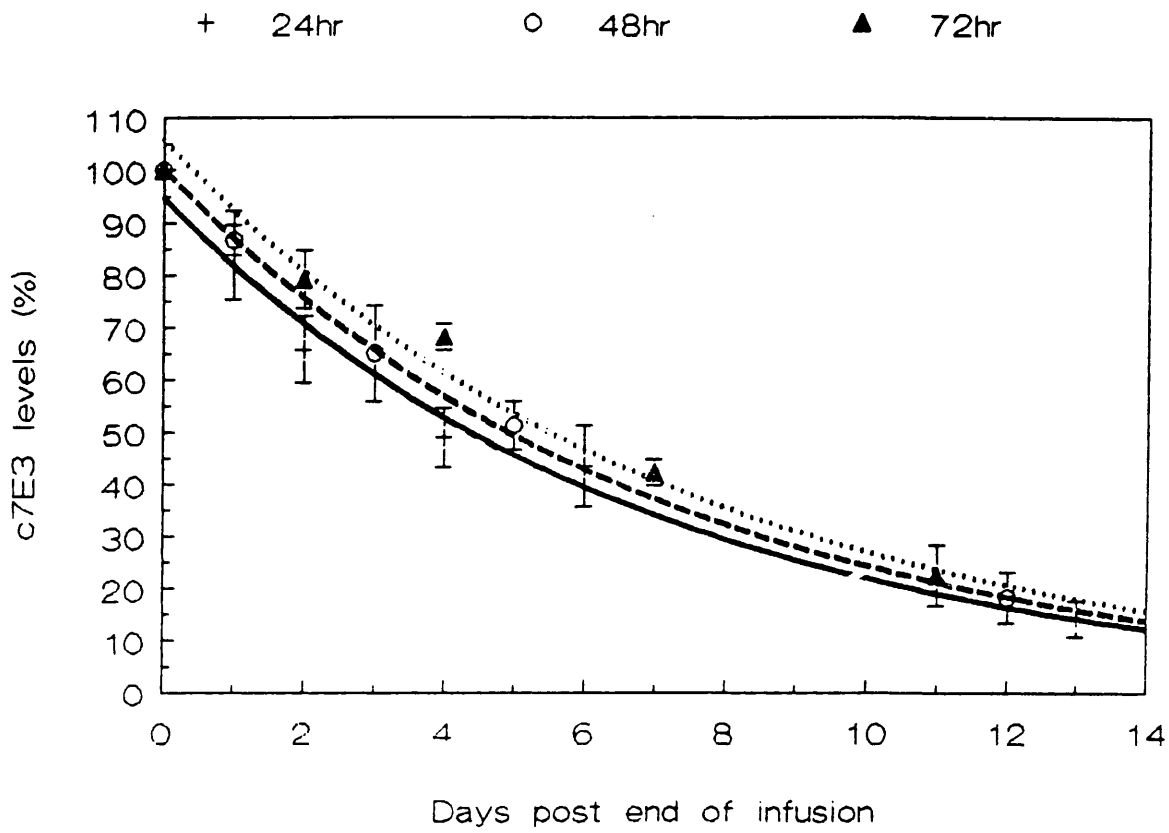


FIGURE 5.25 An exponential curve gave the best fit for c7E3-Fab clearance from the platelet surface following 24 (—), 48 (- -) or 72-hour (...) c7E3-Fab infusions.

blocking the fibrinogen receptor) while the response to ADP is absent, and there is a strong negative correlation between levels of surface-bound 7E3 and response to ADP following the infusion. The shift of some c7E3-Fab to areas accessible only after disruption of the continuity of the platelet membrane is supported by the significant increase in antibody detectable by FC following treatment of the platelets with TX-100 although it is difficult to make assumptions regarding the location of the additional 7E3 measured in this way. As purified Fab fragments of the chimaeric antibody were used in the infusions, crosslinking of receptors through bivalent binding of antibody and Fc binding to the FcγRII receptors (Spycher and Nydegger 1986) can be discarded as possible mechanisms mediating internalization of antibody-IIb-IIIa complexes.

The finding that the number of free 7E3 binding sites on the platelet surface could return to the pre-infusion levels while a significant amount of antibody remained surface-bound (5.3.3) suggests that intracellular IIb-IIIa, not exposed to the antibody, had become exteriorized or/and that platelets bearing higher levels of IIb-IIIa had emerged. The observation that, in 5 patients who had c7E3-Fab-binding capacity measured by FC on day 28, this was significantly increased compared to baseline ($p < 0.02$ by paired t-test, Fig. 5.16), raises the possibility that some form of up-regulation of IIb-IIIa receptors might be taking place as a response to the blocking effect of 7E3. In view of the small number of patients studied, this needs further investigation in future studies.

Finally, the correlations of flow cytometric measurements of platelet-bound c7E3-Fab and free c7E3-Fab binding sites with the simultaneously assessed responsiveness to ADP confirmed the previously observed

relationships between blockade of fibrinogen receptors and impairment of platelet function. The best correlation was between logarithm of available binding sites and aggregation response to 5 or 20 μ M ADP ($r=0.9$, $P<0.0001$). This is in agreement with the finding that the logarithm of the bleeding time was inversely proportional ($r=0.73$) to the residual IIB-IIIa receptors in patients given bolus injections of 7E3-F(ab')₂ (Gold *et al* 1990). It appears that about half of the IIB-IIIa receptors on the platelet surface must be blocked before any appreciable effect on aggregability. This is consistent with the typical lack of demonstrable aggregation abnormalities in heterozygotes for Glanzmann's thrombasthenia, whose platelets bear about 50% of the normal number of IIB-IIIa molecules. There seems that gpIIB-IIIa is present in "excess" of that necessary for haemostasis but whether reductions in the number of receptors to levels not affecting standard platelet function tests afford any long term protection against thromboembolic disease remains to be investigated.

Appendix to Chapter 5

PATIENT No	c7E3-IgG BINDING SITES (THOUSANDS)						
	b/line	24hrs	48hrs	72hrs	5 days	7 days	10 days
24hr infusions							
1	37.82	7.50	25.42		25.17	35.63	
2	51.70	6.15	20.89	35.71	30.21	50.61	
3	47.30	12.98	26.69	25.09	34.58	41.42*	
4	34.55	21.11	17.84	18.83	20.69	44.20	
5	28.89	15.06	16.08	20.56	32.16	32.13	
48hr infusions							
6	36.42	4.59	9.92	9.67	18.27	19.05	
7	50.15	3.71	3.74	11.44	16.61	11.60	
8	37.86	4.51	4.71	9.89	15.72	18.00	
9	38.81	7.78	10.04	11.36	22.88	35.67	
10	40.06	14.20	5.08	9.35	22.14	30.39	
16	55.38	3.85	4.39	8.93	17.27	24.60	
18	29.92	3.58	1.92	1423	13.62	35.77	18.40 ⁺
72hr infusions							
11	44.70	7.78	6.81	5.95	14.97	19.24	
12	53.64	7.42	5.83	6.35	15.54	13.36*	
13	46.04	5.54	4.66	4.26	11.28	25.41	
14	37.92	4.06	3.98	3.17	14.09	22.67	34.22
15	25.44	5.89	3.70	2.97	10.64	21.19	26.66
17	36.05	3.01	3.26	2.35	9.01	14.65	

* day 8, ⁺ day 11

TABLE V.1 RIA estimation of available c7E3-IgG binding sites on platelets at baseline and various time points during and after c7E3-Fab infusions.

PATIENT No	Response to ADP 5 μ M/20 μ M (as percent increase in light transmittance of PRP)					
	b/line	24hrs	48hrs	72hrs	5 days	7 days
24hr infusions						
1	69/72	22/30	46/66	60/69	68/76	74/85
2	76/86	21/38	48/74	70/97	90/85	82/87
3	87/89	36/44	64/65	61/69	43/88	86/86*
4	68/66	22/36	62/78	90/82	81/70	59/81
5	48/64	14/19	27/44	32/58	48/54	61/65
48hr infusions						
6	70/85	9/14	6/12	30/49	47/62	40/49
7	66/74	7/10	7/8	43/71	56/73	>100
8	62/85	10/13	10/18	29/56	63/90	45/74
9	78/92	10/6	0/6	18/39	44/60	66/77
10	56/75	0/7	0/0	15/35	36/58	26/49
16	51/69	0/0	0/0	30/65	41/60	58/80
18	70/83	0/10	0/0	32/56	55/ND	70/93
72hr infusions						
11	85/98	ND	0/0	13/17	58/82	56/87
12	61/96	10/9	4/7	7/9	46/66	84/96*
13	45/71	6/7	7/8	2/10	29/55	53/80*
14	83/84	7/18	5/7	0/7	44/70	67/77
15	60/73	7/16	0/6	10/13	40/66	81/90
17	84/97	0/0	0/0	0/0	29/57	54/91

* day 8, ND: not done

TABLE V.2 Aggregation responses to ADP at baseline and various time points after c7E3-Fab infusion.

PLATELET SURFACE IgG IN PATIENTS RECEIVING INFUSIONS OF CHIMAERIC 7E3-Fab

6.1 INTRODUCTION

Reductions in the circulating platelet count have been observed in association with anti-gpIIb-IIIa MoAb administration to animals and human volunteers. Hanson *et al* (1988) reported a dose-dependent reduction (20-46%) in the platelet count over 24 hours following IV injection of the whole IgG or F(ab')₂ fragments of MoAb AP2 but not LJ-CP8 (another anti-gpIIb-IIIa MoAb) in baboons. Gold *et al* (1990) noted a 16±10% reduction occurring within 1 hour following injection of 0.2 mg/kg 7E3-F(ab')₂ in humans, with the counts normalizing by 24 hours. Although Takami *et al* (1987) reported that whole IgG and F(ab')₂ fragments but not Fab fragments of a MoAb to IIb-IIIa (PP3-3A) caused thrombocytopenia in pigs, episodes of asymptomatic thrombocytopenia (platelet count <50% of baseline) have been seen following single injections or continuous infusions of Fab fragments of both murine and chimaeric 7E3 to humans (Machin *et al* 1990 and 1991 and Centocor, data on file). Thrombocytopenia usually occurred early (within a few hours) and resolved spontaneously over a few days following discontinuation of exposure to 7E3. As with all cases of acute drug-induced thrombocytopenia, an immune aetiology was considered. While simple agglutination through bridging of IIb-IIIa molecules on neigh-

bouring platelets could be a possible mechanism in cases of whole IgG or F(ab')₂ administration, this mechanism could not be operating in 7E3-Fab-associated thrombocytopenia. Furthermore, human-anti-murine antibodies (HAMA), which could theoretically induce immune destruction of 7E3 coated platelets, could not be detected by ELISA in patients who developed thrombocytopenia following murine 7E3-Fab or 7E3-F(ab')₂ administration (Centocor, data on file).

The purpose of the studies described in this chapter was to use the experience gained from the application of flow cytometric analysis in thrombocytopenic states (Chapter 4) in an effort to elucidate the underlying mechanisms in 7E3-associated thrombocytopenia.

6.2 FLOW CYTOMETRIC MEASUREMENT OF PSiGg IN PATIENTS RECEIVING INFUSIONS OF c7E3-Fab.

While using FC to assess cross-reactivity of a polyclonal anti-IgG with the human portion of the platelet-bound Fab fragment in two patients receiving synchronous infusions of c7E3-Fab, it was noted that the level of IgG detectable on the platelets of one of these patients was significantly higher compared to that of the other. The patient with disproportionately raised PSiGg had shown a significant fall (>50% of baseline) in his platelet count, which had occurred within 2 hours after the onset of the infusion and had led to its early termination according to the trial protocol. This finding raised the possibility of IgG recruitment onto the platelet surface in some patients treated with c7E3-Fab and flow cytometric measurements of PSiGg at baseline and various time points during and following the infusion were undertaken.

6.2.1. Method

Measurement of PSiG was performed as described in 2.3.4 but exposure of platelets to EDTA was avoided in order to prevent displacement of platelet-bound 7E3-Fab from its epitope. For this reason, PRP was prepared from citrate-anticoagulated blood and all washes were in citrate-PGE₁ buffer.

Monoclonal and polyclonal antibodies directed against the Fc region of human IgG (Sigma, Table 2.1) were assessed. For the reasons described in section 4.2.3 (significantly higher sensitivity), the polyclonal anti-IgG (Fc-specific) was selected. PSiG was expressed as Relative Fluorescence Intensity (RFI) as defined in Chapter 4 (section 4.2.4). In selecting the FITC-conjugated anti-IgG to use in this application, a basic requirement was that it did not react with the human Fab of the chimeric 7E3-Fab coating the platelets. Lack of such crossreactivity was confirmed with the following experiment:

Platelets isolated from citrate-anticoagulated blood from normal volunteers were washed three times in citrate-PGE₁ buffer and, after resuspension in PBS pH 7.2 at a concentration of $300 \times 10^9/l$, were incubated for 45 minutes with saturating concentrations ($10 \mu\text{g/ml}$) of either c7E3-Fab or c7E3-IgG. After two further washes, saturation of c7E3-binding sites was confirmed by incubating the platelets with FITC-c7E3-Fab which resulted in the same degree of minimal uptake of fluorescence by both c7E3-Fab and c7E3-IgG coated platelets, compared with the uptake by the same platelets before c7E3 coating (Fig. 6.1). The so coated platelets had their PSiG measured using the FITC-conjugated, Fc-specific anti-IgG. Washed platelets from the same donor that had not been coated with c7E3 served as control. The results of a typical experiment are shown in Fig. 6.2 and demonstrate the lack of crossreactivity of the anti-Fc probe with the Fab fragment.

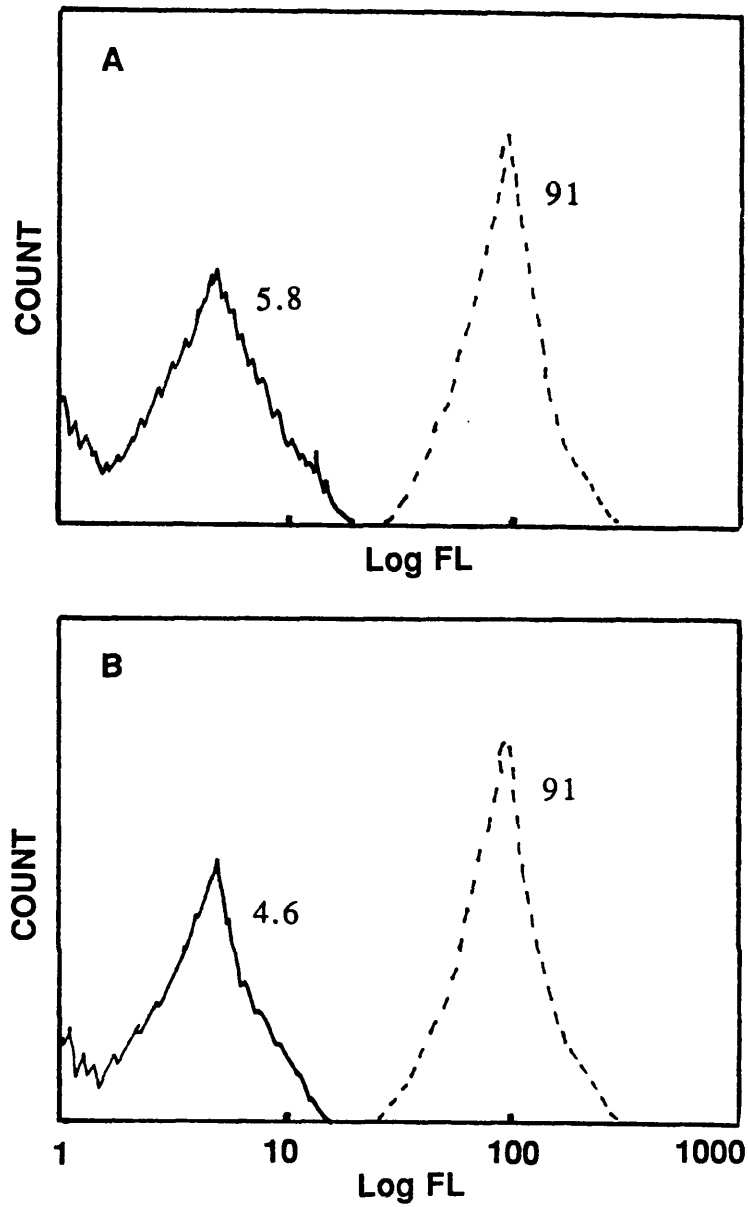


FIGURE 6.1 Uptake of FITC-c7E3-Fab by platelets before (broken line) and after (solid line) saturation with c7E3-Fab (A) or c7E3-IgG (B). Mean fluorescence channel numbers are noted by the histograms.

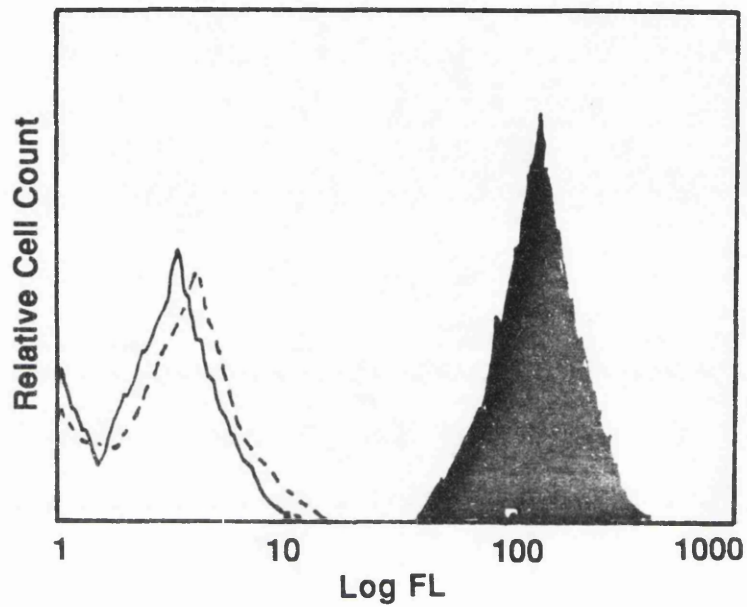


FIGURE 6.2 Measurement of PSiG in control platelets before (solid line) and after coating with c7E3-Fab (broken line) and c7E3-IgG (shaded histogram) to demonstrate lack of crossreactivity of the anti-IgG (Fc-specific) probe with the Fab fragment of chimaeric 7E3.

6.2.2 Results

Nine consecutive patients receiving 48, 72 or 96 hour infusions were studied. The results of PSiG detection (expressed as RFI) at baseline, 24 hours into the infusion and on days 7, 14 and 28 are shown in Table 6.1. Results of a number of random PSiG measurements performed on these and other patients at various time points are not shown; they were all consistent with the results presented here.

There was a statistically significant elevation of PSiG at 24 hours with a mean RFI of 2.73 (SE=0.48) compared to 1.56 (SE=0.12) at baseline ($P<0.01$ by Wilcoxon's signed rank test, $P=0.042$ by Student's paired t-test). The mean RFI on day 7 had risen to 3.88 (SE=0.51), which was significantly higher compared to the RFI at 24 hours ($P<0.01$ by Wilcoxon's signed rank test or $P=0.03$ by Student's paired t-test). By day 14 the mean RFI had fallen to 1.88 (SE=0.1), which was not significantly different from the baseline and by day 28 PSiG had returned to the baseline level (mean RFI=1.53, SE=0.12). The results are presented graphically in Figs. 6.3 and 6.4.

As can be seen from Fig. 6.4, one of the patients studied (No 12) showed a much more marked rise in his PSiG compared to all the rest. The levels of PSiG in this patient were significantly higher than 3 standard deviations above the mean of the remaining eight patients at both the 24 hour and day 7 time points. While his baseline RFI was normal, a marked elevation of PSiG was already present in a sample taken 30 minutes after the onset of c7E3-Fab infusion. The time course of PSiG and platelet-bound c7E3-Fab (PS7E3) levels in this patient is shown in Fig. 6.5. It can be seen that fluctuations in PSiG are in the same direction as fluctuations in PS7E3 up to day 5 but, on day 8, the level of PSiG has remained high despite a fall in PB7E3 to <50% of the level reached

PATIENT		RFI				
No	INF.	baseline	24 hours	day 7	day 14	day 28
11	72	1.89	3.12	2.62	2.43	2.21
12	72	1.80	6.47	7.97	1.66	1.37
13	72	1.90	2.73	3.53	2.20	N/A
14	72	1.28	1.59	3.74	1.75	1.81
15	72	1.13	2.07	3.90	1.80	1.73
16	48	1.10	1.25	3.35	1.59	1.21
17	72	1.15	3.12	4.15	1.64	1.12
18	48	1.89	1.96	2.88	N/A	1.29
19	96	1.91	2.23	2.83	N/A	N/A

INF.: Infusion duration, N/A: Not measured

TABLE 6.1 PSiG levels expressed as Relative Fluorescence Intensity (RFI) before, during and following infusion of chimaeric 7E3-Fab.

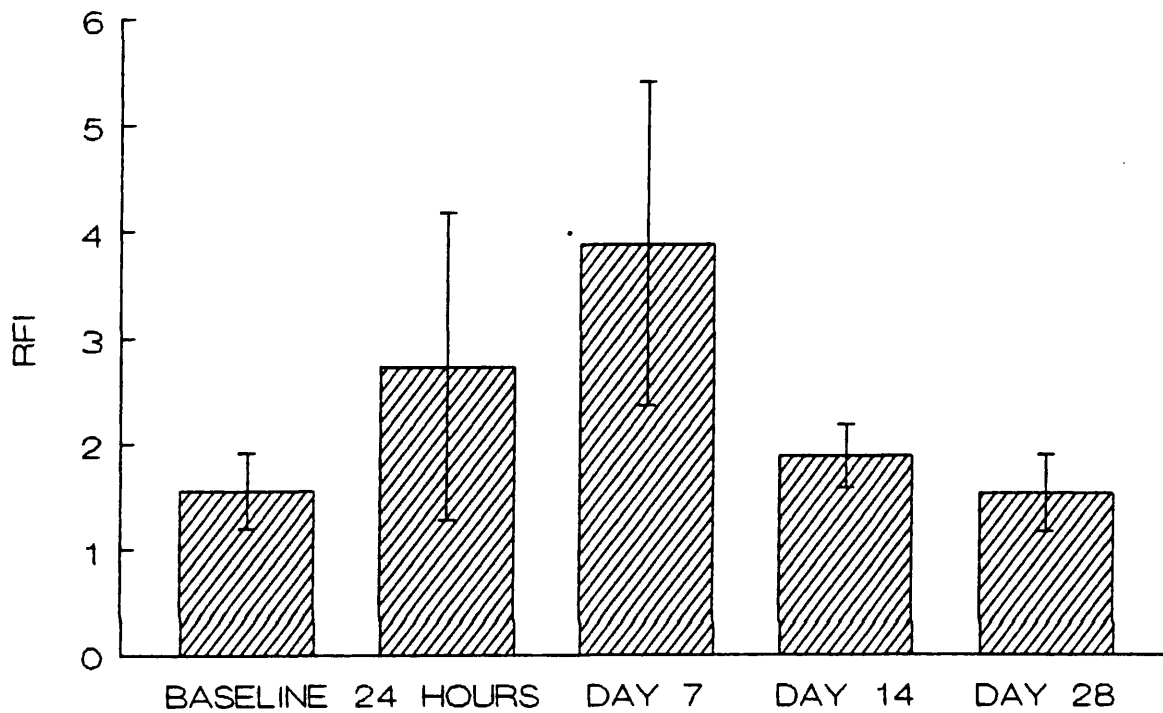


FIGURE 6.3 Mean PSiG expressed as RFI (Relative Fluorescence Intensity as defined in Chapter 4) at various time points of 48 (n=2), 72 (n=6) or 96 (n=1) hour infusions of c7E3-Fab. The thin vertical bars represent SD of the mean. See also Fig. 6.4.

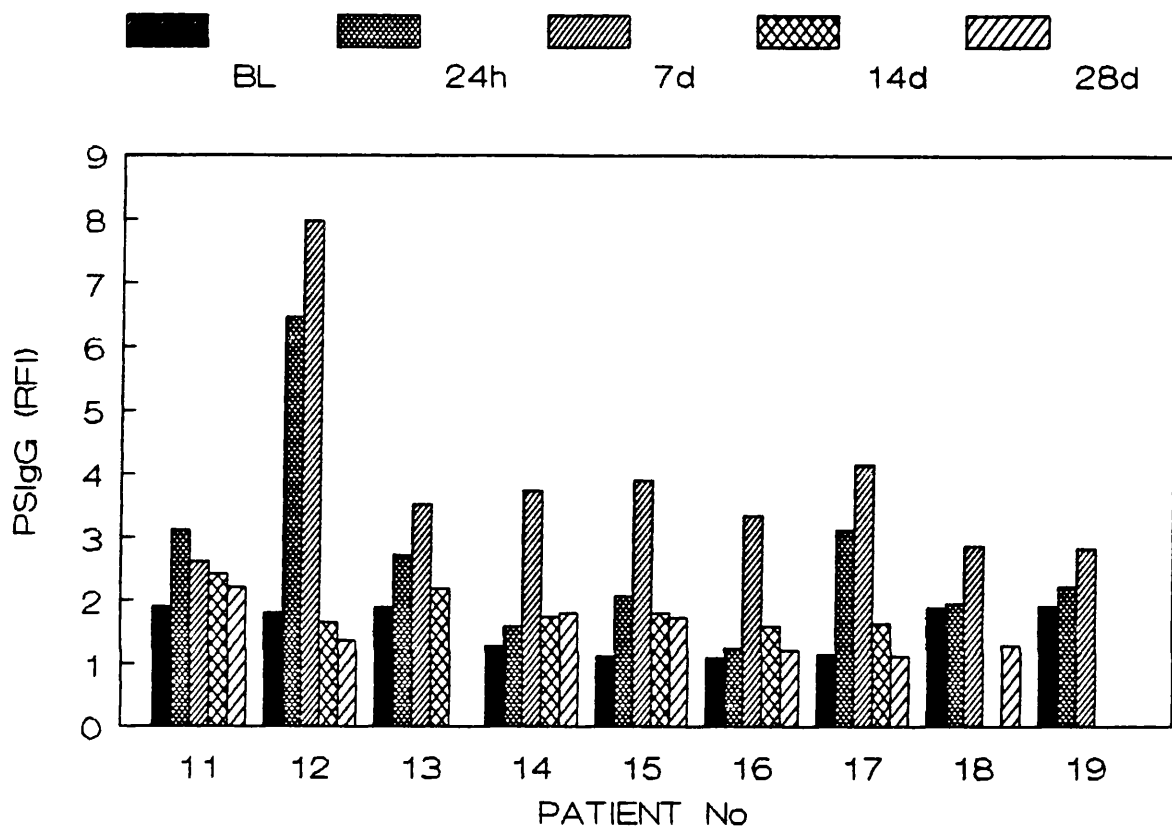


FIGURE 6.4 Fluctuations of PSiG measured by a FITC-conjugated polyclonal goat-anti-human IgG (Fc-specific) in 9 consecutive patients given c7E3-Fab infusions. Patients No 11,12,13,14,15 and 17 received 72-hour infusions; patients No 16 and 18 received 48-hour infusions and patient No 19 received a 96-hour infusion.

RFI: Relative Fluorescence Intensity.

BL: Baseline sample, h: hours, d: days.

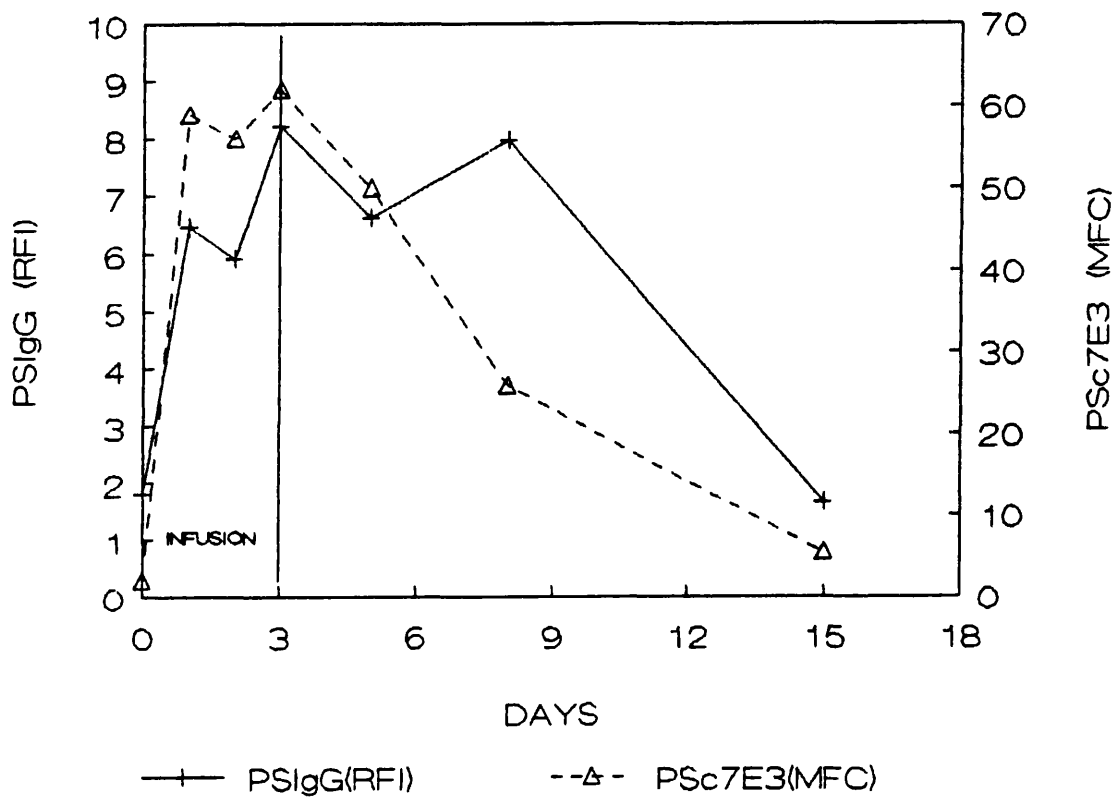


FIGURE 6.5 Platelet surface IgG (PSiG) and c7E3-Fab (PSc7E3) levels expressed as Relative Fluorescence Intensity (RFI) and Mean Fluorescence Channel (MFC) respectively in the patient who showed a marked, infusion-related elevation of PSiG.

at the end of the infusion. Using a formula derived as described in Chapter 4 (section 4.2.7.2), it was estimated that, on day 8, this patient's platelets were bearing *ca.* 12,000 IgG molecules on their surface (mean baseline level=1570, SE=128), whereas the magnitude of PSiG elevation seen at the same time point in the other patients was of 2 to 3 times the baseline level.

6.2.2.1 Correlation of PSiG with the clearance rate of platelet-bound c7E3-Fab

In Fig. 6.6 levels of platelet-bound c7E3-Fab (measured or estimated from the individual clearance curves shown in Chapter 5) 10 days after the end of the infusion have been plotted against levels of PSiG at 24 hours. It can be seen that the patient (No 12) who showed marked early recruitment of IgG onto his platelets had the lowest levels of platelet-bound c7E3-Fab on the 10th post infusion day. (more than 2 standard deviations below the mean of the remaining eight patients). This was not due to restricted access of the fluorescent probe to platelet-bound c7E3-Fab through steric hindrance by PSiG, because the level of the latter was no longer increased at that point. An identical picture was seen when the effect of day 7 PSiG on the clearance of platelet-bound c7E3-Fab was examined.

6.2.2.2 Platelet counts

None of the 19 consecutive trial patients on whom serial flow cytometric studies were performed showed reductions in the circulating platelet

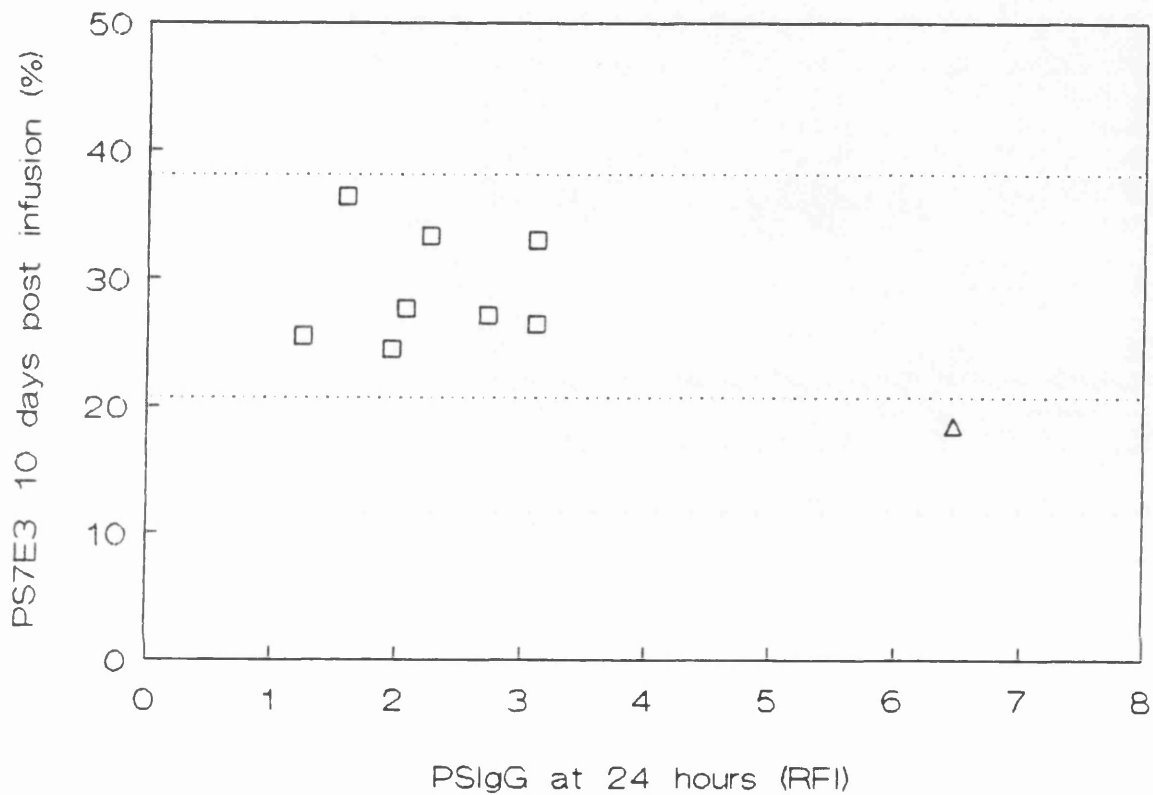


FIGURE 6.6 Levels of platelet surface c7E3-Fab (PS7E3) on the 10th post infusion day (expressed as % of the levels reached at the end of the infusion) plotted against levels of PSIgG (expressed as RFI) at 24 hours. The patient who showed marked elevation of PSIgG is represented by an open triangle. The dotted lines represent the range (mean \pm 2SD) defined by the remaining 8 patients (open squares).

count greater than 30% of baseline. There was no detectable correlation between fluctuations in platelet count and levels of PSiGg including the case of patient No 12 who showed marked elevation of PSiGg (but see Appendix to this chapter).

6.3 RECRUITMENT OF IgG TO c7E3-Fab COATED PLATELETS - IN VITRO STUDIES

The results of ex vivo flow cytometric analyses presented in the above sections suggest clearly that in vivo recruitment of IgG onto platelets coated with c7E3-Fab through I.V. infusion of the antibody is frequently taking place. The timing of IgG recruitment suggests the pre-existence, in the majority of patients, of immunoglobulins bindable to the surface of platelets coated with c7E3-Fab. The data presented so far do not provide much information regarding specificity of this IgG for the chimeric Fab. The in vitro studies that follow were performed in order to:

- a. Establish the frequency with which such immunoglobulins are expected to be found in a group of patients comparable to that analysed in the c7E3 infusion study.

- b. Examine the specificity of these immunoglobulins for platelets coated with the chimaeric Fab fragment.

6.3.1 Patients and methods

Samples from 21 patients (13 males, 8 females, median age 64 years) with ischaemic heart disease admitted to The Middlesex Hospital for coronary angiography were

investigated. None of them had a history of previous treatment with murine or chimaeric MoAbs. These patients had been advised to avoid taking aspirin one week prior to catheterization but continue their cardiac medication. The tests described here were performed on an anonymous basis using surplus blood from samples sent to UCH Coagulation Laboratory for routine clotting screen and, as any results could not conceivably affect patient management or prognosis, no patient consent was sought.

Citrate-anticoagulated blood was spun for preparation of PPP which, after removal of the volume required for the requested clotting tests, was frozen at -20°C until used (within 3 weeks off sampling). Washed control platelets were prepared from GpO donors as described in the general methods chapter (section 2.1.6, using citrate-PGE₁ to wash the platelets) and were divided into three aliquots: Aliquot A ("plain" platelets) served as control. Aliquots B and C were incubated for 45 minutes with saturating concentrations (10µg/ml) of murine and chimaeric 7E3-Fab respectively. Following two further washes of all three aliquots in citrate-PGE₁ buffer, the platelets were resuspended in PBS pH 7.2 at a concentration of 150x10⁹/l. Maximum saturation of 7E3 binding sites was demonstrated as described in 6.2.1. Testing of the plasmas for the presence of platelet bindable IgG was performed as described in 2.3.5, using the FITC-conjugated, Fc-specific, polyclonal goat-anti-human IgG to label platelet-bound IgG (see 6.2.1). All the intervening washes were in citrate-PGE₁.

6.3.2 Results

The mean fluorescence channel (MFC) number obtained with the fluorescent Fc-specific probe was used as a measure of IgG recruited onto the surface of control platelets (plain or coated with either murine or chimaeric 7E3-Fab) following incubation with the under investigation plasmas. The results were also expressed as "Fab-specific fluorescence" (FabSF), defined as the ratio of MFC obtained with the 7E3-Fab-coated control platelets over the MFC obtained with the plain control platelets.

The results of the 21 patient samples tested are shown in Table 6.2. A plasma was classified as positive for IgG bindable to 7E3-Fab-coated platelets when the level of IgG recruited onto 7E3-Fab-coated control platelets incubated with it exceeded the upper limit (mean+2SD) of the "normal" range defined by the PSiG levels of plain control platelets incubated with each of the 21 test plasmas.

Only one (patient No 3) of the 21 plasmas showed clear reactivity with m7E3-Fab-coated platelets. It is worth noting that, when this single sample was excluded, the average IgG recruitment onto m7E3-Fab-coated platelets was less than that onto plain platelets, although the difference did not reach statistically significant levels ($0.05 < P < 0.1$ by Wilcoxon's signed rank test). This probably reflects reduced non-specific uptake of plasma IgG and/or the fluorescent probe, resulting from steric hindrance by the m7E3-Fab. Therefore, *in vitro* recruitment of IgG onto m7E3-Fab-coated platelets which is clearly in excess of that binding to plain platelets is likely to represent true reactivity rather than non-specific uptake.

In contrast to the m7E3-Fab-coated platelets, 14 of the 21 plasmas contained IgG bindable to c7E3-Fab-coated platelets (Fig. 6.7). The increased IgG binding was highly significant ($P < 0.001$ by Student's paired t-test and Wilcoxon's signed rank test), compared to the binding to plain platelets. In only two of them were the results borderline (between 2 and 3 standard deviations above the mean of the "normal" range). Comparative results of individual patients are shown graphically in Fig. 6.8.

PATIENT No	SEX/ AGE	MFC			FabSF- MUR.	FabSF- CHIM.
		PLAIN	MUR.	CHIM.		
1	M/56	4.69	4.16	12.96	0.89	2.76
2	M/74	4.16	4.25	20.03	1.02	4.81
3	M/59	2.42	28.42	11.70	11.74	4.83
4	F/70	3.16	2.60	3.55	0.82	1.12
5	F/75	5.51	3.39	4.16	0.62	0.75
6	M/55	4.06	5.62	4.92	1.38	1.21
7	F/61	5.51	3.12	9.10	0.57	1.65
8	M/64	2.71	2.75	16.01	1.01	5.91
9	M/54	4.35	3.49	15.72	0.80	3.61
10	F/67	2.91	3.10	16.72	1.07	5.75
11	M/27	3.10	3.07	6.86	0.99	2.21
12	M/64	3.04	3.10	5.88	1.02	1.93
13	F/60	2.60	2.63	4.12	1.01	1.58
14	M/66	2.98	2.86	3.09	0.96	1.04
15	M/64	2.65	2.66	8.98	1.00	3.39
16	M/51	3.54	3.48	5.77	0.98	1.63
17	F/75	3.33	3.53	30.14	1.06	9.05
18	F/58	3.15	3.27	25.55	1.04	8.11
19	M/79	2.69	2.62	4.56	0.97	1.70
20	M/39	2.49	2.31	9.38	0.93	3.77
21	F/78	5.99	3.67	4.22	0.61	0.71
MEAN		3.57	4.50	10.64	1.45	3.22
SD		1.05	5.40	7.43	2.30	2.35

MFC: Mean Fluorescence Channel, Mur.: Platelets coated with m7E3-Fab, Chim.: Platelets coated with c7E3-Fab, FabSF: Fab-specific fluorescence (see text).

TABLE 6.2 Recruitment of IgG (expressed as Mean Fluorescence Channel) onto plain, m7E3-Fab- and c7E3-Fab-coated platelets incubated with a range of plasmas.

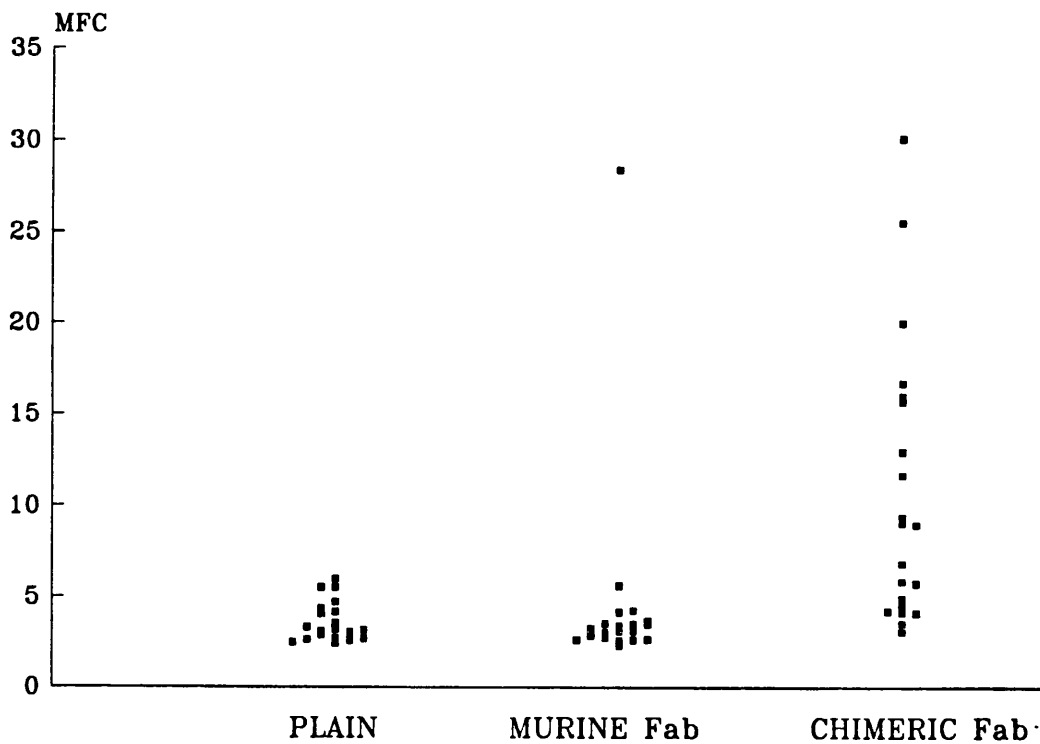


FIGURE 6.7 Levels of plasma IgG (expressed as MFC) bindable to control platelets before (PLAIN) and after coating with murine or chimaeric 7E3-Fab fragments. Plasma samples from 21 patients with ischaemic heart disease were studied; the experimental details and expression of results are explained in section 6.3.1 and 6.3.2.

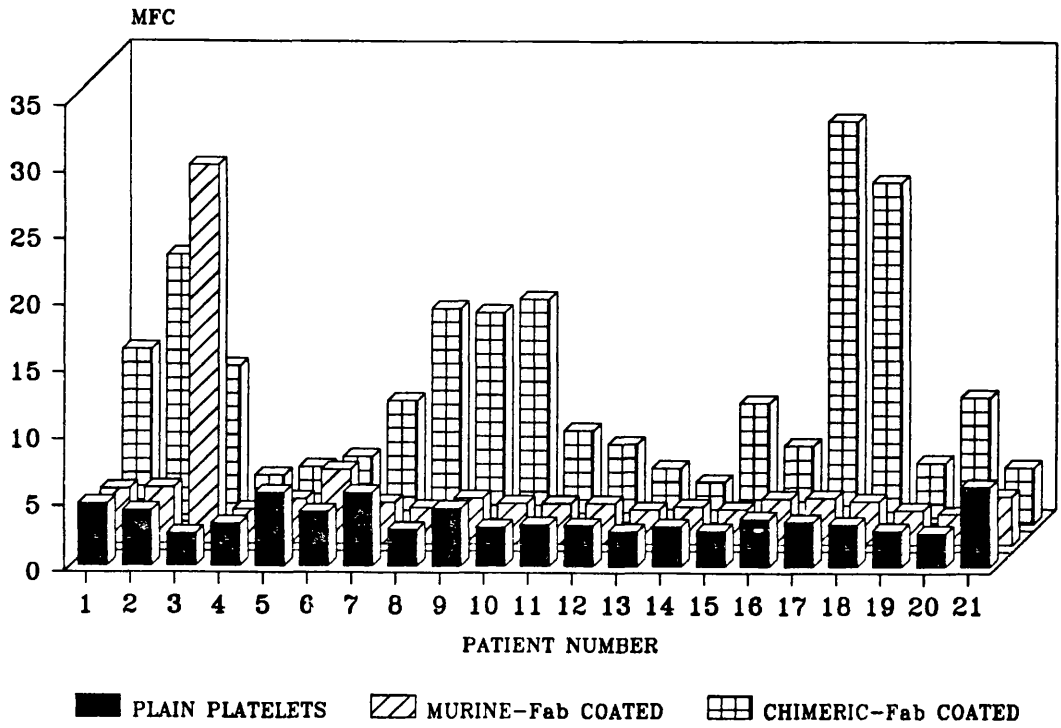


FIGURE 6.8 Graphical representation of the results shown in Table 6.2: comparison of anti-murine 7E3-Fab and anti-chimaeric 7E3-Fab activities (expressed as MFC) in individual patients.

6.3.3 The specificity of IgG recruited onto c7E3-Fab-coated platelets.

The data presented above suggest that chimeric but not murine 7E3-Fab coating of platelets is associated with high incidence of IgG recruitment onto their surface upon incubation with plasmas from individuals not previously sensitized with either murine or chimaeric proteins. It is therefore more likely that the determinant that attracts the IgG is the chimaeric fragment itself rather than some antigen exposed as a result of 7E3-Fab binding to IIb-IIIa. If the murine component of cFab is excluded based on the lack of IgG attraction by the mFab, possible determinants include:

- (a) The human-murine junction.
- (b) The site of cleavage of IgG by papain.
- (c) Determinants formed by conformational changes in the Fab molecule, associated with the presence of (a) and/or (b).

In order to differentiate between the above possibilities, platelets coated with c7E3-Fab (containing both [a] and [b]) and platelets coated with c7E3-IgG (containing only [a]) were used to adsorb the anti-c7E3-Fab activity from a plasma containing IgG bindable to c7E3-Fab-coated platelets:

1 ml aliquots of washed platelets ($300 \times 10^9/l$) from a GpO donor were coated with saturating concentrations of either c7E3-Fab or c7E3-IgG as described in 6.2.1. The same platelets without c7E3 coating (plain) served as control. After two washes in citrate-PGE₁ buffer, 7E3-Fab-coated, 7E3-IgG-coated and plain platelets were pelleted by centrifugation and resuspended in equal volumes (200 μ l) of plasma. Following one hour's incubation at RT the platelets were pelleted again and the upper 50 μ l of the

supernatant plasma were tested for the presence of IgG bindable to c7E3-Fab-coated platelets as described in 6.3.2.

The results of duplicate experiments are shown in Fig. 6.9. There was statistically significant partial absorption (25.8%, $P < 0.02$ by Student's t-test) by the c7E3-Fab- but not the c7E3-IgG-coated platelets, suggesting that the anti-chimaeric Fab activity was associated with the papain-cleaved region of the chimaeric molecule rather than the human-murine junction.

6.3.4. Effect of anti-c7E3-Fab IgG on the binding of c7E3-Fab to platelets.

Washed GpO control platelets were resuspended in 5 plasmas containing anti-c7E3-Fab activity (within the range of the 21 samples reported in section 6.3.1) and 5 plasmas with no detectable activity. All plasmas had been preincubated for 1 hour with the same concentration of FITC-conjugated c7E3-Fab (subsaturating for the final concentration of platelets in the suspension). There was no significant difference in FITC-7E3-Fab binding between high and low anti-c7E3-Fab activity plasmas. The same results were obtained when FITC-7E3-Fab and control platelets were added to the plasma simultaneously (i.e. without preincubation of the plasma with FITC-c7E3-Fab).

6.3.5. Comparison of ELISA and FC assays for detection of human-anti-chimaeric activity in serum/plasma (HACA).

A small number of clinically significant samples from patients who had taken part in earlier stages of the chimaeric 7E3-Fab trial were tested with both the ELISA assay described in 2.7 and the flow cytometric method described in 6.3.1. Clinical information and results are given in Table 6.3. Although the apparent higher pick up rate of FC might simply be due to the different definition of normal range, the flow cytometric

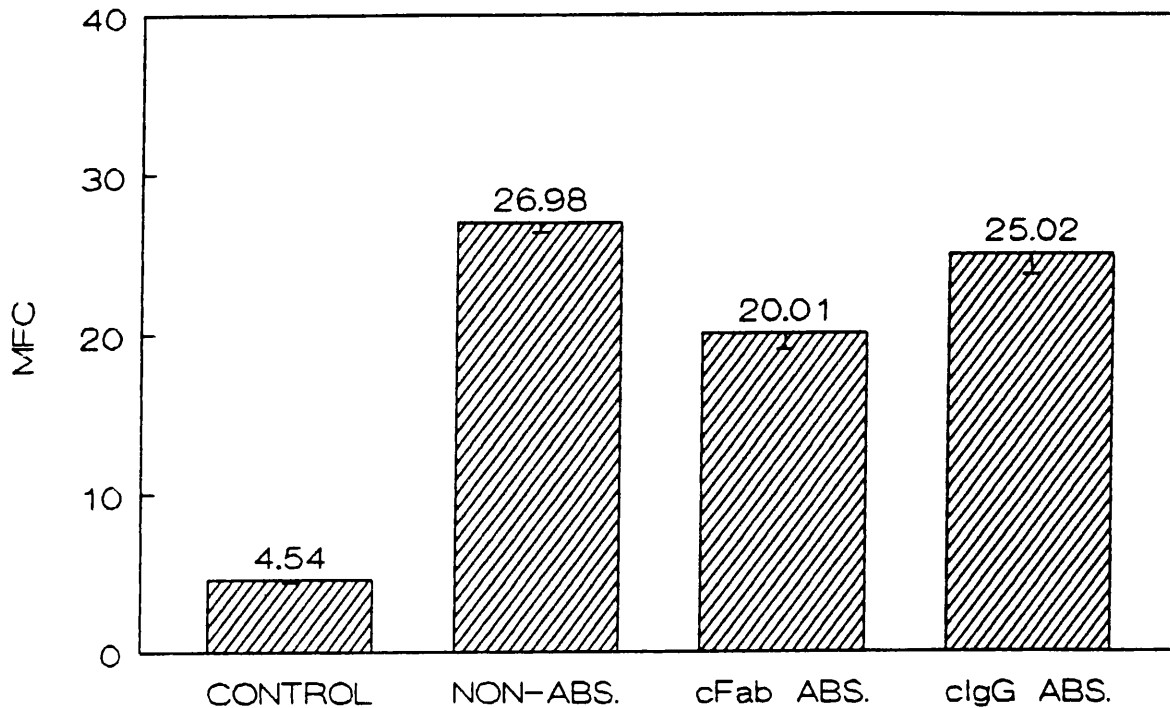


FIGURE 6.9 Adsorption of anti-c7E3-Fab activity from plasma (see section 6.3.3 for experimental details). MFC is the mean fluorescence channel of control platelets labelled with FITC-anti-IgG (Fc-specific) following incubation with:

CONTROL: PBS (negative control).

NON-ABS: Plasma containing anti-c7E3-Fab activity (positive control).

cFab ABS: The same plasma after adsorption with c7E3-Fab-coated platelets.

cIgG ABS: The same plasma after adsorption with c7E3-IgG-coated platelets.

The thin vertical bars represent SD of the mean.

TABLE 6.3 Some results of HACA detection obtained with ELISA and FC.

THROMBOCY- TOPENIA	TIME OF SAMPLE	SERUM/PLASMA HACA	
		ELISA (TITRE) N.Range < 20	FC (MFC) N.Range < 6.7
Mild. Platelets fell to 44% of baseline 4 hours after onset of c7E3-Fab infusion.	6 weeks	< 20	12.2
Severe. Platelets 35.6% of baseline at 2 hours falling to 10% at 12 hours after bolus c7E3-Fab.	b/line	< 20	26.8
Mild. Platelets 42.6% of baseline 2 hours after bolus c7E3-Fab.	b/line	> 800	48.2
None	6 weeks	800	65.1
None	4 weeks	100	19.9
None	b/line	200	51.1

HACA: Human-anti-chimaeric "antibodies", MFC: Mean Fluorescence Channel.

method is theoretically more physiologically relevant, as it employs c7E3-Fab coating intact platelets rather than microtiter wells.

6.4 CONCLUSIONS

The *ex vivo* measurement of PSiGg before, during and following I.V. infusions of c7E3-Fab showed that, in the majority of the patients studied, the infusion resulted in recruitment of variable, usually small (2-3 times the baseline level), amounts of IgG to the platelet surface. The increase in PSiGg occurred early (in less than 24 hours), which suggests the preexistence in the patients' serum of an IgG bindable to c7E3-Fab-coated platelets. Although this recruitment of IgG depended on the presence of c7E3-Fab on the platelet surface, the post infusion course of PSiGg levels did not parallel that of platelet-bound chimaeric Fab (exponential fall) as PSiGg reached its maximum several days after the end of the infusion. This timing is reminiscent of an anamnestic immune response and weakens the possibility that the observed IgG recruitment was due to non-specific binding of serum IgG or circulating Fc fragments to platelet-bound chimaeric Fab.

In vitro flow cytometric studies demonstrated that IgG bindable to c7E3-Fab- but not m7E3-Fab-coated platelets occurs commonly in the plasma of individuals not previously exposed to chimaeric Fab. This is consistent with the results of ELISA assays (R Jordan, personal communication) showing the presence of IgG that binds specifically to chimaeric Fab in the serum of at least 13 of 40 subjects. Preliminary adsorption experiments suggested that the antigenic determinant attracting the anti-c7E3-

Fab activity was exposed as a result of the enzymatic (papain) cleavage of the human component of the chimaeric IgG molecule.

Antibodies against the Fab region of IgG were first described by Osterland Harboe & Kunkel (1963) who reported that sera from rheumatoid arthritis (RA) patients contained antibodies to $F(ab')_2$ fragments derived by pepsin treatment of IgG. These antibodies became known as "serum agglutinators". In contrast to the anti-Fc antibodies (rheumatoid factors) which are almost always IgM globulins and bind to both isolated Fc fragments and the Fc region of intact IgG, serum agglutinators are almost always IgG and only bind to Fab or $F(ab')_2$ fragments produced by enzymatic cleavage of the IgG molecule. Anti-FAB (anti-Fab or anti- $F(ab')_2$) specificity was found to depend on the enzyme used for hydrolysis (papain, pepsin, trypsin etc.). Such anti-FAB activities were detectable in most healthy individuals and it was reported (Waller *et al* 1971) that anti-Fab titres were usually stable over many years but could rise in response to certain infections. Normal individuals were found to differ in their patterns and titres of anti-FAB (Osterland *et al* 1963, Kormeier *et al* 1968, Waller *et al* 1968).

Those first assays for anti-FAB detection were based on the ability of positive sera to agglutinate sensitized cells (Rh positive red cells coated with FAB fragments of an anti-Rh IgG). More recently, other investigators employing ELISA assays have confirmed the existence of anti-FAB antibodies, often in the form of immune complexes, in the clinical settings of RA or other autoimmune disorders, infections, post immunization and also in normal subjects (Nasu *et al* 1980, Ling and Drysdale 1981, Heimer *et al* 1982, Davey and Korngold 1982, Birdsall and Rossen 1983). Persselin and Steven (1985) reported that when anti-FAB IgG was found in normal sera, this was usually of the IgG4 subclass. The same

group of workers (Hunt-Gerardo *et al* 1990) have reported significant heterogeneity in the determinants of the FAB molecule that could be recognized by different anti-FAB; moreover, some anti-FAB antibodies could also react with the Fc region of IgG.

Although the biological function -if any- of these antibodies remains unknown, binding of naturally occurring anti-Fab IgG to the human component of the chimaeric 7E3-Fab fragment appears to be a plausible explanation for the elevations in PSiGg frequently associated with c7E3-Fab infusions. The fact that these elevations are usually mild is consistent with the low titres of anti-Fab usually present in normal subjects.

The observation that the patient who showed the most marked elevation in PSiGg was also found to have the fastest clearance of platelet-bound c7E3-Fab suggests the possibility of increased platelet turnover induced by accelerated removal from the circulation of IgG-coated platelets. The plasma of this patient and of all three patients with a history of c7E3-Fab related thrombocytopenia contained anti-Fab IgG detectable by FC. It has been known for a long time that small (e.g. a few hundred molecules) increases in PSiGg can cause thrombocytopenia in certain settings (Shulman 1964) but, beside the amount, other characteristics of the PSiGg (e.g. subclass) could be important in determining development of this complication. Finally, the finding of much lower incidence of naturally occurring antibodies to the murine 7E3-Fab might be the reason for the apparently lower frequency of thrombocytopenia in patients who received infusions of m7E3-Fab (Machin 1991, Centocor, data on file).

Appendix to Chapter 6

6.A c7E3Fab/EDTA-DEPENDENT PSEUDOTHROMBOCYTOPENIA

Platelet counts of patients participating in the c7E3-Fab infusion trial were routinely monitored using fresh EDTA-anticoagulated samples. During control experiments aimed at optimizing the method for PSiG measurement in the trial setting, it was observed that an EDTA sample taken 30 minutes after the onset of the infusion showed a time-dependent fall in the platelet count, whereas the counts of the baseline EDTA sample remained stable (Fig. 6.10). Examination of blood films made from the 30min EDTA sample revealed the presence of many platelet clumps which accounted for the thrombocytopenia; no clumps were present in the baseline EDTA sample. The same phenomenon was subsequently noticed in EDTA samples from several other patients during and up to 10 days after the infusion and soon became apparent that its intensity varied remarkably between patients. There was no fall in the platelet count of citrate-anticoagulated samples but subsequent addition of 5mM EDTA to those samples resulted in time-dependent platelet clumping.

Flow cytometric measurement of PSiG in platelets isolated from EDTA samples of 2 patients with 7E3-Fab-related pseudothrombocytopenia (pseudoTCP) showed significant, time-dependent (standing at RT) increases in platelet-bound IgG which were not present in simultaneously taken citrate-anticoagulated samples or EDTA samples taken before the

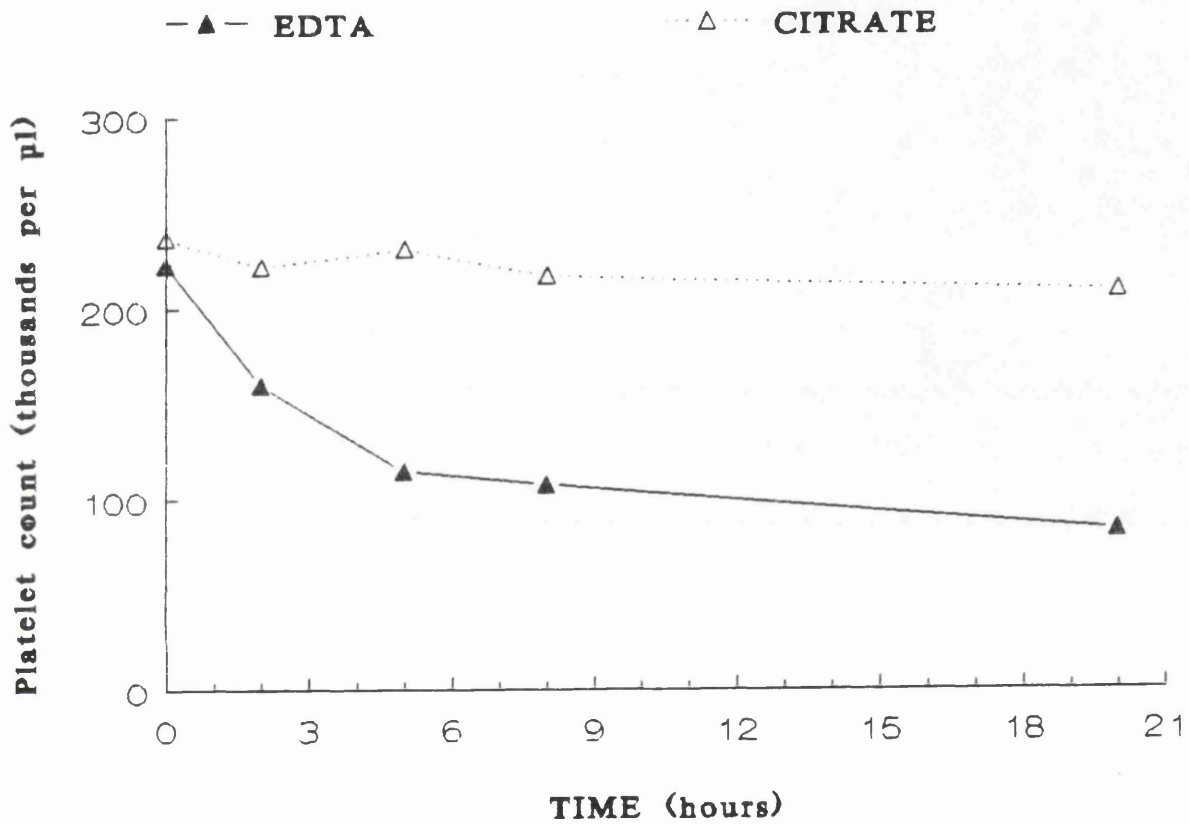


FIGURE 6.10 Platelet counts in EDTA- and citrate-anticoagulated blood at various time points after sampling. The sample was taken 30 minutes after the onset of c7E3-Fab infusion and was kept at RT.

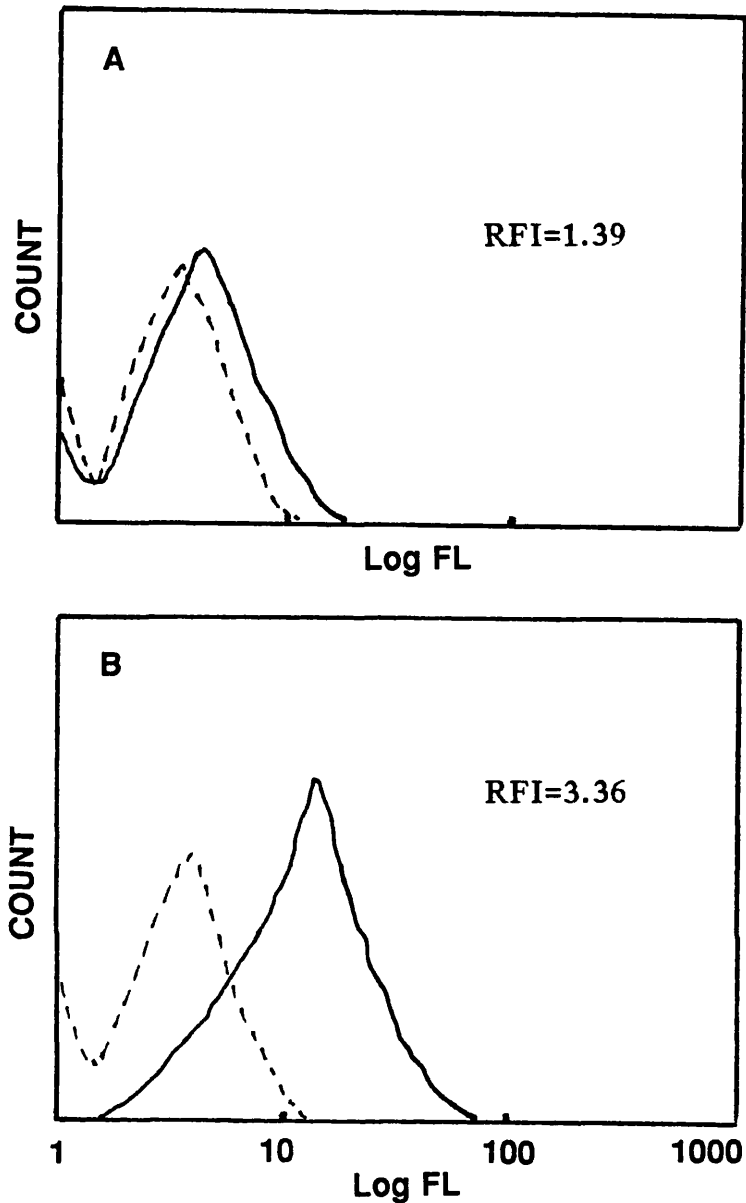


FIGURE 6.11 PSiGg (expressed as RFI) of platelets isolated from citrate (A) and EDTA (B) anticoagulated samples left to stand overnight at RT. The patient was 12 hours into the c7E3-Fab infusion and a previous EDTA sample had shown mild c7E3/EDTA-dependent pseudoTCP. The continuous-line fluorescence histograms were obtained with FITC-conjugated goat-anti-human IgG (Fc-specific) and the broken-line ones with FITC-conjugated non-immune goat IgG serving as control. RFI (relative fluorescence intensity) is defined in Chapter 4.

onset of the infusion (Fig. 6.11). This suggests that IgG mediated agglutination might be the cause of platelet clumping.

To examine whether EDTA-dependent, c7E3-Fab-related pseudoTCP was reproducible with in vitro c7E3-Fab-coated platelets, the following experiment was performed:

Ten weeks after a 48 hour c7E3-Fab infusion, a citrate-anticoagulated sample was taken from a patient who had shown c7E3Fab/EDTA-dependent pseudoTCP during the infusion. A synchronous (10 weeks post infusion) sample from a patient who had not shown this phenomenon served as control. Platelets (in PRP) from both samples were incubated for 40 minutes with nearly saturating concentrations of either c7E3-Fab or m7E3-Fab following which the platelet concentrations were measured. K_3 -EDTA at a final concentration of 5mM was added to aliquots of c7E3-Fab- or m7E3-Fab-coated platelets and following a 2 hour incubation the platelet counts were repeated. Only the c7E3-Fab-incubated, EDTA-treated aliquot from the patient with the history of c7E3-Fab-related pseudoTCP showed a fall in the platelet count (to 66% of baseline) associated with numerous platelet clumps on a MGG-stained film.

The result of this experiment suggests that, at least in this patient, the EDTA-dependent in vitro platelet agglutination was specific for the chimaeric Fab fragment of 7E3.

Interestingly, among the 19 consecutive patients studied by flow cytometry, the patient who showed the most marked pseudoTCP (Fig. 6.12) was also the one who had the highest levels of IgG recruited to his platelets in response to c7E3-Fab infusion (patient No 12, see Table 6.1). This is unlikely to represent simple coincidence ($P=0.052$) and suggests that the factor responsible for the increased PSiGg (?naturally occurring anti-Fab antibodies) might also be participating in the mechanism of c7E3Fab/EDTA-dependent pseudoTCP.

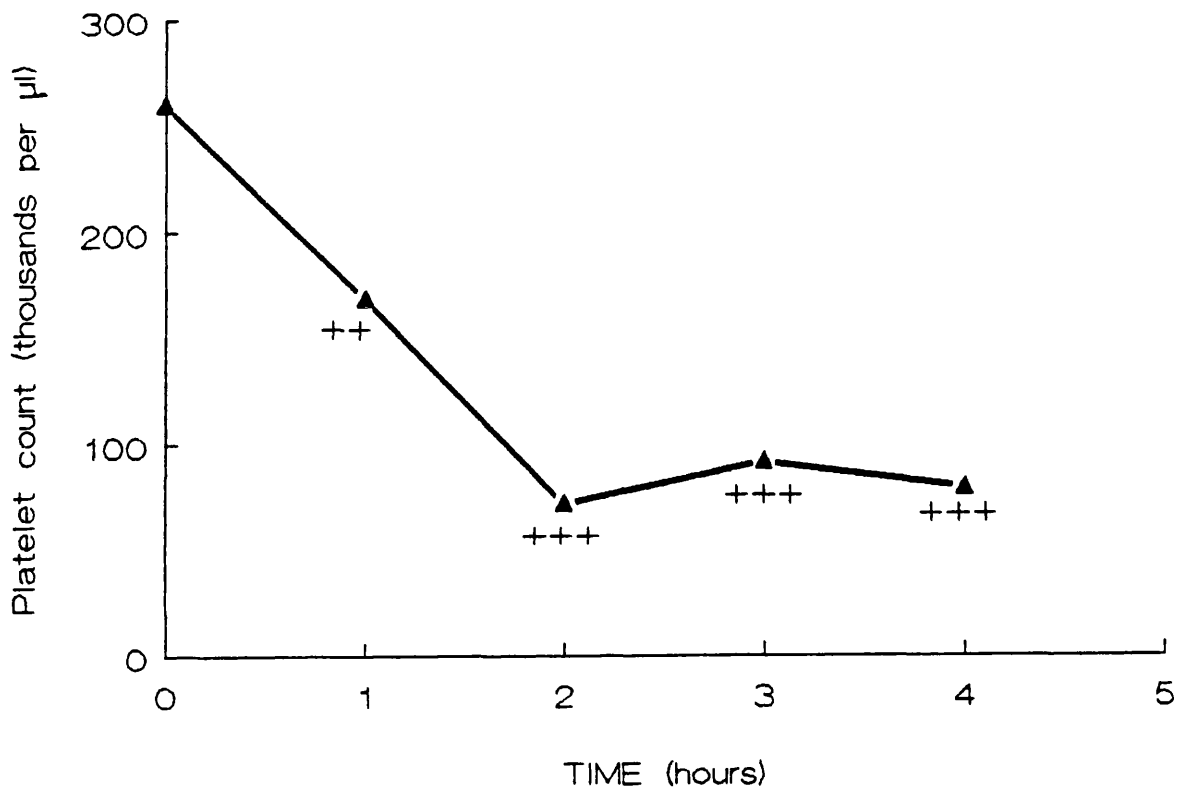


FIGURE 6.12 Marked c7E3Fab/EDTA-dependent pseudoTCP in a EDTA-anticoagulated sample taken 7 days after the end of a 72-hour c7E3-Fab infusion.

The presence of platelet clumps is marked with (+).

6.A.1 Proposed mechanism of c7E3Fab/EDTA-dependent pseudoTCP

The data presented above allow the definition of the following characteristics for this new type of pseudoTCP:

- a. It only occurs in the presence of EDTA.
- b. It only occurs when the platelets exposed to EDTA are coated with c7E3-Fab.
- c. It requires a factor present in the plasma (or on the platelet surface) of some individuals. Indirect evidence (elevated PSiGg) suggests that this factor might be IgG mediating the agglutination of platelets when conditions (a) and (b) are fulfilled.

In classical EDTA-dependent pseudoTCP, *in vitro* exposure of platelets to EDTA induces the expression of a cryptoantigen on the platelet surface. This is probably formed as a result of dissociation of the calcium-dependent IIb-IIIa heterodimer through the calcium chelating effect of EDTA (Pidard *et al* 1986). The rare individuals possessing antibodies to this cryptoantigen will develop platelet agglutination in EDTA blood samples. The agglutinating factor is usually IgG directed to some epitope on the IIIa molecule (Pegels *et al* 1982) but IgM (Pegels *et al* 1982, Van Vliet *et al* 1986, Hoyt and Durie 1989) and even IgA (Pegels *et al* 1982, Imai *et al* 1983) agglutinins have also been described.

The mechanism of c7E3Fab/EDTA-dependent pseudoTCP observed in the setting of c7E3-Fab infusion is likely to be different from that of classical EDTA-dependent pseudoTCP because:

- a. There is no platelet agglutination in baseline EDTA samples of the same patients (i.e. before c7E3-Fab coating).

b. It is much more frequent (more than 100 times) than classical EDTA-dependent pseudoTCP, the frequency of which has been reported to be 0.068% in a caucasian population (Vicari *et al* 1988).

c. c7E3-Fab coating tends to hinder rather than facilitate EDTA-dependent agglutination of control platelets by the plasma of patients with classical EDTA-dependent pseudoTCP and high titres of IgG antibody to a cryptoantigen located on the IIB-IIIa complex (personal unpublished observations). This is probably due to either simple steric hindrance (caused by proximity of the cryptoantigen to the 7E3 binding site) or to 7E3, partially preventing EDTA from splitting the IIB-IIIa complex (and therefore exposing the cryptoantigen).

It is known that treatment with EDTA can displace 7E3 bound to gpIIB-IIIa (Coller *et al* 1986 and personal observations - see Fig.6.13). The rate of loss of platelet-bound 7E3 following exposure to EDTA varies significantly between individuals and the same applies to the residual amount of 7E3 that remains on the platelet after incubation with EDTA (personal observations). In EDTA-anticoagulated blood/PRP containing platelets precoated with c7E3-Fab, the latter will exist as both platelet-bound and free cFab fragments. If the plasma also contains anti-cFab immunoglobulins (a frequent occurrence as shown in 6.3) capable of binding to multiple epitopes on the Fab fragment, then long molecular bridges (consisting of cFab-anti-cFab complexes) could be formed, resulting in platelet agglutination by binding to residual platelet-bound c7E3-Fab and/or the platelet Fc γ RII receptors (1.3.2).

Whatever its exact mechanism, c7E3Fab/EDTA-dependent pseudoTCP remains an *in vitro* phenomenon which, like classical EDTA-dependent pseudoTCP is unlikely to have any clinical significance. Its importance

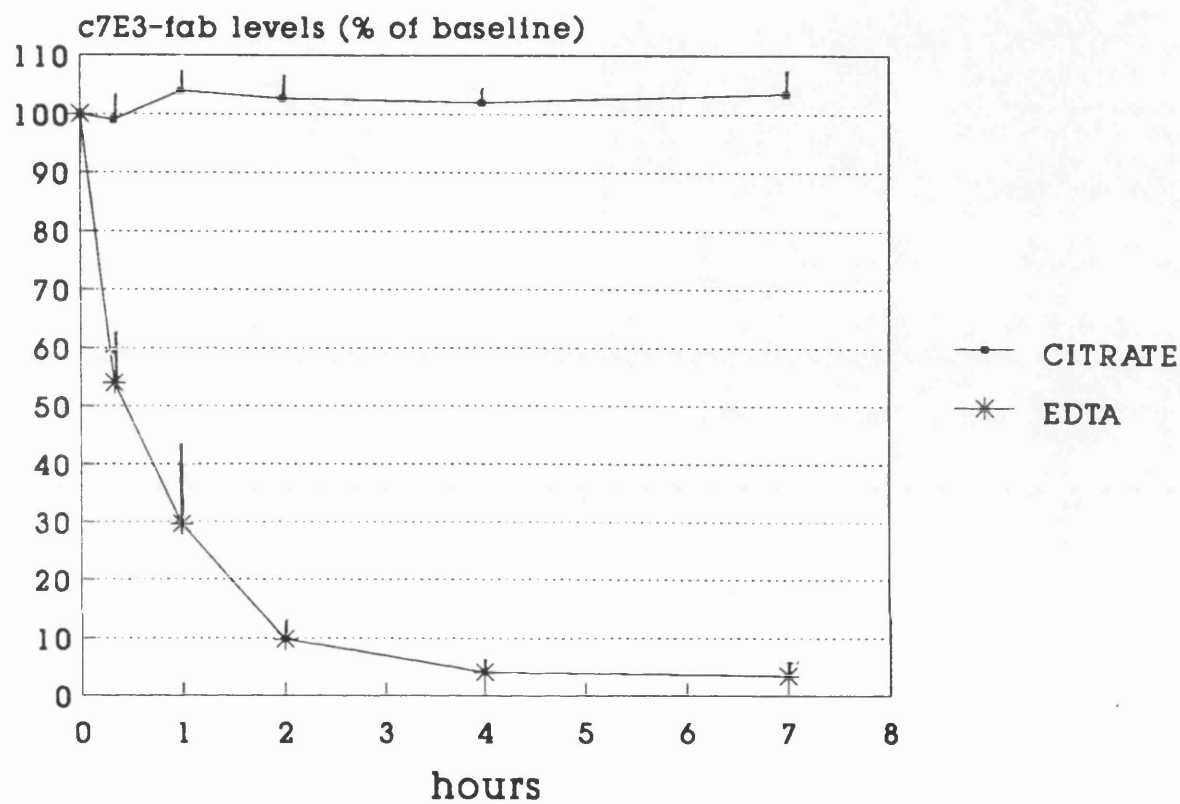


FIGURE 6.13 Effect of EDTA on platelet-bound c7E3-Fab: Citrate-anticoagulated platelets (in PRP) were incubated with saturating concentrations of FITC-conjugated c7E3-Fab. Addition of 5mM disodium EDTA resulted in time-dependent loss of platelet fluorescence (i.e. FITC-c7E3-Fab bound to the platelet surface), while the fluorescence of the same platelets not exposed to EDTA remained stable. The means and SDs (vertical lines) of three independent experiments are shown.

lies in the fact that, in the setting of patients receiving c7E3-Fab (and possibly IgG fragments of other chimaeric anti-platelet antibodies) thrombocytopenia in EDTA anticoagulated samples might be spurious and should always be confirmed by examination of blood films to exclude the presence of platelet clumps and by repeating the platelet count on a citrate-anticoagulated sample.

Chapter 7

GENERAL DISCUSSION - THE FUTURE

The studies included in this thesis have explored the use of fluorescence flow cytometry (FC) as a tool with the potential of providing new information about the structure and function of normal and pathological platelets and platelets exposed *in vivo* to therapeutically administered anti-platelet monoclonal antibodies. To this end they have achieved the following:

- Established a flow cytometric method for quantitative measurement of PSiG. A novel approach, exploiting recent advances in monoclonal antibody (MoAb) technology, was used to create reference curves and assess method sensitivity. The method was applied to study PSiG in normal individuals and patients with a variety of thrombocytopenic states.
- Introduced the use of flow cytometry in monitoring platelet-bound antibody and residual antibody-binding sites in patients receiving trial infusions of an anti-gpIIb-IIIa chimaeric MoAb (7E3). This yielded information - unobtainable by other methods - that improved understanding of the *in vivo* behaviour of the Fab fragments of the particular antibody and might prove of help in designing future trials of the same or similar MoAbs.
- Revealed the existence of naturally occurring human-anti-chimaeric Fab activities which might have important implications in the clinical

application of chimaeric antibodies in general. Preliminary evidence was provided suggesting that these activities are similar - if not identical - to the previously described "serum agglutinators".

- Defined a new type of EDTA-dependent pseudothrombocytopenia.

Flow cytometry for quantitative detection of PSiG.

Although there have been several reports describing the use of FC to detect PSiG (Table 4.3), it is the first time that a flow cytometric method has been used to calculate the number of IgG molecules on the platelet surface of healthy and thrombocytopenic subjects. A novel approach, employing platelets coated with known amounts of a monoclonal chimaeric IgG (cIgG), was used to create reference curves. This approach bypasses potential sources of error present in most other popular quantitative methods; namely, the use of soluble (or bound to plastic) IgG standards or stoichiometric assumptions regarding the binding of the anti-IgG probe to platelet-bound IgG (for review see Schwartz 1988). From this point of view, the model of monoclonal cIgG-coated platelets employed in the present studies moves toward the ideal calibration standard. The availability of a chimaeric MoAb to gpIIb-IIIa allows the sensitivity of the flow cytometric method - and, indeed, any method of PSiG detection - to be assessed objectively, by varying the number of IgG molecules coating the platelets. It can thus be envisaged that, by using this "realistic" platelet-bound IgG model, it will be possible to directly compare various methods currently in use and reach definitive conclusions regarding their performance. Although there is no evidence that quantitative PSiG detection methods have any practical

advantages over qualitative ones in the routine diagnostic work-up of thrombocytopenic states, the expression of results as molecules per platelet can be useful in various research settings.

What then is the true number of IgG molecules "bound" to the surface of normal platelets?

The calibration method used in the present study produced an estimate of *ca.* 1,500 IgG molecules/platelet. This, while being in good agreement with many different methods employing polyclonal anti-immunoglobulins (Schwartz 1988), is apparently at variance with the estimates of methods based on the use of radiolabelled monoclonal anti-IgG (LoBuglio *et al* 1983 & modifications), which usually measure <200 molecules/platelet. Why methods employing MoAbs could be underestimating PSiG has been discussed before (section 4.3). Nevertheless, any underestimation is unlikely to be the sole reason for the 10-fold difference. The epitope of the anti-IgG MoAb is in the Fc region of IgG and, therefore, the MoAb might have no access to it in the case of IgG bound to the platelet Fc receptor. As platelets appear to possess several thousands of Fc receptors (see section 1.3.2), it is possible that a significant amount of PSiG detectable by a polyclonal antibody, which recognizes multiple epitopes on the IgG molecule, could pass undetectable by the MoAb. The same could happen with IgG bound to the platelet membrane through both the Fab (specifically directed to a platelet surface antigen) and the Fc region (binding to the FcγRII receptor, see Fig. 7.1). The latter type of binding has recently been shown to be responsible for the platelet activating effect of many anti-platelet Moabs (Horsewood *et al* 1991, Anderson *et al* 1991). In view of the emerging functional importance of the platelet Fc receptor, IgG bound to the platelet surface through its Fc region should not be simply considered as "background" or irrelevant. In

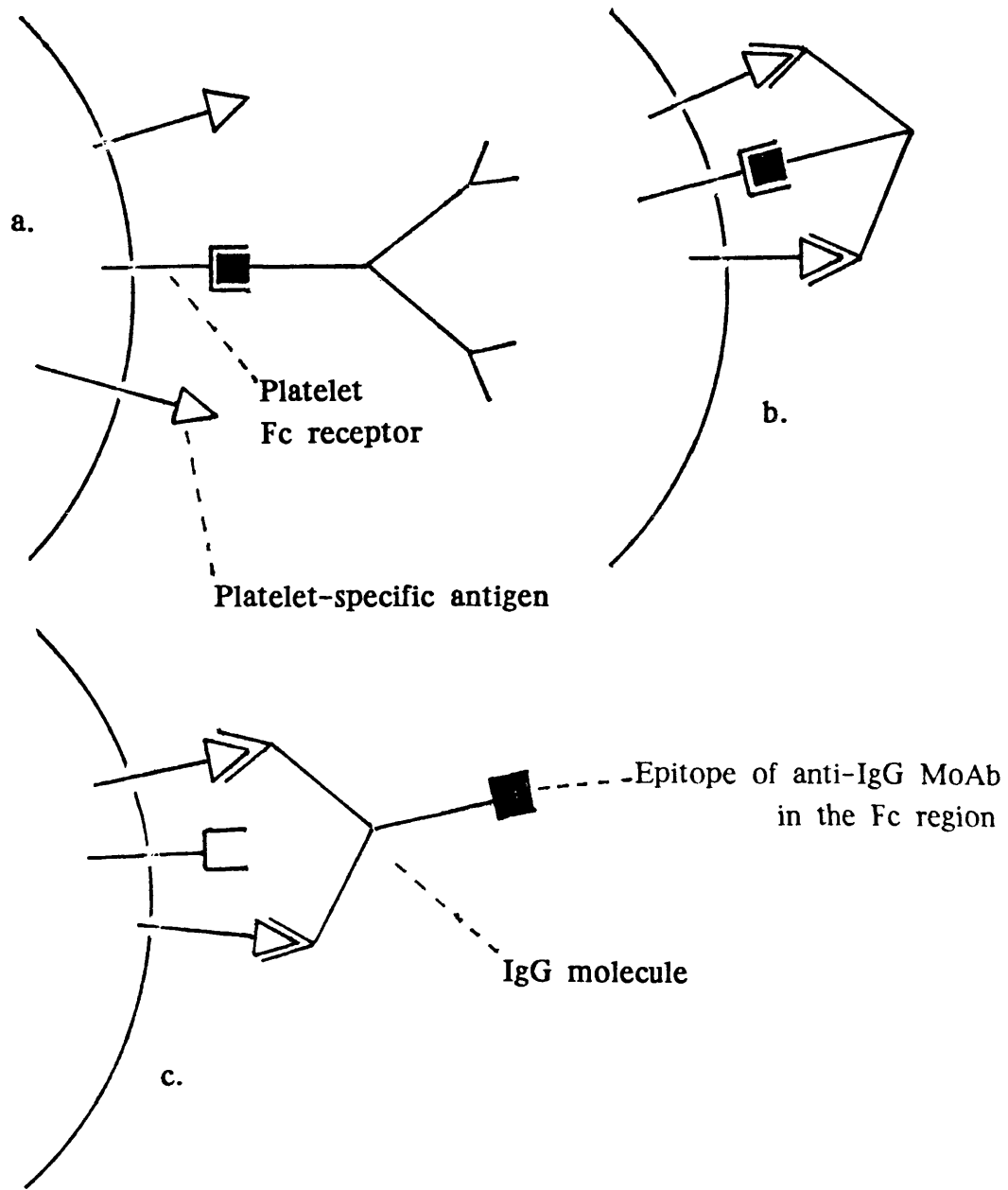


FIGURE 7.1 Three possible ways of IgG binding to the platelet surface. Only IgG bound as shown in c. will be recognized by the monoclonal anti-IgG (Fc-specific) antibody, the epitope of which is marked as black square.

the light of these comments it is easier to reconcile the apparently conflicting PSiG estimates obtained by the polyclonal anti-IgG employed in the present studies and those reported with the monoclonal (Fc-specific) anti-IgG.

Application of the method to measure PSiG in a small group of patients with the clinical diagnosis of immune-mediated TCP produced results (Fig. 4.3) corroborating the recently renewed claim (Tijhuis *et al* 1991) that ITP without detectable elevation in PSiG definitively exists. The prospective study of PSiG in a group of 62 consecutive patients with TCP of unknown aetiology was mainly aimed at determining the frequency of positive results that can be anticipated in a referral laboratory rather than at investigating the biological relevance of raised PSiG. In this group, the detection of a significant proportion of patients with markedly elevated PSiG (Fig. 4.3, Table 4.2) highlights the potential usefulness of this measurement, which, even if it is not always helpful in therapeutic decision making, can contribute to the elucidation of the factors that govern the interactions of immunoglobulins with the platelet membrane.

It is the author's view that, with the acquisition of user-friendly, bench-top flow cytometers by an increasing number of clinical laboratories, flow cytometric methods for PSiG detection will gain in popularity. This will be helped by the development of even more sensitive and more automated cytometers. Nevertheless, even with the present levels of sensitivity, the existence of cell autofluorescence - resulting in background "noise" - is more important than instrument-related factors in defining the lowest antigen concentrations detectable on the surface of the platelet (or any other cell) by FC. Regarding the automation factor,

it should be noted that, even the latest cytometers require a certain instrument-operator interaction; this calls for considerable experience on the part of the operator for both performing and interpreting cytometric analyses, particularly of low density antigens, as is usually the case with PSiGg.

Flow cytometric observations in patients receiving infusions of Fab fragments of chimaeric MoAb 7E3.

The major findings from the application of FC to monitor platelet-bound c7E3-Fab were long detection times post-infusion (exceeding the platelet lifespan) and uniform distribution of the antibody in the platelet population at all post-infusion time points; these lead to the inevitable conclusion that in vivo transfer of antibody to platelets not exposed to it during the infusion was taking place. The in vitro mixing experiments suggested that this transfer might have simply been the result of antibody movement between platelets in the direction of the concentration gradient, made possible by the looser (monovalent) binding of the c7E3-Fab fragment, as opposed to the bivalent binding of the whole molecule or F(ab')₂ fragments of the antibody (Jordan *et al* 1991). Although the available data do not exclude other factors contributing to antibody transfer in vivo, they provide evidence that the in vivo kinetics of 7E3-Fab fragments might be significantly different from those of 7E3-IgG or 7E3-F(ab')₂. As this was the first time that FC was used in this setting, it would be interesting to perform FC analyses in future in vivo studies of 7E3 employing F(ab')₂ and compare the findings with those obtained with the Fab fragments. In the setting of treatment with

antiplatelet MoAbs, transfer of antibody to newly released platelets would appear to be an undesirable phenomenon as:

a. It results in persistence of antibody on the platelet surface long after its anti-aggregatory effect has ceased to exist; gpIIb-IIIa receptor blockade >50% appeared to be required in order to prevent recurrence of chest pain in the study by Gold *et al* (1990) employing 7E3-F(ab')₂ in unstable angina. Prolonged MoAb circulation might result in increased immunogenicity.

b. Exposes new platelets to the risks that could be associated with antibody coating, e.g. recruitment of patient IgG onto the platelet surface (see below).

On the other hand, antibody transfer between cells might be advantageous in different settings. In a hypothetical example where a MoAb aims at cell lysis, one could consider the use of a bispecific construct (Songsivilai & Lachmann 1990) binding to the target cell through one of its Fab arms, the second arm being of irrelevant specificity (or carrying an appropriate cytotoxic or other agent); a constant region with the desirable biological activity (e.g. regarding complement recruiting capacity) could also be selected. Such a construct could combine the *in vivo* kinetic behaviour of Fab fragments with some of the biological effects of the whole IgG MoAb molecule.

Combined monitoring of platelet-bound c7E3-Fab and residual c-7E3-Fab binding sites showed that the latter could return to baseline levels while a significant proportion of the former remained detectable on the platelet surface. This suggests that the number of surface IIb-IIIa receptors should not be considered constant in a given individual. The suggestion that blockade of IIb-IIIa receptors could lead to up-regulation with emergence of platelets bearing higher concentrations of gpIIb-IIIa (Fig. 5.16) deserves further investigation, as these platelets might have

increased potency to aggregate; a significant (24%) increase in platelet surface gpIIb-IIIa (and a 39% increase in gpIb) has been reported in platelets of diabetic patients and this could be aetiologically related to the increased functional properties reported for platelets of such patients (Tschoepe *et al* 1990a).

The amplitude of *ex vivo* turbidometric aggregation responses to ADP was shown to be a linear function of the logarithm of available fibrinogen receptors. Although approximate halving of the receptor number was required before any consistent effect on standard aggregometry, it is probably an over-simplification to state that fibrinogen receptors exist in excess of those required for platelet function. While it appears difficult to dissociate the anti-thrombotic from the anti-haemostatic effect of receptor blocking in acute thrombotic phenomena (see section 1.5.5.2), moderate chronic reduction in receptor number might have an effect on the progress of slowly evolving thrombogenic processes. The only model available for study of this possibility is the heterozygote for Glanzmann's thrombasthenia (with gpIIb-IIIa levels *ca.* 50% of normal) and studies assessing the incidence of occlusive arterial disease in such individuals might add to our understanding of the effects of chronic blockade of fibrinogen receptors.

The results of immunoenzymatic staining of gpIIb-IIIa presented in Chapter 3, combined with the findings of other studies (Isenberg 1987 and review in section 3.4) suggest that the bulk of this gp is located on the platelet membrane and in membrane-associated structures - probably the SCCS. A significant increase in flow cytometrically detectable 7E3-Fab binding sites following TX-100 membrane premeabilization (Fig.3.3) suggests that submembrane gpIIb-IIIa is not accessible to 7E3-Fab in intact platelets after 1 hour's incubation at RT. The presence of

continuous cycling of gpIIb-IIIa molecules between surface and intracellular pools (Wencel-Drake 1980) permits the hypothesis that, with prolonged infusions, antibody will bind to an increased proportion of total platelet gpIIb-IIIa, i.e. 7E3-bearing surface receptors will move intracellularly, while intracellular receptors will be exteriorized becoming available for antibody binding. Internalization of 7E3 is supported by the TX-100 permeabilization experiments (section 5.4.7) described here and the electron microscopy studies reported by Harrison *et al* (1990).

Combined FC monitoring of platelet-bound antibody and residual antibody-binding sites during and following infusions of antiplatelet MoAbs is technically simple and provides novel information unobtainable by other methods; the author believes that it should be used in all future therapeutic trials of anti-glycoprotein MoAbs.

Naturally occurring anti-chimaeric Fab activities

Flow cytometric monitoring of PSiGg in patients receiving infusions of c7E3-Fab revealed an infusion-related recruitment of IgG onto the surface of c7E3-Fab-bearing platelets. This, combined with the demonstration in the plasma of normal subjects of IgG bindable to c7E3-Fab-coated platelets in vitro, suggests the possibility of an immune mechanism to explain the thrombocytopenia reported in some patients receiving c7E3-Fab infusions. It also predicts that this complication is likely to be seen with other chimaeric antiplatelet MoAb fragments. Although characterization of this naturally occurring activities was outside the scope of the present studies, preliminary experiments suggest that they are specific for the chimaeric Fab and are directed to some determinant(s) in the area of papain cleavage of the parent chimaeric IgG

molecule; this is probably why, at least at the concentrations tested, they did not appear to interfere with c7E3-Fab binding to platelets (i.e. with the function of the murine component of the fragment). The specificity and frequency of these activities strongly suggest that they are caused by "serum agglutinators" (discussed in section 6.4). Therefore, while (as discussed in section 1.5.6) chimaeric antibodies appear to have less anti-murine immunogenic potential than their parent murine MoAbs, the chimaeric Fab - or other proteolytically derived fragments - are theoretically more likely than their murine counterparts to result in immediate recruitment of IgG onto the surface of the target cell. This is supported by the low incidence of naturally occurring anti-murine-Fab activities observed in the present study (1/21) comparable to that (1/16) reported by Gold *et al* (1990) for the F(ab')₂ fragment of murine 7E3. It would be advisable for future clinical trials involving fragments of chimaeric MoAbs to assess various enzymatically derived fragments and select those with the lowest IgG-recruiting potential - if such an effect is undesirable - before embarking on in vivo administration. Flow cytometric analysis of cells coated with the under investigation fragments offers perhaps the most physiologically relevant method for assessment of such activities.

Epilog

The development of new antiplatelet agents is an area of rapidly expanding research. Interest in this research is fuelled by the public's hope for effective treatment of "the sudden thrombosis and the slowly hardening artery" and by the pharmaceutical industry's anticipation of big profits, guaranteed for any new agent with wide applicability in the

clinical setting of coronary or cerebrovascular disease. Progress in MoAb technology and genetic engineering is making available "designer" MoAb molecules combining powerful antiplatelet effects and reduced immunogenicity. The observations described in this thesis introduce additional variables, that are likely to play a part in deciding whether and in which form will anti-gp MoAbs become acceptable therapeutic agents.

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