COMPARATIVE EFFECT OF HAEMODYNAMIC VARIABLES AND RHEOLOGICAL PROPERTIES OF THE BLOOD ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

By

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A thesis submitted to the The University of Birmingham
for the degree of **DOCTOR OF PHILOSOPHY**

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February 2010

ABSTRACT

The work in this thesis used an *in vitro* flow system, which incorporated a capillary viscometer, to systematically determine the effects that haemodynamic variables, or rheological properties of the perfused, fluorescently-labelled blood, had on the behaviour of leukocytes and platelets. By varying shear rate and stress, blood haematocrit, and aggregation and deformability of red cells, we obtained evidence that leukocyte adhesion was dictated by the number of free-flowing cells 'marginated' near the vessel wall and the force experienced by their initial adhesive bonds. Platelet adhesion was dependent on the velocity of the free-flowing cells near the vessel wall and the force experienced by the platelets once adhered. In contrast to leukocytes, variation in platelet adhesion appeared less dependent on variation in the number of free-flowing cells near the vessel wall, but dictated more by the width of the peripheral plasma layer; a thinner plasma layer promoting more efficient platelet adhesion. Overall, this thesis provides evidence that the differential margination and adhesion of leukocytes and platelets is largely a result of the difference in their size. Thus, it seems that the sizes of RBC, leukocytes and platelets are all adapted to provide efficient immune and haemostatic responses in different regions of the circulation.

DEDICATION

This thesis is dedicated, with much love, to my Mom, Anita, my Dad, Peter, my Brother, Leigh and my Grandparents, Alfred and Frances.

ACKNOWLEDGEMENTS

The work described in this thesis was performed within the School of Clinical and Experimental Medicine, College of Medical and Dental Sciences at The University of Birmingham, during the period October 2006 to September 2009. Except where stated, it is the original work of Tim Watts. This thesis has not been submitted, in part or *in toto* to any other university.

First and foremost I would to like to thank my supervisor Professor Gerard Nash, who was always there for me, whether it be for a quick question at his door or an in depth scientific discussion. It was definitely privileged to have such a great (and patient) scientist as my supervisor.

I would like extend my thanks to all the members of the of the Cardiovascular Rheology Group within the Medical School for making my Ph.D enjoyable, and providing me with many litres of blood over the years to enable me to undergo the experimental work within this thesis. I would also like to thank the Blood group within the School of Chemical Engineering, particularly Prof. Mostafa Barigou, for their support throughout my Ph.D.

Lastly, I'd like to thank my family for their unwavering love and support throughout the years, especially towards the end when the going got really tough. Mom, Dad, Leigh, I know it wasn't easy but thanks for putting up with me, particularly over the past few months, I am eternally grateful.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: EFFECT OF VESSEL SIZE AND WALL SHEAR RATE ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

CHAPTER 6: EFFECT OF HAEMATOCRIT ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

CHAPTER 7: EFFECT OF RED BLOOD CELL DEFORMABILITY ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Leukocyte and platelet interactions with the vessel wall are imperative for human survival. Leukocytes must interact with adhesion molecules on the endothelial cells lining blood vessels to allow migration into tissue that has been infected and to allow them to perform their protective immune functions (Springer, 1995). Collectively leukocytes can engulf invading micro-organisms, kill infected cells and help produce antibodies against specific antigens. Platelets are required for the arrest of bleeding and maintenance of blood volume (Savage et al., 1996). They adhere to subendothelial collagen and von Willebrand factor (vWF) exposed at the site of trauma to a vessel anywhere in the circulatory system. This leads to activation of the platelets, presentation of clotting factors and ultimately the formation of a clot at the site of damage in a vessel wall.

There are various differences and similarities in the behaviour of leukocytes and platelets in the circulation. Leukocyte adhesion predominantly occurs in a defined region in the circulation, namely post capillary venules, where the shear rate is low (Lipowsky, 1988). Platelets however are able to adhere to any damaged blood vessel wall. Vessel trauma, especially in arteries, can lead to the rapid loss of blood; therefore platelets have developed the ability to adhere to essentially any vessel even at high shear rate (Tangelder et al., 1988).

Both leukocytes and platelets undergo similar processes during adhesion; margination in the blood stream brings them into close proximity to the wall, where receptors on the cells bind their ligands on the vessel wall. These initial bonds form and break very quickly resulting in adherent cells rolling on the vessel wall (Springer, 1995; Savage et al., 1996). This rolling slows the velocity of the cells down enough for a slower, activation dependent, firm adhesion to take place. Once firmly adherent, the migration of leukocytes and the build up of platelet thrombi can begin.

In this introduction, the composition of blood will be described followed by the description of a Newtonian fluid flowing in a cylindrical tube. RBC properties and interactions will be described, along with the non-newtonian behaviour of the blood they induce. The multi-step systems of leukocyte and platelet adhesion to the vessel wall will be described, along with the adhesion molecules and their ligands involved. Finally the effects of haemodynamic and rheological variables and their manipulation on the margination and adhesion of leukocytes and platelets will be considered.

1.1 Composition of blood

The blood consists of cellular components; erythrocytes (red blood cells; RBC), leukocytes (white blood cells; WBC), and platelets, that are suspended in the plasma. The main physical properties of these cellular components and the plasma will be described. Numerical data presented in this section was taken from (Bain, 1989;Blann, 2006).

1.1.1 Red blood cells

Red blood cells (RBC) make up around 99% of all cells by volume in the blood. There are on average 5 x 10^9 RBC/ml of blood. The percentage of the total blood volume made up by RBC is known as the haematocrit (HCT) and is normally between 40-45%, being lower in females than males on average. The physiological function of the RBC is gas transport and their properties are well adapted for this function. RBC have no nucleus or other intracellular organelles, but contain a concentrated viscous solution of haemoglobin which carries about 95% of oxygen in the blood. They have a viscoelastic membrane which incorporates a thin protein skeleton that maintains their biconcave disc shape with a diameter of 7-8μm (Nash and Gratzer, 1993). The flexible nature of the membrane, along with their unique shape (which has a high surface area-to-volume ratio) allows them to enter vessels with a diameter

as small as 3μm. Within these vessels the RBC are deformed into a parachute shape, where the regions of RBC membrane in contact with the vessel wall travel through the vessel behind the more axial regions. This is facilitated by the redistribution of the haemoglobin solution within the folded membrane of the cell (Gaehtgens, 1980). The shape also maximises the surface area for gaseous exchange.

Within the context of this project, RBC can affect the vessel wall interactions of the other cells in the blood. Variations in the rheological properties of RBC and their effects on the flow distributions and adhesion of leukocytes and platelets will be discussed in depth in sections 1.4 and 1.6.

1.1.2 Leukocytes

These are the least numerous cells in the blood with approximately 5 x 10^6 cells/ml, although the number can range between 4-10 x 10^6 /ml even in the healthy. There are 5 main types of leukocyte that can be placed into two groups according to their nuclear morphology (segmented or spherical/kidney-shaped) and cytoplasmic granularity. These main sub-groups are; polymorphonuclear granulocytes (neutrophils, basophils and eosinophils) and mononuclear cells (lymphocytes and monocytes). All leukocytes have the ability to adhere to and migrate through the vessel wall, as part of immune surveillance and response to infection.

1.1.2.1 Granulocytes

Granulocytes are so named due to the prominent granules within their cytoplasm. They also have characteristic multilobed nuclei. They are involved in the innate immunity and can migrate towards and phagocytose invading pathogens.

Neutrophils

In adults the neutrophil is the most numerous leukocyte with between 2-8 x 10^6 cells/ml. They make up around 60% of all circulating leukocytes. In their resting state they are spherical with a highly convoluted membrane and a diameter of 8-10μm. After adhesion to vascular endothelial cells that have expressed adhesion receptors in response to infection, the neutrophils migrate into the tissue space where they phagocytose and destroy invading pathogens. Neutrophils are the first line of defence in innate immunity. In the circulation a neutrophil will circulate between 4-10 hours (Bishop et al., 1971), and once it has migrated into the tissue it will undergo apoptosis and be engulfed by tissue macrophages upon phagocytosing the invading pathogen (Savill, 1997).

Basophils

Less than 1% of all leukocytes are basophils. They are involved in inflammatory, hypersensitivity and anaphylactic reactions. Their granules contain heparin, histamine and platelet activating factor.

Eosinophils

Eosinophils form about 3% of circulating leukocytes and typically have large granules and bi-lobed nuclei. Their main physiological function is defence against parasitic infestation and they are also implicated in allergic reactions. Eosinophils granules are a source of histamine and hence they contribute to the inflammatory process. They circulate in the blood stream for about 5 hours.

1.1.2.2 Mononuclear cells

Lymphocytes

Lymphocytes contribute around 30% of all circulating leukocytes and can be further classified into T and B-lymphocytes. The typical diameter of a lymphocyte is around 7-8μm. Lymphocytes have a large nucleus around which there is only a small amount of cytoplasm visible. Lymphocytes contribute to antigen-specific immunity. They allow the immune system to memorise specific pathogens and fight subsequent infections of the same pathogen more effectively. Some 'memory' lymphocytes therefore have a life span in the order of years. The majority of lymphocytes are trafficking continually through the lymphoid system with relatively few circulating in the blood.

Monocytes

Monocytes constitute around 5% of circulating leukocytes and have a diameter of between 10-12μm. They have a characteristic kidney shaped nucleus with a small number of discrete granules containing hydrolytic enzymes. Monocytes live for approximately 10 hours in the blood and once migrated into the tissue, they can mature into phagocytically active tissue macrophages or into antigen presenting dendritic cells.

In this thesis, whole blood was used in all studies, and no distinction was made between the different classifications of leukocyte when quantifying leukocyte adhesion.

1.1.3 Platelets

Platelets are the second most numerous cell type in the blood at around 250 x 10^6 cells/ml and can range from 100-400 x 10^6 cells/ml. However their contribution to total blood volume is small as these discoid cells are only 2-3μm in diameter and 1μm thick. The primary role of blood platelets is to stop bleeding at sites of vessel trauma, and hence to maintain blood

volume. They bind to components of the extracellular matrix exposed in the wall of damaged vessels, becoming activated, spreading and forming aggregates with other platelets. They have granules in their cytoplasm which contain adhesive proteins fibrinogen and P-selectin (Johnston et al., 1989).

1.1.4 Plasma

Plasma is the liquid medium in which all blood cells are circulated. It constitutes the remaining 55-60% of the blood volume depending on the haematocrit of the individual. At physiological temperature of 37°C plasma has a viscosity of around 1.2 mPa.s (compared to ~0.7mPa.s for water (Robert C.Weast, 1975)). The viscosity of the plasma is relatively constant among the healthy population, being governed by the concentration of proteins in the plasma. The most abundant proteins in the plasma are: albumin, which is used as a non-specific transporter of substances such as bilirubin; fibrinogen, that is important in blood coagulation; the immunoglobulins which are released into the blood by plasma cells and recognise foreign antigens. The plasma also contains extracellular cations, such as Na^{+}, K^{+}, Mg^{2+} and Ca^{2+}, with Mg^{2+} and Ca^{2+} being essential for the operation of several of the adhesion receptors required for leukocyte or platelet adhesion.

1.2 Flowing Newtonian fluids

To be able to understand the complex nature of flowing blood *in vivo*, the simplest case of tube flow must first be described. Some basic principles must also be established.

1.2.1 Laminar flow

Fluid flow over a surface can be thought of as a number of infinitesimally thin adjacent layers or laminae, flowing parallel over each other. Due to the no slip boundary condition, the lamina at the wall of the vessel has zero velocity. The velocity of the adjacent lamina increases with increasing distance from the wall (Goldsmith and Turitto, 1986).

1.2.2 Shear rate

Shear rate (γ) is defined as the gradient of velocity in a flowing fluid ($\gamma = \frac{dV}{dx}$, units = s^{-1}). It can be thought of as the change of velocity between adjacent laminae of fluid passing over each other divided by their separation. The velocity gradient at the wall is the wall shear rate (γ_w) which is notionally the velocity difference between the stationary lamina at the wall and the adjacent lamina, divided by their separation.

1.2.3 Shear stress

Shear stress (τ) is defined as the force acting per unit area between adjacent laminae (units=Pa). The shear stress at the wall (τ_w) is the force per unit area acting at the wall. It can be thought of as the drag force between laminae.

1.2.4 Viscosity

Viscosity can be defined as the internal resistance to flow, or friction between laminae in the flow. For a fluid, viscosity (η) is defined in terms of shear rate (γ) and shear stress (τ):

$$\eta = \frac{\tau}{\gamma} \qquad \textbf{Equation 1}$$

A Newtonian fluid has a viscosity that is independent of shear rate or stress. Particulate suspensions typically have viscosity that decreases as shear rate increases (i.e., non-newtonian, shear thinning).

1.2.5 Poiseuille Flow

The simplest model of blood flow is the steady, laminar flow of a newtonian fluid (which has a viscosity that is independent of flow conditions) through a non-distensible cylindrical tube with infinite length (no end effects). Fluid flow in a cylindrical tube can be described as consisting of a number of infinitesimally thin concentric cylinders (or laminae) that slide over each other. Due to the no slip boundary condition, the lamina at the wall of the vessel has zero velocity. The velocity of the adjacent lamina increases with increasing radial distance from the wall, becoming a maximum at the centre of the cylinder (Goldsmith and Turitto, 1986). This is sometimes known as Poiseuille flow.

For a newtonian fluid in a steady state (non-accelerating) flow in a rigid, straight tube of a known length (L) and radius (R), the force provided by the pressure gradient along the tube must be equal to the frictional forces exerted on the fluid.

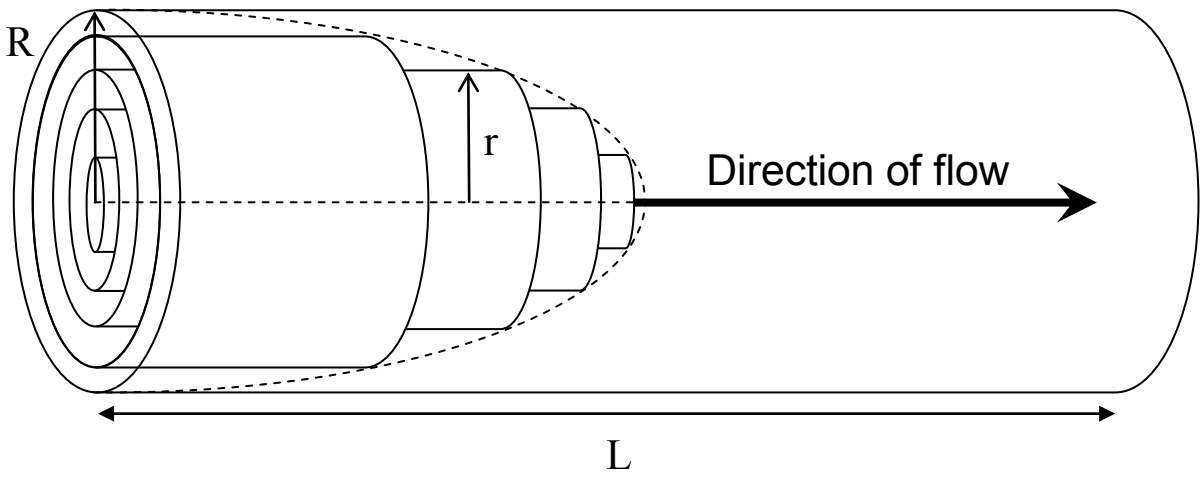


Figure 1.1 Laminar flow in a cylindrical vessel

If one considers a cylinder of radius r within the flow (fig 1.1), then:

$$\Delta P \pi . r^2 = \tau_r 2\pi . rL$$

where ΔP is the pressure drop over the length of the tube and τ_r is the shear stress at the surface of the cylinder of radius r within the flow.

The shear stress at any radial position in the tube can thus be expressed as

$$\tau_r = \frac{\Delta P . r}{2L} \qquad \textbf{Equation 2}$$

Combining equation 1 with equation 2 gives an expression for shear rate, where a minus sign is introduced because dv/dr is always negative with the origin at the centre of the vessel:

$$\frac{dv}{dr} = -\frac{\Delta P . r}{2\eta L} \qquad \textbf{Equation 3}$$

By integrating equation 3 with respect to r and applying the no-slip boundary condition (v=0 at r=R), the fluid velocity as a function of the radius r (V_r) can be derived, and the equation for parabolic flow is obtained:

$$\int_{V_r}^{0} dv = -\int_{r}^{R} \frac{\Delta \mathrm{Pr}}{2\eta L} . dr$$

$$V_r = \frac{\Delta P}{4\eta L}\left(R^2 - r^2\right) \qquad \textbf{Equation 4}$$

To calculate the volumetric flow rate (Q) in Poiseuille flow, where fluid velocity is a function of radial distance from the centre of the tube, one needs to integrate V_r multiplied by the area of the annulus (2πr.dr) over r.

$$Q = \int_0^R V_r .2\pi .r dr$$

$$Q = \frac{\Delta P \pi}{2\eta L} \int_0^R R^2 r - r^3 dr$$

$$Q = \frac{\Delta P \pi R^4}{8\eta L} \qquad \textbf{Equation 5}$$

This is the well known Poiseuille equation for the pressure-flow relation. Substituting equation 5 into the equation 2 and using equation 1 yields:

$$\tau_r = \frac{4Q\eta .r}{\pi R^4} \qquad \textbf{Equation 6}$$

$$\gamma_r = \frac{4Q.r}{\pi R^4} \qquad \textbf{Equation 7}$$

At the wall r =R so the wall shear rate and stress can be expressed as:

$$\tau_w = \frac{4Q\eta}{\pi R^3} \qquad \textbf{Equation 8}$$

$$\gamma_w = \frac{4Q}{\pi R^3} \qquad \textbf{Equation 9}$$

These parameters are particularly useful when considering the adhesion of cells flowing near the wall of a vessel (see below).

When considering a viscous Newtonian fluid flowing through a vessel, the development of the parabolic flow profile upon entering the vessel has to be considered. When entering the vessel, fluid is flowing with a uniform velocity across the vessel, known as plug flow. The vessel wall has an immediate effect on the fluid lamina closest to the vessel wall which is

stationary. The inner adjacent laminae are influenced by the outer laminae through viscous forces. As the fluid near the vessel wall is decclerated, the flow in the centre of the vessel is accelerated by the pressure difference across the vessel. The region of the flow near the vessel wall where the viscosity of the fluid alters the velocity profile within the vessel is known as the boundary layer (Shetz and Fuhs, 1999).

In laminar flow, the thickness of this boundary layer increases with distance from the entrance of the vessel due to the continued action of the frictional force due to the vessel wall acting on the fluid through its viscosity. At the point where the boundary layer reaches the centre of the vessel the flow is said to be fully developed and the velocity profile will be parabolic. The axial length required for flow to be fully developed is known as the entrance length (L_e) and in a cylindrical vessel is given by the equation:

$$L_e = 0.06d.R_e \qquad \textbf{Equation 10}$$

Where d is the diameter of the vessel and R_e is the Reynolds number of the fluid.

1.2.5.1 Adaptation of Poiseuille flow for vessels of rectangular cross section

These equations apply to a cylindrical vessel. In this thesis the experiments described will use glass capillaries (microslides) with a rectangular cross section, so the above equations need to be modified in order to provide relevant values of wall shear rate and stress.

The above method has to be applied to a rectangular fluid element of width w and height x within the rectangular vessel

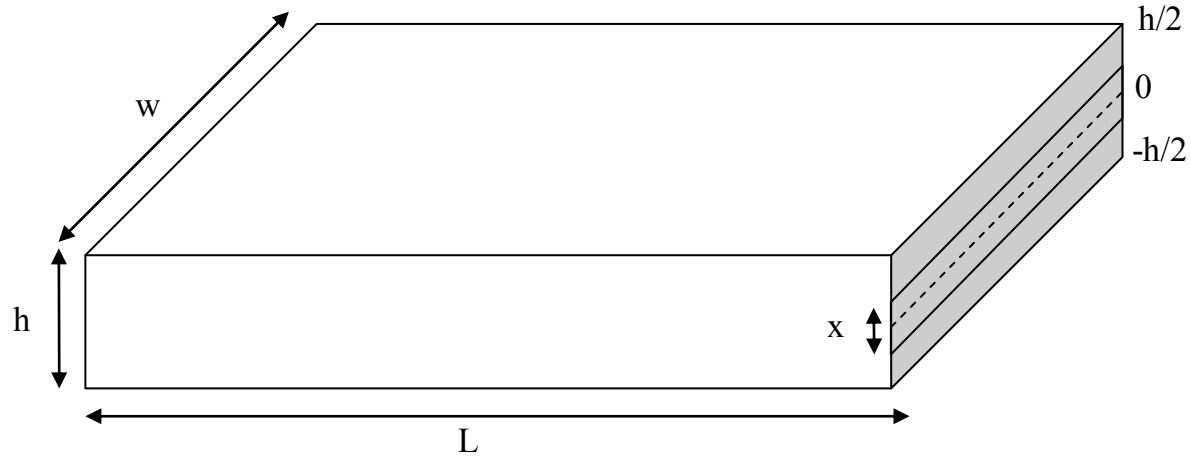


Figure 1.2 Schematic representation of a microslide

Within the derivation the approximation is first made that the shear stress is uniform around the surface of the element. This is linked with the assumption (see below) that w»h, so that effectively the drag on the side walls is negligible and ignored. Flow is thus eventually considered as between infinitely wide, parallel plates. Considering the fluid element of height x, either side of the centreline of the microslide.

$$\Delta P.x.w = \tau_x.2(w+x)L$$

$$\tau_x = \frac{\Delta P x w}{2(w+x)L}$$

Assuming w»x then the equation becomes:

$$\tau_x = \frac{\Delta P x}{2L} \qquad \textbf{Equation 11}$$

Substituting equation 1 into equation 11 results in an expression for shear rate:

$$\frac{dv}{dx} = -\frac{\Delta P x}{2\eta L} \qquad \textbf{Equation 12}$$

Following the equivalent steps for the cylindrical derivation, the equation for pressure-flow relation becomes:

$$Q = \frac{\Delta P w h^3}{12 \eta L}$$ **Equation 13**

Substituting equation 13 into the equation 11 and using equation 1 and applying the conditions that at the wall that x becomes h/2, the wall shear rate and stress in a rectangular vessel become:

$$\tau_w = \frac{6Q\eta}{wh^2}$$ **Equation 14**

$$\gamma_w = \frac{6Q}{wh^2}$$ **Equation 15**

As noted above, the equations neglect the contribution made by the side walls of the rectangular tube to the drag force, and describe flow in parallel plates of infinite width. However, within the microslides the side walls will affect the flow profile as the width is only 10 times the height. For experimental calculations of nominal wall shear rate and stress in the microslides equations 14 and 15 provide a good approximation. The studies of adhesion performed in this thesis analysed fields along the centreline of the vessel, to minimise deviations from the predicted flow parameters created by the side walls.

The entrance length within the microslides needed to be calculated to ensure that flow was fully developed in the regions where leukocyte and platelet behaviour would be analysed. Within the microslides, the entrance length (L_e) can be calculated using the equation (Shetz and Fuhs, 1999):

$$L_e = (0.25 + 0.015.R_e).D_h$$ **Equation 16**

Where R_e is the Reynolds number and D_h is the hydraulic radius of a rectangular vessel of cross sectional area (A) and perimeter of the cross section (P) and is given by:

$$D_h = \frac{4.A}{P} \qquad \textbf{Equation 17}$$

The maximum entrance lengths were calculated using equations 16 and 17 for the 100µm and 300µm microslides and were 3mm and 7mm respectively. Therefore fields were not observed near the inlet of the capillaries, where the flow may not have been fully developed.

1.2.5.2 Correction for side-wall effects in a rectangular vessel

A more accurate derivation of equations of flow in rectangular vessels has been described (White, 1991). The volumetric flow rate in tubes with rectangular cross section is given by the equation:

$$Q = \frac{\Delta P w h^3}{12\eta L}\left[1 - \frac{h}{w}\left(\frac{192}{\pi^5}\sum_{n=1,3,5}^{\infty}\frac{1}{n^5}\tanh\left(\frac{n\pi w}{2h}\right)\right)\right] \qquad \textbf{Equation 18}$$

Equation 18 will collapse to the equation for infinitely wide plates where w»h.

For the rectangular microslides used in this thesis the side wall affects the calculated viscosity of fluids flowing within the microslides. Substituting w=10h into equation 18 and re-arranging, the viscosity of a fluid flowing through a microslide is:

$$\eta = \frac{\Delta P}{Q}\frac{w h^3}{12 L}(0.9373)$$

For the adhesion and margination studies equation 15 was used to calculate the wall shear rates. As this assumed parabolic flow within the vessel, it was only an approximation. Blood tends to flow with a plug flow profile (see section 1.4.3), so that the actual shear rate at the

wall can be higher than expected (Tangelder et al., 1988). When fluid viscosity was to be measured, equation 13 would give an overestimation of viscosity even for a newtonian fluid, and so equation 18 was used in the calculation of viscosity from measured pressure differences.

1.3 Red blood cell properties influencing blood flow

Before attempting to explain the complex behaviour of flowing blood, it is necessary to introduce some concepts regarding the rheology of the red cells. Red cells may aggregate in blood and also have an intrinsic resistance to deformation that can affect blood flow and also influence the distribution and adhesion of leukocytes and platelets.

1.3.1 Red blood cell aggregation

RBC aggregation is a transient interaction between RBC resulting in the formation of rouleaux, which are tube-like aggregates of RBC stacked with the sides of largest area adjacent to each other (fig 1.3). The aggregation of RBC has been recognised as being dependent on the presence of fibrinogen and other macromolecules within the blood (Fahraeus, 1929). Reducing the fibrinogen concentration of the plasma decreases the propensity of the RBC to aggregate (Rampling and Sirs, 1972). Aggregation can also be artificially manipulated using dextrans of different molecular weights (Chien and Jan, 1973). At present there are two theories suggesting possible mechanisms of RBC aggregation. The bridging model and the depletion model (Baumler et al., 1996). There is evidence for both models resulting in rouleaux formation.

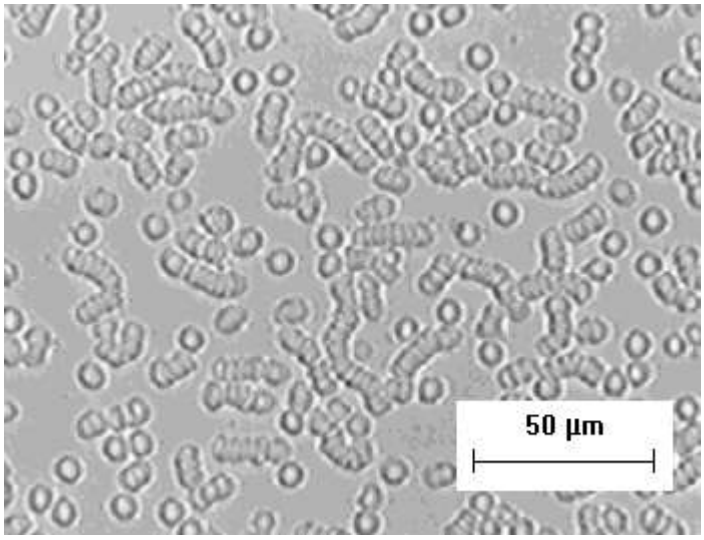


Figure 1.3 Red blood cell aggregation: Rouleaux formation in a thin film of stationary blood
From microscopic observations assessing RBC aggregation (see section 2.2.3).

1.3.1.1 Mechanisms of red cell aggregation

The effects of the neutrally charged macromolecule dextran of different molecular weights on RBC aggregation have been extensively studied. They were used in the studies leading to the conception of the bridging model for RBC aggregation (Chien and Jan, 1973). Using a molecular weight of up to 500kDa it was shown that the tendency to form aggregates was increased with increasing molecular weight of dextran. Together with electron microscopy it was demonstrated that the gaps between the RBC in a rouleau were parallel along the whole length of adjacent RBC. This gap increased in size as the molecular weight of the dextran molecule used increased. The gap created by Dx500 was ~30nm. It was suggested that the interaction of one macromolecule of dextran with 2 RBC (one at each end) needed to be strong enough and/or to provide a large enough spacing to overcome the electrostatic repulsion created by the negatively charged RBC, along with any mechanical and bending forces applied to the surfaces. To corroborate this model, the same group showed that using neurominidase to cleave the negatively charged sialic acid off the RBC membrane resulted in enhanced aggregation with all molecular weight dextrans used (20-80 kDa) (Jan and Chien,

1973). With normal RBC no aggregation was seen with addition of Dx20 as it was too short to overcome the repulsive electrostatic force. Once treated, the neutral RBC could come in much closer proximity to each other and therefore aggregation was increased with a low molecular weight dextran (Jan, 1979).

The depletion model suggests that an osmotic attractive force is present between RBC of close proximity due to a macromolecule depletion layer near the surface of the RBC (van Oss et al., 1990). There is a thin layer around each RBC that has a low concentration of macromolecules. When two surfaces are juxtaposed, the higher concentration of macromolecules outside the contact zone effectively provides an osmotic pressure holding the surfaces together. This model has been supported recently by Neu et al who showed that the propensity for RBC to aggregate was not linearly dependent on the molecular weight of the macromolecule used (Neu and Meiselman, 2002). Dx500 was found to be the optimal molecular weight dextran for inducing RBC aggregation in the range of 70-28000 kDa (Neu et al., 2008).

1.3.1.2 Aggregation in flowing blood

Within the blood the association of increased RBC aggregation with increased plasma fibrinogen levels (normal levels: 0.2-0.3g/100ml (Chien et al., 1970)) has been known for some time (Rampling and Sirs, 1972). In blood flow the degree of aggregation present in any given vessel is a result of the balance of the force of the transient aggregation between the RBC, which is dependent on fibrinogen concentration, and the fluid shear stress on the cells acting to disaggregate the RBC rouleaux. Rouleaux can form in three ways, by single RBC interactions, single RBC-rouleau interactions or rouleau-rouleau interactions (Barshtein et al., 2000). Various mathematical models have been used to describe the aggregation and disaggregation of RBC. Rouleau size is predicted to increase with time to an equilibrium

level, and this equilibrium size decreases with increasing shear rate (Murata and Secomb, 1988). This shear dependent disaggregation is not dependent on haematocrit (Snabre et al., 1987). Although RBC aggregates form over a wide range of surface adhesion energies (Skalak et al., 1981), at high shear the low contact time between RBC and the high stress experienced by any interaction formed results in no aggregates forming (Skalak and Zhu, 1990). Experimentally, it has been shown that RBC aggregation will occur in a region of any vessel below a shear rate of around $50s^{-1}$ (Goldsmith and Turitto, 1986). This suggests that aggregates may form near the centre of vessels even when wall shear rates are above those allowing aggregation.

1.3.1.3 RBC aggregation in disease

RBC aggregation can be altered in disease, where it is often increased due to an increase in plasma fibrinogen concentration (Korotaeva et al., 2007). In chronic hypertension RBC have an increased propensity to aggregate (Bogar, 2002). This increased propensity to aggregate has been shown to be a result of an increased plasma fibrinogen concentration (Blattler et al., 1979). Diabetes has been associated with the increased RBC aggregation (Babu and Singh, 2004). Plasma fibrinogen is increased and the RBC have a reduced surface charge reducing the repulsive force on the RBC in the blood and therefore increasing aggregation. RBC aggregation is also increased in sickle cell anaemia when compared to a healthy donors (Tripette et al., 2009) however this increase in aggregation is not due to an increase in fibrinogen levels as no difference was found between the two sets of donors.

1.3.2 Red blood cell deformability

The term RBC deformability represents the cell's response to external applied forces (Evans, 1989). It depends on a number intrinsic and extrinsic factors: the surface area to volume ratio, the geometry of the cell, the properties of the cytoplasm and the elastic and viscous properties of the cell membrane (Schmid-Schonbein and Gaehtgens, 1981). The ~8μm diameter biconcave disc shape of the RBC has a volume of ~90fl and a surface area of ~140μm^2. To contain 90fl inside a sphere the surface area would be ~97μm^2. The excess surface area enables the cell to deform at constant volume and surface area, and to transit the smallest capillaries (~3μm) and the thin slits in splenic sinosoids (Chien, 1987). This would not be possible if the RBC membrane and cytoplasm did not also have low resistance to deformation (see below). The mechanical properties not only govern the flow in capillaries but also in larger vessels. In a vessel with high shear rate the normal RBC will align with the flow and elongate in the direction of the flow. To maintain cell orientation in flow without tumbling, the shear stress applied to the membrane rotates it around the cytoplasm of the cell. This is known as tank-treading (Schmid-Schonbein and Gaehtgens, 1981;Fischer et al., 1978b). The extent of the elongation and deformation is dependent on the shear forces applied to the RBC, and on the viscous and elastic components of its structure (Chien et al., 1978). The RBC will absorb some energy at the onset of flow and dissipate energy once flow is ceased (Kumar, 1976)

The shape and viscoelastic properties of the red cell are maintained by the membrane protein skeleton, that comprises mainly of spectrin, actin, and protein 4.1 (Boivin, 1984). Spectrin dimers consisting of parallel α and β subunits form tetramers end to end with other dimers. These tetramers interact with actin and protein 4.1 at each end (Chabanel et al., 1989). The protein 4.1 modulates the spectrin-actin interaction. These interactions form a largely hexagonal lattice that underpins the lipid membrane. The underlying protein skeletal lattice is

maintained by a strong interaction of spectrin and ankyrin (Bennett, 1985). Ankyrin then forms a bridge with a transmembrane protein band 3, providing the main association of the lipid bilayer membrane with the underlying protein skeleton. The flexibility of the oligomeric structure of the cytoskeleton is dependent on these protein interactions, as a reduction in protein 4.1 has been shown to reduce the elastic modulus of the RBC (Waugh, 1983). Also protein cross linking agents such as diamide have been shown to increase the elastic modulus of RBC without altering size or shape (Chasis and Mohandas, 1986). The underlying protein skeleton alone has a lower elastic modulus than the membrane of a normal RBC (Svoboda et al., 1992). However the lipid layer of the membrane itself is intrinsically unstable and if the underlying protein skeleton is destroyed the membrane will spontaneously vesiculate (Elgsaeter et al., 1986). This shows that the association between skeleton and lipid is important in maintaining the correct membrane properties for normal function. It appears that any constraint of the lipid bilayer and its integral proteins to move freely over the spectrin skeleton has a major influence on the RBC deformability (Nash and Gratzer, 1993).

Deformation of the RBC membrane occurs in three independent ways. Dilation, extension (at constant area, or shearing) and bending (without dilation or extension) (Evans, 1989). The elastic moduli of these different forms of deformation are very different. The higher the elastic modulus, the larger the force required to deform the membrane in the particular mode of deformation. The elastic moduli for dilation, extension and bending are 0.03-0.05 N/m^2 (Evans and Waugh, 1977), 5-7 $x10^{-7}$ N/m^2 (Waugh and Evans, 1979;Nash and Meiselman, 1983) and 10^{-16} N/m^2 (Evans, 1983;Schneider et al., 1984) respectively. Thus the RBC membranes resistance to bending is small and it will usually deform at constant area because its resistance to dilation is great. The membrane behaves like a visco-elastic solid, as it will recover its shape once an applied force has been removed, but the rate of recovery depends on the membrane viscosity (Evans and La Celle, 1975).

The intracellular rheological properties of the RBC are dominated by the highly concentrated haemoglobin solution within the cell. As the RBC membrane is permeable to water, the RBC maintains its intracellular osmoliarity by regulating its intracellular cation concentration using Na^+/K^+ pumps. The Na^+/K^+ pump, transports $3Na^+$ ions out of the cell in exchange for $2K^+$ ions being transported into the cell. As the RBC membrane has a low permeable to Na^+ ions an osmotic gradient is created that counterbalances the gradient induced by the highly concentrated intracellular haemoglobin concentration and thus acts to maintain the volume of the RBC (Ney etal., 1990). At normal concentration of 32g/dl the viscosity of the intracellular fluid is around 7 mPa.s which is around 6 times more viscous than plasma alone (Chien, 1987). It has been found that older RBC in the circulation have a smaller volume and surface area than younger cells, but the surface area to volume ratio remains constant (Nash and Wyard, 1980). The older cells do have an increased haemoglobin concentration in the cytoplasm that could result in a decrease in deformability of the RBC due to an increase in viscosity to about 50mPa.s (Williams and Morris, 1980). This might increase the time taken to transit splenic sinusoids and therefore increase the probability of the older RBC being removed from the blood and phagocytosed.

1.3.2.1 Experimental manipulation of red cell deformability

There are a number of ways to manipulate the deformability of RBC experimentally. Altering the osmolality of the suspending medium can increase or decrease the volume of the RBC (Reinhart and Chien, 1985). Decreasing the osmolality of the suspending medium will increase the cell volume at constant membrane area resulting in a lower surface area to volume ratio (Linderkamp and Meiselman, 1982). Thus a reduction in medium osmolality results in a decreased filterability of the RBC through 2.6μm pores. Chlorpromazine has also been used as a shape changing agent for RBC. Agents which alter RBC shape, such as

chlorpromazine, reduce RBC deformability (Meiselman, 1978). Echinocytes are RBC that have become spherical with numerous spicules evenly distributed over their surface, this change in morphology can be induced by ATP-depletion. Stomatocytes are RBC that have a bowl shaped morphology with a single concavity and this shape change can be induced by chlorpromazine. Both echinocytes and stomatocytes have an increased resistance to membrane extension using the micro-pipette technique (Chabanel et al., 1987)

Ham reported on the effect of heat on RBC deformability; incubation at 49°C for 15min prevented the passage of RBC through 8μm pores (Ham et al., 1968). There was no shape change or volume change. This also resulted in an increased apparent viscosity at low shear rate. Rakow and Hochmuth (Rakow and Hochmuth, 1975) found that incubation of RBC for 6 minutes at 48.8°C or 47°C showed a significant increase in RBC rigidity, measured by a reduced elongation index. Nash found that heating RBC to 48°C increased the shear elastic modulus of the RBC membrane two-fold but only increased bulk viscosity by 12%, again with no shape change or volume change (Nash and Meiselman, 1985). The fixative glutaraldehyde has been shown to decrease RBC membrane deformability monotonically with time of incubation and concentration of glutaraldehyde in the incubation medium (between 0.05-0.25%) (Yee and Mel, 1978). Low concentrations of glutaraldehyde (0.0125%) have been shown to rigidify the RBC without shape or volume change (Sevick and Jain, 1991). The elastic modulus of the RBC membrane was gradually decreased as the concentration of glutaraldehyde in the incubation medium was increased (0.1-1.0%) (Reinhart et al., 1990). Diamide has also been used to decrease the deformability of RBC; using a transparent cone-plate viscometer the elongation index of diamide treated RBC decreased by 50% with the modification of just 5% of the membrane spectrin (Fischer et al., 1978a). Incubation of RBC with diamide has also shown to reduced the filterability of RBC suspensions (Stone et al., 1990).

1.3.2.2 RBC deformability in disease

The deformability of RBC can be altered if one or more of the factors mentioned above are modified in disease, e.g., through genetic modification or as a result of infection. In sickle cell disease, haemoglobin S solution within the RBC turns to a gel when deoxygenated, increasing the resistance of the cell to deformation and inducing gross shape-change or 'sickling' (Nash and Meiselman, 1985). There is also an accumulated increase in membrane rigidity arising from repeated sickling (Nash et al., 1988). The deformability of the RBC also decreases after infection with the malarial parasite Plasmodium falciparum (Nash et al., 1989;Cooke et al., 2004). These parasitised cells showed a reduction in their surface area to volume ratio and increase in membrane rigidity, which resulted in an increased pressure needed and time taken for the infected cells to enter 3μm tubes when compared to uninfected controls. RBC from diabetic patients have also demonstrated increased bending stiffness (McMillan et al., 1983) and loss of filterability (Symeonidis et al., 2001), which may be directly related to the degree of glycosylation of haemoglobin.

1.4 Characteristics of blood flow

Fluids that are particulate or emulsions, such as paint or blood, do not follow the behaviour of a newtonian fluid. They are described as non-newtonian as they exhibit viscosities that vary with shear rate. The particulate nature of blood causes its non-newtonian behaviour, since the plasma itself is a newtonian fluid with an average viscosity of 1.2mPa.s at 37°C (Robert C.Weast, 1975). Variations in the plasma viscosity affect the viscosity of the blood, but they do not directly influence the non-newtonian behaviour of the blood. The other main determinants of blood viscosity, which affect non-newtonian behaviour, are the RBC

concentration (haematocrit), the tendency of the RBC to form aggregates and the deformability of the RBC.

1.4.1 Viscosity of blood

In the circulation, the physical behaviour of the blood and its resistance to flow is affected by different factors depending on the vessel diameter. In capillaries the flow is mainly affected by the individual cells as they are of a comparable size to the diameter of the vessel and have to deform to enter, effectively one-by-one. The concept of fluid viscosity does not strictly apply. In large vessels (~1mm and above), blood behaves largely as a homogeneous fluid, its bulk viscosity is important, and properties of the blood such as red cell aggregation, deformability, haematocrit and the plasma viscosity all influence its apparent viscosity (Nash, 1991). In intermediate sized vessels (~100μm and below) the apparent viscosity of the blood decreases with decreasing vessel diameter (see section 1.4.2) due to a cell-free plasma layer at the periphery of the vessel becoming a larger proportion of the cross section of the smaller vessel. The term apparent viscosity is used when the viscosity is measured based on the Poiseuille equation, because in tube flow the shear rate and the cell distribution, and therefore the 'viscosity' are not constant across the tube. The apparent viscosity calculated is thus the viscosity of the newtonian fluid which would give the same pressure-flow ratio at the chosen flow rate. This apparent viscosity will, for example, typically decrease for blood as flow rate is increased. In rectangular capillaries, equation 18 (see page 14) can be used to determine the apparent viscosity in pressure-flow experiments.

1.4.1.1 Haematocrit

Using a couette viscometer, Chien showed that increasing the haematocrit from 20% to 60% resulted in a linear increase of the log of apparent viscosity (Chien et al., 1966). Studies assessing the apparent viscosity of blood in tube viscometers also showed that as haematocrit was increased the apparent viscosity of the blood was increased exponentially (Gupta and Seshadri, 1977;Haynes and Burton, 1959;McKay and Meiselman, 1989;Stadler et al., 1990).

1.4.1.2 Suspending phase viscosity

Plasma viscosity is highly dependent on the concentration of large proteins and macromolecules. As the concentrations of proteins such as albumin and fibrinogen increase, the plasma viscosity will increase. The presence of increased amounts of immunoglobulins in the blood can also increase the viscosity of the plasma. In certain disorders, where there is a high concentration of fibrinogen or globulins in the blood, the viscosity of the plasma will increase (Kesmarky et al., 2008). In particular Waldenstrom's syndrome and B cell myeloma (Waldenstrom, 1944) have reported high levels of plasma viscosity. Waldenstrom's syndrome is a plasma hyperviscosity syndrome that is caused by cancerous, bone marrow derived lymphocytes that over produce the antibody immunoglobulin M (IgM) which has a molecular mass of 900kDa. The over production of IgM results in an increased plasma viscosity, that can be upto five times the normal physiological level. In these studies the syndromes were associated with increased blood viscosity compared to a control at a specific haematocrit (Waldenstrom, 1944). Mannick showed that plasma viscosity was increased proportional to the concentration of globulins in the plasma (Mannik, 1974). Both Mannik (Mannik, 1974) and Schmid-Schonbein (Schmid-Schonbein et al., 1968) found aggregation of RBC to be present at over $100s^{-1}$, which is higher than the reported maximum shear rate at which rouleaux occur in normal blood. This could be attributed to the increased macromolecular concentrations in the plasma.

1.4.1.3 Shear rate and RBC aggregation

Studies of blood viscosity are typically performed at a range of shear rates. In cone-plate or couette rotational viscometers which apply uniform shear to blood samples, the viscosity of the blood increases as the shear rate experienced by the blood decreases (Merrill et al., 1963;Schmid-Schonbein and Wells, Jr., 1971;Chien, 1970). This increase in apparent viscosity may be due to a greater amount of RBC aggregation present at lower shear rates. At high shear rates the deformable RBC will tend to elongate and align with the flow which will tend to reduce the apparent viscosity of the blood. Increasing the RBC aggregation within the blood by adding fibrinogen or high molecular weight dextran, will increase the apparent viscosity of the blood at shear rates below $1s^{-1}$ (Chien et al., 1970;Chien et al., 1967b). At high shear rate, where RBC aggregation does not occur, the addition of fibrinogen or high molecular weight dextran had no effect on the apparent viscosity of the blood relative to its suspending phase viscosity. If the RBC are suspended in saline (Chien et al., 1967b) or serum (Chien et al., 1970), where aggregation is abolished due to the absence of fibrinogen, the steep increase in apparent viscosity seen at low shear rates does not occur.

In tube flow at low shear the measured apparent viscosity is orientation dependent; in horizontally perfused vessels at low shear the apparent viscosity will increase due to the increased propensity of the RBC to form aggregates and sediment (Reinke et al., 1987). However in vertically perfused tubes the apparent viscosity will decrease as the shear rate is decreased as a result of the increased inward migration of the larger RBC aggregates (Gaehtgens, 1987;Reinke et al., 1986;Palmer and Jedrzejczyk, 1975). In horizontal tubes, at low shear rates the apparent viscosity of whole blood increased due to a sedimentation effect (Alonso et al., 1989) that was exacerbated with time of flow (Alonso et al., 1995).

Thus, in summary the effects of shear rate and RBC aggregation on blood viscosity are inter-dependent and complex. Depending on how the viscosity is measured one may obtain different shear rate dependences. In rotational viscometers, a decrease in shear rate, or an increase in RBC aggregation at low shear rates will increase the apparent viscosity. Increasing the propensity for RBC aggregates to form may result in a sedimentation related increase in apparent viscosity in horizontally perfused, or a decrease in apparent viscosity in vertically perfused capillary viscometers.

1.4.1.4 RBC deformation

In a couette viscometer Chien showed that the viscosity of blood containing glutaraldehyde fixed RBC was greater than blood containing unfixed RBC at all shear rates at 45% haematocrit (Chien et al., 1967a). Blood containing normal RBC showed a reduction in the viscosity at higher shear rates, but fixed RBC suspensions showed no dependence on shear rate. Fixed RBC do not align with the flow or elongate at high shear as normal RBC do. This alignment acts to reduce viscosity. As hardened RBC do not deform, but rotate and tumble, the viscosity-reducing effects of the deformation of RBC are lost (Schmid-Schonbein et al., 1969). An increase in the apparent viscosity of blood cell suspensions with reduced RBC deformability was also reported in cone plate viscometers (Whitmore, 1981;Nash and Meiselman, 1985;Meiselman, 1981). Decreasing RBC deformability, either by shape change, osmotic volume increase or heat treatment, resulted in an increase in viscosity at all shear rates used when compared to unmodified deformability.

Chan reported that the apparent viscosity in 48μm diameter tubes increased as deformability of the RBC was reduced by heat treatment and further reduced by glutaraldehyde fixation (Chan et al., 1982). In another study, the effect of reduced deformability of RBC was measured in tubes of diameter ranging from 30-200μm (Seshadri et al., 1979). As the

deformability of the RBC was decreased, the apparent viscosity of the whole blood was increased. This effect became larger as the diameter of the tube decreased from 200μm to 30μm. It was suggested that the rigid RBC did not migrate towards the centre of the vessel as efficiently, resulting in a smaller plasma layer around the periphery of the RBC core.

1.4.2 Fahraeus and Fahraeus-Lindqvist effects

In smaller blood vessels of around 300μm and particularly at diameters less than 100μm, the increasing size of the peripheral plasma layer relative to the red cell core has a pronounced effect on blood flow. Robin Fahraeus, in 1929, observed that in capillary tubes of less than 100μm in diameter, the haematocrit of the flowing blood in the tube was less than that in the reservoir of blood feeding the perfusion. This is known as the Fahraeus effect (Fahraeus, 1929). This is explained by the axial RBC travelling more quickly than the marginal plasma, and so RBC are delivered more quickly than the liquid phase. The Fahraeus effect was increased by increasing the flow rate through a given vessel *in vitro* (Gaehtgens et al., 1978a).

The principle of the Fahraeus effect in reverse has been used to determine the average radial position of leukocytes (Goldsmith and Spain, 1984) and platelets (Goldsmith et al., 1989) in tube flow. An increase in the tubular concentration of leukocytes or platelets compared to the feed reservoir is taken as a measure of greater radial displacement (or margination) within the vessel (so that the leukocytes or platelets effectively travel slower than the red cells on average).

The Fahareus-Lindqvist effect (Fahraeus and Lindqvist, 1931) is the phenomenon that the apparent viscosity of blood flowing through a tube decreases as the tube diameter decreases. The apparent viscosity of blood with identical feed haematocrit decreased with decreasing vessel diameter down to a diameter of vessel comparable with the diameter of a RBC (8μm)

(Pries et al., 1992). The reduction in viscosity can be thought of as a lubricating effect of the peripheral plasma layer created by the movement of the blood cells towards the axial centre, or as due to the Fahraeus effect (reduction in haematocrit) in these tubes.

1.4.3 Blunted flow profile of flowing blood

In Poiseuille flow the velocity profile of a liquid flowing through a tube is parabolic. As blood is a non-newtonian particulate suspension, a parabolic flow profile is not always developed as the blood flows through a vessel. The main process that contributes to a deviation of the flow profile from parabolic is RBC aggregation, which is shear rate dependent. In tube flow, assuming a parabolic velocity profile, shear rate is highest at the wall of the vessel and is lowest at the central axis of the tube. In the central region of the vessel where the shear rate experienced by the cells is at its lowest, RBC aggregation is able to occur. Aggregation will make cells flow as a body rather than individual particles, and thus disturb the parabolic flow profile. The flow profile that is developed is a blunted, plug-like flow profile. For plug flow at a given volumetric flow rate (and hence average velocity) the maximal velocity reached by the flowing blood is decreased, but is maintained to a greater radial distance from the axis of the tube compared to parabolic flow (Gaehtgens et al., 1978b;Reinke et al., 1986). The plug-flow causes the reduction from maximal velocity to zero at the wall, to occur over a shorter distance, and so effectively increases the wall shear rate and the velocity of particles close to the wall. In arterioles, the wall shear rate was estimated to be twice that predicted for parabolic flow based on measurements of the velocity profile of fluorescent platelets (Tangelder et al., 1988). In blood perfused through glass capillaries, measurements of the changes in velocity of white cells at the wall as RBC haematocrit was increased yielded a similar estimate for the increase in wall shear rate caused by plug flow (Abbitt and Nash, 2003). While the effects of non-uniform flow on shear rate can be estimated from direct velocity measurements, effects on the wall shear stress are hard to

quantify, because the local viscosity is not defined (Gaehtgens et al., 1970). However estimates of average wall shear stress, or of relative changes in shear stress when blood properties are modified, can be calculated from the pressure drop along the vessel as this effectively represents the wall shear stress integrated over the vessel surface. Thus effectively, the measured apparent viscosity is also a measure of the relative wall shear stress.

1.5 Molecular basis of leukocyte and platelet adhesion

1.5.1 Leukocyte adhesion

In the inflammatory and immune responses leukocyte recruitment occurs in post-capillary venules and follows a series of well-defined steps: margination to the cell wall from free flow; capture from flow; rolling adhesion; stationary adhesion; migration on the endothelium and subsequent transmigration through the endothelium into tissue space (Springer, 1995). Different stages are mediated by specific adhesion molecules expressed on the leukocytes and endothelial cells lining the venules. All leukocytes undergo a similar process but the following is based mainly on the adhesive interactions of neutrophils. The steps underlying the adhesion of platelets are also similar, but involve different receptors, and platelet aggregation occurs rather than migration.

Margination occurs when leukocytes or platelets are displaced radially towards the vessel wall by the centrally-flowing red cells. The extent of this margination varies throughout the circulation and differs between leukocytes and platelets. The factors affecting the margination of leukocytes and platelets are discussed in section 1.6. The important point is that once marginated, the cells are in close proximity to the vessel wall and able to form adhesive bonds with it.

Specialised adhesion receptors are used to mediate different stages of leukocyte adhesion to the endothelium. The selectins, of which there are 3 types (P, E and L-Selectin; (Bevilacqua et al., 1989); (Johnston et al., 1989); (Lasky et al., 1989)), mediate capture from flow and rolling adhesion due to their high association and dissociation rates with their carbohydrate ligands presented on membrane glycoproteins. P-selectin is stored in Weibel-Palade bodies of endothelial cells and can be rapidly moved to the cell surface in response to inflammatory agonists. E selectin is produced by de novo synthesis and typically takes a few hours to be up-regulated after cytokine treatment of endothelial cells. L-selectin is found on leukocytes, and recognises endothelial ligands best described in lymphoid tissue. The selectins share a common structure; an N-terminal domain homologous to the Ca^{2+} dependent lectins, a short epidermal growth factor-like domain, short consensus repeats, a transmembrane domain and a short cytoplasmic tail. Once neutrophils are adjacent to the vessel wall they can be captured from free flow by bonds formed between selectin molecules on the endothelial cells and their carbohydrate ligands on the leukocyte. The endothelial up regulation of selectins to the luminal surface of the vessel can be induced by many agents such as cytokines (interferon γ, interleukins and tumour necrosis factor α) and various bacterial components (lipopolysaccharide or fMLP) ((Hattori et al., 1989); (Wyble et al., 1997)). The leukocytes roll along the wall of the vessel under the shear stress experienced as the bonds break quickly at the trailing edge of the cell but new bonds form at the leading edge. Rolling of leukocytes is important in slowing their velocity down from a free flow velocity of $mm.s^{-1}$ to a rolling velocity in the order of $\mu m.s^{-1}$ (Firrell and Lipowsky, 1989).

Stationary adhesion is mediated by leukocyte integrins. The integrins are a family of transmembrane glycoproteins. They are heterodimers consisting of a large α subunit in a non-covalent association with a β subunit (Springer, 1995). The integrins associated with leukocyte adhesion are constitutively expressed but must undergo a conformational change in

order to bind to their ligand (Diamond and Springer, 1993). The ligands for integrins include cellular adhesion molecules up-regulated on endothelium during inflammation, such as inter-cellular adhesion molecules (ICAM-1 and ICAM-2) (Nortamo et al., 1991).

The slow rolling leukocytes are in close proximity to the endothelium, allowing them to receive signals from activating agents expressed by the endothelium, such as the chemokine interleukin-8 (IL-8). Upon activation, stationary adhesion is rapidly induced via β_2 integrins interacting with their endothelial ligands. Leukocyte activation also causes a redistribution and increase in the content of F-Actin which leads to shape change and migration (Howard and Oresajo, 1985). The leukocytes migrate over the endothelium and transmigrate through endothelial cell layer into the tissue. To migrate, the leukocyte must continually form new integrin bonds at the leading edge of the cell and break bonds at the rear of the cell (Rochon et al., 2000). Transmigration of the leukocytes through the endothelial surface layer occurs at cellular junctions and preferentially at tri-cellular junctions (Gopalan et al., 2000).

1.5.2 Platelet adhesion

Platelets, unlike leukocytes, have the ability to be captured over the full range of physiological shear rates. Under high shear platelet recruitment from bulk flow is dependent on the interaction between von Willebrand Factor (which is itself bound to collagen on the damaged vessel wall) and the glycoprotein GP Ib-IX-V complex on the platelet surface. The bond formation between these molecules is very fast enabling them to form bonds at very high shear rate (Dopheide et al., 2001). The glycoprotein GP Ib-IX-V complex, a member of the leucine rich protein family, is a constitutively expressed platelet membrane receptor for von Willebrand Factor, a multimeric adhesive glycoprotein found in the plasma and in the extracellular matrix underlying endothelial cell lining of the vessel wall. Due to the fast association and dissociation of the vWF-GP Ib-IX-V interactions platelets may initially

translocate over the vWF matrix. Activation of members of the integrin family is required to mediate firm adhesion (Savage et al., 1996). The interaction of vWF and GP Ib-IX-V causes intracellular signalling that promotes the activation of integrins expressed on the platelet membrane. The main activator, however, is thought to be the interaction of platelet GPVI with collagen (Moroi et al., 1996). Binding of GpVI to collagen and vWF-GP Ib interaction can cause a conformational change in the integrin $\alpha_{IIb}\beta_3$, which mediates strong adhesion to vWF and fibrinogen (Ruggeri et al., 2006). Once bound and activated, platelets rapidly change from their discoid shape to a flattened form, extending long filopodia, and also secrete platelet-activating agents such as ADP or thromboxane. The formation of a platelet monolayer is followed by the addition (or aggregation) of platelets from the circulation. The activated integrins on two platelets can bind fibrinogen, a dimeric plasma protein, and thus become attached to each other. This platelet-platelet adhesion helps form platelet aggregates that contribute to thrombus formation on the damaged vessel wall.

1.5.3 Endothelial glycocalyx

Within the circulation, the endothelial cells luminally express a network of membrane-bound proteoglycans and glycoproteins referred to as the endothelial glycocalyx (Reitsma et al., 2007). The exact distance that the glycocalyx protrudes in to the blood vessels within the circulation is uncertain, the glycocalyx have been measured *in vitro* to be around 50-200nm (Rostgaard and Ovortrup, 1997) but may be as long as 500nm *in vivo* and vary in length within the circulation itself (Vink and Duling, 1996). Platelet adhesion is unlikely to be affected by the glycocalyx as it only occurs in regions where the vessel wall is damaged. Leukocyte adhesion however may be affected by the endothelial glycocalyx within post capillary venules. The P-selectin-PSGL-1 bond has been shown to bridge plasma membranes that are separated by 100nm. The micro-villi in the leukocyte membrane can protrude a further 300nm (Springer, 1995). Selectin ligands have been shown to be non-uniformly

distributed on the leukocyte membrane and show association with these micro-villi (Sasaki et., 1998). As the glycocalyx is estimated to have low stiffness (Weinbaum et al., 2003), it is likely that the leukocyte microvilli, expressing the selectin ligands, protrude relatively easily into the glycocalyx to interact with the selectins expressed on the endothelial surface and facilitate leukocyte adhesion to the vessel wall.

1.6 Effects of rheological variables on the margination and adhesion of leukocytes and platelets

To enable fast flowing leukocytes and platelets to adhere to blood vessel walls both cell types have specialised adhesion molecules expressed on their surface, which can rapidly bind to their receptors on the vessel wall. The key factors at this stage in adhesion are the margination of the cells (so they are close enough to allow binding to the vessel wall) and the shear rate and stress at the wall (which will influence the efficiency of the bond formation and survival). These factors are, in turn, influenced by the rheological properties of the blood (see below). Once a cell is travelling close to the vessel wall, its free flowing velocity is determined by the wall shear rate and the distance of the centre of the cell to the vessel wall (x) (Goldman et al., 1967).

$$v \approx \gamma_w . x \qquad \textbf{Equation 19}$$

As cell velocity increases, the time available for a bond to form is reduced. The velocity of the cell thus influences the probability of a bond forming between the receptor-ligand pair on the surfaces of the cell and the vessel wall.

The wall shear stress and surface area of the cell (of radius a), will determine the force experienced by an attached cell, and hence the formed adhesive bonds between the cell and the vessel wall (Goldman et al., 1967).

$$F \approx 32.\tau_w.a^2 \qquad \textbf{Equation 20}$$

The greater the force applied to the bond by the fluid flow the greater the likelihood or the rate at which the bond will break (Orsello et al., 2001).

The following sections review the effects of rheological variables on the adhesion and margination of leukocytes and platelets.

1.6.1 Particle size

In a simple, dilute suspension, cells will migrate away from the vessel wall towards the axis (Goldsmith and Turitto, 1986). The tendency for cells to migrate towards the vessel axis in tube flow is increased with increasing deformability of the cell, increasing flow rate and increasing cell diameter to tube diameter ratio. In the absence of RBC, rigid particles exhibit the tubular pinch effect, where the highest concentration of flowing cells is at 0.6 of the radius of the vessel from the centre (Segre and Silberberg, 1962). In blood, however, due to their relative sizes the different cellular elements migrate away from the wall at different rates. In decreasing order, the tendency of the constituents of the blood to migrate towards the centre of the vessel was RBC aggregates, leukocytes, single RBC and platelets (Palmer, 1967). The rate at which the separate constituents migrate will dictate their distribution within the vessel, and so, for example, platelets are likely to be more peripherally situated (or marginated) compared to the other constituents, and RBC aggregates to be most central.

One of the first experiments that focused on the margination of different sized particles in blood was performed by Phibbs and Dong (Phibbs and Dong, 1970). They perfused beads ranging from 7.5-80μm in diameter into the aorta of a rabbit and flash froze the femoral artery to assess the distribution of these different sized beads within this vessel. The larger spheres were more concentrated near the centre of the vessel, with the distribution becoming nearly uniform for the smaller microspheres (Phibbs and Dong, 1970).

To study margination *in vitro*, human blood at 15% haematocrit was perfused through rectangular vessels. Latex beads of different sizes were suspended in the blood. The term used in this experiment to quantify the lateral position of platelet-sized beads within the vessels was the near wall excess (NWE), which represents a higher concentration of beads at the vessel wall when compared to the central region of the vessel. At a shear rate of $1630s^{-1}$ no NWE was seen with 1.0μm beads but NWE was evident when beads with a diameter of 2.2μm and above were introduced into the blood. Beads of 2.2μm perfused in larger vessels showed a larger NWE when perfused at a wall shear rate (WSR) of $400s^{-1}$ compared to $50s^{-1}$. The higher NWE at higher WSR was taken to suggest that a lack of RBC aggregation and an increased alignment of RBC with the flow caused the platelets to be preferentially marginated. The smaller (1μm) beads were thought to have a very high diffusion coefficient and so to be able to diffuse uniformly across the radius of the vessel (Eckstein etal., 1988)

Margination of platelets is likely to be dependent on their size relative to red cells. Aarts et al studied this indirectly be analysing how altering the size of the RBC in the blood affected platelet adhesion (Aarts et al., 1983). Under controlled conditions, the change in adhesion was likely to be associated to a change in margination of the platelets. Human platelets were perfused over everted human umbilical artery subendothelium with increasing haematocrits of goat (mean cell volume (MCV) ≈ 25fl, cell diameter ≈ 3μm) rabbit (mean cell volume (MCV)

≈ 70fl, cell diameter ≈ 6μm) and human (mean cell volume (MCV) ≈ 95fl, cell diameter ≈ 8μm) RBC. The goat RBC showed no augmentation of platelet adherence at increasing haematocrit. Human RBC showed a linear dependence of platelet adherence with haematocrit. Rabbit RBC showed an intermediate response. Larger RBC (mean cell volume (MCV) ≈ 110fl, cell diameter ≈ 11μm) from newborn donors or patients with known macrocytosis showed a stronger dependence of platelet adhesion on haematocrit than normal human RBC. It was concluded that RBC size was an important factor in the development of a near wall excess of flowing platelets.

Apart from its effect on margination, cell size will affect the near-wall velocity of cells and the forces they experience, as noted above, and hence adhesion. Within the circulation the wall shear rate and stress vary over a wide range (wall shear rates of approximately 100 to 5000s^{-1} corresponding to wall shear stresses of around 0.2 to 10Pa) (Tangelder et al., 1988; Lipowsky,1988). Leukocyte adhesion is restricted to areas of low shear rate which are largely found in the post capillary venules. Platelets on the other hand can adhere on the arterial side of the circulation where the shear rates and stresses are considerably higher. This difference in adhesive behaviour of leukocytes and platelets is likely to be linked to their difference in size. The platelets are smaller and effectively travel closer to the vessel wall; this will reduce their near-wall flow velocity compared to leukocytes (by a factor of about 4). Their small size also reduces the force applied to the adhesive bonds formed (by a factor of about 16). One would thus predict that flowing platelets could adhere to the vessel wall over a much wider range of shear rates and stresses even though their adhesion receptors have similar intrinsic kinetics (Doggett et al., 2002).

1.6.2 Peripheral plasma layer

The peripheral plasma layer is a thin layer of plasma at the vessel wall that is present during blood flow. Its formation is a result of the tendency of RBC and their aggregates to migrate away from the vessel wall. This inward migration in RBC is opposed by cellular collisions that tend to push the cells towards the vessel wall (Bishop et al., 2001). Thus the width of the peripheral plasma is a result of the equilibrium between these forces and decreases as the haematocrit is increased (Kim et al., 2007).

The width of the cell free layer can be altered by the manipulation of rheological variables in the blood and by vessel size, but it is always between 2-6.5μm (Uijttewaal et al., 1993). It has been established that a reduction in shear rate is accompanied by an increase in the width of the plasma layer (Goldsmith and Spain, 1984;Reinke et al., 1987). This increase in the plasma layer width is due to the increased tendency of RBC aggregates to migrate across the streamlines towards the centre of the vessel. Increasing or decreasing RBC aggregation by adding dextran or suspending RBC in saline resulted in an increase or decrease in the width of the plasma layer respectively (Goldsmith et al., 1999).

The inward migration of RBC at high haematocrits is limited by the maximum packing density that can be achieved, and so the width of the plasma layer is expected to be smaller at higher haematocrit (Goldsmith and Spain, 1984). Indeed, it was shown that blood perfused through a 100μm tube at a haematocrit of 20% yielded a wider plasma layer than at a haematocrit of 40%.

In a study assessing the effect of increasing RBC rigidity on the flow behaviour of blood, tube haematocrit of blood containing heat treated RBC (heated at 50°C for 1 minute) was closer to the reservoir haematocrit than normal blood in tubes ranging in size from 30-200μm in

diameter (Seshadri et al., 1979). The effect of the heat treated RBC was decreased with increasing vessel diameter. The above study implied that, as the Fahraeus effect is due to the increasing proportion of the plasma layer in smaller vessels, increasing the rigidity of the RBC decreased the width of the plasma layer in these tubes.

Intravital observations in mesentery of the rabbit directly showed that the plasma layer increased with decreasing haematocrit, and decreased as RBC deformability was decreased (Tateishi et al., 1994). In the rat cremaster muscle, in vessels between 10-50μm, the width of the plasma layer increased as the diameter increased (Kim et al., 2007).

Although the cell free plasma layer was not directly measured in this thesis, the above findings may help explain how changes in the layer width could affect the margination and adhesion of both leukocytes and platelets when blood rheology was manipulated.

1.6.3 Shear rate

1.6.3.1 Effects on leukocyte margination

Initial *in vivo* observations of the tendency for leukocytes to be marginated in small vessels (<100μm) were made by Vejlens (Vejlens, 1938). He noted that a decrease in the shear rate within the post capillary venules resulted in a greater number of leukocytes being marginated towards the vessel wall. These observations were supported *in vitro* by measurements of the radial distribution of leukocytes in suspensions of red cell ghosts in plasma (Goldsmith and Spain, 1984). The distribution of the cells was measured at the medium plane of the vessels across the diameter, which varied between 100-180μm. At mean shear rates of over $100s^{-1}$ the leukocytes flowed centrally, but at mean shear rates of below $50s^{-1}$ there was a net outward migration of leukocytes towards the periphery.

Nobis et al perfused blood containing fluorescently labelled leukocytes through a vertically orientated glass tube of 69μm in diameter, and showed that as wall shear stress was increased from 0.1-2.5Pa, the position of the maximal radial concentration of leukocytes moved inward from around 5.8μm to 17.4μm from the vessel wall (Nobis et al., 1985). Very few cells were found at the centre of the vessel at the low shear stresses. Between 30-50% of all cells were found in the centre of the vessel above a wall shear stress of 2.0Pa. The outward movement with decreasing shear stress was abolished when saline was used as the suspending phase (i.e., when red blood cell aggregation was absent). This implied that the decrease in shear rate resulted in larger RBC aggregates displacing the leukocytes towards the periphery of the vessel as larger particles tend to flow more centrally (Phibbs and Dong, 1970).

The above suggests that decreasing shear rate in a vessel results in an increased proportion of marginated leukocytes in the presence of RBC, but only when red cell aggregation occurs. Rouleaux formation in the centre of the vessel, where shear rate is at a minimum, appears to facilitate the increased radial displacement of leukocytes towards the vessel wall, rather than changes in shear rate per se.

1.6.3.2 Effects on platelet margination

As direct observation of platelet margination is difficult, a method was devised to deduce the radial position of platelets within tube flow by comparing the count in the tube with the feed reservoir count. The flowing blood perfused within the vessel was collected (by blocking one end of the tube and stopping perfusion immediately) and the cell numbers counted. An increase of platelet count in the tube relative to the reservoir was labelled the reverse Fahraeus effect and signified an increase in margination (Goldsmith et al., 1989). Other direct studies

on platelet margination added fluorescent platelets sized beads to blood. The distribution of these beads within the flow was taken to replicate that of native platelets.

Beck and Eckstein used the reverse Fahraeus effect for platelets to assess the effect of shear rate on platelet distribution within flowing blood (Beck, Jr. and Eckstein, 1980). The tubular concentration of flowing platelets increased with shear rate up to a wall shear rate of $500s^{-1}$ and remained constant from 500 up to $10{,}000s^{-1}$. In tubes of internal diameter 90-210μm it was shown that tubular concentration of platelets was greater than reservoir concentration at all shear rates tested ($80\text{-}8000s^{-1}$) with the maximal increase at shear rates of around $800s^{-1}$ for all vessel sizes (Corattiyl and Eckstein, 1986). This study showed again at low shear rate that the tubular platelet concentration increased with respect to the reservoir concentration as shear rate was increased. At wall shear rates higher than $800s^{-1}$, in contrast to the study performed by Beck and Eckstein, the results showed a decrease in the platelet tubular concentration. The results from the two studies above suggest that platelet margination increases to a maximum value at a wall shear rate of around $800s^{-1}$ and increasing the wall shear rate above this results in no further increase in platelet margination.

Unlike leukocyte margination, platelet concentration near the vessel wall was increased in flowing blood in non-aggregating suspensions (Tilles and Eckstein, 1987). The number of fluorescent platelet-sized beads was counted every micron as the focal plane was moved into the vessel from the wall. Wall shear rates were varied from $50\text{-}3180s^{-1}$; there was no significant near wall excess of platelet-sized beads up to a wall shear rate of $430s^{-1}$, however at all wall shear rates above this a significant near wall excess was present. This near wall excess showed no shear rate dependence above $430s^{-1}$ (Tilles and Eckstein, 1987). In a similar system, human blood at 15% haematocrit was perfused through rectangular vessels (Eckstein et al., 1988). Platelet-sized beads of 2.2μm perfused in these vessels showed a near

wall excess of 2.5 times the concentration found in the centre of the vessel at $50s^{-1}$, 7 times at $210s^{-1}$ and 8 times at $400s^{-1}$. The higher near wall excess at higher flow rates suggested that a lack of RBC aggregation and an alignment of RBC with the flow caused the platelets to be preferentially marginated (Eckstein et al., 1988).

Using liquid nitrogen to freeze a tube during blood perfusion and sectioning the frozen tube, it was found that increasing wall shear rate ($250\text{-}1220s^{-1}$) resulted in a significant change in platelet margination (Yeh and Eckstein, 1994). Platelet margination was more pronounced at $560s^{-1}$ than it was at $250s^{-1}$ or $1220s^{-1}$. Over a similar range of shear rates, it was shown that increasing shear rate from $240\text{-}1260s^{-1}$ increased the concentration of fluorescently-labelled platelets flowing close to the wall in 3mm tubes (Aarts et al., 1988). In long polyethylene tubes of 200μm diameter, platelet margination in flowing blood was increased as shear rate was increased from $100\text{-}800s^{-1}$ (Uijttewaal et al., 1993).

The *in vitro* studies have been verified *in vivo*. It was shown by Tangelder et al (Tangelder et al., 1985) that, in the rabbit mesenteric arteriole, the distribution of platelets labelled with acridine red was non-uniform. On average, when the vessel was divided up into 6 regions across the diameter, the platelet count in the region near the wall was twice that found in the central regions of the vessel. The wall shear rate in these arterioles was comparable to the shear rates used in the above *in vitro* studies, in the order of $1000s^{-1}$. In other studies, platelet density near the wall was found to be significantly higher in arterioles than in venules of similar diameter (Woldhuis et al., 1992).

In summary, platelet margination is increased as shear rate is increased but occurs at all shear rates. The high shear rates are expected to result in the RBC flowing singly, aligning with

flow in the vessel and moving inward. As a result there might be more lateral displacement of the platelets.

1.6.3.3 Effects on leukocyte adhesion

Atherton and Born (Atherton and Born, 1973) first showed that the free-flowing blood velocity was much greater than the velocity of granulocytes rolling at the vessel wall in hamster cheek pouch venules. The rolling velocity of these granulocytes showed a linear increase with the mean blood flow velocity in different vessels. Since these observations were made, other *in vivo* studies have been performed and have assessed the role of shear rate on the adhesive characteristics of leukocytes. Reducing the shear rate (800-50s^{-1}) in mesenteric venules (diameter 20-60μm) of a rat, by occlusion with a blunt probe, led to a non-linear increase in the percentage of rolling cells in these venules (Firrell and Lipowsky, 1989). Leukocyte rolling velocity was independent of the size of the vessel and the RBC velocity in these vessels in this study. Two studies from a different group showed an increase in the number of adherent rolling leukocytes as shear rate was decreased in the mesenteric venules of a cat (diameter 25-40μm) (Bienvenu and Granger, 1993;Perry and Granger, 1991). In these studies the wall shear rate was decreased by tightening an adjustable screw clamp on the arterial side of the venules. Perry and Granger (Perry and Granger, 1991) also showed that a reduction in the shear rate resulted in a decrease in the adherent leukocyte rolling velocity.

In rat mesenteric venules, the wall shear rate was altered between 30-2000s^{-1} by occlusion of upstream branches (Ley and Gaehtgens, 1991). At less than 100s^{-1}, around 47% of all leukocytes flowing through the venules became adherent, at 400s^{-1} this had dropped to 24%, showing that as the shear rate increased the capture of leukocytes decreased. The rolling velocity of these leukocytes increased as wall shear rate was increased.

In vitro, most of the work on assessing the effect of shear rate on leukocyte adhesion has been done in isolated leukocyte suspensions. Lawrence et al performed two similar studies assessing neutrophil adhesion to cytokine-activated human umbilical vein endothelial cells (HUVEC) at various shear rates using a parallel plate flow chamber (Lawrence et al., 1987;Lawrence et al., 1990). Both studies showed that as shear rate was increased from 280 to $430s^{-1}$ the number of adherent neutrophils decreases by around a factor of 30. The earlier study (Lawrence et al., 1987) showed that as shear stress was increased the rolling velocity of the neutrophils also increased.

Lawrence and Springer used selectins coating a parallel plate flow chamber to assess effects of wall shear rate on leukocyte adhesion (Lawrence and Springer, 1991;Lawrence and Springer, 1993). The number of rolling neutrophils bound to a planar lipid bi-layer containing P-selectin decreased dramatically as the suspension was perfused at increasing wall shear stresses from 0.03Pa to a maximum of 0.35Pa (equivalent to shear rates of approximately 30-350 s^{-1} at room temperature) where no rolling leukocytes were seen (Lawrence and Springer, 1991). The rolling velocity of the adherent leukocytes increased approximately 3 fold as the shear stress was increased from 0.03 to 0.35Pa. When E-selectin at different concentrations was used as the adhesive substrate, again an increase in shear stress resulted in a decrease in the rolling adhesion of neutrophils on all E-selectin concentrations. Adhesion was essentially absent by a shear stress of 0.4 Pa (Lawrence and Springer, 1993). There was also an increase in the rolling velocity of the adherent neutrophils as shear stress was increased. However, the rolling velocity was reduced as the concentration of E-selectin was increased.

The effect of increasing shear rate on the adhesive behaviour of leukocytes in whole blood has only been studied *in vitro* once (Abbitt and Nash, 2001). The number of leukocytes adhering to a rectangular glass capillary (0.3 x 3mm internal dimensions) coated with P-Selectin

decreased as the wall shear rate was increased, and was nearly absent by a shear rate of $280s^{-1}$. This limit of shear rate was similar to that found in the studies using isolated leukocyte suspensions. The rolling velocity of the adherent cells was not significantly affected by the changes in shear rate, which disagreed with the *in vivo* data and the isolated suspension data *in vitro*.

The above data demonstrate that increasing the shear rate results in a reduction in the adhesion of leukocytes *in vivo* and *in vitro*. The rolling velocity of the adherent leukocyte *in vivo* is increased at higher shear rates, and this is also the case *in vitro* when isolated leukocyte suspensions are perfused over HUVEC or immobilised selectins. The only *in vitro* study using whole blood however showed no shear rate-dependence on rolling velocity. The *in vitro* studies used chambers much deeper than the diameter of venules, and more work needs to be done *in vitro* using whole blood in vessels of a size closer to venules.

1.6.3.4 Effects on platelet adhesion

The effect of shear rate on the adhesion of platelets has been investigated both *in vivo* and *in vitro*. Rabbit aorta subendothelium was exposed to reconstituted human blood in an annular perfusion system (Baumgartner, 1973). All shear rates allowed the eventual 100% coverage of the subendothelium, but at shear rates of $800s^{-1}$ and lower, the time taken to achieves 100% coverage increased as shear rate was decreased (Turitto et al., 1980). Platelet adhesion (percentage coverage) increased with increasing wall shear rate after 5 or 10 minutes exposure to shear rates between 50-$800s^{-1}$ but between 800 and $2600s^{-1}$ adhesion remained constant (Weiss et al., 1986). Results in this study were not corrected for the increased blood flow and hence platelet flux at higher shear rate. If one applies a simple correction for the amount of blood delivered, then based on this experiment, platelet adhesion (per ml) would decrease with increasing shear rate over the whole range used.

When blood containing prostaglandin E1 (PGE_1) to inhibit platelet activation was perfused over purified von Willebrand Factor (vWF) or fibrinogen, strong but different shear rate-dependence of platelet adhesion was found (Savage et al., 1996). Glass cover slips were coated and inserted into a perfusion chamber. The number of platelets adhered to the vWF increased with increasing shear rate but the increase in adhesion began to plateau between $920s^{-1}$ and $1500s^{-1}$. However, when fibrinogen was used as the adhesive substrate, as the shear rate was increased from 50 to $1500s^{-1}$ the number of adherent platelets decreased monotonically to almost zero. Zaidi et al (Zaidi et al., 1996) also demonstrated a steady decrease in platelet adhesion to fibrinogen as shear rate was increased from 250 to $2000s^{-1}$. Moroi et al also showed that when whole blood was perfused through a chamber coated with type III bovine collagen, platelet coverage increased with increasing shear rate (Moroi et al., 1996). As shear rate was increased from 200-$3200s^{-1}$ the percentage coverage in the first 100 seconds was increased from around 2.5% to around 17.5%. The adhesion to different types of collagen was investigated in a parallel plate flow chamber at various shear rates (Ross et al., 1995). As with collagen type III, collagen type I demonstrated an increasing percentage coverage of platelets as the shear rate was increased between 100-$1000s^{-1}$. However, with type VI collagen, platelet coverage decreased as shear rate was increased from 100-$1000s^{-1}$.

None of the above results were corrected for platelet flux and as such the adhesion results may be misleading, if they are taken to suggest that an increase in the intrinsic ability to adhere occurs with increasing shear rate or stress. The adhesion of platelets to vWF and collagen types I, III and IV seem to increase with increasing shear rate, but if the adhesion results are corrected for the delivery of platelets, the adhesion actually stays relatively constant until shear rates of around $1000s^{-1}$. It then drops off as coverage doesn't increase with shear rate but platelet flux does. In the case of fibrinogen and collagen type VI, the

adhesion drops with increasing shear rate even without the correction, and so is clearly highly sensitive to shear.

1.6.3.5 Summary

Studies have shown a distinct difference in the effect of increasing shear rate on the margination and adhesion of leukocytes and platelets. Leukocyte margination and adhesion have both been shown to decrease monotonically with increasing shear rate, and adhesion occurs up to about $300s^{-1}$ *in vitro*. Platelet margination has been shown to increase up to shear rates of around $1000s^{-1}$ and to be relatively constant at even higher shear rates. Platelet adhesion on collagen and vWF is probably nearly constant with increasing shear rate up to around $1000s^{-1}$ once one corrects for platelet flux, although this has not been formally shown. At higher shear rates adhesion becomes less efficient. The outcome of increasing shear rate for either cell type must represent some combination of the effects on margination and on the adhesion process itself. One must bear in mind also that at a given shear rate or stress, the velocities of platelets and forces acting on them at the wall will be lower than for leukocytes. Overall, platelets can adhere to the vessel wall over a much wider range of shear rates than leukocytes, and e.g., *in vivo* leukocyte adhesion was possible up to a WSR of around $300s^{-1}$ but platelet adhesion could occur at the maximum shear rates measured in arterioles *in vivo* (~$5000s^{-1}$ (Tangelder et al., 1988)).

1.6.4 Suspending phase viscosity

The viscosity of the blood plasma has rarely been considered as a factor affecting the margination and adhesion of leukocytes and platelets. The viscosity of blood plasma *in vivo* is governed by the concentration of macromolecules within the plasma such as fibrinogen or globulins. The concentration of these macromolecules, and thus the plasma viscosity, can be

increased in times of infection or in some cancers and myelomas. The obvious effect of increasing plasma viscosity is to increase blood viscosity and the shear stresses experienced by all blood cells at a given shear rate. This will result in higher forces on adhesive bonds formed at the vessel walls. The increased plasma viscosity will increase the shear forces experienced by RBC, and could act to break up rouleaux and increase elongation and alignment with the flow. Increased plasma viscosity might also decrease the rate of lateral redistribution of all cells within a given vessel. While these factors could modify the margination and adhesion of leukocytes and platelets in principle, literature on the subject is sparse.

1.6.4.1 Effects on platelet margination

The effect of changing plasma viscosity on the distribution of platelet-sized beads has been studied in channels of 50μm in height. Blood at 15% haematocrit was perfused at a wall shear rate of $210s^{-1}$. Increasing the plasma viscosity from 1.2mPa.s to 3.9mPa.s by adding dextran (molecular weight 15-21kDa) resulted in a higher number of platelet-sized beads flowing within 5μm from the vessel wall. The distribution of platelet-sized beads was more uniform across the vessel at the lower plasma viscosity (Eckstein et al., 1988).

1.6.4.2 Effects on leukocyte adhesion

The effect of increasing the suspending medium viscosity on leukocyte adhesion has only been studied for isolated cells. Not surprisingly, for constant shear rate, increasing the viscosity caused fewer flowing neutrophils to bind to P-selectin, presumably because of the increase in wall shear stress (Nash et al., 2001). Chen and Springer showed that, in isolated neutrophil suspensions perfused across P-selectin in a parallel plate flow chamber, increasing

the medium viscosity decreased the bond survival time effectively decreasing neutrophil adhesion (Chen and Springer, 2001).

1.6.4.3 Effects on platelet adhesion

A study investigating the effect of plasma viscosity on platelet adhesion in whole blood was performed by van Breugel using human serum albumin to increase the viscosity of the plasma (van Breugel et al., 1992). Reconstituted blood using glutaraldehyde fixed RBC (to abolish any effect that increasing plasma viscosity would have on RBC deformation) was perfused at 40% haematocrit and a shear rate of $300s^{-1}$over a cover slip coated with endothelial cell matrix pre-incubated with von Willebrand factor. The percentage coverage of platelets decreased over the range of plasma viscosity from 0.89mPa.s to 2.00mPa.s.

1.6.4.4 Summary

The literature suggests that increasing plasma viscosity at low shear rate increases the NWE of platelet-sized beads, although this was tested at a low haematocrit of 15%. Also the adhesion of platelets to endothelial matrix decreased with increasing plasma viscosity but the range of plasma viscosities used was limited. Suspending phase viscosity affects leukocyte adhesion in isolated suspensions, but equivalent studies have not been done with blood. This thesis will analyse how increasing the plasma viscosity in the blood influences the margination and adhesion of both leukocytes and platelets in the same vessels. A wide range of viscosities will be used, since in diseases such as Waldenstrom's syndrome plasma viscosity can be 5 times the normal value (Waldenstrom, 1944).

1.6.5 RBC aggregation

1.6.5.1 Effects on leukocyte margination

In early *in vitro* studies the addition of high molecular weight dextran to enhance RBC aggregation resulted in leukocytes flowing more peripherally in vertically orientated glass tubes of diameter 30μm, when compared to blood samples without dextran (Palmer, 1967). During vertical perfusion of blood containing labelled leukocytes in 69μm tubes, abolishing RBC aggregation by using saline as the suspending medium increased the centreline concentration of leukocytes at all shear rates (Nobis et al., 1985). When a 1% solution of high molecular weight dextran was used to enhance RBC aggregation almost no leukocytes were seen at the centre and a large number were found in peripheral flow regions (Nobis et al., 1985). In vertically-perfused tubes Goldsmith and Spain showed that when red cells were suspended in 0.35% bovine serum albumin in phosphate buffered saline (PBSA) (Goldsmith and Spain, 1984) the increase in tubular leukocyte concentration found with decreasing shear rate was abolished. The ratio of tubular leukocyte concentration to that of the reservoir (Fahraeus effect for leukocytes) at high shear rate ($465s^{-1}$) was no different to that at low shear rate ($34s^{-1}$). At similar haematocrits and flow rates in un-manipulated blood there was a significant increase in the tube to reservoir ratio of leukocytes at the lower flow rate. This showed that abolishing RBC aggregation reduced the effect of flow rate on the margination of leukocytes.

Recent *in vitro* studies of leukocyte margination in blood perfused through rectangular glass capillaries showed that the addition of high molecular weight dextran, to enhance RBC aggregation, increased the number of free flowing cells near the wall (Abbitt and Nash, 2003). However there was no significant difference in margination between plasma and low molecular weight dextran (which abolished aggregation whilst keeping plasma viscosity

constant). The measured velocity of leukocytes near the wall did not change under the different manipulations.

1.6.5.2 Effects on platelet margination

When high molecular weight dextran was infused into rabbits, the concentration of platelets near the wall of mesenteric arterioles decreased and the number of those flowing more centrally increased (Woldhuis et al., 1993). Infusion of low molecular weight dextrans had no effect on the distribution in arterioles. In venules, it increased near-wall platelet concentration and reduced central flowing concentration. The shear rates in the arterioles are higher than in venules and thus the effect of reducing aggregation in arterioles might be expected to be smaller. In venules, where shear rates are lower and RBC aggregation is more prevalent in the axial region, the addition of low molecular weight dextrans might have a more pronounced effect. Thus in venules, the axial region where RBC aggregation occurs would be abolished upon addition of the low molecular weight dextran. Although the authors did not consider that changes in red cell aggregation explained their results, no other clear mechanism was suggested.

1.6.5.3 Effects on leukocyte adhesion

Direct studies measuring the effect of RBC aggregation on the adhesion of leukocytes have been sparse. *In vivo*, aggregation can be modified by the injection of high or low molecular weight dextrans (Pearson and Lipowsky, 2000). Increasing or decreasing red blood cell aggregation in this way resulted in an increase or decrease respectively in the proportion of rolling leukocytes in the rabbit mesentery, but this effect was only evident at artificially reduced shear rates (below $350s^{-1}$). Pearson and Lipowsky showed that increased RBC aggregation resulted in at least a 4 fold increase in the number of rolling leukocytes at a wall

shear rate of less than $100s^{-1}$. The addition of low molecular weight dextran to reduce RBC aggregation resulted in half the rolling leukocyte flux when compared to normally aggregated RBC at around $100s^{-1}$. Thus, at low shear rate, enhancement of the RBC propensity to aggregate promoted the adhesion of leukocytes; this may have been due to increased margination within the flow. However this cannot be said with certainty because rolling and margination were not independently measured.

In vitro, the effects of RBC aggregation on adhesion were quantified using vertically perfused tubes coated with P-selectin (Abbitt and Nash, 2003). At 20% haematocrit, increasing RBC aggregation above that found in native plasma was associated with increased adhesion, while inhibiting RBC aggregation was associated with a reduction in adhesion.

The effects of RBC aggregation on leukocyte adhesion may reflect the effects on leukocyte margination, although effects on the width of the plasma layer may also contribute. In general, leukocyte adhesion and margination are augmented when RBC aggregation is increased. Only one study has assessed the effect of RBC aggregation on the margination and adhesion of leukocytes in the same system (Abbitt and Nash, 2003).

1.6.5.4 Effects on platelet adhesion

There are no direct studies measuring the effect of the manipulation of RBC aggregation on platelet adhesion to our knowledge.

1.6.5.5 Summary

As with shear rate, the results above indicate different effects of RBC aggregation on leukocyte and platelet margination, and possibly also adhesion, although this has not been studied. Increasing RBC aggregation artificially, independently of shear rate, results in the inward migration of the RBC aggregates as they form. The experiments outlined above demonstrated that the margination of leukocytes was highly dependent on the aggregation of RBC. Free flowing leukocytes flowed more peripherally as RBC aggregation was increased. A reduction in near wall leukocyte concentration was seen when RBC aggregation was abolished. This effect was more pronounced at low shear rates and as shear rate and RBC aggregation are linked it may be hard to distinguish the effects of these two variables in whole blood. RBC aggregation has a similar effect on leukocyte adhesion to the vessel wall as it does on leukocyte margination. The limited studies of the effect of RBC aggregation on platelet margination suggest that increased levels of aggregation inhibit platelet margination. However, it is difficult to be certain. Specific studies into platelet margination need to be made to clarify the role of RBC aggregation.

This thesis will study the effects of shear rate and of RBC aggregation on the margination and adhesion of both leukocytes and platelets using microslides of the same dimensions. It will verify or otherwise the earlier leukocyte data and show for the first time, *in vitro*, how the margination and adhesion of platelets is affected by manipulations in RBC aggregation. These results will be associated with tube viscometry in the same system to gain insight into how non-newtonian behaviour of blood and wall shear stress is affected.

1.6.6 Haematocrit

1.6.6.1 Effects on leukocyte margination

To investigate the initiation of margination, Bagge et al (Bagge et al., 1983) created a stenosis of 10μm diameter in 50μm glass tubes. When a suspension of leukocytes was perfused through the stenosed tube they remained close to the centre of the vessel, after passing through the stenosis. In the presence of RBC the distribution of leukocytes changed. The interactions with RBC redistributed the leukocytes from the centreline of the flow to a more peripheral position. In these small tubes, blood cell suspensions at 10% haematocrit behaved similarly to 40%, with the latter being only slightly more efficient in displacing the leukocytes.

In whole blood, leukocytes had a tendency to migrate outwards towards the vessel wall at low shear rates. Increasing the haematocrit from 20-60% resulted in no significant change in leukocyte tubular concentration when compared to the reservoir count, however at the lower shear rate used (~$50s^{-1}$) the tubular count increased above the reservoir count which was attributable to the leukocytes flowing more peripherally (Goldsmith and Spain, 1984). In rectangular vessels of height 300μm the margination of leukocytes at haematocrits of 10-50% was measured by direct observation at the vessel wall (Abbitt and Nash, 2003). Here, increasing the haematocrit did not significantly affect the number of free flowing leukocytes at the vessel wall, although it strongly increased their flow velocity, indicating that plug flow developed progressively.

In the experiments outlined above, the addition of the quite low concentrations of RBC resulted in a significant increase in leukocyte margination, but the exact dependence of leukocyte margination on haematocrit remains unclear.

1.6.6.2 Effects on platelet margination

Tubular platelet concentrations of isolated platelet suspensions perfused through tubes of diameter <300μm were 60% of the reservoir concentration (Beck, Jr. and Eckstein, 1980). This was presumably because the platelets flowed preferentially in the central fast moving fluid. The presence of RBC increased the concentration of platelets within the vessel from 60% (when no RBC were present) to around 200% (at haematocrits of 10-50%) of the platelet concentration in the reservoir. The increase in the tubular concentration with respect to the reservoir indicates an increase in the number of platelets flowing in the periphery of the vessel. In these experiments there was no increase in platelet margination as haematocrit was increased from 10% to 50%. In similar experiments, platelet rich plasma perfused at a shear rate of $1000s^{-1}$ in tubes of internal diameter ranging from 50-210μm showed a reduced tubular concentration when compared to the reservoir (Corattiyl and Eckstein, 1986). Again, red blood cells were required for the tubular platelet concentration to exceed that of the reservoir. The tubular concentration in the flow, and hence the radial displacement, increased up to a haematocrit of 38% and then remained constant between haematocrits of 38- 77%.

Aarts et al (Aarts et al., 1988) showed that the concentration of platelets flowing within 500μm of the wall of a 3mm diameter vessel was increased as the haematocrit of ghost RBC was increased. This was associated with a decrease in the number of platelets seen flowing in the central 200μm.

At a wall shear rate of $1630s^{-1}$ in a 50μm channel, perfused platelet-sized beads showed a uniform distribution at 0% haematocrit (Tilles and Eckstein, 1987). When RBC were added, there was no increase in near wall concentration of platelet-sized beads until a haematocrit of 15% was reached. Then, above 15% haematocrit there was no further increase in platelet-sized beads near the wall as haematocrit was increased up to 45%. In 200μm diameter

polyethylene tubes, the tubular concentration of platelet-sized beads increased when the haematocrit was varied from 0-40%, and then remained relatively constant from 40% up to 60% (Uijttewaal et al., 1993).

In studies using horizontally perfused polyethene tubes with blood at 0%, 15% and 40% haematocrit, the degree of lateral displacement of platelets towards the wall was significantly different for the three haematocrits (Yeh and Eckstein, 1994). This was quantified using a freeze capture method, viewing the tube end on and taking sections down the tube. At 0% haematocrit an approximately uniform concentration of platelet-sized beads was seen, with an exclusion zone seen near the wall. A near wall excess of platelet-sized beads was induced by the presence of the red blood cells, and this effect was more pronounced at 40% than at 15% haematocrit.

In another study, washed human red cells and platelet-sized beads were suspended in a dextrose solution to abolish RBC aggregation. Suspensions at haematocrits of 7% and 15% were perfused through tubes with an internal diameter of 210μm at a shear rate of $500s^{-1}$ (Bilsker et al., 1989). No near-wall excess was seen at 0% and 7 %, but at 15% haematocrit a near wall excess of 2.5μm beads was seen. This suggested that a haematocrit of 15% was needed for a significant excess of platelet-sized beads to be seen at the vessel wall.

From the above literature it is clear that RBC are required to produce a higher concentration of platelets near the vessel wall than in the centre. Results are not highly consistent between studies, but the general trend is that an increase in haematocrit results in an increase in the concentration of platelets flowing near the vessel wall. Margination tended to increase up to 40% haematocrit, but increases over 40% yielded little or no increase in the platelet near wall concentration.

1.6.6.3 Effects on leukocyte adhesion

Initial studies into the effect of haematocrit on the adhesive behaviour of leukocytes were done by Munn et al (Munn et al., 1996). In a vertically-perfused, parallel plate flow chamber with a mean depth of 78μm it was shown that increasing haematocrit increased the number of adherent lymphocytes rolling on human umbilical vein endothelial cells. This study showed a haematocrit of 5% was enough to increase the amount of rolling adhesion. Adhesion reached a maximum at 30% and was not changed as haematocrit was increased to 60%. This result was supported by a systematic study to measure the effects of rheological and haemodynamic parameters on leukocyte adhesion (Abbitt and Nash, 2003). It was found that in rectangular microslides (300 x 3000μm cross section) perfused vertically, leukocyte adhesion to P-selectin was increased as haematocrit was increased up to 30% and then remained constant as it was increased from 30% to 50%.

1.6.6.4 Effects on platelet adhesion

Initial studies compared adhesion for platelet suspensions with or without added RBC. Turitto and Baumgartner (Turitto and Baumgartner, 1975) used an annular perfusion chamber to perfuse blood across rabbit aortic sub-endothelium at a wall shear rate of $840s^{-1}$. The rate of deposition of platelets was 57 times higher in blood at a haematocrit of 38% than in a platelet-only suspension of equal platelet concentration. After 4 minutes of blood perfusion the percentage coverage was around 40%, but when perfusing the platelet suspension the platelet coverage only reached 20% after 100min perfusion. Karino and Goldsmith (Karino and Goldsmith, 1979) found that reconstituted blood at 20% haematocrit resulted in 4 times the number of adherent platelets bound to a collagen-coated glass tube of 3mm diameter when compared to a platelet suspension alone. The only study to use a range of haematocrits was

performed by Turitto and Weiss (Turitto and Weiss, 1980). Platelet adhesion and thrombus formation (platelet build up more than 5μm into the vessel) were measured on everted rabbit aortic subendothelium. At haematocrits of between 10% and 70 % adhesion increased until the haematocrit was 40% and remained constant up to 70% at low shear rates ($200s^{-1}$). At high shear rates ($2600s^{-1}$), there was a linear increase in adhesion in the whole range of haematocrits studied. This was coupled to an increase in thrombus formation at higher haematocrit (40-70%).

1.6.6.5 Summary

In the absence of RBC, leukocytes and platelets will flow more centrally within a vessel, and this is verified by the tubular counts of both cell types being lower than the feed reservoir counts. This would result in very low levels of adhesion of leukocytes and platelets, in vertically perfused vessels at least, where the effect of sedimentation is abolished. Increasing the haematocrit increases the margination of platelets. Although it is clear that the presence of RBC also results in leukocytes flowing more peripherally, the exact dependence of leukocyte margination on haematocrit is unclear. An increase in haematocrit increases the efficiency of adhesion of platelets and leukocytes. Both increase linearly at low haematocrits and plateau at physiological haematocrits (30-40%) at low shear rates. On the other hand, at high shear rate, platelet adhesion continues to increase as haematocrit increases well past the normal physiological range. Thus, this thesis will attempt to define haematocrit dependence for leukocyte and platelet margination, and to test any link this has to the adhesion of both cell types as haematocrit is altered. It should be kept in mind that haematocrit will affect wall shear stress as well as margination through its effects on viscosity, and will affect flow distribution (development of plug flow) and the plasma layer width, and hence wall shear rate. Interpretation of effects of haematocrit are thus not necessarily straight-forward, and this

thesis will be the first attempt to quantify the effects of haematocrit on margination and adhesion of both leukocytes and platelets in the same system.

1.6.7 RBC deformability

1.6.7.1 Effects on leukocyte margination and adhesion

In a recent study, mathematical modelling predicted that the tendency of more deformable particles to flow along the centreline in tube flow would laterally displace more rigid particles, such as leukocytes, towards the wall, at low haematocrit at least (Munn and Dupin, 2007). A large scale perfusion model (tube internal diameter 9.7mm) using flexible discs (3mm diameter) representing erythrocytes and rigid spheres (3mm diameter) for leukocytes suspended in glycerine oil showed that the discs had a faster centreline flow than the spheres (Schmid-Schonbein et al., 1980). The faster flowing flexible discs interacted with the slower flowing rigid spheres and the hydrodynamic interaction caused radial displacement of the rigid spheres towards the vessel wall.

From information obtained in the above studies it is possible to suggest that increasing red cell rigidity might be expected to reduce leukocyte margination seen in tube flow, as the more rigid RBC may not displace the leukocytes towards the vessel wall as efficiently. Increased rigidity of red cells can also inhibit close-packing of red cells, expand the width of the central flowing column and narrow the plasma layer (Seshadri et al., 1979). However, no experimental studies on the effects of modifying red cell deformability on leukocyte margination or adhesion have been carried out to our knowledge.

1.6.7.2 Effects on platelet margination

In rectangular tubes perfused at 15% haematocrit at wall shear rates of $210s^{-1}$ or $1630s^{-1}$, the near wall excess of 2.47μm beads was reduced when glutaraldehyde-fixed RBC were used compared to normal RBC (Eckstein et al., 1988). Uijttewaal et al (Uijttewaal et al., 1993) used heat treatment or incubation with glutaraldehyde to make RBC become slightly less deformable or rigidly-fixed respectively. Platelet margination in blood at 37% haematocrit perfused at a wall shear rate of $400s^{-1}$ in a tube of diameter 200μm was assessed from counts in the tube compared to counts in the sample reservoir. The tubular platelet count was decreased from 150% of reservoir concentration with normal RBC, to approximately equal to reservoir count with fixed RBC, and an intermediate value was obtained with heat-treated cells. These results suggest that the platelets flowed more centrally the more rigid the RBC.

1.6.7.3 Effects on platelet adhesion

Aarts et al (Aarts et al., 1984) used isoxsuprine and chlorpromazine to increase RBC deformability and diamide and chlolestrol to decrease RBC deformability. The effects on platelet adhesion were then assessed. Umbilical artery subendothelium was exposed to the blood in an annular perfusion chamber that could be varied in width to obtain various wall shear rates. The experiments showed that, over the entire range of RBC deformabilities used in this experiment, platelet adhesion was increased as RBC deformability was decreased. This effect was evident at wall shear rates of $300s^{-1}$ and $1800s^{-1}$ but was more pronounced at the higher shear rate. The effects of the treatments on RBC deformability in these studies were deduced from changes in the apparent viscosity of the blood, and hence were effectively linked with changes in shear stress. No direct measurements of flow resistance of the individual cells were made.

1.6.7.4 Summary

The above suggests that decreasing the RBC deformability tends to decrease margination but increase the adhesion of platelets. There are no studies of the effect of manipulating RBC deformability on leukocyte behaviour to date. In this thesis, the effect of increasing RBC rigidity on the margination and adhesion on leukocytes and platelets will be assessed using the same experimental system and the same manipulations for the first time.

1.7 Methods for modelling leukocyte and platelet adhesion

In principle, the effects of rheological variables on leukocyte and platelets adhesive behaviour can be studied *in vivo* or *in vitro*. *In vitro* models offer the ability to control a wide range of variables individually or together, although it is difficult to reproduce the situation in very small microvessels. This thesis uses a flow model that has been adapted and improved from a previous system (Buttrum et al., 1993;Abbitt and Nash, 2003), to investigate a wide range of physical variables that could influence the behaviour of leukocytes and platelets. The use of rectangular microslides provides a good quality image for analysis. The microslides are easily coated with adhesive substrates or non-adhesive albumin to facilitate the adhesion and margination experiments respectively. Different sized microslides are available, and ones with depth of 300μm or 100μm were used here. The latter allowed production of high shear rates using relatively small volumes of blood. Blood rheological parameters could be manipulated independently by specific protocols which would not be possible using *in vivo* models. With the use of a camera capable of very short exposure times, the near wall free-flowing behaviour of leukocytes and platelets could be assessed, using the same setup as for adhesion experiments. In a new development, we measured pressure differences across the microslides so that the apparent viscosity of the blood samples could be measured as a function of shear rate in the same system. This allowed assessment of non-newtonian behaviour and of changes

in wall shear stress. This versatile model thus provided a controlled environment for all of the rheological and haemodynamic variables to be manipulated and assessed.

1.8 Hypothesis and Aims of this thesis

The hypothesis of this thesis is that haemodynamic and rheological variables influence the margination and adhesion of leukocytes and platelets differently, in part at least because of their different cell sizes. The aim of this thesis is therefore to carry out a systematic, comprehensive, comparative assessment of the effects of haemodynamic and rheological variables on leukocyte and platelet behaviour under conditions that are as nearly identical as possible. This thesis aims to provide a more complete understanding of the physical factors which influence adhesion and thus function of leukocytes and platelets in different parts of the circulation and under the influence of different perturbations which might be relevant to physiological or pathological responses to tissue insult.

There have been numerous investigations into the effects of haemodynamic and rheological variables on the margination and adhesion of leukocytes and platelets. However, these studies have not been systematic and have rarely covered the effects of more than two variables in the same system. The literature contains only a few studies that have directly measured the margination and adhesion of leukocytes or platelets in the same vessel. No study has assessed the affect of any variable on both leukocyte and platelet behaviour in flow, whether it be margination or adhesion. There are also many gaps in the literature. For instance, the effects of altering suspending phase viscosity or RBC deformability on leukocyte margination or adhesion have been largely ignored. Also no studies have been performed assessing the effect of RBC aggregation on platelet adhesion. This thesis aims to address such shortcomings.

CHAPTER 2: MATERIALS AND METHODS

2.1 Blood collection

Blood was withdrawn from the anticubital vein of healthy adult volunteers with informed consent, and according to procedures approved by the University Research Ethics Committee. The blood was immediately transferred into tubes containing the anticoagulant citrate phosphate dextrose adenine (CPDA) (Sigma-Aldrich Company Ltd). CPDA was used as it does not chelate all of the Ca^{2+} within the blood. Both leukocyte adhesion to P-selectin and platelet adhesion to collagen is Ca^{2+} dependent. When anticoagulated with CPDA the concentration of free Ca^{2+} remaining in the blood was sufficient to facilitate this adhesion (Geng et al., 1992;Dopheide et al., 2001). 9ml of blood was added to 1ml CPDA pH 7.4. In all experiments, apart from those assessing platelet adhesion, the tube also contained 200µl of theophylline (Sigma-Aldrich Company Ltd) dissolved in PBS at a concentration 0.35M. This resulted in a final concentration of theophylline of 7mM in the blood (see section 2.5.2).

2.2 Manipulation and measurement of rheological parameters

2.2.1 Blood cell counts

Cell counts were performed using a Coulter Counter (Counter Multisizer II, Coulter Electronics Ltd, Luton, UK). The concentration of RBC was determined using a 200,000 times serial dilution of the blood in Isoton® II (Beckman Coulter, High Wycombe, UK). Leukocyte counts were determined by using a 200 times dilution of the blood in Isoton® II followed by the addition of a drop of Lyzerglobin™ (Becton Dickinson, Heiselberg, Germany). The addition of the Lyzerglobin lysed the RBC rapidly, and counts were taken immediately after 3 inversions of the sample, to ensure red cell lysis but before any shrinking of the leukocytes could occur. The volume distribution display on the Coulter Counter showed two distinctive peaks corresponding to smaller lymphocytes and larger neutrophils

and monocytes. This allowed separate counts to be made in addition to a total leukocyte count. The number of leukocytes in whole blood was never manipulated to a specific count. The count was recorded and a correction for the number of cells in the blood was made in any analysis performed. Platelet counts in whole blood or manipulated samples were measured using a Pentra 60 haematology cell counter (Horiba ABX, Northampton, UK).

2.2.2 Haematocrit

2.2.2.1 Measurement of haematocrit

The haematocrit of the blood was measured using the microhaematocrit method. After thorough mixing, the blood was drawn into glass capillary tubes (Bilbate Ltd, Daventry, UK) under capillary action. One end of the tube was then sealed using plasticine (Hirschmann Laborgerate, Eberstadt, Germany). The capillary tube containing the blood was then centrifuged at 15,000g for 5 minutes. The centrifugal force created by the centrifuge was great enough to separate the RBC from the plasma and pack them tightly at one end. The haematocrit was calculated by dividing the length of the column containing RBC only, by the total length of the blood in the column. No correction was made to allow for the small amount of plasma trapped in the closely packed RBC.

2.2.2.2 Manipulation of haematocrit

The haematocrit of the blood was manipulated in various ways depending upon the experiment. To alter haematocrit without altering platelet count, several tubes containing 10ml of blood of known haematocrit were centrifuged at 550g for 10 minutes. After centrifugation the platelet-rich plasma in these tubes contained approximately the same platelet concentration as that in the original blood, verified by measurements using the Pentra 60 haematology cell counter. An amount of plasma was removed from one tube to leave

blood of 50% haematocrit. Platelet-rich plasma was then added to this blood to obtain haematocrits of 10, 20, 30, 40 and 50% whilst keeping platelet count constant.

It had been noted in experiments where centrifugation of the blood had been attempted, that leukocyte adhesion was reduced. Therefore, to alter the haematocrit in the leukocyte adhesion studies, centrifuging the blood was avoided. Autologous plasma was added to the blood of known haematocrit, to obtain haematocrits of 10, 20, 30, and 40%. Leukocyte count was different in each of these samples. However this was corrected for in the data analysis of the experiment.

When altering the haematocrit, the blood volume was increased or decreased with autologous plasma according to the formula:

$$V_R = \frac{H_O V_O}{H_R} \qquad \textbf{Equation 21}$$

H_O = Original haematocrit
V_O = Original volume of blood
H_R = Required haematocrit
V_R = Final blood volume, achieved by adding or removing plasma, to obtain the required haematocrit.

All adjusted haematocrits were confirmed by microhaematocrit centrifugation.

2.2.3 Red blood cell aggregation

Red blood cell aggregation was manipulated in two different ways. Initially 20% haematocrit was used for leukocyte and platelet experiments, as this required no centrifugation of the blood and reflected the likely haematocrit in the microcirculation due to the Fahraeus effect (Lipowsky et al., 1980). However at 20% haematocrit, and at a low shear rate, leukocyte

adhesion was affected by a strong sedimentation effect (see section 6.3.1). Thus in addition, leukocyte and platelet adhesion experiments were also performed at 40% haematocrit.

In initial RBC aggregation experiments, a volume of blood at 40% haematocrit was diluted 1:1 to produce a final haematocrit of 20%. Diluents were: autologous plasma (to maintain red cell aggregation at a control level), low molecular weight dextran at a concentration of 3.25% (40kDa: to dilute plasma fibrinogen and reduce red blood cell aggregation; Dx40) or high molecular weight dextran at a concentration of 1.6% (500kDa; to increase red blood cell aggregation; Dx500) (Sigma-Aldrich Company Ltd, Dorset, UK).

When the experiments were to be performed at 40%, the blood was first adjusted to this haematocrit. Then the blood was centrifuged for 10min at 250g for leukocyte, and 750g for platelet adhesion experiments. Then a volume of plasma was removed and replaced with diluent. The volume was chosen to give the same final concentration of dextran in the plasma as above, where the ratio of plasma to diluent was 0.6:1, or the final suspending medium was 6/16 plasma and 10/16 diluent. The following equation was used to determine the volume of plasma to be replaced by each diluent:

$$V_r = V_{40} \times 0.6 \times {}^{10}\!/_{16} \qquad \textbf{Equation 22}$$

V_r = Volume of plasma to be replaced with diluent
V_{40} = Initial total volume of blood at 40%
0.6 = Proportion of plasma in the whole blood at 40% haematocrit
10/16 = Required proportion of diluent in the suspending phase in the final manipulated blood sample.

The effects of the diluents on red cell aggregation are illustrated in figure 2.1. These micrographs show that Dx40 abolished aggregation while Dx500 enhanced RBC aggregation compared to plasma.

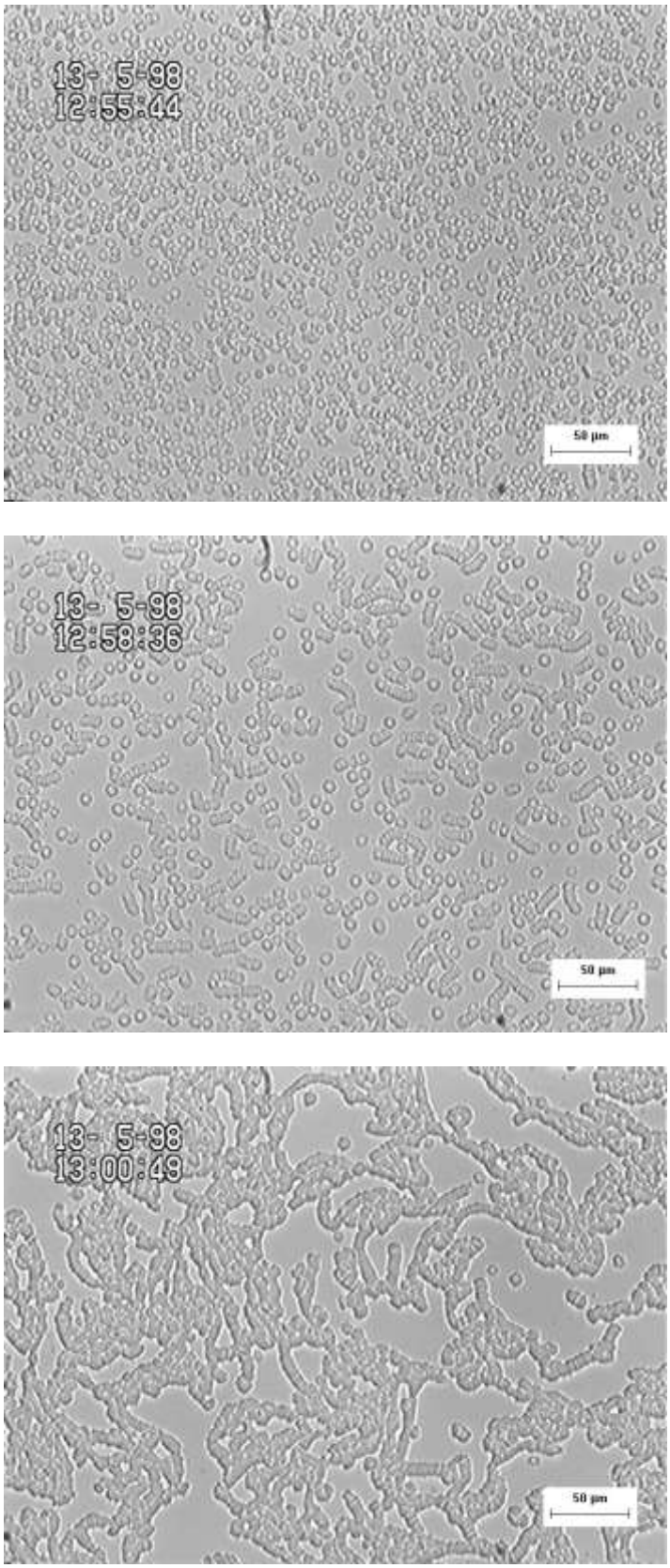


Figure 2.1 The effect of adding dextran to blood on RBC aggregation
Top: Dx40 at 3.25% was added to whole blood to abolish aggregation
Middle: Autologous plasma was added to blood to maintain aggregation
Bottom: Dx500 at 1.6% was added to whole blood to enhance aggregation

To ensure the viscosity of the suspending phase was not altered by the addition of the dextran, it was necessary to add dextran solutions with the same viscosity as the plasma (approximately 1.2mPa.s at 37˚C; (Kesmarky et al., 2008)). Solutions of 3.25% Dx40 and 1.6% Dx500 were made and their viscosities were verified by tube viscomerty as described in section 2.8 as being 1.176 and 1.171mPa.s respectively. The addition of Dx500 at this concentration achieved a final concentration in the blood of 1%, near that previously described as being optimal for red cell aggregation (Nash et al., 1987). The resultant concentration of Dx40 in the blood was 2%. By perfusing isolated neutrophils over an adhesive substrate it was previously verified that dextran added at these concentrations had no direct effect on leukocyte adhesion (Abbitt and Nash, 2003).

To test whether there was any effect on platelet function, platelet aggregometry was performed in the presence of Dx40 and Dx500 at these concentrations. Using collagen at 1μg/ml as an agonist, the dextrans had no effect on platelet aggregation. There was no significant effect on the increase in light transmission through PRP in the aggregometer after the addition of collagen (fig 2.2).

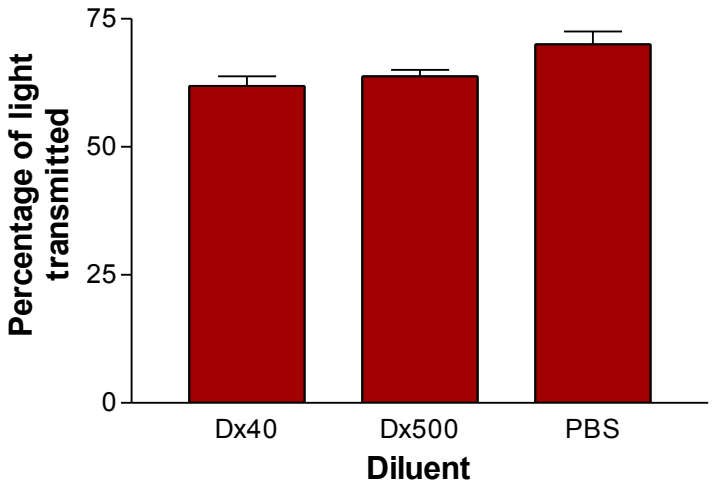


Figure 2.2 Effect of dextran on the aggregation of platelets in PRP at concentrations used in adhesion experiments
The aggregation of the platelets in PRP containing Dx40, Dx500 or PBS was assessed by measuring the light transmission as a proportion of transmission through autologous platelet poor plasma. Three minutes after the addition of the agonist (collagen at 1μg/ml) the percentage of light transmission in the presence of the dextran solution showed no difference between Dx40 and Dx500. Data are means ± SEM from 2 experiments.

2.2.4 Suspending phase viscosity

Suspending phase viscosity was altered by adding low molecular weight dextran solutions (dissolved in PBS) of equal volume but increasing concentration to blood that had been normalised to 40% haematocrit using autologous platelet poor plasma. Initial platelet adhesion experiments were performed at 20% haematocrit. Here the Dx40 solution was added directly to the whole blood 1:1. For the platelet and leukocyte adhesion studies performed at 40% haematocrit, the blood was initially centrifuged at 250g for leukocytes and 750g for platelets and the appropriate amount of plasma was removed and replaced with the Dx40 solution (see equation 22). In the leukocyte experiments the percentage of Dx40 in PBS that was added to the blood was 0, 3.25, 5, 7.5 and 10%. For platelet experiments the concentrations used were 3.25, 10, 12.5, 15, 17.5 and 20%. As Dx40 was added to the blood, aggregation was abolished in these experiments.

Upon completion of the experiments performed to assess the effect of suspending phase viscosity on adhesion, the apparent viscosity of the blood samples was measured using the tube viscometer, details of which are found in section 2.8. The blood samples were then centrifuged at 1000g for 10 minutes and the suspending phase was removed. The viscosity of the suspending phase of each sample was also measured using the tube viscometer.

2.2.5 Red blood cell deformability

2.2.5.1 Manipulation of RBC deformability

Incubating red blood cells with glutaraldehyde (Sigma-Aldrich Company Ltd, Dorset, UK) has been shown to reduce the deformability of RBC by forming cross links between membrane proteins including the spectrin cytoskeleton (Heusinkveld et al., 1977;Goldsmith, 1971b). To assess the influence of RBC deformability on the adhesion of leukocytes and

platelets, washed red blood cells were incubated with glutaraldehyde, and then re-combined with isolated platelets and leukocytes

For platelet experiments, whole blood was centrifuged at 250g for 10 minutes to obtain platelet rich plasma. The PRP was removed and stored at room temperature. For experiments involving leukocytes the blood was centrifuged at 1000g for 10 minutes to obtain a buffy coat. This buffy coat, along with a few adjacent RBC, was suspended in 2ml of autologous plasma. Each 2ml sample was added to a 5ml tube and laid on its side (horizontally) for 15 minutes. The plasma was then pipetted off the top by holding the tube at a slight angle. This was then centrifuged at 1000g for 10 minutes and the resultant buffy coat was re-suspended in 1ml of plasma. Samples obtained in this way had approximately the same proportion of different leukocytes as found in the whole blood (Mikita et al., 1986).

PBS was added to the remaining blood which was centrifuged at 1000g for 10 minutes and the cells resuspended in 10ml PBS. This was repeated three times. The buffy coat was discarded between each wash to leave packed red blood cells only, free of plasma proteins. These red cells were then incubated for 15 min with PBS (control), 0.005% glutaraldehyde in PBS (Sigma-Aldrich Company Ltd, Dorset, UK) (reduced deformability) or 0.01% glutaraldehyde in PBS (rigid) to obtain a range of deformabilities of red cells. These cells were then centrifuged at 1000g for 10 min and re-suspended in PBS. This process was repeated three times to remove the glutaraldehyde.

A mix of plasma containing platelets or leukocytes and Dx40 (proportions equal to that used in the RBC aggregation and suspending phase studies) were added to a known volume of packed red blood cells. The Dx40 was used to abolish RBC aggregation. The haematocrit and red cell count were measured. Alteration in RBC deformability may alter centrifugal packing

efficiency (Ham et al., 1968), so that haematocrit appears greater for a given cell count or mean cell volume. Here, even using 0.01% glutaraldehyde had no detectable effect on packing efficiency judged by the comparisons shown in Table 1. The average measured haematocrit, RBC count and mean cell volume (haematocrit/count) of the blood after reconstitution showed no significant difference between samples.

Incubation medium	Haematocrit (%)	RBC count (10^9/ml)	Mean cell volume (fl)
PBS	39.2±1.10	4.09±0.18	96.1±0.54
0.005% Glutaraldehyde	38.5±0.82	3.96±0.14	98.2±0.53
0.01% Glutaraldehyde	38.6±0.67	3.95±0.17	98.4±0.54

Table 1 Effect of glutaraldehyde treatment on haematocrit, RBC count and mean cell volume in reconstituted blood

2.2.5.2 Measurement of RBC deformability

A small aliquot of each manipulated RBC sample was diluted to a haematocrit of 10% in PBS. The filterability of the RBC suspensions was measured using a St.Georges filtrometer (Carri-Med, Dorking, UK). Polycarbonate filters with an average pore diameter of ~5µm were used (Nuclepore Corporation, Pleasanton, USA). The filtrometer measures the flow rate of the suspensions through the filter at a constant pressure (4cm of H_2O in this study) (Dormandy et al., 1985). The rate of flow is measured for three consecutive volumes of 20µl after an initial 20µl run-in, and expressed relative to the flow rate for PBS alone. The initial relative flow rate (IRFR) and rate of decrease of flow rate (clogging rate; proportion decrease per ml) are calculated by linear regression. Red cell transit time (RCTT) is the relative resistance of a pore filled with cells compared to an empty pore and represents the resistance to flow corrected for haematocrit. Clogging particles (CP) represents the concentration of

cells which cause pore blockage and hence progressive reduction in flow rate. They are calculated as:

$$RCTT = \left(\frac{1}{IRFR} - 1 \right) \cdot \frac{1}{HCT} + 1 \qquad \textbf{Equation 23}$$

$$CP = CR.N_P \qquad \textbf{Equation 24}$$

where HCT = haematocrit as a proportion (i.e., 0.1).

The RBC incubated in 0.005% glutaraldehyde had a significantly increased transit time compared to RBC incubated in PBS (fig 2.3). The RBC incubated with 0.01% glutaraldehyde rapidly blocked the filter so that not even the flow rate of the first 20µl could be measured. There was no significant difference between the number of clogging particles between the RBC incubated in PBS and 0.005% glutaraldehyde.

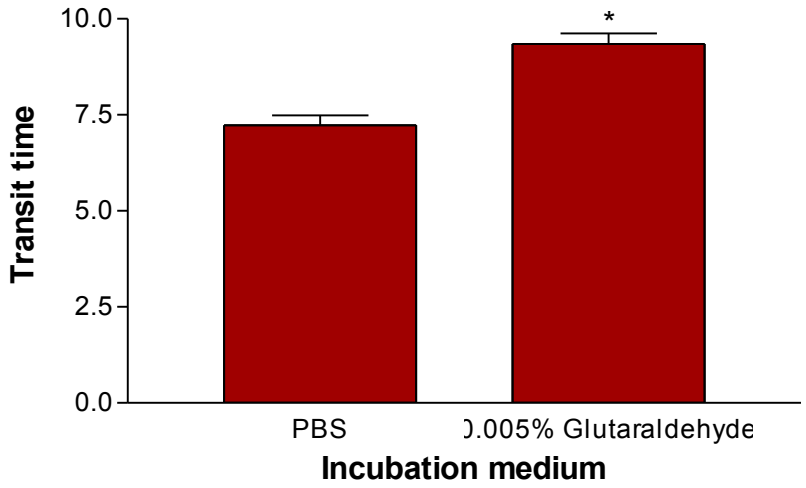


Figure 2.3 Effect of incubating RBC with 0.005% glutaraldehyde on transit time through 5µm pores
RBC at ~10% haematocrit in PBS were perfused through a filter of 260,000 5µm pores. The St George's filtrometer houses the filter and controls the perfusion pressure across it. Results shown are means ± SEM from 3 experiments. The measured transit time of the blood through the filter was significantly higher for RBC incubated with glutaraldehyde at a concentration of 0.005% when compared to those that had been incubated in PBS. * $P<0.05$ compared to PBS control by paired t-test.

2.3 Microslides

A microslide (CamLab Ltd., Cambridge, UK) is a rectangular glass capillary. In the flow-based adhesion assays described in section 2.5, microslides were used with internal dimensions of: 5cm (length), 3mm (width) and 300μm (height); 5cm (length), 1mm (width) and 100μm (height). A cross-section of the smaller microslide is shown in Figure 2.4.

2.3.1 APES coating

Microslides were coated with 3-aminopropyltriethoxysilane (APES; Sigma-Aldrich Company Ltd, Dorset, UK) as described, to produce a surface which bound the protein adhesive substrates (Cooke et al., 1993;Prooijen-Knegt et al., 1982). The microslides were immersed in 50% nitric acid (BDH Laboratory Supplies) overnight to clean the internal surface. The microslides were then washed by running tap water over them for 4 hours.

In the case of the 300μm microslides, a suction pump was used to remove any excess water from within the microslides. After being left to dry, around 50 microslides were placed in a 50ml polystyrene tube (Becton, Dickinson and Company, USA) containing anhydrous acetone (BDH Laboratory Supplies) and inverted and straightened repeatedly for 30 seconds. The acetone was discarded and this step was repeated. After being blotted dry the microslides were placed in a 50ml tube containing 4% APES in anhydrous acetone. They were inverted and straightened repeatedly for 60 seconds to ensure adequate coating of the internal surface of the microslide. After the microslides were blotted dry this process was repeated. After blotting dry again, the microslides were rinsed in anhydrous acetone once more. Sterile, distilled water was then aspirated through the microslides three times using a pump. The microslides were allowed to dry and were autoclaved at 121˚C for 11 minutes. They were then stored in sterile conditions indefinitely.

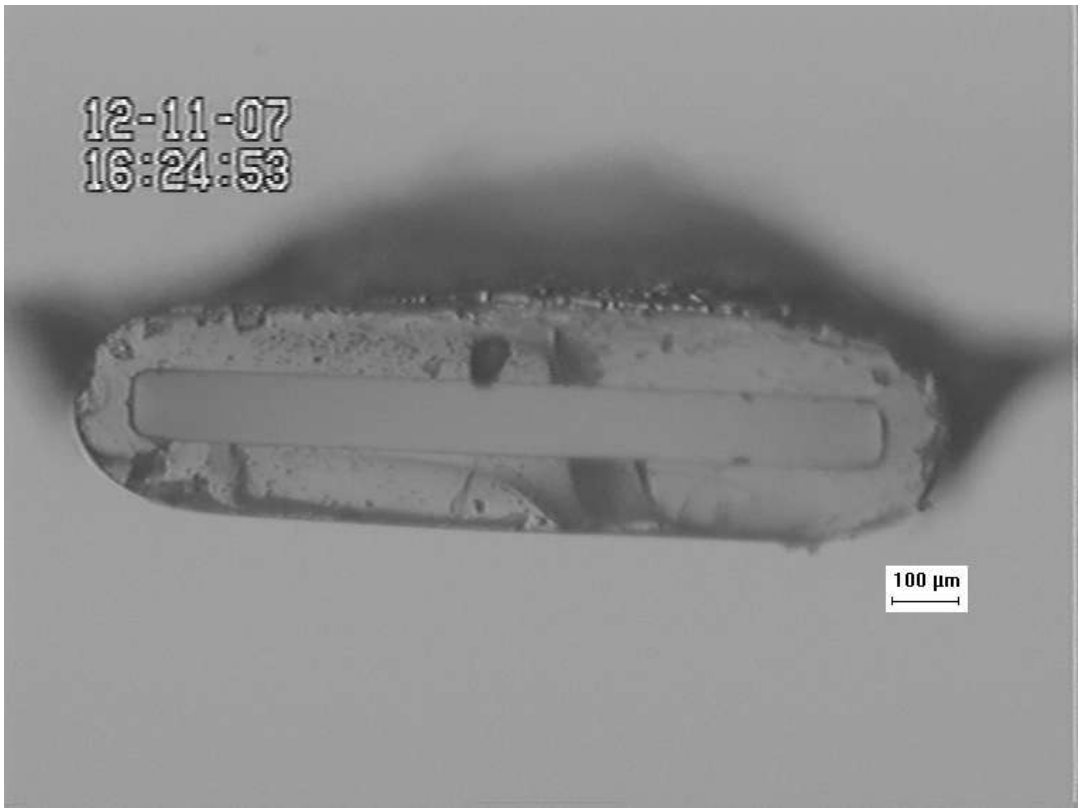


Figure 2.4 Microslide viewed end-on by light microscopy.
The cross section of a microslide with dimensions of 1 x 0.1mm clearly shows the rectangular shape of the internal cross section.

In the case of the smaller 100µm microslides, after being left to dry after the acid wash, a small piece of silicone tubing of internal diameter 1mm and external diameter of 3mm (Fisher Scientific UK Ltd, Loughborough, UK) was attached to one end of each microslide with double sided sticky tape (3M United Kingdom Plc, Bracknell, UK). All steps were completed as with the 300µm microslides but instead of using 50ml tubes; acetone, APES and water were drawn into individual microslides and pushed out using a pipette. This was necessary as, due to the small dimensions and presence of bubbles, reagents did not enter the microslide efficiently without the use of a pipette.

2.3.2 Coating microslides with appropriate substrates

For experiments involving leukocyte adhesion, purified P-selectin (R&D, Abingdon, Oxfordshire, UK) dissolved in PBS was diluted to the required concentration and pipetted into microslides. After 1 hour incubation at room temperature, non-specific binding sites were blocked by replacing the P-selectin with 2% bovine serum albumin (BSA) in PBS (2% PBSA) and incubating for a minimum of one further hour (Abbitt et al., 2000;Rainger et al., 1997). Leukocyte adhesion was tested at various concentrations of P-selectin and 10µg/ml was chosen for all further experiments as it was the lowest concentration to yield a sufficient number of adherent leukocytes for analysis (fig 2.5).

For experiments involving platelet adhesion, an equine tendon derived prepartation of collagen type I fibrils called Horm collagen (Axis-Sheild, Dundee, UK) at a concentration of 500µg/ml was pipetted into the microslides and incubated for 1 hour. Non-specific binding was blocked by replacing the Horm collagen with 2% PBSA and incubating for a minimum of one further hour (Butler et al., 2007;McCarty et al., 2005).

Experiments to assess the near wall concentrations and velocities of both cell types required a non-adhesive surface. 2% PBSA was drawn into microslides and left for a minimum of 2 hours to block any non-specific binding.

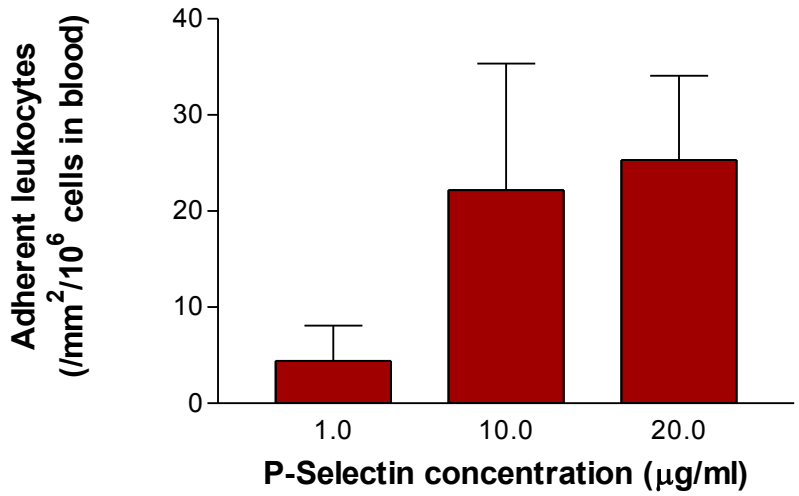


Figure 2.5 Effects of changing P-selectin coating concentration on the number of adherent leukocytes.
Whole blood, anti-coagulated with CPDA, was perfused through microslides of 300μm in height that had been coated with purified P-selectin at various concentrations. The blood was un-manipulated with an average haematocrit of 41.7% and the adherent leukocytes were counted on the lower surface of the microslides. The count was corrected for the area and for the number of leukocytes in the blood. Data are means ± SEM from 3 experiments.

2.4 Flow system

The flow system used in this thesis is represented in Figure 2.6. At the centre of this assay is the microslide, whose shape allows for good optical quality when viewed perpendicular to the widest side. Using different volumetric flow rates the wall shear rate can be changed. A microslide coated with the appropriate substrate was glued across the width of a glass microscope side so the smaller dimension (height) was vertical. Each end of the microslide was protruding over the microscope slide by 1cm. This allowed enough length to apply double-sided adhesive tape around the protrusion. One end of the microslide was connected via a rigid plastic tube to a glass syringe, with a luer fitting, with volume of either 50ml or 2ml (Harvard Apparatus Ltd, Kent, UK) depending on the size of the microslide and the shear rate required. The syringe was placed in a withdrawal pump (Harvard Apparatus Ltd, Kent, UK) that could withdraw liquid through the microslide at the desired flow rate, corresponding to the wall shear rate required (see equation 15). The opposite end of the microslide was connected to an electronic valve via a small piece of silicon tubing to reduce dead-space. The electronic valve was directly attached to a sample reservoir, and to a wash-buffer reservoir (both made from sterile disposable syringe barrels) and switched input between them. The microslide and microscope slide were placed on the stage of a fluorescence microscope; either an Olympus BX51 (upright) or an Olympus IX70 (invert) for observation of upper or lower surfaces of the microslide respectively. The stage, microslide, valve and reservoirs were enclosed in a Perspex housing maintained at 37 °C.

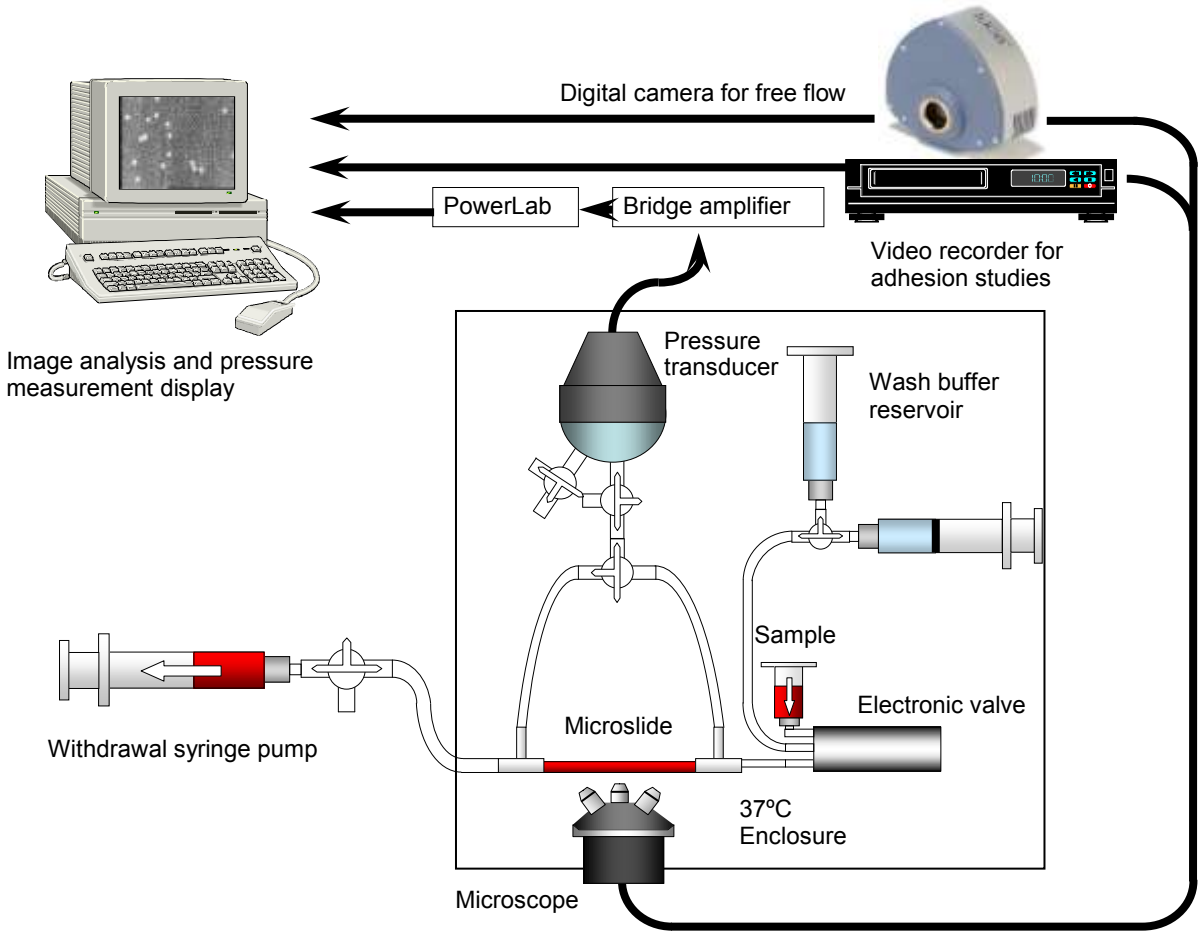


Figure 2.6 Schematic diagram of the flow-based adhesion, free flow and viscometry apparatus
The apparatus was used to assess the adhesion of leukocytes and platelets in flowing blood. The number and velocity of cells flowing close to the wall could also be measured. The system was also used to measure the pressure drop across the microslide, so that fluid viscosity could be calculated.

2.5 Adhesion assay

The wall shear rates used in the adhesion experiments were calculated using the following equation for flow between infinitely wide parallel plates:

$$\gamma_w = \frac{6Q}{wh^2}$$

This assumes that the flow profile is parabolic and neglects the effects of the side walls on the wall shear rate (section 1.2.5). The volumetric flow rate was chosen to supply the wall shear rate required.

The fluorescent dye Rhodamine 6G (R6G) was added to the blood at 5µg/ml to label leukocytes and platelets. This dye has been previously shown to have no effect on the number and rolling velocity of adherent leukocytes at this concentration (Abbitt et al., 2000). Leukocytes and platelets were easily distinguishable from RBC which do not fluoresce with this dye. The dye is a mitochondrial stain and RBC do not possess any mitochondria and hence do not fluoresce (Gear, 1974).

2.5.1 Leukocyte adhesion

To measure the adhesion of leukocytes to P-selectin, blood was perfused through the microslide at the appropriate flow rate for 4 minutes from the time the blood first exited the microslide. The adhesive interactions of leukocytes were recorded in eight random fields, each recorded for 15 seconds along the centreline of the flow in the microslide, between the second and fourth minute of perfusion. The fields were recorded onto a video cassette using a high sensitivity video camera (JVC TK-S350, Japan) to be analysed after the completion of the experiment.

Blood was perfused at a wall shear rate of $140s^{-1}$ for all experiments except those assessing the effect of wall shear rate on leukocyte adhesion. This is comparable to the wall shear rate experienced in post capillary venules *in vivo* (Lipowsky, 1988).

2.5.2 Analysis of leukocyte adhesion

Using Image Pro software (Media Cybernetic, Inc, Bethesda, MD, USA), ten images were captured from each recorded video sequence, one second apart. Adherent cells were bright spheres, nearly all of which were rolling slowly across the field, and easily distinguished from free flowing cells near the wall which were elongated blurs (fig 2.7). The number of adherent leukocytes present in the first frame was counted, this was repeated for each of the 8 fields recorded, and the mean was calculated for each sample. This average value was normalised using the known field area, to a cell count per mm^2. As the number of adherent cells per field increased with the time of blood perfusion, and the leukocyte count varied between samples, the number of adherent cells was also corrected for number of leukocytes perfused. The data were collected between 2 and 4 minutes of blood perfusion. Thus the average adhesion count over the eight recorded fields was normalised by the total number of leukocytes that had been perfused by the midpoint of the observation (3 minutes) based on the known blood leukocyte count and flow rate. The data was thus expressed as adherent leukocytes/$mm^2/10^6$ cells perfused.

For all leukocyte adhesion experiments, the method of normalisation was chosen as it corrected for any slight differences in field size between microscopes and for any difference in leukocyte count between donors. The normalisation was performed using the number of cells perfused by the mid point of observation to directly compare leukocyte adhesion experiments at a range of wall shear rates, as at higher shear rates the number of leukocytes perfused through the microslide was increased. At increasing shear rates the time that the

blood was flowing within the microslide was reduced, thus a normalisation procedure that accounted for the residence time of the blood within the microslide may have also been appropriate. The normalisation procedure that expressed leukocyte adhesion as leukocytes/$mm^2/10^6$ cells perfused was chosen as it was considered to be more appropriate when compaing changes in wall shear rate on leukocyte adhesion.

The rolling velocity was calculated by identifying and recording the location of the cells in the first frame of the ten recorded frames for each field. The position of the leading edge was marked using the Image Pro software, the frames were then advanced for nine seconds, the leading edge re-marked and the distance that the cells had travelled was used to calculate their rolling velocity. A very small proportion of cells were stationary; another small proportion of cells were transiently detaching and reattaching, rolling for a short while and skipping along. Cells that displayed these types of adhesion were not included in the measurement of rolling velocity, but were included in the count of the adherent leukocytes if present in the first frame.

2.5.3 Platelet adhesion

Blood was perfused at the chosen wall shear rate for up to 6 minutes after the microslide was first filled with blood. Three images of random fields along the centre-line of the microslide were video-recorded in quick succession at chosen times. Figure 2.8 shows a typical field recorded after 4 minutes of perfusion of blood containing fluorescent platelets (fig 2.8). Images were analysed off-line.

2.5.4 Analysis of platelet adhesion

The 3 fields that had been recorded at each time point were analysed using Image Pro Plus 6.2.1 (Media Cybernetic, Inc, Bethesda, MD, USA). The software set a threshold of intensity on a grey scale between 0 and 255; if the pixel intensity was less than the threshold it would be black or above the threshold would be white. A visual verification of the threshold level was always performed to ensure the area coverage of the platelets was represented accurately in the thresholded image. The area covered by platelets was expressed as a percentage of the total area of the field. Normalisation for variation in platelet count of donors was not performed as it was difficult to quantify the number of platelets in the non-planar thrombi that formed. However, normalisation was performed to account for different volumetric flow rates used in experiments by dividing the percentage coverage by the volume of blood that had been perfused by the given time point. In experiments comparing different conditions, the platelet count was the same in all samples.

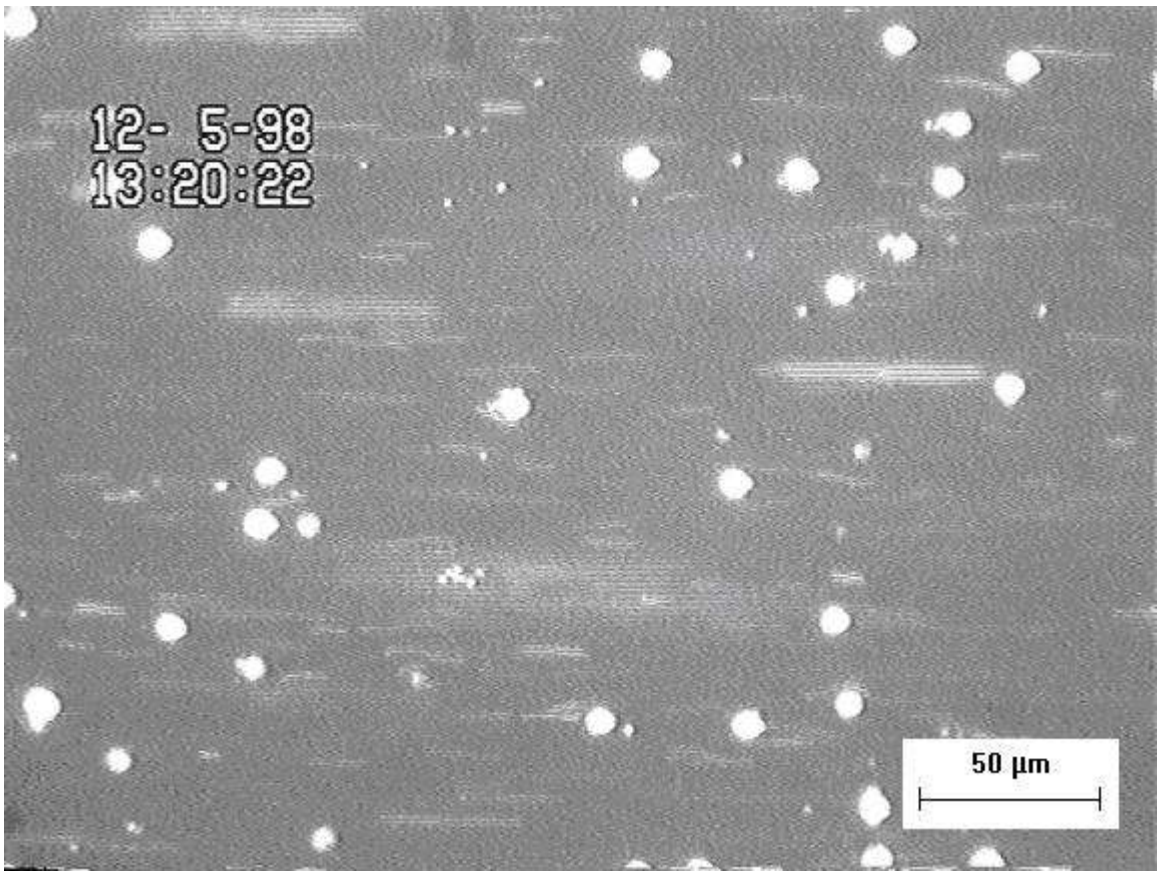


Figure 2.7 Fluorescent leukocytes adherent on P-selectin
This single frame shows the adherent fluorescent leukocytes along with the blur of free-flowing near wall leukocytes from blood perfused from left to right, demonstrating how they were easily differentiated.

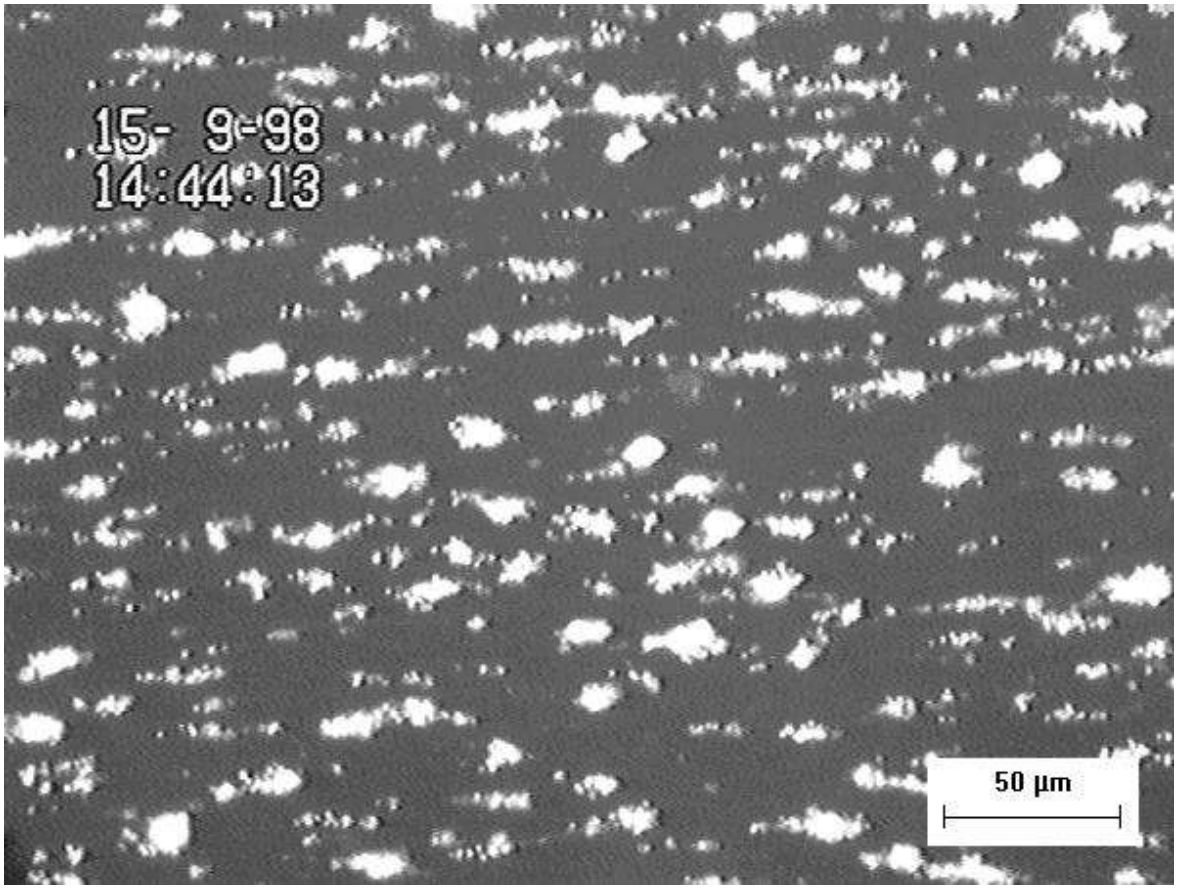


Figure 2.8 Fluorescent platelets adherent on collagen
This single frame represents a typical field recorded during a platelet adhesion experiment after 4 minutes perfusion of fluorescently labelled blood from left to right. The areas of platelet thrombi are clearly distinguishable from the areas free from platelet adhesion.

2.6 Optimisation of adhesion experiment protocols

2.6.1 Effects of storage, centrifugation and use of theophylline

It had previously been found that the number of leukocytes adherent on P-selectin decreased as the time the blood was stored at room temperature was increased (Abbitt and Nash, 2001). Here it was noticed, in experiments that required more time for manipulations, that the effect of time on leukocyte adhesion was not negligible and could be as large as the effect of any blood manipulations on leukocyte adhesion. It was necessary to overcome this time dependence of leukocyte adhesion in order for leukocyte adhesion to be quantified accurately. A possible explanation of this time dependent decrease in adhesion could be activation of the leukocytes which impairs selectin binding. Theophylline is believed to be a phosphodiesterase inhibitor which would increase cAMP levels, and thus inhibit activation of leukocytes and platelets. Therefore an experiment to assess the effect of storage time on leukocyte adhesion was performed with and without theophylline using 100μm microslides. Figure 2.9(a) shows the decrease in adhesion with time in a sample without theophylline compared to a sample with theophylline over the time course of a typical experiment. The leukocyte adhesion in the sample including theophylline shows no time dependence. To see how long this effect extended, another experiment was performed including theophylline over a 4 hour time period (fig 2.9(b)). This showed no time dependence so the inclusion of theophylline would reduce errors in adhesion due to any possible variation in the duration of blood storage.

It was also observed that blood that had been previously centrifuged yielded a lower number of adhesive leukocytes than blood without centrifugation, presumably because the packing in the buffy coat promoted activation. The effect of the centrifugation was tested with and without addition of theophylline (fig 2.9(c)). Without theophylline both spinning the blood and increasing the storage time resulted in decreased adhesion. No decrease in adhesion was

seen as the blood was centrifuged in the presence of theophylline or as storage time was increased.

Thus theophylline was included in all experiments apart from those assessing platelet adhesion, where inhibition of activation would inhibit thrombus formation. If theophylline did inhibit spontaneous neutrophil activation, this would only tend to reduce the already low level of transformation of rolling to stationary adhesion. From this stage on, we no longer observed effects of order of treatment on results of leukocyte adhesion assays.

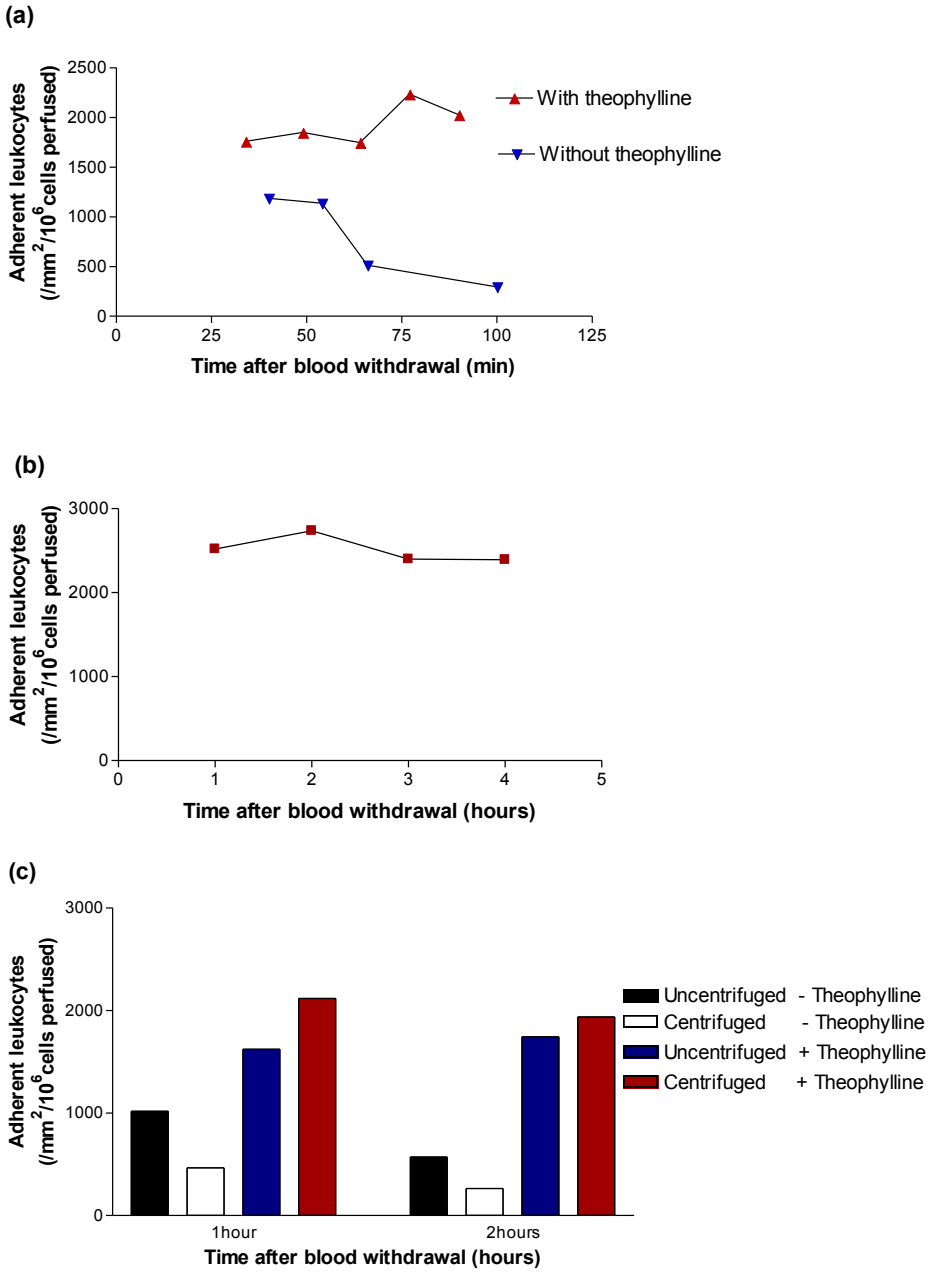


Figure 2.9 Effect of theophylline on the time dependence of leukocyte adhesion to P-selectin

Whole blood, anti-coagulated with CPDA, was perfused (at a wall shear rate of $140s^{-1}$) through microslides of 100µm in height that had been coated with 10µg/ml of purified P-selectin. The blood was corrected to a haematocrit of 40% and the adherent leukocytes were counted on the lower surface of the microslides. Data shown are from single experiments. (a) A comparison of leukocyte adhesion with and without theophylline over the time course of a typical experiment. (b) Effect of storage time on leukocyte adhesion over 4 hours. (c) Effect of spinning the blood on leukocyte adhesion including theophylline and over a time course of 2 hours. The count was corrected for the area and for the number of leukocytes in the blood.

2.6.2 Effects of illumination on adhesion

Initial leukocyte adhesion experiments yielded an interesting phenomenon; during continuous illumination adherent leukocytes would break free from the adhesive P-selectin surface and get washed away. This reduction in adhesion was thought to be due to the formation of superoxide free radicals within the leukocytes as a result of the illumination. On the upper surface of the microslide at 37 °C nearly all adherent cells detached within 40 seconds (fig 2.10(a)). When the perspex enclosure was raised to 42 °C, this decrease was seen within 10 seconds (fig 2.10(b)).

A neutral density filter was placed between the light source and the microslide to only allow 25% of the incident light transmission. At both 37°C (fig 2.10(c)) and 42°C (fig 2.10(d)) the reduction in adhesion with time was abolished.

In initial platelet free flow experiments, illumination with full light intensity resulted in platelet activation and adhesion on the surface of the albumin coated microslide. This was also abolished when the 25% neutral density filter was used.

Thus a 25% neutral density filter was used in all further experiments and prolonged illumination of the same field for long periods of time was avoided. Leukocytes and platelets were still clearly visible when the filter was in place.

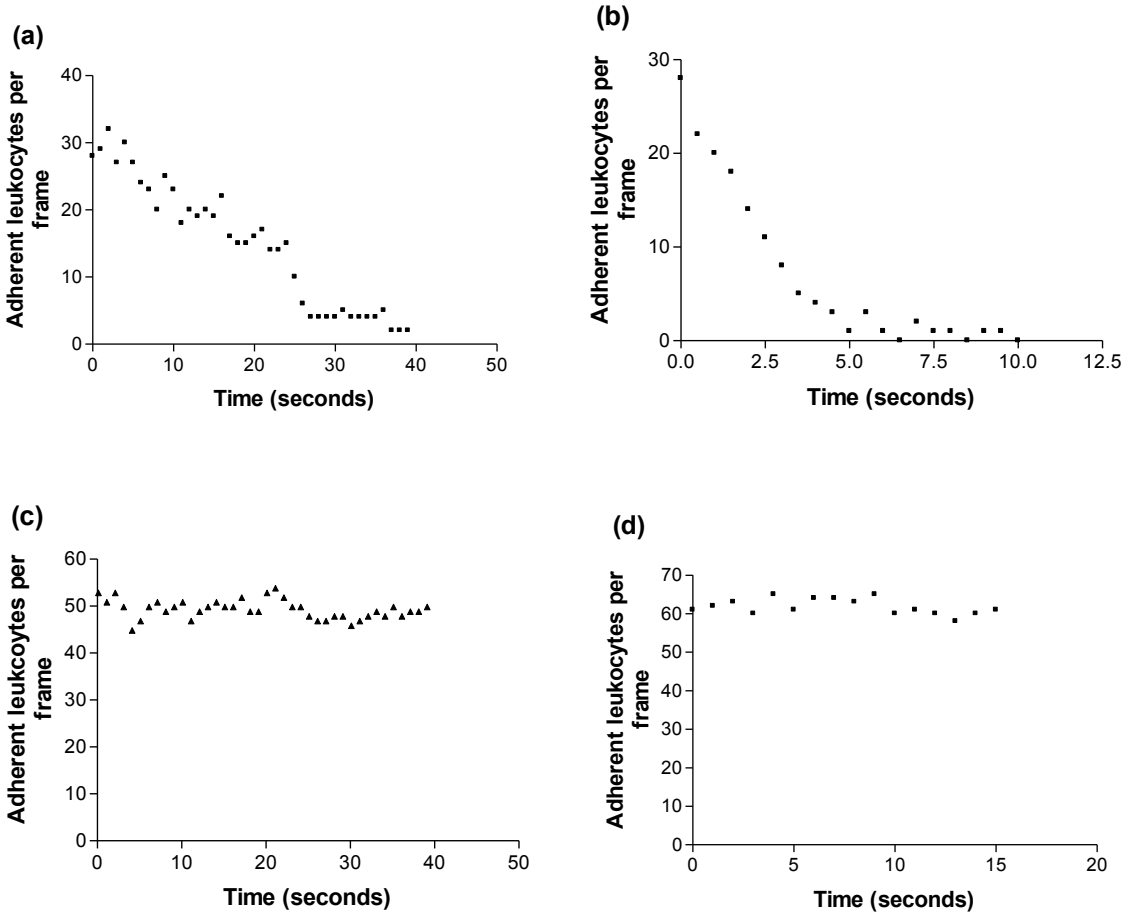


Figure 2.10 Effect of time and illumination on leukocytes remaining adherent to P-selectin

Whole blood, anti-coagulated with CPDA, was perfused through microslides of 100µm in height that had been coated with 10µg/ml of purified P-selectin, at (a,c) 37°C or (b,d) 42°C. Either full intensity (a,b) illumination was used or a 25% of full intensity (c,d) produced by a neutral density filter. The blood was corrected to a haematocrit of 40% and the adherent leukocytes were counted on the upper surface of the microslides at frequent intervals. Data shown are from single experiments. (a) At 37°C the number of leukocytes in the frame were counted every second and after 40 seconds of continuous illumination the number of adherent leukocytes had dropped off to nearly zero. (b) At 42°C the number of leukocytes in the frame was counted every second and after 10 seconds of continuous illumination the number of adherent leukocytes had dropped off to zero. This drop in adhesion at (c) 37°C and (d) 42°C was not seen when the 25% neutral density filter was used.

2.7 Near wall cell concentration and velocity measurements

Manipulating the rheological properties of blood along with haemodynamic variables is likely to affect the distribution of leukocytes and platelets within the flow. To assess the cell distribution in the same size microslides in which we had assessed the adhesion, a similar setup to the adhesion assay was used. The numbers of free flowing cells near the wall were measured along with the velocities of these cells. Microslides were coated with 2% PBSA (as mentioned in section 2.3.2.) to avoid non-specific binding. Theophylline was added to the blood to avoid activation of the platelets or leukocytes. This maintains the cells morphology and further reduces the chances of any non-specific binding. Using a Luca EM-CCD camera (ANDOR Luca DL-658M-TIL, Belfast, UK) that was capable of very short exposure times, rapid frame rates and single photon detection, videos were recorded digitally straight to a computer. The short exposure time (0.00047seconds) resulted in images of free flowing cells that were not blurred. The high sensitivity of the camera resulted in images of high signal to noise ratio at these short exposure times. The quick frame rate (36 Hz for full field, 95 Hz when one third of the field was recorded) allowed the same cell to be tracked over several frames. With the rate of capture known, the distance travelled by the free flowing cell could be used to calculate their velocity. An oil immersion lens with 25x magnification and a numerical aperture of 0.8 (Zeiss Objective LD LCI "Plan-Apochromat" 25x/0.8, Carl Zeiss Ltd, Herts, UK) allowed the field captured to have a thin depth of focus (~2μm), but had a low enough magnification to enable the capture of enough cells in a frame for reliable statistical analysis to be performed. Images were recorded with the objective focussed at 0, 5 and 10μm from the vessel wall to quantify the change in the number of leukocytes and platelets as the focal plane was moved into the vessel lumen. This was done by initially aligning the focal plane of the lens at the vessel wall; transient adherent platelets helped the

alignment. The distance of the focal plane of the lens from the vessel wall was measured using the calibrated fine focus on the microscope.

2.7.1 Free flowing leukocytes

2.7.1.1 Near-wall concentration

Five digital image sequences, each of 100 frames (frame rate 36.258 Hz) were recorded at depths 0, 5 and 10μm into the vessel for each manipulation in the experiment. The images were analysed using Image Pro Plus 6.2.1 (Media Cybernetic, Inc, Bethesda, MD, USA). The software set a threshold of intensity on the image (see section 2.5.4). Leukocytes being much brighter than the background were left visible after the threshold had been imposed. Those that appeared to be very large were out of focus, and an area filter was set manually to count only leukocytes that were in focus and also to discriminate against the occasional smaller platelet that was bright enough to be above the threshold.

After verifying the intensity threshold and setting the area filter, a cell count was done every ten frames by the Image Pro software and averaged to obtain the free-flowing near-wall number density. The number was normalised for frame size and leukocyte concentration in the blood sample, resulting in data expressed as free flowing leukocytes per mm^2 per 10^6 cells in the blood.

2.7.1.2 Near-wall velocity

The videos recorded to assess the free flowing near wall concentrations at different depths were used to measure the free flowing velocity of the leukocytes near the wall. The leukocytes counted every tenth frame were tracked over several frames in the same manner as

rolling leukocytes (see section 2.5.2) and the velocity of these cells was calculated by dividing the distance they had travelled by the time taken to travel that distance.

2.7.2 Free flowing platelets

2.7.2.1 Near-wall concentration

Five digital image sequences, each of 200 frames (frame rate 95.147 Hz) were recorded at depths of 0, 5 and 10μm into the vessel for each manipulation in the experiment. Platelets were counted using software written in MatLab (The MathWorks, Inc, Natick, MA, USA) by Dr. Ali Tonddast (School of Chemical Engineering, University of Birmingham, UK) that assumed fluorescent particles emit light with a Gaussian distribution in spatial intensity. As platelets are not point particles, there was an allowance made for a degree of deviation from a perfect Gaussian distribution of light intensity. A restriction on the upper limit of the radius of the light emitted from the platelet was also included. These parameters could be altered manually. Initially several frames were analysed and the parameters were altered to match the software analysis with human observations; the values were saved and used in all subsequent platelet near wall concentration analysis. This allowed the platelets in focus to be counted objectively in a large number of images. The platelets that were out of the plane of focus appeared larger and had a lower correlation to a Gaussian light distribution, and so were not counted. The leukocytes, that were also labelled, were too big to be counted by the software. The number of platelets in each frame was counted and the average over the 200 frames was determined. The numbers of platelets per frame, along with the platelet concentration in the blood sample, were used to obtain a normalised value of the marginated platelets. The final normalisation was free flowing platelets per mm^2 per 10^8 cells in the blood. This normalisation was appropriate because the individual flowing platelets could be counted. This

was not the case in the adhesion experiments where thrombus build up contained an undetermined number of platelets and area coverage was the analysed parameter.

2.7.2.2 Near-wall velocity

The images recorded to measure platelet concentration were used to measure platelet velocity. To obtain a frame rate that was sufficient to capture the same platelets in several successive fields at high flow rates, the top and bottom thirds (horizontally) of the field were not recorded and only the middle third was used. The sequences were 200 frames long and in every 20^{th} frame, three random cells were tracked as for leukocytes in 3-5 successive frames. From the distance they had travelled between the frames their velocity was calculated. Above a wall shear rate of $300s^{-1}$, image quality decreased significantly, as a result only this shear rate could be used for the assessment of free-flowing platelets near the vessel wall.

2.8 Viscometry

The flow system was adapted to measure the apparent viscosity of the blood flowing through the microslide. T-pieces were attached adjacent to the inlet and outlet of the microslide (Harvard Apparatus Ltd, Kent, UK) and connected to a pressure transducer (type 4-4222 Bell & Howell Ltd., UK) using silicone tubing. The pressure transducer was attached to MacLab/2s via a MacLab bridge amplifier (AD Instruments Ltd) and the pressure was recorded by MacLab Chart software (AD Instruments Ltd). This enabled the pressure of the inlet and the outlet to be determined individually. The inlet pressure was recorded for 30 seconds prior to one minute of outlet pressure recording at each flow rate used. This was done to ensure any changes in inlet pressure associated with changes in the level of the blood sample in the reservoir were recorded and the true pressure difference could be calculated at each flow rate. Being aware of the potential effects on flow resistance of sedimentation of

blood at low flow and in samples with enhanced aggregation (Alonso et al., 1989) it was necessary to avoid prolonged perfusion of the microslide at low flow rates. This was achieved by setting the flow rate to a high value equivalent to a wall shear rate of $1500s^{-1}$ on the withdrawal pump for 10 seconds, after the inlet pressure had been recorded for 30 seconds, to effectively mix the blood. The flow rate was then reset to the initial value to enable the outlet pressure to be recorded. This ensured a minimal effect of sedimentation on pressure measurements in the horizontally orientated microslides. The shear rates at which the blood was perfused in the tube viscometer covered the entire range of shear rates used in all adhesion experiments ($70\text{-}1500s^{-1}$). Using the values of pressure difference between inlet and outlet pressure, the volumetric flow rate and the dimensions of the microslide, the apparent viscosity of the blood, and wall shear stress, at each nominal wall shear rate could be calculated using equation 18 (See section 1.2.5.2.).

2.8.1 Validation of the viscometer

Upon construction of the tube viscometer it was necessary to ensure that the calculated values of fluid viscosity were accurate. The pressure transducer was calibrated daily using a mercury sphignomanometer to ensure accurate pressure readings. A two point calibration was used, setting zero pressure to be atmospheric pressure and attaching a sphignomanometer directly to the pressure transducer and applying a fixed pressure corresponding to the upper limit of measured outlet pressure for the experiment. To check the calculated viscosity of liquids flowing through the microslides simple non-newtonian fluids were used. At 37°C the viscosity of water is 0.692mPa.s (Robert C.Weast, 1975). The viscometer was first validated using distilled water at 37°C and a calibration oil at 25°C (with a viscosity of 5.1mPa.s). The viscosity was measured and was constant over a range of shear rates (fig 2.11). The value obtained for the viscosity of water at 37°C averaged 0.693mPa.s over the range of shear rates tested. The viscosity of the calibration oil at 25°C average 5.1mPa.s. Dextran solutions with a

range of concentrations were also tested. These newtonian fluids covered the full range of viscosities used in this thesis. The gradients of viscosity vs. shear rate did not deviate significantly from zero, demonstrating that over the full range of flow rates and viscosities, the viscometer maintained its performance. Thus any deviation from a flat line subsequently observed when plotting apparent viscosity of the blood against shear rate was a result of the particulate, non-newtonian nature of the blood and not an artefact of the viscometer.

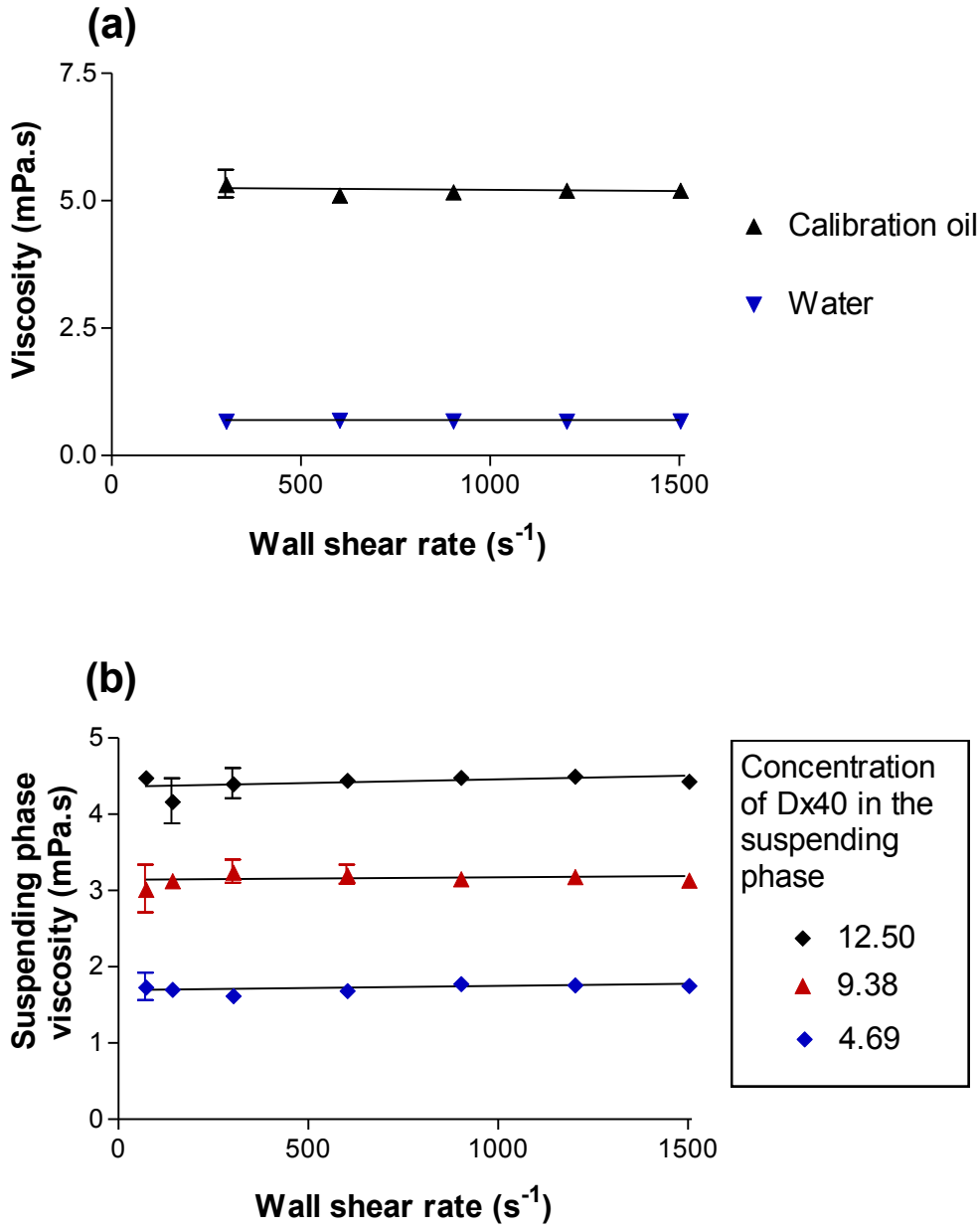


Figure 2.11 Viscosities of (a) water and (b) dextran solutions measured at a range of wall shear rates at 37°C.
In (a) water at 37°C and a calibration oil at 25°C were perfused through a microslide of 100μm in height. In (b), whole blood mixed with dextran 40 solutions with different concentration (see section 2.2.4) was centrifuged and the suspending phase retrieved and perfused through a microslide of 100μm in height. Data shown are means ± SEM from 3 experiments. Numbers represent concentration of Dx40 in blood plasma. In all cases, the viscosity of the medium showed no dependence on shear rate (slope of lines not significantly different from zero by linear regression). The viscosity of the suspending phase however increased significantly as the concentration of dextran increased ($P<0.0001$, one way ANOVA).

2.9 Statistical analysis

Graphically presented data in this thesis are the mean ± SEM of n independent experiments using n different donors for margination, adhesion and tube viscometry experiments for both leukocytes and platelets. Variation between multiple treatments was evaluated using one-way or two-way analysis of variance (ANOVA) as appropriate, followed by Dunnett's test for comparison to control or by Bonferroni multiple comparison post-test between treatments. In some experiments evaluating effects of continuous variables, linear regression was used to evaluate significance of trends. For studies consisting of only 2 samples, the variation between them was analysed using a paired t-test.

CHAPTER 3: EFFECT OF VESSEL SIZE AND WALL SHEAR RATE ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

3.1 Introduction

Investigations were made into the effects of the size of the vessel and the wall shear rate on leukocyte and platelet margination and adhesion in the same assay. Previous studies to quantify leukocyte rolling adhesion in whole blood were performed in 300μm depth microslides (Abbitt and Nash, 2001;Abbitt and Nash, 2003) which is comparable to a small artery but large compared to the diameter of post-capillary venules in which leukocyte rolling adhesion typically occurs (Lipowsky, 1988). Here, smaller microslides of 100μm depth were also used, which is closer to the size of post-capillary venules. Experimentally this allowed a small volume of blood to be used to produce the wall shear rates required. In the larger 300μm microslides a maximum shear rate of $560s^{-1}$ was applied and in the smaller 100μm microslides a range of wall shear rates up to $1500s^{-1}$ could be obtained without the use of excessive volumes of blood. Although various vessel sizes have been used in the past to quantify leukocyte and platelet adhesion, a direct comparison of the efficiency of adhesion in different-sized vessels has not been reported.

The apparatus used in this thesis did not have the capability to perfuse the microslides vertically. It was therefore necessary to determine whether there was any effect of sedimentation within the microslide. This was done by quantifying the adhesion of leukocytes and platelets on the upper and lower surfaces of the microslides. Adhesion studies have largely ignored the effects of sedimentation. When isolated suspensions of leukocytes are perfused horizontally, the rate of sedimentation strongly affects leukocyte adhesion to the lower surface of a vessel (Munn et al., 1994). In blood, the sedimentation of all cell types could potentially affect levels of adhesion.

We hypothesised that, due to their difference in size, and their likely difference in response to RBC aggregation, adhesion and margination of platelets would be less sensitive to shear rate

than leukocytes. Our aims for this study were therefore, for the first time, to study the effect of shear rate on leukocyte and platelet margination and adhesion in the same vessels perfused in an identical system.

3.2 Methods

3.2.1 Blood collection and preparation

Venous blood was anticoagulated with CPDA. In studies of leukocyte adhesion using 100μm microslides, we noticed adhesion tended to decrease with time of storage of the blood even over 1 to 2 hours. Thus the order of measurements was rotated in the studies of varying shear rate. During studies of leukocyte adhesion with 300μm microslides, we discovered that inclusion of theophylline greatly reduced the storage effect. Thus theophylline (final concentration of 7mM) was included in the blood for these studies, which were nevertheless done in rotation. In adhesion experiments, blood was perfused at the native haematocrit (mean of 42.5% in these experiments). In the margination experiments, the blood was adjusted to 40% haematocrit by the addition of autologous plasma, as part of experiments which analysed the effects of modifying plasma constituents at constant haematocrit (see Chapter 5).

3.2.2 Microslide coating

For experiments on leukocyte adhesion, microslides were coated in purified P-selectin at a concentration of 10μg/ml. For experiments on platelet adhesion, microslides were coated with Horm collagen at 500μg/ml. For margination and tube viscometry experiments, the microslides were coated with albumin to provide a non-adhesive surface.

3.2.3 Adhesion assay

Blood was fluorescently labelled with 5μg/ml R6G. To measure the adhesion of leukocytes to P-selectin, blood was perfused at shear rates of $70s^{-1}$ to $280s^{-1}$ in either the smaller or the larger microslides for a total of 4 minutes from the time the microslide was first filled with

blood. To measure platelet adhesion to collagen, the blood was perfused at shear rates ranging from 70-560s^{-1} in the larger microslides and 300-1500s^{-1} in the smaller microslides. Adhesion was measured up to 4 minutes after the microslide was first filled with blood.

3.2.4 Assessment of Margination

Margination was analysed by perfusing fluorescently labelled blood through albumin-coated microslides. The microslides were perfused horizontally and the free flowing cells were measured at the upper surface of the microslide. Leukocyte experiments were performed at the same range of shear rates as the adhesion experiments (70-280s^{-1}) in the 100μm microslides. A comparison was made between the leukocyte margination in the smaller 100μm microslides and the larger 300μm microslides at a wall shear rate of 140s^{-1}. Platelet margination was assessed at 300s^{-1}; higher rates could not be used because images of flowing platelets at higher shear rates were not distinct enough to allow tracking.

3.2.5 Tube viscometry

The wall shear rates at which the blood was perfused through the tube viscometer covered the entire range of 70-1500s^{-1}. Using the values of pressure difference between inlet and outlet, the flow rate and the dimensions of the microslide, the apparent viscosity of the blood at each nominal wall shear rate was calculated using equation 18 (see page 14).

3.3 Results

3.3.1 Effects of vessel size, sedimentation and wall shear rate on leukocyte adhesion

Initially the smaller microslides were used to assess the effect of sedimentation and shear rate on leukocyte adhesion. On both the upper and lower surface of the microslide, the number of adherent leukocytes was significantly decreased with increasing shear rate, but there was no significant difference in adhesion between the upper and lower surfaces at any wall shear rate (fig 3.1(a)). All adherent leukocytes were seen rolling on the P-selectin coating on the vessel wall. The rolling velocity of the adherent leukocytes showed no shear rate-dependence (fig 3.1(b)). There was no overall difference between the rolling velocities of the leukocytes on the lower or on the upper surface.

Experiments were repeated in the larger 300μm microslides to assess whether the vessel size affected the efficiency of adhesion, rolling velocity and sedimentation-dependence of leukocyte adhesion. Again, adhesion was significantly decreased as shear rate was increased and there was no significant difference in leukocyte adhesion between the upper and lower surfaces of the microslides at any wall shear rate (fig 3.2(a)). The rolling velocity of the adherent leukocytes showed no dependence on wall shear rate and no difference between the upper or lower surface of the microslide (fig 3.2(b)).

After correcting the values of adhesion for differences in the volumetric flow rate and internal surface area of the microslides of different size, comparing leukocyte adhesion in the 100μm and 300μm microslides showed that leukocyte adhesion was much more efficient in the 100μm microslides (fig 3.3). The rolling velocity of adherent leukocytes was slower in the larger microslides. This may have been due to the use of theophylline in the 300μm microslide experiments which would delay unwanted neutrophil activation during blood

storage. Activation has been associated with impairment of binding to selectins (Davenpeck et al., 2000), and may have been the cause of the faster rolling in 100μm microslides where theophylline was not used. Such activation could not, however, explain the much greater efficiency of adhesion in the 100μm microslides.

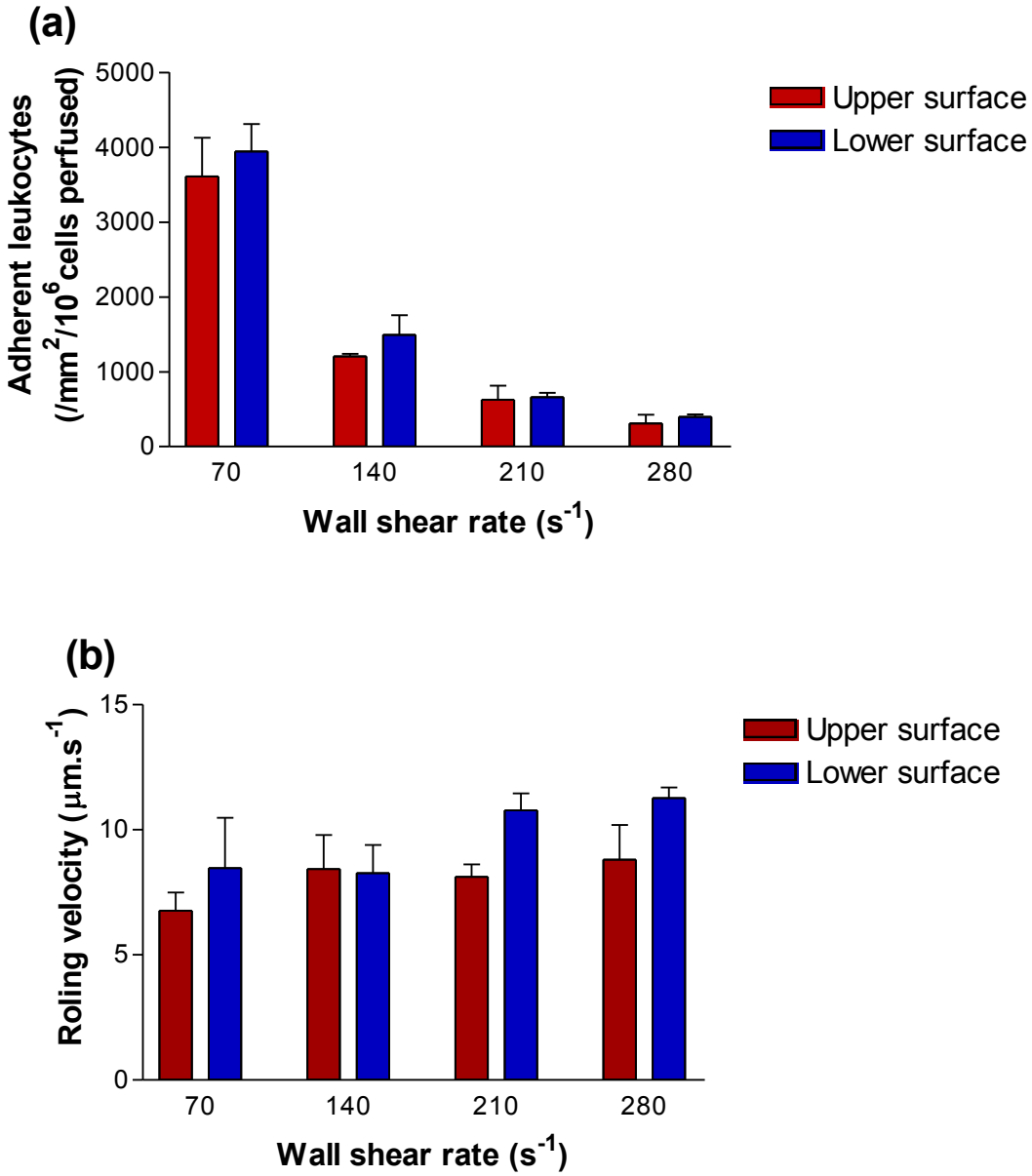


Figure 3.1 Effect of wall shear rate on the number of leukocytes adhering and their rolling velocity on P-selectin in 100µm microslides

Whole blood was perfused through microslides of 100µm in height that had been coated with P-selectin. The blood had an average haematocrit of 42.6%. The numbers of adherent leukocytes were counted on the upper or lower surface of the microslides. Data shown are means ± SEM from 3 experiments. Leukocyte adhesion was normalised per 10^6 cells perfused. (a) ANOVA showed no significant difference between the upper and lower surface, but a significant effect of wall shear rate ($P<0.0001$). (b) ANOVA showed no significant effect of surface or shear rate on rolling velocity of leukocytes.

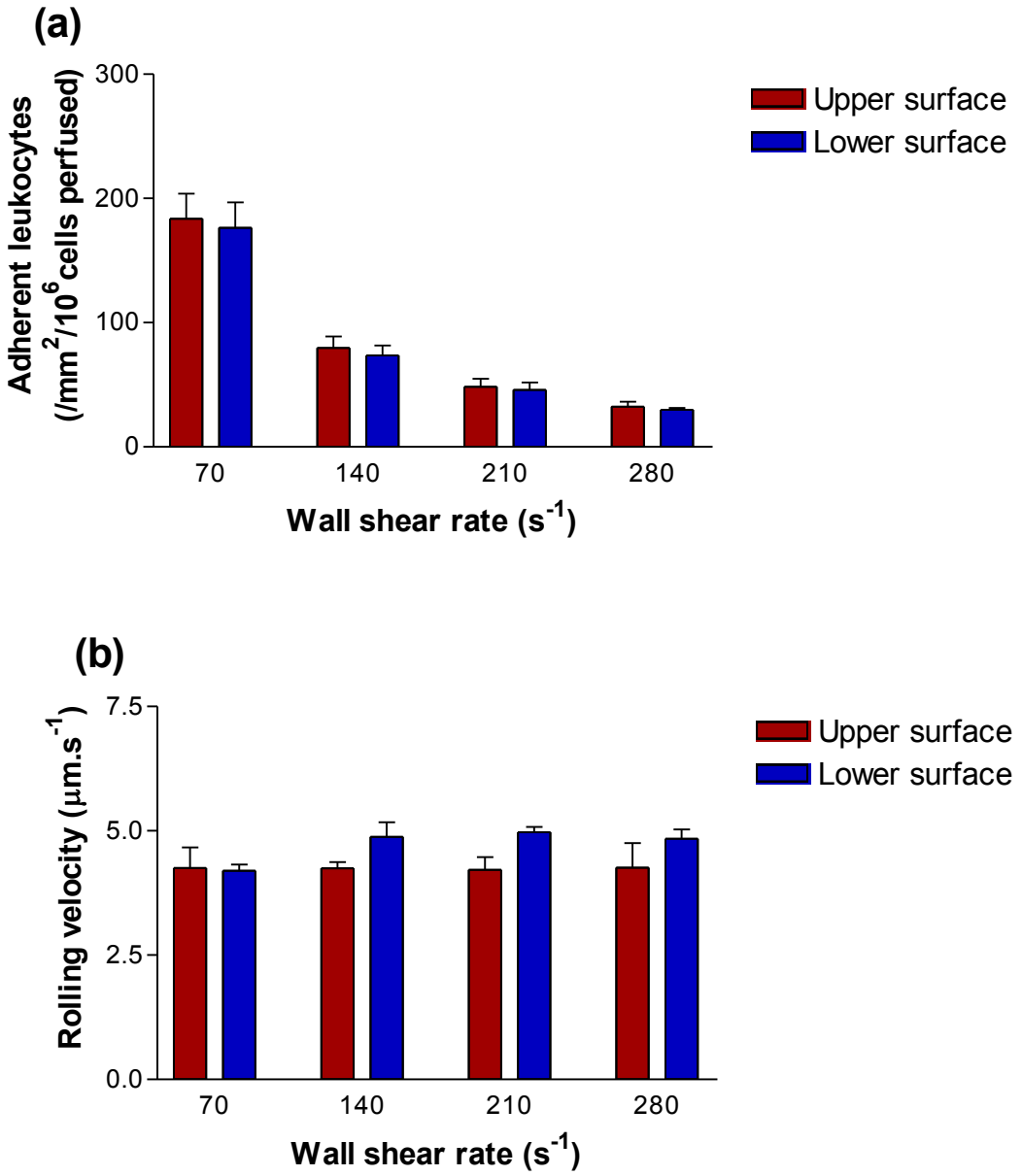


Figure 3.2 Effect of wall shear rate on the number of leukocytes adhering and their rolling velocity on P-selectin in 300µm microslides
Whole blood was perfused through microslides of 300µm in height that had been coated with P-selectin. The blood had an average haematocrit of 42.3%. The numbers of adherent leukocytes were counted on the upper or lower surface of the microslides. Data shown are means ± SEM from 3 experiments. Leukocyte adhesion was normalised per 10^6 cells perfused. (a) ANOVA showed no significant difference between the upper and lower surface, but a significant effect of wall shear rate ($P<0.0001$). (b) ANOVA showed no significant effect of surface or shear rate on rolling velocity of leukocytes.

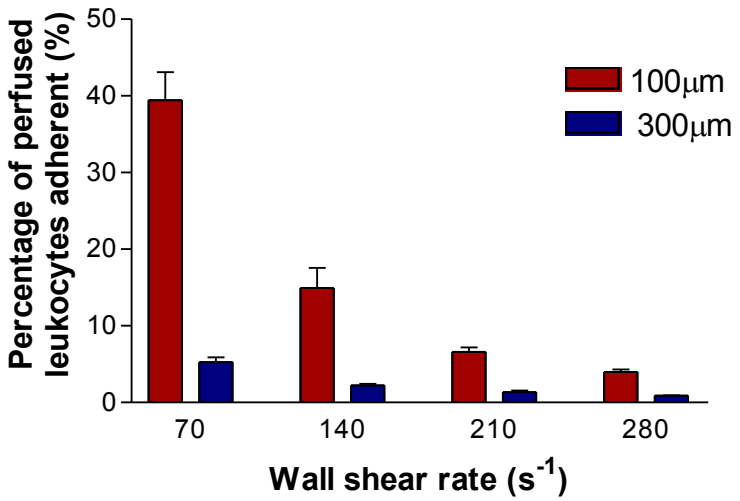


Figure 3.3 Comparison of leukocyte adhesion in 100μm and 300μm microslides. Whole blood was perfused through microslides of 100μm or 300μm in height that had been coated with P-selectin. The blood had an average haematocrit of 42.8%. The numbers of adherent leukocytes were counted on the lower surface of the microslides. Data shown are means ± SEM from 3 experiments in both the 100μm and 300μm microslides. Total leukocyte adhesion per microslide was normalised as a percentage of total perfused leukocytes, to account for differences in flow rate and surface area. There was a significant decrease in the number of adherent leukocytes as shear rate was increased and significantly more efficient binding in the smaller microslides than the larger (both $P<0.0001$ by ANOVA).

3.3.2 Effects of vessel size, sedimentation and wall shear rate on platelet adhesion

Initially platelet adhesion was studied in the larger 300μm microslides. The percentage coverage of platelets increased with time of perfusion in both vessel sizes at all shear rates used (fig 3.4). In addition, at the end of the bolus (after 4 minutes), there was no significant difference between coverage of the lower and upper surfaces of the microslide. Perfusing whole blood through 300μm microslides was limited to a maximum wall shear rate of $560s^{-1}$ due to the volumes of blood needed. Platelet coverage (i.e., percentage of the surface covered with fluorescent platelets) after 3 minutes, when corrected for the volume of blood perfused, showed no significant change as shear rate was increased (fig 3.5(a)).

Assessing platelet adhesion in the smaller microslides allowed larger shear rates to be produced. The smaller cross section of 100μm x 1000μm allowed much smaller volumes to be used. Wall shear rates on the arterial side of the circulation can be over $1000s^{-1}$ which resulted in the choice of wall shear rates used in these experiments. Altering wall shear rate from 300-$1500s^{-1}$ showed a downward trend in the adhesion of platelets to collagen in the 100μm vessels after 3 minutes when the percentage coverage had been normalised for volume of blood delivered (fig 3.5(b)). Upon completion of blood perfusion (after 4 minutes), flow was stopped and the microslide was disconnected form the perfusion system. In both sizes of microslide the percentage coverage of platelets was recorded from the lower surface and the microslide was then inverted and the platelet coverage of the upper surface was recorded. There was no significant difference in the platelet adhesion to the upper or lower surface of the 100μm microslides (fig 3.6(a)) or the 300μm microslides (fig 3.6(b)) at any shear rate. However, these final levels of adhesion did decrease significantly with increasing shear rate for the 100μm microslides.

To directly compare platelet adhesion in the different vessel sizes, the actual area covered (in mm^2) per ml of blood perfused was calculated. The platelet count in the blood was not used for normalisation as the number of platelets in each adherent platelet thrombi could not be quantified. Comparison of the platelet coverage on the lower surface showed that platelet adhesion tended to be more efficient in the smaller microslides (fig 3.7). There was limited overlap in the shear rates used in the two different microslides, and the difference in efficiency of adhesion was not as great as for leukocytes.

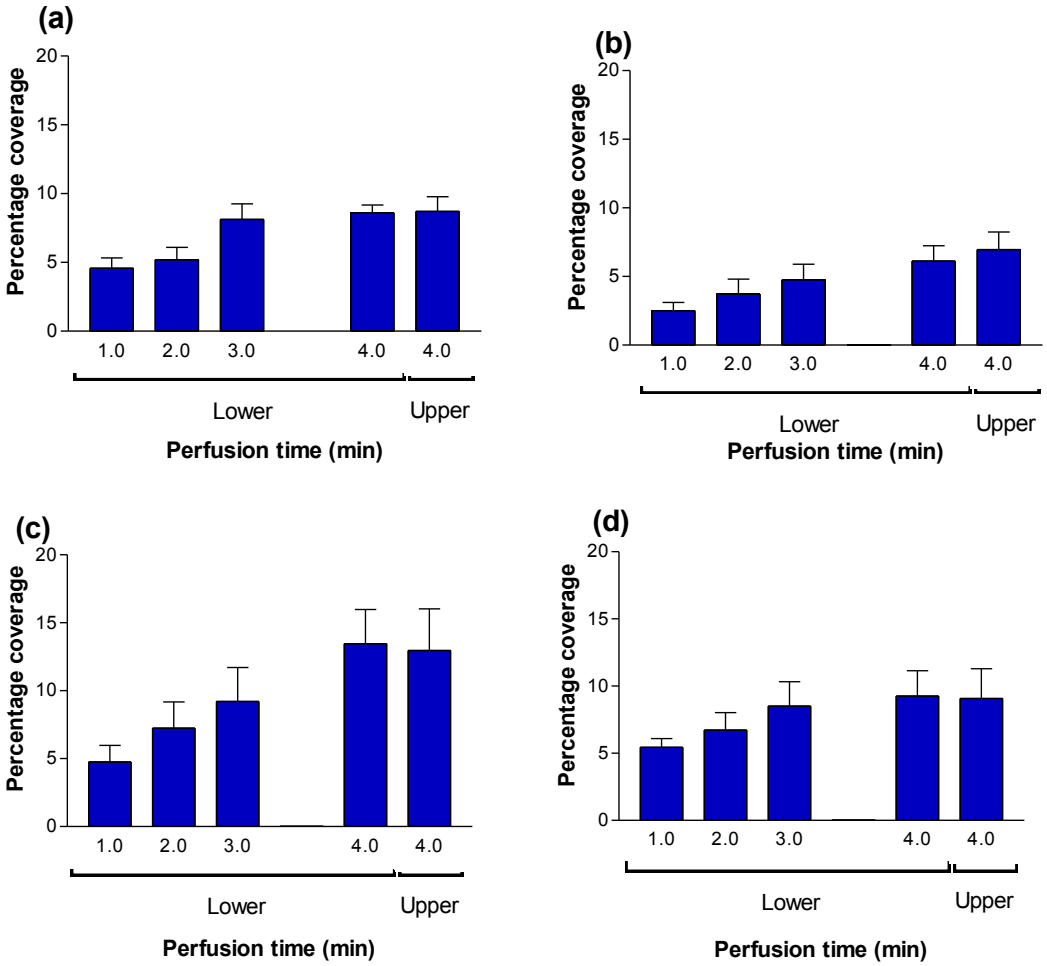


Figure 3.4 Time course of platelet adhesion at various wall shear rates
Whole blood was perfused through microslides that had been coated with collagen. Data shown are means ± SEM of the platelet coverage at 1 minute intervals from 3-4 experiments. The percentage coverage of the upper and lower surface of the microslide after completion of the perfusion experiment are also shown. The shear rate and vessel sizes used were respectively; (a) $280s^{-1}$ in 300µm, (b) $300s^{-1}$ in 100µm, (c) $900s^{-1}$ in 100µm and (d) $1500s^{-1}$ in 100µm.

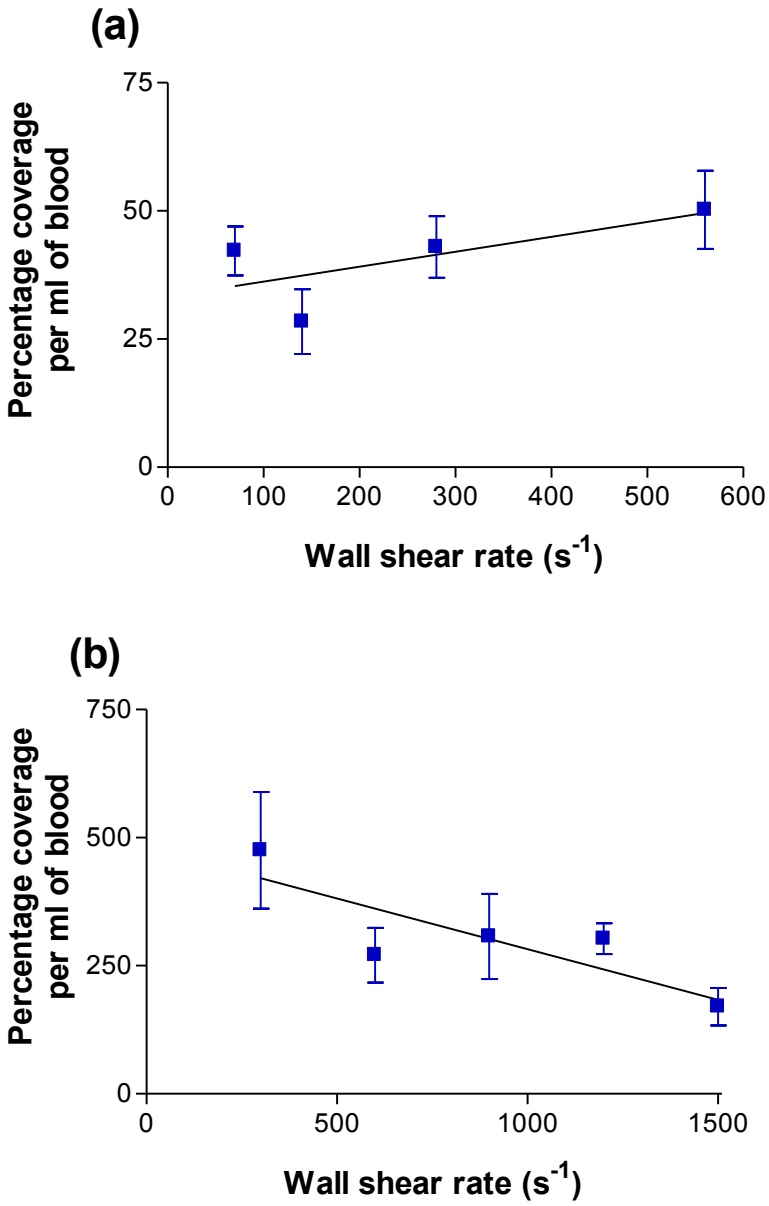


Figure 3.5 Effect of wall shear rate on platelet adhesion in 300µm and 100µm microslides

Whole blood was perfused through microslides of (a) 300µm or (b) 100µm in height that had been coated with collagen. The blood had an average haematocrit of (a) 41.9% or (b) 43.2% and the platelet coverage was quantified every minute. Data shown are means ± SEM of the percentage coverage of platelets at 3 minutes from 3-4 experiments. (a) No significant effect of wall shear rate was found when percentage coverage was corrected for volume of blood perfused. The line of best fit is shown, based on linear regression. (b) The effect of wall shear rate was of marginal statistical significance when percentage coverage was corrected for volume of blood perfused (P=0.08 by ANOVA). However, linear regression showed a significant correlation between coverage and shear rate, and a negative gradient on the line of best fit to the data (P=0.019).

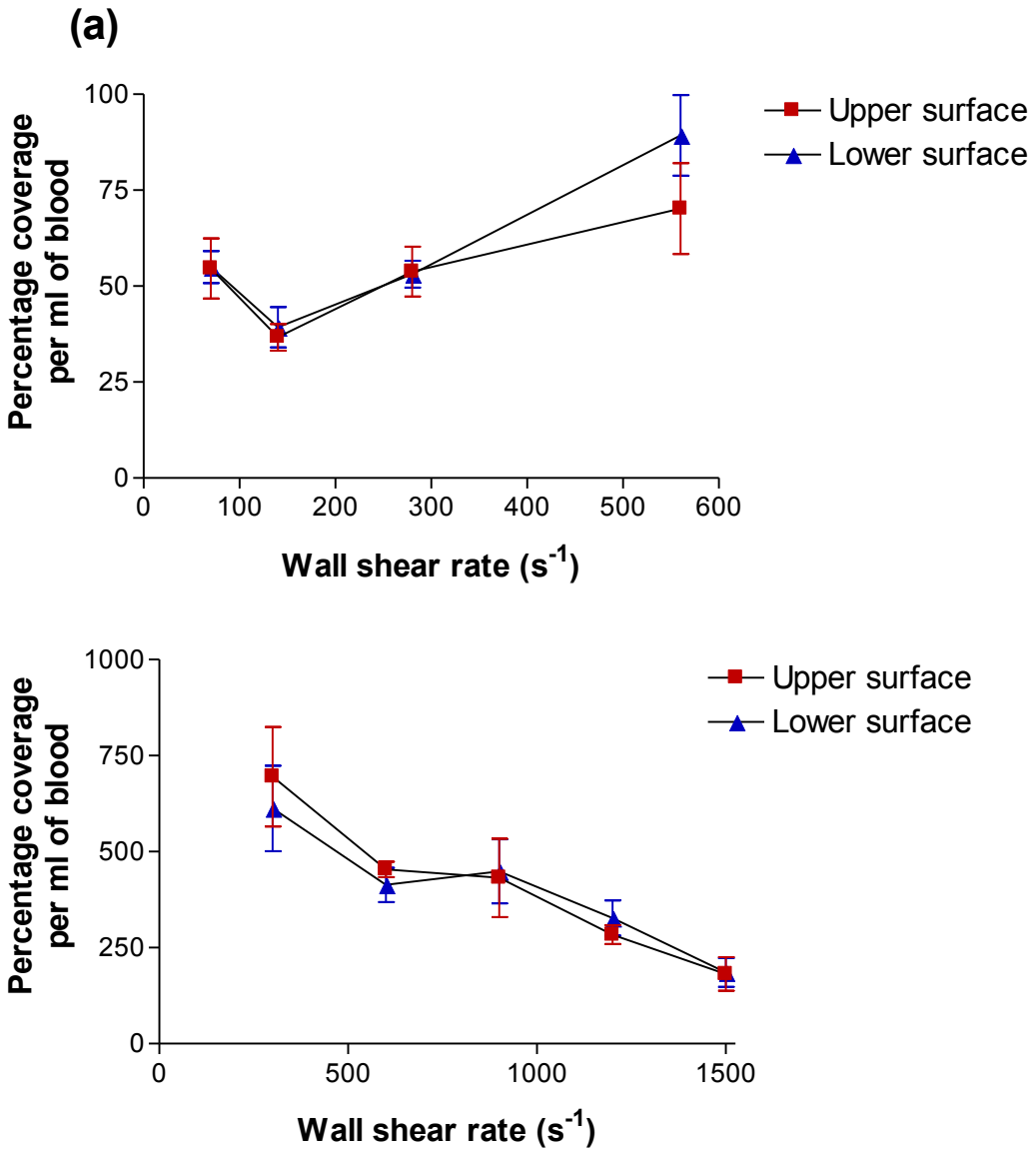


Figure 3.6 Comparison of platelet coverage on the upper and lower surfaces of (a) 300µm microslides and (b) 100µm microslides
Whole blood was perfused through microslides of (a) 300µm or (b) 100µm in height that had been coated with collagen. The blood had an average haematocrit of (a) 41.9% and (b) 43.2%. Data shown are means ± SEM of the percentage coverage of platelets from 3-4 experiments from the upper and lower surface of the same microslide upon completion of perfusion (after 4 minutes). No significant difference in adhesion to the upper and lower surface of the microslide was found in either size of microslide. In (b) there was a significant effect of shear rate on coverage ($p<0.01$ by ANOVA).

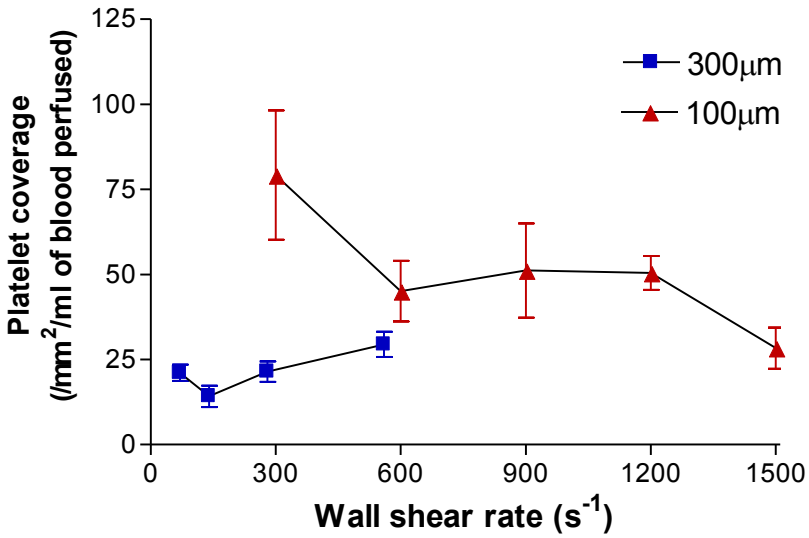


Figure 3.7 Comparison of platelet adhesion in 100µm and 300µm microslides
Whole blood was perfused through microslides of 100µm or 300µm in height that had been coated with collagen. The blood had an average haematocrit of 42.6%. Data shown are means ± SEM from 3-4 experiments. The percentage coverage of platelets on the lower surface of the microslides, after 3 minutes perfusion, was normalised per mm^2 per ml of blood perfused to account for differences in vessel size and flow rate. Although there was limited overlap of the shear rates used, adhesion appeared more efficient in the smaller microslides.

3.3.3 Comparison of the effects of wall shear rate on platelet and leukocyte adhesion

Direct comparison in the same system, using 100μm microslides, showed the differing effects of shear rate on leukocyte and platelet adhesion. It is clear that leukocytes are much more sensitive to changes in wall shear rate than platelets. Where leukocyte adhesion dropped dramatically up to a wall shear rate of $280s^{-1}$, platelet adhesion showed a weaker downward tend and remained efficient over the full range of shear rates used in these experiments (300-$1500s^{-1}$) (fig 3.8).

3.3.4 Effect of vessel size and wall shear rate on leukocyte margination

3.3.4.1 Effect of wall shear rate on leukocyte margination

In 100μm microslides, there was a significant decrease in the number of free flowing leukocytes adjacent to the vessel wall and at 5μm into the vessel lumen as the shear rate was increased from 70 to $140s^{-1}$ and no change thereafter (fig 3.9(a)). At a distance of 10μm from the vessel wall, changes in the number of free flowing leukocytes were less marked as shear rate was increased. Expressed differently, there tended to be more leukocytes visible in the flow immediately adjacent to the wall, than at 5 or 10μm into the bloodstream at the lowest shear rate, but the numbers evened out as shear rate was increased (fig 3.9(a)). Thus, overall, margination towards the wall was greater at the lowest shear rate. The velocity of the free flowing leukocytes at the vessel wall showed a linear increase with increasing shear rate (fig 3.9(b))

3.3.4.2 Effect of vessel size on leukocyte margination

There was no significant difference in the concentration of free-flowing leukocytes adjacent to the vessel wall in the 100μm and 300μm microslides at a wall shear rate of $140s^{-1}$. At

distances of 5 and 10μm from the vessel wall, however, the number of free-flowing leukocytes was slightly but significantly higher in the smaller 100μm microslides (fig 3.10(a)).

The velocity of the free flowing leukocytes was significantly higher in the larger 300μm microslides in the layers close to the wall (fig 3.10(b)). Free flowing velocity increased further from the vessel wall, as expected. Therefore in subsequent experiments only the velocity of the free flowing cells adjacent to the wall will be presented as this is directly relevant to the adhesion of the leukocytes or platelets.

3.3.5 Platelet margination

The volume of blood needed to perform the margination experiments in the 300μm microslides was impractically large. Therefore only the 100μm microslides were used in platelet margination experiments. Due to the imaging and software limitations mentioned above (see section 2.7.2.2) platelet margination could only be quantified at $300s^{-1}$. At $300s^{-1}$ the average platelet count at the vessel wall was 1075 cells/mm^2/10^8 cells in the blood and the average velocity was 1242 μms^{-1}. In subsequent chapters, changes in margination will be presented for experiments manipulating blood rheology, all at $300s^{-1}$.

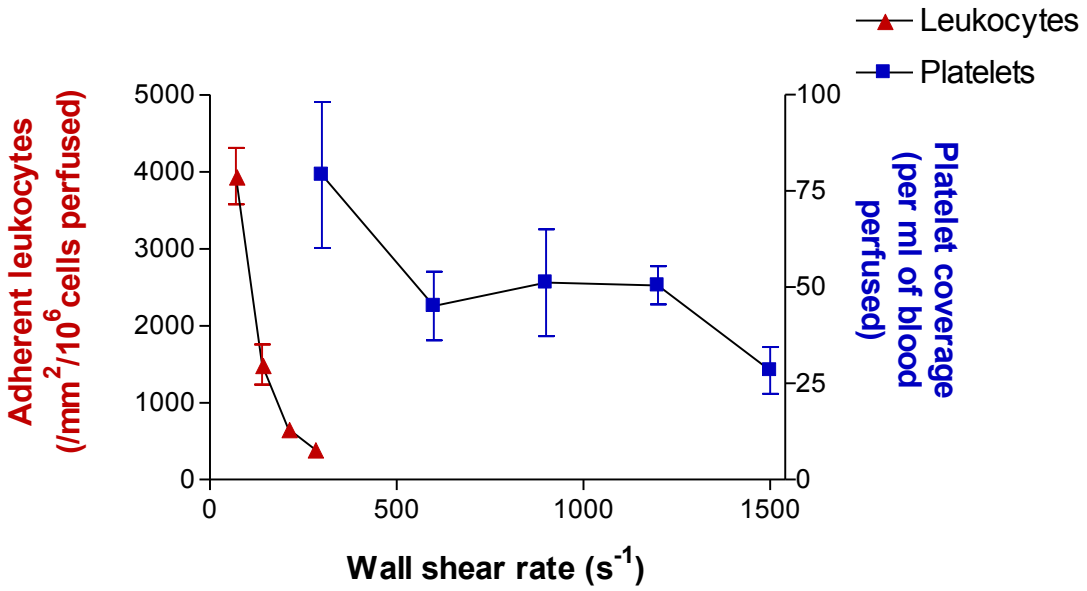


Figure 3.8 Comparison of the effects of wall shear rate on leukocyte and platelet adhesion

Whole blood was perfused through microslides of 100µm in height that had been coated with P-selectin or Horm collagen for assessment of leukocyte or platelet adhesion respectively. Data are means ± SEM of 3 experiments for leukocytes and 4 experiments for platelets. Leukocyte adhesion decreased rapidly between 70 and 280s^{-1}. Platelet adhesion was much less sensitive to the increase in wall shear rate.

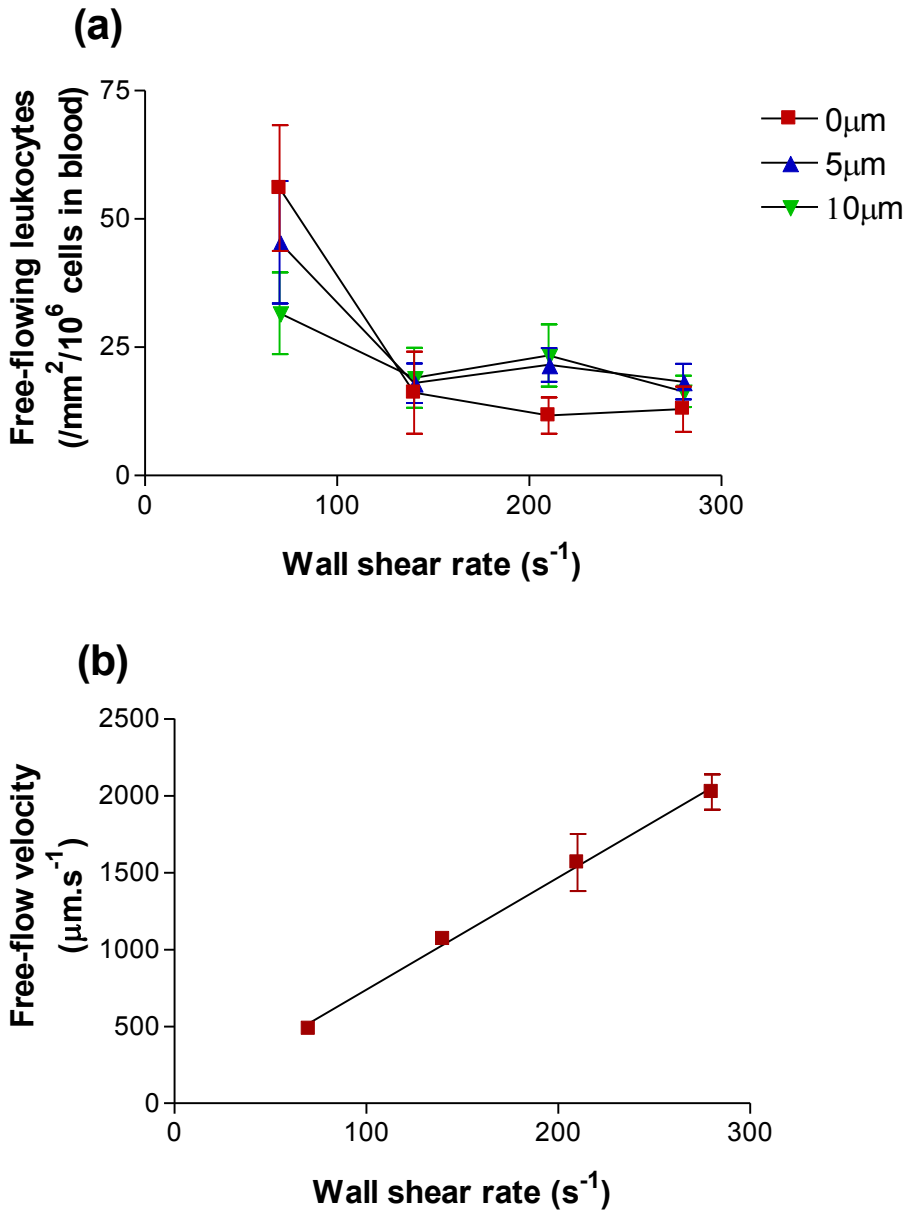


Figure 3.9 Effect of wall shear rate on the number and velocity of leukocytes flowing near the vessel wall

Blood, adjusted to 40% haematocrit, was perfused through microslides of 100μm in height that had been coated with albumin. Data shown are means ± SEM from 3 experiments assessing; (a) the number of free-flowing leukocytes adjacent to the vessel wall (0μm), 5μm and 10μm into the vessel lumen, and (b) the velocity of free-flowing leukocytes adjacent to the vessel wall (0μm). (a) The decrease in the number of free flowing leukocytes adjacent to the wall as wall shear rate was increased was significant ($P<0.01$ by ANOVA). However there was no significant difference between numbers at wall shear rates of 140-280s^{-1}. (b) ANOVA showed that increasing the shear rate resulted in a significant increase in the velocity of the leukocytes flowing adjacent to the vessel wall (0μm) ($P<0.001$). Linear regression fitted a line with intercepts on x and y axes very close to and not significantly different from zero.

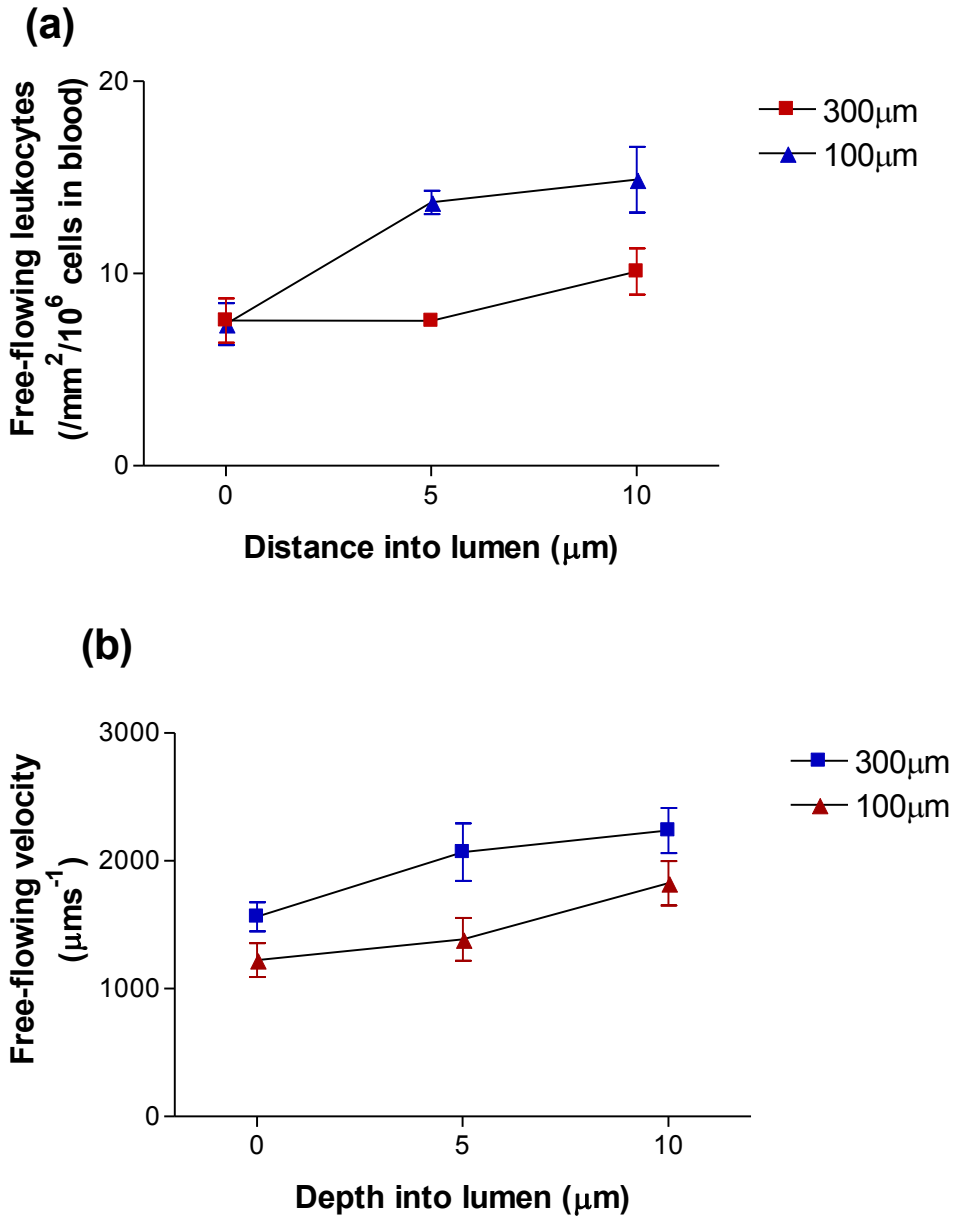


Figure 3.10 Effect of vessel size on the number and velocity of leukocytes flowing near the wall at a wall shear rate of 140s^{-1}
Blood, adjusted to 40% haematocrit, was perfused through 300μm or 100μm microslides that had been coated with albumin. Data shown are means ± SEM from 3 experiments assessing (a) the number of free flowing leukocytes at various depths from the vessel wall and (b) the velocity of these cells. (a) ANOVA showed significant effects of vessel size and distance from the wall on cell number ($P<0.01$ in both cases). At the vessel wall there was no difference in the concentration of leukocytes, however the leukocyte concentration was significantly higher in the 100μm microslides at 5μm ($P<0.01$ Bonferroni post test) and 10μm ($P<0.05$, Bonferroni post test). (b) ANOVA showed significant effects of vessel size and distance from the vessel wall on velocity of the leukocytes ($P<0.01$).

3.3.6 Effect of shear rate and vessel size on the apparent viscosity of the blood

The apparent viscosity of the blood flowing within the microslides of different size was measured to compare non-newtonian behaviour and the average wall shear stress at the same nominal wall shear rates. The full range of shear rates used in leukocyte and platelet adhesion experiments were applied. Pressure differences across the microslides were measured and increased with increasing wall shear rate (fig 3.11(a)). The apparent viscosity of the blood calculated for the 300μm microslides was significantly higher than that calculated for the smaller microslides (fig 3.11(b)). In both sizes of microslide, the apparent viscosity of the blood increased at the lowest shear rates.

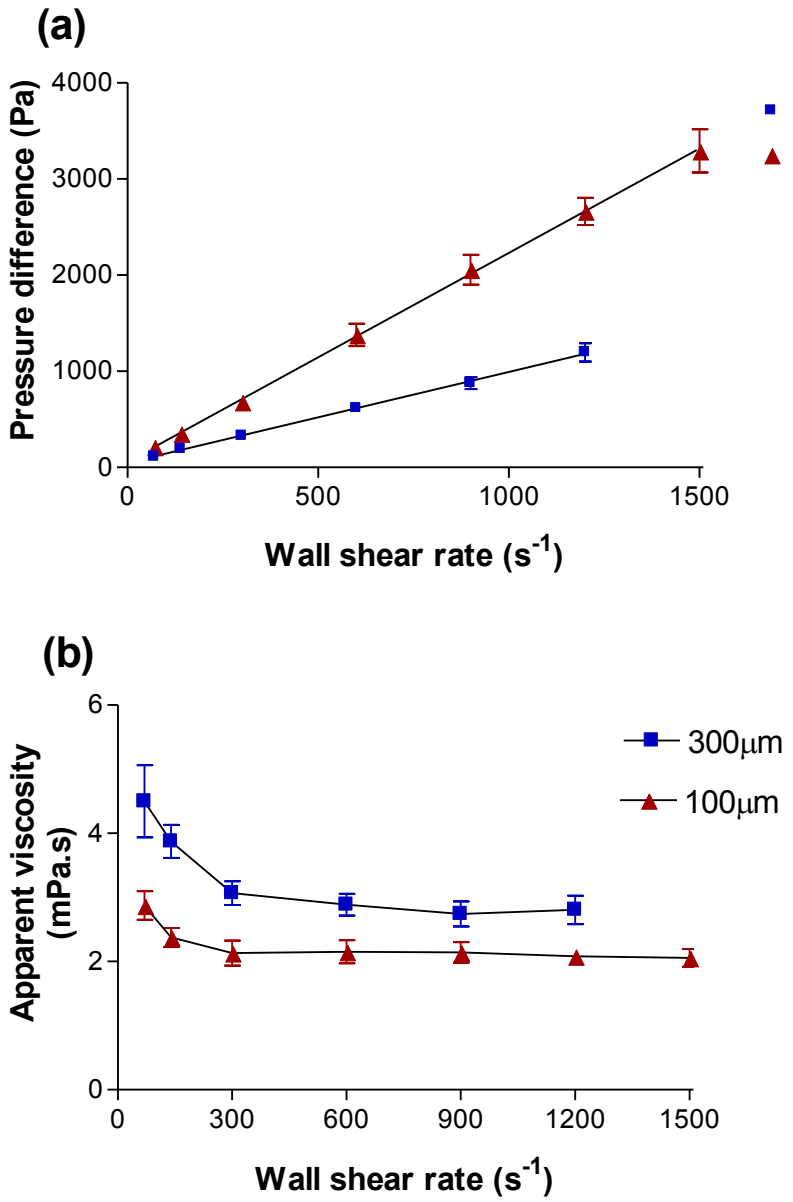


Figure 3.11 Effects of wall shear rate and vessel size on the pressure difference across the microslides and the apparent viscosity of the blood
Blood, adjusted to haematocrit of 40%, was perfused through microslides coated with albumin. (a) The pressure difference across the microslides was significantly greater for the 100µm microslides compared to the 300µm microslides ($P<0.0001$ by ANOVA).
(b) The apparent viscosity of the blood within the microslide was calculated using equation 18 (section 1.2.5.2). The apparent viscosity of the blood was significantly higher in the larger microslide ($P<0.0001$ by ANOVA)

3.4 Discussion

Here for the first time the shear-dependence of leukocyte and platelet adhesion has been quantified in the same experimental model. Furthermore we investigated the effects of vessel size and sedimentation in horizontal tubes. The shear-dependence of leukocyte margination was also quantified in the same assay used to quantify leukocyte adhesion. We found that increasing shear rate decreased leukocyte adhesion between 70-280s^{-1} in both vessel sizes. The shear sensitivity of platelets was much lower than for leukocytes, as platelet adhesion was still high at a wall shear rate of 1500s^{-1}. No differences in adhesion between upper and lower surfaces were found in any experiments at any shear rates, showing that there was little or no effect of sedimentation in this assay. The adhesion of both cell types was more efficient in the smaller microslides, but the adhesion of leukocytes appeared more sensitive to vessel size.

3.4.1 Effect of sedimentation on the adhesion of leukocytes and platelets

The system used here could not be perfused vertically and so the possible effects of sedimentation of cells on the adhesion of leukocytes and platelets needed to be measured. Leukocyte adhesion in 300μm or 100μm microslides was essentially the same on the upper or lower surface of the microslide at any shear rate used. This showed that sedimentation of the RBC as they travelled along the microslide at native haematocrit did not affect the adhesion of leukocytes. We know from work with isolated neutrophils, that these cells do not adhere to the upper surface of horizontal microslides (G. Nash and colleagues; unpublished observations). Thus leukocyte sedimentation is important in inducing contact with the lower vessel wall when RBC are absent. In vertical microslides, there is very little leukocyte adhesion on any surface in the absence of RBC (Abbitt and Nash, 2003), showing that in this orientation, RBC are required for leukocyte margination. In either case, it is clear that RBC have a strong influence on leukocyte contact with the vessel wall.

Platelet experiments were performed mainly at higher flow rates than the leukocyte experiments, so the blood transit times within the microslides were shorter, and the potential for any effect of sedimentation was much lower. Platelets themselves are smaller than leukocytes and as a result their tendency to sediment will be much lower than that of leukocytes. In the 300μm microslides, platelet adhesion to the upper and lower surface was not significantly different. In the 100μm microslides, where the smaller cross section might result in an increased effect of sedimentation, there was no difference in platelet adhesion to the upper or lower surface of the microslide showing that there was no effect of sedimentation on platelet adhesion in this flow assay.

The results above suggest that in flowing blood at native haematocrit, dispersive force generated by collisions with flowing RBC within the vessel dominate the effect of gravity that pulls the cells towards the lower surface of the microslide. As sedimentation was shown not to affect leukocyte and platelet adhesion, and presumably distributions of these cells across the vessels, the adhesion was only assessed on the lower surface of the microslide in subsequent chapters.

3.4.2 The effect of increasing shear rate on the margination of leukocytes

As blood of 40% haematocrit was perfused through the 100μm microslides, the number of leukocytes flowing near the vessel wall was significantly reduced as wall shear rate was increased from 70 to $140s^{-1}$, but changed little between $140s^{-1}$ and $280s^{-1}$. As wall shear rate increased, there was a linear increase in the free flowing velocity of the leukocytes near the vessel wall.

In general, leukocyte margination is due to RBC aggregates forming in the centre of the vessel, where shear rate is lower, displacing the leukocytes towards the vessel wall. The radial distribution of particles within a particulate suspension in tube flow such as blood is governed by the size of the particles themselves (Phibbs and Dong, 1970). Larger particles or aggregates of particles will tend to flow more towards the central axis of the vessel, with smaller particles flowing more peripherally. The transient aggregation of RBC is shear rate dependent and is abolished above shear rates of ~$50s^{-1}$ (Goldsmith et al., 1999). At a wall shear rate of $70s^{-1}$ in our experiments, the proportion of the central vessel region that experienced a shear rate low enough for RBC aggregates to form would be larger than at the higher shear rates, and hence more of the leukocytes were displaced towards the periphery of the vessel. At higher wall shear rates, RBC aggregation was presumably reduced in the centre of the vessel, so the leukocytes flowed more centrally, as they are larger than individual RBC.

The shear-dependence of margination and lack of effect above $140s^{-1}$ are consistent with previous studies of the effect of shear rate on the margination of leukocytes in cylindrical vessels. Goldsmith and Spain (Goldsmith and Spain, 1984) used suspensions of ghost RBC to directly observe that leukocytes flowed more centrally as mean shear rate was increased from $50s^{-1}$ to $100s^{-1}$. Nobis et al. (Nobis et al., 1985) used fluorescently labelled leukocytes to assess the effect of shear rate on leukocyte margination. The vessel was viewed end on and the radial position of each leukocyte was recorded. As the wall shear stress was increased from 0.1-2.5Pa (equivalent to a range in wall shear rate of 50-$1250s^{-1}$) the leukocytes flowed more centrally. The number of leukocytes flowing in the outer most regions of the vessel decreased rapidly between wall shear rates of $50s^{-1}$ to $500s^{-1}$. Between $500s^{-1}$ and $1250s^{-1}$ the number of leukocytes flowing in this region was consistently low. Thus the present data, and the previous studies, indicate that margination of leukocytes is efficient over quite a narrow range of shear rates.

3.4.3 The effect of shear rate on leukocyte adhesion

Adhesion of leukocytes to the vessel wall is dependent on the number of free flowing leukocytes in close enough proximity to allow adhesive interactions, as well as the velocity of cells by the wall and shear the stress they experience. The time that the selectin-ligand pair is close enough to form a bond is reduced as shear rate increases, and when a bond is formed, the force exerted on the bond is increased.

In both the 100μm and 300μm microslides, increasing the wall shear rate from 70 to $280s^{-1}$ resulted in a marked decrease in leukocyte adhesion, which was nearly absent by the highest shear rate. At $70s^{-1}$ there were a large number of rolling adherent leukocytes because there were a large number of marginated leukocytes flowing near the vessel wall at a relatively low velocity. From 70-$140s^{-1}$ the number of adherent leukocytes decreased dramatically as the number of marginated leukocytes decreased and their velocity increased. Then, from 140-$280s^{-1}$ the number of free flowing leukocytes near the vessel wall showed no significant change but the adhesion of the leukocytes continued to decrease. The velocity of the free flowing leukocytes doubled between nominal wall shear rates of $140s^{-1}$ and $280s^{-1}$. So the effects of shear rate on adhesion reflect effects on margination and on the adhesion process itself. At low shear rate, adhesion is dominated by the effects of shear rate on margination and at higher shear rates leukocyte adhesion is affected not by a change in leukocyte margination but by an increase in the velocity of the marginated leukocytes and in the force they experience when attaching.

The results obtained here are in good agreement with previous studies assessing the effect of shear rate on leukocyte adhesion. *In vivo* studies in the mesentery of the rat (Firrell and Lipowsky, 1989;Ley and Gaehtgens, 1991) and the cat (from $50s^{-1}$ to $1000s^{-1}$) (Bienvenu and

Granger, 1993;Perry and Granger, 1991) all observed an increase in leukocyte adhesion as shear rate was decreased in the appropriate vessels. In the rat Firrell and Lipowsky found a non-linear increase in the percentage of rolling adherent leukocytes in venules as shear rate was decreased (Firrell and Lipowsky, 1989). At $50s^{-1}$, 30% of all flowing leukocytes were performing rolling adhesion and by $800s^{-1}$ only around 5% of all perfused leukocytes were adherent. Ley and Gaehtgens found that the percentage of leukocytes performing rolling adhesion within venules decreased as shear rate was increased from 30-$2000s^{-1}$ (Ley and Gaehtgens, 1991).

In vitro studies assessing the effect of shear rate on leukocyte adhesion show a strong decrease in leukocyte adhesion as shear rate is increased. Those in isolated suspensions using HUVEC (Lawrence et al., 1987;Lawrence et al., 1990) and P-selectin (Lawrence and Springer, 1991) all found that at shear rates up to $250s^{-1}$ the number of rolling adherent leukocytes decreased sharply. The only other study using whole blood *in vitro* (Abbitt and Nash, 2001) also showed that leukocyte adhesion decreased as wall shear rate was increased markedly from 35-$280s^{-1}$. The results shown here in the smaller 100μm microslides agree well with the behaviour in blood vessels. In these smaller microslides the efficiency of leukocyte adhesion was much greater than in 300μm microslides and was very close to that found in rat post-capillary venules (Ley and Gaehtgens, 1991). At shear rates of $<100s^{-1}$ 47% of all leukocytes perfused were found to be rolling adherent in the venules, compared to around 40% at $70s^{-1}$ in our *in vitro* system.

3.4.4 The effect of shear rate on platelet margination and adhesion

The shear rate-dependence of the margination of free flowing platelets could not be measured in our system, where assessment of cell numbers and velocities was restricted to a shear rate of $300s^{-1}$. Previous studies were made by measuring tube versus reservoir blood platelet

counts (Beck, Jr. and Eckstein, 1980;Corattiyl and Eckstein, 1986;Uijttewaal et al., 1993) or by direct near wall platelet counts (Tilles and Eckstein, 1987;Yeh and Eckstein, 1994;Aarts et al., 1988).

The tubular concentration of flowing platelets increased with shear rate up to a wall shear rate of $500s^{-1}$ and remained constant from 500 up to $10,000s^{-1}$ (Beck, Jr. and Eckstein, 1980). In tubes of internal diameter 90-210μm it was shown that tubular concentration of platelets was greater than reservoir concentration at all shear rates tested ($80-8000s^{-1}$) with the maximal increase at shear rates of around $800s^{-1}$ for all vessel sizes (Corattiyl and Eckstein, 1986). This study showed again at low shear rate, that the tubular platelet concentration increased with respect to the reservoir concentration as shear rate was increased. In long polyethylene tubes of 200μm diameter, platelet margination in flowing blood was increased as shear rate was increased from $100-800s^{-1}$ (Uijttewaal et al., 1993). These results from the two studies using relative tubular platelet concentration to assess platelet margination suggest that it reaches a maximum value at a wall shear rate of around $800s^{-1}$ and any further increase in shear rate results in no additional increase in platelet margination.

Studies using fluorescent platelets and platelet-sized beads have yielded varying results for platelet margination. As wall shear rates were varied from $50-3180s^{-1}$, there was no significant near wall excess of platelet-sized beads up to a wall shear rate of $430s^{-1}$ (Tilles and Eckstein, 1987). A near wall excess was present at $430s^{-1}$, but at higher shear rates there was no further increase. Platelet-sized beads of 2.2μm perfused in blood at 15% haematocrit found that the near wall excess of platelets was increased up to $400s^{-1}$ (Eckstein et al., 1988). Using liquid nitrogen to freeze a tube during blood perfusion and sectioning the frozen tube found that increasing wall shear rate ($250-1220s^{-1}$) resulted in a significant change in platelet margination (Yeh and Eckstein, 1994). Platelet margination was more pronounced at $560s^{-1}$

than it was at $250s^{-1}$ or $1220s^{-1}$. Over a similar range of shear rates, it was shown that increasing shear rate from 240-$1260s^{-1}$ increased the concentration of fluorescently-labelled platelets flowing close to the wall in 3mm tubes (Aarts et al., 1988).

From the literature reports, one would have expected that in our system, as the shear rate increased, the concentration of free flowing platelets near the wall would increase initially up to a shear rate of between 400-$1200s^{-1}$ and tend to plateau at the higher wall shear rates used.

In our studies of platelet adhesion, the larger vessels allowed analysis of shear rates up to $560s^{-1}$, while shear rates up to $1500s^{-1}$ were used in the smaller microslides. The platelet adhesion was normalised for the flow rate (i.e. per ml of blood perfused) to allow for its effects on delivery of platelets. Normalisation also allowed comparison of adhesion within the different sized microslides (see section 3.3.2). The percentage coverage showed no significant change in adhesion as shear rate was increased to $560s^{-1}$ in the larger microslides. In the smaller microslides, over a wider range of wall shear rate there was a tendency for the adhesion to decrease with increasing wall shear rate.

The sensitivity of platelet adhesion to increasing shear rate may depend on the balance of several factors. With increasing shear rate, as with leukocytes, platelets will travel faster and experience greater forces at the wall, and so adhesion itself should be less effective. At higher shear rates the amount of RBC aggregation occurring in the centre of the vessel will decrease. This will in turn increase the width of the RBC core and reduce the width of the RBC free plasma layer adjacent to the vessel wall. Platelets resident in this layer will experience more RBC-platelet collisions and be forced into contact with the wall more efficiently, helping to maintain the percentage coverage per ml of perfused blood with increasing shear rate, at least

for the lower range of shear rates we observed (up to $560s^{-1}$). An increase in shear rate will also increase platelet margination up to around $800s^{-1}$ after which it remains at a maximum level (Corattiyl and Eckstein, 1986). As the shear rate continues to increase past these intermediate levels, the percentage coverage per ml of blood perfused starts to decrease, presumably because the effects of shear on the adhesion process itself has now come to dominate.

Superficially, our results do not appear in direct agreement with the previous literature. Previous studies showed an increase in adhesion as shear rate was increased to $800s^{-1}$ and then it remained constant up to $2600s^{-1}$ (Turitto et al., 1980;Weiss et al., 1986). Savage (Savage et al., 1996) found that adhesion increased up to $920s^{-1}$ and plateaued between 920 and $1500s^{-1}$. An increase in adhesion to collagen was seen between shear rates of 100-$1000s^{-1}$ (Ross et al., 1995). However, none of these studies corrected for the increase in delivery of platelets with increasing flow rate, i.e., they did not report on efficiency of adhesion as was done here. If this is taken into account, broadly all reports are consistent with a low sensitivity of adhesion to increasing shear rate until about $1000s^{-1}$, and a fall off thereafter.

3.4.5 The effect of vessel size on the adhesion of leukocytes and platelets

One of the components of this study was an investigation of the effect of vessel size on the adhesion of leukocytes and platelets. Leukocyte adhesion in the smaller 100μm microslides was found to be much more efficient than in the larger 300μm microslides. Investigations into the number of free flowing leukocytes at the vessel wall found that there was no difference between the two sizes of microslide. The velocity of the free flowing leukocytes near the vessel wall in the larger microslides was significantly higher. Tube viscometry of blood at 40% haematocrit showed that the apparent viscosity of the blood was significantly

higher in the 300μm microslides at all shear rates, indicating that the wall shear stress was higher for a given shear rate. The higher velocity and higher apparent viscosity of the blood in the larger microslides would make the formation of adhesive bonds less likely and would result in a larger force on the formed selectin-ligand bonds, leading to an increased chance of the bond breaking before the initiation of stable rolling adhesion. The volume of blood used to produce the nominal wall shear rates used in this study was 27 times higher in the larger microslides and their surface area was only 3 times higher than that of the 100μm microslides. Thus a lower proportion of the blood was close to the vessel wall, again resulting in decreased adhesion efficiency.

For platelets, although efficiency of coverage was greater in the smaller microslides, the effect was not so marked as for leukocytes. One would expect that there would be similar numbers of platelets at the vessel wall in both vessel sizes, as with leukocytes, however as the platelets are smaller than leukocytes their velocity would not increase as significantly in the 300μm microslides. This could account for a smaller increase in adhesion efficiency between the 300μm and 100μm microslides. The higher apparent viscosity of the blood in the larger microslides would again result in a larger force on the formed adhesive bonds, leading to an increased chance of the bond breaking before the initiation of firm adhesion. The increased apparent blood viscosity in the 300μm microslides coupled with the smaller proportion of blood in contact with the vessel wall could account for the reduced adhesion efficiency in these vessels. The small size of the platelets might therefore contribute to the smaller reduction in adhesion efficiency between the two vessel sizes when compared to leukocytes. In addition, it is possible that the much higher number of platelets in blood than leukocytes makes the surface area to volume consideration less critical. As even with a uniform distribution (no margination) there would be a high number of platelets flowing near the vessel wall.

The effect of vessel size on the apparent viscosity of the blood has been widely studied (Chan et al., 1982;Reinke et al., 1987;Pries et al., 1992;Stadler et al., 1990;Gupta and Seshadri, 1977). Results obtained here are consistent with the well known Fahraeus-Lindqvist effect, which is the decrease in the apparent viscosity of blood flowing through narrow tubes (Fahraeus and Lindqvist, 1931). Although differently sized vessels and perfusion chambers have been used previously in the investigation of leukocyte and platelet adhesion, the study performed here is the first to our knowledge that has directly, quantitatively compared adhesion efficiency in different vessel sizes at equal shear rates.

3.4.6 Comparison of the effect of shear rate on leukocytes and platelets

It is clear that leukocyte adhesion is more sensitive to an increase in shear rate than platelet adhesion (fig 3.7). Leukocyte adhesion drops off significantly between 70 and 280s^{-1} whereas platelet adhesion shows a slight decrease up to a shear rate of 1500s^{-1}. These trends reflect the behaviour of the cells *in vivo*, as platelet adhesion can occur anywhere in the circulation where vessel trauma has occurred, whereas leukocyte adhesion is largely restricted to post-capillary venules where the shear rates experienced are low.

Platelets are the smallest cells in the blood and as a result will travel predominantly in the periphery of the vessel at all shear rates (Phibbs and Dong, 1970). The small size of the platelets will allow them to approach closer to the vessel wall than the larger leukocytes, as the closest the centre of a cell can get to the wall is one cell radius. Thus platelets adjacent to the wall travel more slowly than leukocytes (ideally by about four-fold) at the same shear rate. As the kinetics of the receptor-ligand bonds involved in leukocyte and platelet adhesion are comparable (Doggett et al., 2002), the reduced velocity contributes to an explanation of how

the platelets can adhere at much higher shear rates than leukocytes. Once adhered to the vessel wall, the leukocytes protrude a greater distance into the vessel, and their larger cross section and surface area increases the shear force the leukocytes and hence the adhesive bonds experience (ideally by about sixteen-fold) compared to the platelets. This will contribute to maintaining platelet adhesion at higher shear rates than leukocytes.

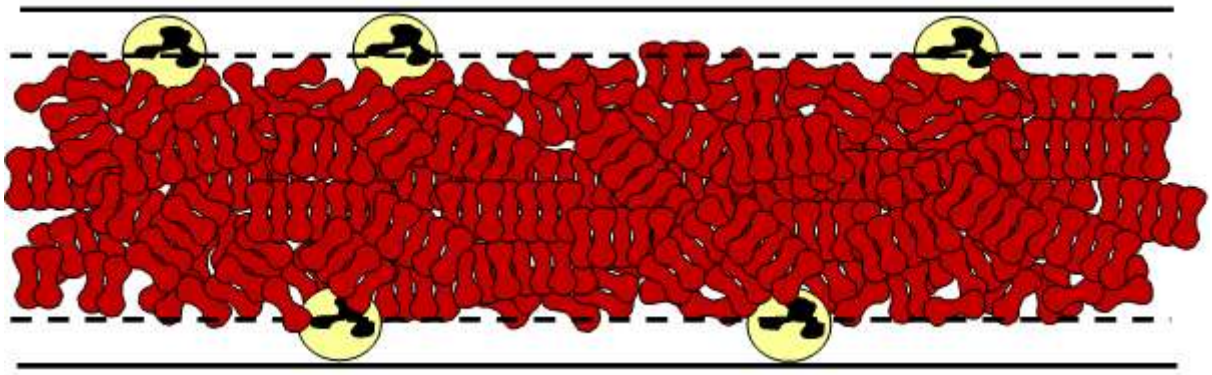
Figures 3.12 and 3.13 seek to summarise effects of shear, margination and the peripheral plasma layer on leukocytes and platelets. The main shear-dependent determinants of leukocyte adhesion are the number and velocity of marginated leukocytes flowing in close enough proximity to form adhesive bonds with the vessel wall. Due to the size of leukocytes the number is heavily dependent on the amount of RBC aggregation present in the vessel. At low shear rates (~$70s^{-1}$ in this system) the region in which RBC aggregation can occur is large resulting in increased margination and therefore adhesion of the leukocytes. At higher shear rates the amount of RBC aggregation is lower and the larger leukocytes flow more centrally within the vessel and thus leukocyte adhesion is reduced (fig 3.12).

Proposed here for the first time, is that the narrowing of the peripheral plasma layer due to a reduction in RBC aggregation at high shear rate is of particular importance in the modification of platelet adhesion. As the shear rate is increased the plasma layer decreases in width and the volume of plasma that the peripherally flowing platelets can occupy is reduced. This results in an increased number of RBC-platelet collisions that facilitate platelet adhesion. This facilitation will reach a maximum level as the plasma layer reaches a minimum. As the adhesion of platelets showed a tendency to decrease as shear rate increased, the plasma layer in this system may reach a minimum thickness between 400 and $1200s^{-1}$. The effect of shear rate on the adhesion of platelets is less dependent on the size related redistribution on the cells because platelets are smaller than individual RBC or aggregates, therefore one may predict

relative insensitivity to shear-dependent aggregation (fig 3.13). Thus reduction in the plasma layer thickness may play a part in the low sensitivity of platelet adhesion to shear rate.

In summary, this study has provided, for the first time, a direct comparison of the effect of shear rate on the adhesion of leukocytes and platelets within the same sized vessels. While margination affects shear-dependent leukocyte adhesion, the comparison has suggested that variation in the width of a plasma layer may help explain how shear rate differently affects platelets adhesion. The latter concept will be returned to in subsequent chapters when attempting to explain the effect of changes in other rheological variables on platelet adhesion.

(a)



(b)

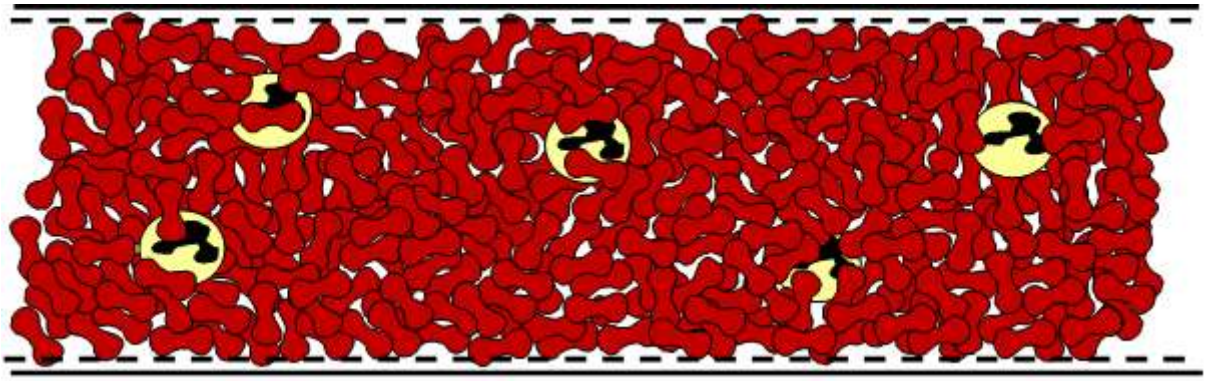
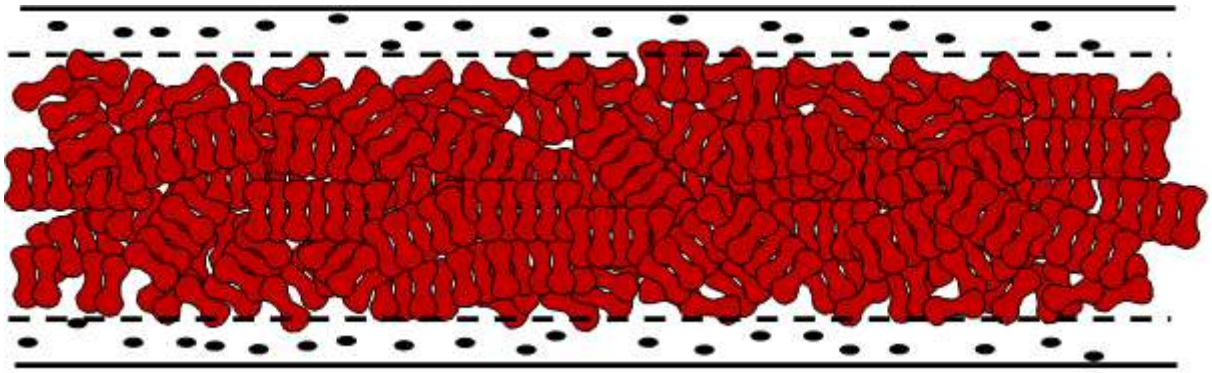


Figure 3.12 Effect of shear rate on leukocyte distribution within a blood vessel
This figure illustrates how the shear rate may affect the margination of leukocytes and formation of a plasma layer at the periphery. (a) At a low shear rate (e.g a wall shear rate of $70s^{-1}$), extensive RBC aggregation occurs in the central region of the vessel, causing the leukocytes to be displaced radially towards the vessel wall. This acts to increase leukocyte adhesion. The leukocytes are bigger than the plasma layer. (b) At a high shear rate (e.g a wall shear rate of $280s^{-1}$), RBC aggregation does not occur in the central regions of the vessel and therefore the larger leukocytes flow more centrally reducing leukocyte adhesion. The plasma layer is also narrower.

(a)



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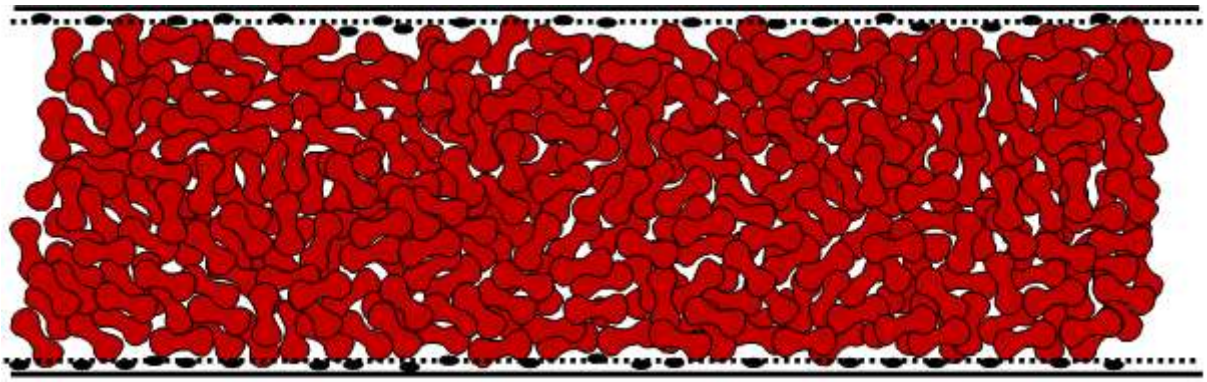


Figure 3.13 Effect of shear rate on the distribution of platelets within a blood vessel
This figure illustrates how the shear rate may affect the distribution of platelets and formation of a plasma layer at the periphery. (a) At a low shear rate, RBC aggregation occurs in the central region of the vessel, and increases the width of the plasma layer in which the small platelets can flow. (b) At a high shear rate, RBC aggregation does not occur and the plasma layer is also narrower. The number of RBC-platelet collisions that cause the platelets to come into contact with the vessel wall and facilitate platelet adhesion will increase. Platelet margination (or number density near the wall) may itself be similar at the two rates.

CHAPTER 4: EFFECT OF SUSPENDING PHASE VISCOSITY ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

4.1 Introduction

The effects of variation in suspending phase viscosity on the margination and adhesion of leukocytes and platelets have not been well defined. To our knowledge there has been no attempt to assess the effect of suspending phase viscosity on the margination or adhesion of leukocytes in blood. For isolated neutrophils, an increase in suspending phase viscosity at constant shear rate caused leukocyte adhesion to P-selectin to decrease (Chen and Springer, 2001;Nash et al., 2001). Limited studies on the margination and adhesion of platelets have been performed. In rectangular channels, 50μm in height, increasing suspending phase viscosity caused an increase in the near wall excess of platelets at a shear rate of $210s^{-1}$ (Eckstein et al., 1988), although only normal (1.2mPa.s) and high (3.9mPa.s) plasma viscosities were used in this study. The only study assessing the effect of suspending phase viscosity on platelet adhesion showed a decrease of platelet adhesion with increasing plasma viscosity over a small range (0.89-2.00mPa.s) (van Breugel et al., 1992).

The viscosity of the plasma can be increased in various disease states. In some B Cell myelomas an increase in plasma immunoglobulin levels can increase the plasma viscosity 5-fold (Waldenstrom, 1944). This condition has been associated with mucosal bleeds which suggest a possible effect on haemostasis and platelet adhesion. In general, increased fibrinogen concentration and hence plasma viscosity are markers of the acute phase response to tissue injury and of most inflammatory conditions (Stuart and Whicher, 1988). Changes in plasma viscosity may have an effect on leukocyte and platelet adhesion in such disorders, but sufficient studies in this area have not been performed.

For this study, the hypothesis was that leukocyte adhesion would be more sensitive to an increase in suspending phase viscosity than platelets. Work from the previous chapter on

shear rate at constant viscosity suggested that the two cell types could be affected by an increase in shear stress differently. The work described in this chapter attempted to assess the effect of suspending phase viscosity on the margination and adhesion of leukocytes for the first time and to verify previous studies on platelet margination and adhesion. All experiments were performed in vessels of the same size, allowing direct comparison. By varying suspending phase viscosity using increasing concentrations of Dx40, the effect of increasing plasma viscosity was studied in the absence of RBC aggregation. Therefore the direct effect of increasing suspending phase viscosity was measured and not the secondary effects of modified aggregation. We could not devise a method for varying suspending phase viscosity over a wide range where red cell aggregation would be retained at a level consistent with that found physiologically at each suspending phase viscosity.

4.2 Methods

4.2.1 Blood collection

Venous blood was anticoagulated with CPDA. Theophylline was included in all experiments, apart from those assessing platelet adhesion, at a final concentration of 7mM in the blood. Adhesion and margination experiments were performed at a normalised haematocrit of 40%. Platelet adhesion experiments were also performed at 20% haematocrit.

4.2.2 Blood manipulation

The suspending phase viscosity in all blood samples in this section was manipulated by adding equal volumes of Dx40 solutions with different concentrations to the blood. For the platelet adhesion experiments performed at 20% haematocrit the Dx40 was added to the blood directly. In the samples with normalised haematocrit of 40%, blood was centrifuged and the appropriate amount of plasma was removed prior to the addition of the Dx40, resulting in a final haematocrit of 40%.

4.2.3 Microslide coating

For experiments on leukocyte adhesion, microslides of 100μm in height were coated in purified P-selectin at a concentration of 10μg/ml. For experiments on platelet adhesion, microslides were coated with Horm collagen at 500μg/ml. For margination and tube viscometry experiments, the microslides were coated with albumin to provide a non-adhesive surface.

4.2.4 Adhesion assay

Blood was fluorescently labelled with 5μg/ml of R6G. To measure the adhesion of leukocytes to P-selectin, blood samples, containing different concentrations of Dx40, were perfused at a shear rate of $140s^{-1}$ for a total of 4 minutes from the time the microslide was first filled with blood. To measure platelet adhesion to collagen, the blood was perfused through the microslides at a shear rate of $900s^{-1}$. Adhesion was measured up to 4 minutes after the microslide was first filled with blood.

4.2.5 Assessment of margination

Margination was analysed by perfusing fluorescently labelled blood through albumin coated microslides. The microslides were perfused horizontally and the free-flowing cells were measured on the upper surface of the microslide. Leukocyte experiments were performed at a shear rate of $140s^{-1}$. Platelet margination was assessed at a wall shear rate of $300s^{-1}$.

4.2.6 Tube viscometry

The wall shear rates at which the blood was perfused through the tube viscometer covered the entire range of $70\text{-}1500s^{-1}$. Using the values of pressure difference between inlet and outlet, the flow rate and the dimensions of the microslide, the apparent viscosity of the blood and the viscosity of the separated suspending phase of the blood at each nominal wall shear rate was calculated using equation 18 (see page 14).

4.3 Results

4.3.1 Effect of suspending phase viscosity on leukocyte adhesion

By adding Dx40 at increasing concentrations to the blood, the viscosity of the plasma was varied. At the same time, RBC aggregation was abolished, judged by microscopic observations (see section 2.2.3). As the viscosity of the medium was increased from 0.85mPa.s to 2.1mPa.s there was a significant drop in leukocyte adhesion (fig 4.1(a)). Whilst the increase in plasma viscosity had a dramatic effect on leukocyte adhesion it had no significant effect on the rolling velocity of the adherent leukocytes (fig 4.1(b)).

4.3.2 Effect of suspending phase viscosity on platelet adhesion

The effect of augmenting suspending phase viscosity on platelet adhesion was assessed over a wider range of plasma viscosities than the leukocyte adhesion experiments. The platelet experiments were initially performed at 20% haematocrit to avoid spinning the blood unnecessarily. At 20% haematocrit, platelet adhesion showed no change as suspending phase viscosity was increased between 1 and 2mPa.s. As suspending phase viscosity was further increased there was a rapid decrease in adhesion until at about 4mPa.s, platelet adhesion was almost completely abolished (fig 4.2(a)).

The platelet adhesion experiments were also performed at 40% haematocrit where the same trend in adhesion was visible. However, the drop off seen above about 2mPa.s was not as sharp as that seen at 20% haematocrit (fig 4.2(b)). We also compared the adhesion of platelets on the upper and lower surface of the microslide in these experiments. We found that after washout (after 6 minutes perfusion) percentage coverage was essentially the same on the two surfaces (fig 4.2(c)). This suggests that there was no effect of sedimentation and that the slight increase in density of the higher concentration dextran solutions did not promote adhesion on the upper surface.

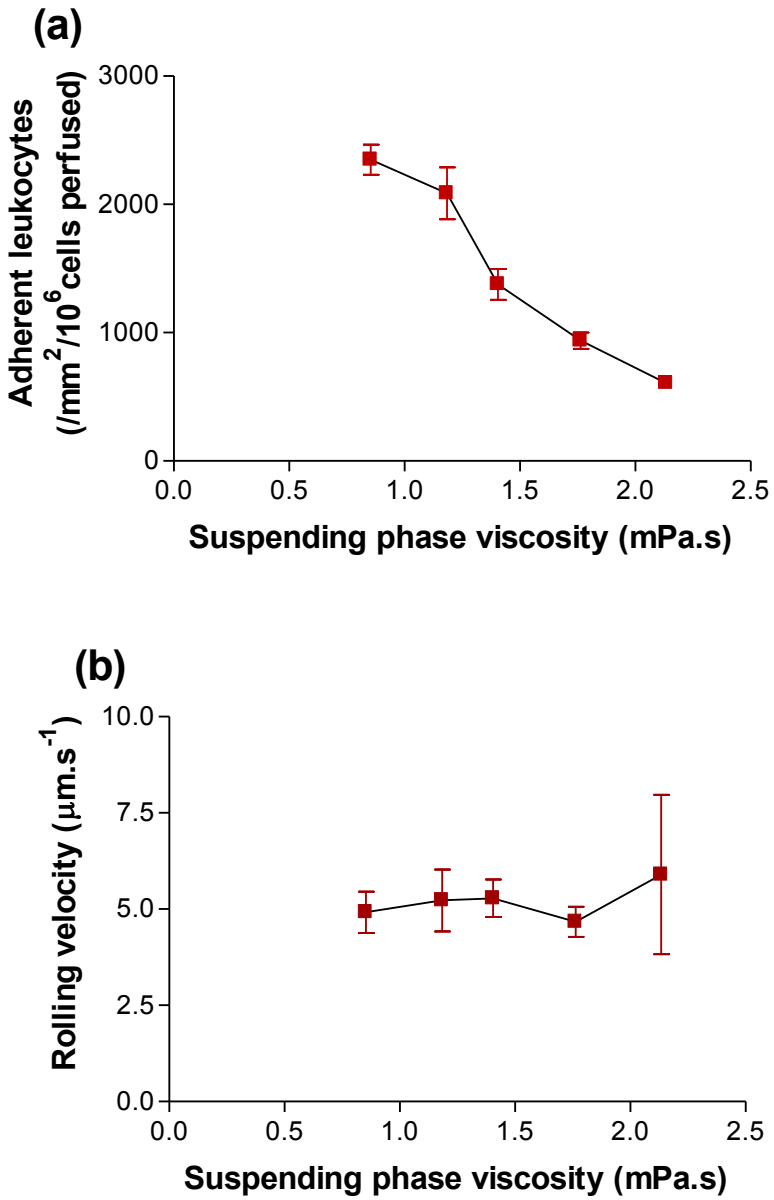


Figure 4.1 Effect of suspending phase viscosity on (a) the number of leukocytes adhering and (b) their rolling velocity on P-selectin
Blood was perfused through microslides of 100µm in height that had been coated with P-selectin. The blood was manipulated to increase suspending phase viscosity and keep haematocrit at 40%. The number of adherent leukocytes were counted on the lower surface of the microslides. Data shown are means ± SEM from 4 experiments. Leukocyte adhesion was normalised per mm^2 per 10^6 cells perfused. (a) ANOVA showed a significant effect of suspending phase viscosity on adhesion ($P<0.0001$). (b) ANOVA showed no significant effect of suspending phase viscosity on rolling velocity of leukocytes.

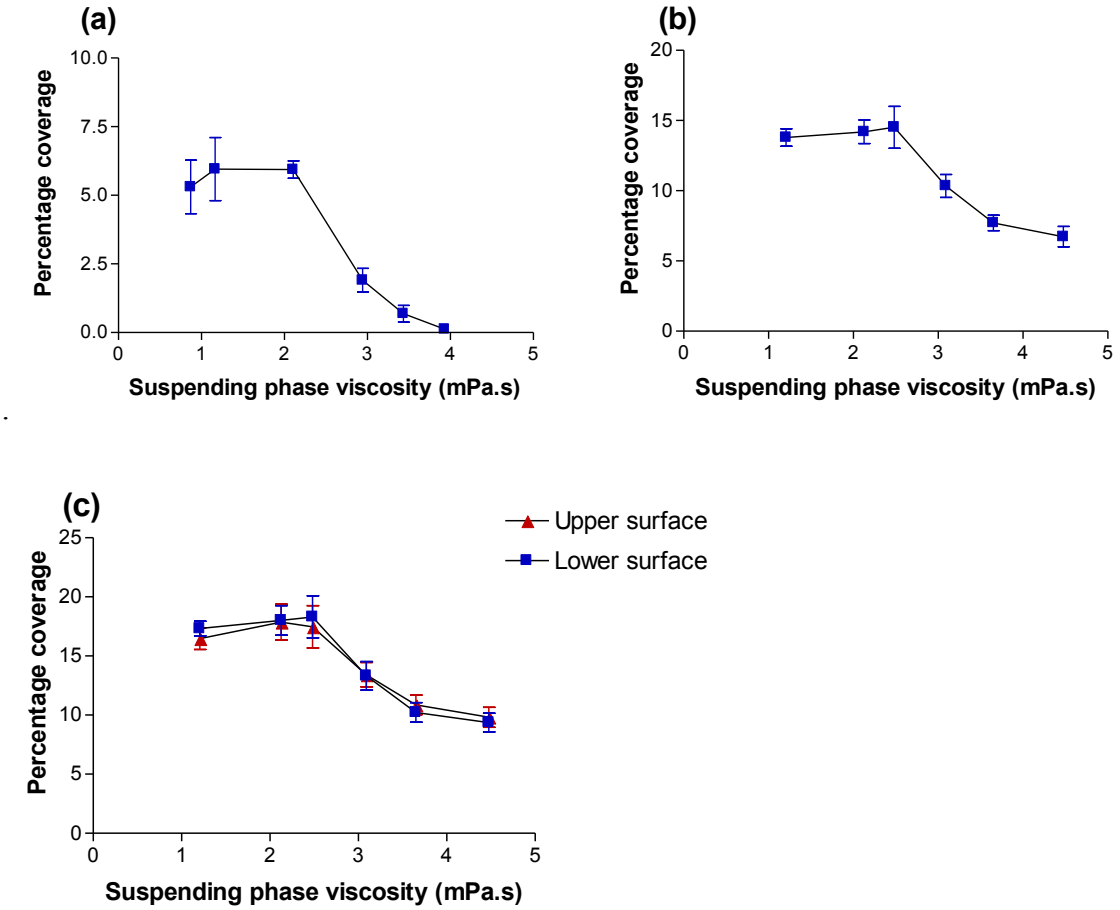


Figure 4.2 Effect of suspending phase viscosity on platelet adhesion at haematocrit of (a) 20%, (b) 40% and (c) 40% on the upper and lower surface
Blood was perfused through microslides of 100μm in height that had been coated with collagen. The blood was manipulated to increase suspending phase viscosity and keep haematocrit at (a) 20% or ((b) and (c)) 40%. Data shown are means ± SEM of the percentage coverage of platelets after ((a) and (b)) 4 minutes or (c) 6 minutes blood perfusion from 4 experiments. In (a), the overall change in adhesion seen as suspending phase viscosity was increased was significant ($P<0.0001$ by ANOVA). ANOVA showed no change in platelet adhesion below a suspending phase viscosity of 2.1mPa.s, but showed a significant effect of suspending phase viscosity at viscosities of 2.1mPa.s and above ($P<0.0001$). In (b) the effect of suspending phase viscosity on platelet adhesion was significant ($P<0.0001$ by ANOVA). ANOVA showed no change in the platelet adhesion below a suspending phase viscosity of 2.4mPa.s but showed a significant effect of suspending phase viscosity at viscosities of 2.4mPa.s and above ($P<0.01$). In (c) the percentage coverage on the upper and lower surfaces of the microslides was essentially the same.

4.3.3 Comparison of the effects of suspending phase viscosity on leukocyte and platelet adhesion

A direct comparison of leukocyte and platelet adhesion in the same system at 40% haematocrit showed differing effects of suspending phase viscosity which can be explained by the differing forces experienced by the cells (fig. 4.3). The results show that leukocytes are much more sensitive to changes in suspending phase viscosity than platelets. As plasma viscosity was increased up to 2.1mPa.s leukocyte adhesion dropped rapidly, but platelet adhesion showed no significant decrease in this range (fig 4.3(a)). Platelet adhesion showed a less pronounced but significant decrease as suspending phase viscosity was increased to around 4.5mPa.s. Given that the two cells were tested at different wall shear rates, the data can be re-plotted as a function of wall shear stress (i.e., wall shear rate multiplied by apparent viscosity of the blood measured for the various suspending phase viscosities - see below). Leukocyte adhesion then showed even greater sensitivity to wall shear stress (fig 4.3(b)), with leukocyte adhesion decreasing rapidly at shear stresses up to 1Pa. Platelet adhesion on the other hand only showed a significant decrease at wall shear stresses of 4-6Pa. However, when the force experienced by the cells adhering to the wall was calculated using the Goldman equation (Equation 20; chapter 1), the critical force that the bonds could withstand before their efficiency started to decrease was around 0.15nN for both cell types (fig 4.3(c)).

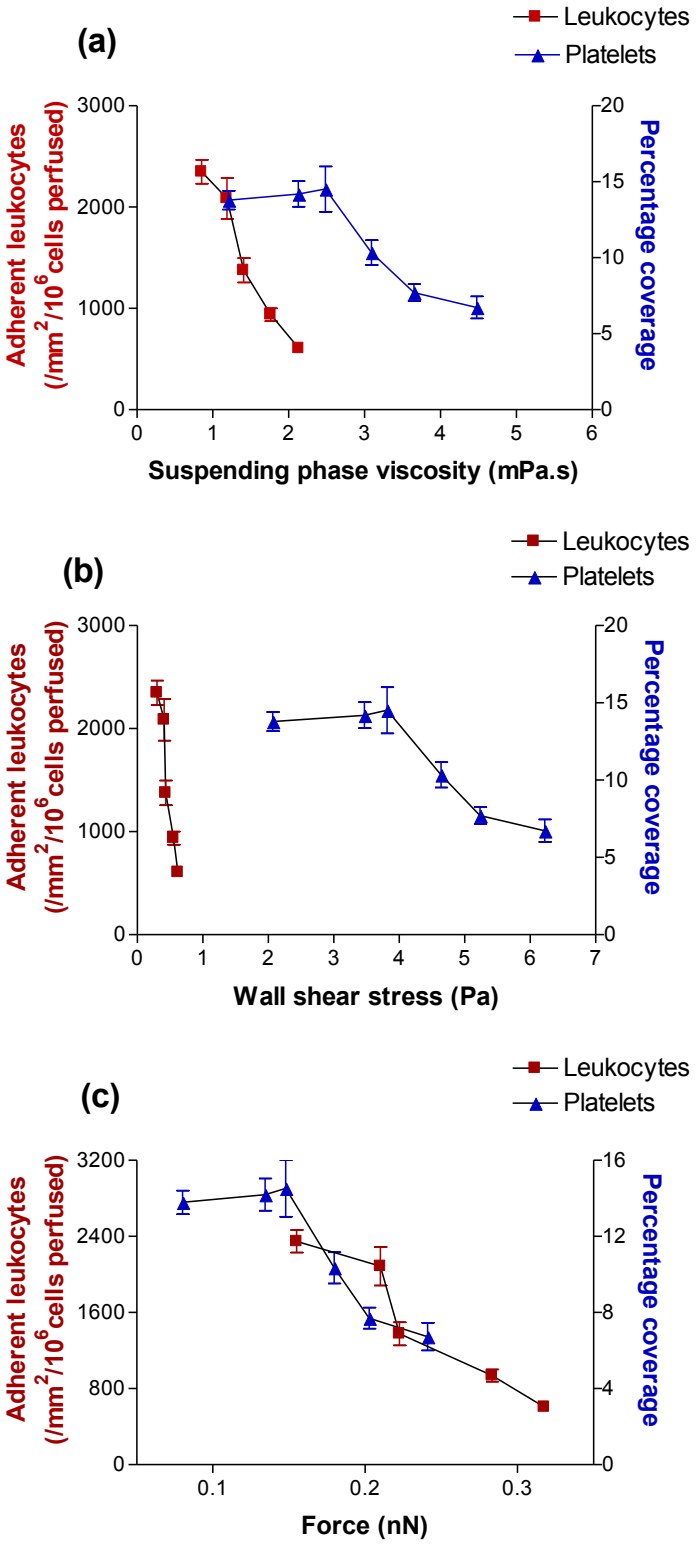


Figure 4.3 Comparison of variations in leukocyte and platelet adhesion as functions of (a) suspending phase viscosity, (b) wall shear stress, (c) force applied to an adherent cell. Blood was perfused through microslides of 100µm in height that had been coated with P-selectin or Horm collagen for assessment of leukocyte or platelet adhesion respectively. Data are means ± SEM of 4 experiments. For calculations, see text.

4.3.4 Effect of suspending phase viscosity on leukocyte margination

The distribution of free-flowing leukocytes was quantified to help understand how increasing suspending phase viscosity affected leukocyte adhesion. After correcting for leukocyte count within the blood, it was found that there was no significant change in the number of free-flowing leukocytes at or near the vessel wall as suspending phase viscosity was increased (fig 4.4(a)). In these experiments, in the absence of RBC aggregation, there were fewer free-flowing leukocytes adjacent to the wall than there were further into the vessel, which suggests that there was no margination of leukocytes in this system. The velocity of the free-flowing leukocytes adjacent to the vessel wall showed no significant change as the viscosity of the suspending phase was increased, although there was a slight downward trend (fig 4.4(b)).

4.3.5 Effect of suspending phase viscosity on platelet margination

Platelet margination experiments were performed at a haematocrit of 40% to provide a direct comparison to the leukocyte margination studies. The number of free-flowing platelets near the vessel wall showed no significant change as the suspending phase viscosity was increased at all depths into the blood stream (fig 4.5(a)). In these experiments, there were more free-flowing platelets adjacent to the wall than there were further into the vessel. The velocity of free-flowing platelets adjacent to the vessel wall decreased significantly as the viscosity of the suspending phase was increased (fig 4.5(b)).

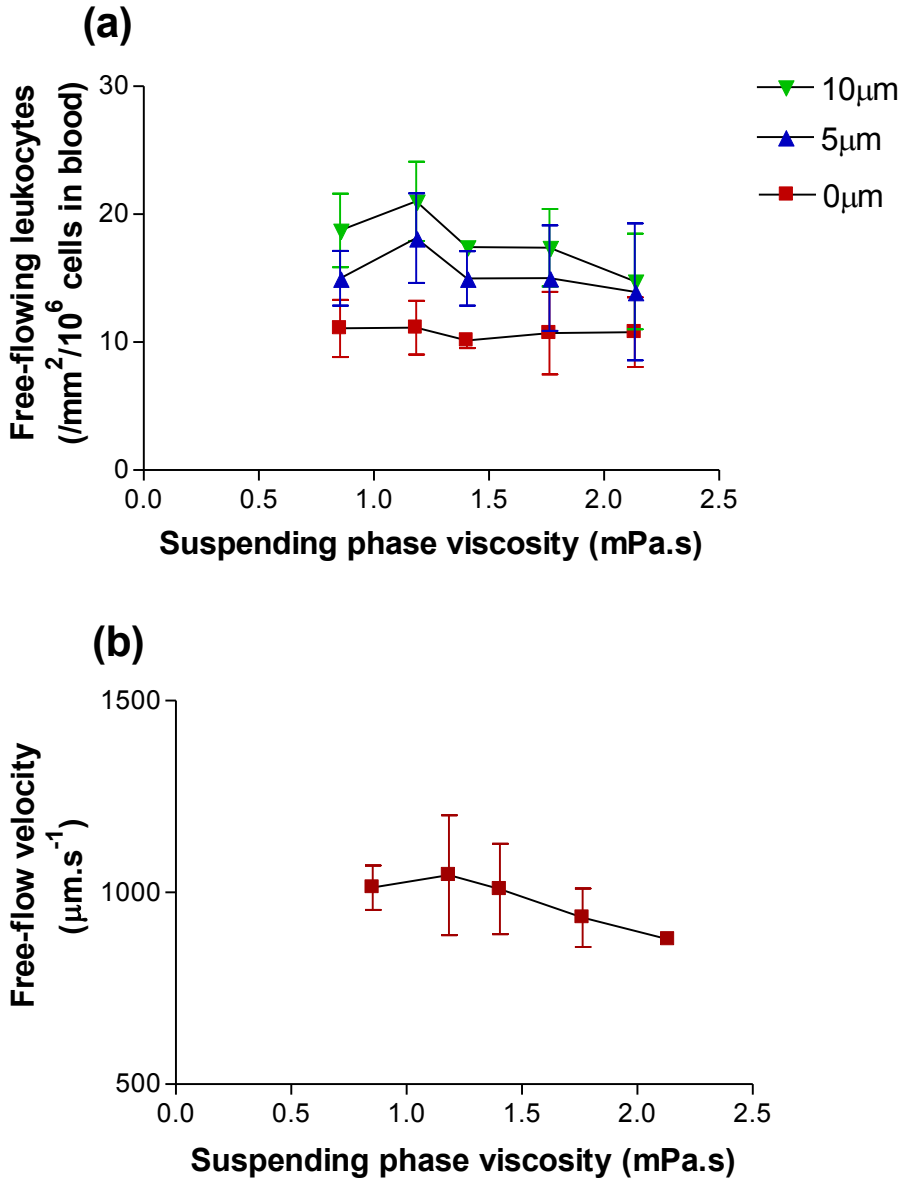


Figure 4.4 Effect of suspending phase viscosity on the number and velocity of leukocytes near the vessel wall at a wall shear rate of 140s^{-1}
Blood, adjusted to 40% haematocrit, was perfused through microslides of 100μm in height that had been coated with albumin. Data shown are means ± SEM from 3 experiments assessing; (a) the number of free-flowing leukocytes adjacent to the vessel wall (0μm), 5μm and 10μm into the vessel lumen, and (b) the velocity of free-flowing leukocytes adjacent to the vessel wall (0μm). (a) ANOVA showed there was a significant variation in the number of free-flowing leukocytes with depth into the vessel ($P<0.0001$), but not with viscosity. (b) There was no significant variation in the free-flowing leukocyte velocity at the vessel wall as suspending phase viscosity was increased.

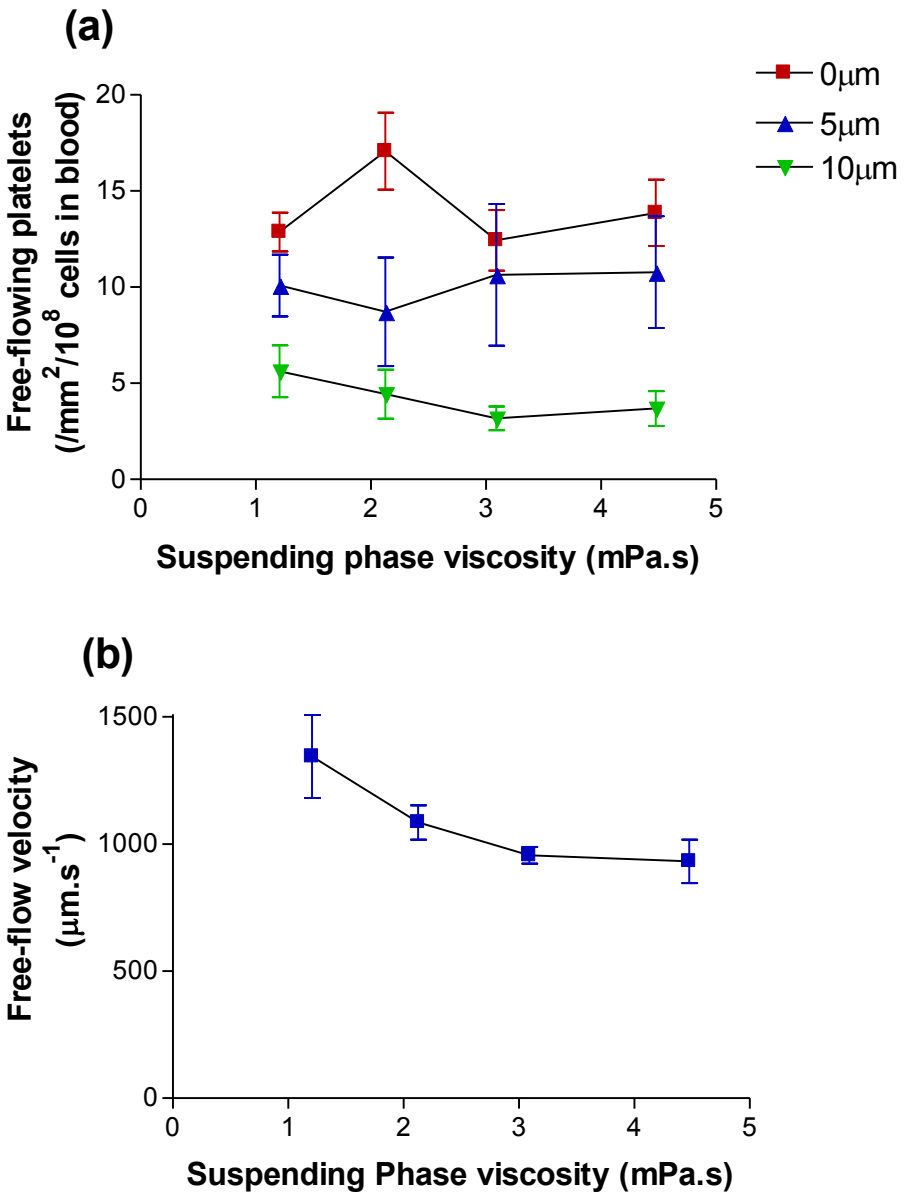


Figure 4.5 Effect of suspending phase viscosity on the number and velocity of free-flowing platelets near the vessel wall
Blood, adjusted to 40% haematocrit, was perfused through microslides of 100μm in height that had been coated with albumin. Data shown are means ± SEM from 3 experiments assessing; (a) the number of free-flowing leukocytes adjacent to the vessel wall (0μm), 5um and 10μm into the vessel lumen and (b) the velocity of free-flowing leukocytes at the vessel wall (0μm). (a) ANOVA showed no significant effect of suspending phase viscosity but a significant effect of depth on the number of free-flowing platelets ($P<0.0001$). (b) The free-flowing velocity of platelets at the vessel wall decreased significantly as suspending phase viscosity was increased ($P<0.05$ by ANOVA).

4.3.6 Effect of dextran concentration on the suspending phase viscosity and the apparent viscosity of the blood

The viscosities of the suspending phases retrieved from the blood samples were measured. There was no dependence of the calculated viscosity of the suspending phase on wall shear rate (see section 2.8.1). Increasing the concentration of the dextran solution significantly increased the viscosity of the suspending phase (fig 4.6).

The pressure difference across the microslides being perfused with blood increased as the wall shear rate was increased and as the dextran concentration was increased (fig 4.7(a)). The apparent viscosity of the blood flowing within the microslides was calculated to allow comparisons of the average wall shear stress at varying plasma viscosities (fig 4.7(b)). Blood viscosity increased with increasing concentration of dextran. When blood viscosity at 40% haematocrit was plotted against suspending phase viscosity at the same shear rate for the various dextran concentrations, there was a linear relation (fig 4.7(c)) indicating that blood viscosity was directly proportional to suspending phase viscosity. Stated differently, the relative apparent viscosity of the blood remained constant with varying suspending phase viscosity.

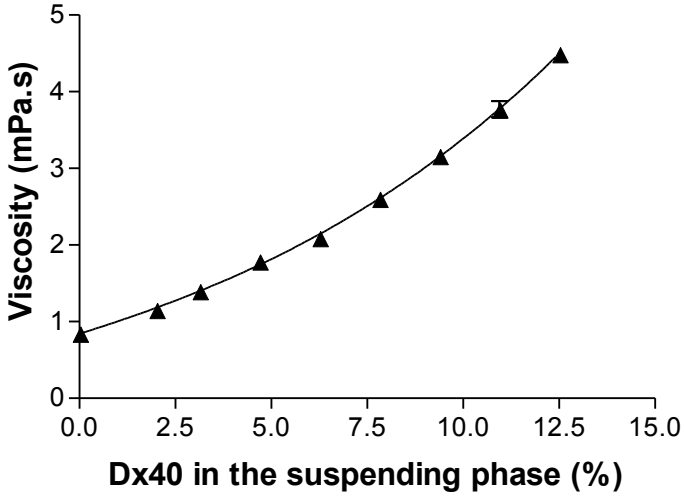


Figure 4.6 Effect of increasing Dx40 concentration on viscosity of the suspending phase
Blood was centrifuged and the suspending phase was perfused at $900s^{-1}$ across microslides of 100μm in height that had been coated with albumin. The suspending phase viscosity increased significantly with increasing Dx40 concentration ($P<0.0001$ by ANOVA).

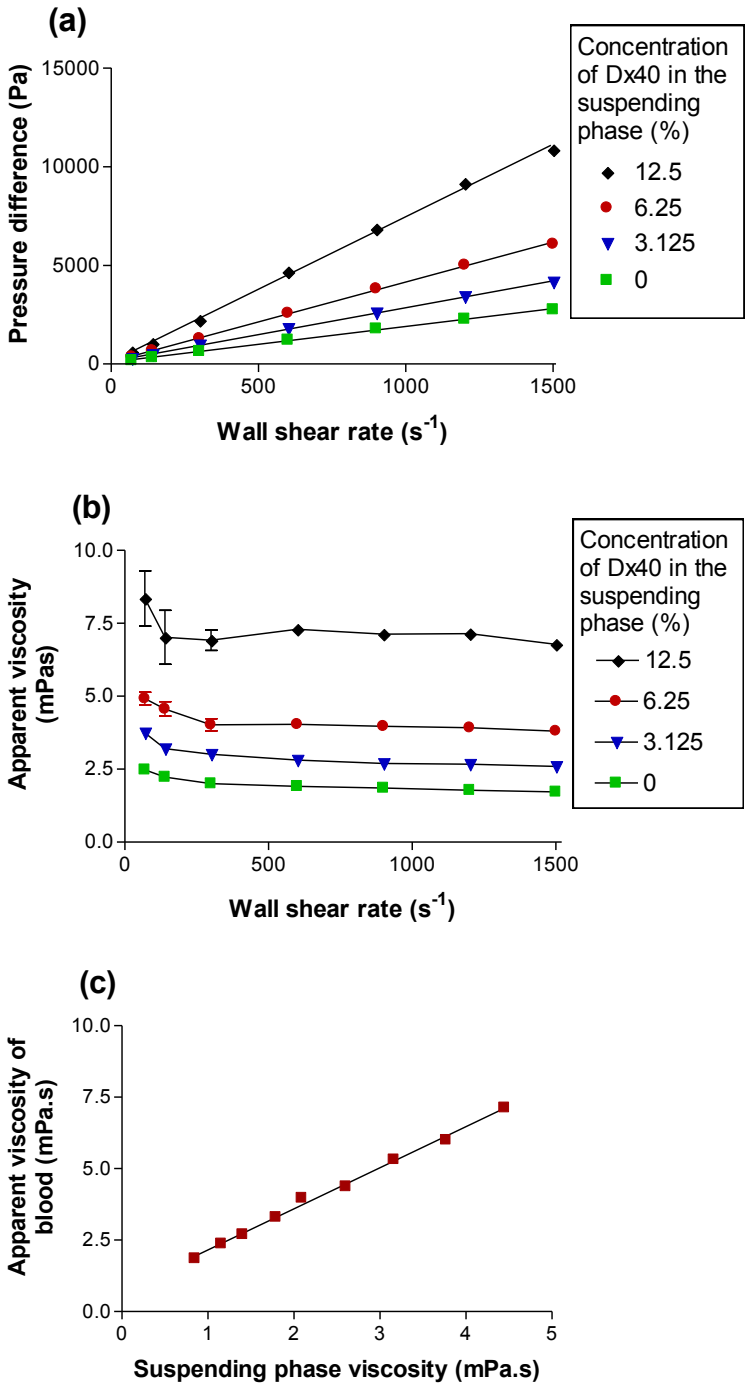


Figure 4.7 Effect of dextran concentration or suspending phase viscosity on (a) the pressure difference across a microslide; (b, c) the apparent viscosity of the blood
Blood, adjusted to a haematocrit of 40%, was perfused through microslides. (a) ANOVA showed significant effects of Dx40 concentration and wall shear rate on pressure difference ($P<0.0001$ for both). (b) ANOVA showed there was a significant effect of dextran concentration and shear rate on the apparent viscosity of the blood ($P<0.0001$ for both). (c) Linear regression showed a significant proportional increase in the apparent viscosity of the blood as the suspending phase viscosity increased ($P<0.0001$). Both were measured at wall shear rate of $900s^{-1}$.

4.4 Discussion

The effect of increasing suspending phase viscosity on leukocyte margination and adhesion has not previously been studied in blood. Only limited information was available on the effect of suspending phase viscosity on the margination and adhesion of platelets. Here for the first time the comparative effect of suspending phase viscosity on the adhesion of leukocytes and platelets has been assessed. In all blood samples in this study the addition of dextran 40 resulted in the abolition of RBC aggregation, judged by microscopic observation. Leukocyte adhesion decreased significantly as suspending phase viscosity was increased up to 2mPa.s. The sensitivity of platelet adhesion to suspending phase viscosity was lower than for leukocytes, with adhesion unaffected up to about 2mPa.s, but decreasing from 2 to 4mPa.s. The margination of both cell types was unaffected by suspending phase viscosity, although it was notable that in the absence of aggregation, leukocytes were more numerous in layers further from the wall, whereas platelets were most numerous adjacent to the wall. However, the free-flowing velocity of platelets (and to a lesser extent leukocytes) adjacent to the vessel wall did tend to decrease with increasing viscosity.

4.4.1 The effect of increasing suspending phase viscosity on the margination of leukocytes

As blood, normalised to a haematocrit of 40%, containing increasing concentrations of Dx40 was perfused through the 100μm microslides at a wall shear rate of $140s^{-1}$, there was no change in the number of the free-flowing leukocytes near the vessel wall. Thus, at constant shear rate and in the absence of aggregation, the distribution of the leukocytes was constant as suspending phase viscosity and shear stress in the blood increased. There was a slight trend for the velocity of the free-flowing leukocytes near the vessel wall to decrease as suspending

phase viscosity was increased although this effect was not significant. The number of free-flowing leukocytes near the vessel wall actually increased with depth into the lumen, which suggests that there were no preferentially marginated leukocytes in this system. As RBC aggregation had been abolished in these experiments this was to be expected. Single RBC are smaller than leukocytes, so that the leukocytes would have a tendency to flow more centrally in the vessel, resulting in fewer leukocytes near the vessel wall. The constant number of free-flowing leukocytes with increasing suspending phase viscosity at all depths into the vessel suggests that, although suspending phase viscosity should affect the deformation of RBC in flowing blood by providing a larger shear stress, the dispersion of leukocytes throughout the vessel was not affected.

The slight trend in the free-flowing velocity to decrease as suspending phase viscosity increased may have been due to the increased shear stress within the system. The particulate nature of blood leads to a plug-like velocity flow profile within a blood vessel. The aggregation of RBC in the centre of the vessel usually acts to increase this effect. In this system all RBC aggregation had been abolished and hence one would expect a more parabolic profile. The increased shear stress with increasing suspending phase viscosity would act to increase the alignment of the highly deformable RBC within the flow. The alignment would bring the flow-profile more closely to a parabolic profile. As a more plug-like flow acts to increase the near-wall shear rate and free-flowing velocity, the effect of increasing the suspending phase viscosity would result in a lower shear rate at the wall and hence a lower free-flowing leukocyte velocity. As the actual increase in suspending phase viscosity was quite small in the leukocyte margination experiments, the reduction of the free-flowing velocity of leukocytes was slight.

4.4.2 The effect of increasing the suspending phase viscosity on leukocyte adhesion

Leukocyte adhesion decreased significantly as the suspending phase viscosity of the blood was increased from a viscosity of 0.85mPa.s to 2.1mPa.s. As there was no change in the number of the leukocytes in free flow near the vessel wall, and velocity tended to decrease, the drop in leukocyte adhesion was likely to have been due to the increasing force experienced by the adhering cells. At a constant shear rate, a similar number of P-selectin-ligand bonds might form initially between flowing leukocytes and the vessel wall. However, as the suspending phase became more viscous, the force acting on any formed bonds would increase and the number of bonds surviving and resulting in continuous rolling adhesion would decrease.

Using the data from chapter 3 as a comparison it was possible in this system to show the effects of increasing shear stress on leukocyte adhesion in two ways. The first, from chapter 3 was to increase the shear rate at a constant viscosity; the second from this chapter was to alter the viscosity at a constant shear rate. This provided a comparison of the leukocyte adhesion when the shear stress and hence force on the P-selectin-ligand bond was increased, one with increasing leukocyte velocity and the other with nearly constant velocity (see fig 4.8). The data in each case were normalised relative to the value at a shear stress of 0.33Pa, at which point the suspending phase viscosity and shear rate in both studies were the same. In general the free-flowing velocity is expected to affect the rate of formation of the adhesive bonds and the force experienced by the bonds to affect the rate of dissociation (Chen and Springer, 2001;Orsello et al., 2001). Below 0.33Pa, one would expect that the adhesion would be higher for the lower shear rate for two reasons; the cells were travelling more slowly and in the native plasma, RBC aggregation was present and margination was effective. It was clear that a decrease in wall shear stress increased leukocyte adhesion; as data from both constant

shear rate and constant viscosity studies followed a similar trend (fig. 4.8). Above 0.33Pa, significant leukocyte margination was not observed in either study therefore the only unequal factor affecting adhesion was the velocity of the free-flowing leukocytes. If the cell velocity was the dominating factor, the adhesion at constant viscosity would have decreased more rapidly than the adhesion at constant shear rate. However, this was not the case and in fact adhesion decreased at a similar rate under the two different conditions. As the adhesion of the leukocytes in both studies decreased at the same rate with increasing shear stress, the predominant factor achieving stable leukocyte attachment over this range of shear stresses appears to have been the force on the P-selectin-ligand bond.

4.4.3 The effect of increasing suspending phase viscosity on the margination of platelets

At the vessel wall there was no significant variation in the number of free-flowing platelets over the full range of viscosities used in the platelet adhesion study. The free-flowing platelet velocity at the wall was significantly decreased as the suspending phase viscosity was increased. A greater number of free-flowing platelets were seen adjacent to the vessel wall when compared to greater depths into the lumen. This shows that there was margination of the platelets, although there was no effect of suspending phase viscosity on this margination. This suggested that the effect of increased viscosity in this system was not enough to inhibit the dispersive effects of the flowing RBC. The high dispersive force of the RBC would act to keep the number of platelets available for thrombus formation in inflammatory disorders high, where plasma viscosity is increased.

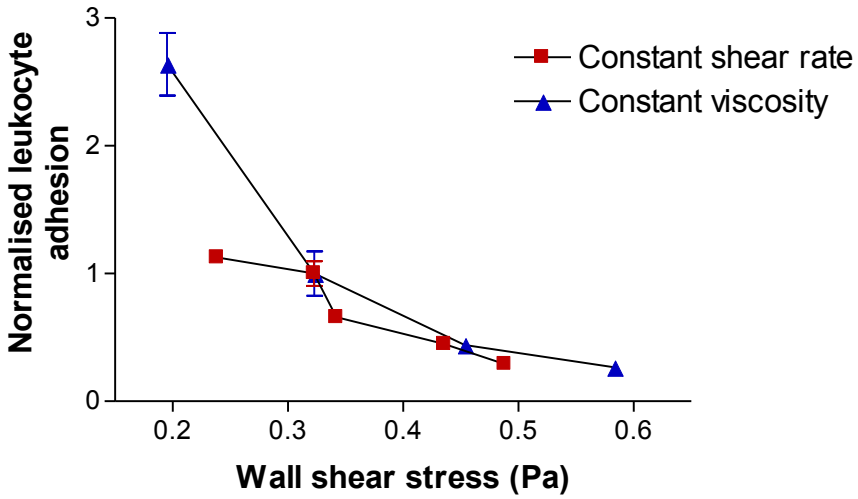


Figure 4.8 Effect of wall shear stress on leukocyte adhesion
Blood was perfused through microslides of 100μm in height that had been coated with P-selectin. Wall shear stress, calculated from pressure differences across the microslides, was varied at constant shear rate ($140s^{-1}$) by increasing the suspending phase viscosity, or was varied at constant viscosity (2mPa.s) by varying the shear rate. The number of adherent leukocytes were counted on the lower surface of the microslides. Data shown are means ± SEM from 4 experiments. Leukocyte adhesion was normalised to that at 0.33Pa.

As the suspending phase viscosity was increased, the decrease in the free-flowing velocity of platelets adjacent to the vessel wall may have been due to the increased shear stress across the vessel leading to the deformable RBC becoming more aligned with the blood flow. This would cause a more parabolic flow profile than that seen at low suspending phase viscosity, which would be more plug-like due to less alignment of the RBC in the flow. The more parabolic flow would result in a lower wall shear rate, and hence the cells adjacent to the vessel wall would travel more slowly.

The only previous study assessing the effect of suspending phase viscosity on platelet margination did not agree with our results entirely (Eckstein et al., 1988). Blood normalised to 15% haematocrit was perfused through rectangular vessels of 50μm in height at a wall shear rate of $210s^{-1}$. It was found that an increase in suspending phase viscosity from 1.2-3.9mPa.s resulted in the development of a greater near wall excess of platelet-sized beads. However, there was little difference between the relative number of platelet-sized beads between the two suspending phase viscosities close to the vessel wall. Deeper into the vessel the relative number of platelet-sized beads decreased, which matched our observations. However, in the higher viscosity suspending phase this decrease was larger up to 10μm from the vessel wall in the earlier study. RBC aggregation was abolished in both studies by the addition of low molecular weight dextran. The use of a higher haematocrit and larger range of suspending phase viscosities used in our study provided a more comprehensive overview of the effect of suspending phase viscosity on platelet margination.

4.4.4 The effect of suspending phase viscosity on the adhesion of platelets

At haematocrits of 20% or 40%, as the suspending phase viscosity was increased the percentage coverage with platelets remained constant until a suspending phase viscosity of about 2.5mPa.s was reached. Upon further increase of the suspending phase viscosity, the percentage coverage of platelets showed a significant decrease, with the decrease being sharper at the lower haematocrit. With a lower number of RBC flowing through the vessel the peripheral plasma layer would be larger. As the viscosity increased, the force perpendicular to the direction of flow provided by the RBC at the lower haematocrit would be less effective at facilitating platelet adhesion, as there would be fewer RBC-platelet collisions that would promote platelet-vessel wall interactions. Thus, the less efficient effect of RBC radial displacement of platelets at the lower haematocrit may have resulted in platelet adhesion being more sensitive to an increase in suspending phase viscosity.

The platelet margination studies found no difference in the number of marginated platelets as suspending phase viscosity was increased, although their velocity adjacent to the vessel wall did significantly decrease. The decrease in velocity would result in lower flux of platelets available to form thrombi and might have contributed to the reduction in adhesion seen at high suspending phase viscosities. The reduced velocity may have had an effect on platelet adhesion directly, but this would not have caused the significant drop in adhesion seen. The major effect on adhesion is likely to be the wall shear stress, which increased as the suspending phase viscosity was increased. Increasing the shear stress will increase the force experienced by any platelet-vessel wall bond. Initially, increasing the suspending phase viscosity had no effect on platelet adhesion as the force experienced by the bonds was low. As the suspending phase viscosity was increased further, the force on the adhesive bonds continued to increase. Upon reaching a certain limit, presumably at a suspending phase viscosity of around 2.5mPa.s, the force experienced by the adhesive bonds was large enough

to decrease the probability of bond survival and the adhesion was decreased (fig 4.3(c)). This suggests the existence of a threshold force for efficient bond survival.

Using the data from chapter 3 as a comparison, it was possible to show the effects of increasing shear stress on platelet adhesion in two ways. The first, from chapter 3 was to increase the shear rate at a constant viscosity; the second from this chapter was to alter the viscosity at a constant shear rate. This provided a comparison of platelet adhesion when the shear stress and hence force on the platelet-collagen bond was increased, one with increasing platelet velocity and the other with nearly constant velocity (fig 4.9). The data in each case were normalised around a shear stress of 2.1Pa, at which point the suspending phase viscosity and shear rate in both studies were the same.

Platelet free-flowing velocity is expected to affect the rate of formation of the adhesive bonds and the force experienced by the bonds to affect the rate of dissociation (Orsello et al., 2001). Below a wall shear stress of 2.1Pa, one would expect that platelet adhesion would be higher for the lower shear rate as the cells were travelling more slowly. There was no overlap at shear stresses below 2.1Pa, however platelet adhesion did increase as shear rate was decreased (fig. 4.9). Above 2.1Pa, the only unequal factor affecting adhesion was the velocity of the free-flowing platelets. Platelet adhesion at constant viscosity decreased more rapidly than at constant shear rate. The predominant factors influencing platelet adhesion with increasing shear stress appears to have been the rate of formation of the platelet-collagen bonds initially, and then the force acting on the bonds.

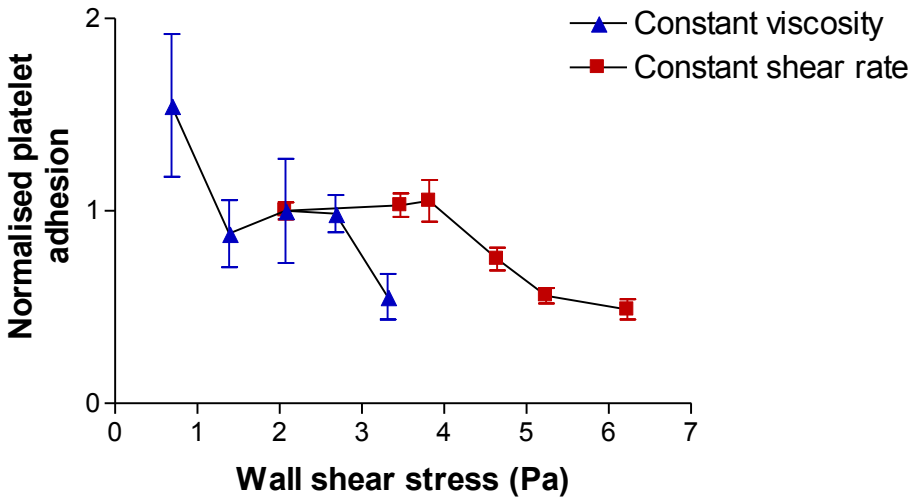


Figure 4.9 Effect of wall shear stress on platelet adhesion
Shear stress was varied at constant shear rate ($900s^{-1}$) by increasing the suspending phase viscosity, or was varied at constant viscosity (2mPa.s) by varying the shear rate. Percentage coverage was measured on the lower surface of the microslides. Data shown are means ± SEM from 4 experiments. Platelet adhesion was normalised to that at 2.1Pa.

The results presented here do not agree closely with the only previous study on the effect of suspending phase viscosity on platelet adhesion (van Breugel et al., 1992). Although both studies found a decrease in platelet adhesion as the suspending phase viscosity of the blood was increased, the range over which this was found was different. The previous study found a decrease in platelet adhesion with increasing viscosity between 0.89-2.0mPa.s (van Breugel et al., 1992), but adhesion was constant in this range in our study. In the previous study, glutaraldehyde fixed RBC were used. In addition, albumin was used to increase suspending phase viscosity. These differences may account for the difference in results obtained between both studies, perhaps because the rigid RBC also increased the shear stress.

4.4.5 Comparison of the effects of increasing suspending phase viscosity on adhesion of leukocytes and platelets

In this study, platelet adhesion persisted at a higher suspending phase viscosity than leukocyte adhesion. Leukocyte adhesion decreased significantly up to a suspending phase viscosity of 2mPa.s whilst platelet adhesion was constant over the same range (fig 4.3(a)). The platelet experiments were performed at a higher wall shear rate than the leukocyte experiments, hence when leukocyte and platelet adhesion were compared as a function of shear stress, platelet adhesion was present at far greater wall shear stresses than leukocyte adhesion (fig 4.3(b)). We considered whether the increased sensitivity of leukocyte adhesion to wall shear stress when compared to platelet adhesion was due to the size difference of the cells. Leukocytes are larger than platelets and as a result experience a much greater force at the wall for a given shear stress when adhesive bonds are formed. All adhesion is initiated by one or two bonds that lead to further bond formation and stable adhesion. The force experienced by these initial bonds can dictate the outcome of the adhesion process. As wall shear stress was increased,

the force experienced by the adhesive bonds was increased. Above some critical force the probability of bond survival will be dramatically decreased. As leukocytes are much larger than platelets, once an adhesive bond is formed the force experienced by leukocyte bonds will be much greater that those on a platelet at a given wall shear stress. Using the Goldman equation (Equation 20; Chapter 1) the force on the adhesive bonds was calculated for leukocytes and platelets (fig 4.3(c)). It was found that the critical force that the adhesive bonds could withstand before adhesion decreased markedly was around 0.15nN for leukocytes or platelets and above this value, adhesion dropped off at similar rates for the two cell types. Thus the difference in sensitivity of leukocyte and platelet adhesion to an increase in suspending phase viscosity appears to be due to the leukocytes being larger in size, and therefore the adhesive bonds formed with the vessel wall experiencing a greater force at a given wall shear stress.

4.4.6 Physiological relevance of findings

The results obtained in this study may be relevant to physiological changes in leukocyte and platelet adhesion in the circulation as plasma viscosity is increased. The viscosity of the plasma increases in most chronic inflammatory conditions and within a day or so of acute trauma or infection (Stuart and Whicher, 1988), as a result of increased fibrinogen within the plasma. The increase in plasma viscosity is not large in these conditions (similar to the range used in leukocyte adhesion experiments) and so would not affect platelet adhesion, but might affect leukocyte adhesion. However the increase in fibrinogen concentration in the blood will increase the aggregation of RBC , which might act to increase leukocyte adhesion *in vivo* (Pearson and Lipowsky, 2004) and thus overcome the effect of the more viscous plasma.

In disorders such as Waldenstrom's syndrome, where plasma viscosity can increase to around 5 times the normal value, both leukocyte and platelet adhesion may be affected. A symptom of this high viscosity disorder is mucosal bleedings which suggests platelet adhesion is indeed inhibited (Mannick.,1974).

In summary, this study has provided, for the first time, a direct comparison of the effect of suspending phase viscosity on the adhesion of leukocytes and platelets within the same sized vessels. This, coupled with analysis of the effects of suspending phase viscosity on the margination of leukocytes and platelets, helped to better understand the adhesive behaviour of both cell types. This comparison has shown that leukocytes are more sensitive to an increase in suspending phase viscosity then platelets, and suggests that this increased sensitivity is due to the difference in size between leukocytes and platelets.

CHAPTER 5: EFFECT OF RED BLOOD CELL AGGREGATION ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

5.1 Introduction

The effects of varying RBC aggregation on leukocyte and platelet margination and adhesion were quantified. Previous studies on the effects of modifying RBC aggregation on the margination and adhesion of leukocyte and platelets have been sparse. Leukocyte margination was clearly affected by RBC aggregation, with both *in vivo* (Vejlens, 1938) and *in vitro* (Palmer, 1967;Nobis et al., 1985) experiments showing an increase in the number of leukocytes travelling in the periphery of the vessel as RBC aggregation was increased. Adding high molecular weight dextran to increase the propensity of RBC to aggregate increased the number of adherent leukocyte at low shear rates *in vivo* (Pearson and Lipowsky, 2000) and *in vitro* (Abbitt and Nash, 2003). Similarly, abolishing aggregation by using low molecular weight dextran reduced adhesion in both studies. The previous *in vitro* study to assess leukocyte adhesion was performed in 300μm microslides; here microslides of depth 100μm were used as they were closer in size to post-capillary venules, where leukocyte adhesion occurs *in vivo*. Only a single study has assessed the effects of adding dextran to the blood on platelet margination *in vivo* (Woldhuis et al., 1993). It showed, in the rabbit mesentery, that the infusion of high molecular weight dextran increased the number of platelets flowing more centrally in the arterioles and decreased the number at the vessel wall. The infusion of low molecular weight dextran had no effect on platelet distributions in arterioles. However, RBC aggregation was not measured in this study and the effects on platelet margination were not attributed it. To our knowledge, the effect of RBC aggregation on platelet adhesion has not been studied to date. In this thesis the 100μm microslides were used to study platelet margination and adhesion.

We hypothesised that, due to the difference in cell size between leukocytes and platelets, their margination and hence adhesion would be affected differently by RBC aggregation. Our aims for the study were, for the first time, to study the effect of RBC aggregation on leukocyte and platelet margination and adhesion in the same vessels perfused in an identical system.

5.2 Methods

5.2.1 Blood collection

Venous blood was anticoagulated with CPDA. Theophylline was included in all experiments, apart from those assessing platelet adhesion, resulting in a final concentration of 7mM in the blood. Experiments were performed at a normalised haematocrit of 40%, except platelet adhesion was also performed at 20% haematocrit.

5.2.2 Blood manipulation

The RBC aggregation was manipulated by adding equal volumes of Dx500, Dx40 or autologous plasma to the blood, (e.g. see Figure 2.1). The dextrans were at concentrations that would not alter plasma viscosity. For the platelet adhesion experiments performed at 20% haematocrit, the dextrans or plasma were added to the blood directly. In all other experiments, the blood was centrifuged and the appropriate amount of plasma was removed prior to the addition of the dextrans or autologous plasma to normalise the blood to a haematocrit of 40%.

5.2.3 Microslide coating

For experiments on leukocyte adhesion, microslides were coated in purified P-selectin at a concentration of 10μg/ml. For experiments involving platelet adhesion, microslides were coated with Horm collagen at 500μg/ml. For margination and tube viscometry experiments the microslides were coated with albumin to provide a non-adhesive surface on the inside of the microslide.

5.2.4 Adhesion assay

Blood was fluorescently labelled with 5μg/ml of R6G. To measure the adhesion of leukocytes to P-selectin, blood was perfused through the microslide at a shear rate of $140s^{-1}$ for a total of 4 minutes from the time the microslide was first filled with blood. To measure platelet adhesion to collagen, the blood was perfused through the microslides at a shear rate of 300 or $900s^{-1}$. Adhesion was measured up to 4 minutes after the microslide was first filled with blood.

5.2.5 Assessment of margination

Margination was analysed by perfusing fluorescently labelled blood through horizontal, albumin-coated microslides. The free-flowing cells were measured at the upper surface of the microslide. Leukocyte experiments were performed at a shear rate of $140s^{-1}$. Platelet margination experiments were performed at a wall shear rate of $300s^{-1}$.

5.2.6 Tube viscometry

The apparent viscosities of blood samples containing Dx40, autologous plasma or Dx500 were analysed as previously.

5.3 Results

5.3.1 Effect of RBC aggregation on the leukocyte adhesion

Dextrans of different molecular weight were used to assess the effect of RBC aggregation on leukocyte adhesion (Dx40 to abolish, and Dx500 to enhance RBC aggregation). The number of adherent leukocytes was significantly increased as RBC aggregation was increased (fig 5.1(a)). The rolling velocity of the adherent leukocytes showed no dependence on RBC aggregation, although the rolling velocity in plasma was slightly higher than in the blood samples containing dextrans (fig 5.1(b)).

5.3.2 Effect of manipulating RBC aggregation on the platelet adhesion

Initially, to avoid centrifugation of the blood, the platelet adhesion experiments were performed at a haematocrit of 20%, at wall shear rates of $300s^{-1}$ and $900s^{-1}$. As RBC aggregation increased, platelet adhesion was significantly decreased at both wall shear rates (fig 5.2).

The platelet adhesion experiments were also performed at 40% haematocrit, where the same trends in adhesion were visible at both shear rates. The percentage coverage of platelets significantly decreased as RBC aggregation was increased (fig 5.3). There was a higher percentage coverage of platelets at the higher shear rate as no correction was made for the volume perfused in these data.

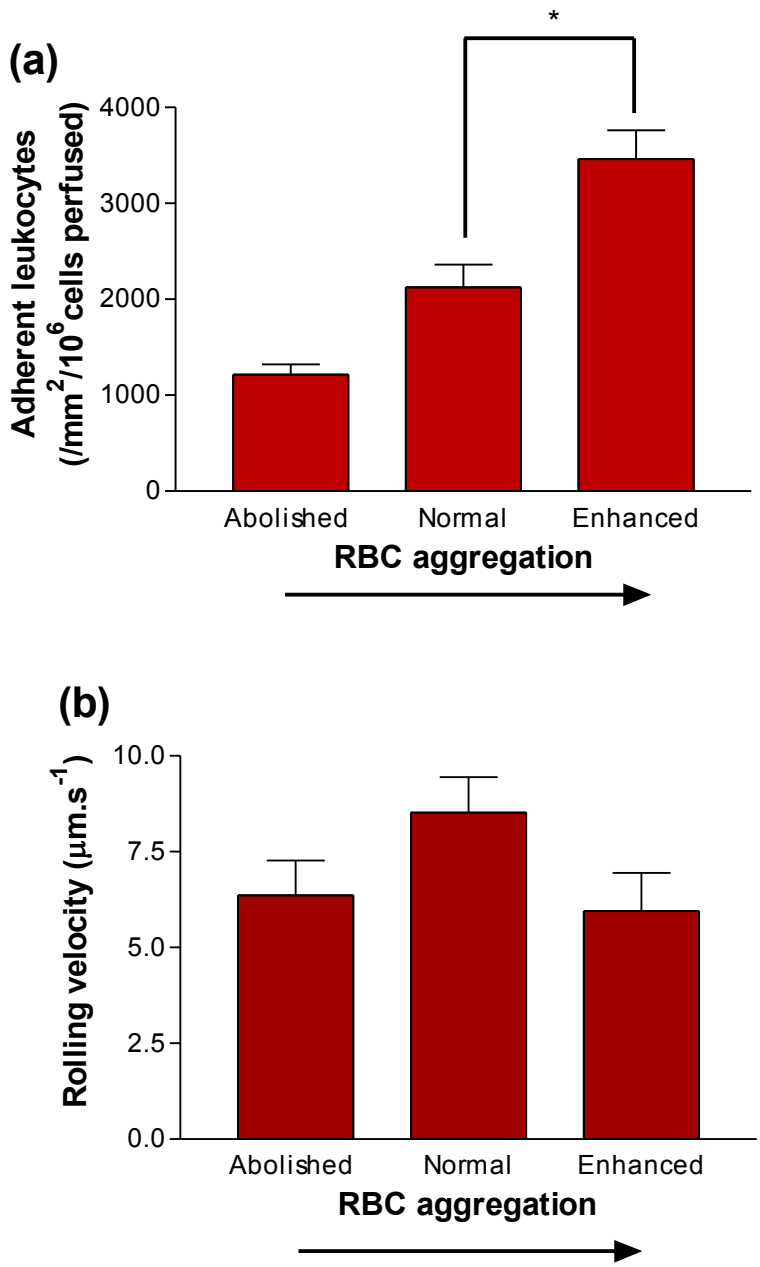


Figure 5.1 Effect of manipulating RBC aggregation on (a) leukocyte adhesion and (b) rolling velocity of adherent cells
Blood was perfused through microslides of 100μm in height that had been coated with P-selectin. The blood was manipulated to alter RBC aggregation and keep haematocrit at 40% and plasma viscosity at ~1.2mPa.s. Data shown are means ± SEM from 4 experiments. Leukocyte adhesion was normalised per 10^6 cells perfused. (a) ANOVA showed a significant effect of RBC aggregation on adhesion ($P<0.01$). * = $P<0.05$ compared to normal (control) RBC aggregation by Dunnett's test. (b) ANOVA showed no significant effect of RBC aggregation on the rolling velocity of leukocytes.

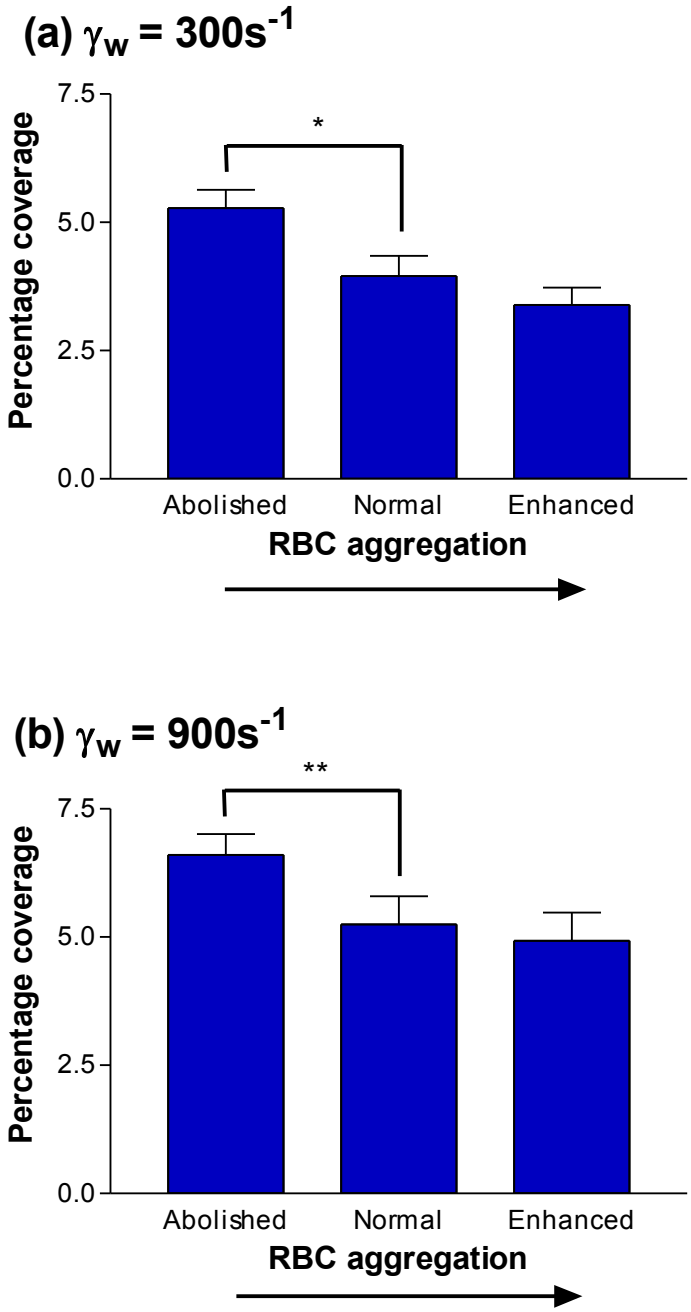


Figure 5.2 Effect of manipulating RBC aggregation on platelet adhesion at 20% haematocrit

Blood was perfused through microslides of 100μm in height that had been coated with collagen. The blood was manipulated to alter RBC aggregation and keep haematocrit at 20% and plasma viscosity at ~1.2mPa.s. Data shown are means ± SEM of the percentage coverage of platelets after 4 minutes perfusion at wall shear rates of (a) 300 s^{-1} or (b) 900s^{-1} from 4 experiments. ANOVA showed a significant effect of RBC aggregation on adhesion at both wall shear rates ($P<0.01$ in both cases). * = $P<0.05$, ** = $P<0.01$ compared to normal (control) RBC aggregation by Dunnett's test.

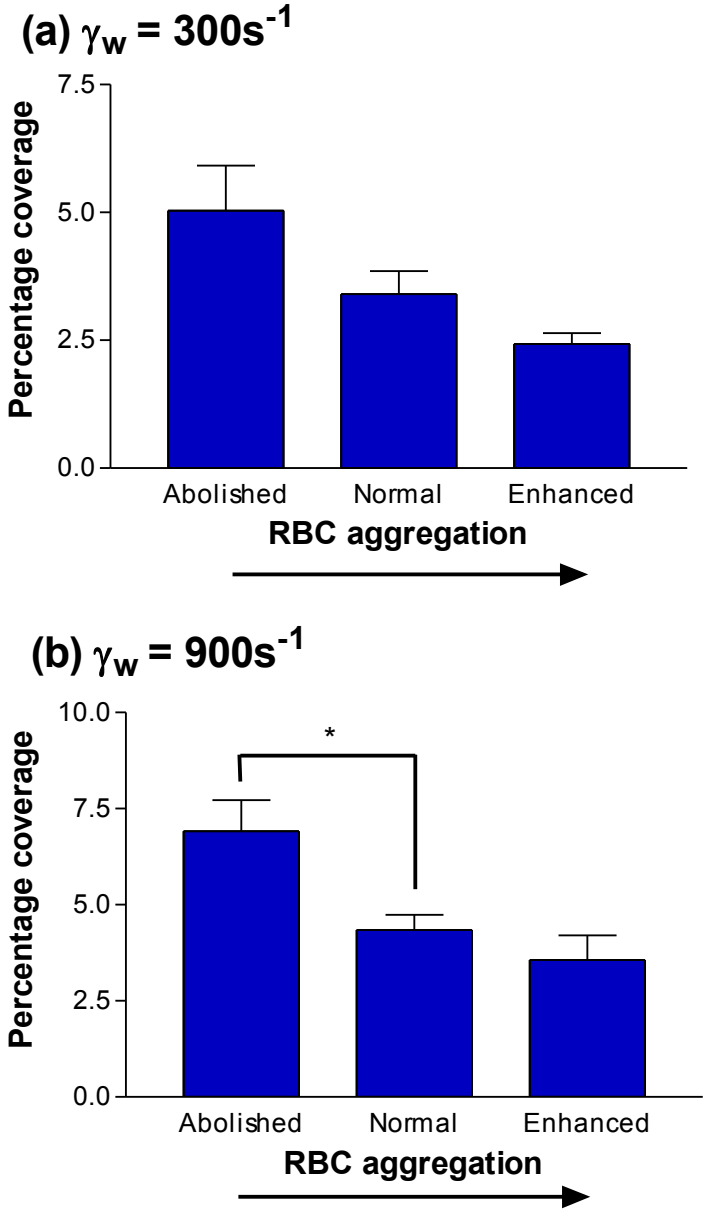


Figure 5.3 Effect of manipulating RBC aggregation on platelet adhesion at 40% haematocrit

Blood was perfused through microslides of 100μm in height that had been coated with collagen. The blood was manipulated to alter RBC aggregation and keep haematocrit at 40% and plasma viscosity at ~1.2mPa.s. Data shown are means ± SEM of the percentage coverage of platelets after 4 minutes perfusion at wall shear rates of (a) 300 s^{-1} or (b) 900s^{-1} from 4-5 experiments. ANOVA showed a significant effect of RBC aggregation on coverage at both wall shear rates ($P<0.05$ in both cases). * = $P<0.05$ compared to normal (control) RBC aggregation by Dunnetts's test.

5.3.3 Comparison of the effects of RBC aggregation on leukocyte and platelet adhesion

Direct comparison of the adhesion trends observed in this study are shown in Figure 5.4. As RBC aggregation was increased, the trends in leukocyte adhesion (at $140s^{-1}$) or platelet adhesion (at $900s^{-1}$) were in similar proportions but opposite in direction.

5.3.4 Effect of RBC aggregation on leukocyte margination

After correcting for the number of leukocytes within the blood it was found that there was a significant increase in the number of free-flowing leukocytes adjacent to the vessel wall as RBC aggregation was increased (fig 5.5(a)). At 5μm and 10μm into the vessel lumen, the number of free-flowing leukocytes did not vary significantly as RBC aggregation was manipulated. However there was a trend for the number to increase as RBC aggregation was increased. The velocity of the free-flowing leukocytes at the vessel wall was increased slightly in the blood samples with increased RBC aggregation, but this effect was not statistically significant (fig 5.5(b)).

5.3.5 Effect of RBC aggregation on platelet margination

Near the vessel wall, as RBC aggregation was increased at 40% haematocrit there was no significant change in the number of free-flowing platelets (fig 5.6(a)). However, there was a significant variation with distance into the lumen, with the highest number of platelets observed adjacent to the wall. The free-flowing velocity of the platelets adjacent to the vessel wall was not affected by changes in RBC aggregation (fig 5.6(b)).

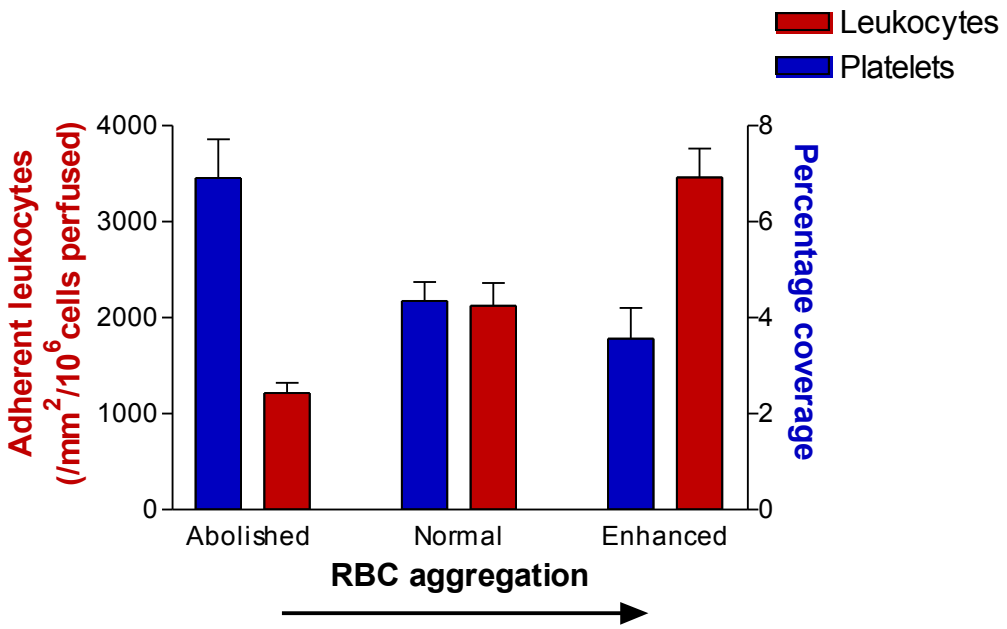


Figure 5.4 Comparison of the effect of RBC aggregation on leukocyte and platelet adhesion

Blood was perfused through microslides of 100µm in height that had been coated with P-selectin or Horm collagen for assessment of leukocyte or platelet adhesion respectively. Data are means ± SEM of 4 experiments at shear rates of 140 s^{-1} or 900s^{-1} for leukocytes or platelets respectively.

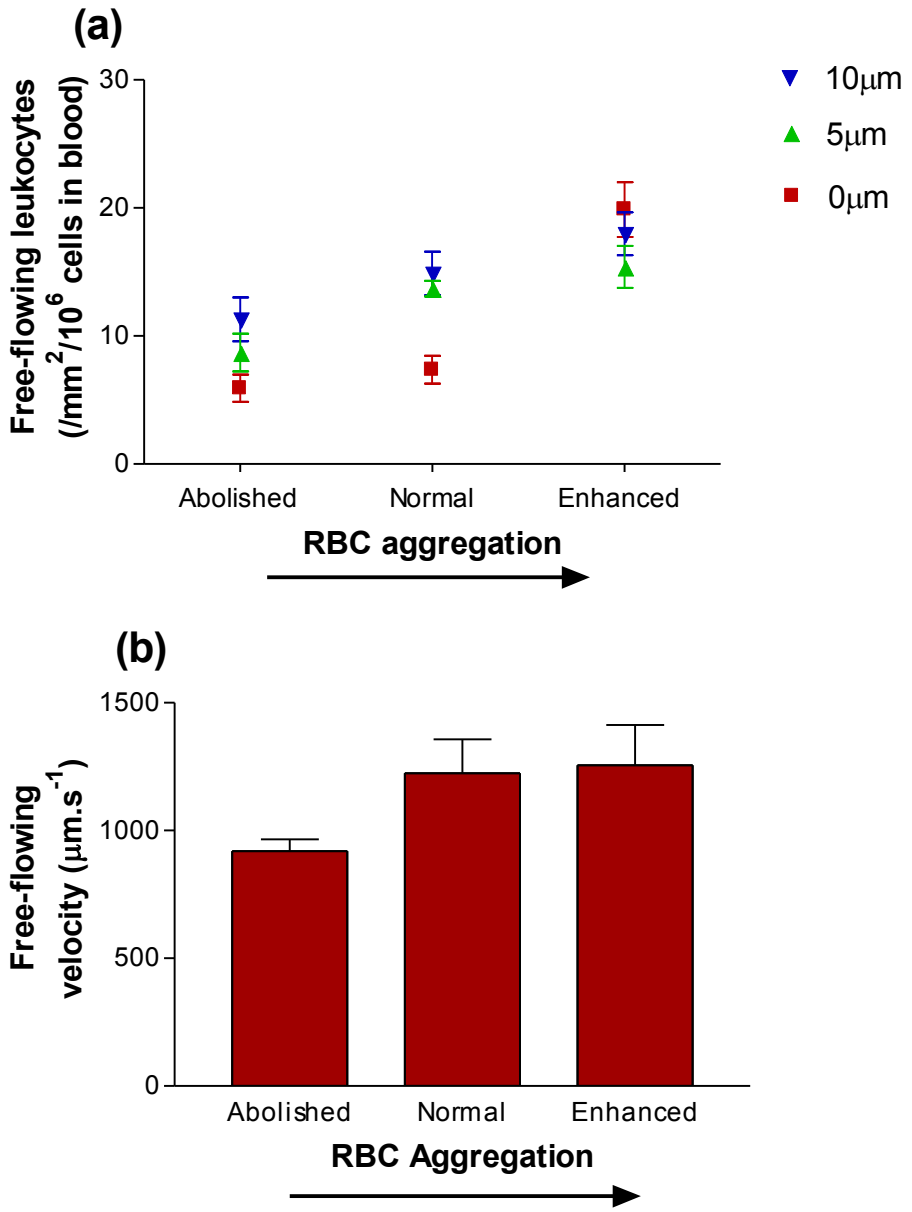


Figure 5.5 The effect of RBC aggregation on the number and velocity of free-flowing leukocytes near the vessel wall

Blood was perfused through microslides of 100μm in height that had been coated with albumin. The blood was manipulated to alter RBC aggregation and keep haematocrit at 40% and plasma viscosity at ~1.2mPa.s. Data shown are means ± SEM from 3 experiments assessing; (a) the number of free-flowing leukocytes adjacent to the vessel wall (0μm), 5μm and 10μm into the vessel lumen, and (b) the velocity of the free-flowing leukocytes adjacent to the wall (0μm). (a) ANOVA showed that RBC aggregation significantly affected the number of free-flowing leukocytes near the vessel wall ($P<0.01$) and depth had a significant effect on the number of free-flowing leukocytes ($P<0.05$). Subsequent analysis showed that RBC aggregation only had a significant effect on the number of leukocytes adjacent to the vessel wall ($P<0.01$). Bonferroni post tests showed a significant difference between enhanced RBC aggregation and the other two samples ($p<0.01$ in each case). (b) There was no significant effect of RBC aggregation on the free-flowing velocity of leukocytes adjacent to the vessel wall.

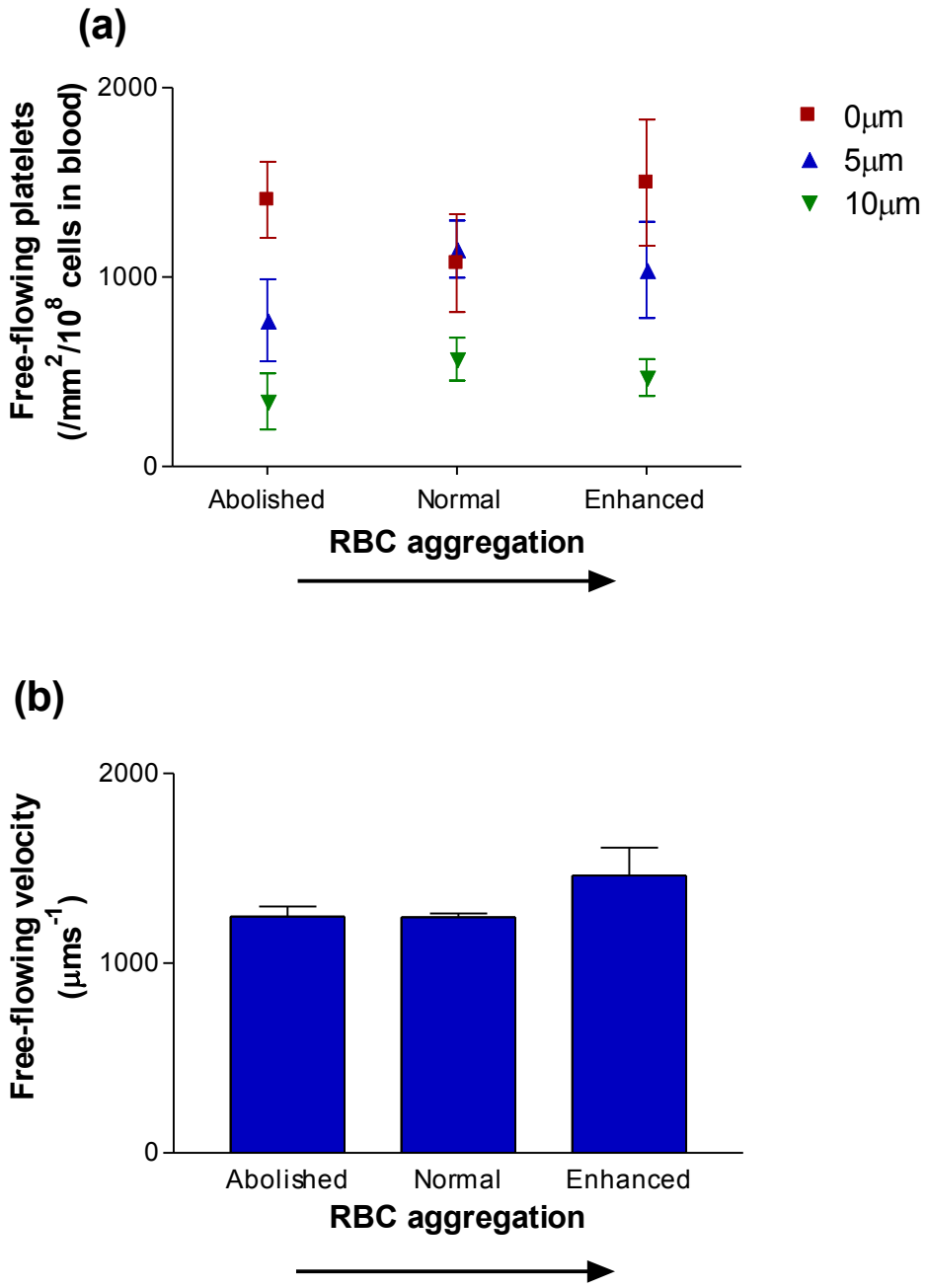


Figure 5.6 Effect of RBC aggregation on the number and velocity of free-flowing platelets near the vessel wall

Blood, was perfused through microslides of 100μm in height that had been coated with albumin. The blood was manipulated to alter RBC aggregation and keep haematocrit at 40% and plasma viscosity at ~1.2mPa.s. Data shown are means ± SEM from 3 experiments assessing; (a) the number of free-flowing platelets adjacent to the vessel wall (0μm), 5um and 10μm into the vessel lumen and (b) the velocity of free-flowing leukocytes at the vessel wall (0μm). (a) ANOVA showed a significant effect of depth on the number of free-flowing platelets ($P<0.001$). (b) There was no significant effect of RBC aggregation on the free-flowing velocity of platelets adjacent to the vessel wall.

5.3.6 Effect of RBC aggregation on blood viscosity

The concentrations of Dx40 and Dx500 which were suitable for altering RBC aggregation were previously defined (Abbitt and Nash, 2003). As the viscosity of the plasma had a significant effect on the adhesion of both leukocytes and platelets, it was important that the diluents used in the above experiments were the same viscosity as the plasma. Prior to starting the aggregation experiments, the viscosity of the diluents used in the manipulation of RBC aggregation were shown to be not significantly different from the viscosity of plasma; it was also verified that viscosity did not vary with shear rate (confirming newtonian behaviour of the diluents) (fig 5.7). The mean values of viscosity of the dextran solutions were 1.176 ± 0.034 and 1.171 ± 0.101mPa.s for Dx40 and Dx500 solutions respectively and plasma was 1.105 ± 0.063mPa.s. After analysis of the diluent and plasma viscosity, the tube viscometer was used to determine the viscosity of the 40% blood samples with manipulated RBC aggregation.

The manipulation of RBC aggregation had a significant effect on the pressure difference across the microslides being perfused with blood (fig 5.8(a)). The calculated apparent viscosity of the blood showed no significant variation at shear rates between $140s^{-1}$ and $1500s^{-1}$ (fig 5.8(b)). As the wall shear rate dropped below $140s^{-1}$ the apparent viscosity of all the samples increased significantly. In addition there was a significant difference between the sample with increased RBC aggregation and the other two samples. Thus, non-newtonian behaviour was evident in the blood and appeared exaggerated when RBC aggregation was increased.

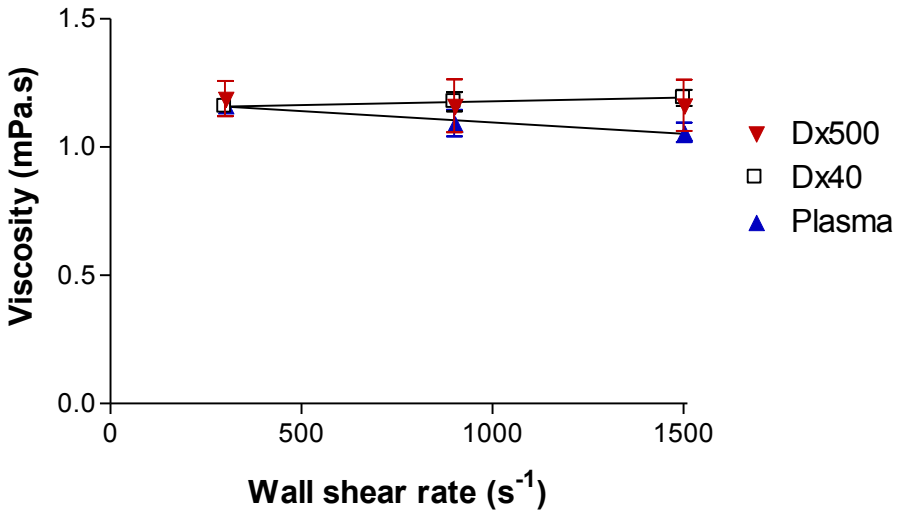


Figure 5.7 Viscosity of dextran solutions and plasma
Dextran solutions at the appropriate concentrations and plasma were perfused through microslides 100μm in height coated with albumin. Linear regression showed no effect of shear rate on viscosity for all solutions. The viscosities of the Dx40 and Dx500 solutions and plasma were not significantly different from each other. The mean values of the viscosities of the Dx40, Dx500 solutions and plasma were 1.176, 1.171 and 1.105mPa.s respectively.

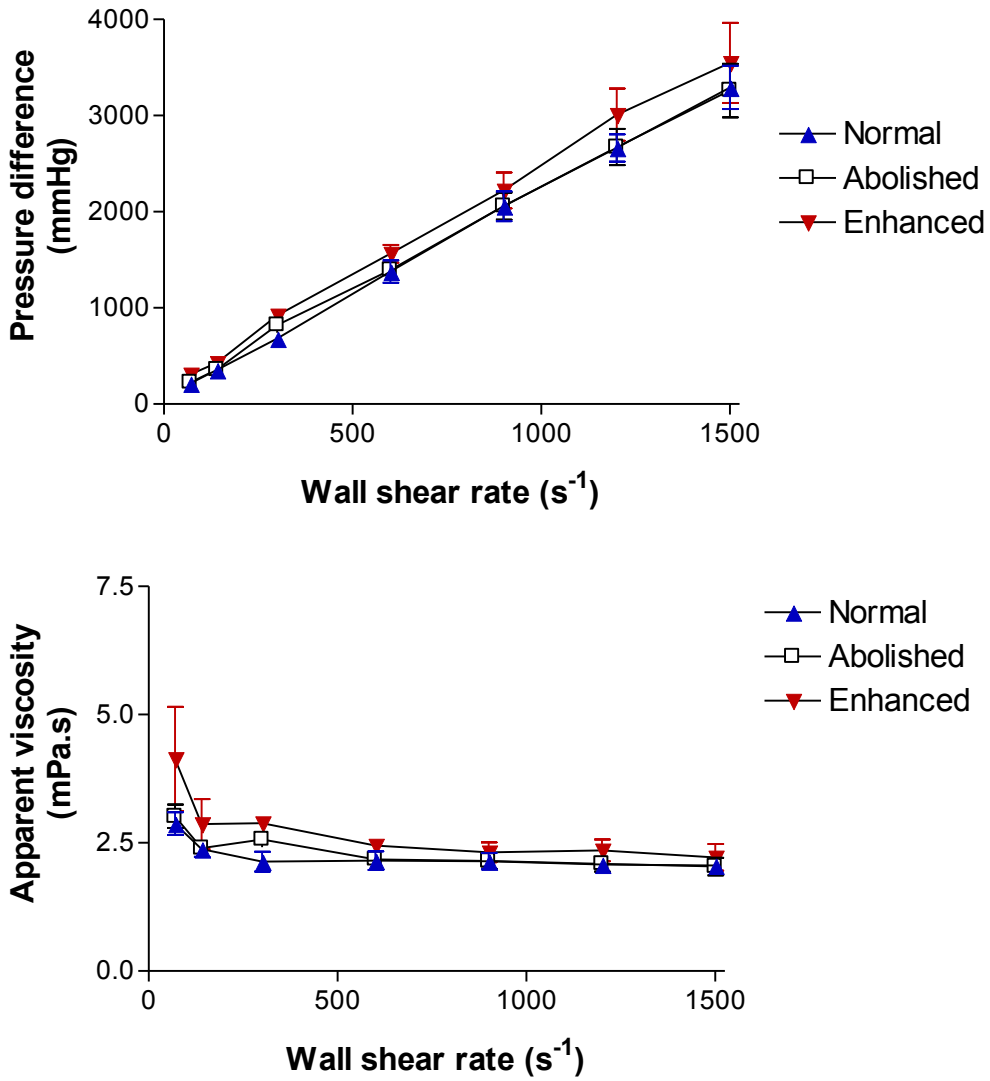


Figure 5.8 Effect of RBC aggregation on the pressure difference across microslides and the apparent viscosity of the blood

Blood was perfused through microslides of 100μm in height coated with albumin. The blood was manipulated to alter RBC aggregation and keep haematocrit at 40% and plasma viscosity at ~1.2mPa.s. In (a) there was a significant effect of RBC aggregation on the pressure difference across the microslides ($P<0.001$ by ANOVA). In (b) ANOVA showed a significant effect of RBC aggregation on the apparent viscosity of the blood within the microslides ($P<0.001$). The apparent viscosity of the blood sample with enhanced aggregation was significantly higher than normal and abolished samples ($P< 0.01$ and $P< 0.05$ respectively, Bonferroni post test). Wall shear rate had no effect on the apparent viscosity of blood between 140-1500s^{-1} but had a significant effect below 140s^{-1} ($P<0.05$ by ANOVA).

5.4 Discussion

Here for the first time the effect of RBC aggregation on the adhesion of leukocytes and platelets has been directly compared in the same experimental model. We found that increasing RBC aggregation increased the leukocyte adhesion at a wall shear rate of $140s^{-1}$, but reduced the percentage coverage of platelets at $300s^{-1}$ and $900s^{-1}$. Increasing RBC aggregation increased the number of free-flowing leukocytes flowing adjacent to the vessel wall but had no significant effect on the number of platelets. When RBC aggregation was enhanced above normal, the viscosity of the blood, and hence wall shear stress, was elevated at low shear rates.

5.4.1 Effect of RBC aggregation on leukocyte margination

Increasing RBC aggregation in this system increased the number of free-flowing leukocytes adjacent to the vessel wall. This increase was not maintained at 5μm or 10μm into the vessel lumen. There was a trend for the velocity of the free-flowing leukocytes at the vessel wall to increase with RBC aggregation, but this effect was not statistically significant.

The radial distribution of blood cells in flow is size dependent (Phibbs and Dong, 1970). When RBC aggregation is enhanced, it is expected that the larger aggregates will tend to flow in the axial regions of the vessel and push the smaller leukocytes towards the vessel wall. When RBC aggregation is abolished and RBC flow singly, the leukocytes become the largest flowing cellular component in the blood and are expected to flow more centrally. Our data conform to these expectations. The size of the leukocytes is such that it allows them to be preferentially marginated when the aggregation of RBC is increased. *In vivo*, the low shear rate experienced in post-capillary venules should result in a high degree of RBC aggregation

occurring in the central region of the vessel, increasing the number of leukocytes flowing near the wall. Upon inflammation, the endothelial up-regulation of selectins will promote adhesion and a greater number of leukocytes will be able to respond to invading pathogens. During inflammation, acute phase plasma proteins including fibrinogen are released, and this will tend to increase RBC aggregation. This could further promote adhesion.

The results obtained in this study are in good agreement with the literature. Palmer (Palmer, 1967) performed the first *in vitro* study to assess the effect of adding dextran to the blood on leukocyte distribution within a vessel. Using 30μm diameter tubes, which diverged into 5 smaller tubes, he showed that the addition of high molecular weight dextran to the blood caused leukocytes to flow more peripherally than in normal blood, so that they preferentially entered the outer tubes. Nobis et al (Nobis et al., 1985) used high and low molecular weight dextran to enhance and abolish RBC aggregation respectively. Using fluorescently labelled leukocytes and viewing the blood flow 'end-on' they showed that abolishing RBC aggregation increased the central concentration of leukocytes within the vessel and that enhancing the RBC aggregation resulted in almost no leukocytes flowing in the central region of the blood vessel and a large number flowing in the periphery. Goldsmith and Spain (Goldsmith and Spain, 1984) showed, by measuring relative tubular leukocyte concentration, that when RBC were suspended in buffered albumin to match viscosity but abolish RBC aggregation, the increase in tubular leukocyte concentration at low shear rate was abolished. Thus, RBC aggregation was responsible for radial displacement of leukocytes at low shear rate.

Recent studies in rectangular vessels (300μm in height), found an increase in the number of free-flowing leukocytes near the vessel wall as high molecular weight dextran was used to enhance RBC aggregation (Abbitt and Nash, 2003). However no difference was seen

between normal blood and blood with abolished RBC aggregation. No effect of aggregation on the velocity of the free-flowing leukocytes near the wall was detected. Here, in smaller tubes, the tendency for leukocyte velocity to increase as RBC aggregation was enhanced suggested that, at high levels of aggregation, there was increased plug-like flow and blunting of the flow profile of the blood. This would result in a higher shear rate at the wall and hence a higher free-flowing velocity.

5.4.2 Effect of RBC aggregation on leukocyte adhesion

In this system, increasing RBC aggregation resulted in a significant increase in the number of rolling adherent leukocytes. The free-flowing velocity of the leukocytes adjacent to the vessel wall showed a trend to increase as RBC aggregation was increased and the apparent viscosity was significantly higher in the blood samples with enhanced RBC aggregation; both of these effects would be expected to decrease adhesion. Thus the main determinant of the effect of manipulating RBC aggregation on leukocyte adhesion was presumably the increased number of free-flowing leukocytes at the vessel wall. Indeed, the proportional increases in free-flowing cells and in adhesion were similar if the results for reduced aggregation and increased aggregation were compared.

The results obtained here are in good agreement with previous studies of RBC aggregation on leukocyte adhesion both *in vivo* and *in vitro*. Pearson and Lipowsky (Pearson and Lipowsky, 2000) showed, in rat mesenteric venules, at shear rates below $100s^{-1}$ that the number of adherent rolling leukocytes on the vessel wall was increased four-fold when high molecular weight dextran was infused into the blood to increase RBC aggregation, compared to around a two-fold increase in this study. The number of adherent leukocytes was decreased to around half the normal value when low molecular weight dextran was infused to abolish RBC aggregation; this matched the results found in our study.

In vitro, rectangular vessels have been used to assess the margination and adhesion of leukocytes in the same system (Abbitt and Nash, 2003). Although the blood was normalised to 20% haematocrit in the previous study (compared to 40% here) and 300μm microslides were used, the results obtained followed the same trend as those reported here.

Along with the increased number of free-flowing leukocytes near the vessel wall, as RBC aggregation was increased, the width of the peripheral plasma layer was probably increased, due to the increased tendency of the large RBC aggregates to migrate towards the axis of the vessel. This may also have been a factor in the increase in adhesion seen as the RBC aggregation was increased. Any adherent leukocytes experience a force, parallel to the vessel wall, from the flowing blood. When the plasma layer is at its widest, the number of collisions between flowing RBC and adherent leukocytes that will result in an increased force experienced by the adherent leukocyte, in a direction parallel to the vessel wall, with the potential to detach an adhesive leukocyte will be low. As the plasma layer is at its narrowest when RBC aggregation is abolished, the number of RBC collisions with adherent leukocytes in a direction parallel to the vessel wall will be increased, this may result in a higher detachment rate and a subsequent decrease in leukocyte adhesion.

Leukocytes are optimally sized to be affected by RBC aggregation in blood flow. They are larger than a single RBC but smaller than RBC aggregates. As these aggregates form more readily at low shear rates, experienced in post-capillary venules, it is here where leukocyte adhesion is facilitated. This process presumably enhances the innate immune response to infection.

5.4.3 Effect of aggregation on the margination of platelets

Within this system, manipulating the RBC aggregation had little effect on the number of free-flowing platelets adjacent to the vessel wall. The number of free-flowing platelets seen, decreased with increasing depth into the vessel lumen. The velocity of the platelets was not significantly changed, although there was a slight increase as RBC aggregation was increased. Due to the size-dependent migration of platelets towards the periphery of the vessel, the marginated platelets in the blood will flow in the peripheral plasma layer near the wall, and the number of platelets near the vessel wall was always higher than the number at 5μm or 10μm into the vessel. This showed that platelet margination occurred in the presence or absence of RBC aggregation. Thus, manipulating the RBC aggregation had no effect on platelet margination. The slight increase in platelet free flow velocity suggests that the flow profile of the blood may have been more plug-like as RBC aggregation was increased.

Only one previous study relevant to the effects of RBC aggregation on the margination of platelets has been performed. Woldhuis et al (Woldhuis et al., 1993) showed, in the rabbit mesentery, that infusing high molecular weight dextran increased the number of platelets flowing more centrally in the arterioles and decreased the number at the vessel wall. The infusion of low molecular weight dextran had no effect on platelet distributions in the arterioles. Aggregation was not evaluated in that study, and in fact, responses were not attributed to this phenomenon. The results found in the present study partly match the data presented by Woldhuis et al, since both observed little effect on platelet distribution when dextran 40 was added. *In vivo*, the mean shear rates in the arterioles was around $130s^{-1}$ when low molecular weight dextran was infused and $85s^{-1}$ when high molecular weight dextran was infused. These values were lower than the shear rates used in our study, especially for high molecular weight dextran. The lower shear rate would probably have resulted in a higher degree of RBC aggregation in the central region of the vessel *in vivo*. This could, in turn

increase the width of the peripheral plasma layer, lowering the concentration of platelets flowing within it and result in a lower observed count near the vessel wall. In our study, the high shear rates used may not have yielded such a great effect on the plasma layer. Thus, platelet margination showed a lack of dependence on RBC aggregation in our study.

5.4.4 Effect of aggregation on platelet adhesion

In our studies we found that, as RBC aggregation was increased, the percentage coverage of platelets decreased markedly and significantly at wall shear rates of $300s^{-1}$ and $900s^{-1}$. As RBC aggregation had no significant effect on the number of free-flowing platelets or their velocity adjacent to the vessel wall, the drop in platelet adhesion seen as RBC aggregation was increased must have been due to other factors.

Since the number of free-flowing platelets adjacent to the vessel wall was essentially constant regardless of RBC aggregation, we propose that the increase in platelet coverage as RBC aggregation was reduced was due to a change in the width of the peripheral plasma layer. Measurements of the peripheral plasma layer have shown that when RBC aggregation is enhanced the plasma layer increases in width and vice versa, and that the width can vary between 2-6.5μm (Uijttewaal et al., 1993). Thus, as leukocytes are larger in diameter than the maximum width of the plasma layer, it has very little effect on leukocytes. Platelets, being discoid shaped cells of around 2μm diameter and 1μm thickness, are smaller than the peripheral plasma layer, even at its thinnest. This results in platelet adhesion being significantly affected by the peripheral plasma layer. As RBC aggregation is decreased, the plasma layer becomes thinner and the number and velocity of free-flowing platelets adjacent to the vessel wall remains constant. The reduced distance between the RBC-platelet interactions and the vessel wall would result in more efficient platelet-vessel wall contact and

adhesion as a result of the platelets experiencing a force in a direction perpendicular to the vessel wall due to collisions with flowing RBC. In other words, the marginated platelets in the narrow plasma layer would be more efficiently forced into contact with the wall. The opposite would be true in blood samples with enhanced RBC aggregation, as the distance from the edge of the flowing RBC core and the vessel wall would be increased. This increased distance would result in fewer RBC-platelet collisions driving adhesive interactions between the platelet and the vessel wall. Thus, modifying RBC aggregation in this system may affect platelet adhesion indirectly by altering the width of the peripheral plasma layer.

The blood samples with enhanced RBC aggregation had a significantly higher apparent viscosity than the other samples. Studies performed assessing the effect of suspending phase viscosity on the adhesion of platelets in the same system (see chapter 4) showed that, at 40% haematocrit; platelet adhesion was constant up to apparent blood viscosities of around 4.5mPa.s. This value is in excess of the apparent blood viscosity measured in the aggregation experiments. Thus the changes in apparent viscosity with aggregation, which are consistent with previous reports from studies using horizontal tubes (Alonso et al., 1989), are unlikely to explain adhesion responses seen here.

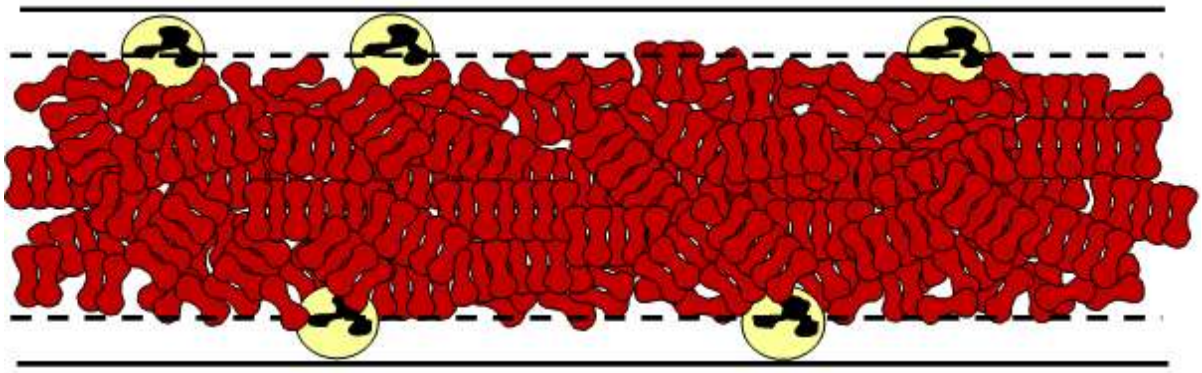
To our knowledge there have been no previous studies assessing the effect of RBC aggregation on platelet adhesion, and none attempting to link adhesion and margination. As a result this study showed for the first time that increasing RBC aggregation significantly decreases the percentage coverage of adherent platelets on collagen. Combined with platelet margination studies in the same system, we suggest here for the first time that, with regards to platelet adhesion, it is not the margination of the free-flowing platelets that determines adhesion but rather the relative size of the peripheral plasma layer containing the marginated free-flowing platelets.

5.4.5 Comparison of the effect of RBC aggregation on leukocytes and platelets

It is clear that RBC aggregation has opposite effects on leukocyte and platelet adhesion (fig 5.4). An increase in RBC aggregation caused an increase in leukocyte margination and no change in platelet margination. Adhesion is affected differently, with an increase in RBC aggregation resulting in an increase in leukocyte adhesion and a decrease in platelet adhesion.

The events underlying these phenomena are summarised schematically in Figures 5.9 and 5.10. Again, the size of the cells appears to dictate how RBC aggregation affects cell behaviour within blood flow. Platelet margination was unaffected by a change in RBC aggregation as platelets are the smallest cells in the blood. Leukocytes, being smaller then RBC aggregates but larger than individual RBC are only radially displaced towards the periphery of the vessel when aggregation is present (fig 5.9). The peripheral plasma layer around the flowing RBC core is always smaller than the diameter of the leukocytes and hence they are unaffected by changes in the plasma layer thickness. Thus, it is the direct increase in the number of free-flowing leukocytes adjacent to the vessel wall that results in the increase in leukocyte adhesion as RBC aggregation is increased. Platelet adhesion is modified by a different mechanism as their free-flowing distribution is unaffected by RBC aggregation. In contrast to leukocytes, the increase in platelet adhesion seen when RBC aggregation is decreased is due to narrowing of the peripheral plasma layer. The width of the plasma layer, at its thinnest is larger than the diameter of a platelet, thus a decrease in the width of the plasma layer, induced by abolishing RBC aggregation, would result in marginated platelets being forced into contact with the vessel wall more efficiently (fig 5.10).

(a)



(b)

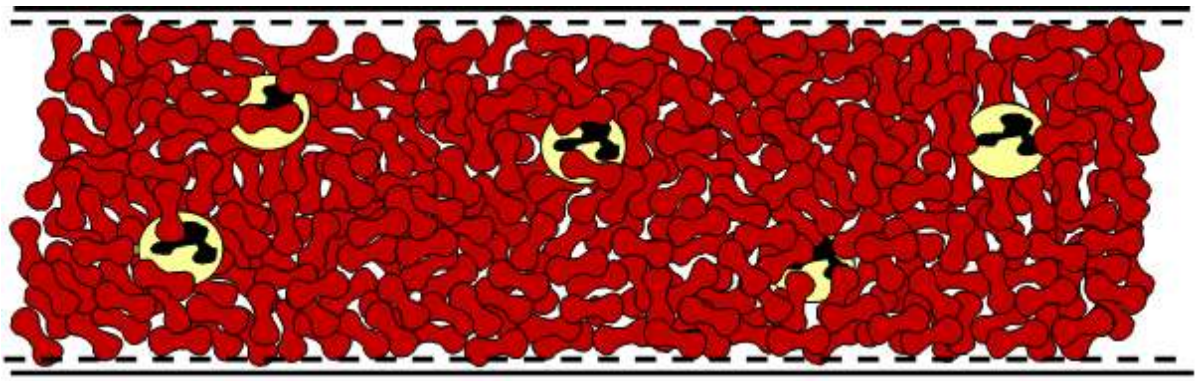
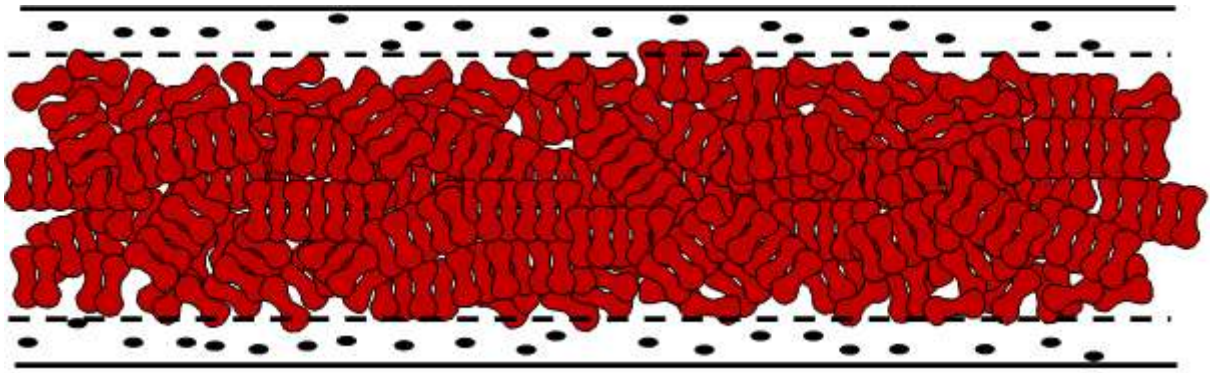


Figure 5.9 Effect of RBC aggregation on leukocyte distribution within a blood vessel
This figure illustrates how RBC aggregation may affect the margination of leukocytes and the plasma layer around the periphery. (a) When RBC aggregation is enhanced, the central flow of RBC is contracted and leukocytes are displaced radially towards the vessel wall. This acts to increase leukocyte adhesion. At its largest the plasma layer is still smaller than the leukocyte diameter. (b) When RBC aggregation is abolished, leukocytes are not radially displaced outward as they are larger than single RBC, therefore leukocytes flow more centrally, reducing leukocyte adhesion.

(a)



(b)

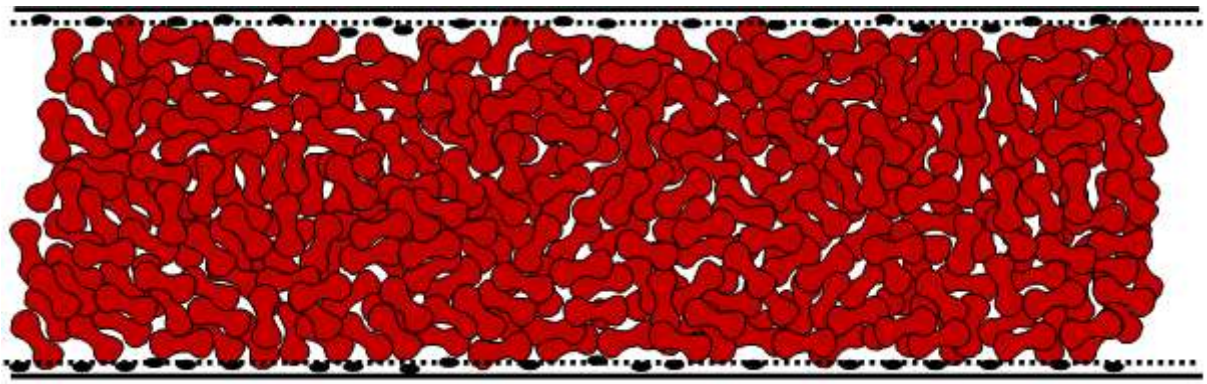


Figure 5.10 Effect of RBC aggregation on the distribution of platelets within the blood
This figure illustrates how RBC aggregation may affect the distribution of platelets and the plasma layer around the periphery. (a) When RBC aggregation is enhanced, the central flow of RBC is contracted. Platelets are displaced radially towards the vessel wall but the plasma layer is wider than the platelet diameter and they are less effectively forced against the wall. (b) When RBC aggregation is abolished, the plasma layer becomes narrower and comparable to the platelet size. The number of RBC-platelet collisions that cause platelets to come into contact with the vessel wall and facilitate platelet adhesion increases, even though platelet margination (or number density near the wall) may itself be similar whether RBC aggregation is enhanced or abolished.

In summary, studies of red cell aggregation, in conjunction with studies of shear rate dependence, support the concept that the efficiency of platelet adhesion depends on the width of the plasma layer near the wall. Leukocyte adhesion, in contrast depends more on aggregation- and shear rate-dependent margination. *In vivo*, the extent of RBC aggregation depends mainly on the local shear rate. Leukocyte adhesion occurs in post-capillary venules where the shear rate is low (Pearson and Lipowsky, 2000), facilitating RBC aggregation and in turn leukocyte margination and thus adhesion. In inflammation, the concentration of fibrinogen within the plasma increases (Stuart and Whicher, 1988), this will also act to increase RBC aggregation and promote leukocyte adhesion within the post-capillary venules. Efficient platelet adhesion in the small vessels with low shear rates on the venous side of the circulation is not so important or indeed desirable. This study found that platelet adhesion tended to decrease as RBC aggregation was increased. Efficient platelet adhesion is much more desirable in the high shear rate environment within the arterial side of the circulation, as due to the high pressure and flow rate of the blood in these regions blood loss could occur very rapidly. Within the arterial side of the circulation the high shear rates experienced result in low levels of RBC aggregation and as a result the width of the peripheral plasma layer is small. This will promote platelet adhesion within the regions of the circulation in which it is most important. Thus it seems that the sizes of both leukocytes and platelets are well suited to help promote adhesion within the areas of the circulation in which they are most important.

CHAPTER 6: EFFECT OF HAEMATOCRIT ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

6.1 Introduction

Investigations were made into the effect of haematocrit on leukocyte and platelet margination and adhesion. Previous studies had shown that leukocyte margination required RBC but was not critically dependent on the haematocrit level. Although Bagge et al showed that leukocytes flowed more peripherally in the presence of RBC than in their absence (Bagge et al., 1983), studies performed by Goldsmith and Spain (Goldsmith and Spain, 1984) and Abbitt and Nash (Abbitt and Nash, 2003) both showed a lack of dependence of leukocyte margination on haematocrit above a minimum level.

Platelet margination however does show a haematocrit dependence over a wider range. Much of the work performed to assess the effect of increasing haematocrit on the margination of platelets has used the ratio of tubular count to that of the reservoir, where a higher concentration of platelets within the tube than in the reservoir indicates that platelets flowed more peripherally. Although the exact distribution of the platelets cannot be determined by this method, it is an indicator of platelet margination. It was shown that an increase in haematocrit increased the tubular concentration, up to a haematocrit of around 40%, but further increases in haematocrit did not yield further increases in tubular platelet count (Corattiyl and Eckstein, 1986;Uijttewaal et al., 1993). Increasing haematocrit from 0 % to 40% resulted in increased radial distribution of platelet-sized beads at a range of shear rates (520-1220s^{-1}) in a similar model (Yeh and Eckstein, 1994). The only study of platelet margination using direct observations was done by Tilles and Eckstein (Tilles and Eckstein, 1987). Their results showed an increase in haematocrit from 15-40% resulted in an increase in the number of platelet-sized beads flowing more peripherally.

Studies on the effect of haematocrit on adhesion of leukocytes or platelets are limited. Previous studies have shown increasing haematocrit up to 30% increased leukocyte adhesion

with no significant further increase above 30% (Abbitt and Nash, 2003). *In vivo* observations in the rat mesentery have shown that at low shear, haematocrits above 50% resulted in an increase in leukocyte adhesion in post capillary venules (Firrell and Lipowsky, 1989).

Here, we used fluorescently labelled blood to provide a direct comparison of the margination and adhesion of leukocytes and platelets in the same sized vessels. We hypothesised that an increase in haematocrit would not affect leukocyte margination but would increase the margination of platelets and the adhesion of both cell types.

6.2 Methods

6.2.1 Blood collection

Venous blood was anticoagulated with CPDA. Theophylline was included in margination experiments only, resulting in a final concentration of 7mM in the blood.

6.2.2 Blood manipulation

The haematocrit of the blood was manipulated by centrifuging the blood and removing the appropriate amount of plasma to result in 50% haematocrit. Autologous plasma with a platelet count the same as the blood at 50% was added to obtain haematocrits of 10, 20, 30, 40 and 50%. In leukocyte experiments, the haematocrit of the blood was manipulated by adding autologous plasma to un-manipulated blood to obtain haematocrits of 10, 20, 30 or 40%.

6.2.3 Microslide coating

For experiments on leukocyte adhesion, microslides were coated in purified P-selectin at a concentration of 10μg/ml. For experiments on platelet adhesion, microslides were coated with Horm collagen at 500μg/ml. For all margination and tube viscometry experiments the microslides were coated with albumin to provide a non-adhesive surface.

6.2.4 Adhesion assay

Blood was fluorescently labelled with 5μg/ml of R6G. To measure the adhesion of leukocytes to P-selectin, blood samples at various haematocrits, were perfused at a shear rate of $140s^{-1}$ for a total of 4 minutes from the time the microslide was first filled with blood. To measure platelet adhesion to collagen, the blood was perfused through the microslides at a

shear rate of $900s^{-1}$. Adhesion was measured up to 4 minutes after the microslide was first filled with blood.

6.2.5 Assessment of margination

Margination was analysed by perfusing fluorescently labelled blood, with manipulated haematocrit, through albumin coated microslides. The microslides were perfused horizontally and the free-flowing cells were measured adjacent to the upper surface of the microslide. Leukocyte margination was not systematically quantified as they experienced marked sedimentation at haematocrits below 30% (see section 6.3.1). Platelet margination experiments were performed at a wall shear rate of $300s^{-1}$.

6.2.6 Tube viscometry

The apparent viscosities of blood samples with manipulated haematocrit were analysed as previously.

6.3 Results

6.3.1 Effect of haematocrit on leukocyte adhesion

The effect of varying haematocrit on leukocyte adhesion was previously assessed in our laboratory using 300μm microslides, vertically perfused (Abbitt and Nash, 2003). In that case, the trend was for adhesion to increase with increasing haematocrit. Here, using horizontal 100μm microslides, the number of rolling leukocytes at 10% haematocrit was much higher than at 20% haematocrit, and this level was still higher than at 30 or 40% haematocrit (fig 6.1(a)). The rolling velocity of these adherent leukocytes showed no significant change overall as haematocrit was increased (fig 6.1(b)). The large amount of leukocyte adhesion seen at 10-20% haematocrit was apparently due to the sedimentation of the leukocytes onto the lower surface. No adherent cells were observed on the upper surface of the microslides at 10%. On the other hand, it was observed that at 40% haematocrit the leukocyte adhesion to the upper and lower surface of the microslide was the same (see Chapter 3). Below 20% haematocrit the results do not reflect a direct effect of haematocrit on adhesion, but an indirect effect through allowing rapid leukocyte sedimentation. We checked whether this phenomenon was specific to the smaller 100μm microslides. It was clear that the reduction in adherent leukocytes on the lower surface of the microslides as haematocrit was increased from 20% to 40% was similar for 300μm microslides (fig 6.2). Thus, the effect of low haematocrit in horizontal tubes was not dependent on vessel size.

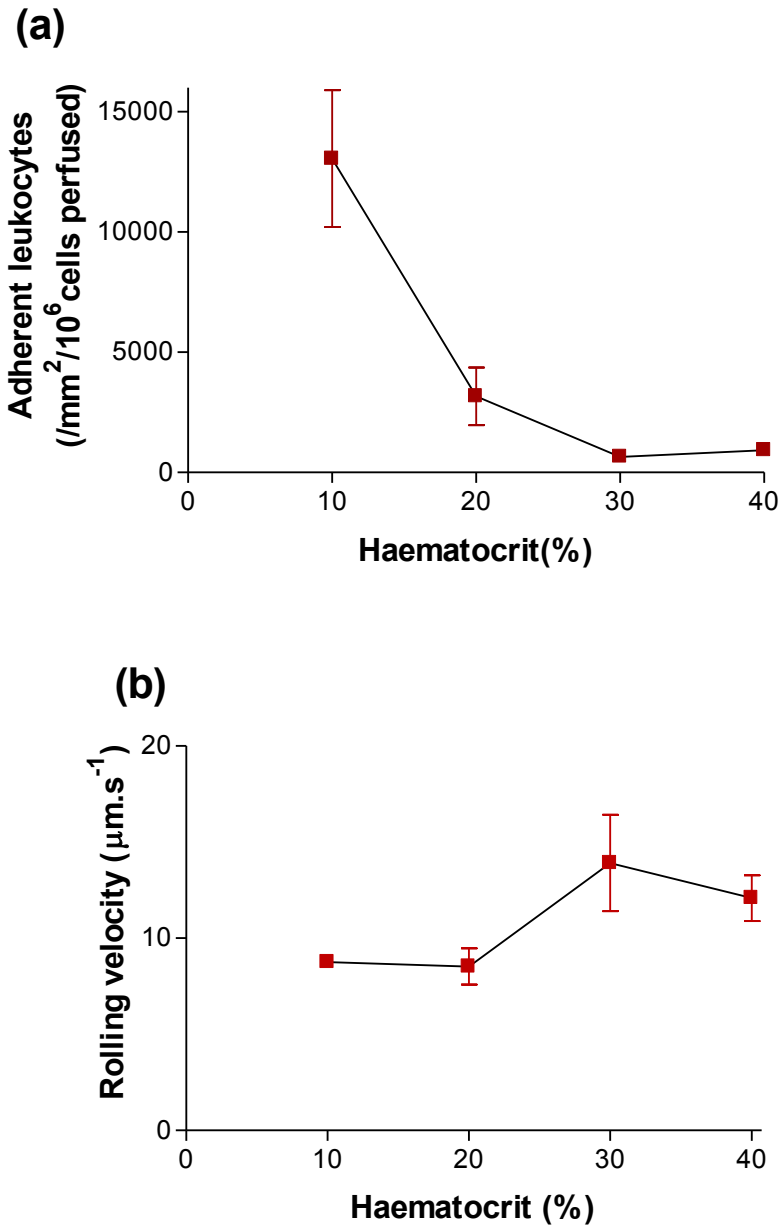


Figure 6.1 Effect of increasing haematocrit on leukocyte adhesion
Blood was perfused through microslides of 100µm in height that had been coated with P-selectin. The blood was manipulated to vary haematocrit between 10-40%. The number of adherent leukocytes were counted on the lower surface of the microslides. Data shown are means ± SEM from 3 experiments. Leukocyte adhesion was normalised per 10^6 cells perfused. (a) ANOVA showed a significant effect of haematocrit on adhesion ($P<0.01$). (b) ANOVA showed no significant effect of haematocrit on rolling velocity of leukocytes.

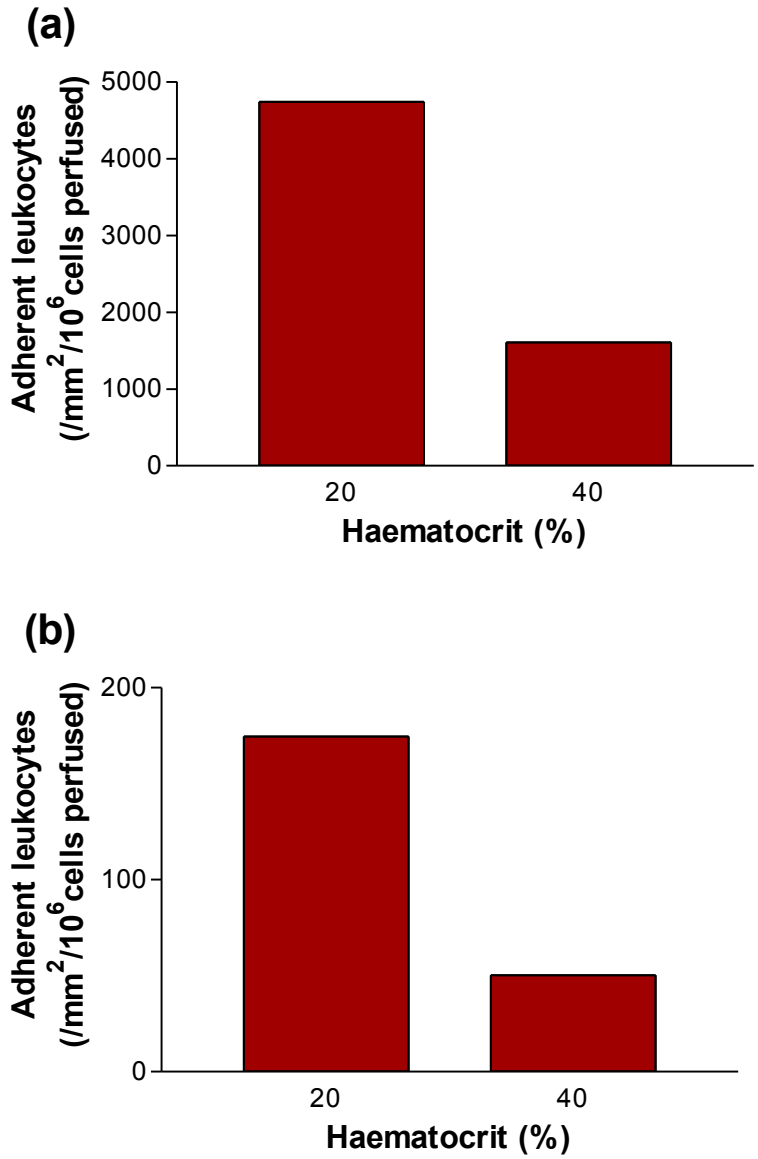


Figure 6.2 Comparison of adhesion of leukocytes at 20 and 40% haematocrit in 300µm or 100µm microslides

Blood was perfused through microslides of (a) 100µm or (b) 300µm in height that had been coated with P-selectin. The blood was manipulated to have haematocrit of 20% or 40%. The number of adherent leukocytes was counted on the lower surface of the microslides. Data shown is from one experiment. Leukocyte adhesion was normalised per 10^6 cells perfused. There was a large decrease in adhesion as haematocrit was increased from 20% to 40% in both sizes of microslide.

6.3.2 Effect of haematocrit on platelet adhesion

The perfusion of blood through microslides of 100μm in height, at a wall shear rate of $900s^{-1}$, showed a significant, nearly linear increase in platelet coverage as haematocrit was increased from 10-50% (fig 6.3).

6.3.3 Comparison of the effects of haematocrit on the adhesion of leukocytes and platelets

As the haematocrit of the blood perfused was increased, leukocyte adhesion was significantly decreased between 10-30%, but the adhesion was constant between 30-40%. Platelet adhesion showed a significant increase across the whole range of haematocrits used (10-50%) (fig 6.4). It was clear that the results, when assessing the effect of haematocrit on leukocyte adhesion, were strongly influenced by sedimentation as leukocyte adhesion to the lower surface of the microslide was extremely high at 10% haematocrit, but negligible to the upper surface. Platelet adhesion was not augmented at low haematocrit.

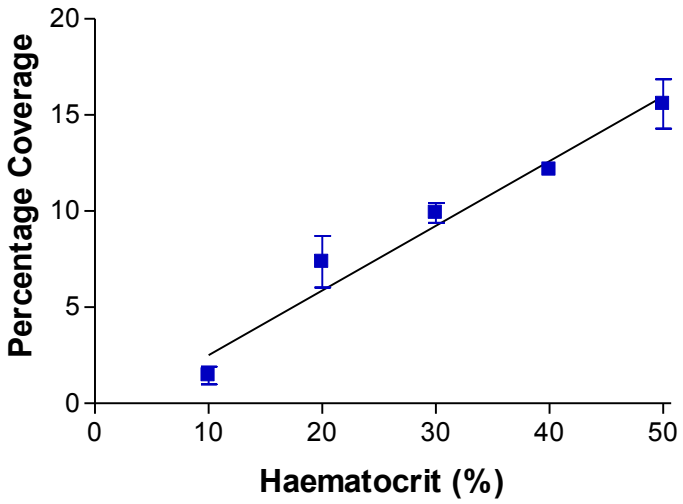


Figure 6.3 Effect of increasing haematocrit on platelet adhesion
Blood was perfused through microslides of 100μm in height that had been coated with collagen. The blood was manipulated to obtain haematocrits of 10-50%. Data shown are means ± SEM of the percentage coverage of platelets after 4 minutes blood perfusion from 4 experiments. ANOVA showed a significant effect of haematocrit on platelet coverage ($P<0.0001$).

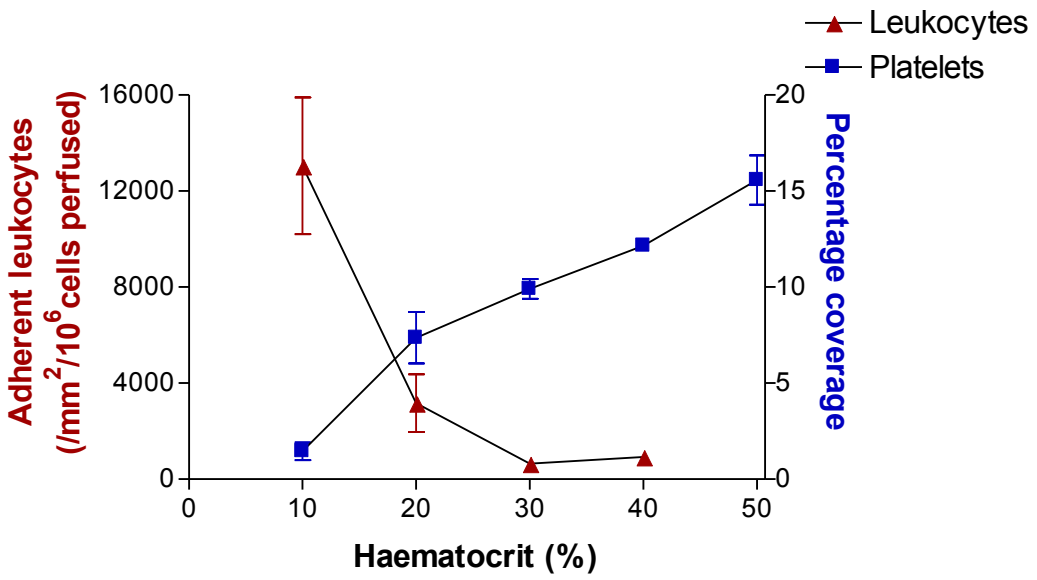


Figure 6.4 Comparison of the effects of haematocrit on leukocyte and platelet adhesion
Blood was perfused through microslides of 100μm in height that had been coated with P-selectin or Horm collagen for assessment of leukocyte or platelet adhesion respectively. The blood was manipulated to obtain haematocrits of 10-50%. Data are means ± SEM of 3-4 experiments.

6.3.4 Effect of haematocrit on platelet margination

As the horizontal perfusion of blood lead to leukocyte sedimentation, margination experiments were not performed for leukocytes. At 10% haematocrit within the leukocyte adhesion experiments, no rolling adherent leukocytes or fluorescent streaks (typical of free-flowing leukocytes flowing near the vessel wall in this system) could be seen on the upper surface of the microslide.

Adjacent to the vessel wall and at 5μm into the vessel lumen, the number of free-flowing platelets was significantly increased between 20 and 50% haematocrit. However at 10μm into the vessel, there was no significant change in the number of free-flowing platelets with haematocrit (fig 6.5(a)). Thus, the near wall excess of platelets tended to increase with haematocrit. The velocity of the free-flowing platelets adjacent to the wall of the microslide showed no significant change as the haematocrit was increased from 20 to 50% (fig 6.5(b)).

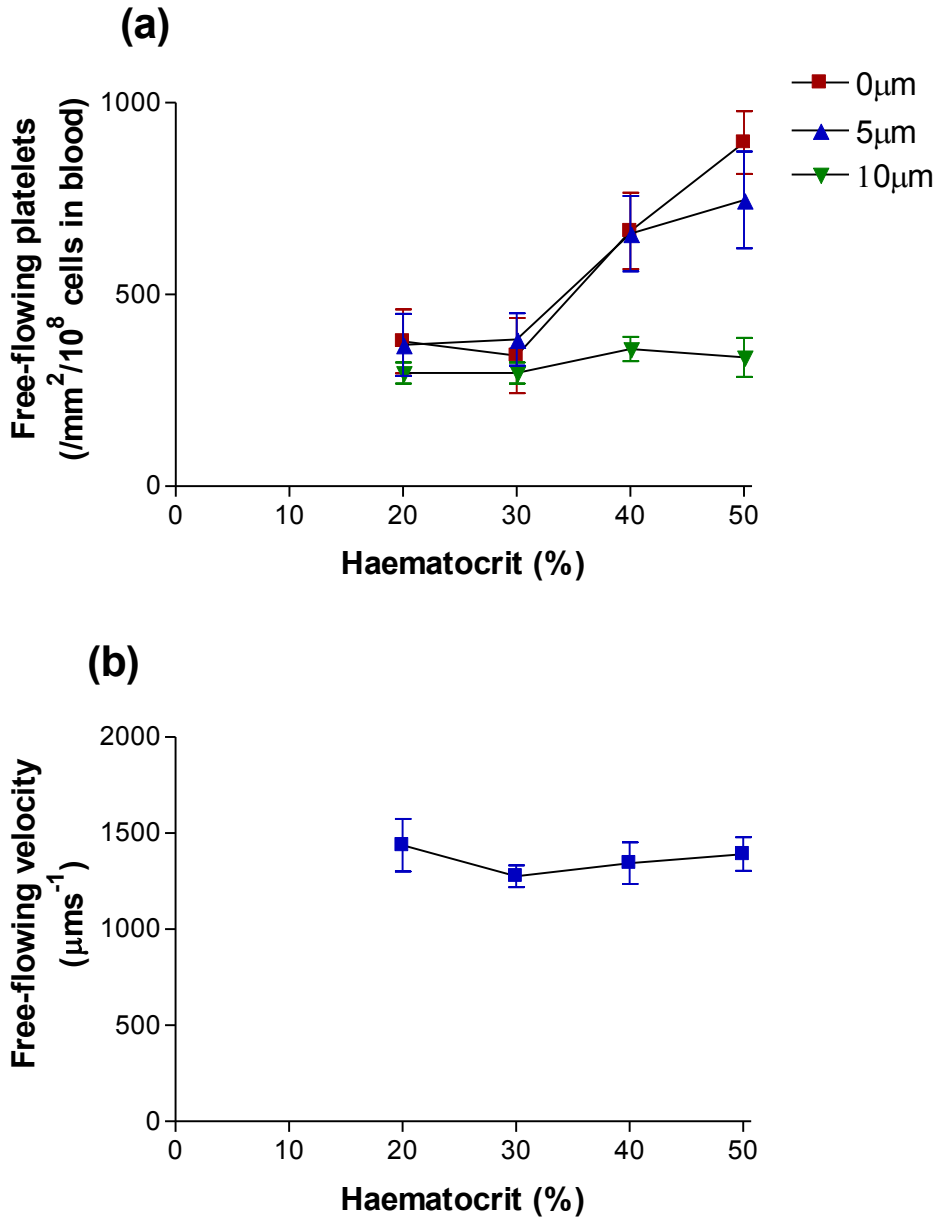


Figure 6.5 Effect of haematocrit on number and velocity of free-flowing platelets near the vessel wall

Blood, was perfused through microslides of 100μm in height that had been coated with albumin. The haematocrit of the blood was varied from 20-50%. Data shown are means ± SEM from 3 experiments assessing; (a) the number of free-flowing leukocytes adjacent to the vessel wall (0μm), 5μm and 10μm into the vessel lumen and (b) the velocity of free-flowing leukocytes adjacent to the vessel wall (0μm). (a) There was a significant effect of haematocrit on the number of free-flowing platelets at 0 and 5μm from the vessel wall ($P<0.01$ and $P<0.05$ respectively by ANOVA). ANOVA showed a significant effect of depth on the number of free-flowing leukocytes ($P<0.0001$). (b) There was no significant effect of haematocrit on the free-flowing velocity of platelets adjacent to the vessel wall.

6.3.5 Effect of haematocrit on apparent viscosity of blood

The pressure difference across the microslides being perfused with blood increased as the wall shear rate increased and as the haematocrit increased (fig 6.6(a)). The apparent viscosity of the blood flowing within the microslides was calculated to compare the average wall shear stress at varying haematocrit. As the haematocrit was increased, the apparent viscosity of the blood was significantly increased (fig 6.6(b)). The effect of shear rate on viscosity appeared to be less marked at the lower haematocrits (fig 6.6(b)). Comparing the effect of haematocrit on apparent viscosity at low shear rate ($70s^{-1}$) and high shear rate ($900s^{-1}$), we found that the apparent viscosity increased supralinearly with haematocrit at both shear rates. At the lower shear rate the calculated apparent viscosity was higher than at the higher shear rate (fig 6.7). However, the variation in viscosity with haematocrit was similar for the two shear rates.

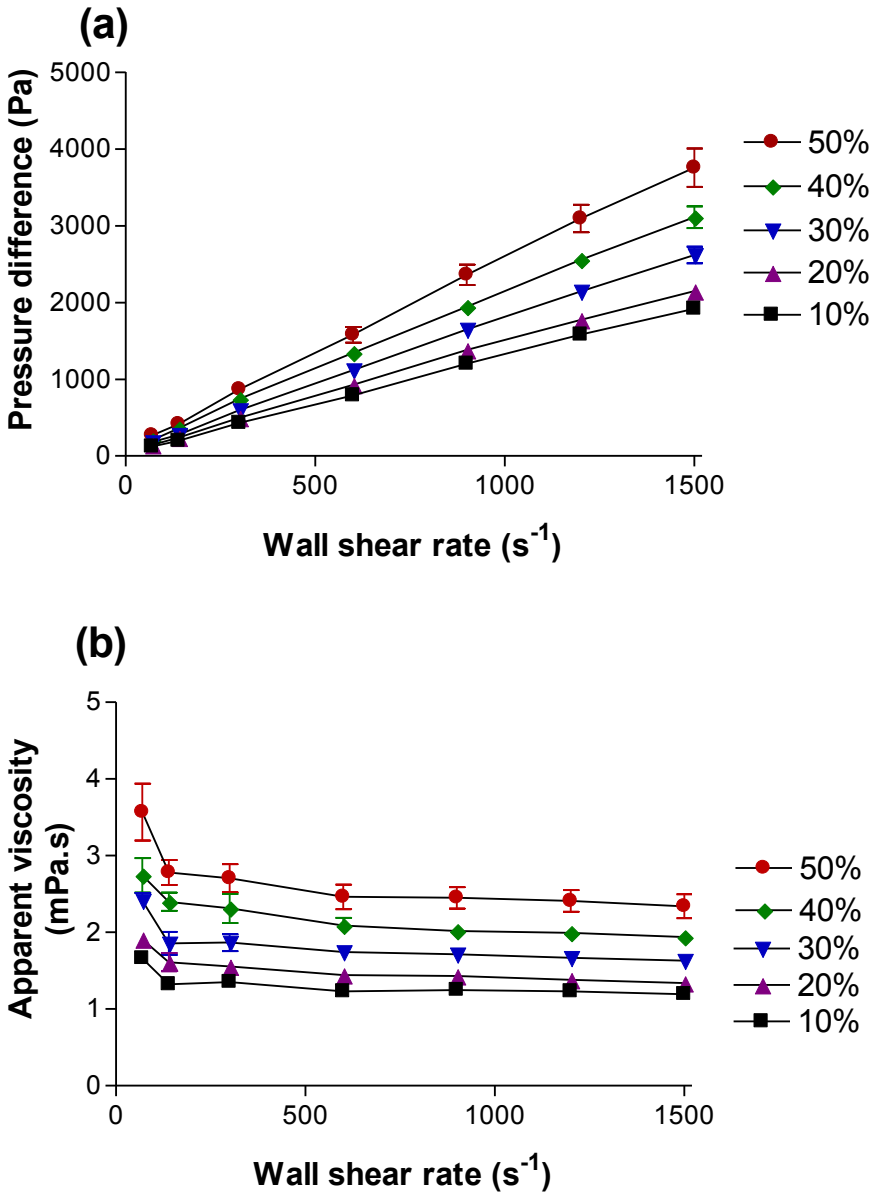


Figure 6.6 Effect of haematocrit on the pressure difference across a microslide and the apparent viscosity of the blood

When blood, of varying haematocrit, was perfused through microslides (a) the pressure difference across the microslides was significantly increased as the haematocrit was increased ($P<0.0001$ by ANOVA). (b) The apparent viscosity of the blood within the microslide was calculated using equation 18 (section 1.2.5.2). ANOVA showed a significant effect of haematocrit and wall shear rate on the apparent viscosity of the blood ($P<0.0001$ and $P<0.01$ respectively).

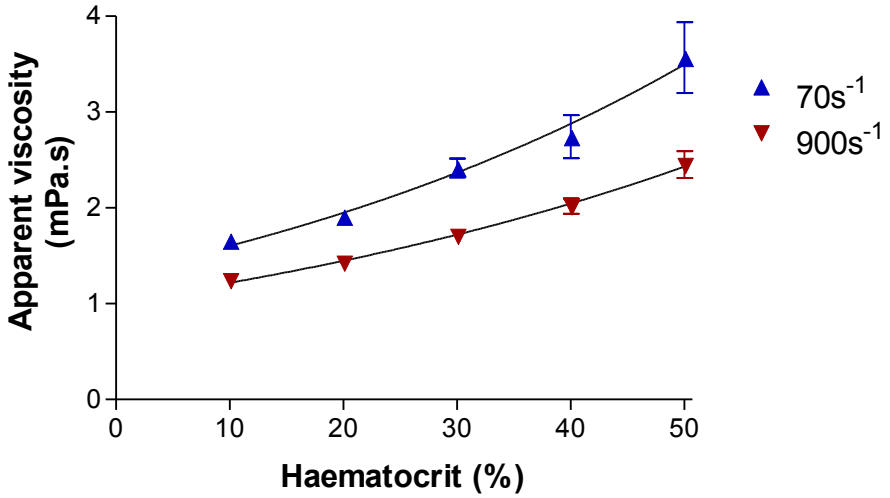


Figure 6.7 Effect of haematocrit on apparent blood viscosity at wall shear rates of $70s^{-1}$ versus $900s^{-1}$

The apparent viscosity of the blood increased supralinearly with increasing haematocrit at either shear rate ($P<0.0001$ by ANOVA). The apparent viscosity was higher at the lower shear rate, and the shear rate induced increase in apparent viscosity showed no variation with haematocrit.

6.4 Discussion

Despite the extensive work performed on the haematocrit dependence of leukocyte (Bagge et al., 1983;Goldsmith and Spain, 1984), and particularly platelet (Tilles and Eckstein, 1987;Yeh and Eckstein, 1994;Aarts et al., 1988;Corattiyl and Eckstein, 1986) margination, there are relatively few studies on the effect of haematocrit on adhesion, and no comparative studies between leukocytes and platelets. In this thesis we have studied both leukocytes and platelets in the same microslides, providing a good basis for comparison of the haematocrit-dependence of their margination and adhesion. During this study we found that increasing haematocrit from 10% initially had opposite effects on the adhesion of leukocytes and platelets. At a wall shear rate of $140s^{-1}$ leukocyte adhesion had a very high value on the lower surface of the microslide at 10% haematocrit, compared to the adhesion of leukocytes from whole blood. However, there was essentially no adhesion to the upper surface. As haematocrit was increased to 30%, leukocyte adhesion decreased. Between 30% and 40% adhesion changed little, but now adhesion was the same on the lower and upper surface. Platelet margination remained constant between 20-30% haematocrit and increased significantly between 30-50% showing no plateau. Platelet adhesion increased as haematocrit was increased between 10-50% and showed a trend that was almost linear. There was no difference observed in adhesion to upper and lower surfaces of the microslide. The apparent viscosity also increased as haematocrit was increased; the trend was close to linear but the apparent viscosity increased slightly more rapidly than the haematocrit.

6.4.1 Effect of haematocrit on leukocyte margination

Leukocyte margination was not quantitatively measured in this system because in the range 10 to 30% haematocrit the dominant effect was linked to sedimentation. At the lower

haematocrits, leukocytes were not visible at the upper surface of the microslide, where margination was usually assessed.

Previous studies show that RBC are essential in the promotion of leukocyte margination in the absence of sedimentation (Munn et al., 1996). At zero haematocrit, in the absence of sedimentation, flowing leukocytes will migrate away from the wall (Goldsmith and Turitto, 1986). Thus RBC are required to facilitate leukocyte margination in vertically perfused vessels, where sedimentation does not occur. Within horizontally perfused vessels, leukocytes sediment towards the lower surface in the absence of RBC. This effect allows leukocyte adhesion from isolated suspensions perfused through horizontal flow chambers (Lawrence et al., 1987;Munn et al., 1994). The presence of RBC at haematocrits of around 40% promotes margination towards the upper and lower surfaces of horizontally perfused microslides equally; at these haematocrits leukocyte adhesion is also equal on the two surfaces (see chapter 3).

Bagge et al showed that the presence of RBC caused a radial displacement of leukocytes after perfusing the blood through a stenosis (Bagge et al., 1983). This effect was present at both 10% and 40% haematocrit, with only a slight increase in displacement at 40% haematocrit. Goldsmith and Spain (Goldsmith and Spain, 1984) found that the ratio of tubular leukocyte count to reservoir count increased when RBC were added up to a haematocrit of 20%, but did not change significantly when haematocrit was increased further from 20 to 60%. Abbitt and Nash (Abbitt and Nash, 2003) also found little dependence of the number of free-flowing leukocytes near the vessel wall at haematocrits ranging from 10-50%.

6.4.2 Effect of haematocrit on leukocyte adhesion

Figure 6.8 compares the results of the previous study on leukocyte adhesion, in vertically perfused microslides of 300μm in height (Abbitt and Nash, 2003), to the results obtained in this thesis for horizontal 100μm microslides. It is clear that there was an effect of orientation of the microslide on the effect of haematocrit on leukocyte adhesion, especially at low haematocrit (fig 6.8). In the current horizontal microslides, the number of adherent leukocytes rolling on P-selectin at 10% haematocrit was much higher than at 20% haematocrit. At 20% haematocrit the number of adherent leukocytes was again much higher than at 30% or 40%, which were nearly equal. The trend in the previous study was for leukocyte adhesion to increase as haematocrit was increased. The number of adherent leukocytes increased significantly between haematocrits of 10-30%, with no significant increase between haematocrits of 30 and 50%. When compared to the previous study, the absolute levels of adhesion found in this thesis were much higher due to the increased efficiency of leukocyte adhesion within the smaller vessels, as noted in Chapter 3.

As haematocrit was increased, the apparent viscosity would increase within both the vertically- and the horizontally-perfused microslides, increasing the wall shear stress and hence the force on any adhesive bonds forming at the wall. This would act to decrease leukocyte adhesion as haematocrit was increased. At low haematocrit, the low concentration of flowing RBC would result in fewer RBC-leukocyte collisions that promote the radial displacement of leukocytes towards the vessel wall. In vertically perfused microslides, between 10-30% the increased dispersive or normal force provided by the increased concentration of RBC presumably outweighs the effects of increased shear stress.

Here, no adherent cells were observed on the upper surface of the horizontally perfused microslides at a haematocrit of 10%, and the large number of adherent leukocytes seen at 10-

20% haematocrit must have been promoted by the sedimentation of the leukocytes onto the lower surface. It is known that RBC sedimentation itself increases as haematocrit decreases, as observed when the erythrocyte sedimentation rate is measured clinically. Presumably, both RBC and leukocytes are sedimenting within the horizontal microslide, but at the lower haematocrit the reduced number of RBC within the system results in more efficient sedimentation of the leukocytes. Thus, in this study, the more efficient leukocyte sedimentation ultimately resulted in the large number of adherent leukocytes on the lower surface of the microslides at haematocrits of 10-20%.

At haematocrits of over 30% leukocyte adhesion remained relatively constant with haematocrit in both studies. Presumably the increase in shear stress and the increase in dispersive and normal forces provided by the increase in haematocrit were balanced resulting in little change in leukocyte adhesion above 30% haematocrit in the previous study, performed in vertical microslides. Within this thesis, at 30-40% haematocrit the dispersive force of the large number of flowing RBC within the vessel was presumably great enough to counteract any sedimentation of the leukocytes within the horizontal microslides. Thus, an equal distribution of cells and hence adhesion was seen.

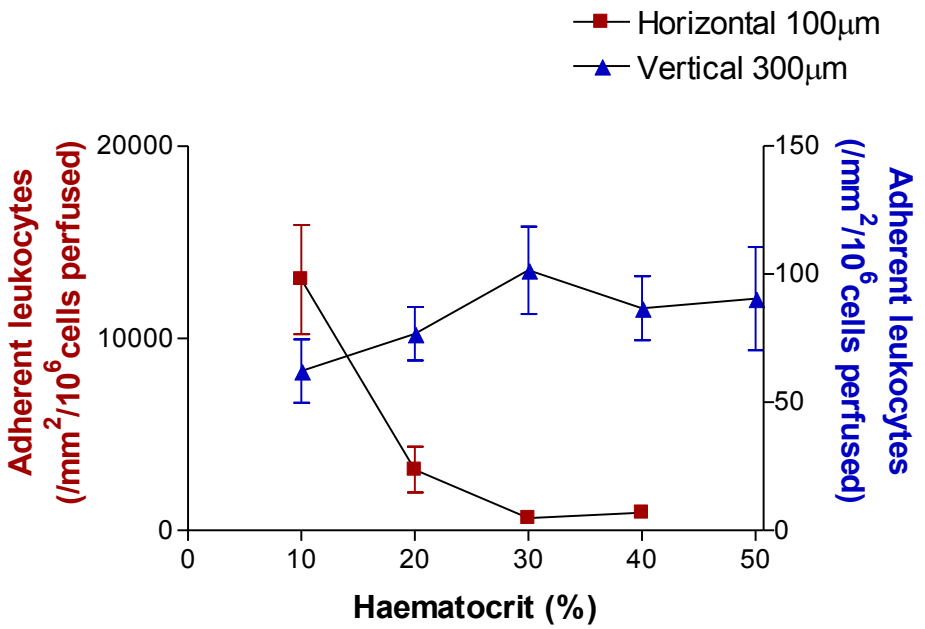


Figure 6.8 Effect of orientation of microslides on the effect of haematocrit on leukocyte adhesion

Blood was manipulated to obtain haematocrits of 10-50%. Data are means ± SEM of 3-6 experiments. In the horizontally perfused microslides of 100μm in height, adhesion decreased significantly, from very high values, as haematocrit was increased. Vertically perfused microslide leukocyte adhesion data are from Abbitt and Nash (Abbitt and Nash, 2003). In the vertically perfused microslides leukocyte adhesion tended to increase as haematocrit was increased, increasing significantly from 10-30% and remaining constant from 30-50%.

6.4.3 Effect of haematocrit on platelet margination

Adjacent to the vessel wall and 5μm into the vessel the number of platelets was significantly increased with increasing haematocrit between 30-50%, although between 20-30% there was no increase. Haematocrit had no effect on the number of free-flowing platelets 10μm into the vessel lumen. Thus, as haematocrit increased there was increasing margination in the area close to the wall. The velocity of the platelets adjacent to the vessel wall showed no dependence on haematocrit.

As the RBC concentration was increased, the proportion of the vessel occupied by flowing RBC would increase, and the width of the plasma layer around the periphery of the vessel would decrease. This decrease in width may have contributed to the increase in concentration of platelets close to the vessel wall that we observed. Platelet margination itself may increase with haematocrit, as the platelets become more efficiently displaced towards the periphery of the vessel by the flowing RBC.

The literature broadly suggests that as the haematocrit reaches a level of around 40% the margination of platelets reaches a maximum. Here, margination appeared to continue to increase at higher haematocrit. The presence of RBC increased the tubular platelet concentration from 60% of the concentration in the feed reservoir (when no RBC were present) to around 200% at haematocrit of 10% (Beck, Jr. and Eckstein, 1980). This showed that the presence of RBC resulted in a more peripheral distribution of platelets within the vessel, but above 10%, increasing haematocrit had little effect on this redistribution. In similar experiments, the tubular concentration, and hence the radial displacement, increased up to a haematocrit of 38% and then remained constant between haematocrits of 38- 77% (Corattiyl and Eckstein, 1986).

Tilles and Eckstein (Tilles and Eckstein, 1987) showed that as haematocrit was increased there was no increase in near wall concentration of platelet-sized beads until a haematocrit of 15% was reached. Above 15% haematocrit there was no further increase in platelet-sized beads near the vessel wall as haematocrit was increased up to 45%. In 200μm diameter polyethylene tubes, the tubular concentration of platelet-sized beads increased when the haematocrit was varied from 0-40%, and then remained relatively constant from 40% up to 60% (Uijttewaal et al., 1993). In horizontally perfused polyethene tubes the lateral displacement of platelets towards the wall was significantly increased as haematocrit was increased from 0% to 15% and again from 15% to 40% (Yeh and Eckstein, 1994). This was quantified using a freeze capture method, viewing the tube end on and taking sections down the tube. A near wall excess of platelet-sized beads was induced by the presence of the red blood cells, and this effect was more pronounced at 40% than at 15% haematocrit.

In our study the number of free-flowing platelets near the vessel increased up to 50% with no appreciable plateau. Haematocrits of above 50% were not studied in this thesis, as they were considered to be in excess of normal physiological conditions. If the haematocrit was increased further, the width of the peripheral plasma layer would eventually reach a minimum. However in this system this didn't appear to have been occurred by a haematocrit of 50%.

6.4.4 Effect of haematocrit on platelet adhesion

In this system platelet coverage was increased over the full range of haematocrits used (10-50%). The platelet free-flowing distributions near the vessel wall showed a significant increase in concentration as haematocrit was increased (adjacent to (0μm) and 5μm from the vessel wall). There was no increase in the free-flowing near wall velocity of these platelets.

This results in a higher near wall platelet flux at the higher haematocrits. This could account for the increase in adhesion of platelets seen in this experiment, in part at least. However, the increase in platelet flux did not match the increase in platelet adhesion exactly. Platelet free flow concentration remained relatively constant until 30% haematocrit and then increased up to 50%. The adhesion of platelets increased almost linearly.

As platelets are the smallest cells in the blood they will be displaced radially by the RBC at all haematocrits. As the haematocrit was increased, this displacement presumably became more effective. However, when discussing the adhesive behaviour of platelets with increasing haematocrit, the width of the flowing RBC core and in turn the peripheral plasma layer has to be considered. The reduction in width of the plasma layer, with increasing haematocrit, increases the number of RBC-platelet interactions in free flow. Also as haematocrit is increased these collisions occur in a closer proximity to the vessel wall. Thus, as a result, the probability that a RBC-platelet collision will cause a platelet-vessel wall interaction increases. Therefore an increase in haematocrit will promote platelet adhesion in this way too.

The results obtained here correlate well to previous studies assessing the effect of haematocrit on platelet adhesion. Turitto and Baumgartner (Turitto and Baumgartner, 1975) used an annular perfusion chamber to perfuse blood across rabbit aortic sub-endothelium. The rate of deposition of platelets was 57 time higher in blood at a haematocrit of 38% than in a pure platelet suspension of equal platelet concentration. Karino and Goldsmith (Karino and Goldsmith, 1979) found that use of reconstituted blood at 20% haematocrit resulted in 4 times the number of adherent platelets bound to a collagen-coated glass tube of 3mm diameter when compared to a platelet suspension alone. In the only other study of incrementally increasing haematocrit, Turitto and Weiss (Turitto and Weiss, 1980) found, at haematocrits of between 10% and 70 %, that platelet adhesion increased until the haematocrit reached 40% and

remained constant up to 70% at low shear rates ($200s^{-1}$). At high shear rates ($2600s^{-1}$), there was a linear increase in adhesion in the whole range of haematocrits studied. The study performed in this thesis agreed with that of Turitto and Weiss (Turitto and Weiss, 1980). Both studies found a linear increase in platelet adhesion at high shear rate as haematocrit was increased between 10-50%.

Although the effect of haematocrit on the margination and adhesion of platelets have been studies previously, our study was the first to assess the effect of haematocrit on platelet margination and adhesion in the same system, and thus to be able to directly link the two phenomena.

6.4.5 Comparison of the effect of haematocrit on leukocyte and platelet adhesion, and physiological implications

In this system, as the haematocrit of the blood perfused was increased, leukocyte adhesion significantly decreased between 10-30% and the adhesion was constant between 30-40%. Platelet adhesion showed a significant increase across the whole range of haematocrits (10-50%) (fig 6.4). As there were no adherent leukocytes observed on the upper surface of the microslides at low haematocrit, the high levels of adhesion were attributed to sedimentation of the leukocytes. No similar increase in adhesion was seen in the platelet experiments at lower haematocrits, suggesting that platelets did not sediment in the same way. As leukocyte and platelet adhesion was quantified in the same vessels at the same haematocrits, the only two factors that differed in these experiments were the wall shear rate and the size of the cells in question. Both will impact on the rate of sedimentation of the cells within the blood in the microslide.

Stoke's law (Fahraeus, 1929) states that the terminal velocity (v_s) of a sedimenting sphere, with radius (R) and density (ρ_p), in a viscous medium, of viscosity (η) and density (ρ_f), where g is the acceleration due to gravity, is given by equation 25.

$$v_s = \frac{2}{9}\frac{(\rho_p - \rho_f)}{\eta} g.R^2 \qquad \textbf{Equation 25}$$

This can be used to approximate the sedimentation rate of leukocytes and platelets within flowing blood.

The apparent viscosity of the blood increased as haematocrit was increased, but the viscosity of the plasma was not varied between blood samples. It is likely that sedimentation would effectively increase at the lower haematocrit because the overall suspension viscosity decreased, but this would be the same for leukocytes and platelets. The leukocytes, having a radius approximately 4 times larger than that of platelets, would therefore have a sedimentation rate of around 16 times that of the platelets. This would contribute to the difference in sedimentation of the leukocytes and platelets in the horizontal perfusion of blood within our system. Along with the increased sedimentation rate due to size, the density of neutrophils (but not lymphocytes or monocytes) is greater than platelets, judging from density gradients methods used to fractionate the cells (Butler et al., 2005), further increasing the relative sedimentation rate of the neutrophils at least. The higher wall shear rate used in the platelet adhesion experiments would also act to reduce the effect of sedimentation within the microslide. To produce a wall shear rate of $900s^{-1}$ in the platelet experiments the flow rate was 6.5 times faster than the flow rate used to produce a wall shear rate of $140s^{-1}$ in the leukocyte experiments and so the platelets were travelling within the microslides for a much shorter time than the leukocytes, providing less time for sedimentation to occur. Thus at low haematocrit and low flow rate the sedimentation of leukocytes was much higher than that of

platelets at the higher shear rate. If there was any effect of sedimentation on platelet adhesion it was small, and would have acted to increase adhesion to the lower surface at low haematocrit. Even with this potential sedimentation of platelets, platelet adhesion increased significantly with increasing haematocrit, showing that the other effects of haematocrit were dominant in this system.

Within the microcirculation, due to the Fahraeus effect (Fahraeus, 1929), the haematocrit can commonly be around 20%. Intra-vital studies are often performed on vascular beds that have been exteriorised and laid flat for observation. Within the lower haematocrit and low shear rate environment, sedimentation of the leukocytes would occur. However, in the analysis of intra-vital studies only adherent cells are counted and no attention is paid to whether the leukocyte is rolling on the top or the bottom of the venule (Pearson and Lipowsky, 2000). Physiologically, no vessel is horizontal or vertical for any great distance. Within the microcirculation, it is rare to see a micro-vessel free from bifurcations for more than a few millimetres (Lipowsky, 1988). Even if vessels were long and straight, the movement of the body would change the orientation in which the vessels were perfused. Therefore in the body, the extent to which sedimentation of leukocytes will affect their adhesion is uncertain, due to the factors mentioned above. The presence of RBC at a haematocrit of 20% is high enough to promote leukocyte margination (Munn et al., 1996) and thus adhesion in vertical vessels. Platelet adhesion however, does not appear to be affected significantly by sedimentation, as platelets are small. As vessels are in all orientations within the body and continually changing during movement, the ability of the platelet to form thrombi should not be affected, reducing the potential for blood loss in the event of vessel trauma. In vessels larger than around 300μm the Fahraeus effect is negligible (Fahraeus, 1929). As a result in most vessels on the arterial side of the circulation, where the potential for blood loss is high, platelet adhesion will be high as the blood is at the systemic haematocrit for that individual. Within smaller vessels,

where the Fahraeus effect may reduce the haematocrit to values as low as 20%, platelet adhesion will be reduced. This may act to lower the potential risk of vessel occlusion within the smaller vessels of the microcirculation.

In summary, the effects of haematocrit on the margination and adhesion of platelets within identical vessels have been studied together for the first time. An increase in haematocrit increased the number of marginated platelets and platelet adhesion between 10-50%. Leukocyte adhesion was influenced by the tendency for leukocytes to sediment at low haematocrit in the horizontal vessels, and so the effect of haematocrit on adhesion was quite different from previous studies with a vertically perfused vessel (Abbitt and Nash, 2003). It is not clear whether the very high levels of adhesion seen in the horizontal vessel at low haematocrit are reproduced in the microcirculation where orientation of the vessels is not constant.

CHAPTER 7: EFFECT OF RED BLOOD CELL DEFORMABILITY ON THE MARGINATION AND ADHESION OF LEUKOCYTES AND PLATELETS

7.1 Introduction

The abilities of RBC to transit capillaries that have a diameter smaller than their own, and to elongate, align and pack closely in shear flow arise from their low resistance to deformation. This study investigated how variation in the deformability of the RBC would affect the margination and adhesion of leukocytes and platelets in small vessels.

Although much work has been done on artificially altering RBC deformability and its affect on their flow properties (see section 1.3.2), literature on the effects on leukocyte and platelet margination and adhesion is scarce. The results from the limited studies on platelet margination are consistent, and indicate that as red cell deformability is decreased, platelet distribution in flowing blood becomes more uniform. At a haematocrit of 15%, decreasing the deformability of the RBC resulted in a decrease in the near wall excess of flowing, fluorescent, platelet-sized beads (Eckstein et al., 1988). Uijttewaal investigated the effect of graded reductions in the RBC deformability on tubular platelet count (Uijttewaal et al., 1993). It was found that as the deformability was decreased, the tubular platelet count relative to the feed platelet count (and hence margination) decreased.

We hypothesised that reducing RBC deformability would decrease margination of leukocytes as well as platelets. While this might cause a decrease in adhesion, we also hypothesised that narrowing of the peripheral plasma layer might offset this effect for platelets at least. Our aims for this study were thus to show for the first time, how decreasing RBC deformability influences leukocyte free-flowing distribution and adhesion, and to assess the effect on platelet margination and adhesion. With margination and adhesion studied for both cell types in the same system, the mechanisms by which both cell types are affected by the deformability of RBC might be better understood.

7.2 Methods

7.2.1 Blood collection

Venous blood was anticoagulated with CPDA. Theophylline was included in all experiments, apart from those assessing platelet adhesion, at a final concentration of 7mM in the blood.

7.2.2 Blood manipulation

The RBC deformability was manipulated by incubating RBC in glutaraldehyde at concentrations of 0.005% or 0.01% and the effects on filterability were assessed using a St. George's Filtrometer (see section 2.2.5.2). The lower concentration increased the transit time moderately but significantly, but the higher concentration caused rapid pore blockage. The blood was reconstituted from the RBC and autologous plasma containing Dx40 (to abolish RBC aggregation) and the appropriate cell type for the experiment at a known concentration. In all experiments the samples were reconstituted to a haematocrit of 40%.

7.2.3 Microslide coating

For experiments involving leukocyte adhesion, microslides were coated in purified P-selectin at a concentration of 10μg/ml. For experiments involving platelet adhesion, microslides were coated with Horm collagen at 500μg/ml. For all margination and tube viscometry experiments the microslides were coated with albumin to provide a non-adhesive surface.

7.2.4 Adhesion experiments

Blood was fluorescently labelled with 5μg/ml of R6G. To measure the adhesion of leukocytes to P-selectin, blood samples, with manipulated RBC deformability, were perfused at a shear rate of $140s^{-1}$ for a total of 4 minutes from the time the microslide was first filled

with blood. To measure platelet adhesion to collagen, the blood was perfused through the microslides at a shear rate of $300s^{-1}$ or $900s^{-1}$. Adhesion was measured up to 4 minutes after the microslide was first filled with blood.

7.2.5 Assessment of margination

Margination was analysed by perfusing fluorescently labelled blood, with manipulated RBC deformability, reconstituted at 40% haematocrit, through albumin coated microslides. The microslides were perfused horizontally and the free-flowing cells were measured near the upper surface of the microslide. Leukocyte experiments were performed at a shear rate of $140s^{-1}$. Platelet margination experiments were performed at a wall shear rate of $300s^{-1}$.

7.2.6 Tube viscometry

The apparent viscosities of blood samples with manipulated RBC deformability were analysed as previously.

7.3 Results

7.3.1 Effect of RBC deformability on leukocyte adhesion

Leukocyte adhesion initially increased significantly from its control value as RBC deformability was decreased moderately, but when the RBC were fully rigidified the leukocyte adhesion decreased back to a level comparable to that measured with un-manipulated RBC (fig 7.1(a)). The rolling velocity of the adherent leukocytes did not vary significantly as the RBC deformability was decreased (fig 7.1(b)).

7.3.2 Effect of RBC deformability on platelet adhesion

As RBC deformability was decreased the percentage coverage of the platelets on the microslide increased significantly. This increase in adhesion was seen at wall shear rates of $300s^{-1}$ and $900s^{-1}$ (fig 7.2).

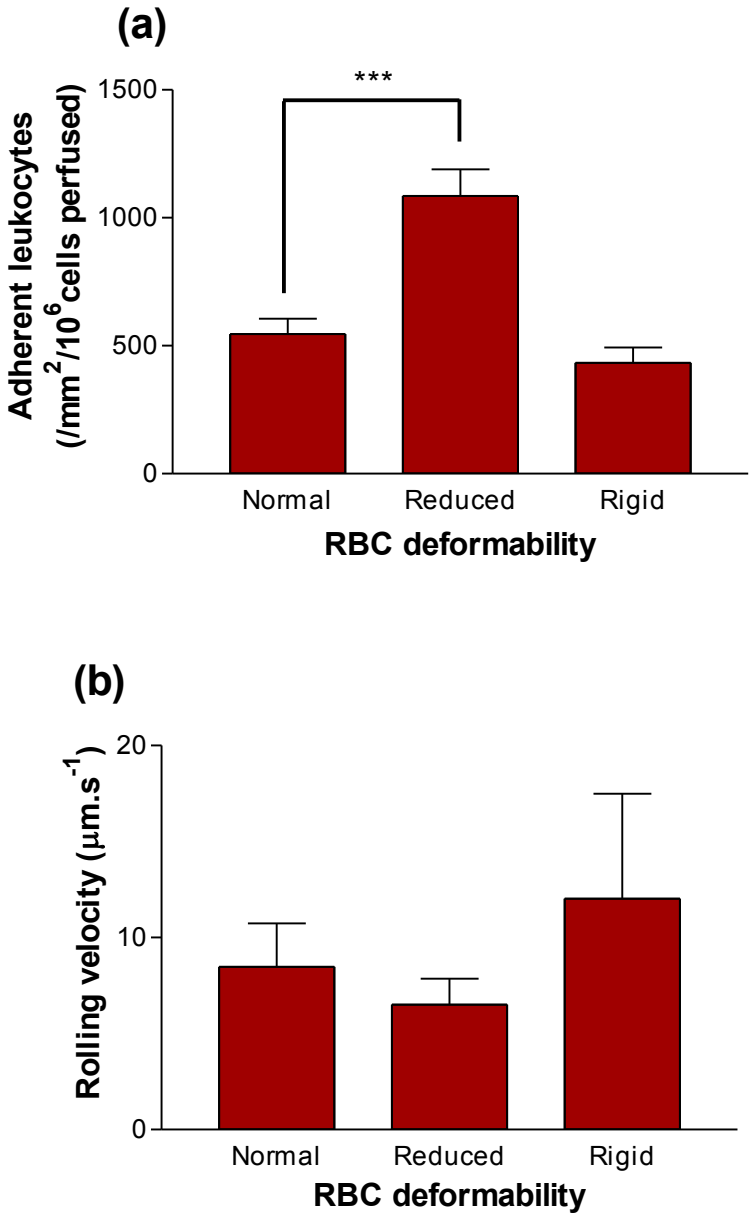


Figure 7.1 Effect of reducing RBC deformability on leukocyte adhesion
Blood, normalised to 40% haematocrit with manipulated RBC deformability, was perfused through microslides of 100µm in height that had been coated with P-selectin. Data shown are means ± SEM from 3 experiments. (a) ANOVA showed a significant effect of treatment on adhesion ($P<0.05$). ***=$P<0.001$ compared to normal (control) by Dunnett's test. (b) There was no significant variation in the rolling velocity of the adherent leukocytes as RBC deformability was decreased.

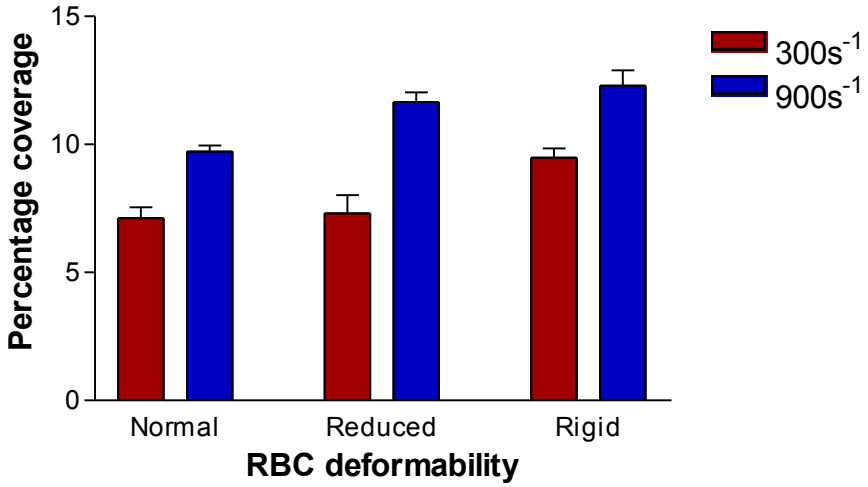


Figure 7.2 Effect of reducing RBC deformability on platelet adhesion
Blood, normalised to 40% haematocrit with manipulated RBC deformability, was perfused through microslides of 100μm in height that had been coated with collagen. Data shown are means ± SEM of the percentage coverage of platelets at 4 minutes from 4 experiments. There was a significant effect of treatment on platelet adhesion at both shear rates ($P<0.05$ by ANOVA for each).

7.3.3 Effect of RBC deformability on leukocyte margination

Upon correction for the number of leukocytes within the blood it was shown that there was a significant effect of RBC deformability on the number of free-flowing leukocytes adjacent to, and at 5μm from the vessel wall (fig 7.3(a)). The numbers of cells near the wall decreased as the RBC were made less deformable. There was no significant effect on the number of free-flowing leukocytes at 10μm from the vessel wall. The free-flowing velocity of the leukocytes at the wall increased significantly with decreasing RBC deformability (fig 7.3(b)).

7.3.4 Effect of RBC deformability on platelet margination

There was a decrease in the number of free-flowing platelets at the vessel wall and at 5μm into the vessel lumen as the percentage of glutaraldehyde within the incubation medium was increased from 0-0.01% (fig 7.4(a)). However no significant variation was seen at a depth of 10μm into the vessel lumen. Thus, margination or near-wall excess of platelets tended to decrease with decreasing RBC deformability. The free-flowing velocity of the platelets adjacent to the vessel wall showed a significant increase as RBC deformability was decreased (fig 7.4(b)).

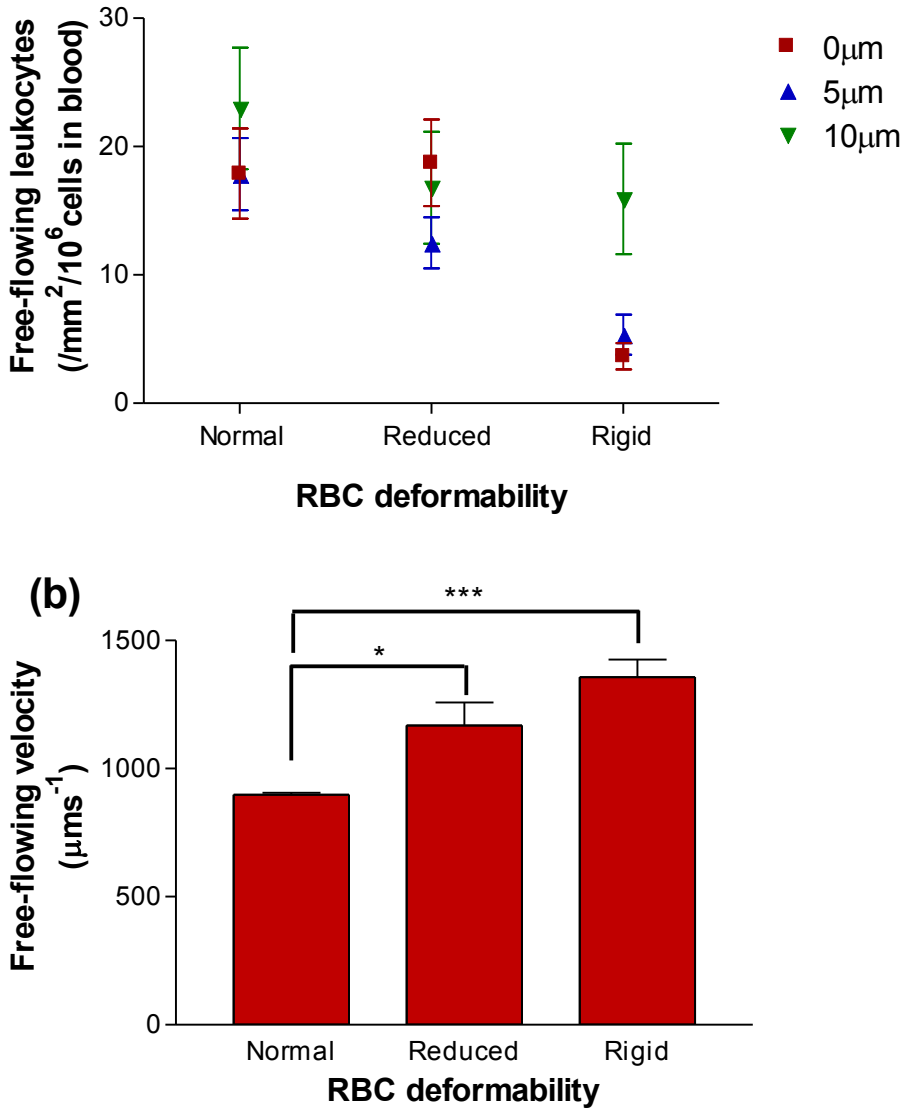


Figure 7.3 Effect of reducing RBC deformability on the number and velocity of leukocytes flowing at the vessel wall
Blood, adjusted to 40% haematocrit with manipulated RBC deformability, was perfused through microslides of 100μm in height that had been coated with albumin. Data shown are means ± SEM from 3 experiments assessing; (a) the number of free-flowing leukocytes adjacent to the vessel wall and (b) the velocity of free-flowing leukocytes adjacent to the vessel wall. (a) ANOVA showed that RBC treatment significantly affected the number of free-flowing leukocytes near the vessel wall ($P<0.05$) . Subsequent analysis showed that that RBC deformability had a significant effect on the number of leukocytes adjacent to, and at a depth of 5μm the vessel wall ($P<0.01$ in each case). Adjacent to the vessel wall, Bonferroni post tests showed a significant difference between rigid RBC and the other two samples ($p<0.05$ in each case). (b) There was a significant effect of treatment on the velocity of free-flowing leukocytes at the vessel wall ($P<0.05$ by ANOVA). * = $P<0.05$, *** = $P<0.001$ compared to normal (control) RBC deformability by Dunnett's test.

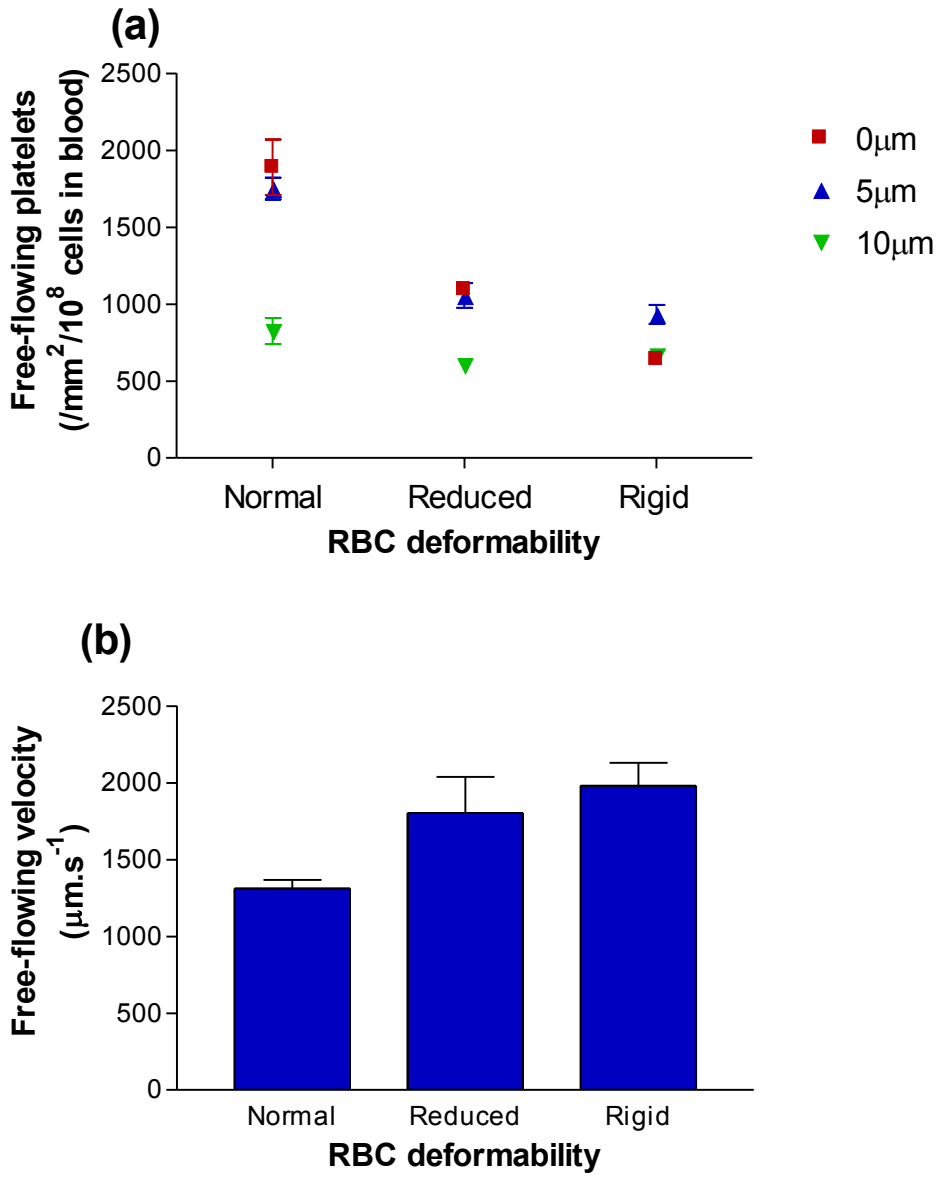


Figure 7.4 Effect of reducing RBC deformability on the number and velocity of free-flowing platelets near the vessel wall

Blood, adjusted to 40% haematocrit with manipulated RBC deformability, was perfused at a wall shear rate of $300s^{-1}$ through microslides of 100μm in height that had been coated with albumin. Data shown are means ± SEM from 4 experiments assessing; (a) the number of free-flowing platelets adjacent to the vessel wall (0μm), 5μm and 10μm into the vessel lumen and (b) the velocity of free-flowing platelets adjacent to the vessel wall (0μm). (a) ANOVA showed that RBC treatment significantly affected the number of free-flowing platelets near the vessel wall ($P<0.0001$). Subsequent analysis showed RBC deformability had a significant effect on the number of free-flowing platelets adjacent to, and 5μm from the vessel wall ($P<0.001$ by ANOVA in both cases). Adjacent to the vessel wall, Bonferroni post tests showed a significant difference between normal RBC and the other two samples ($p<0.01$ compared with reduced RBC and $p<0.001$ compared with rigid RBC). (b) There was a significant effect of treatment on the velocity of free-flowing platelets at the vessel wall ($P<0.05$ by ANOVA).

7.3.5 Effect of RBC deformability on blood viscosity

The pressure difference across the microslides perfused with blood increased as the wall shear rate increased and as the RBC deformability decreased (fig 7.5(a)). The apparent viscosity of the blood flowing within the microslides was calculated to compare the average wall shear stress at varying RBC deformability. As the RBC deformability was decreased, the apparent viscosity of the blood was significantly increased (fig 7.5(b)). There was no significant effect of shear rate on the ratio of apparent viscosity of the blood with reduced or rigid RBC to the viscosity with normal RBC (fig 7.5(c)). The ratio of apparent viscosity was significantly higher in the rigid RBC when compared to the reduced RBC across the whole range of shear rates used. Subsequent analysis showed at shear rates below $300s^{-1}$ there was no significant difference between these ratios.

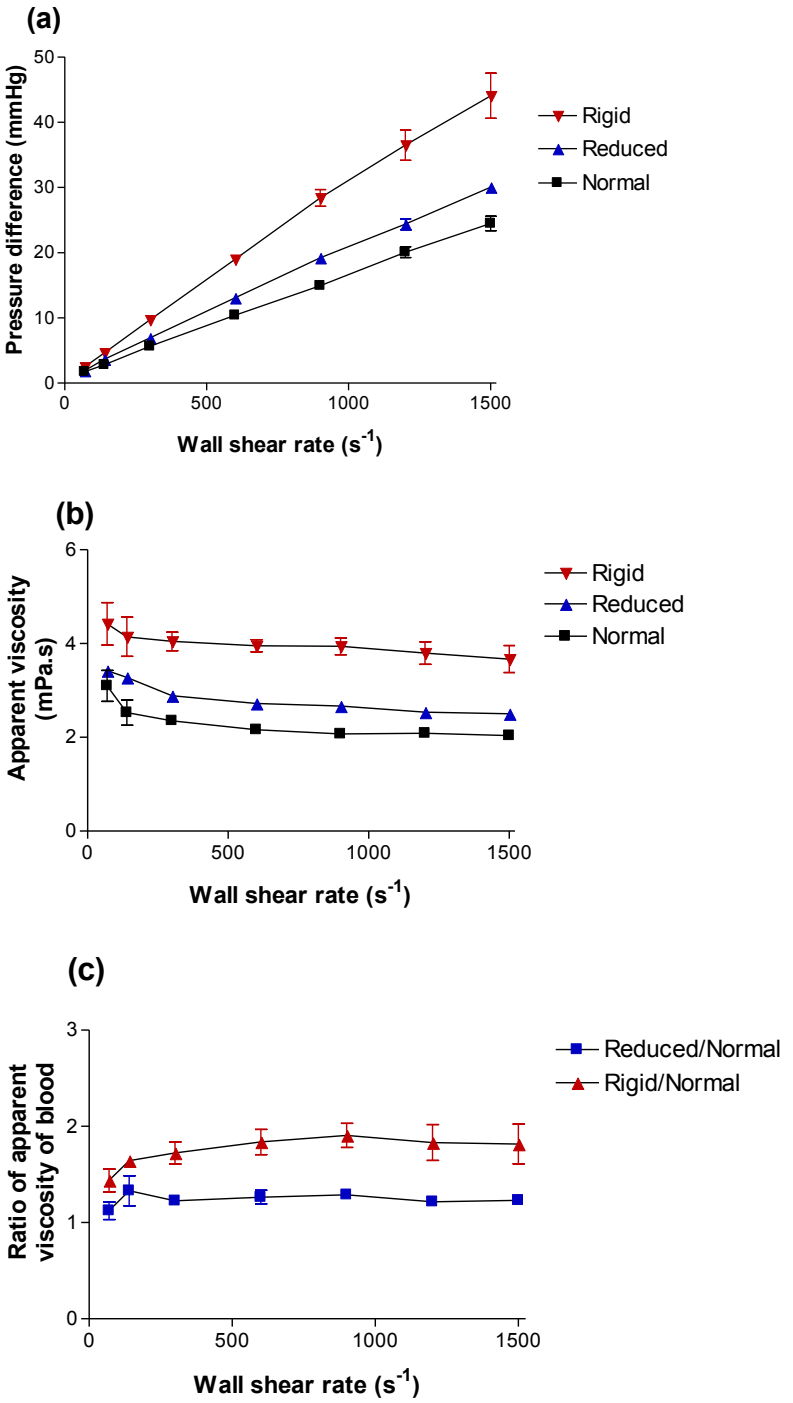


Figure 7.5 Effect of decreasing RBC deformability on the pressure difference across a microslide and the apparent viscosity of the blood
Blood, adjusted to a haematocrit of 40%, was perfused through microslides. (a) The pressure difference across the microslides was significantly increased as RBC deformability was decreased ($P<0.0001$ by ANOVA). (b) The apparent viscosity of the blood within the microslide was calculated using equation 18 (section 1.2.5.2). There was a significant effect of RBC treatment on the apparent viscosity of the blood ($P<0.0001$ by ANOVA). (c) ANOVA showed that there was a significant difference in the ratio of apparent viscosity with reduced deformability or rigid RBC to viscosity with normal RBC across the whole range of shear rates used ($P<0.0001$). Bonferroni post tests showed that this difference was only significant at shear rates of $300s^{-1}$ and above. There was no significant effect of shear rate on the apparent viscosity ratios.

7.4 Discussion

The effect of RBC deformability on leukocyte margination and adhesion has not previously been studied. Only limited information was available on the effect of RBC deformability on the margination and adhesion of platelets (Munn and Dupin, 2007;Uijttewaal et al., 1993;Eckstein et al., 1988,Aarts et al.,1984). In all blood samples in this study the addition of dextran 40 resulted in the abolition of RBC aggregation, judged by microscopic observation. Any effects seen are therefore the effect of the reduced deformability of the RBC and not due to its influence on RBC aggregation. We used two levels of modification by glutaraldehyde. Treatment with 0.005% glutaraldehyde caused a 30% increase in transit time through 5µm pore filters that was comparable to that seen in conditions such as diabetes that have been shown to moderately decrease the deformability of RBC (Symeonidis et al., 2001). Treatment with 0.01% glutaraldehyde caused much greater rigidification, so that filters clogged immediately. This effect was probably beyond that seen except in the most severe conditions, such as de-oxygenation of sickle cells (Nash et al., 1988) or infection with mature malarial parasites (Cooke et al., 2004).

Leukocyte adhesion at a wall shear rate of $140s^{-1}$ increased significantly as RBC deformability was initially reduced and then decreased back to the 'normal' level upon further reduction in deformability. Platelet adhesion at a wall shear rate of $300s^{-1}$ or $900s^{-1}$ was increased progressively as RBC deformability was reduced. The number of free-flowing leukocytes and platelets adjacent to the wall was significantly reduced as RBC deformability was reduced overall, but for leukocytes reduction in margination was only evident for the most rigid RBC. The free-flowing velocities of both leukocytes and platelets near the wall were increased as the RBC deformability was decreased. As the RBC deformability was

reduced, the apparent viscosity of the blood was significantly increased, which agrees with previous studies (Whitmore, 1981;Meiselman, 1981).

7.4.1 Effect of RBC deformability on leukocyte margination

The number of free-flowing leukocytes adjacent to the vessel wall was constant for normal and reduced deformability and dropped off significantly as the deformability of the RBC was reduced further still. In general, as the deformability of the RBC decreases, their tendency to migrate towards the centre of the vessel will be reduced (Goldsmith, 1971a;Goldsmith, 1971b). In addition, as the deformability of the RBC was reduced, their elongation and alignment with the flow should have decreased; this should be accompanied by less efficient packing of the RBC and possibly widening of the plug flow and narrowing of any cell-free outer layer. Here, when the RBC deformability was reduced by a small amount, the RBC were still much more deformable than the leukocytes, and there was no detectable effect on the leukocyte distribution within the vessel. As the deformability of the RBC was reduced further still, the RBC tendency to migrate towards the centre of the blood vessel would have been considerably reduced relative to the leukocytes. It appears that as the RBC core widened and the peripheral plasma layer was reduced, the leukocytes that would normally be flowing close to the vessel wall were displaced by the RBC. This resulted in very few free-flowing leukocytes near the vessel wall, and as the number of free-flowing leukocytes increased considerably at 10μm into the vessel lumen, presumably they were travelling more centrally. The increase in free-flowing leukocyte velocity as RBC deformability was decreased may have been due to an increase in the width of the central RBC core. This would have reduced the distance over which the velocity would decrease near the wall, producing a larger velocity gradient. The leukocytes closer to the vessel wall would then flow more quickly.

There are no previous data on the effect of RBC deformability on the margination of leukocytes. Thus, we have shown here for the first time that decreasing the deformability of RBC can lead to a decrease in the number of marginated leukocytes. This decrease in the number of free-flowing leukocytes adjacent to the vessel wall only appeared when RBC deformability was drastically reduced. Therefore it is unlikely that any reduction in leukocyte margination would occur *in vivo*, except in cases of extreme reduction in RBC deformability, such as sickle cell disease.

7.4.2 Effect of RBC deformability on leukocyte adhesion

Leukocyte adhesion increased as RBC deformability was moderately decreased, and then decreased again as RBC were made rigid. With equal numbers of leukocytes flowing near the vessel wall in the two samples with the more deformable RBC, the only observed differences between them were the slightly increased velocity of the leukocytes near the wall and the increased wall shear stress (apparent viscosity) in the sample with reduced deformability. There may also have been reduced axial migration of the less-deformable RBC. The increased shear stress and velocity would tend to decrease the number of adherent leukocytes. Reduced axial migration of the less deformable RBC might result in a greater number of RBC-leukocyte collisions; in turn these collisions could result in a greater number of leukocyte-vessel wall interactions, resulting in increased leukocyte adhesion. It is also possible that the less deformable RBC more effectively forced the leukocytes onto the wall, as Munn et al (Munn et al., 1996), suggested that the normal force exerted by RBC is a critical factor in promotion of leukocyte adhesion.

As the deformability of the RBC was further reduced, the drop in adhesion can be explained by the effect on margination. The number of free-flowing leukocytes near the wall was markedly reduced in the samples with the least deformable RBC; this may have been due to

the reduced tendency for less deformable cells to migrate away from the wall. The decreased number of free-flowing leukocytes adjacent to the vessel wall was presumably more important than any further increase in collision efficiency or force caused by the rigid RBC. The increased free-flowing velocity would have reduced the time available for bond formation. However in this system it appears that the force on the P-selectin-ligand bonds is the governing factor in adhesion (see chapter 4). The increased apparent viscosity of the blood with the least deformable RBC would in turn increase the wall shear stress and the force experienced by the bonds. This may also have resulted in reduction in the level of leukocyte adhesion.

To our knowledge this is the first study that has assessed the effect of manipulating RBC deformability on the adhesion of leukocytes in flowing blood. Overall, a slight decrease in RBC deformability may act to increase leukocyte adhesion. Within sickle cell disease, oxygenated RBC are less deformable than normal RBC (Nash et al., 1988). The level of leukocyte adhesion may be increased if RBC are oxygenated within post-capillary venules. However, although we found here that the level of leukocyte adhesion may not be affected as RBC are made rigid, this may not be physiologically appropriate. As sickle cells are deoxygenated they become extremely rigid and become sickle shaped, due to the gelation of the haemoglobin (Nash and Meiselman, 1985), and occlude the vessels within the microcirculation. Thus as the rigid RBC within this study blocked 5μm rapidly the effect on leukocyte adhesion may not be physiologically relevant as vascular occlusion would be the main result of such rigidification.

7.4.3 Effect of RBC deformability on platelet margination

As the deformability of the RBC was reduced, there was a significant decrease in the number of platelets flowing adjacent to the vessel wall and at 5μm into the vessel, however at 10μm

there was no change in the number of free-flowing platelets. Platelets usually flow in the periphery of the vessel as they are the smallest cells in the blood and are preferentially marginated. As the RBC become less deformable it is expected that their tendency to migrate towards the centre of the vessel will decrease and that their packing efficiency will decrease. Thus the peripheral plasma layer should decrease in thickness. Presumably, the reduction in the number of free-flowing platelets near the vessel wall was a result of the reduced RBC migration away from the vessel wall.

There have been two previous studies that have assessed the effect of RBC deformability on the margination of platelets. Eckstein et al (Eckstein et al., 1988) found that using glutaraldehyde-fixed RBC resulted in a decreased near wall excess of platelet-sized beads when compared to normal RBC. Uijttewaal et al (Uijttewaal et al., 1993) used heat treatment and glutaraldehyde fixation to reduce RBC deformability. The tubular platelet count was decreased from 150% of reservoir concentration with normal RBC, to approximately equal to the reservoir count when glutaraldehyde fixed RBC were used. An intermediate value was obtained with heat-treated RBC. As the heat-treated RBC were not as rigid as those fixed with glutaraldehyde, these results suggest that the platelets flowed more centrally the more rigid the RBC. These results thus agree broadly with the findings of the current study.

7.4.4 Effect of RBC deformability on platelet adhesion

Platelet adhesion showed a significant increase as RBC deformability was decreased even though reducing RBC deformability resulted in a decrease in the number of free-flowing platelets at the wall. The decrease in the number of free-flowing cells was accompanied by an increase in free-flowing platelet velocity and an increase in shear stress (judged from the changes in apparent viscosity). The reduced tendency of the RBC with low deformability to migrate away from the vessel wall may well have played a part in the increase of platelet

adhesion. As the RBC deformability was decreased, the peripheral plasma layer is likely to have decreased in width. Even though the number of free-flowing platelets adjacent to the vessel wall was reduced, the reduction in the width of the plasma layer may have resulted in a greater number of RBC-platelet collisions that resulted in platelet-vessel wall interactions. Thus the decrease in the number of free-flowing platelets at low RBC deformability was presumably overcome by a narrowing of the plasma layer and an increase in the efficiency of interaction of the platelets with the wall.

There has been one study on platelet adhesion and RBC deformability, performed by Aarts et al (Aarts et al., 1984). They showed, at wall shear rates of 300-1800s^{-1}, as RBC deformability was decreased, platelet adhesion to umbilical vein subendothelium was increased. The changes in deformability were inferred from measured increases in apparent viscosity of the blood and it is difficult to assess whether the effects of the treatments used were comparable to those applied here. Nevertheless, the results obtained here are in agreement with the existing literature, and indicate that abnormalities in red cell deformability could in fact promote platelet adhesion.

7.4.5 Comparison of the effects of RBC deformability on leukocytes and platelets

The reduction in the deformability of the RBC had similar effects on the margination of both leukocytes and platelets. Both showed a reduction in the number of free-flowing cells adjacent to the vessel wall as the deformability of the RBC was reduced. Initially both leukocyte and platelet adhesion was increased as the deformability of the RBC was reduced. Upon further reduction, leukocyte adhesion dropped back down to the levels seen with 'normal' RBC and platelet adhesion continued to increase.

The reduced tendency of the RBC to migrate away from the vessel wall as they become less deformable accounts for the behaviour of the margination of both cell types. Leukocytes and platelets flowing more peripherally were displaced by the flowing RBC. However this effect was not significant for leukocytes until the deformability was reduced to the minimum level used in this experiment. This is because, due to their larger size, leukocytes have a higher tendency to migrate towards the vessel axis than platelets. Normal RBC, due to their highly deformable nature, have the highest tendency to migrate towards the axis of the vessel of any cell in the blood. Presumably it was only as the RBC were made rigid that their inward migration tendency was reduced to such an extent that the number of free-flowing leukocytes near the vessel wall was decreased (as they migrated away from the wall more rapidly than the RBC).

The initial reduction in RBC deformability increased both leukocyte and platelet adhesion. Leukocyte adhesion may have been facilitated by an increased number of near-wall RBC-leukocyte collisions facilitated by the reduced tendency of less deformable RBC to migrate away from the vessel wall. The same was true of platelet adhesion; reducing RBC deformability reduced the width of the peripheral plasma layer which acts to facilitate platelet adhesion. Upon further reduction of RBC deformability, leukocyte adhesion dropped significantly, presumably due to the decrease in the number of marginated leukocytes, where as platelet adhesion continued to increase. A continued decrease in the width of the plasma layer would have facilitated platelet adhesion once more. The increased shear stress provided by the increased apparent viscosity of the blood with less deformable RBC would also have contributed to the decrease in the amount of leukocyte adhesion but would not have as much effect on platelet adhesion (see chapter 4).

7.4.6 Physiological significance of effects associated with abnormal RBC deformability

Relatively mild changes in RBC deformability are associated with diseases such as hypertension and diabetes (Chien, 1987). More severe changes occur in sickle cell disease (Nash et al., 1988) and upon infection with the malarial parasite Plasmodium falciparum (Cooke et al., 2004). Sickle cell disease is associated with blockage of the blood vessels due to the reduced deformability of the RBC (Stuart, 1984). Malarial parasite infection is associated with sequestration of the rigid and adhesive infected cells in microvessels (Nash et al., 1989) although this may have more to do with the parasitic up-regulation of adhesive receptors on the surface of the RBC than with the reduction of the deformability of the RBC. In milder diseases, such as hypertension or diabetes one would predict that platelet adhesion would be increased, and that any response to infection would be enhanced due to an increase in leukocyte adhesion upon infection. In severe diseases, the occlusion of blood vessels causes the main symptoms and changes in leukocyte and platelet adhesion might not be of critical importance. However, the decrease in the deformability of RBC, if severe, would have little effect on leukocyte adhesion in the response to infection. The reduction in RBC deformability could cause an increase in platelet adhesion, resulting in larger thrombi forming in the instance of vessel trauma; in smaller vessels this could lead to reduced blood flow. Indeed, there is an increased thrombotic tendency in individuals with sickle cell anaemia although this may arise from other causes as well as from the reduced deformability of their RBC (Stuart, 1984).

All of the studies assessing the effect of RBC deformability on the margination and adhesion of leukocytes and platelets were performed in the absence of RBC aggregation. Physiologically, if the RBC deformability happened to decrease in plasma, the ability of the RBC to form aggregates would be impaired (Goldsmith, 1971b). As RBC aggregation was abolished within these studies it is important to relate the results here to what we would

expect to happen *in vivo*. Leukocyte margination was decreased as the deformability of the RBC was decreased; the effect of RBC aggregation on leukocyte margination (see chapter 5) in plasma may result in elevated leukocyte margination with normal RBC and similar levels with rigid RBC, compared to the results from our study. This effect would lead to a more significant decrease in leukocyte margination as RBC deformability was decreased. As leukocyte adhesion is dependent on the number of marginated leukocytes, leukocyte adhesion would be affected in a similar way to margination, had RBC aggregation not been abolished, a similar level of adhesion with rigid RBC and a higher level of adhesion with normal RBC may have been seen. Platelet margination decreased as RBC deformability was decreased; the associated decrease in RBC aggregation would not have had any further effect on platelet margination, as it had shown no dependence on RBC aggregation (see chapter 5). Within plasma, at normal deformability, the RBC would form aggregates more readily than was the case within this study, and thus the width of the peripheral plasma layer may have been increased, resulting in a decrease in platelet adhesion when compared to the levels of adhesion found here. In the case of rigid RBC there may not be any difference in adhesion as the peripheral plasma layer would not have been affected, thus the increase in platelet adhesion with decreasing RBC deformability would be more pronounced if RBC aggregation had not been abolished.

This study has shown how different levels of RBC deformability modulate the behaviour of leukocytes and platelets in flow. Reducing the deformability of RBC by an intermediate amount resulted in no change in leukocyte margination but a reduction in platelet margination, and an increase in both leukocyte and platelet adhesion. A further reduction of deformability showed a decreased in both leukocyte and platelet margination. In turn leukocyte adhesion was decreased back to similar levels seen with normal RBC and platelet adhesion saw a further increase. This study was the first to assess the effect of RBC deformability on the margination and adhesion of leukocytes, and to provide a comparison between platelet margination and adhesion in the same model.

CHAPTER 8: GENERAL DISCUSSION

8.1 Main findings

In this thesis, the margination and adhesion of leukocytes and platelets have been assessed within the same experimental system for the first time. The flow assay used allowed us to study the effects of various haemodynamic variables and rheological properties of the blood on the margination of both leukocytes and platelets, along with their adhesion to P-selectin and collagen respectively. The main findings of this study are summarised below.

8.1.1 Shear rate and vessel size

Blood was perfused through microslides of 100μm or 300μm in height over a range of shear rates. We found that:

- Leukocyte adhesion was much more efficient in the smaller microslides for a given shear rate. Platelet adhesion was more efficient in the smaller microslides although the shear rates used did not overlap to a large extent.
- The measured apparent viscosity of the blood was lower in the 100μm than in the 300μm microslide, presumably due to the Fahraeus-Lindqvist effect (Fahraeus and Lindqvist, 1931).
- Within both sizes of microslide, at native haematocrit, the adhesion of leukocytes and platelets to the upper and lower surfaces of the microslides was not significantly different at any shear rate used.
- At wall shear rates of 70-280s^{-1} within the 100μm microslides, margination and adhesion of leukocytes decreased as the shear rate was increased.
- At wall shear rates of 70-1500s^{-1} within the microslides, platelet adhesion was shown to be approximately constant initially, once corrected for the perfused volume, and then tended to decrease between shear rates of 300-1500s^{-1}.

- Within the smaller microslides, leukocyte adhesion was much more sensitive to an increase in shear rate than platelet adhesion, as it dropped off rapidly between wall shear rates of 70 and 280s^{-1} whereas platelet adhesion only showed a decrease at a shear rate of 1500s^{-1}.

8.1.2 Suspending phase viscosity

The suspending phase viscosity of the blood was varied at a constant haematocrit of 40% (also 20% in platelet adhesion experiments), in the absence of RBC aggregation, by adding equal volumes of Dx40 solutions with different concentrations to the blood. When blood was perfused at constant shear rate we found that:

- Increasing suspending phase viscosity within the blood had no effect on the number of marginated leukocytes or platelets but significantly decreased the adhesion of both.
- Leukocytes were more sensitive to a change in suspending phase viscosity than platelets; at 40% haematocrit platelet adhesion was relatively constant up to a viscosity of 2mPa.s and leukocyte adhesion decreased significantly within this range. Platelet adhesion, however did decrease at higher suspending phase viscosities of between 2-4.5mPa.s.
- The apparent viscosity of the blood increased as the viscosity of suspending phase was increased. Blood viscosity was found to be linearly dependent on the viscosity of the suspending phase.
- The results from the shear rate and suspending phase viscosity studies were used in combination to show that the critical force needed to decrease the initiation of adhesion of both leukocytes and platelets was very similar.
- Leukocyte adhesion dropped off more rapidly than platelet adhesion as shear stress was increased, this was due to the larger size of the leukocytes. At a given shear stress, the

force experienced by the adhesive bonds is much higher for leukocytes when compared to platelets.

8.1.3 RBC aggregation

Using high or low molecular weight dextrans to increase or decrease RBC aggregation respectively, at constant haematocrit and suspending phase viscosity, we found that:

- Increasing RBC aggregation had no effect on platelet margination but acted to increase leukocyte margination.
- Adhesion of leukocytes followed a similar trend to that of margination; as RBC aggregation was increased, adhesion was increased.
- Platelet adhesion decreased as RBC aggregation was increased, and did not correlate with the lack of dependence of platelet margination on RBC aggregation.
- Increasing RBC aggregation was associated with increasing apparent viscosity of the blood in the horizontal capillaries.
- These results support the concept that platelet adhesion is facilitated by the reduction in the width of the plasma layer associated with a decrease in RBC aggregation. Where as leukocyte adhesion is more dependent on the number of marginated leukocytes.

8.1.4 Haematocrit

Manipulating the haematocrit of the blood in this system, by adding different volumes of autologous plasma to blood at a haematocrit of 50% we found that:

- Leukocyte adhesion decreased, from a very high value at 10% haematocrit, as haematocrit was increased. The behaviour of leukocyte adhesion was explained by sedimentation of the leukocytes at low haematocrit.

- Platelet margination remained constant between 20-30% haematocrit and increased significantly between 30-50% showing no plateau.
- Platelet adhesion showed a nearly linear increase as haematocrit was increased between 10-50%.
- The apparent viscosity of the blood increased almost linearly with haematocrit, although the viscosity of the blood tended to be disproportionately increased at higher haematocrits.
- The behaviour of platelets again suggests that the increase in the width of the RBC core and in turn, the decrease in the width of peripheral plasma layer brought about by an increasing haematocrit facilitates platelet adhesion.
- Leukocyte adhesion results suggest uneven adhesion within the microcirculation and could be very efficient at low haematocrit within horizontally orientated vessels.

8.1.5 RBC deformability

Reducing the deformability of the RBC in a graded manner, at constant haematocrit in the absence of RBC aggregation, we found that:

- The margination of both leukocytes and platelets was decreased as the deformability of RBC was decreased; for leukocytes this decrease was only seen as RBC were made very rigid.
- Leukocyte adhesion increased initially as RBC deformability was decreased and decreased back to control levels as deformability was further decreased.
- Platelet adhesion seemed to increase despite a decrease in the number of free-flowing platelets near the vessel wall.
- The apparent viscosity of the blood measured within the microslide was increased as the deformability of the RBC was decreased.

- It appears that the narrowing of the plasma layer associated with decreasing RBC deformability facilitates platelet adhesion, even as the number of free-flowing platelets near the vessel wall is decreased.

8.1.6 Effects of cell size and peripheral plasma layer

Taken together, these findings provide strong evidence that the size of the cells within the blood and the width of the peripheral plasma layer are very important in determining the different behaviours of leukocytes and platelets within blood flow. These factors have rarely been considered previously when quantifying leukocyte and platelet margination and adhesion. Thus the results of this thesis suggest that:

- Margination of blood cells is size dependent. The largest cells or aggregates of cells will flow more centrally and the smaller cells will flow more peripherally.
- Leukocyte margination only occurs when RBC aggregates are present as they are larger than the leukocytes.
- Platelets will always flow in the periphery of the vessel as they are smaller than individual RBC.
- As a change in platelet adhesion was seen at constant near wall concentration and cell velocity, the width of the peripheral plasma layer plays an important role in the adhesion of platelets.
- As platelets always flow within the peripheral plasma layer, changes in its width can alter platelet adhesion. As the plasma layer is decreased in width the platelets are forced into the vessel wall more efficiently.
- Leukocytes are larger then the plasma layer and are consequently not significantly affected by it.

8.2 Effects of rheology of the blood on margination and adhesion of leukocytes and platelets; relation to previous findings

The effects of RBC and their properties on the rheology of blood are well understood. However, their effects on the adhesion of leukocytes and platelets have not been considered to as great a depth. The rheological properties of RBC can have a significant effect on the levels of adhesion of both leukocytes and platelets. The *in vitro* assay used in this thesis has provided insights into how RBC might modify leukocyte and platelet adhesion *in vivo*.

Adhesion of leukocytes to the vessel wall is dependent on the number of free flowing leukocytes in close enough proximity to allow adhesive interactions, as well as the shear stress they experience. The time that each selectin-ligand pair is close enough to form a bond is reduced as shear rate increases, and when a bond is formed, the force exerted on the bond is increased. The frequency of the collisions of leukocytes with the vessel wall can influence the amount of adhesion, and these collisions are promoted by a normal force provided by the flowing RBC (Munn et al., 1996). The modification of leukocyte adhesion by the manipulation of haemodynamic variables and rheological properties of the blood can all largely be attributed to the factors above. The difference with platelets when compared to leukocytes is that platelet adhesion was seen to be modified at constant near-wall free-flowing concentration. Thus platelet adhesion was dependent on more than just the number of marginated platelets. As the number of platelets flowing at the vessel wall was always high due to the large number of platelets within the blood and their margination by larger RBC, an important determinant of the efficiency of platelet adhesion was the width of the cell-free peripheral plasma layer.

The wall shear rate experienced by the blood plays an important role in the adhesion of leukocytes and platelets. As the wall shear rate was increased in this thesis, the number of

adherent leukocytes decreased significantly and there was a trend for platelet adhesion to decrease. This was similar to previous studies assessing the effect on leukocyte adhesion in whole blood *in vitro* (Abbitt and Nash, 2001), with this thesis using smaller microslides than the previous study to more closely mimic the microcirculation. Upon correction for the delivery of platelets, platelet adhesion, showed a tendency to decrease as shear rate was increased with the smaller microslides. Previous studies did not correct adhesion for the increased delivery of platelets seen with increased flow rate (Turitto et al., 1980;Weiss et al., 1986;Savage et al., 1996). Broadly, all previous studies are consistent with a low sensitivity of platelet adhesion to increasing shear rate until about $1000s^{-1}$, with a decrease thereafter.

In this thesis we found that increasing the suspending phase viscosity of the blood decreased leukocyte adhesion. As leukocyte margination was not affected by the increase in suspending phase viscosity, the decrease in adhesion was a result of the increased force on initial adhesive interactions. Comparing shear rate and suspending phase viscosity studies performed here it was possible to show how an increase in wall shear stress affected leukocyte adhesion in two ways. We increased wall shear stress by increasing wall shear rate at constant viscosity or by increasing viscosity at constant shear rate. Apart from at the lowest shear rate, where RBC aggregation increased the number of free-flowing leukocytes near the vessel wall, there was no effect on leukocyte margination within either study. Therefore the only unequal factor affecting adhesion between the two studies was the increasing velocity of the free-flowing leukocytes within the shear rate study. The adhesion at constant viscosity did not decrease more rapidly with shear stress than the adhesion at constant shear rate. Thus the predominant factor in achieving stable, rolling leukocyte adhesion over the range of shear stresses used here appears to be the force on the P-selectin-ligand bonds.

Increasing the suspending phase viscosity of the blood had no effect on platelet adhesion up to 2.5mPa.s and decreased at higher viscosities. Comparing shear rate and suspending phase viscosity studies performed within this thesis it was possible to show how an increase in wall shear stress affected platelet adhesion in two ways. Wall shear stress was increased by increasing wall shear rate at constant viscosity or by increasing viscosity at constant shear rate. As a result, we were able to compare the effect of increasing shear stress on platelet adhesion, with increasing, or nearly constant velocity of the near wall free-flowing platelets. At high shear stresses (above around 2mPa.s) the only unequal factor affecting adhesion was the velocity of the free-flowing platelets. As platelet adhesion at constant viscosity decreased more rapidly than at constant shear rate it was clear that platelet adhesion with increasing shear stress appears to be dependent on the rate of formation of the platelet-collagen bonds initially, and then the force acting on the bonds.

The effects of suspending phase viscosity observed here did not closely match results of the only previous experiments on its effects on platelet adhesion (van Breugel et al., 1992). In the range of plasma viscosities over which a significant decrease in adhesion was seen in that study, we saw no decrease. However our study showed a decrease at higher suspending phase viscosities. The increased sensitivity of platelet adhesion to suspending phase viscosity found in the previous study could have been due to the use of glutaraldehyde fixed RBC. We have shown in this thesis that using fixed RBC results in a much higher wall shear stress than found with normal RBC, the increase in shear stress could have been enough to reduce platelet adhesion in the previous study at a lower suspending phase viscosity than was seen here.

Platelet adhesion was present at much larger wall shear stresses than leukocyte adhesion. Leukocytes, being larger than platelets, protrude further into the vessel and have a larger

surface area and thus, experience a larger force at a given wall shear stress upon the initiation of adhesion. The force experienced by the initial adhesive bonds can alter the outcome of the initial adhesive interactions of the cells. Although leukocyte adhesion was much more sensitive to an increase in shear stress than platelet adhesion, the force experienced by the adhesive bonds at which leukocyte and platelet adhesion began to decrease was similar. Thus, as the bond kinetics are very similar for leukocyte and platelet adhesion (Doggett et al., 2002) the difference in the effect of suspending phase viscosity appeared to arise from the differences in the sizes of the cells.

Shear rate not only affects the velocity of cells and the shear stress which they experience, but it also affects the extent to which RBC aggregation occurs within a given vessel (Goldsmith and Turitto, 1986). The number of marginated leukocytes at the lowest shear rate used in this thesis ($70s^{-1}$) was significantly higher than at higher shear rates, and adhesion was clearly dependent on the number of marginated leukocytes. This was also the case within the RBC aggregation studies, agreeing with studies performed within vertically perfused tubes (Nobis et al., 1985). An increase in RBC aggregation was found to increase the number of free-flowing leukocytes near the vessel wall, which correlated to an increase in adhesion. This agreed with previous studies on leukocyte adhesion (Abbitt and Nash, 2003). It was the studies of platelet adhesion that provided an insight into the effects of the width of the peripheral plasma layer. Unlike leukocytes, the number of free-flowing platelets at the vessel wall did not change as RBC aggregation was manipulated. This agreed with the only other study to assess platelet margination using dextran (Woldhuis et al., 1993), which showed that in arterioles, the addition of low molecular weight dextran had no significant effect on platelet margination. An increase in RBC aggregation, although not affecting the number of free-flowing platelets at the vessel wall, should have increased the width of the plasma layer as the large RBC aggregates migrated towards the centre of the vessel. As the width of the plasma

layer was increased, the distance from the edge of the flowing RBC core and the vessel wall would be increased. As a result there would be fewer RBC-platelet collisions facilitating adhesive interactions between the platelets and the vessel wall, resulting in a decrease in platelet adhesion.

The width of the plasma layer will also have been dependent on the haematocrit of the blood perfused, and would have decreased as haematocrit was increased. The dependence of platelet adhesion on haematocrit obtained in this thesis was consistent with previous studies (Turitto and Weiss, 1980). However our study also quantified platelet margination within the same system allowing for a greater understanding of the processes behind the effect of haematocrit on platelet adhesion. This enabled us to identify that the increase in platelet adhesion as haematocrit was increased was due to both, an increase in the number of free-flowing platelets adjacent to the vessel wall and the associated decrease in the width of the plasma layer. The only two previous studies assessing the effect of haematocrit on leukocyte adhesion were done in vertical perfusion systems (Munn et al., 1996;Abbitt and Nash, 2003). Here, the horizontal perfusion of the microslides showed how sedimentation of leukocytes within a vessel at low haematocrit could increase leukocyte adhesion. Leukocyte adhesion decreased significantly from a very high level at 10% and remained constant as haematocrit was increased from 30% to 40%. We predicted that an increase in haematocrit would increase leukocyte adhesion due to an increase in the normal force of the RBC facilitating leukocyte contact with the vessel wall. Due to sedimentation, the results did not conform to our expectations; however the increase in normal force at the higher haematocrits maintained the symmetry of leukocyte adhesion to the upper and lower surfaces of the microslides seen at native haematocrit.

Reducing the deformability of the RBC, will lead to a reduced ability to migrate towards the vessel axis within flowing blood (Goldsmith, 1971a). The tendency for leukocytes to migrate away from the vessel wall is low and will only surpass that of RBC when the RBC are very rigid. In this thesis, leukocyte adhesion was increased as RBC deformability was moderately decreased and then returned back to control levels as RBC were made rigid. Reduced axial migration of the less deformable RBC would result in a decrease in the width of the peripheral plasma layer and possibly a greater normal force exerted by the poorly deformable RBC on the leukocytes, resulting in increased leukocyte adhesion. As RBC deformability was reduced further, the drop in adhesion back to control levels can be explained by the reduction in margination seen. Decreasing RBC deformability led to an increase in platelet adhesion despite a decrease in the number of free-flowing platelets near the vessel wall, suggesting that the width of the peripheral plasma layer had been reduced, as expected. These results are in line with previous studies involving platelets (Aarts et al., 1984) that also reported an increase in platelet adhesion as RBC deformability was decreased.

8.2.1 Physiological relevance of this study

Physiologically, the shear rate and RBC aggregation within a vessel are linked, and RBC aggregation is abolished at shear rates of above around $50s^{-1}$ (Goldsmith and Turitto, 1986). The studies of effects of shear rate and RBC aggregation on leukocyte and platelet adhesion within this thesis can be compared to *in vivo* findings, and thus the relevance of our *in vitro* studies to leukocyte and platelet adhesion *in vivo* can be tested.

Leukocyte adhesion occurs in post-capillary venules where the shear rate is low (Firrell and Lipowsky, 1989). At low wall shear rates ~$50s^{-1}$, 30% of all leukocytes were adherent and at $800s^{-1}$ adhesion dropped to around 5%. Ley and Gaehtgens also found (Ley and Gaehtgens, 1991), at shear rates of <$100s^{-1}$ that 47% of all leukocytes were adherent in the venules.

Within the flow assay used in this thesis, we found leukocyte adhesion to be around 40% at $70s^{-1}$, and this dropped to around 5% at a wall shear rate of $280s^{-1}$. This suggests that our *in vitro* assay replicated leukocyte behaviour within the microcirculation *in vivo*.

In inflammation, RBC aggregation is increased by an increase in the concentration of fibrinogen in the plasma (Stuart and Whicher, 1988). This increase in RBC aggregation might act to promote leukocyte adhesion in post-capillary venules after infection. Efficient platelet adhesion is desirable on the arterial side of the circulation, as there is a potential for rapid blood loss. Within the arterial side of the circulation, the high shear rates experienced result in low levels of RBC aggregation and as a result, the width of the peripheral plasma layer is small. This will promote platelet adhesion within the regions of the circulation in which it is most important. On the venous side, where shear rates and stresses are low, RBC aggregation would tend to inhibit platelet adhesion, perhaps lowering risk of unwanted venous thrombosis. Thus it seems that the sizes of both leukocytes and platelets, and their responses to RBC aggregation are well suited to help promote adhesion within the areas of the circulation in which they are most important.

In most chronic inflammatory conditions, plasma viscosity is increased. Within our study of the effects of plasma viscosity, RBC aggregation was abolished. However within this context, it is likely that the ability of the RBC to form aggregates may well be enhanced *in vivo*, as the increase in plasma viscosity is due to an increase in fibrinogen concentration (Stuart and Whicher, 1988). Any decrease in leukocyte adhesion with increasing plasma viscosity is likely to be counteracted by the increased RBC aggregation. With regards to platelet adhesion, the increase in the plasma viscosity will most probably be within the range that saw no effect on platelet adhesion within this study. In disorders such as Waldenstrom's syndrome (Waldenstrom, 1944) where plasma viscosity can be increased up to 5 times normal

values, it is probable that both leukocyte and platelet adhesion would be affected. A decrease in platelet adhesion would lead to an impaired haemostatic capability of the host and indeed one of the symptoms of this hyperviscosity syndrome is bleeding within the mucosal membranes. In Waldenstom's syndrome, as plasma viscosity is increased by increasing the concentration of the immunoglobulin IgM, it has been shown that the tendency for RBC to aggregate will also increase (Dintenfass and Somer, 1975). Thus, the expected decrease in leukocyte adhesion with increasing plasma viscosity may not be as pronounced as found in this thesis, as the resulting RBC aggregation will act to increase leukocyte adhesion.

This thesis has shown that horizontal blood perfusion at low haematocrit can result in very high levels of leukocyte adhesion to the lower surface of a blood vessel. Within the body, due to the nature of the vasculature and normal movement, no vessel stays in one orientation for very long. Therefore in the body, the extent to which sedimentation of leukocytes will have on their adhesion is uncertain. Even if the leukocytes adhere to the lower surface of the vessels within the microvasculature, upon transmigration through the vessel endothelium the cells will continue along the chemotactic gradient to engulf invading pathogens. Thus, *in vivo* the fact that adhesion will be on the upper or lower surface of a blood vessel is not of major importance, although our results suggest that very high levels of adhesion can occur in the horizontal vessels. In the small blood vessels within the body, the local haematocrit will be low compared to the systemic haematocrit due to the Fahraeus effect and could reach values as low as 20% (Lipowsky et al., 1980). The presence of RBC at a haematocrit of 20% is high enough to promote leukocyte margination (Munn et al., 1996) and thus adhesion (Abbitt and Nash, 2003) in vertically orientated vessels. Therefore, in vertically orientated vessels, leukocyte adhesion will be promoted by the RBC. If a vessel happens to be horizontally orientated, where the shear rate is low and sedimentation may occur, this will act to promote

leukocyte adhesion. Thus at the haematocrit found within the microcirculation leukocyte adhesion is facilitated in either orientation.

The highly deformable nature of RBC enables them to transit the smallest capillaries which can be smaller than the diameter of the cell. Within larger vessels, the deformability of the RBC influences their alignment and packing, and the distribution of the different cell types within the blood. The RBC, being highly deformable, tend to migrate towards the axis of any vessel more readily than the less deformable leukocytes and the smaller platelets. This tendency to migrate is one of the factors involved in the formation of the peripheral plasma layer. The local shear rate within a vessel will also affect the orientation of the RBC; at high shear the deformable cells will elongate and align with the flow, tending to reduce the width of the plasma layer. In some disorders that effect the rheology of the blood, such as diabetes and hypertension, the deformability of the RBC has been shown to be slightly reduced (Chien, 1987). The deformability of the RBC is greatly reduced in conditions such as sickle cell disease (Nash et al., 1988) or infection of the RBC with the malarial parasite Plasmodium falciparum (Cooke et al., 2004). Thus, through the modification of RBC deformability, these conditions could affect the adhesion of leukocytes and platelets. Severe reductions in RBC deformability will most likely result in the occlusion of small vessels and the effects on adhesion may not be of much concern. However, from our results it would appear that, in vessels that do not become occluded, leukocyte adhesion may be unaffected whilst platelet adhesion may actually increase. Mild reductions in RBC deformability, such as those seen in diabetes (Chien, 1987) or in oxygenated sickle cells (Nash et al., 1988) may result in an increase in both leukocyte and platelet adhesion.

8.3 Future work

The model used in this thesis could be used and adapted to facilitate further studies to understand the effects of rheological variables on the margination and adhesion of leukocytes and platelets.

The method devised for increasing suspending phase viscosity of the blood and for reducing the RBC deformability were both performed in the absence of aggregation. This was due to the fact that we specifically wanted to assess the effects that these variables had on leukocyte and platelet margination and adhesion, and not the secondary effects that these properties have on RBC aggregation which we manipulated independently. It was also experimentally easier to normalise the RBC aggregation to be abolished, than to try and replicate the *in vivo* situation exactly. It would, however, be interesting to be able to perform the suspending phase viscosity and RBC deformability experiments including RBC aggregation, as *in vivo*. Increasing suspending phase viscosity or making RBC less deformable would both probably inhibit RBC aggregation in flowing blood.

Within this system, the measurement of platelet margination was very time consuming and limited as to shear rate. Micro-Particle Image Velocimetry (μPIV) could be utilised within a similar assay to provide an efficient way of analysing platelet free-flowing velocity. The low number of leukocytes means that μPIV could not be used for these cells. μPIV will only work for a known, substantial number of small tracer particles, such as platelets. Two images are recorded in quick succession and the displacement of these tracer particles between the two images will give a statistical velocity field across the microslide. This method would yield an accurate velocity profile of the plane being analysed and may give an insight into platelet velocity within a vessel. Work was ongoing within our group to develop this method. However the system was not ready for the present study, and therefore analysis of velocity

was performed manually. Developing the velocimetry method would enable a less time consuming and more substantial analysis of platelet velocity to be performed.

Our analysis of the margination of leukocytes and platelets was limited to the region of the vessel near the wall due to the opacity of the flowing RBC. It would be interesting to quantify the behaviour of the free-flowing cells further into the vessel, preferably into the centre. Dyes that emit in the infrared range of the electromagnetic spectrum rather than visible light should lead to less absorption within the blood and better visualisation of the labelled cells further into the blood flow. Scattering of light would still occur. However, such dyes are under development by collaborators in Birmingham, and they may enable information about the free-flowing distribution of the cells to be obtained from deeper within the vessel.

One the most interesting concepts to arise from this thesis was the size-dependent differential behaviour during the margination of leukocytes and platelets. An interesting future study would be to investigate the effects of the various rheological manipulations on the margination of fluorescent beads of different sizes, e.g., between the large leukocytes of 8-10μm diameter and the small platelets of around 2μm diameter. It would be very interesting to find whether the extent to which the beads were marginated was directly related to their size in relation to RBC and their aggregates.

In this thesis the cell-free peripheral plasma layer, and specifically its effect on platelet adhesion, have been discussed extensively. No actual measurements of the width of the plasma layer were made in this thesis. In future studies, if the width of the peripheral plasma layer could be measured, then the effect that rheological properties of blood have on its thickness could be quantified more directly. Thus a definitive explanation of the effect of the

width of the peripheral plasma layer on the margination and adhesion of leukocytes and platelets could be obtained. This might be done by viewing the wall of a microslide that has been rotated 90º along its length whilst perfusing blood with specific rheological manipulations to see whether the width of the plasma layer is changed. As the thickness of the peripheral layer fluctuates, measuring the width over a long period of time would enable the accurate mean width of the layer to be established and compared between manipulations.

8.4 General conclusions

The adhesive processes of leukocytes and platelets are governed by the receptor-ligand binding of specific adhesion molecules at the vessel wall. Haemodynamic factors, such as shear rate and shear stress, and particularly rheological factors such as RBC aggregation, deformability, haematocrit and plasma viscosity, play an important role in the adhesion processes of leukocytes and platelets

This thesis assessed each of these factors individually, to systematically determine their effect on the margination and adhesion of leukocytes and platelets. In general, the presence of RBC is essential for leukocytes and platelets to come into contact efficiently with the vessel wall. In the absence of RBC and sedimentation effects, the leukocytes and platelets would rarely come into contact with the vessel wall. All rheological variables had differing effects on the margination and adhesion of leukocytes and platelets (summarised above). Ultimately the manipulation of leukocyte adhesion could be dictated by only a few important properties, namely; the number of free-flowing cells near the vessel wall and the force experienced by the initial adhesive interactions between the leukocytes and the vessel wall. An increase in the wall shear stress would result in an increased force experienced by any adhesive interactions. The number of adhesive interactions resulting in stable rolling adhesion would therefore be

reduced. Platelet adhesion was dependent on the velocity of the free-flowing cells near the vessel wall and the force experienced by the platelets once adhered. Platelet adhesion appeared to be less dependent on the number of free-flowing cells near the vessel wall than leukocyte adhesion, and seemed to be dictated by the width of the peripheral plasma layer, with a thinner plasma layer resulting in more efficient platelet adhesion.

Overall, these differences can be considered as an adaptation to function. The platelets, being small, will always flow in the periphery of the vessel, with the distribution not being dependent on shear rate or aggregation. This adaptation allows platelet adhesion and thrombus formation to occur rapidly at even the highest of shear rates and stresses found within the circulation, thus keeping any potential blood loss to a minimum. The size of the leukocytes and the ability of the RBC to form aggregates together provide an effective system for any response to infection. The size of leukocytes results in more central flow in the high shear environment of the arterial circulation, where the shear rates and stresses will far exceed the limits for leukocyte adhesion. Within post-capillary venules it appears that leukocytes, and indeed the blood as a whole, has been adapted to facilitate efficient leukocyte adhesion in the response to infection. Within the post-capillary venules the shear rate is low and the vessels are small (<100μm). The low shear rate environment will induce the formation of RBC aggregates and result in leukocyte margination. The low shear rate will result in a relatively low velocity of the free-flowing leukocytes near the vessel wall and more importantly a low shear stress which we have shown here will maximise leukocyte adhesion. The small size of the vessels will also result in more efficient leukocyte adhesion per unit volume of blood perfused. Thus the blood properties have been adapted to the anatomy of the circulation to provide the body with efficient haemostatic and immune responses.

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