BLOOD RHEOLOGY IN DIABETES MELLITUS AND ITS COMPLICATIONS: ASSESSMENT OF NEW METHODS

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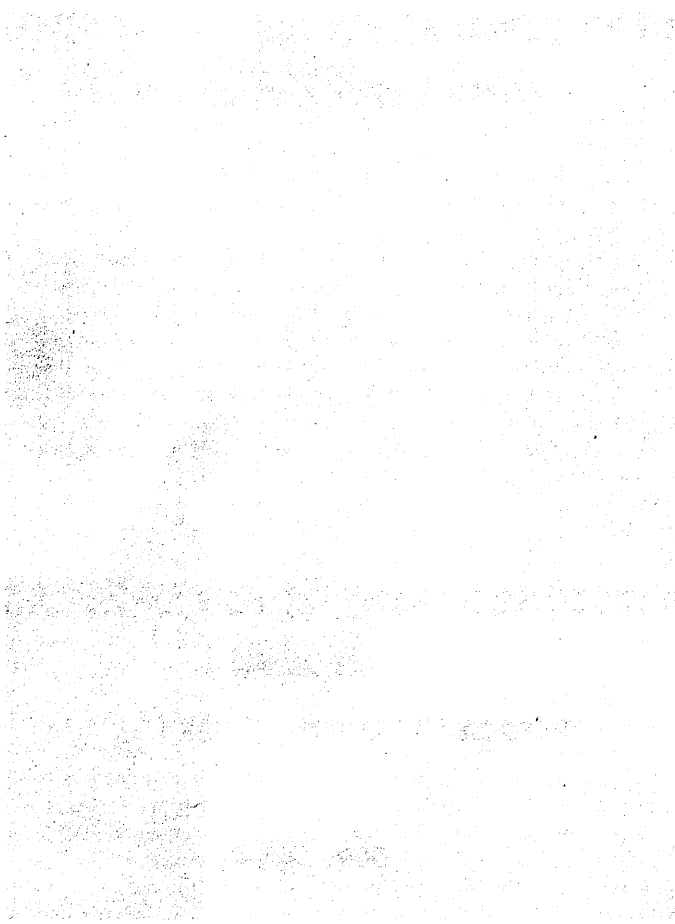
This thesis is dedicated

to

Phil



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DECLARATION

The initiation and design of all the studies described in this thesis was my own work, as was the analysis of the results. The work was carried out in the rheology laboratory of the University Department of Medicine at Glasgow Royal Infirmary between the years 1986 and 1990.

I personally carried out all venepunctures and patient assessments and, after a period of training in laboratory techniques in the rheology laboratory, I performed all the red cell aggregation and fibrinogen assays.

Some of the studies described have been published or are in press in peer review journals. These include:-

Association of hypertension with blood viscosity in diabetes. S M MacRury, M Small, A C MacCuish, G D O Lowe. Diabetic Medicine 1988; 5: 830-834.

Evaluation of red cell deformability by a filtration method in type 1 and type 2 diabetes mellitus, and association with vascular complications. S M MacRury, M Small, J Anderson, A C MacCuish, G D O Lowe. Diabetes Research (in press).

Relation of haematological and biochemical variables to red cell deformability in normal individuals. S M MacRury, M Small, J Anderson, G D O Lowe. Clinical Hemorheology (in press).

SUMMARY

This thesis reviews blood rheology and its known associations with diabetes mellitus and vascular complications in diabetic patients. The relationship between blood viscosity and two conditions which are common in diabetes, namely hypertension and peripheral neuropathy, was examined for the first time. Type 2 (non-insulin dependent) diabetics with hypertension were found to have increased blood viscosity compared with normotensive type 2 diabetics. Blood viscosity and red cell deformability were measured in diabetic patients with peripheral neuropathy. When compared with diabetics who have no evidence of neuropathy but were matched for other microvascular complications , no differences were found.

Using the recently-introduced Carri-Med filtrometer, red cell deformabiltiy was assessed by filtration through Nuclepore membranes in a large group of type 1 (insulin-dependent) and type 2 diabetic patients. Compared with healthy control subjects, deformability was impaired in all diabetic patients, but to a greater extent in type 1 patients. In the control population, red cell filterability was related to mean cell volume; while in diabetic patients, it was related to mean cell haemoglobin concentration. Within the diabetics, red cell filtration was not significantly different in patients with microvascular or macrovascular complications.

Red cell aggregation was measured in the new Myrenne photometric aggregometer and found to be increased in both type 1 and type 2 diabetic patients, particularly hypertensive type 2 diabetics. Aggregation was found to be related to plasma triglyceride and very low density lipoprotein levels.

Deformability of white cell subpopulations was measured by a filtration method in type 2 diabetics, and although non significant differences were found when compared with non-diabetic control subjects, a correlation of both mononuclear and polymorphonuclear cell filtration pressure was demonstrated with glycaemic control.

The implications of the findings in these studies are discussed, and suggestions for further rheology studies in diabetic patients are proposed.

CHAPTER 1

BLOOD RHEOLOGY

1.1 Introduction

Rheology is the study of deformation and flow of matter, and haemorheology is the study of deformation and flow of blood (1). Blood flow depends on the pumping action of the heart, the resistance to flow imposed by the blood vessels and the resistance to flow of the blood itself. Blood flow, therefore can be altered not only by diseases of the heart or blood vessels but also by abnormalities of the constituents of the blood.

Blood is basically a suspension of red and white cells and platelets in plasma, and plasma is a suspension of proteins and smaller molecules in water. Macrorheology describes the flow of blood as a bulk fluid, and microrheology the flow of individual cells.

Shearing within a liquid is the telescopic sliding of theoretical "layers" over each other (streamlines) and shear stress is the force required per unit area to cause shearing, while shear rate is the velocity gradient between adjacent layers. The shear rate is determined by a dynamic interaction of flow rate and vessel radius, and mean shear rate can be determined by the equation :

$$\text{Mean shear rate } (s^{-1}) = \frac{4 \times \text{Velocity (m/s)}}{\text{Radius (m)}}$$

The viscosity of a liquid (η) is the measure of its resistance to flow and is defined as the ratio of shear stress (τ) to shear rate (γ):

$$\text{Viscosity (mPa.s)} = \frac{\text{Shear stress (mPa)}}{\text{Shear rate } (s^{-1})}$$

Thus as the viscosity of a liquid increases, a greater force is required to achieve the same shear rate and flow rate.

Sir Isaac Newton's hypothesis (1686) stated that fluids had a constant viscosity at constant temperature i.e. the shear rate was directly proportional to the shear stress. This certainly holds true for fluids such as water and plasma and these are therefore known as Newtonian fluids. However whole blood viscosity is dependent on the shear conditions and blood is therefore Non-Newtonian (2). The relationships between shear rare and viscosity for plasma and blood are shown in Figure 1.1.

1.2 Interaction of flow conditions and blood rheology

The flow rate of a liquid is inversely related to its viscosity and is directly related to driving pressure. In a straight tube, the length and radius of the tube determine resistance to flow. The Hagen-Poiseuille equation shows the relationship between these variables:-

$$\text{Flow rate } \alpha \frac{\text{pressure gradient x tube radius}^4}{\text{tube length x fluid viscosity}}$$

In the circulation, blood flows along an energy gradient from high energy, high flow rate arteries to the low energy, low flow rate venous system, and its rheological behaviour depends not only on flow conditions but also on its viscosity, which is determined by frictional interactions between its

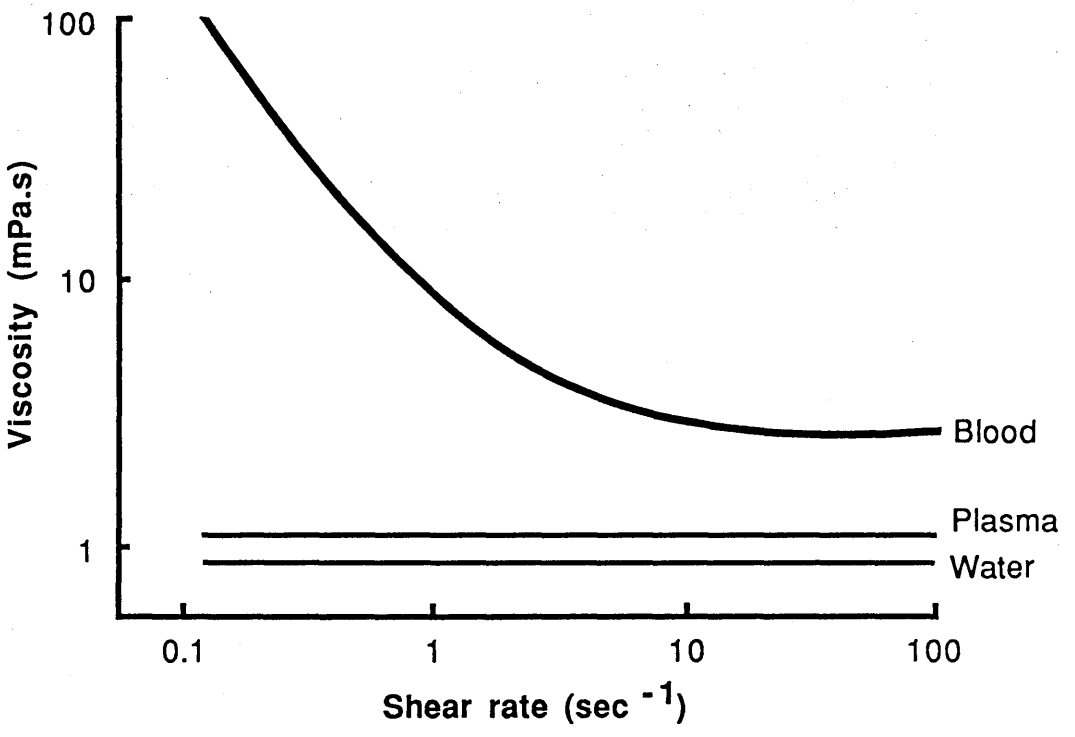


Figure 1.1

Dependence of blood viscosity on shear rate. Normal blood shows non-Newtonian behaviour, with a marked increase in viscosity at low shear rates. Plasma and water are Newtonian fluids, i.e. they show no shear dependence.

component parts, i.e. the cells and plasma. Blood flow to an organ therefore depends on the driving blood pressure gradient, the vascular resistance of the vessel and the viscosity of the blood.

Abnormalities of vascular resistance can be encountered in both the arterial and the venous circulation: stenosis due to atherosclerosis, thromboembolism, spasm or arteritis will increase arterial resistance, while venous thrombosis and increased tissue pressure which collapses veins will increase venous resistance. Because low flow rates increase blood viscosities, rheological factors will probably be of greater importance in the presence of any of these vessel abnormalities, or in the presence of a generalised fall in driving pressure e.g. in circulatory shock.

1.3 Rheological factors

The determinants of blood viscosity include the plasma viscosity, the volume ratio of cells to plasma (determined by red cell count and red cell volume), and by other properties of the cellular constituents.

1.3.1 *Plasma Viscosity*

The viscosity of plasma is about 1.6 times that of water, because the flow streamlines of plasma water are being interrupted by large plasma proteins such as fibrinogen and globulins (3,4). Fibrinogen is one of the largest and the most asymmetrical of the plasma proteins, and thus has an important effect on viscosity despite a lower concentration than either serum globulins or albumin. Serum globulins in turn have a greater effect than albumin, although again lower in concentration, (Table 1.1). The general composition of plasma is shown in Table 1.2. The viscosity of plasma is

Table 1.1

Contribution of plasma proteins to the increase of the mean normal plasma viscosity over the viscosity of water.

	Mean concentation (g/l)	% of Total	% Effect on viscosity	Characteristics of protein molecules			
				Molecular weight	Molecular dimensions (Å)		
					Length (L)	Diameter (D)	L/D ratio
Protein	78		99				
Albumin	45	58	36	69000	150	38	3.95
Globulin	30	38	41	160000	235	44	5.34
Fibrinogen	3	3.8	22	341000	700	38	18.4
Non-proteins			1.2				

(From Harkness J. Measurement of plasma viscosity. In Lowe G D O, Barbenel J C, Forbes C D. Eds. Clinical aspects of blood viscosity and cell deformability. Berlin: Springer-Verlag 1981;79)

Table 1.2

The protein and lipid constituents of plasma

Material	concentration (g/dl)	molecular weight $x10^3$	molecular dimensions (nm)
proteins:			
Albumin	3.3 - 5.0	69	15 x 4
α_1 - globulins	0.31 - 0.32	44 - 200	
α_2 - globulins	0.48 - 0.52	150 - 300	
β - globulins	0.78 - 0.81	90 - 1300	
γ - globulins	0.66 - 0.74	160 - 320	23 x 4
Fibrinogen	0.20 - 0.43	400	50-60 x 3-8
Cholesterol	0.14 - 0.27	0.39	
Triglyceride	0.002 - 0.015	0.9	
VLDL			50
LDL			21
HDL			12

(Modified from Caro C G, Pedley T J, Schroter R C, Seed W A. The mechanics of the circulation. Oxford: University Press 1978.)

independent of shear rate i.e. it is Newtonian (3) and can be measured in capillary viscometers by using the Hagen-Poiseuille equation. In pathological states, where there is an increase in large asymmetrical plasma proteins (e.g. paraproteinaemia) the plasma viscosity will be increased, as will the whole blood viscosity because the viscosity of the suspending medium (plasma) contributes to the viscosity of the suspension (blood). Plasma viscosity exhibits a degree of shear rate dependence in a few cases of paraproteinaemia, due to complexing of paraproteins (5).

1.3.2 *Whole Blood Viscosity*

When erythrocytes, which constitute the largest percentage of blood cells, are added to plasma, the whole blood viscosity increases logarithmically with a linear increase in packed cell volume (6). To determine if differences in blood viscosity are due to factors other than haematocrit (Hct), viscosity at a standard haematocrit (0.45) can be calculated from the following equation (7):

$$\frac{\text{Blood viscosity (Hct 0.45)}}{\text{Plasma viscosity}} = \left[\frac{\text{Blood viscosity (native Hct)}}{\text{Plasma viscosity}}\right]^{\frac{0.45}{\text{native Hct}}}$$

Whole blood viscosity is haematocrit dependent at any shear rate. At high shear rates, deformation of erythrocytes into ellipsoids allows them to become orientated in parallel with flow streamlines, thereby reducing the bulk viscosity (8). At shear rates of greater than 50/s the cell membrane may exhibit a "tank-treading " motion around the cytoplasm and it has been suggested that this could also reduce the suspension viscosity (9). At low shear rates, shear deformation of erythrocytes is reduced and erythrocytes

are joined by plasma protein bridges, (which overcome their natural electrostatic mutual repulsion) to form linear aggregates causing disruption of the flow streamlines and increasing bulk viscosity (10).

The determinants of blood viscosity are therefore plasma viscosity, haematocrit, red cell deformability and red cell aggregation. The contribution of cell deformation and aggregation to the rheological behaviour of blood may be estimated by calculation of relative blood viscosity, which is the ratio of blood viscosity at a standard haematocrit to plasma viscosity:

$$\text{relative viscosity} = \frac{\text{whole blood viscosity (45\% hct)}}{\text{plasma viscosity}}$$

At high shear rates, relative viscosity is a measure of the degree by which red cells elevate plasma viscosity due to lack of deformation. At low shear rates, relative viscosity is a measure of the degree by which red cells elevate plasma viscosity due to aggregation.

The relationships between blood viscosity, shear rate, red cell deformation and red cell aggregation and viscosity are shown in Figure 1.2. Fibrinogen depletion reduces red cell aggregation and hence low shear viscosity. Hardening of cells with aldehydes reduces their deformability and aggregation and abolishes shear dependence.

The viscosity of all fluids is temperature dependent, an increase in temperature resulting in decreased molecular interactions and hence in decreased viscosity. This temperature dependence is seen in both plasma and whole blood viscosity (11)

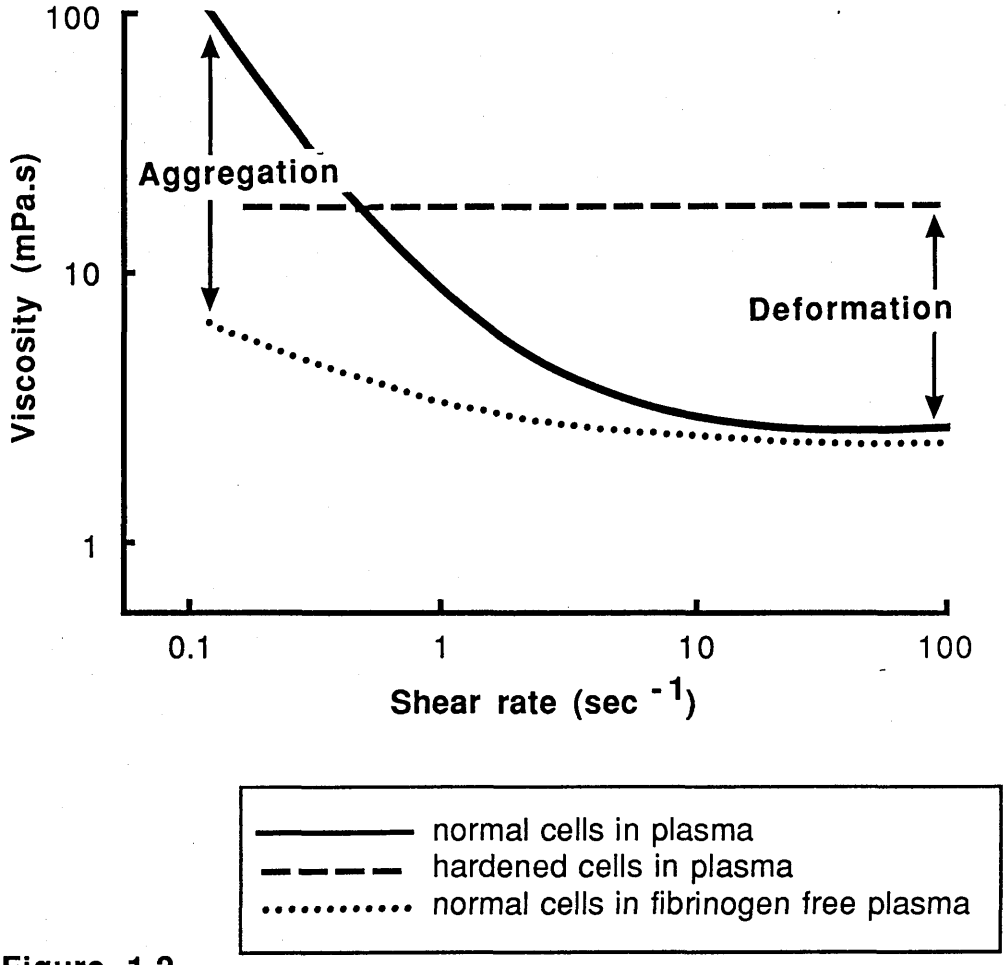


Figure 1.2

The effects of red cell aggregation and red cell deformation on the shear dependence of blood viscosity

1.4 Red cell deformability

In the resting state erythrocytes are biconcave discs with a diameter of c. 7.5μm. Deformation is important in the macrocirculation because it contributes to the low bulk viscosity of blood at high shear rates, and also in the microcirculation where this property allows the cells to traverse nutritive capillaries (mean diameter 3-5μm). The excess surface area to volume ratio of biconcave erythrocytes facilitates deformation, and conditions in which this ratio is reduced (e.g. spherocytosis) result in a reduction of red cell deformability (12). Transformation of cells to alternative shapes (such as stomatocytes) by chemical stresses will also cause a loss of deformability (13). The other main determinants of deformability are cell age, (older cells are less deformable than younger cells (14)), cell membrane fluidity (15), internal viscosity - determined largely by the mean cell haemoglobin concentration, MCHC (16); and cell size i.e. mean cell volume (MCV), the deformability being reduced by increased MCV (16, 17).

The intracellular fluid viscosity increases non-linearly with increasing cell haemoglobin concentration and thus the deformability falls (13). Abnormal haemoglobins (eg in sickle cell disease) are also associated with increased intracellular viscosity for a given haemoglobin concentration (18, 19).

The red cell membrane consists of a phospholipid bilayer matrix. Lecithins and sphingomyelins are on the outside and phosphatidyl 6-ethanolamines and phosphatidylserine lipids on the inside. The proteins spectrin, actin and band 4.1 on the surface provide mechanical strength in the membrane. Within the lipid bilayer the ratio of cholesterol to phospholipid has an important control on fluidity (20, 21). Changes in this lipid bilayer or in protein structures will affect membrane microviscosity, which will increase

the resistance to deformation and impair shape recovery after deformation (22).

1.5 Red cell aggregation

At low shear rates normal erythrocytes aggregate to form rouleaux which disturb flow streamlines and cause an increase in whole blood viscosity. Aggregation of cells is reversibly dependent on the cell to cell protein bridges (23) which are created by large plasma proteins such as fibrinogen and α_2-macroglobulin (24). These proteins are absorbed to the cell surface and are capable of overcoming the natural repulsive forces of the negatively charged red cells (25,26). The exact binding forces are unknown but may be due to hydrogen bonding (van der Waal's attraction) (27). The paraproteinaemias are associated with an increase in both plasma viscosity and red cell aggregation, due to production of abnormal globulins.

An increase in haematocrit up to 40 to 50% will also increase aggregation, but at very high haematocrits the close proximity of the cells reduces aggregation (24). At normal haematocrit and plasma protein levels, aggregates are easily dispersed by an increase in shear stress (23), the cells become orientated and deformed in the direction of the flow streamlines. Other factors which exert an influence on red cell aggregation are cell size and age: the older and smaller the cell the greater the degree of aggregation (28), and the rigidity of the cell - increased rigidity being associated with reduced rate of aggregate formation (29).

1.6 White cell deformability

White blood cells are less in number than red blood cells by a factor of about 700, they have similar diameters to red blood cells (6.2-7.5 μm) but due to their spherical shape have twice the volume. Their spherical shape, increased internal viscosity (about three orders of magnitude higher than red cells), and reduced elasticity render them less deformable than red cells (30). White cell subpopulations differ in their rheological properties, with monocytes being the least deformable followed by polymorphonuclear cells and lymphocytes (31,32).

White blood cells contribute little to the bulk resistance of blood to flow in the macrocirculation, but it is in microcirculatory blood flow that their poor ability to deform is most important, since they have greater difficulty entering and traversing nutritive capillaries. At low shear rates in small diameter vessels, white cells can cause a transient arrest in blood flow and red cells will tend to build up behind these impacted cells thus increasing viscosity by increasing red cell aggregation (33). The flow of other white cells becomes marginal in venules, which will facilitate their adherence to the endothelial surface, reducing venular diameter and causing a further increase in flow resistance (34).

1.7 Platelet aggregation

The small diameter of platelets (2-4 μm) means that they have negligible effects on bulk viscosity. However aggregation of platelets and adhesion of such aggregates to vascular subendothelium (e.g. at arterial stenosis) alters blood flow. High haematocrit levels and high shear rates cause

diffusion of platelets towards the vessel wall (35), thus facilitating adhesion (36). Increased red cell volume (37) and decreased red cell deformability (38) increase platelet adhesion. Shear induced release of adenosine diphosphate (ADP) from red cells causes platelet activation which in turn results in increased platelet aggregation, and such release of ADP may be increased by reduced red cell deformability (39).

1.8 Flow conditions and blood rheology in disease

1.8.1 *Macrovascular disease*

Flow separation areas (e.g. bifurcations) are associated with low shear rates (40), as are the axial regions of large vessels (41). These areas allow accumulation of red cell aggregates. Flow separation areas are also the sites at which lipids may accumulate and predispose to atherosclerosis, leading to stenosis. Stenosis formation will also increase shear stresses upon the blood as it passes through and thus activate platelets and enhance the formation of thrombi. Red cell aggregation will significantly increase blood viscosity at low perfusion pressures causing low shear conditions, thus reducing blood flow.

1.8.2 *Microvascular disease*

Vessel radius, determined by vascular morphology, is the most important factor controlling blood flow to an organ according to the Hagen-Poiseuille equation. Abnormalities of microvessels may not only have a direct adverse effect on microrheology, but also when combined with a primary

rheological abnormality there will be further compromise to an organ's vital blood flow.

Red cell aggregation is least likely to occur in the capillaries due to their narrow geometry, high shear rates and low haematocrit (Fåhraeus effect), (42), thus maintaining a low viscosity. It is in the low shear environment of the post-capillary venule that red cell aggregation influences flow in the microcirculation; an increase in viscosity in the post-capillary venule due to red cell aggregation will increase capillary back pressure, promote fluid transudation and haemoconcentration, and reduce flow (43). This effect on blood viscosity will be enhanced by elevated levels of haematocrit or plasma proteins which favour red cell aggregation.

The high shear forces existing in normal capillaries (Figure 1.3) aid the deformation of the red cell, thus facilitating its passage through the narrow organ capillaries for oxygen delivery. Narrowing or occlusion of these capillaries will impede red cell flow and may lead to ischaemic organ damage.

In high flow states in the arterioles, the red blood cells tend to flow axially, displacing white cells peripherally and when white cells reach the nutritive capillary first, they will raise the local resistance and cause preferential entry of red cells into other capillary branches. Hence white cells exert a significant influence on microcirculatory flow of red cells (44). Alterations in deformability of red or white cells leading to impaired entry to the capillary will have important effects on tissue oxygen delivery.

In low flow states in post-capillary venules, red cell aggregation results in white cells being pushed towards the vessel wall which facilitates leucocyte adhesion and further narrowing of the vessel lumen with reduction in vessel conductivity (33).

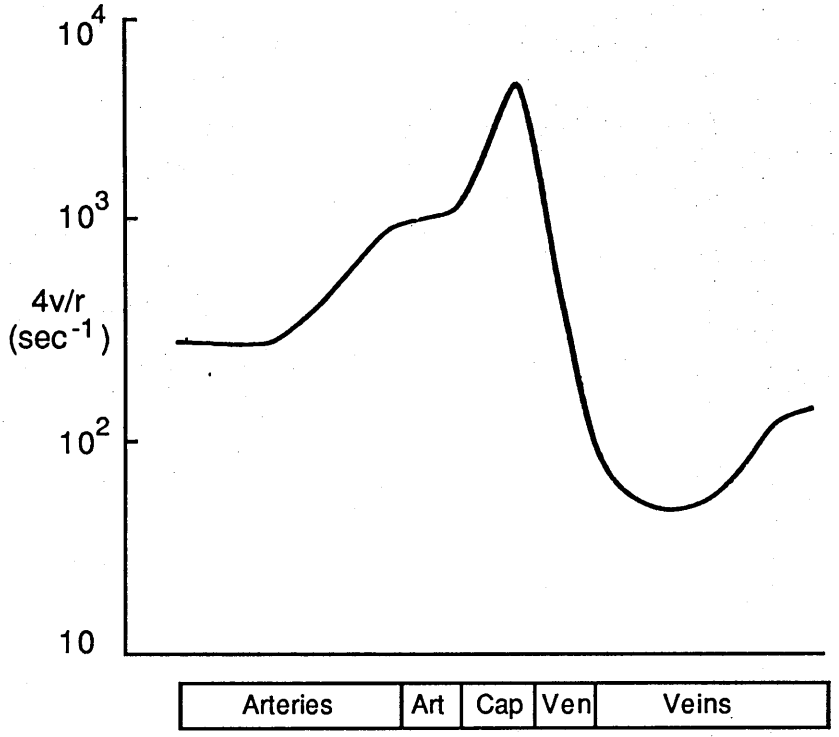


Figure 1.3

Variation in the parameter 4v/r in various parts of the circulation.

Diabetes mellitus is classically associated with altered microvessel geometry, and thus studies of blood rheology are of particular significance in this condition.

1.9 Review of methodology for measuring blood rheology

1.9.1 *Blood viscosity*

a) Capillary viscometers: These instruments are relatively simple and consist of tubes through which a column of liquid is passed, which is sheared by flow past the wall of the tube. A constant pressure is usually applied e.g. the standard height column of the liquid in the Ostwald (U-shaped) viscometer (3), the flow rate is calculated, and viscosity estimated from the equation:

$$\text{viscosity} = \frac{\Delta P \times r^4}{Q \times L \times 8}$$

ΔP=pressure gradient

r=tube radius

Q=flow rate

L=tube length

b) Semi-automatic capillary viscometers: These are more sophisticated instruments and have standardised capillary dimensions, constant driving pressures and accurate temperature control e.g. the Coulter-Harkness viscometer (45). These viscometers are commonly used for plasma or serum viscosity measurements, but can also be used for measuring whole blood viscosity at high shear rates

c) Rotational viscometers: Here the test liquid is sheared between two closely adjacent, concentric surfaces (one fixed, the other moving rotationally). This creates a uniform shear rate, across the test fluid which can not be achieved in a capillary tube and, rotational viscometers are therefore useful for non-Newtonian fluids such as whole blood. In these viscometers the rotational shear is usually calculated from the torque measurement and the shear rate is pre-set by choosing the rotational speed, hence the viscosity can be determined ($\eta = \tau/\gamma$). There are a number of different rotational viscometers available. The cone-plate variety e.g.Wells-Brookfield, consists of a cone which is rotated on the surface of a flat plate with the fluid lying in between (46). The cone applies the shear and also measures the torque. In the cone-in-cone variety the fluid is placed between the two cones, and the torque exerted on the inner cone is measured optically (47). Coaxial or couette viscometers consist of a cylindrical cup in which a bob is suspended (the fluid lies between bob and cup), and the torque measured electromagnetically. These viscometers are useful for measuring viscosity accurately at both high and low shear rates e.g. the Contraves LS 30 viscometer (48). Finally the controlled stress rheometer utilies the principle of varying the shear stress and measuring the shear rate, which may be a more physiological approach e.g. the Carri-Med CS viscometer (49).

1.9.2 *Red cell deformability*

a) High shear viscometry: After correcting for the effects of haematocrit and plasma viscosity (e.g. calculating relative high shear viscosity), high shear viscometry can be used as an indirect measure of bulk red cell deformability. Red cell suspensions can also be studied. This method however can not be used to estimate red cell deformability in the microcirculation, where red cells deform individually. Filtration methods are preferred for this purpose.

b) Bulk filtration: This involves the filtration of large numbers of red cells through micropores, usually 3-5 μm in diameter, and is a more direct method of measuring the ability of the cell to adapt and pass through channels of a similar size to those encountered in the capillary microcirculation in vivo. In general the flow rate of the cells is estimated and expressed relative to the flow rate of buffer. Cells can be filtered through the membrane by positive pressure e.g. in the Erythrometre (50), by gravity e.g. in the Hemorheometre (51) or by negative pressure e.g. the Carri-Med filtrometer (52). Using these instruments, the white cells and platelets usually have to be reduced in number to prevent clogging of the filters. However in the Carri-Med filtrometer extrapolation of the clogging rate to calculate the initial flow rate is insensitive to residual leucocytes (52).

c) Red cell subpopulations: It can be argued that it is preferable to measure the ability of individual red cells to deform. This normally involves the examination of single cells e.g. in micropipettes. These usually have a tube diameter of 3-5 μm and the pressure changes required to oscillate the cell can be measured (53). If smaller diameter tubes are used, then the deformability of the membrane alone can be measured. The single

erythrocyte rigidometer is a varient on the filtrometers described above, and estimates the time taken for individual cells to pass through a single pore in a plastic membrane of 5-6μm diameter (54). Another apparatus, the Cell Transit Time Analyser, has recently been developed, which uses a 20-pore membrane.

The main disadvantages of these filtration techniques are lack of standardisation, large variability and sensitivity to mean cell volume especially with 3 μm pores. They may be used to study cell fractions, e.g. by density gradient separation old and young cells can be examined separately.

d) The ektacytometer is a unique instrument which measures red cell elongation at a standard shear stress but changing osmolality, so that not only can membrane rigidity be estimated, but the effects of mean cell haemoglobin concentration and cell surface area / volume ratio can be taken into account (55).

1.9.3 *Red cell aggregation*

a) Low shear viscometry: An indirect estimation of red cell aggregation can be made from measuring relative blood viscosity at low shear rates.

b) Erythrocyte sedimentation rate: This is a very simple way of estimating aggregation, by measuring the descent of the red cell column in whole blood with time. Its main disadvantages being that it is sensitive to both age of the sample and haematocrit.

c) Direct microscopic observation: This is the most direct way of measuring

red cell aggregation, but is tedious and poorly standardised. An indexing system can be used to measure the average size of rouleaux (56).

d) Rheoscopy: This consists of a cone-plate viscometer with attached microscope, which shears the sample at high shear rates. After stopping rotation, the rate of rouleaux formation can be observed, as can the size of the aggregates and resistance to shear stress (57).

e) Photometry: This employs the principle that there is increased light transmission through areas of cell free plasma when the red cells aggregate. The erythrocyte aggregometer (Myrenne) is a new apparatus which is a highly automated cone-plate viscometer, which again shears the blood at high shear rates and measures the degree of aggregation over time after stopping rotation (58).

1.10 Summary

In recent years more attention has been focussed on the individual determinants of blood viscosity, and their influence on blood flow. Abnormal rheology may not only contribute to vascular complications in certain disease states (e.g. paraproteinaemias) but also in combination with blood vessel abnormalities may present additional flow impairment leading to ischaemic organ damage. Although much is known about bulk blood rheology, and macrorheology can be adequately determined using current viscometers, the measurement of microrheology is influenced by individual cellular properties and a wide variety of techniques is currently used. The newer instruments require further evaluation, in particular the Carri-Med filtrometer for measuring red cell deformability, and the

Erythrocyte aggregometer for measuring red cell aggregation. There is also a need to develop systems for measuring white cell deformability because despite their relatively small number compared to red cells, further impairment of their normally poor deformability could have a greater potential for disturbing flow in the microcirculation.

CHAPTER 2

BLOOD VISCOSITY IN DIABETES MELLITUS

2.1 Introduction

Diabetes mellitus is a common disorder which affects about 1% of the population. It is generally divided into two basic types, although it may be the consequence of other acquired or genetic diseases. Both types of diabetes have similar symptoms and signs at presentation and are associated with similar long-term complications but have quite distinct aetiologies. Type 1 diabetes, also known as insulin-dependent, ketosis-prone or juvenile onset diabetes, is associated with HLA- DR3 and -DR4 haplotypes (59) and is thought be be the result of islet cell destruction by autoantibodies (60). Affected individuals tend to develop diabetes in childhood or young adulthood. These diabetics therefore have insulopenia and eventually have no endogenous insulin secretion. Type 2 diabetics , also known as non-insulin-dependent, non-ketosis-prone, or maturity onset diabetes, on the other hand are older, usually overweight and often have high insulin levels, especially at the onset of diabetes (61). The hyperinsulinaemia is associated with a degree of insulin resistance and insulin-receptor down-regulation (62), although eventually insulin secretion may fall and in some cases treatment with exogenous insulin is required. This type of diabetes is familial and is the commoner of the two basic types by a factor of 4 : 1.

Diabetes is associated with several specific complications due mainly to disturbance of the microcirculation, namely retinopathy and nephropathy, and neuropathy which is generally assumed to have a metabolic origin. However all microvascular complications have been related to both duration of disease (63) and to glycaemic control (64), and as a consequence of the former, type 1 diabetics are obviously more prone to these complications.

Macrovascular disease i.e. ischaemic heart disease, cerebrovascular disease and peripheral vascular disease are all commoner in diabetic patients (65), both type 1 and type 2, but due to the greater age of the type 2 patients large vessel disease tends to be more prevalent in this group.

2.2 Complications in diabetes mellitus

2.2.1 *Microvascular*

2.2.1 (i) Retinopathy

Diabetic retinopathy rarely develops before 10 years duration of diabetes, but in type 2 diabetics may present apparently before this time due to the condition being undiagnosed for several years. Generally speaking, after about 5-20 years duration of diabetes the prevalence of diabetic retinopathy is about 67-100% (66,67).

In health a blood-retinal barrier exists, formed by the tight junctions between endothelial cells (68). In diabetic patients this barrier is imperfect due to various mechanisms (69); one of the capillary abnormalies is endothelial cell proliferation which may contribute to breakdown of the blood-retinal barrier (70). Basement membrane thickening in capillaries is a widespread feature of diabetes (71), thought to be due to augmentation of the membrane by entrapped plasma proteins after leakage through the endothelial cell junctions (70). Other suggested mechanisms contributing to endothelial damage include reduced prostacyclin activity (72) and reduced fibrinolytic activity (73).

The cause of retinopathy is unknown but the clinical manifestations are due to vascular occlusion i.e. non-perfusion leading to ischaemic haemorrhages and neovascularisation and ultimately in some cases a degree of visual loss. The initial lesions consist of microaneurysms, "dot and blot" haemorrhages and hard exudates - this is known as background retinopathy. In many patients there is little progression beyond this stage but others develop ischaemic or pre-proliferative retinopathy. This is characterised by large blotch haemorrhages, soft exudates (cotton-wool spots) and venous abnormalities such as beading and the formation of loops. Fluorescein angiography shows capillary non-perfusion. New fragile capillaries develop in the areas of occlusion in response to release of retinal vasoproliferative factors and are liable to rupture and cause vitreous haemorrhage.

2.2.1 (ii) Nephropathy

Diabetic renal disease is the other main manifestation of microvascular disease in diabetes. It affects about 25% of all diabetics after 15-25 years duration of disease (74), thereafter the risk appears to be reduced presumably due to protective genetic factors (75).

The underlying pathological lesion in diabetic nephropathy is glomerulosclerosis (76). As in retinopathy basement membrane thickening of the glomerulus occurs in diabetes (77). This is thought to be due to defective removal of basement membrane in addition to entrapment of plasma proteins and leads to their accumulation in the mesangium (78). The changes in the glomerulus lead to increased permeability and protein loss. Other contributing factors may be fibrin deposition (79), and leakage of plasma collagenase inhibitors (80,81).

The first clinical sign is an intermittant increase in microprotein excretion detected in the urine. This becomes more persistent and the degree of protein excretion increases until the stage of macroproteinuria. This stage may last for years and since renal function is preserved patients are usually asymptomatic. Progression to renal failure is variable thereafter and can again be very slow; the serum creatinine rises, oedema and hypertension develop, and the serum albumin level falls. Although end-stage renal failure may be treated with dialysis or transplantation the main cause of death in these patients is from arterial disease (82).

2.2.1 (iii) Neuropathy

Both the somatic and automatic nervous systems may be involved in diabetes mellitus. The commonest form of somatic neuropathy is a peripheral sensorimotor neuropathy affecting the limbs in a "glove and stocking" distribution with the legs being predominantly affected. The true prevalence of this condition is difficult to establish because several studies have shown that patients may have neurophysiological or clinical evidence of neuropathy but are asymptomatic (83) and conversely some studies have shown that many patients with symptoms have no evidence of neuropathy on conventional testing (84). Prevalence certainly increases with age and duration of disease being about 50% after 25 years (63).

Diabetic neuropathy is often worse during periods of poor control and this has led to the assumption that the aetiology is mainly metabolic; possibly due to intraneural accumulation of sorbitol and fructose from increased polyol pathway activity (85) or reduced levels of myoinositol (86). Isolated nerve lesions occur in diabetes and are thought to have a focal ischaemic cause (87), and because the prevalence of neuropathy tends to follow that of other microvascular complications it has been suggested that micro

vascular lesions may contribute to diabetic peripheral neuropathy (88).

Patients with peripheral neuropathy present with a variety of symptoms including pain, paraesthesiae, numbness and signs of loss of vibration and position sense, absent ankle reflexes and impaired cutaneous sensation. Most patients experience problems for months or years and in some cases progress to loss of pain appreciation, which in conjunction with local joint subluxation can result in neuropathic ulceration of the feet. In a few cases widespread joint disruption of the foot results in neuropathic arthropathy (Charcot's arthropathy). Autonomic neuropathy is less common and may present with diarrhoea, gastroparesis, postural hypotension, erectile impotence and cardiac denervation. The latter may result in fatal cardiorespiratory arrest.

2.2.2 *Macrovascular*

Morbidity and mortality due to abnormalites of the coronary, cerebral and peripheral arteries are increased in diabetic patients (89,90,91). Atheroma is generally more extensive in diabetics compared to non-diabetics (65) which may account for some of the increased risk. Disease of the microcirculation may also contribute to vascular occlusive disease (92,93) as may hypertension (89).

2.3 Abnormal rheology in diabetes mellitus

2.3.1 *Plasma and serum viscosity*

Serum viscosity (i.e. plasma viscosity less the effect of fibrinogen) was first measured in diabetic patients by Cogan et al, using a capillary viscometer, and was found to be elevated compared to healthy controls due to elevations of several serum proteins (94). Elevation of plasma viscosity in diabetes has also been confirmed by many investigators (95-99). In most of these studies diabetics had raised fibrinogen levels as well as serum globulin levels, particularly α_2-macroglobulin, haptoglobin and caeruloplasmin (100), i.e. a disturbance in the average molecular size or shape of plasma proteins may be causing an increase in plasma viscosity (101). However in other studies no difference was detected between diabetic and control subjects (102-104).

2.3.2 *Whole blood viscosity*

Because the concentration and mechanical properties of the red cells also contribute to whole blood viscosity, several viscometers have been developed to measure whole blood viscosity at different shear rates to reflect the range found in the vascular system. Skovborg first studied whole blood viscosity in diabetic patients, and throughout a variety of shear rates, he found that viscosity was about 20% higher in diabetic patients compared to controls (105). Increased whole blood viscosity has since been documented at both high and low shear rates in diabetes, in some cases related to a higher haematocrit (106) and in others the increase was maintained after correction to a standard haematocrit (96,97). In some

studies, a higher viscosity was related to increased fibrinogen levels (99,107,108). The difference in blood viscosity between diabetic and non-diabetic subjects is more marked at low shear rates (104,108-111) implying that increased red cell aggregation is an important determinant of viscosity increase in diabetes. There have been a few studies showing no difference in whole blood viscosity at any shear rate between diabetic and control subjects (98), however this may have been due to the use of older, less sensitive viscometers.

The measurement of plasma and whole blood viscosity in diabetes has therefore given some conflicting results. Several factors may account for this, eg difference in apparatus (some older viscometers being less sensitive), patient selection, and confounding factors affecting blood viscosity which is for example increased in males compared to females, and in smokers compared to non-smokers (112).

2.2.3 *Red cell deformability*

Impaired red cell deformability in diabetic patients compared to non-diabetic control subjects has been found by several workers using filtration techniques (96,98,104,113-116) and also by others using micropipettes (117) However several other studies have found no difference in red cell deformability between diabetics and non-diabetics (103,109,118). It has been suggested that decreased whole blood filtration may reflect leucocytosis in diabetics (103,119). Increased microviscosity of the membrane has been reported in diabetes using techniques such as fluorescent probes (120,121). It has been suggested that increased glycosylation of the membrane may cause stiffening but although this has been demonstrated in old cells (122,123) there appears to be no difference

in deformability between old diabetic and old non-diabetic cells (124).

The cell membrane contains a fluid phophorylated protein, spectrin, in which the enzymes Na^+/K^+ ATPase and Ca^{2+}/Mg^{2+} ATPase are located. Increased spectrin glycosylation has been reported in diabetic red cells, but this was not associated with membrane stiffening (125). It may alternatively be reduced spectrin phosphorylation that is responsible for the increased membrane viscosity in diabetic patients (126).

Reduced ATP levels have also been associated with impaired red cell deformability: this may be due to either calcium accumulation within the red cell or spectrin cross-linkage (127).

Other work has suggested that an imbalance of the cholesterol/phospholipid ratio in the membrane may increase its microviscosity (128,129).

Further evidence for a red cell membrane defect in diabetes comes from the study of Wautier et al, who found increased adhesion of diabetic erythrocytes to endothelial cells, an abnormality also present in sickle cells (130).

2.3.4 *White cell deformability*

Leucocytosis has been reported in diabetic patients (131) and Stuart et al have reported reduced whole blood filterability in diabetic patients to be related to a raised white cell count (103). Newer filtration instruments such as the Carri-Med filtrometer allow the initial red cell filtration rate to be measured independently of the residual white cell count (52). Because this apparatus can also measure the clogging rate of the filter, it is also possible to determine white cell deformability. Reduced white cell filterability has been found in diabetic patients using this system (116,132)

2.3.5 *Red cell aggregation*

By direct observation, Ditzel first described increased red cell aggregation in the microcirculation of diabetic patients (133) but since then there have been few studies measuring red cell aggregation directly in diabetic patients. One study found no increase in aggregation in microchambers implanted in skin flaps of diabetic patients, though the numbers involved were small and the contribution of the endothelium was excluded (134). Schmid-Schönbein found an increased resistance to dispersion of aggregates at high shear rates in vitro in diabetic patients, which was associated with their abnormal plasma proteins (96). Other workers have confirmed increased aggregation in diabetic compared to non-diabetic subjects (135,136).

Diabetes therefore appears to be associated with increased red cell aggregation compared to non-diabetics. This is associated with increased plasma proteins, particularly fibrinogen, but may also be associated with reduced red cell size (28).

2.4 Glycaemic control and rheology

Very high levels of blood viscosity are found in diabetic ketoacidosis (137) due both to dehydration (causing haemoconcentration) and increase in acute phase proteins. It might therefore be expected that viscosity is related to glycaemic control. This, however, has only been found in a few studies (95,100) although changes in viscosity have been observed with improvement in glycaemic control (106,138).

Impaired red cell deformability in diabetes has been related to poor

glycaemic control (96,98,106,120), but incubating red cells, both diabetic and non-diabetic, with glucose to increased intracellular sorbitol levels produced only a minor reduction in deformability (115). Other studies have found no relationship with glycated haemoglobin (HbA1) or blood glucose levels (113,116,117,119). Juhan et al found that deformability of normal red cells was reduced after incubation in plasma from uncontrolled diabetic patients, despite a lack of correlation of deformability with glycaemic control (139). They subsequently showed that reduced membrane fluidity in diabetic cells could be corrected by insulin in vitro (140). Filterability of red cells was improved in one longitudinal study after insulin infusion for three months (141).

Many large serum proteins as well as fibrinogen may be elevated in diabetic patients, often as part of an acute phase reaction to infection or during periods of poor glycaemic control (131). Bauersachs et al found increased red cell aggregation associated with increased fibrinogen in uncontrolled type 2 diabetic patients, which was not improved by short term insulin treatment (142).

2.5 Rheology and microvascular complications in diabetes

2.5.1 *Blood viscosity*

Non-diabetic conditions such as myeloma and Waldenstrom's macroglobulinaemia which are associated with increased blood viscosity, are also associated with retinopathy. It is possible therefore that the increased viscosity of diabetes could contribute to the progression of diabetic retinopathy. Dintenfass measured viscosity in a group of diabetic

patients with retinopathy and compared them with a group of patients with non-diabetic retinopathy, but found the viscosity to be highest in the non-diabetic male patients (143). Lowe et al found no difference in viscosity in a group of male diabetics with background diabetic retinopathy compared with a similar group having no retinopathy (97), but in a subsequent study blood viscosity was increased, especially at low shear rates in patients with proliferative retinopathy compared with those having minimal change or no retinopathy (99). Other investigators have confirmed an increase in low shear rate viscosity in patients with retinopathy although the grade of retinopathy was not always stated (107,109,111). In a prospective study, Barnes et al have found that abnormal blood rheology was predictive of deterioration in retinopathy over a three year follow-up period (144).

There has been little work on blood viscosity in diabetic nephropathy. Nevertheless, Simpson has suggested that increased viscosity in diabetes may cause an increased perfusion pressure in the glomerulus, thus leading to proteinuria (145). Hill et al, however showed that in diabetic children viscosity was not related to microalbuminuria (108) . Similarly it has been suggested that altered blood rheology could contribute to decreased flow in nerve capillaries (146) but there is little evidence to support this hypothesis.

2.5.2 *Red cell deformability*

Abnormal red cell deformability could in theory have a detrimental effect on diabetic retinopathy by reducing blood flow and contributing to hypoxia in the microcirculation. There have been conflicting reports of red cell deformability in association with microvascular complications ; several studies have found reduced deformability (103,109,113,114) and others no difference (96,108). Sewchand found no difference in red cell membrane

properties between healthy non-diabetics and diabetic patients with retinopathy (147). Lowe et al found no statistically significant difference in red cell filterability between diabetics with proliferative retinopathy and those with background or no retinopathy (99), although a trend to impaired filterability in the former group was apparent

2.5.3 *White cell deformability*

Leucocytosis and decreased white cell deformability in diabetic patients could contribute to impaired blood flow in the microcirculation, possibly promoting the increased microvascular complications seen in diabetes. Using the Carri-Med filtrometer and white cell suspensions, Vermes et al. found increased clogging of filters, presumably due to reduced white cell deformability, in diabetic patients with retinopathy compared to those with no retinopathy (132).

There have been few studies of white cell filtration in diabetic patients as a whole compared with non-diabetics.

2.5.4 *Red cell aggregation*

An increase in red cell aggregation would increase blood viscosity at low shear rates, which may promote blood stasis. This could induce local hypoxaemia and endothelial damage and in diabetic patients this could be yet another mechanism contributing to microvascular complications.

In Ditzel's study he found increased red cell aggregation in the microcirculation of diabetic patients known to have microvascular complications, though there was no difference between patients who had proliferative retinopathy alone and those who also had nephropathy (133) Increased red cell aggregation has been reported in diabetic patients with

retinopathy (136) but other studies have found no difference in aggregation between patients with retinopathy and those without, although the degree of retinopathy was not always specified (96,135)

As in studies of viscosity and red cell deformability in diabetic nephropathy, there is little published work on red cell aggregation in this subgroup of patients. Non-diabetic patients with nephrotic syndrome appear to have increased red cell aggregation which was not related to renal function, but which was related to increased fibrinogen levels (148). It is therefore likely that diabetic patients who have increased fibrinogen levels (101) will have a further increase in aggregation if they develop nephropathy.

2.6 Rheology and macrovascular complications in diabetes

In large vessel disease the narrowing of the lumen due to atherosclerosis will eventually impede flow, and since blood viscosity is inversely related to blood flow a rise in whole blood viscosity would therefore further reduce the blood supply to vital organs. In the capillaries, impaired red and white cell deformability may cause further ischaemia as may increased red cell aggregation in venules. Diabetic patients with abnormal rheology have therefore an additional risk factor for vascular complications. The increased plasma viscosity and impaired cellular deformability may be partly a secondary phenomenon due to acute and/or chronic phase responses induced by damage to vessels or tissues. Such responses are characterised by increased fibrinogen and serum protein levels and a leucocytosis, which will increase plasma and whole blood viscosity and reduce blood filterability thus exacerbating the degree of ischaemia.

2.6.1 *Cerebrovascular disease*

Increased haematocrit and blood viscosity have been associated with reduced cerebral blood flow, while venesection causes a fall in viscosity and increase in cerebral blood flow (149). Reduced red cell deformability has been described in stroke patients and may be an cause of ischaemic damage (150). In diabetic patients reduced vasodilatory reserve in the cerebral circulation (151) coupled with raised viscosity may partly expain why patients with diabetes mellitus have an increased risk of stroke. Rheological studies of diabetic patients with cerebrovascular disease are awaited.

2.6.2 *Ischaemic heart disease*

Studies of patients with ischaemic heart disease have found increased blood viscosity associated with a raised haematocrit (152) and raised fibrinogen levels (153), whereas others have shown no difference in the individual determinants of viscosity between patients with stable angina and those with no heart disease (154,155). In patients who have suffered a myocardial infarction, reduced blood filterability was found within the first 12 hours after infarction, along with increased plasma and whole blood viscosity (156). In patients with abnormal rheology this may contribute to extension of infarct size. Conversely, reduction of infarct size by thrombolytic therapy may reflect reductions in plasma fibrinogen, plasma viscosity and blood viscosity as well as lysis of coronary thrombi.

Diabetic patients with abnormal rheology again would be more susceptible to ischaemic damage with resultant poor ventricular function, and this may be relevant to the increased incidence of cardiac failure found in these

patients (157). There have been few studies looking at viscosity and deformability in diabetic patients with ischaemic heart disease. Activation of white cells causes a reduction in their deformability (158) and recently reduction in filterability of white cells due to activation has been demonstrated in post-myocardial infarct patients (159)

Hypertension is a major cause of both cerebrovascular and cardiovascular disease and the incidence of hypertension is increased in diabetic patients (89,160). Hypertension has also been associated with raised viscosity in non-diabetic patients (161) and could be one mechanism leading to increased vascular complications in these patients. Hypertension may reflect increased cardiac output against a raised viscosity which has increased the peripheral resistance.

2.6.3 *Peripheral vascular disease*

Peripheral vascular disease is a major problem in the elderly, especially smokers and diabetic patients. In the latter the problem is compounded by microvascular disease and by peripheral neuropathy. Any increase in viscosity, reduction in red or white cell deformability or increase in red cell aggregation could reduce the blood supply to tissues which are already ischaemic. Reduced filterability of whole blood has been shown in patients with peripheral vascular disease in association with a raised white cell count which is probably due to a chronic phase response (162). In diabetic patients better healing of amputations is found in the patients with the lowest pre-operative haemoglobin levels, suggesting a deleterious effect of haematocrit on blood flow (163). Whether therapeutic reduction of haematocrit increases healing of amputations in diabetics is not established.

2.7 Summary

Both plasma and whole blood viscosity are elevated in diabetic patients due mainly to increased plasma proteins causing increased plasma viscosity and increased red cell aggregation at low shear rates. Abnormalities of red and white cell deformability are more controversial and depend on the methodology used (164), but it does seem that there are cell membrane abnormalities associated with the diabetic state which could well increase cell rigidity.

The contribution of rheological abnormalities to specific diabetic complications requires to be established.

2.8 Scope of the present thesis

1. Using established techniques for measuring whole blood viscosity, it was aimed to assess whether or not abnormal viscosity in diabetes was related to complications not previously studied, i.e. hypertension and diabetic neuropathy.

2. It was aimed to evaluate the new Carri-Med filtrometer for the measurement of red cell deformability, and the Myrenne erythrocyte aggregometer for red cell aggregation, in diabetic patients compared with non-diabetic control subjects and to determine if any abnormalities detected were related to vascular complications.

3. With a recently developed method for separating white blood cell subpopulations and a modified filtration technique, it was aimed to measure white cell deformability in diabetic patients compared with non-diabetic control subjects.

CHAPTER 3

METHODOLOGY

3.1 Preparation of blood samples

The experimental work in this thesis as far as possible followed the guidelines of the International Committee for Standardisation in Haematology (ICSH) for blood sampling and handling (165,166).

Venous blood was used in all studies, this was collected in the morning after minimal venostasis from rested subjects using a large bore (to avoid high shear damage to cells), sterile needle. For all rheological procedures, routine haematology and glycated haemoglobin samples, 5 ml aliquots of blood were anticoagulated with dry potassium edetate (EDTA, 1.5mg/ml). This particular anticoagulant was used to minimise platelet aggregation, and because it has least effect on red cell morphology and plasma viscosity and avoids dilution of samples which is an additional variable with liquid anticoagulants (3). EDTA is recommended for anticoagulation in measurements of blood viscosity, red cell deformability and red cell aggregation (167,168)

Samples were analysed within 2 hours of venesection, and 2ml of plasma supernatant was used on the same day for analysis of plasma viscosity (3.3). An aliquot of citrated plasma was stored at -20°C for later analysis of plasma fibrinogen, and aliquots of serum were stored for serum protein measurements (3.9).

3.2 Haematocrit

Blood was drawn from the EDTA tube into duplicate 1mm diameter glass capillaries, and after the end was heat-sealed, they were centrifuged at 13000 xg for 5 minutes in a Hawksley Microcentrifuge (Gallenkamp, Glasgow). Microhaematocrit was read as the percentage red cell pack to

that of the whole sample, the reading used being the mean of two samples.

3.3 Plasma viscosity

Plasma viscosities were measured in the Coulter-Harkness semi-automatic capillary viscometer (Coulter Electronics Ltd., Harpenden, Hertfordshire). This instrument was chosen for ease of handling, accuracy, speed of measurement and small sample volume requirement (45).

After calibration using distilled water as the standard fluid, 0.5ml of plasma was pipetted into the sample cup at one end of the horizontal capillary. It is drawn through the capillary (0.38 mm in diameter) using a constant head of pressure applied by a mercury column. The time taken for the sample to travel is recorded by the mercury meniscus moving in a capillary parallel to the sample activating an electronic timer. The reading on the timer is made equivalent to the value of the sample's viscosity and is displayed on a register in mPa.s. The capillary system is immersed in a thermostatically controlled water bath at 37°C.

The mean of duplicate readings was taken. The coefficient of variation within samples was less than 1%.

3.4 Whole blood viscosity

3.4.1 *Contraves LS 30 rotational viscometer*

Low and high shear viscometry was performed in the Contraves LS 30 rotational viscometer (Contraves Industrial Products Ltd., Ruislip, Middlesex), which is a coaxial cylinder (couette) viscometer (48).

Measurements were made with the 2T bob and cup system on 2.5ml of whole blood. The shear rate is a function of the cup's geometry and rotational speed. Rotating the cup produces a torque by the sample on the bob. The bob is attached to a torsion wire which is linked to a mirror and tilting system and the torque transmitted from the sample on the bob is sensed in the reflected light source and monitored by a photo-electrical system. An equal and opposite torque is produced from a regulating current passing from a differential amplifier through electromagnetic coils concentric with a ferrite core attached to the torsion wire. The strength of the current is proportional to the torque from the sample and the sample's viscosity can be calculated from the following equation:

$$\text{Viscosity} = \frac{\text{shear stress}}{\text{shear rate}} = \frac{\text{torque x K}}{\text{rotational speed}}$$

(K = conversion factor determined by the system geometry and evaluated using standard Newtonian silicone oils).

A Rheoscan 20 programming unit was used allowing measurements over an accelerating shear rate ramp. This was used for the studies of diabetic patients with hypertension (chapter 4), while a constant shear rate measurement was used for the studies of diabetic patients with peripheral neuropathy (chapter 6).

3.4.2 *Experimental procedure*

The machine was calibrated with standard silicone oils (viscosities 7.6, 13.6, and 20.3mPa.s at 37°C). The sample was manually mixed using the bob for 5 seconds and the measurement procedure started. The constant

shear measurements were performed during the next 15 seconds with the sample at 37°C. Low shear viscosity was measured at 0.945 s^{-1} and high shear at 94.5 s^{-1}. High shear viscosity measurements were made first, since high shear mixing of the sample for 10 seconds is the most convenient way of breaking up red cell aggregates (169). The accelerating shear programme also measured high shear rate (94.5s^{-1}) viscosity first, then low shear rate (0.945s^{-1}) viscosity. Viscometry results were recorded as chart traces of torque reading as a function of time using a Bryans 27000 chart recorder and taking the peak value for constant shear measurements as recommended by the ICSH (166). Blood viscosity was measured at native haematocrit and a mathematical method of correction to a standard haematocrit of 45% (0.45) was used (7):

$$\frac{\text{Blood viscosity (Hct 0.45)}}{\text{Plasma viscosity}} = \left[\frac{\text{Blood viscosity (native Hct)}}{\text{Plasma viscosity}}\right]^{\frac{0.45}{\text{native Hct}}}$$

This equation is based on the linear relationship between the logarithm of relative blood viscosity and haematocrit (170). The coefficient of variation at a shear rate of 94.5s^{-1} was 2.5% and at 0.945s^{-1} was 2.2%. The contributions of red cell factors (deformation and aggregation) and plasma viscosity at the standard haematocrit were assessed by calculating the ratio of blood viscosity to the measured plasma viscosity, i.e. relative viscosity.

3.5 Red cell Deformability

3.5.1 *Carri-Med filtrometer*

Red cell deformability was measured using the St. George's filtrometer (Carri-Med Ltd., Dorking, UK), (Figure 3.1). This system utilises a vertically positioned filter, to minimise red cell sedimentation causing filter blocking. The red cells are drawn through the filter by a constant but adjustable negative pressure of water. The rate of filtration is measured by the times taken for the sample meniscus to flow past 4 light detectors and this information is stored on an interfacing BBC computer. Three subsequent steps of 20 μl sample volume flow are used, and the initial filtration rate is determined by extrapolating back to time zero. The clogging rate of the filter can also be determined. The initial filtration rate of red cells is independent of white cell clogging (52).

3.5.2 *Preparation of samples*

5 mls of EDTA-anticoagulated venous blood was centrifuged at 1500 xg for 10 minutes in a Mistral 4L centrifuge at ambient temperature. The plasma, buffy coat and upper 10% of red cells were discarded. Two ml from the middle of the red cell column were suspended in pre-filtered phosphate buffer saline (pH 7.4, 290 mOsm/kg) at a haematocrit of 10%. (Sensitivity to individual cell properties is optimal at haematocrits less than 15%).

3.5.3 *Filtration procedure*

Phosphate-buffer saline was filtered through a Nuclepore polycarbonate filter (pore diameter 5μm, Nuclepore Corporation, Pleasanton, CA, USA;

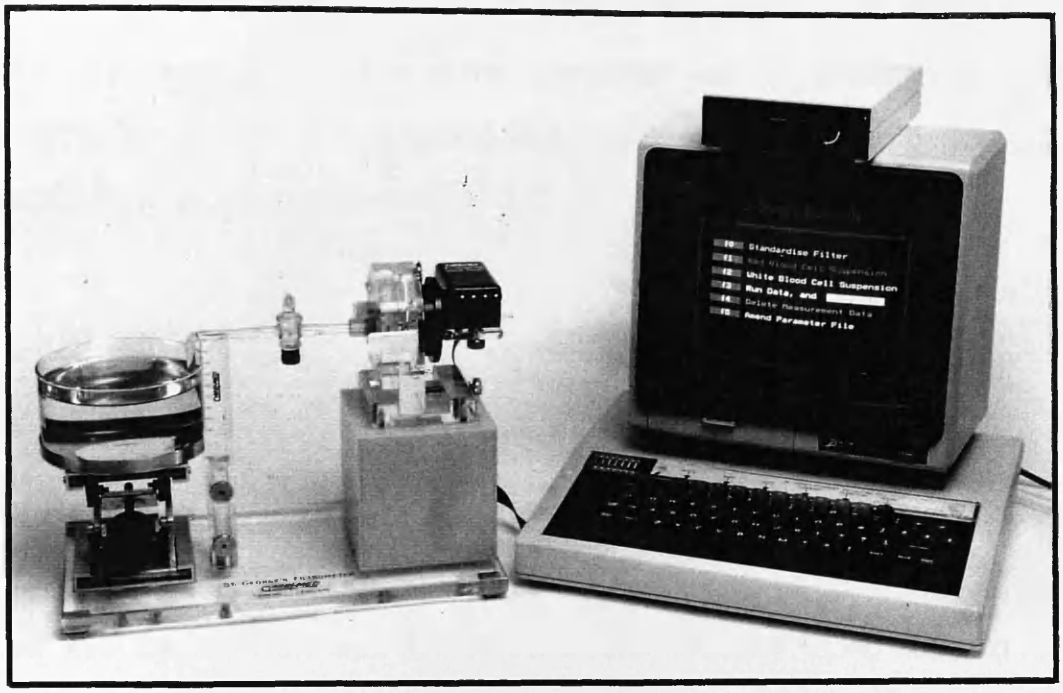


Figure 3.1 Carri-Med filtrometer

constant batch number 54B4D10) and the initial filtration rate recorded on the computer. The red cell suspension was then introduced to the capillary and the filtration rate measured through the same filter. A 3 cm negative filtration pressure was used throughout and studies were performed at 25°C. The initial filtration rate of the suspension was expressed in relation to the buffer filtration rate as a ratio (red cell filtration ratio). Within sample coefficient of variation was 4.5%

3.6 Red cell aggregation

3.6.1 *Myrenne aggregometer*

Red cell aggregation was assessed photometrically using the highly automated Myrenne cone-plate aggregometer (Myrenne GmbH, Roetgen, FRG). Measurement is based on the light transmitting properties of aggregating suspensions. The amount of light transmitted is dependent on the shear rate applied. At high shear rates increased light transmission occurs due to the deformation of cells with flow streamlines, and at low shear rates the increased light transmission with time is due to the cell-free gaps associated with rouleaux formation. (Figure 3.2)

The Myrenne aggregometer was developed from the work of Schmid-Schönbein et al (58) and consists of a transparent perspex cone and plastic plate, the latter being fixed in position and shearing of the sample achieved by rotation of the cone. Infrared light transmitted through the sample is measured by a photometer which produces photovoltages (mV) that are processed in a microprocessor unit incorporated within the machine.

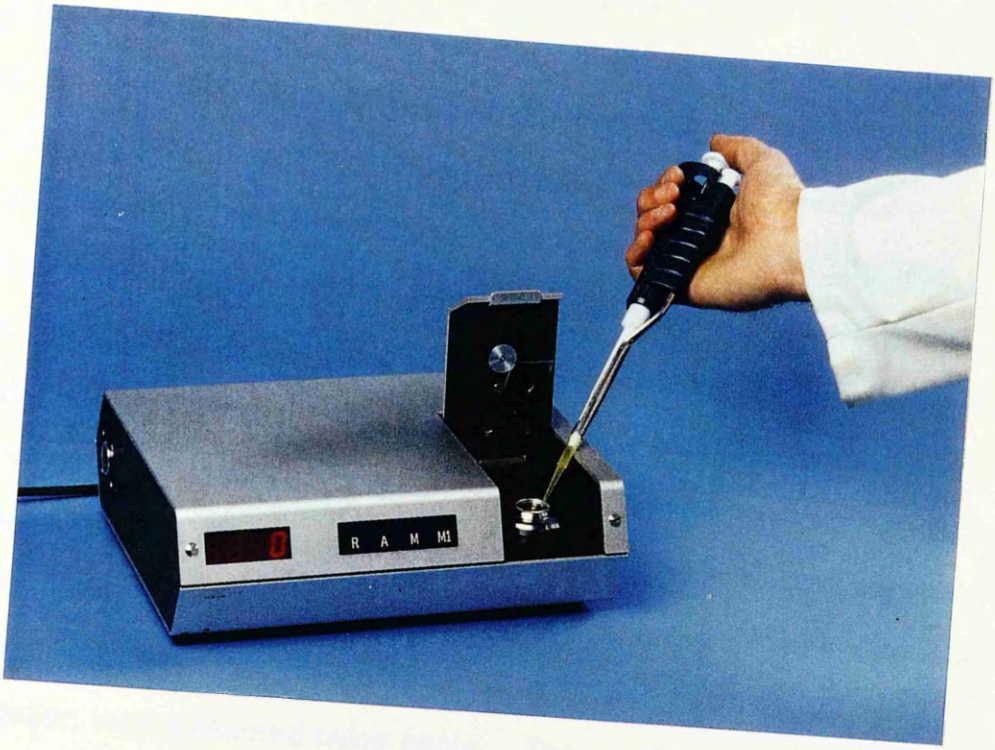


Figure 3.2 Myrenne red cell aggregometer

3.6.2 *Experimental procedure*

The intensity of the light source transmitted through the cleaned cone and plate of the Myrenne aggregometer was logged with the chamber closed and stored by pressing 'A' (adjust) key. At 25°C, 20 µl from an EDTA-anticoagulated blood sample was then pipetted onto the centre of the cone and the plate lowered into position by closing the cover. The sample was sheared at 600/s for 10 seconds to give a baseline reading and the increase in light transmission was then measured over a 5 second period of stasis (M mode) and integrated via the microprocessor unit and displayed digitally as the aggregation index. Aggregation was measured twice for each sample and the average of the duplicate readings taken as the mean aggregation index. The measuring system was cleaned between each new sample. Measurements were made first at native haematocrit, and after samples had been reconstituted to a standard haematocrit of 40% aggregation was measured twice again. The coefficient of variation was 5.2%. Aggregation was measured at standard haematocrit as well because aggregation is haematocrit dependent, increasing between haematocrits of 20-45% and decreasing at haematocrits above 40-45%.

3.7 White cell deformability

The methods used were those of Lennie et al (31).

3.7.1 *Preparation of buffer*

Phosphate buffer saline (PBS) was prepared from 42.5 g sodium chloride, 1 g potassium chloride, 1 g potassium dihydrogen orthophosphate and

5.75 g disodium hydrogen orthophosphate in distilled water at 4°C. pH was adjusted to 7.4 and osmolality to 290 mOsm/kg.

Before use the PBS was mixed with 0.5% w/v bovine serum albumin (BSA, Sigma, UK) and the solution filtered through a 0.45μm bacterial filter (Millipore, UK).

3.7.2 *Separation of white cells*

4 ml of mono-poly resolving medium was prepared from 15.454g sodium metrizoate and 8.182g Ficoll 400 mixed to 100mls with distilled water, giving a solution with a specific gravity 1.114 g/ml and an osmolality of 300 mOsm/kg.

1 ml of lymphoprep, a solution with a specific gravity of 1.114 g/ml and osmolality of 300 mOsm/kg, (Nyegaard, UK) was layered on top of the mono-poly resolving medium. Although mono-poly resolving medium separates mononuclear and polymorphonuclear cells in a one step procedure the resolution of cells was improved by modifying the separation technique with the addition of lymphoprep.

These solutions were placed at the bottom of a plastic tube and 5 ml of anticoagulated blood was layered on top of both. The mixture was centrifuged at 200 xg in a Mistral 4L centifuge for 40 minutes at ambient temperature.

After centrifugation the leucocyte subpopulations appear as two separate bands, the upper layer being the mononuclear cells (monocytes and lymphocytes) and the lower the polymorphonuclear cells (Figure 3.3) Each fraction was aspirated and added to 10 ml PBS/BSA and centrifuged at 200 xg for a further 10 minutes.

The supernatant was discarded and the cells resuspended in a known volume of PBS/BSA to a concentration of 10^5/l.

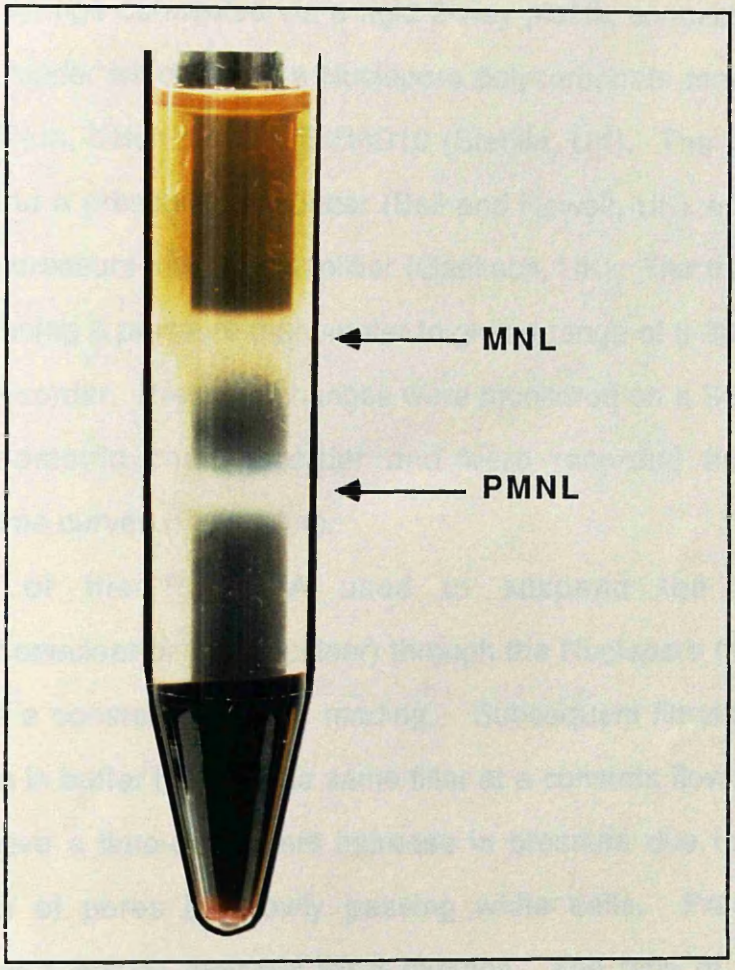


Figure 3.3

Separation technique for white blood cell subpopulations

3.7.3 *Filtration procedure*

The apparatus consisted of a syringe pump (Treonic,Vickers, UK) with a 60 ml plastic syringe connected via a rigid 3-way plastic connector to a "pop-top" filter holder which holds a Nuclepore polycarbonate membrane, pore diameter 5 μm, batch number 54B4D10 (Sterilin, UK). The filter holder is connected to a pressure transducer (Bell and Howell, UK) which in turn is linked to a pressure indicator/amplifier (Gaeltech, UK). The transducer was calibrated using a pressure manometer to give a range of 0-20 cm water on the chart recorder. Pressure changes were monitored on a Servoscribe RE 11 potentiometric chart recorder and were recorded as continuous pressure-time curves (Figure 3.4).

Filtration of the PBS/BSA used to suspend the white cells (polymorphonuclear or mononuclear) through the Nuclepore filter was used to produce a constant pressure reading. Subsequent filtration of the cell suspension in buffer through the same filter at a constant flow of 1.5 ml/min at 25°C gave a time-dependent increase in pressure due to progressive occupation of pores by slowly passing white cells. Pressures were analysed at 1 minute intervals for 6 minutes. The ratio of the pressure produced by the cell suspension to the pressure produced by buffer was calculated.

3.8 Plasma proteins

3.8.1 *Serum Globulins*

Total protein, albumin, and globulins (total protein minus albumin) were determined by SMAC on the Technicon Autoanalyser (Basingstoke), total

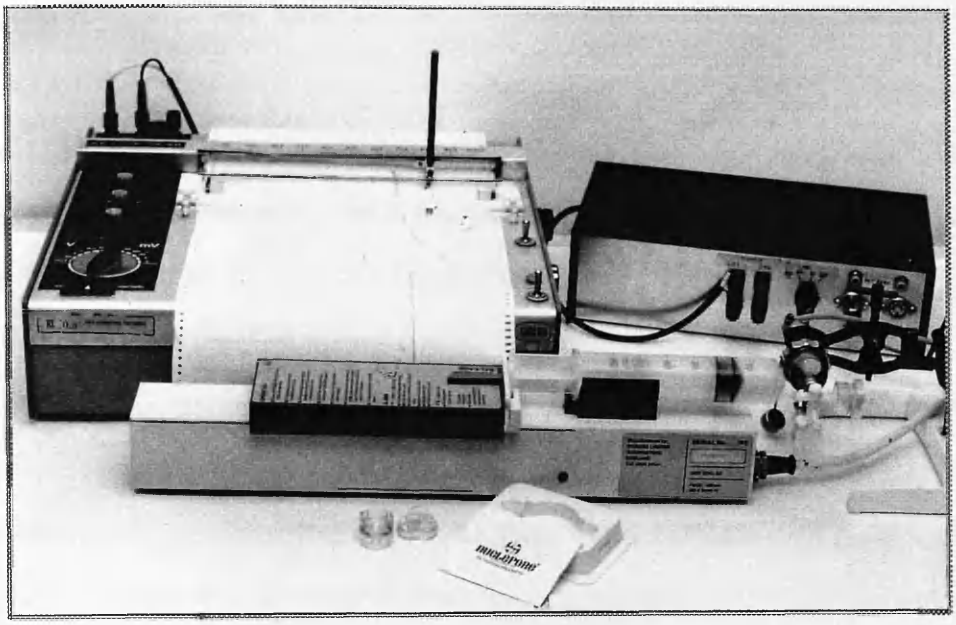


Figure 3.4 White cell filtration apparatus

protein was measured by the biuret method and albumin by the bromocresyl green method, in the hospital biochemistry department.

3.8.2 *Fibrinogen*

Fibrinogen was measured in a citrated plasma from a 5 ml citrated blood sample (4.5 ml blood = 0.5 ml 3.8% tritodium citrate) at 37° C in a Coag-a-mate X2 analyser (General Diagnostics, Morris Plains, NJ, USA) by the clotting time method of Clauss with a photo-optical system, during the viscosity and red cell deformability studies (chapters 4-6). Organon-Teknika reagents and standards were used.

The heat precipitation method (171) was used for estimation of plasma fibrinogen during the aggregation studies (chapter 7). Blood is drawn into microhaematocrit tubes, spun in the Hawksley centrifuge for 5 minutes, immersed in a waterbath at 57° C for 4 minutes and then centrifuged for a further 3 minutes in the Hawksley centrifuge. Using a X70 magnification on a microscope with a moving stage, the length of the fibrinogen precipitate was expressed as a ratio to the original plasma column (ml/100ml).

The mean of duplicate readings was taken.

3.8.3 *Alpha$_2$-macroglobulin and Haptoglobin*

These large plasma proteins were measured by an immunoturbidimetric assay on the Encore centrifugal analyser (Baker Instruments, Pennysylvania). Normal ranges were determined from the healthy population used in the study as described in chapter 7.

3.9 Blood cell indices

Haemoglobin concentration, mean red cell volume (MCV normal range 76-96 fl) and leucocyte count (WCC, normal range 4.0-11.0 x 10^9/l) were measured by Coulter -S -Counter. Mean cell haemoglobin concentration (MCHC) was estimated from haemoglobin concentration divided by the microhaematocrit measurement from the Hawksley centrifuge (g/dl).

3.10 Glycated haemoglobin and Blood glucose

Glycated haemoglobin ($HbA_{1)}$ was measured by agar gel electrophoresis (Corning Glytrac, Palo Alto, California), laboratory normal range 5.5-8.5%.

Blood glucose was measured by the hexokinase method (Hitachi 773 automatic analyser, Boehringer Mannheim, FRG). Normal laboratory fasting range 4.0-5.5 mmol/l

3.11 Plasma lipoproteins

Triglyceride, and total cholesterol, and very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) were measured after overnight density separation ultra-centrifugation of plasma at 35000xg (Beckman, Palo Alto, California) on a Hitachi 704 automatic analyser (Boehringer Mannheim, FRG).

CHAPTER 4

ASSOCIATION OF WHOLE BLOOD VISCOSITY WITH HYPERTENSION IN DIABETES MELLITUS

4.1 Introduction

Arterial blood pressure is determined by cardiac output and by total peripheral resistance, which is determined by both vessel geometry (vascular hindrance), and by blood viscosity In recent years the neglected contribution of raised viscosity to the pathogenesis of hypertension has been realised.

Blood viscosity has been found to be increased in hypertensive individuals (161,172) and this appears to be related partly to a raised haematocrit (161,173), which may be due to a contracted plasma volume from transcapillary shift of extracellular fluid from plasma caused by the increased arterial pressure (174). Loss of extracellular fluid will also cause a rise in plasma protein concentration, which would lead to both an increase in plasma viscosity and an increase in blood viscosity at low shear rates due to increased red cell aggregation. Plasma exchange, which reduces the level of high molecular weight plasma proteins, has been shown to reduce blood pressure (175). Similarly, reduction of haematocrit by venesection reduces blood pressure (176). One of the main plasma proteins contributing to blood viscosity is fibrinogen, and this has been shown to be independently related to the raised blood viscosity in hypertension (161), the increased fibrinogen perhaps being due to a "chronic phase response".
The other main determinant of blood viscosity at high shear rates is red cell deformability and this has also been shown to be reduced in hypertensive subjects (177.178).

Hypertension is common in diabetes mellitus (160,179), has been associated with both micro- and macrovascular disease (180-184), and is a

risk factor for cardiovascular disease (66,91). Because hypertension in people without diabetes has been associated with increased whole blood and plasma viscosity, and because diabetic patients have raised blood viscosity (185), it is possible that there is an additive effect of diabetes and hypertension on viscosity, leading to the increased risk of vascular complications related to hypertension in diabetic patients.

The aim of this study was to assess whole blood viscosity and its determinants in diabetic patients with hypertension, compared to a matched group of diabetic patients without hypertension and a healthy control population, to determine if blood rheology was altered in diabetic patients with hypertension.

4.2 Patients

4.2.1 *Diabetic patients*

86 diabetic out-patients were studied (38 type 1 and 48 type 2). The mean age was 49 years and the range was 18-74 years. There were 41 males and 45 females and mean body mass index was 26.1 ± 3.9 kg/m^2. The other clinical characteristics of the diabetic patients and control subjects are shown in Table 4.1.

Hypertension was defined as blood pressure > 160/95 mmHg on a conventional mercury sphygmomanometer on two occasions after sitting for 10 minutes, or accepted as diagnosed in patients receiving anti-hypertensive treatment. Because only 4 type 1 diabetic patients were hypertensive, these were excluded from further analysis and 34 normotensive type 1 diabetics were compared with 23 normotensive and

Table 4.1

Clinical characteristics and rheology of diabetic patients and normal subjects studied

	Control subjects	Diabetic Patients
n	52	86
Sex (M/F)	32/20	41/45
Smokers	24	37
Age (yr)	46.0 ± 5.0	49.0 ± 14.0
Body Mass Index (kg /m^2)	26.4 ± 4.0	26.1 ± 3.9
HbA_1 (%)	-	10.5 ± 2.3
Haematocrit (%)	44.8 ± 3.3	43.2 ± 4.5
Fibrinogen (gl /l)	3.2 ± 0.7	3.0 ± 0.8
Plasma viscosity (mPa.s)	1.32 ± 0.14	1.45 ± 0.15 [a]
Whole blood viscosity (mPa.s)		
95 s^{-1}	5.0 ± 0.6	5.1 ± 0.7
Corrected [c]	4.9 ± 0.6	5.3 ± 0.5 [a]
0.95 s^{-1}	17.5 ± 3.6	19.4 ± 4.8 [b]
Corrected [c]	17.4 ± 2.6	20.7 ± 2.7 [a]
Relative blood viscosity (mPa.s)		
95 s^{-1}	3.8 ± 0.4	3.8 ± 0.3 [a]
0.95 s^{-1}	13.5 ± 1.9	14.8 ± 1.8

[a] $p < 0.05$, diabetic patients vs control subjects
[b] $p < 0.01$, diabetic patients vs control subjects
[c] Corrected to a standard haematocrit of 45%

25 hypertensive type 2 diabetic patients. 18 patients were on anti-hypertensive medication which included a combination of diuretics, beta-blocking and calcium channel blocking drugs. All patients had normal urea and electrolyte concentrations and except for anti-hypertensive and diabetic medication were receiving no other drug therapy. 13 type 2 normotensive diabetics were on insulin therapy and 10 were taking oral hypoglycaemic agents. In the hypertensive group, 4 were receiving insulin therapy while 11 were taking oral hypoglycaemic drugs and 10 were on dietary therapy only, Table 4.2.

Duration of diabetes was significantly longer in the type 1 diabetics (18 ± 11 years) compared with type 2 diabetics (10 ± 6 years-normotensive and 6 ± 5 years -hypertensive, $p<0.02$). The groups were well matched for glycaemic control (HbA1; 11.1 ± 2.1%-type 1, 10.6 ± 2.6%-type 2 normotensive, 10.6 ± 2.3%-hypertensive, $p>0.05$) and the type 2 diabetics were well matched for sex distribution (10 male/13 female-normotensive and 12 male/13 female-hypertensive), age (58 ± 7 years-normotensive and 58 ± 7 years-hypertensive) and body mass index (26.4 ± 3.9 kg/m^2-normotensive and 27.2 ± 3.9 kg/m^2-hypertensive), (Table 4.3). The vascular complications of all the diabetic patients are shown in Table 4.2.

4.2.2 *Control Subjects*

52 healthy normotensive control subjects were selected from factory employees. They had a mean age of 46 years and range 36-57 years. There were 32 males and 20 females and they were well matched with the diabetic patients for body mass index (26.4 ± 4.0 vs 26.1 ± 3.9 kg/m^2, $p>0.05$), Table 4.1. None were taking any medication and all had normal fasting blood glucose levels.

Table 4.2

Mode of treatment of diabetes, and complications present in diabetic patients studied

	Type 1 diabetes normotensive	Type 2 diabetes normotensive	Type 2 diabetes hypertensive
n	34	23	25
Duration of diabetes (yr)	18±11[b]	10±6[a]	6±5
Treatment of diabetes			
Insulin	34	13	4
Oral hypoglyceamic agents	0	10	11
Diet only	0	0	10
Treatment of hypertension	0	0	18
Diabetic complications			
Background retinopathy	12	10	6
Proliferative retinopathy	3	1	2
Nephropathy	1	2	0
Neuropathy	6	4	0
Macrovascular disease	2	5	10

[a] $p < 0.02$, Type 1 vs Type 2 diabetic patients.
[b] $p < 0.01$, Type 2 normotensive vs Type 2 hypertensive diabetic patients.

Table 4.3

Clinical characteristics of diabetic and control subjects studied

	Control subjects	Diabetic patients		
		Type 1 normotensive	Type 2 normotensive	Type 2 hypertensive
n	52	34	23	25
Sex (M/F)	32/20	17/17	10/13	12/13
Smokers	24	18	11	6
Age (yr)	46.0±5.0	37.0±12.0[a]	58.0±7.0[a,b]	58.0±7.0[a,b]
BMI (kg /m^2)	26.4±4.0	25.2± 4.0	26.4±3.9	27.2±3.9
HbA_1 (%)	-	11.1± 2.1	10.6±2.6	10.6±2.3
BP (mmHg)	128±19/80±10	127±18/82±6	130±13/83±8	177±33[a,d] /97±15[a,c]

[a] $p < 0.01$, diabetic patients vs control subjects.
[b] $p < 0.05$, Type 2 vs Type 1 diabetic patients.
[c] $p < 0.05$, hypertensive vs normotensive diabetic patients.
[d] $p < 0.01$, hypertensive vs normotensive diabetic patients.

4.3 Methods

4.3.1 *Blood samples*

Whole blood viscosity (shear rates 94.5 and 0.945 s^{-1}) and plasma viscosity were measured at 37°C in blood anticoagulated with EDTA within 2 hours of venesection. Whole blood viscosity was measured in a Contraves LS rotational viscometer, and plasma viscosity in a Coulter-Harkness capillary viscometer. Microhaematocrit was measured on the Hawksley microcentrifuge and blood viscosity was corrected to standard microhaematocrit of 45%. Relative blood viscosity was calculated as whole blood viscosity divided by plasma viscosity.

Fibrinogen was measured from a citrated sample at 37°C on the Coag-a-Mate X2 analyser by the clotting time method.

4.3.2 *Statistical methods*

The difference in means was calculated by the Mann-Whitney U-test and correlation by the Spearmann rank test. Values are expressed as mean ± SD.

4.4 Results

Whole blood viscosity (corrected for haematocrit) at high and low shear rates, low shear relative viscosity and plasma viscosity were significantly higher in diabetic patients (5.3 ± 0.5, 20.7 ± 2.7, 14.8 ± 1.8 and 1.45 ± 0.15 mPa.s) compared to control subjects (4.9 ± 0.6, 17.4 ± 2.6, 13.5 ± 1.9 and 1.32 ± 0.14 mPa.s, $p<0.01$). Low shear rate viscosity at native haematocrit

Table 4.4

Blood viscosity and its determinants in diabetic and control subjects

	Control subjects	Diabetic patients		
		Type 1 normotensive	Type 2 normotensive	Type 2 hypertensive
Haematocrit (%)	44.8±3.3	43.8±4.0	43.4±4.4	42.8±4.4
Fibrinogen (g/l)	3.2±0.7	3.2±0.9	3.4±0.8	3.4±0.6
Plasma viscosity (mPa.s)	1.32±0.14	1.43±0.15	1.45±0.20	1.45±0.15
Whole blood viscosity (mPa.s)				
95 s^{-1}	5.0±0.7	5.0±0.7[b]	5.1±0.7[b]	5.2±0.8[b,d,e]
Corrected[f]	4.9±0.6	5.1±0.5[a]	5.2±0.3[a]	5.5±0.4[b]
0.95 s^{-1}	17.6±3.6	19.3±4.2[b]	20.1±4.9[b]	20.5±5.0[b,d]
Corrected[f]	17.4±2.6	19.8±2.9	21.1±2.0	21.9±2.4
Relative blood viscosity (mPa.s)				
95 s^{-1}	3.8±0.4	3.8±0.4	3.7±0.4	3.8±0.2[b,c]
0.95 s^{-1}	13.5±1.9	14.5±2.1	15.0±1.5	15.2±1.5

[a] $p < 0.02$, diabetic patients vs control subjects.
[b] $p < 0.01$, diabetic patients vs control subjects.
[c] $p < 0.05$, Type 2 hypertensive diabetic patient vs Type 1 normotensive.
[d] $p < 0.01$, Type 2 hypertensive diabetic patient vs Type 1 normotensive.
[e] $p < 0.01$, Type 2 hypertensive diabetic patient vs Type 2 normotensive.
[f] Corrected to a standard heamatocrit of 45%

was also significantly higher in diabetic parients compared with control subjects (19.5 ± 4.9 vs 17.6 ± 3.6 mPa.s, $p<0.05$), Table 4.3. There was no significant difference in viscosity or its determinants between type 1 or type 2 normotensive diabetic patients (Table 4.4). Compared with the control group high shear corrected viscosity was 7.1% higher in the normotensive type 2 diabetics (5.2 ± 0.3 vs 4.9 ± 0.6 mPa.s, $p<0.01$) and 12.6% higher in the hypertensive diabetics (5.5 ± 0.4 vs 4.9 ± 0.6 mPa.s, $p<0.01$) and was significantly higher in the hypertensive than in the normotensive diabetics (5.5 ± 0.4 vs 5.2 ± 0.3 mPa.s, $p<0.01$), Table 4.5. There was no difference in high shear rate blood viscosity between hypertensive diabetic patients on treatment (5.5 ± 0.5 mPa.s) and those on no treatment (5.5 ± 0.4 mPa.s).

There was a positive correlation of fibrinogen levels with both corrected high and low shear rate viscosity measurements (high shear, $r=0.37$; low shear, $r=0.49$, $p<0.001$) and of blood pressure with corrected low shear rate viscosity (systolic, $r=0.25$; diastolic $r=0.35$, $p<0.05$). Although no correlation of viscosity was found with HbA1 (high shear, $r=0.05$; low shear, $r=0.18$, $p>0.05$) there was a weak significant inverse correlation of corrected whole blood viscosity with duration of diabetes (high shear, $r= -0.35$, $p<0.01$; low shear, $r=-0.24$, $p<0.05$), (Table 4.5).

Corrected whole blood viscosity at both high and low shear rates was significantly higher in type 1 diabetic patients with retinopathy (background or proliferative retinopathy), (5.4 ± 0.2 mPa.s-high shear, 21.2 ± 1.9 mPa.s-low shear) than in those without (5.0 ± 0.5 mPa.s-high shear, 18.8 ± 3.2 mPa.s-low shear, $p<0.02$), but no difference was observed in type 2 patients (5.4 ± 0.4 vs 5.3 ± 0.4 mPa.s-high shear, 21.8 ± 2.3 vs 21.1 ± 2.1 mPa.s-low shear, $p>0.05$), (Table 4.6).

Table 4.5

Correlation of whole blood viscosity with variables in diabetic patients

	High shear * WBV (r)	Low shear * WBV (r)
Age (yr)	-0.12	-0.01
Blood mass index (kg/m^2)	0.26	-0.09
Duration of diabetes (yr)	-0.35 [b]	-0.24 [c]
Blood pressure - systolic (mmHg)	-0.07	0.25 [c]
Blood pressure - diastolic (mmHg)	-0.07	0.35 [b]
HbA_1 (%)	0.05	0.18
Haematocrit (%)	0.12	0.01
Fibrinogen (g/l)	0.37 [a]	0.49 [a]

* Corrected to haematocrit 45%

a $p < 0.001$

b $p < 0.01$

c $p < 0.05$

Table 4.6

Blood and plasma viscosity in diabetic patients with and without microvascular disease

	Type 1 diabetic patients Microvascular disease		Type 2 diabetic patients Microvascular disease	
	+	-	+	-
n	15	19	29	19
Plasma viscosity (mPa.s)	1.44±0.13	1.42±0.14	1.52±0.23	1.44±0.15
Whole blood viscosity (mPa. s)				
95 s^{-1}	5.3±0.7	4.9±0.6	5.3±0.7	4.9±0.7
Corrected[b]	5.4±0.2[a]	5.0±0.5	5.4±0.4	5.3±0.4
0.95 s^{-1}	20.0±5.3	18.2±3.5	21.3±5.1	18.5±4.5
Corrected[b]	21.2±1.9[a]	18.8±3.2	21.8±2.3	21.1±2.1

[a] $p < 0.02$, Type 1 diabetic patients with microvascular disease vs without.
[b] Corrected to a standard haematocrit of 45%
+ present
- absent

Fibrinogen levels were similar in diabetic and control groups (3.4 ± 0.8 vs 3.2 ± 0.9 g/l, p>0.05). There was no significant difference in fibrinogen between smokers and non-smokers in either the diabetic (3.5 ± 1.0 vs 3.1 ± 0.6 g/l) or the control groups (3.3 ± 0.8 vs 3.2 ± 0.7 g/l).

4.5 Discussion

The present study has not only confirmed the general findings of previous studies (see chapter 1) that blood viscosity and plasma viscosity are elevated in diabetes, but in addition has demonstrated for the first time that the presence of hypertension in diabetes is associated with a further increase in whole blood viscosity at a standard haematocrit, which in hypertensive diabetic patients is over 12% higher than in non-diabetic control subjects. The cause of the increased viscosity is not certain, but it appears to be due to the combined effects of an increased plasma viscosity (although this study was not powerful enough to show a significant difference between hypertensive and normotensive diabetic patients) and a decrease in erythrocyte deformability which is the remaining determinant of high shear viscosity.

Since this study was performed Ramping et al have confirmed higher blood viscosity in hypertensive diabetics (186)

The raised blood viscosity in diabetic patients was associated with a normal fibrinogen level, and although a raised fibrinogen has been suggested to be one cause of the increased viscosity in diabetics, other studies have found normal fibrinogen levels in diabetic patients (187). The higher plasma viscosity in the diabetic patients compared with controls probably reflects an increase in plasma proteins other than fibrinogen, as reviewed

in chapter 1.

It may be argued that the raised blood viscosity is the result of hypertension i.e. the combined effects of raised arterial pressure being an increased haematocrit and increased plasma proteins from transcapillary shift of extracellular fluid. This rise in blood viscosity could lead to an increase in cardiac load and hence promote left ventricular hypertrophy (188). However there is some evidence that increased blood viscosity is implicated in the pathogenesis of hypertension. Chien has shown that mild hypertension and increased blood viscosity are associated with a fall in vascular hindrance i.e. vasodilatation, while severe hypertension is associated with normal vascular hindrance i.e. loss of compensatory vasodilatation (189). This would tend to suggest that the increase in vascular resistance parallels the increase in blood viscosity.

An increase in viscosity was found in type 1 diabetic patients with microvascular complications as in previous studies (95,99,107,101,146). Thus the increased viscosity at high shear rates in hypertensive diabetics may be one mechanism by which diabetic patients with hypertension develop more complications.

4.6 Summary

Whole blood viscosity measured at high shear rate and corrected to a standard haematocrit of 45%, was increased in type 2 hypertensive diabetics. Whole blood viscosity at both high and low shear rates (at standard haematocrit) was increased in type 1 diabetics with microvascular complications.

Prospective studies of the predictive value of blood viscosity for diabetic complications, both microvascular and macrovascular, are required to test the hypothesis that hypertensive diabetics develop more complications than normotensive diabetics, related to greater abnormalities in blood rheology. It would also be of interest to evaluate blood viscosity in hypertensive type 1 diabetic patients.

CHAPTER 5

ASSESSMENT OF RED CELL DEFORMABILITY IN NORMALS AND DIABETICS USING AN IMPROVED FILTRATION METHOD

5.1 Introduction

Impaired red cell deformability (filterability) has been suggested in studies of vascular disease (150,156), sickle cell disease (19), other haemoglobinopathies (190) and diabetes mellitus (185). Such changes may reflect alterations in the red cell membrane, shape or geometry, or an increase in the internal viscosity of the cell (191).

Reduced red cell deformability might impede blood flow in the abnormal microcirculation of diabetic patients, promoting tissue hypoxia, and hence could contribute to the increased incidence of microvascular disease in diabetes.

Previous studies assessing red cell deformability in diabetic patients have given conflicting results, depending partly on the methodologies employed. Filtration techniques in which the red cells are passed through micropore filters with pores of diameter 3-5μm, have found impaired deformability associated with diabetes in some studies, but not all (185). Reduced whole blood filterability has been related to the increased white cell count in diabetic patients, and clogging of the filter by less deformable white cells gives non-specific results in filtration studies (103). As discussed in chapters 1-3, the St. George's filtrometer (Carri-Med) measures the initial filtration rate of red blood cells, independently of filter clogging by rigid red cells.

It was aimed to measure red cell deformability by an improved filtration method (Carri-Med filtrometer) (52), firstly in a group of healthy non-diabetic individuals to determine which red cell properties and which biochemical constituents of the blood, if any, influence red cell filtration rates.

In then comparing groups of diabetic patients and non-diabetic healthy individuals, I aimed to determine if red cell deformability, as suggested by some previous studies, was impaired in diabetic patients. I also wished to evaluate the associations of red cell deformability with type and duration of diabetes and with glycaemic control, and aimed to determine if abnormalities of deformability were related to the extent of vascular disease present in diabetic patients.

5.2 Subjects

5.2.1 *Healthy volunteers*

A fasting blood sample was taken from 66 healthy factory employees (38 male, 28 female), mean age 47 years-male, 46 years-female, range 24-57 years. All were normotensive, matched for body mass index (26.1 ± 2.2-male, 26.9 ± 5.9-female), (Table 5.1), had normal fasting blood glucose, urea and electrolyte concentrations, and were taking no medications.

5.2.2 *Diabetic patients*

69 patients (28 type 1-insulin dependent, 41 type 2-non-insulin-dependent) were recruited from the diabetic outpatient clinic. Mean age was 38 years-type 1, 58 years-type2 ($p<0.001$), range was 18 -70 years. They were matched for ratio of males to females (15 M/13 F-type 1 and 22 M/19 F-type 2), smokers to non-smokers (14/14-type 1 and 17/24-type 2), body mass index (25.8 ± 4.2kg/m^2-type 1 and 26.6 ± 3.7kg/m^2-type 2), and glycaemic control (HbA1; 11.3 ± 2.3%-type 1 and 10.6 ± 2.6%-type 2),
Table 5.2.

Table 5.1

Characteristics of the non - diabetic group, filtration ratios, red cell indices and plasma constituents

	Males (n = 38)	Females (n = 28)
Age (yr)	47 ± 5	46 ± 4
Body Mass Index (Kg/m^2)	26.1 ± 2.2	26.9 ± 5.9
Smokers (n)	13	13
* RCF ratio	0.545 ± 0.04	0.543 ± 0.02
Mean cell volume (fl)	91.6 ± 5.2	93.7 ± 6.2
Mean cell haemoglobin concentration (g/dl)	32.4 ± 1.4	31.3 ± 1.0
Microhaematocrit (%)	45.8 ± 3.2[a]	43.1 ± 3.1
White cell count ($x10^9/l$)	6.4 ± 1.7[b]	7.6 ± 1.7
Globulins (g/l)	25.4 ± 2.9	25.0 ± 3.9
Fibrinogen (g/l)	3.2 ± 0.8	3.2 ± 0.6
Osmolality (mmol/l)	288 ± 3	284 ± 4
Triglyceride (mmol/l)	2.0 ± 1.1[a]	1.3 ± 0.6
Cholesterol (mmol/l)	6.2 ± 1.1	5.9 ± 1.3

[a] $p < 0.01$
[b] $p < 0.05$
] Male volunteers v Female volunteers

* RCF = Red Cell Filtration

Table 5.2

Characteristics of the diabetic and control groups

	Control subjects	Diabetic patients type 1	type 2
n	66	28	41
Age (yr)	44±8	38±12	58±7 [a]
Sex (M/F)	38 / 28	15 / 13	22 / 19
Smokers (Y/N)	26 / 40	14 / 14	17 / 24
BMI (Kg/m^2)	26.2±4.0	25.8±4.2	26.6±3.7
Duration of diabetes (yr)	-	17±11	8±6 [a]
HbA_1 (%)	-	11.3±2.3	10.6±2.6

[a] $p < 0.001$, type 2 v type 1 diabetics or control subjects

Patients were divided into three groups according to vascular complications: no complications, microvascular complications only, or both micro and macrovascular complications.

Microvascular disease was defined as the presence of retinopathy on ophthalmology (performed by myself through dilated pupils), background retinopathy being the presence of microaneurysms, "dot and blot" haemorrhages and hard exudates and/or nephropathy which was defined as persistant proteinuria (albustix positive) in a longstanding diabetic patient. Macrovascular disease was accepted as present in patients with documented evidence of ischaemic heart disease, cerebrovascular disease or peripheral vascular disease.

5.3 Methods

5.3.1 *Blood samples*

The blood was anticoagulated with EDTA and analysed within 2 hours of venesection. Red cell deformability was measured from a 5 ml sample by bulk red cell filtration on the Carri-Med filtrometer as described (chapter 3.6). Samples were analysed for mean cell volume (MCV, normal range 76-96 fl) and white cell count (WCC, normal range 4.0-7.5 $x10^9/l$) on the Coulter-S-Counter (3.10). Mean cell haemoglobin concentration (MCHC) was estimated from the haemoglobin concentration (measured on the Coulter-S-Counter) divided by the microhaematocrit (measured on the Hawksley microcentrifuge, chapter 3.2).

Serum triglyceride and cholesterol concentrations were measured by automated enzymatic colourimetric analysis (Hitachi 704, chapter 3.11), and plasma fibrinogen by the clotting time (Clauss) method (chapter 3.8.2).

Serum osmolality and globulins were measured by SMAC (Technicon, Basingstoke, chapter 3.8.1).

5.3.2 *Statistical methods*

Parametric analysis was utilised when the control subjects were examined independently from the diabetic patients i.e. the Student's independent t-test was used to compare difference in the means, and correlation coefficients were by the least squares method.

Non-parametric analysis was used when diabetic and control subjects were compared and for analysis within the diabetic group alone, since it could not be assumed that the variables examined were normally distributed in diabetic patients. Statistical analysis was by the Mann-Whitney U-test and Spearman rank correlation. Values throughout are expressed as mean ± SD.

5.4 Results

5.4.1 *Healthy volunteers*

In male volunteers compared with female volunteers, triglyceride level and microhaematocrit were significantly higher (2.0 ± 1.1mmol/l vs 1.3 ± 0.6mmol/l, $p<0.01$ and 45.8 ± 3.2% vs 43.1 ± 3.1%, $p<0.01$) whereas white cell count was significantly lower (6.4 ± 1.7 $x10^9$/l vs 7.6 ± 1.7 $x10^9$/l, $p<0.05$, Table 5.1)

There was no difference in red cell filtration ratio between males and females (0.545 ± 0.04 vs 0.543 ± 0.02) nor between smokers and non-smokers (0.545 ±0.03 vs 0.544 ± 0.03).

A weak significant inverse correlation of red cell filtration ratio with mean cell volume was found ($r = -0.28$, $p<0.05$, Figure 5.1), but no correlations were found with age, body mass index, serum lipids, osmolality, plasma fibrinogen, globulins, microhaematocrit, mean cell haemoglobin concentration or white cell count, (Table 5.3).

5.4.2 *Diabetic patients and control subjects*

The diabetic and control groups were well matched for body mass index, and ratio of males to females and ratio of smokers to non-smokers. Type 2 diabetic patients were significantly older than type 1 diabetic patients and control subjects, although the age range of control subjects matched the complete range in the diabetic patients (Table 5.3).

5.4.2 (i) Effect of type of diabetes.

Red cell filtration ratio was significantly decreased in both type 1 (0.470 ± 0.05) and type 2 (0.488 ± 0.06) diabetic patients compared to control subjects (0.549 ± 0.03, $p<0.001$) and in type 1 compared to type 2 diabetics ($p<0.03$), (Figure 5.2).

5.4.2 (ii) Relationship between red cell filtration ratio and red cell and white cell indices.

The difference in filtration ratio was partly related to a higher mean cell haemoglobin concentration in the diabetic patients, which in turn was related to a significantly lower mean cell volume in the diabetic patients compared with the control subjects, (Table 5.4). There was no difference in mean cell haemoglobin concentration or mean cell volume between type 1 or type 2 diabetic patients.

Red cell filtration ratio was inversely related to mean cell haemoglobin

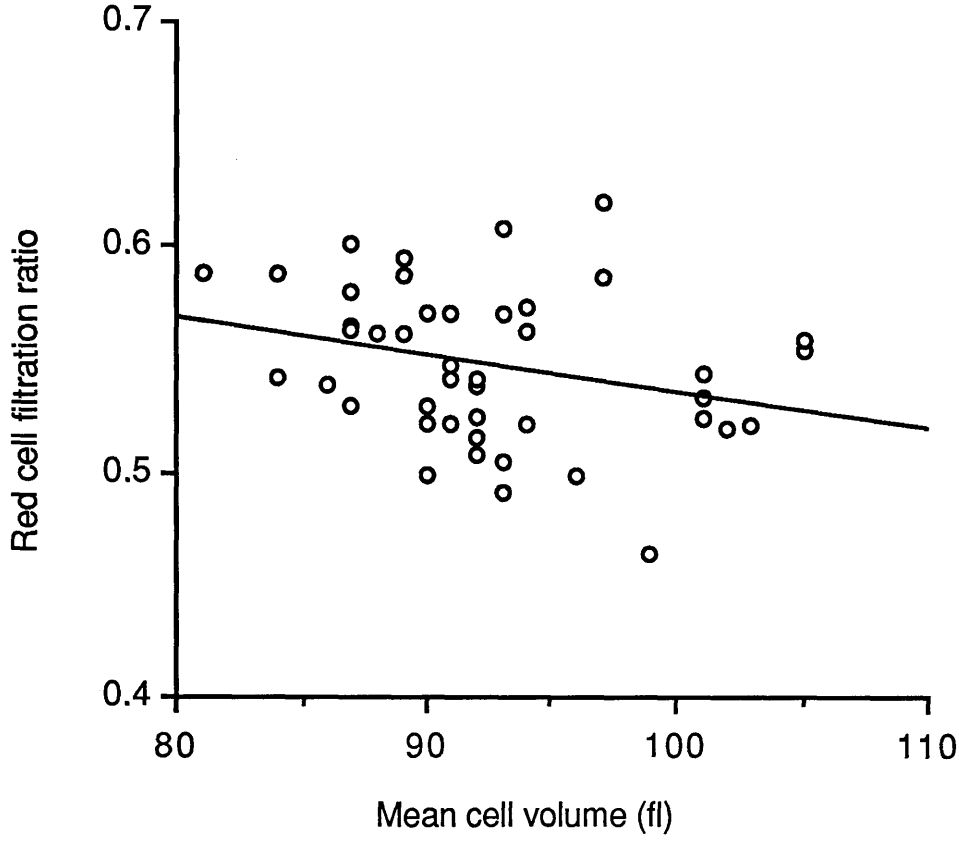


Figure 5.1

Correlation of red cell filtration ratio with mean cell volume in normal individuals

Table 5.3

Red Cell Filtration Ratio in Normal Indivduals: Correlation with variables

	r_s
Age (yr)	0.13
Body Mass Index (kg/m^2)	-0.16
Triglyceride (mmol/l)	-0.17
Cholesterol (mmol/l)	0.06
Haematocrit (%)	0.02
White blood cell count ($x10^9/l$)	0.08
Mean cell volume (fl)	-0.28 [a]
Mean cell haemoglobin concentration (g/dl)	-0.01
Plasma viscosity	-0.17
Fibrinogen (g/l)	0.17
Globulin (g/l)	-0.08

[a] $p < 0.05$

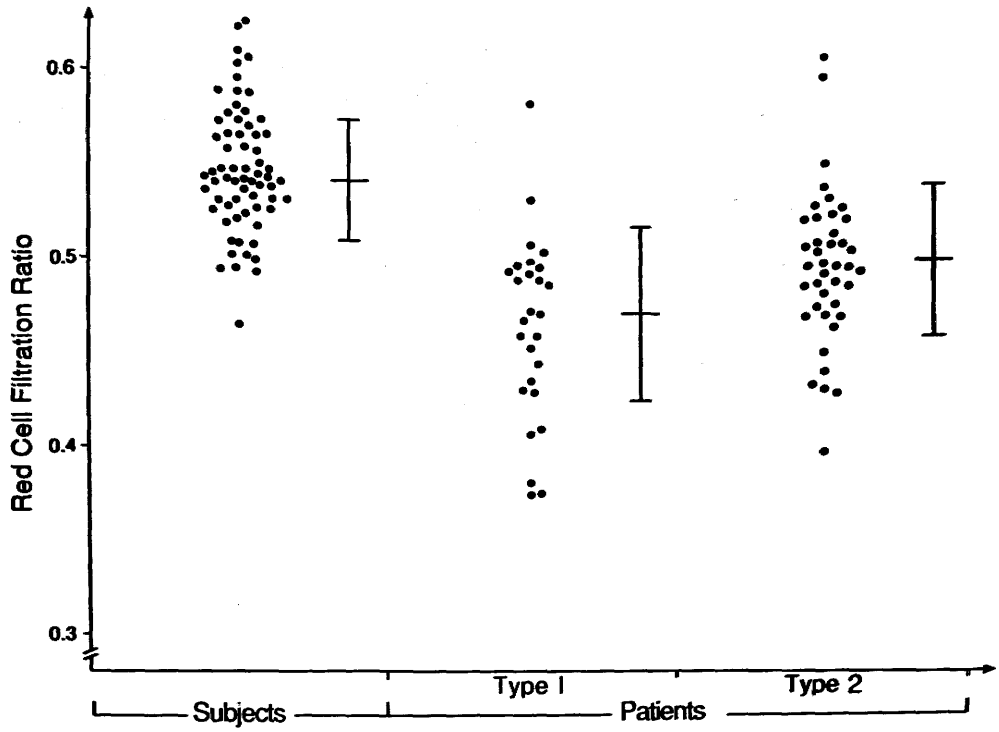


Figure 5.2

Red cell filtration ratios in diabetic and control subjects

Table 5.4

Haematological and biochemical variables in diabetic patients and control subjects

	Control subjects	Diabetic patients type 1	Diabetic patients type 2
White cell count ($x10^9$/l)	6.9±1.8	7.4±1.5	7.3±1.7
Mean cell volume (fl)	92±6	89±4[a]	88±5[a]
Mean cell haemoglobin concentrations (g/dl)	31.9±1.4	33.0±2.6[b]	33.6±1.7[b]
Microhaematocrit (%)	44.7±3.4	43.4±4.8	43.5±4.7
Fibrinogen (g/l)	3.2±0.7	3.3±0.9	3.4±0.7
Globulin (g/l)	25.2±3.3	25.5±3.4	26.3±3.6

[a] $p < 0.001$
[b] $p < 0.0003$
] diabetic patients v control subjects

concentration (r= - 0.43, p<0.01, Figure 5.3) but no correlations were found with white cell count, haematocrit, plasma fibrinogen or globulin level (Table 5.5).

No association of red cell filtration ratio with glycaemic control was found as measured by glycated haemoglobin (r = 0.07) nor with duration of diabetes (r = 0.08).

To assess whether the higher mean cell haemoglobin concentration in the diabetic patients could account for their lower deformability, red cell fitration ratios in diabetics and controls with similar mean cell haemoglobin concentrations were compared: the filtration ratio was still significantly lower in the diabetic patients (0.494 ± 0.05) compared with the control subjects (0.553 ± 0.38, p<0.001).

5.4.2 (iii) Effect of vascular disease.

Diabetic patients with microvascular disease had significantly longer duration of disease than either patients with no complications or patients with both micro and macrovascular disease, although the ratio of type 1 and type 2 diabetics was similar in this group.

Red cell filtration ratio was similar in all three groups (0.487 ± 0.05-no complications, 0.478 ± 0.04-microvascular complications, 0.495 ± 0.04-microvascular + macrovascular complications) and no differences in the determinants of red cell deformability were found between the three groups, (Table 5.6)

5.6 Discussion

Deformability of the red cell was measured by a filtration method in which cells traverse filters with 5μm diameter pores, and from which the initial

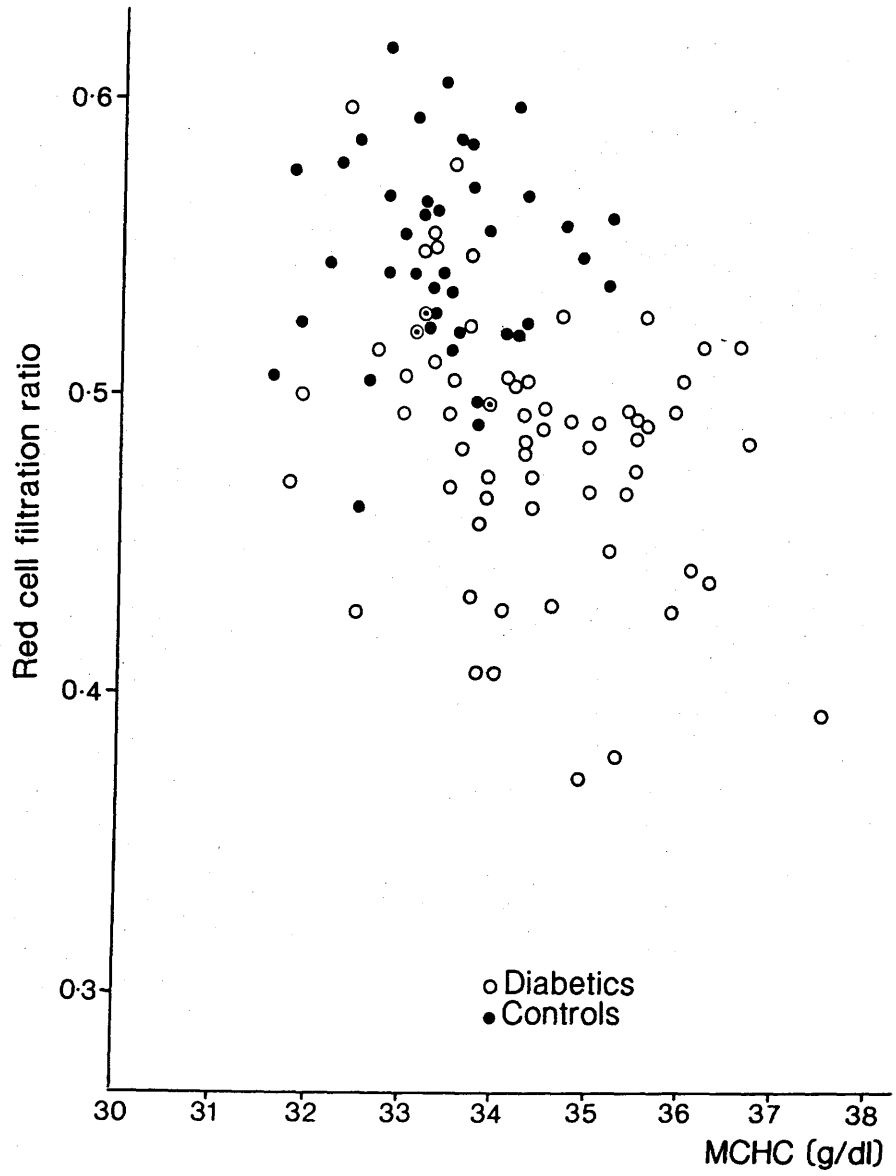


Figure 5.3

Correlation of red cell filtration ratio with mean cell haemoglobin concentration in diabetic and control subjects

Table 5.5

Red cell filtration ratio in diabetics: Correlation with variables

	r_s
Age (yr)	0.20
Body Mass Index (kg/m^2)	0.08
Duration of diabetes (yr)	0.08
Cholesterol (mmol/l)	0.07
Haematocrit (%)	-0.08
White blood cell count ($x10^9/l$)	-0.04
Mean cell volume (fl)	0.02
Mean cell haemoglobin concentration (g/dl)	-0.30[a]
Plasma viscosity	
Fibrinogen (g/l)	-0.22
Globulin (g/l)	0.20

[a] $p < 0.01$

Table 5.6

Association of red cell filterability and other variables with vascular complications in diabetic patients

	No complications (n = 32)	Microvascular complications (n = 20)	Microvascular & macrovascular complications (n = 17)
Red cell filterability ratio	0.487± 0.05	0.478± 0.04	0.495± 0.04
Fibrinogen (g/l)	3.1± 0.8	3.4± 0.9	3.6± 0.6
Microhaematocrit (%)	44.3± 4.6	42.1± 5.2	43.2± 4.0
White cell count ($x10^9$/l)	7.3± 1.5	7.0± 1.4	7.7± 2.0
Globulin (g/l)	26± 4	26± 3	26± 4
HbA_1 (%)	11.0± 2	10.7± 2.3	10.9± 3.0
Duration of diabetes (yr)	8± 6	18± 11[a]	10± 9
Type 1 (n)	14	10	4
Type 2 (n)	18	10	13

[a] $p < 0.02$, patients with microvascular disease v patients with micro and macrovascular disease or no complications

filtration rate of the cells can be extrapolated from the clogging rate of the filter.

No difference in red cell deformability was found between males and females, and no effect of smoking, age or body mass index was observed in either the healthy volunteers or the diabetic patients. In the healthy subjects deformability was inversely related to mean cell volume, but there was no relationship to mean cell haemoglobin concentration (a measure of internal cell viscosity which is a determinant of deformability (191)). This finding may indicate that larger cells have difficulty passing through 5μm pores. These results are similar to those of Bareford et al (17) who correlated the mean cell volume with impaired red cell deformability through 5 μm pore diameter Nucleopore filters in the Hemorheometre ($r = 0.21$, $p<0.05$).

A higher mean cell haemoglobin concentration in association with lower mean cell volume was found in diabetic patients, a finding which has previously been described (124,192). The increased mean cell haemoglobin concentration partly explained the reduced red cell deformability in diabetics but did not account for it completely.

Another determinant of red cell deformability is cell membrane fluidity, which may be related to the phospholipid bilayer (120) and membrane stiffening could therefore reflect alterations in serum lipids eg cholesterol (chapter 1). However the serum cholesterol level of the control group did not correlate with red cell filtration ratio. Lipid levels were not measured in the diabetic patients in this study, and therefore it is not possible to speculate whether the elevated levels of plasma lipids found in diabetes mellitus (89) could be related to impaired red cell filtration in the diabetic patients.

At capillary level deformability of individual red cells is important if they are to pass through small diameter vessels. Filtration methods devised to stimulate this process in vitro have the main disadvantage of progressive clogging of the filters by sub-populations of rigid red and white cells. While the Carri-Med filtrometer is subject to some white cell contamination (165), it allows the clogging rate of the filter to be measured with time, and the initial filtration rate of the red cells can thus be extrapolated. Using this method there was a significant impairment in red cell deformability in diabetic patients compared to control subjects, this abnormality being worse in type 1 (insulin-dependent) diabetic patients.

Red cell deformability was unrelated to duration of diabetes or glycaemic control. Some previous studies have found impaired deformability to be related to glycaemic control (106,140) however incubating either diabetic or non-diabetic red cells with glucose to raise intracellular sorbitol levels produces only minor changes in deformability (118).

If internal cell changes or hyperglycaemia cannot account for the reduced red cell deformability in diabetics it seems likely that a membrane defect may be the major cause. Increased microviscosity of diabetic red cell membranes related to glycaemic control and insulin levels has been reported using fluorescent probes (120,121) and may be related to reduced spectrin phosphorylation or cross-linkage (127) or an imbalance of the cholesterol/phospholipid ratio (128).

Although it has been suggested that red cell deformability may be related to microvascular disease in diabetes (145,146) there have been few studies examining red cell deformability in diabetic patients with macrovascular disease. In this study red cell deformability was equally impaired in

diabetic patients with microvascular complications, in patients with a combination of micro and macrovascular complications and in patients with no complications, which is in agreement with several previous studies of deformability in diabetic patients with microvascular disease (96,99,108).
While reduced deformability may not be directly causative of micro- or macrovascular problems in diabetic patients, it could have a detrimental effect on ischaemic tissue, due to reduced blood flow in the disturbed microcirculation or in collateral vessels. It may also be a factor contributing to the early onset of vascular complications in diabetes mellitus. Further longitudinal studies are required to determine the relationship between red cell deformability, glycaemic control and onset of diabetic complications.

5.6 Summary

Red cell deformability through 5μm pores in normal healthy individuals was related only to mean cell volume. It is possible that conditions associated with macrocytic cells (such as hypothroidism, alcohol abuse or megaloblastic anaemia) may therefore be associated with reduced red cell filterability and this has indeed been suggested by other studies (193).
In diabetic patients red cell deformability assessed by filtration in this relatively new filtrometer was impaired when they were compared with non-diabetic individuals, and this was partly related to mean cell haemoglobin concentration but may also be related to increased membrane rigidity.

CHAPTER 6

THE ROLE OF RHEOLOGICAL VARIABLES IN DIABETIC PERIPHERAL NEUROPATHY

6.1 Introduction

Diabetic sensory peripheral neuropathy is generally accepted to have a metabolic origin, being related to hyperglycaemia and to duration of disease (194). Raised levels of intraneural sorbitol from increased activity of the polyol pathway and reduced levels of myoinositol and Na^+/K^+ ATPase have been implicated in its pathophysiology (85,86). Nevertheless other diabetic complications such as retinopathy and nephropathy which are related to glycaemic control and duration of disease (63), are the result of microvascular abnormalites, and since there is an increased prevalence of microvascular disease in diabetic patients with sensorimotor neuropathy (195) it is possible that there may be a microvascular component leading to intraneural hypoxia in the development of neuropathy.

Simpson has suggested that alterations in haemorheological variables could be one mechanism contributing to decreased flow in nerve capillaries (146), but so far only abnormalities in platelet aggregation have been related to diabetic neuropathy (196). Whole blood viscosity and its determinants, including red cell deformability, was therefore measured in diabetic patients with symptomatic peripheral neuropathy and compared with a closely matched group of patients who had no evidence of neuropathy and with non-diabetic subjects selected from the healthy control population (chapter 5), to assess if diabetics with neuropathy have altered blood rheology.

6.2 Patients

The three groups of patients studied were :

Group A which consisted of 29 diabetic out-patients (19 Male, 10 Female) with clinical evidence of chronic sensorimotor neuropathy of the lower limbs for more than six months duration. All patients had symptoms of pain and/or paraesthesia with nocturnal exacerbation together with signs of absent ankle reflexes, reduced or absent vibration perception to a tuning fork (128 Hz) and in some cases reduced cutaneous sensation to touch (n=12) or thermoreception (n=15). The patients all had normal peripheral arterial pulses and none had a history of excessive alcohol intake. Confirmation of neuropathy was obtained in 18 patients; neurophysiological studies were performed on the common peroneal and sural nerves of the right lower leg using a Medelec Mystro MS6 machine. Compared to the laboratory normal range, conduction velocity was significantly reduced in both nerves in the diabetic patients and distal latency was significantly prolonged in the common peroneal nerve (Table 6.1).

Group B comprised 30 diabetic outpatients (20 Male, 10 Female) matched with group A patients for age (56 years-group A and 55 years-group B), sex (M/F; 19/20-group A and 20/10-group B) and glycaemic control (10.8 ± 2.6%-group A and 10.9 ± 2.7%-group B). There were equal numbers of patients with type 1 diabetes (6 in each group), patients receiving insulin therapy (17 in each group) and patients with microvascular complications (13-group A and 12-group B), (Table 6.2). Diabetic patients in this group had no clinical evidence of neuropathy based on the criteria used to define neuropathy in group A making it unlikely that significant neuropathy was present .

Table 6.1

Neurophysiology Studies

	Value	Normal
Common Peroneal nerve:		
Conduction velocity (m/s)	40.7± 4.3[a]	50.5±4.6
* Distal latency (ms)	7.6± 3.3[a]	3.6±0.5
Sural nerve:		
Conduction velocity (m/s)	31.4±5.3[a]	48.3±5.3
Distal latency (ms)	3.2±0.5	3.6±0.4

* Absent in 7 patients,

[a] $p < 0.001$

Table 6.2

Characteristics of the groups

	Group A	Group B	Group C
Sex ratio (M/F)	19/10	20/10	26/18
Smokers (n)	11	14	21
Age (yr)	56±10	55± 9	47± 8 [a]
Type 1 diabetes (n)	6	6	-
Insulin therapy (n)	17	17	-
Microvascular disease (n)	13	12	-
Body Mass Index (kg/m^2)	28.4± 4.5	26.6± 3.9	26.4± 4.0
Duration of diabetes (yr)	12±10	11±9	-
HbA_1 (%)	10.8±2.6	10.9±2.7	-

[a] $p < 0.001$

Group C was formed from 44 healthy volunteers (26 Male, 18 Female) free of painful or paraesthetic symptoms in their legs and with normal fasting blood glucose levels. They were matched with the diabetic patients with respect to sex ratio (M/F; 26/18) and body mass index (26.4 ± 4.0kg/m^2) but were younger than the diabetic patients (47 years, $p< 0.001$), (Table 6.2).

6.3 Methods

6.3.1 *Blood samples*

Samples for viscosity, red cell deformability, haematocrit and red cell indices were measured in blood anticoagulated with dry potassium edetate (1.5mg/ml) within 2 hours of venesection. Whole blood viscosity was measured in the Contraves LS rotational viscometer (high shear-94.5s^{-1}, low shear-0.945s^{-1}) and plasma viscosity in a Coulter-Harkness capillary viscometer. Blood viscosity was corrected to standard microhaematocrit (Hawksley centrifuge) of 45% .

Red cell deformability was measured in the Carri-Med filtrometer (52) as described in the preceding chapter and chapter 3.5.

Mean cell volume was measured in the Coulter-S-Counter (normal range 75-95 fl) and mean cell haemoglobin concentration was estimated as described (chapter 3.9). Fibrinogen was measured by the Clauss method, and glycated haemoglobin (HbA1) was measured by agar gel electrophoresis (Corning Glytrac).

6.3.2 *Statistical methods*

Statistical methods utilised the Mann-Whitney U-test for difference in means and correlations between variables were analysed by the Spearman rank test. Results are expressed as mean ± SD.

6.4 Results

6.4.1 *Viscosity*

Corrected whole blood viscosity at both high and low shear rates was significantly higher in diabetic patients (5.29 ± 0.51 and 21.10 ± 3.03 mPa.s) compared to control subjects (4.83 ± 0.54 and 17.36 ± 2.78 mPa.s, $p<0.001$), (Table 6.3). Low shear viscosity at native haematocrit was also elevated in the diabetic patients compared with control subjects (19.74 ± 4.72 vs 17.36 ± 3.62 mPa.s, $p<0.02$). The higher whole blood viscosity in the diabetic patients was related to a higher plasma viscosity (1.41 ± 0.13) compared to the control subjects (1.29 ± 0.09 mPa.s, $p<0.001$) but there was no difference in protein levels (either fibrinogen or globulins) between diabetic patients or controls. Relative blood viscosity at low shear rates, was significantly higher in diabetic patients (15.12 ± 1.96) compared to control subjects (13.38 ± 2.02 mPa.s, $p<0.001$) and was similar in diabetic patients with neuropathy compared to those without neuropathy. No differences were found between diabetic patients with neuropathy and those without for whole blood viscosity, plasma viscosity or fibrinogen levels but globulin levels were significantly higher in the neuropathy group compared to the non-neuropathy group (Table 6.3).

Table 6.3

Whole blood, relative and plasma viscosity and protein levels in diabetic patients and control subjects

	Control Subjects	Diabetic Patients		
		All	Neuropathy	No neuropathy
Whole blood viscosity (mPa.s)				
$95s^{-1}$	4.95 ±0.64	5.16 ±0.71	5.03 ±0.82	5.26 ±0.61
Corrected	4.83 ±0.54	5.29 ±0.51[a]	5.20 ±0.64	5.37 ±0.35
$0.95s^{-1}$	17.56 ±3.62	19.74 ±4.72[b]	18.49 ±5.37	20.82 ±3.85
Corrected	17.36 ±2.78	21.10 ±3.03[a]	20.46 ±3.94	21.65 ±1.86
Microhaematocrit (%)	45.0 ±3.4	43.6 ±3.9	43.3 ±4.5	43.9 ±3.4
Plasma viscosity (mPa.s)	1.29 ±0.09	1.41 ±0.13[a]	1.39 ±0.09	1.42 ±0.16
Fibrinogen (g/l)	3.3 ±0.7	3.5 ±0.7	3.4 ±0.7	3.5 ±0.7
Globulins (g/l)	25.2 ±3.5	25.9 ±5.5	27.6 ±6.1[c]	24.3 ±4.4
Relative Blood viscosity				
$95s^{-1}$	3.72 ±0.45	3.78 ±0.38	3.75 ±0.45	3.81 ±0.33
$0.95s^{-1}$	13.38 ±2.02	15.12 ±1.96[a]	14.86 ±2.52	15.34 ±1.37

[a] $p<0.0001$
[b] $p<0.02$
} Diabetic patients vs control subjects

[c] $p<0.03$ Diabetic patients with neuropathy vs diabetic patients with no neuropathy

6.4.2 *Red cell deformability*

Red cell filtration was significantly reduced in both type 1 (0.456 ± 0.04) and type 2 diabetic patients (0.497 ± 0.04) compared to control subjects (0.550 ± 0.03 , $p<0.01$) and was significantly lower in type 1 compared to type 2 diabetic patients (difference 0.04, 95% CI; 0.02 to 0.06, $p<0.02$). There was no difference in red cell filtration rates between diabetic patients with neuropathy and those without neuropathy (0.490 ± 0.05 vs 0.490 ± 0.04 , $p>0.05$), (Figure 6.1).

There was a significant inverse correlation of red cell filtration ratio with mean cell haemoglobin concentration ($r=-0.29$, $p<0.01$) but no correlation of filtration ratio with other haematological variables, glycaemic control, age or duration of diabetes was found.

6.5 Discussion

It has long been thought that persistent hyperglycaemia was responsible for the nerve damage sustained in diabetes and correlations have been shown between both fasting blood glucose and glycated haemoglobin levels and conduction velocities of motor nerves (195,197) and with vibration sense (198). There is also evidence that improving glycaemic control with treatment using continuous subcutaneous insulin infusions over a period of several months can alleviate the symptoms of neuropathy and improve conduction velocity (199).

Mechanisms thought to be involved in the metabolic aetiology of neuropathy include increased activity of the polyol pathway leading to raised levels of insoluble sorbitol and fructose within nerve cells and a

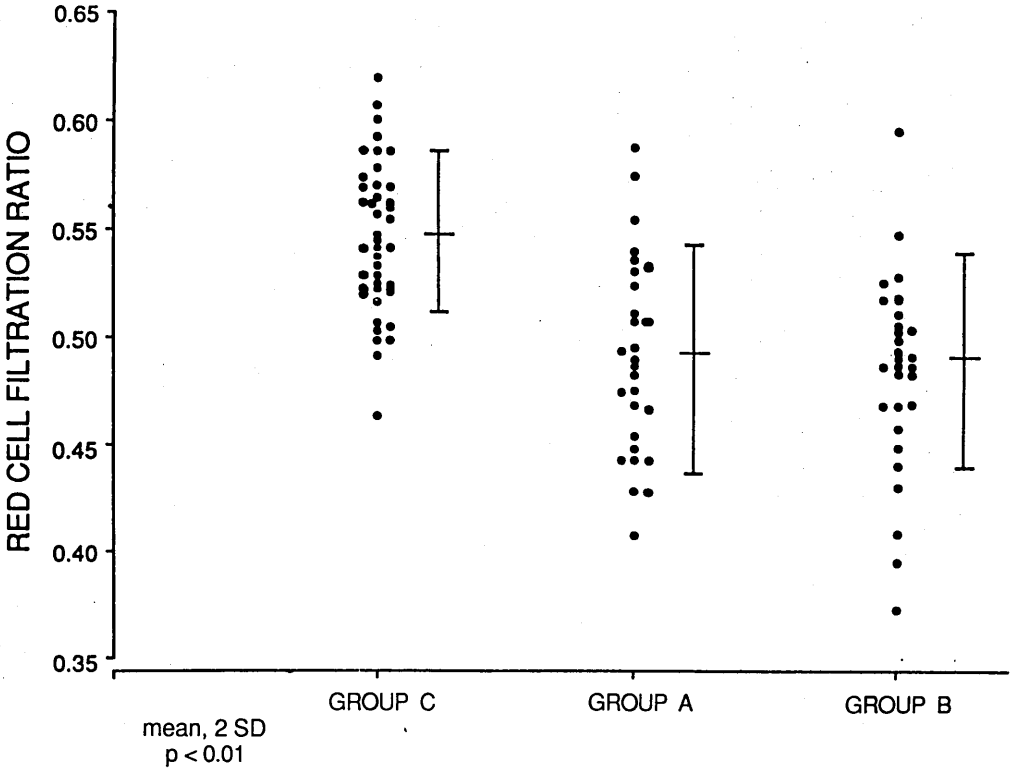


Figure 6.1

Red cell filtration ratios in control subjects and diabetic patients with and without neuropathy

consequent fall in levels of myoinositol and Na^+/K^+ ATPase (85). While increased concentrations of sorbitol have been found in diabetic nerves at biopsy, alterations in myoinositol within the nerve remain controversial (86). Several studies in diabetic patients using aldose reductase inhibitor drugs appear to improve both symptoms of neuropathy and conduction velocities of specific nerves (200,201) .

Fagerberg in 1959 described intraneural vascular lesions in diabetic nerves such as a reduction in capillary size with thickening of the walls, hyalinisation and deposition of PAS positive material (202). Endoneurial capillary disease could therefore be a manifestation of the generalised microangiopathy of diabetes mellitus and due to disruption of blood flow could be responsilble for hypoxic nerve damage and thus neuropathy. Since these observations, further intraneural abnormalities have been described in diabetic patients with a history of neuropathy; capillary plugging with fibrin and thrombus (203,204) and capillary closure due to endothelial hyperplasia and desquamation (205,206). In addition it has been suggested that the pattern of nerve fibre loss in diabetic neuropathy has an ischaemic origin (207). In support of this theory Newrick et al have demonstrated reduced oxygen tension within the sural nerve of diabetic patients suffering from neuropathy (208).

Diabetic patients have rheological abnormalities including an increase in whole blood viscosity, plasma viscosity, red cell aggregation and a reduction in red cell deformability (185). The disturbance in blood flow behaviour has been shown to be more abnormal in patients with proliferative retinopathy (99) and microalbuminuria (108) and this could be one mechanism leading to reduced flow and sludging of blood within intraneural capillaries in diabetic patients with neuropathy. I sought to

determine if patients with diabetic neuropathy could have significant alterations in blood viscosity or its determinants or in red cell deformability compared to patients without neuropathy, which could explain the finding of intraneural ischaemia in diabetic neuropathy.

Although a significant increase in viscosity at high and low shear rates and a reduction in red cell deformability in the diabetic patients as a whole was found, I was unable to demonstrate differences in these variables between patients with neuropathy and those without. It may be argued that subclinical neuropathy was not definitely excluded in patients from group B, but since there was little doubt that patients in group A had clear evidence of neuropathy based on clinical findings (209), with confirmation of large fibre damage as shown by impaired conduction velocities, it is extremely unlikely that patients in group B had nerve damage comparable to that of group A.

It is of interest that patients with neuropathy had higher levels of serum proteins (other than fibrinogen) compared to patients without neuropathy, because large serum proteins such as α_2-macroglobulin would be expected to give rise to greater red cell aggregation (57), a parameter which is indirectly measured by low shear whole blood viscosity. I found no difference in viscosity at these shear rates between the two groups, but more sensitive apparatus such as the Myrenne aggregometer may be able to detect more subtle differences (142).

These findings, although negative, do not exclude abnormal rheology from contributing to nerve ischaemia by acting as a cofactor with intraneural microvascular abnormalities (210) to reduce blood flow in capillaries and

enhance thrombus formation. Increased erythrocyte adhesion to intraneurial capillary endothelial cells in diabetes (130) may be a factor reducing capillary luminal diameter, and it also remains possible that the presence of wider channel arterio-venous shunts in the vicinity of the nerve caused by coincidental autonomic neuropathy (211) could favour the diversion of more viscous diabetic blood from the smaller calibre nutritive capillaries.

6.6 Summary

Thus the current evidence for microangiopathy in diabetic neuropathy is strong, but it seems unlikely that altered rheology in diabetes per se is directly responsible for endoneurial hypoxia. Capillary structural abnormalities in conjunction with alterations in rheology may however lead to nerve ischaemia and along with metabolic factors result in neuropathy.

CHAPTER 7

ASSESSMENT OF RED CELL AGGREGATION IN NON-DIABETIC VOLUNTEERS AND ASSOCIATION WITH VASCULAR COMPLICATIONS IN DIABETES MELLITUS

7.1 Introduction

The mechanism leading to aggregation of red cells is not entirely clear, but many molecules in the red cell membrane have charged head groups which under normal circumstances amounts to an overall negative charge due mainly to sialic acid residues (212). Repulsive electrostatic forces therefore exist between red cells, which dominate over the weaker attraction of the Van der Waal's forces causing a net repulsion (213). The force causing aggregation of red cells must be greater than the repulsive forces: this is achieved by the formation of protein bridges between cells created by long-chain proteins adhering to cells at either end.

The most important plasma protein causing red cell aggregation is fibrinogen, this being a large, elongated and flexible molecule (length 40 nm, molecular weight 340 000). However it is not known what holds fibrinogen to the cell membrane although its adsorption is probably influenced by electrostatic forces (27). Other proteins with high molecular weights will also cause aggregation and it has been suggested that some of these proteins may enhance the aggregating effect of fibrinogen (24).

Rouleaux formation is dependent on shear rate (23): as the shear rate falls the bulk viscosity of blood will increase due partly to red cell aggregation (chapter 1). As shear rate increases viscosity falls until rates of about 100/s, when the aggregates will be totally dispersed. Rouleaux formation affects blood flow in both large and small vessels; in the centre of large vessels, the shear rate is low and thus increased viscosity associated with rouleaux formation will occur, while in microvessels aggregation will be enhanced due to low shear rates in post capillary venules. An increase in haematocrit or serum proteins will increase the extent of aggregation and could

potentially reduce flow in these small vessels.

High levels of fibrinogen may reflect the acute or chronic stress responses which are the body's response to injury, infection or inflammation. They are thought to involve stimulation of monocyte production of Interleukin by fibrin degradation products D and E. This causes both hepatic synthesis of acute phase proteins and release of platelets and white blood cells from bone marrow. Fibrinogen is the most important acute phase protein, and many studies have found raised levels in diabetic patients, possibly due to increased hepatic synthesis (214). The chronic stress response in diabetes (131) could therefore lead to increased red cell aggregation in vivo.

There have been few studies measuring red cell aggregation directly in diabetic patients, but using instruments such as the rheophotometer (96), rheoscope (136) and syllectometry (135) aggregation has been found to be raised in diabetes, usually in association with raised levels of plasma proteins e.g. fibrinogen, α_2 and β-globulins. Because increased aggregation leads to raised blood viscosity and hence to reduced blood flow and possibly vascular stasis, particularly in post-capillary venules, it can be postulated that this could contribute to tissue ischaemia and the increased incidence of vascular complications in diabetes. The main diabetic complication which has been studied in relation to red cell aggregation is diabetic retinopathy, and the results of previous studies have been conflicting, with some workers finding no difference in red cell aggregation between patients with retinopathy and those without (96,135), and others who found a correlation between the extent of aggregation and severity of retinopathy (136,215).

This study was undertaken to compare red cell aggregation, measured by a

new simple technique, in diabetic patients with non-diabetic healthy individuals and to assess mainly the effects of glycaemic control, type of diabetes, and whether vascular complications or hypertension in diabetes are associated with higher levels of aggregation.

Triglyceride's carrier protein, very low density lipoprotein (VLDL) is a large plasma protein (having a diameter of 30-80nm), which could in theory also have an effect on red cell aggregation by creating protein bridges, but to date this has not been studied in any population sample. Diabetic patients generally have higher levels of serum lipids and lipoproteins (89) compared with non-diabetic subjects and it was aimed to examine the relationship between plasma lipids and red cell aggregation in both diabetic and non-diabetic control subjects, to determine if this could be a factor contributing to the reported increased red cell aggregation in diabetes mellitus.

7.2 Patients

7.2.1 *Diabetic Patients*

110 diabetic patients were selected from the out-patient clinic (54 type 1, insulin-dependent and 56 type 2 non-insulin dependent). Sex ratio of the type 1 diabetics was 22 male and 32 female, and of the type 2 diabetics was 29 male and 27 female. Mean age of the type 1 patients was 38 years (range 17-65 years) and 59 years in the type 2 patients (range 43-73 years), $p < 0.001$.

Therapy in the type 2 diabetics consisted of insulin in 26 patients, oral hypoglycaemic agents in 23 patients and dietary therapy in 7 patients. The

type 1 diabetics had significantly longer duration of diabetes (19 ± 10 years) compared with the type 2 diabetics (9 ± 6 years, $p < 0.001$) and significantly lower body mass index (23.8 ± 3.2 kg/m^2) compared with type 2 diabetics (28.3 ± 5.2 kg/m^2, $p < 0.001$). Both groups of patients were matched for glycaemic control as measured by blood glucose (11.9 ± 6.2mmol/l-type1 v 12.3 ± 5.1mmol/l-type 2, $p>0.05$) and glycated haemoglobin (10.8 ± 2.1%-type1 v 10.4 ± 2.1%-type 2, $p>0.05$), (Table 7.1). Macrovascular disease was said to be present if patients had evidence of ischaemic heart disease, cerebrovascular disease or peripheral vascular disease. Microvascular disease was scored as 0 for no complications, 1 for background diabetic retinopathy, 2 for pre-proliferative or proliferative retinopathy and 3 for retinopathy plus nephropathy.

Of the type 1 diabetics two had macrovascular disease, while 15 of the type 2 diabetics had macrovascular complications, five of whom were hypertensive. Microvascular complications were present in 33 type 1 diabetics and 36 type 2 diabetics.

7.2.2 *Control subjects*

100 healthy non-diabetic control subjects were chosen to match as closely as possible with the diabetic patients for age and sex distribution. They were divided into two groups of 50 subjects to compare with the type 1 and the type 2 diabetics. Mean age of group 1 was 34 years (range 21-48 years) and of group 2 was 59 years (range 45-87 years), $p < 0.001$. The two groups were matched for body mass index (23.0 ± 3.8kg/m^2-group 1 vs 24.6 ± 4.7kg/m^2-group2, $p>0.05$) and consisted of 21 males, 29 females in group 1 and 23 males, 27 females in group 2, (Table 7.1).

Table 7.1

Characteristics of the groups

	Diabetics		Controls
	Type 1	Type 2	
n	54	56	100
Age (yr)	38 ± 13	59 ± 7 [a]	47 ± 16 [b,c]
Body Mass Index (Kg/m^2)	23.8 ± 3.2	28.3 ± 5.2 [a]	23.9 ± 4.3 [b]
Sex (M/F)	(22/32)	(29/27)	(44/56)
Duration of diabetes (yr)	19 ± 10	9 ± 6 [a]	-
Blood Glucose (mmol/l)	11.9 ± 6.2	12.3 ± 5.1	-
Hb A_1 (%) [NR 5.5 - 8.5%]	10.8 ± 2.1	10.4 ± 2.1	-

Values are mean ± SD

[a] $p < 0.001$ Type 2 v Type 1 diabetics
[b] $p < 0.001$ Controls v Type 2 diabetics
[c] $p < 0.02$ Controls v Type 1 diabetics

7.3 Methods

7.3.1 *Blood samples*

Red cell aggregation was assessed in the Myrenne red cell aggregometer (chapter 3.7). Briefly, 25 µl of whole blood was dropped onto the centre of the cone and the sample spun at 600 s^{-1} before being stopped abruptly. The extent of aggregation, determined as the change in light transmission over 5 seconds of stasis, was recorded digitally as the aggregation index. The mean of two readings was taken then a further assessment was made on a further sample adjusted to a haematocrit of 40%.

Microhaematocrit was measured with the Hawksley centrifuge and reader, and red and white cell indices on the Coulter-S-Counter. Plasma viscosity was measured on 1ml of plasma in the Coulter-Harkness capillary viscometer (3.3).

Plasma lipoproteins were measured on the Hitachi 704 analyser (Boehringer Mannheim, FRG) after density gradient separation by ultracentrifugation on a Beckman centifuge.
Total proteins, albumin and globulin were measured by SMAC (Technicon, Basingstoke). Fibrinogen was measured by the heat precipitation method (chapter 3.8) and haptoglobin and macroglobulin by immunoturbidimetric analysis on the Encore analyser (Baker Instruments, Pennysylvania).
Blood glucose was measured by the hexokinase method on a Hitachi 737 analyser and glycated haemoglobin by agar gel electrophoresis (Corning Glytrac).

7.3.2 *Urinary protein*

Microproteinuria excretion was measured by radioimmunoassay as albumin excretion rate (AER) and macroproteinuria excretion as total protein excretion per volume in 24 hours. Urinary protein values of between 20 and 200mg/l were accepted as microproteinuria and values of > 200mg/l as macroproteinuria.

7.3.4 *Physical variables*

Blood pressure was measured by an electronic sphygmomanometer (Neissi, Japan) and values greater than 160/95 mmHg were accepted as hypertension. Values less than this were accepted as hypertension if the patients were taking specific antihypertensive treatment. Body mass index was estimated from weight (kg) divided by height squared (m^2).

7.3.5 *Statistical methods*

The Mann-Whitney U-test was used for comparison of means between diabetic patients and controls and within the two groups. Spearman rank correlation was used to determine the association between red cell aggregation index and variables.

7.4 Results

No difference was found in red cell aggregation measured at native haematocrit or at 40% haematocrit in any of the groups of patients studied, and all the following results of red cell aggregation index are therefore

given for haematocrit of 40%.

7.4.1 *Control subjects*

Red cell aggregation index was similar in male subjects (3.4 ± 1.4) compared with female subjects (3.2 ± 1.2, $p>0.05$) and in smokers (3.2 ± 1.2) compared with non-smokers (3.4 ± 1.1, $p>0.05$).

There was a significant correlation of red cell aggregation index with triglyceride level ($r=0.21$, $p<0.02$), fibrinogen level ($r=0.26$, $p< 0.02$) and α_2-macroglobulin level ($r=-0.30$, $p<0.02$) and an inverse correlation with mean cell volume ($r=-0.19$, $p<0.05$), Table 7.2.

7.4.2 *Diabetic patients*

(i) Type of diabetes:

Red cell aggregation index was significantly increased in type 2 diabetic patients compared with type 1 (at corrected haematocrit; 5.5 ± 1.5 vs 4.3 ± 1.3, $p<0.001$), Figure 7.1. Fibrinogen level was higher in type 2 diabetics (4.1 ± 1.2g/l) compared with type 1 diabetics (3.5 ± 1.2g/l, $p<0.03$) while α_2-macroglobulin levels were significantly higher in type 1 diabetics (2.46 ± 0.70g/l) compared with type 2 diabetics (1.71 ± 0.66g/l, $p<0.001$), (Table 7.3).

Triglyceride and VLDL levels were significantly higher in type 2 diabetics (2.64 ± 1.80 mmol/l and 1.06 ± 0.68 mmol/l) compared with type 1 diabetics (1.49 ± 1.16 mmol/l, $p<0.002$ and 0.59 ± 0.34 mmol/l, $p<0.001$) and HDL levels were significantly lower in the type 2 diabetics (1.24 ± 0.41 mmol/l) compared with the type 1 diabetics (1.44 ± 0.39 mmol/l, $p< 0.02$), Table 7.4.

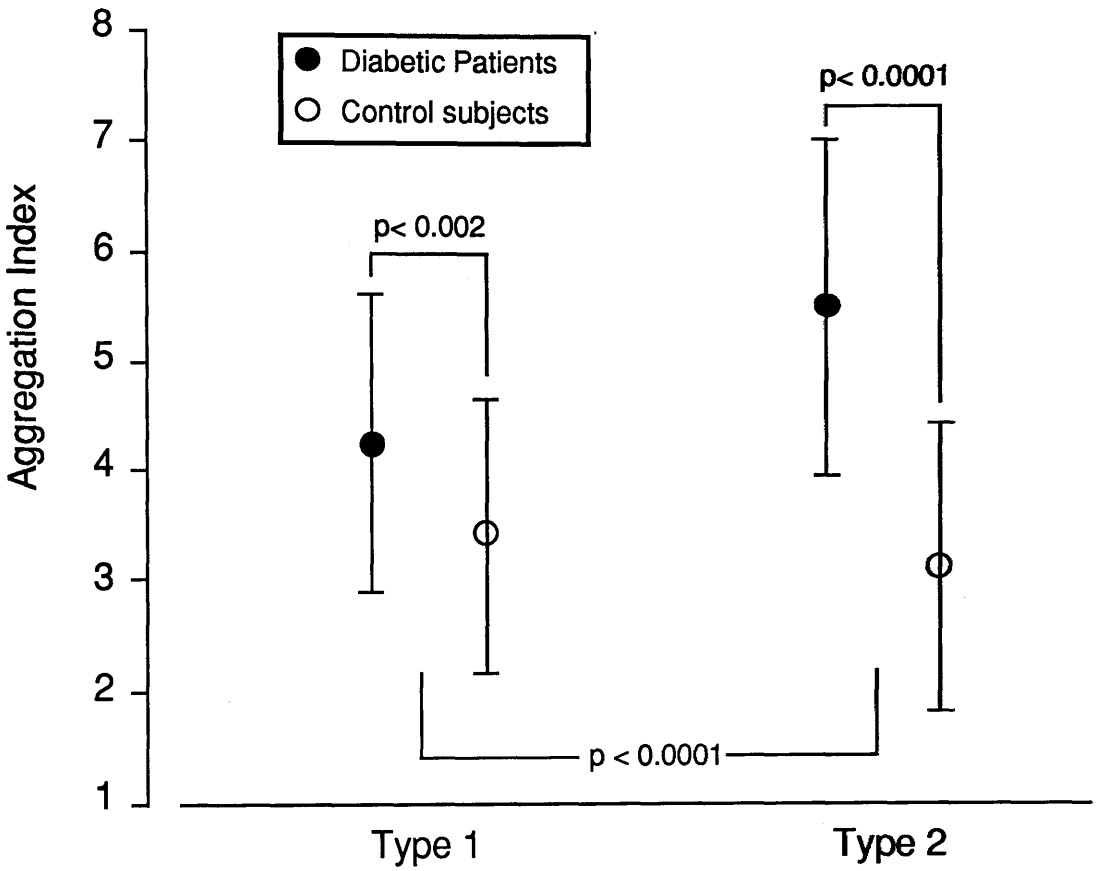
In both type 1 and type 2 patients red cell aggregation index correlated with

Table 7.2

Correlation of red cell aggregation with variables in control subjects.

Variable	r_s
Age (yr)	- 0.07
Body mass index (kg/m^2)	0.16
Plasma viscosity (mPa.s)	0.11
Haematocrit (%)	- 0.15
Mean cell volume (fl)	- 0.19[b]
Globulin (g/l)	0.26[a]
Fibrinogen (g/l)	- 0.04
α_2 macroglobulin (g/l)	- 0.30[a]
Haptoglobin (g/l)	- 0.17
Triglyceride (mmol/l)	0.21[a]
Cholesterol (mmol/l)	- 0.12
VLDL (mmol/l)	0.11
LDL (mmol/l)	- 0.04
HDL (mmol/l)	- 0.08

[a] $p < 0.02$
[b] $p < 0.05$



Values are mean ± SD

Figure 7.1

Red cell aggregation index in diabetic patients and control subjects

Table 7.3

Comparison of plasma viscosity, haematocrit and proteins in diabetic patients with control subjects.

	Type 1	Control Group	p	Type 2	Control Group	p
PV (mPa.s)	1.35 ± 0.97	1.27 ± 0.06	p< 0.001	1.38 ± 0.01	1.30 ± 0.11	p< 0.001
Haematocrit (%)	42.2 ± 4.7	43.0 ± 3.5	p> 0.05	42.5 ± 4.2	43.5 ± 4.2	p> 0.05
Haptoglobin (g/l)	3.5 ± 1.2	2.8 ± 0.9	p< 0.001	4.1 ± 1.2 [a]	3.3 ± 1.2	p< 0.01
α_2-macroglobulin (g/l)	1.48 ± 0.57	0.91 ± 0.35	p< 0.001	1.62 ± 0.98	1.23 ± 0.68	p> 0.05
Fibrinogen (g/l)	2.46 ± 0.70	1.63 ± 0.58	p< 0.01	1.71 ± 0.66 [a]	2.33 ± 0.50	p< 0.001
Globulin (g/l)	25 ± 4	23 ± 3	p> 0.05	25 ± 3	27 ± 3	p> 0.05
Globulin/Albumin ratio	0.59 ± 0.12	0.52 ± 0.10	p< 0.01	0.61 ± 0.13	0.63 ± 0.08	p> 0.05

[a] p< 0.0001 Type 2 v Type 1 diabetics

Table 7.4

Comparison of lipid and lipoprotein levels in diabetic patients with control subjects.

	Type 1	Control Group	p	Type 2	Control Group	p
Triglyceride (mmol/l)	1.49± 1.16	1.05±0.42	p< 0.01	2.64±1.80 [a]	1.23±1.80	p< 0.001
Cholesterol (mmol/l)	5.64± 1.17	5.61±1.24	p> 0.05	6.18±1.71 [b]	5.87±1.71	p> 0.05
VLDL (mmol/l)	0.59± 0.34	0.50±0.24	p> 0.05	1.06±0.68 [a]	0.59±0.68	p< 0.01
LDL (mmol/l)	3.56± 0.89	3.22±0.76	p> 0.05	3.84±1.60	3.54±1.60	p> 0.05
HDL (mmol/l)	1.44± 0.39	1.56±0.37	p> 0.05	1.24±0.41	1.68±0.41	p< 0.001

[a] p< 0.0001
[b] p<0.02
} Type 2 v Type 1 diabetics

plasma triglyceride level ($r=0.36$ and $r=0.41$, $p<0.001$), VLDL level ($r=0.26$ and $r=0.30$, $p<0.02$) and HDL level ($r=-0.26$ and $r=-0.30$, $p<0.02$). However there was no significant association wth other plasma proteins i.e. fibrinogen, α_2-macroglobulin, haptoglobin or with globulin/albumin ratio, Table 7.5. Correlation of red cell aggregation with VLDL is shown in Figure 7.2.

In the type 2 patients there was a significant inverse correlation with duration of diabetes ($r=-0.24$, $p<0.05$) and a positive correlation with body mass index ($r=0.30$, $p<0.02$).

Within the type 2 diabetics there was no difference in red cell aggregation between patients receiving insulin therapy and those receiving oral hypoglycaemic agents or dietary therapy alone (5.2 ± 1.5 vs 6.1 ± 1.5 vs 5.4 ± 1.3, $p>0.05$).

(ii)Type 1 diabetics and controls

Red cell aggregation index was significantly higher in the diabetic patients (4.3 ± 1.3) compared with the control subjects (at corrected haematocrit, 3.4 ± 1.2, $p<0.002$), Figure 7.1.

Plasma viscosity was significantly higher in diabetics compared with the controls (1.35 ± 0.97 vs 1.27 ± 0.06 mPa.s, $p<0.001$). The globulin/albumin ratio was higher in diabetic patients compared with controls (0.59 ± 0.12 vs 0.52 ± 0.10, $p<0.01$) and all three plasma proteins measured were significantly higher in the diabetics compared with the controls (fibrinogen, 3.5 ± 1.2 vs 2.8 ± 0.9 g/l, $p<0.01$; haptoglobin, 1.48 ± 0.57 vs 0.91 ± 0.35 g/l, $p<0.001$; α_2-macroglobulin, 2.46 ± 0.70 vs 1.63 ± 0.58 g/l, $p<0.001$)), (Table 7.3) Of the lipids only triglyceride level was significantly different in diabetic patients compared with control subjects (1.49 ± 1.16 vs 1.05 ± 0.42 mmol/l, $p<0.01$), (Table 7.4).

Table 7.5

Correlation of red cell aggregation with variables in diabetic patients (r_s values)

	Type 1	Type 2
Age (yr)	- 0.16	- .0.07
Body mass index (kg/m^2)	0.15	0.30[b]
Duration of diabetes (yr)	- 0.18	- 0.23[c]
HbA_1 (%)	- 0.19	0.07
Blood glucose (mmol/l)	0.16	- 0.02
Plasma viscosity (mPa.s)	- 0.19	- 0.09
Mean cell volume (fl)	0.19	- 0.05
Globulin (g/l)	- 0.06	0.01
Globulin/Albumin ratio	- 0.17	0.06
Fibrinogen (g/l)	- 0.03	- 0.17
α_2 macroglobulin (g/l)	- 0.11	- 0.15
Haptoglobin (g/l)	0.17	- 0.01
Triglyceride (mmol/l)	0.36[a]	0.41[a]
Cholesterol (mmol/l)	0.07	- 0.08
VLDL (mmol/l)	0.26[b]	0.30[b]
LDL (mmol/l)	0.04	- 0.13
HDL (mmol/l)	- 0.26[c]	- 0.30[b]

a $p < 0.001$
b $p < 0.02$
c $p < 0.05$

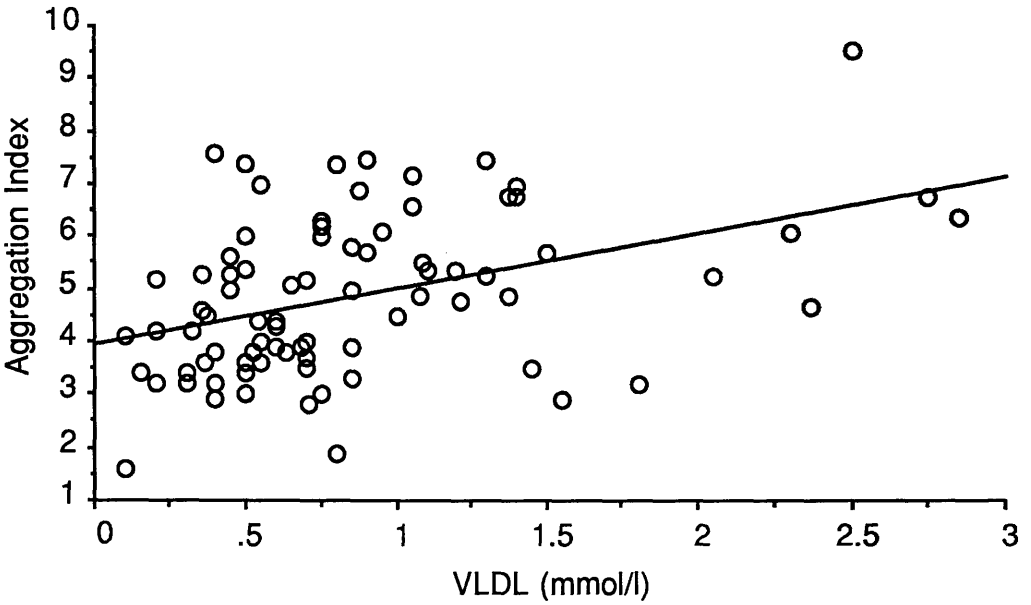


Figure 7.2

Correlation of red cell aggregation index with VLDL in diabetic patients

(iii) Type 2 diabetics and controls

Red cell aggregation index was significantly higher in the diabetic patients compared with the controls (5.5 ± 1.5 vs 3.2 ± 1.3, $p<0.001$), Figure 7.1.

Plasma viscosity was significantly higher in the diabetics compared with controls (1.38 ± 0.10 vs 1.30 ± 0.11, $p<0.001$) as was fibrinogen level (4.1 ± 1.2 vs 3.3 ± 0.8 g/l, $p<0.01$) but α_2-macroglobulin levels were significantly lower in the diabetics (1.71 ± 0.66 vs 2.33 ± 0.50 g/l, $p<0.001$). Both triglyceride and VLDL levels were higher in the diabetic patients compared with the controls (2.64 ± 1.80 vs 1.23 ± 0.59 mmol/l, $p<0.001$ and 1.06 ± 0.68 vs 0.59 ± 0.22 mmol/l, $p<0.01$) and the HDL level was significantly lower (1.24 ± 0.41 vs 1.68 ± 0.42 mmol/l, $p<0.001$), Table 7.4.

(iv) Effect of hypertension

Mean blood pressure in the hypertensive patients was 163 ± 29/89 ± 15 mmHg which was significantly higher than in the normotensive patients 133 ± 20/76 ± 9mmHg, $p<0.001$. Since type 1 and type 2 diabetic patients had differences in red cell aggregation and several other variables they were assessed separately for differences in red cell aggregation between hypertensive and normotensive patients.

There was no significant difference in red cell aggregation index between patients with hypertension (n=9; 4.3 ± 1.4) and patients without hypertension (n=42; 4.3 ± 1.4) in the type 1 diabetics whereas in the type 2 diabetics red cell aggregation index was significantly higher in patients with hypertension (n=15; 6.3 ± 1.8) compared to those without (n=40; 5.2 ± 1.3, $p<0.02$), Figure 7.3.

In the type 2 group the only variable which was significantly different between the hypertensive and normotensive patients was body mass index (31.0 ± 5.2 vs 27.1 ± 4.8 kg/m^2, $p<0.02$).

Both diastolic and systolic blood pressure correlated with red cell

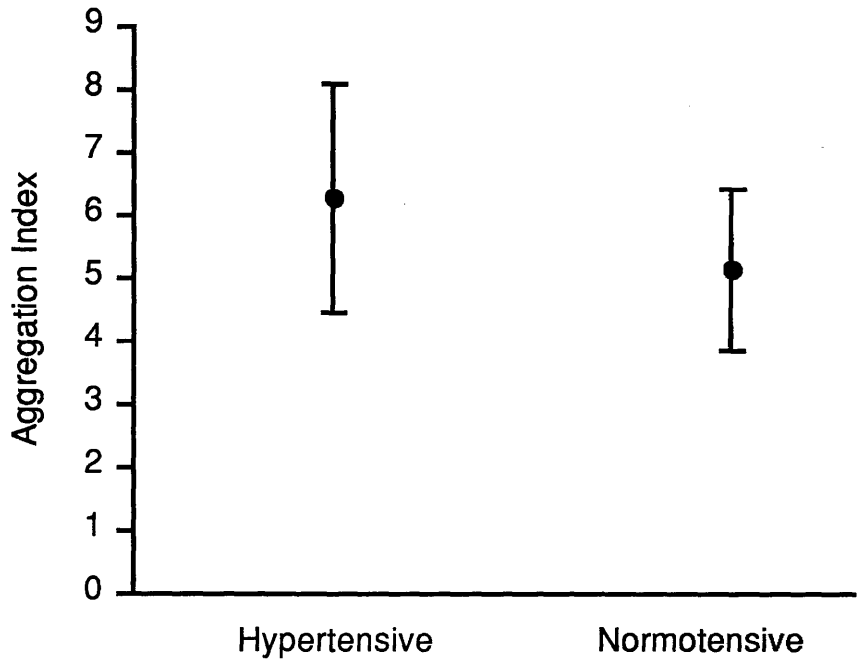


Figure 7.3

Red cell aggregation in hypertensive and normotensive type 2 diabetic patients

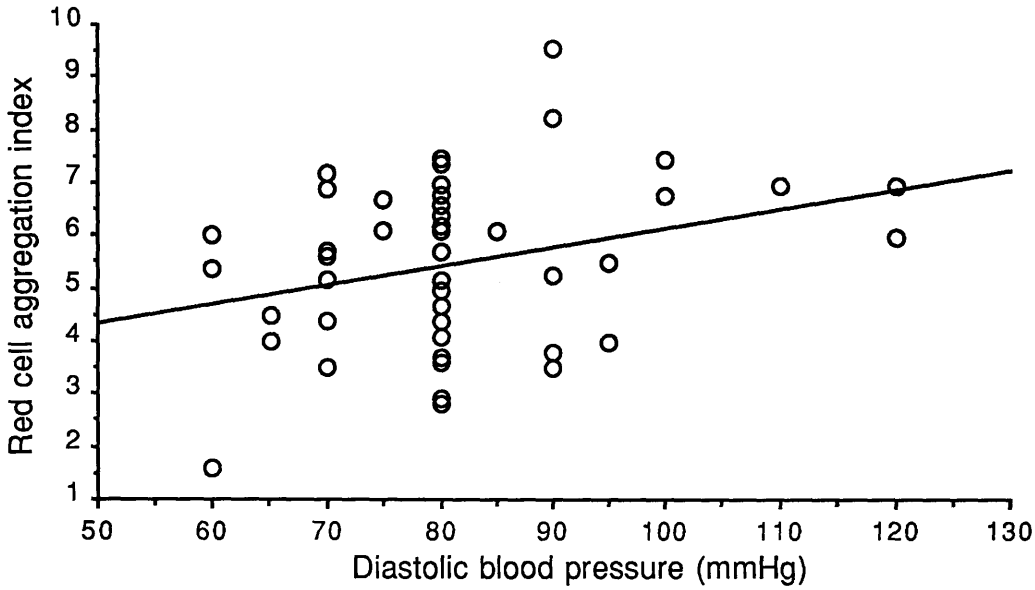


Figure 7.4

Correlation of red cell aggregation index with diastolic blood pressure in type 2 diabetic patients

aggregation in the diabetic population, (Table 7.5) but in the type 2 diabetic patients only diastolic blood pressure correlated with red cell aggregation index ($r=0.23$, $p<0.02$, Figure 7.4).

(v) Effect of macrovascular and microvascular complications

The effect of macrovascular disease was not examined in the type 1 diabetic patients because only two patients had evidence of large vessel complications. Within the type 2 diabetic patients 15 had macrovascular disease and because red cell aggregation was higher in type 2 patients with hypertension these were analysed separately from normotensive type 2 patients. Red cell aggregation index was significantly higher in the hypertensive diabetics with macrovascular disease (7.8 ± 1.2) compared with hypertensive diabetics without macrovascular disease (5.6 ± 1.6, $p<0.02$). In the normotensive patients red cell aggregation index was similar in patients with macrovascular disease (5.1 ± 1.3) and those without (5.2 ± 1.3, $p>0.05$).

The association between microvascular disease and red cell aggregation was assessed in the type 1 diabetics as a whole because there was no difference in those with hypertension and those without and because there were only 9 patients with hypertension. In the type 2 patients differences in red cell aggregation between the various microvascular groups were assessed both for the type 2 patients as a whole and for the subgroup of patients without microvascular disease. The numbers in each microvascular category for patients with hypertension were too small to allow statistical comparisons. Within the type 1 diabetics there was no difference in red cell aggregation index between patients with no complications and those with background retinopathy (4.1 ± 1.2 vs 3.7 ± 1.1, $p>0.05$) but the value was significantly higher in patients with

Table 7.6

Association of red cell aggregation with microvascular complications

	Type 1 diabetics	Type 2 diabetics
No complications	4.1± 1.2	5.2±1.1
Background retinopathy	3.7± 1.1	5.2±1.4
Proliferative retinopathy	5.2± 1.3 [a]	5.6±2.0
Retinopathy and nephropathy	4.3± 1.5	6.8±1.1

[a] $p < 0.02$, type 1 diabetic patients with proliferative retinopathy v type 1 patients with background retinopathy.

proliferative retinopathy (5.2 ± 1.3) compared to those with background changes (3.7 ± 1.1, $p<0.02$). However there was no difference between patients with retinopathy and nephropathy and the other groups (4.3 ± 1.5). In type 2 diabetics as a whole although the red cell aggregation index increased with increasing severity of complications the difference between patients with no complications and those with background or proliferative retinopathy was not significant (5.2 ± 1.1 vs 5.2 ± 1.4 vs 5.6 ± 2.0 vs 6.8 ± 1.1, $p>0.05$), (Table 7.6). When the non-hypertensive diabetics were assessed the results were similar (no complications - 5.1 ± 1.0, background retinopathy - 5.3 ± 1.3 , proliferative retinopathy - 5.1 ± 1.7, $p>0.05$). Only one normotensive type 2 patient had retinopathy and nephropathy and this group was therefore insufficient for comparison.

7.5 Discussion

This study found increased levels of fibrinogen in both type 1 and type 2 diabetic patients compared to their matched controls, and in the type 1 diabetics raised levels of haptoglobin and α_2-macroglobulin compared with controls, as would have been expected from previous reports of these proteins in diabetics (100) although they did not correlate with red cell aggregation as might have been predicted. Interestingly α_2-macroglobulin level was lower in the type 2 patients compared with their matched controls. There is no obvious explanation for this finding, but perhaps it is due to alterations in the balance of plasma globulin levels in type 2 diabetics, because the globulin/albumin ratio was similar in type 2 diabetics compared with controls, in contrast to type 1 diabetics where the ratio was higher compared with controls, (Table 7.3).

There was an increased level of triglyceride in type 1 diabetic patients and increased levels of triglyceride and VLDL with lower levels of HDL in type 2 diabetic patients compared with non-diabetics. Previous studies have suggested that type 1 diabetics often have similar levels of plasma lipids to non-diabetic controls if well-controlled (216), whereas type 2 diabetics have increased lipid levels compared with type 1 diabetics (217) and can have levels of 50-100% greater than those of non-diabetics (218). In particular diabetics have higher levels of triglyceride and VLDL and lower levels of HDL (219). There appears to be increased production and decreased removal of triglyceride in type 2 diabetics leading to increased VLDL production (220) and in type 1 diabetics there is reduced clearance of VLDL, possibly due to decreased lipoprotein lipase activity as a result of insulopenia (221). Why VLDL production is high remains unclear but it may be secondary to relative cellular insulopenia in the presence of hyperinsulinaemia or due to raised non-esterified fatty acid production (222). Qualitative changes in VLDL have also been suggested, viz. triglyceride enrichment leading to increased size of VLDL molecules (223), alterations in apoproteins (224) or glycosylation of apoproteins leading to reduced clearance of VLDL (225). HDL is formed from the hydrolysis of VLDL and insulin deficiency has been blamed for reduced production of HDL in type 1 diabetes (226) and there is thought to be an inverse relationship between plasma insulin level and HDL levels in type 2 diabetic patients (227).

Red cell aggregation was increased in diabetic patients compared with non-diabetic individuals, confirming the findings of previous workers but in this study in a much larger group of patients than has been reported before. Although aggregation of red cells is most commonly related to plasma protein levels, particularly fibrinogen, this was found to be the case only in

the non-diabetics, despite fibrinogen levels being higher in the diabetics. In non-diabetics aggregation was also related to red cell size, as expected (28) but in addition was correlated with plasma triglyceride level which has not previously been described. In the diabetic patients the extent of red cell aggregation not only correlated with triglyceride level but also with VLDL and (inversely) with HDL levels. These findings support the theory that lipoproteins may be capable of aggregating red cells, and in the case of diabetics (especially the older type 2 diabetics where the plasma lipid levels are high compared with non-diabetics), this could be a major factor determining increased red cell aggregation. The Framingham study showed that diabetic patients have an increased morbidity and mortality from vascular diseases (90) and further analysis of the data revealed that triglyceride was an independent risk factor for ischaemic heart disease in diabetics, especially in the presence of a low HDL level (228). In the WHO multinational study, triglyceride was also found to correlate with ischaemic heart disease in several of the centres studied, especially in obese non-insulin-dependent diabetics (219). Thus the raised triglyceride and VLDL levels in diabetes, as well as being an independent risk factor for cardiovascular disease could also contribute to vascular complications by increasing red cell aggregation and leading to reduced flow, stasis or even thrombosis in low shear conditions in blood vessels.

In type 2 diabetic patients the degree of red cell aggregation was related to body mass index and inversely to duration of diabetes, possibly because the shorter the duration of disease the less the extent of weight loss and thus the less likely that good glycaemic control had been established. Triglyceride level tends to be higher in newly diagnosed diabetics (229) and in this study triglyceride was inversely related to duration of diabetes ($r=-0.30$, $p<0.001$).

When the relationship between hypertension and red cell aggregation was studied no effect was observed in type 1 diabetic patients, although the number of patients with hypertension in this group was small. However in type 2 diabetic patients red cell aggregation was higher in hypertensive patients and was positively correlated with blood pressure. Hypertension has been associated with rheological disturbance in non-diabetic subjects (160,177) and one previous study has found whole blood viscosity at low shear rates to be higher in hypertensive type 2 diabetic patients (185) which would be in keeping with the findings for red cell aggregation in the present study. Hypertension is common in type 2 diabetes (160,179) and is a cardiovascular risk factor in diabetes (91). The increased red cell aggregation of diabetes and of hypertension may be additive and relevant to the increased incidence of macrovascular disease in hypertensive diabetics. In a study of non-diabetic patients cardiovascular disease was shown to correlate with red cell aggregation level and it was suggested that red cell aggregation may be a prognostic indicator for ischaemic heart disease (230). In support of this hypothesis this study found that red cell aggregation was higher in hypertensive type 2 diabetics with macrovascular disease compared with hypertensive type 2 diabetics who had no evidence of macrovascular disease, although the numbers in each group were small. Triglyceride and VLDL levels were higher and HDL levels lower in the hypertensive diabetics, and although the difference was not significant when compared with the normotensive patients, possibly due again to the relatively small number of patients with hypertension, increased lipids in hypertensive diabetics may nevertheless be a contributing factor to the enhanced red cell aggregation.

Hypertension has also been demonstrated as a risk factor for microvascular complications (182,184) but the number of patients with hypertension and

microvascular disease was too small to allow comparison of red cell aggregation between patients with and those without hypertension. However it was possible to examine the association of red cell aggregation with microvascular disease in both type 1 and type 2 diabetics. Within the type 2 patients there was no difference in red cell aggregation whether microvascular disease was present or not, the extent of aggregation being higher in all groups compared with type 1 diabetics with similar complications. Type 1 diabetics on the other hand appeared to have increased red cell aggregation in the presence of proliferative retinopathy but there was no additive effect of nephropathy. Ditzel's study showed similar results (133), and it is only possible to speculate why the increasing severity of microvascular damage is not associated with increased red cell aggregation - perhaps alterations in plasma proteins due to protein loss in nephropathy prevent excessive aggregation.

7.6 Summary

Red cell aggregation is increased in diabetic patients and is influenced by the type of diabetes. The greater extent of aggregation in type 2 diabetics per se seems to override the increased red cell aggregation of microvascular disease seen in type 1 patients. Aggregation in diabetes is related to plasma lipids and the higher lipoproteins in type 2 patients may account for the increased red cell aggregation in this group. High levels of lipoproteins may also contribute to the increased aggregation seen in type 2 hypertensive diabetics. The increased incidence of macrovascular disease in diabetes could be related to increased red cell aggregation leading to alterations in blood flow which in combination with structural changes in blood vessels (e.g. due to atheromatous plaques) result in

tissue hypoxia and subsequent organ damage. The increased triglyceride and VLDL levels in diabetes may therefore indirectly contribute to vascular complications by an effect on red cell aggregation. Thus overweight type 2 diabetics with hypertriglyceridaemia (particularly with low HDL levels) are at the greatest risk of complications related to increased red cell aggregation and patients with concomitant hypertension will be further compromised.

To investigate these findings further, longitudinal studies of red cell aggregation in diabetic patients with hypertension and vascular disease are required and it would be of interest to measure red cell aggregation in diabetic patients with different types of hyperlipoproteinaemia.

CHAPTER 8

FILTERABILITY OF WHITE BLOOD CELL SUBPOPULATIONS IN DIABETES

8.1 Introduction

White blood cells have a similar diameter to red blood cells (6-7 μm- lymphocytes, 7-7.5 μm- granulocytes, compared to 7.5 μm for red cells) but deform less readily than red blood cells due to their greater intracellular viscosity (about three times that of red blood cells) and spherical shape (158), Table 8.1. They can however pass through capillaries with diameters as small as 2.6-2.8 μm as with red cells, but deformation takes longer and the force required is about four to five times that necessary to deform a red blood cell (30).

Rheological influence of white blood cells on flow resistance is negligible in large vessels because of the low white cell concentration in normal blood (7 million/ml i.e. 1/700 of red cells) and they account for < 1% of the total volume of blood cells and contribute little to the bulk viscosity of blood. It is in capillaries with diameters less than 5 μm that the white cell exerts its greatest effect on flow resistance (231) and where rheological properties of different white cell subpopulations are most important.

White blood cell subpopulations differ in their rheological properties as shown by both filtration studies and micropipette studies (31,32,232), mononuclear cells being less deformable than polymorphonuclear cells, and monocytes being less deformable than lymphocytes.

White blood cells have a large effect on the distribution of red cells at capillary level and the interaction of red and white blood cells will thus determine oxygen delivery to tissue cells. Entrance of a white cell into a capillary branch will increase the resistance and decrease the flow in that branch causing preferential entry of the subsequently arriving twenty to

Table 8.1

Rheological properties of neutrophils and erythrocytes

	erythrocytes	neutrophils
Time constant for initial deformation (msec)	20 - 120	650
Cellular viscosity (Pa.s)	7	1300

From: Chien S. White blood cell rheology: Clinical Blood Rheology (ED GDO Lowe) 1988:96

thirty red cells into another branch. As red blood cells leave capillaries to enter venules they overtake white cells forcing the latter towards the vessel wall (233) and because the post-capillary venules have the lowest shear stress in the circulation (234) the rouleaux formation from red cell aggregation will also tend to displace the white cells towards the vessel wall (33). There are therefore two mechanisms which cause margination of white cells. At the vessel wall white cells may interact with the endothelium to cause adhesion which will be important for extravasation, but which could also cause narrowing of the vessel diameter and a further increase in flow resistance by large immobile white cells (34), this is particularly the case with granulocytes (44). In addition white cell adhesion and activation in ischaemia could lead to endothelial damage due to the release of toxic oxygen compounds and proteolytic enzymes (33).

An increase in white blood cell count or a decrease in white cell deformability could further reduce flow in microvessels and lead to tissue hypoxia, and as the flow rate is reduced the leucocyte-related obstruction increases in a cycle which may lead to capillary closure.

In diabetic patients interaction between the disturbed microcirculation and alterations in white blood cell rheology may be one mechanism leading to vascular complications. Previous studies of white cell deformability in diabetic patients have involved filtration of total white cell suspensions through Nuclepore filters and increased clogging of the filter, an indirect measure of deformability, has been reported by some investigators (116,132). However no study has attempted to measure the deformability of white cell subpopulations in diabetic patients and therefore we sought to determine if deformability of Mononuclear (Monocytes + lymphocytes, MNL) and Polymorphonuclear (PMNL) subpopulations was altered in type 2 (non-insulin-dependent) diabetic patients compared to non-diabetic control subjects.

8.2 Patients

8.2.1 *Diabetic patients*

19 type 2 (non-insulin dependent) diabetic patients (12 male and 7 female) were selected from the outpatient clinic. Mean age was 56 yrs with a range of 39-73yrs, and all were free of diabetic complications. 12 patients were taking oral hypoglycaemic agents in addition to dietary therapy and 2 patients were receiving dietary therapy alone. Duration of diabetes in these 14 patients was 5 yrs (range 6 mths - 11 yrs). The remaining 5 patients were newly diagnosed and therefore receiving no treatment.
Mean blood glucose level was 14.3 mmol/l (range 4.0-25.0 mmol/l) and glycated haemoglobin 12.9% (range 8.5-17.1%).

8.2.2 *Control subjects*

The diabetic patients were compared with 19 healthy control subjects who were matched for age (mean age 58 years, range 43-73 years) and sex distribution (11 male and 8 female).

8.4 Methods

8.4.1 *Blood samples*

Venous blood was anticoagulated with EDTA (1.5 mg/ml) and 5 mls of whole blood was layered on top of 1 mls Ficoll-Hypaque solution 2 which was layered on top of 4 mls Ficoll-Hypaque solution 1 to achieve separation of the white cell subpopulations (chapter 3.7.2). The Ficoll-

Hypaque solutions were made within the laboratory. The mixture was centrifuged at 200g, 20°C for 40 minutes after which the two layers of white cells were harvested and made up to a concentration of 10^5/l in phosphate buffer saline/bovine serum albumin and used immediately.

Filterability of each white cell subpopulation was measured by pressure generated (cm H_2O) during 6 mins of constant flow of cell suspensions (1.5ml/min) through 5µm pores in a polycarbonate membrane (Nuclepore), relative to the pressure generated by prefiltration with buffer-albumin alone (usually about 1 cm H_2O). Pressures were analysed at 1 min intervals and a time dependent increase in pressure was observed due to progressive plugging of pores by slowly-passing white cells (Figure 8.1).

Differential white cell counts were measured by Coulter-S-Counter, glycated haemoglobin (HbA1) by agar gel electrophoresis (Corning-Glytrac), and blood glucose by the hexokinase method (Hitachi 737, Boeringer Mannheim, FRG).

8.4.2 *Statistical methods*

Differences in mean values between the groups were calculated by Mann-Whitney U-test and correlations by Spearman rank test.
Values are expressed as mean ± SD, or range.

8.5 Results

Relative filtration pressures were significantly higher for MNL compared with PMNL in both diabetic patients (6.96 ± 2.67 vs 4.24 ± 1.75 cmH_2O,

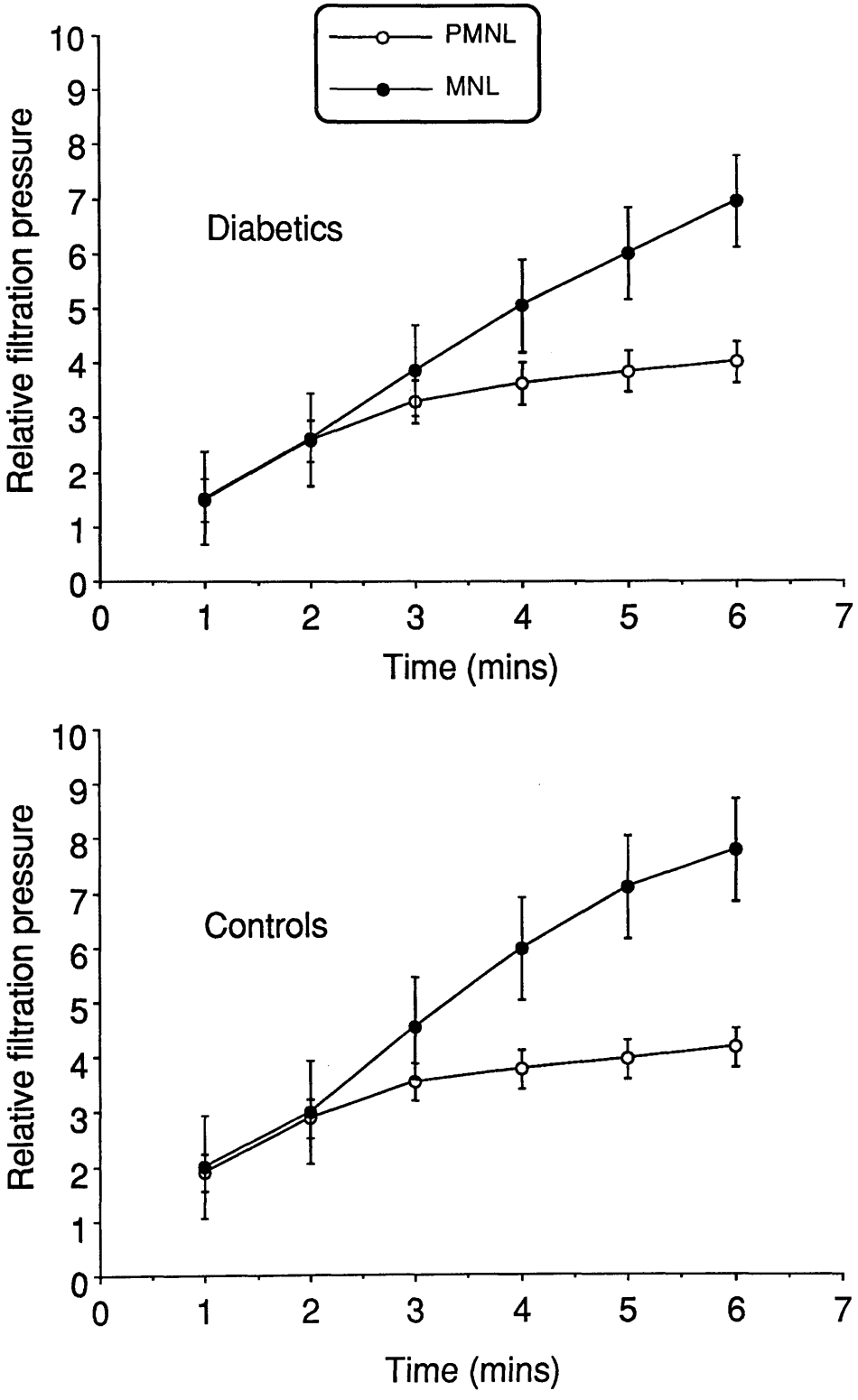


Figure 8.1

Relative filtration pressures (from 1-6 mins) of mononuclear and polymorphonuclear cells in diabetic and control subjects

$p < 0.001$) and control subjects (7.75 ± 1.62 vs 4.20 ± 0.76 cmH_2O, $p < 0.001$), (Figures 8.1 and 8.2).

There was no significant difference in either MNL or PMNL filtration pressures between the diabetic patients or the control subjects at 6 mins (6.96 ± 2.67 vs 7.75 ± 1.62 cm H_2O, $p>0.05$ and 4.24 ± 1.75 vs 4.20 ± 0.76 cmH_2O, $p>0.05$), (Figure 8.2).

A significant inverse correlation of HbA_1 and fasting blood glucose with MNL filtration pressure was found ($r = -0.74$, $p<0.001$ and $r = -0.76$, $p<0.02$), and with PMNL filtration pressure ($r = -0.71$, $p<0.001$, and $r=-0.46$, $p<0.05$), (Figure 8.3). No correlations were found between either MNL or PMNL filtration pressures and duration of diabetes, but there was a weak correlation of age and mononuclear filtration ratio in the population as a whole, Table 8.2. The effect of diabetic therapy on white cell deformability was not examined due to the imbalance of patients on oral hypoglycaemic treatment compared with dietary therapy in the previously diagnosed diabetic patients, and there was no association with sex, or smoking in the group as a whole, Table 8.3.

The correlation between percentage monocytes for individuals and MNL filtration pressures did not reach the conventional level of significance, ($r = 0.40$, $p>0.05$).

8.6 Discussion

Using this improved separation method for white blood cell subpopulations, mononuclear cells were found to be significantly less deformable than polymorphonuclear cells, a finding that partly reflects the more rigid monocytes which form a significant percentage of mononuclear cells,

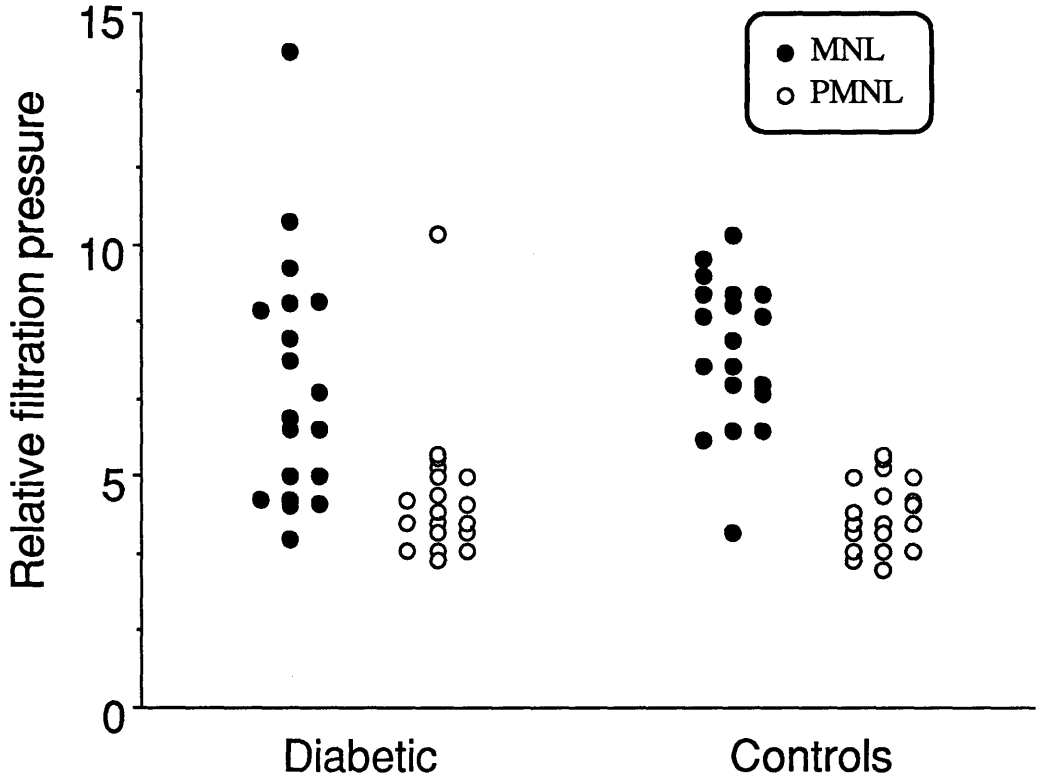


Figure 8.2

Final filtration pressures (at 6 mins) for MNL and PMNL in diabetic patients compared to control subjects.

Table 8.2

Correlation of mononuclear and polymorphonuclear white cell subpopulations with glycaemic control and duration of diabetes. (r_s values)

	mononuclear	polymorphonuclear
Fasting blood glucose (mmol/l)	-0.76[a]	-0.46[b]
Glycated haemoglobuin (%)	-0.74[a]	-0.71[a]
Duration of diabetes (yr)	-0.06	-0.18

[a] $p < 0.001$
[b] $p < 0.05$

Table 8.3

Effect of gender and smoking on mononuclear and polymorphonuclear relative filtration pressures.

		mononuclear	polymorphonuclear
Sex	Male	7.49±2.42	4.52±1.53
	Female	7.17±1.93	3.78±0.08
Smoking	Yes	8.29±2.84	4.52±1.99
	No	7.09±1.83	3.95±0.86

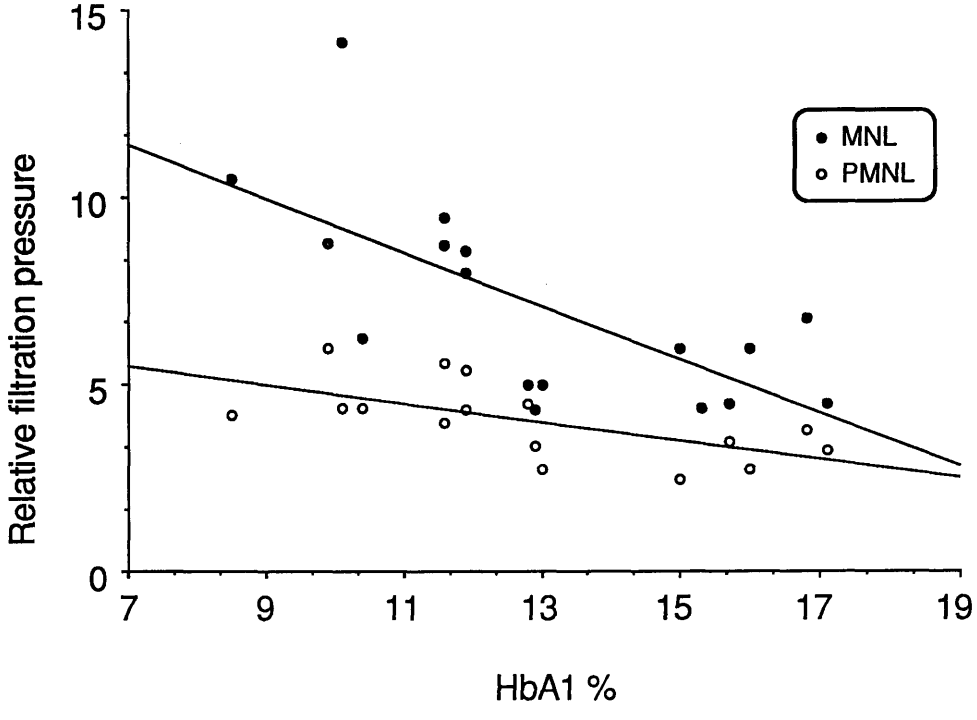


Figure 8.3

Correlation of glycated haemoglobin with mononuclear and polymorphonuclear filtration pressures.

although there was only a poor correlation of filtration pressure with percentage monocytes in whole blood (r=0.40). This finding is in agreement with the results of previous studies of white cell deformability (31,32,233), but in contrast to the two previous filtration studies of white cell deformability in diabetic patients (116,132) no differences were found for either white cell subpopulation studied between diabetic patients or non-diabetic control subjects.

When white blood cells become activated they develop protopods: these are sheet like projections formed at the cell membrane which are a gelation of actin-like and myosin-like proteins. Protopods increase the resistance to deformation at the sites where they form (158) and thus a greater filtration pressure will be required if white cells become activated.

There was an interesting correlation of filtration pressures with glycaemic control in the diabetic patients, particularly with mononuclear cells; well-controlled diabetics had less filterable white blood cells than non-diabetics, whereas poorly-controlled diabetics had more filterable cells. It is possible that the cells become activated during the extraction process, but since samples from both groups were analysed simultaneously the same degree of activation would be expected in all cells. The more activated the cell the greater the pressure required for filtration through micropores, and as diabetic patients with the worst glycaemic control had the most easily deformed cells it may be that these cells were less able to be activated than cells from well-controlled diabetic patients. These findings could have implications for other white cell functions such as chemotaxis and phagocytosis.

Alterations in microvessel geometry in diabetic patients in vivo (i.e. endothelial proliferation and capillary narrowing which are widespread in

diabetic microvascular disease) will have further effects on leucocyte flow. White blood cells being less deformable than red blood cells would be expected to have a major effect in regulating red blood cell flow and distribution in microvascular disease. In addition, the alterations in the vessel wall per se could exert an adverse effect on the white cells by increasing the tendency to adhesion. The combination of these effects may contribute to tissue hypoxia and therefore end-organ damage in diabetes.

8.7 Summary

This study did not detect any differences in white cell deformability between diabetic patients and non-diabetic control subjects, but the significant association of filtration pressure of mononuclear and polymorphonuclear subpopulations with glycaemic control suggests that uncontrolled diabetes may have an effect on white cell function. Further studies of the association between white cell deformability and glycaemic control are indicated, using techniques that avoid cell activation, and also to examine the relationship of diabetic complications to white cell deformability.

It would be of interest to measure white cell deformability in type 1 diabetic patients, to investigate the effects of insulin therapy and glycaemic control.

CHAPTER 9

DISCUSSION

Diabetes mellitus is a metabolic disorder characterised by a high level of plasma glucose, and is associated with specific microvascular complications such as retinopathy and nephropathy. Affected individuals have an earlier onset of macrovascular disease, with increased morbidity and mortality from large vessel complications. A combination of blood flow abnormalities and alterations in vessel geometry probably result in reduced perfusion and tissue oxygenation. Increased blood viscosity has been demonstrated in diabetes and recently attention has been focussed on the contribution of cellular properties to abnormal rheology in diabetic patients. Red blood cell rheology has been examined by a variety of methods and attempts have been made to correlate this with glycaemic control, with treatment and duration of diabetes and with vascular complications. The results of many studies have been conflicting but whole blood viscosity appears to be increased in diabetes due to an increase in plasma proteins which cause an increase in plasma viscosity and red cell aggregation.

Previous methods of measuring red cell aggregation and red cell deformability were non-specific and thus unsuitable for assessing diabetic patients to confirm the extent of the increase in red cell aggregation or the reduction in red cell deformability suggested by viscosity studies. Using the new Myrenne red cell aggregometer, increased red cell aggregation has been confirmed and for the first time a relationship between aggregation and plasma lipoproteins has been demonstrated. A reduction in red cell deformability in diabetic patients has also been confirmed using the new Carri-Med filtrometer.

The findings in this thesis show rheological differences between type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetic patients: red cell deformability was reduced to a greater extent than in type 2 diabetic

patients, whereas red cell aggregation was further increased in type 2 diabetics compared with type 1 diabetics. Abnormalities in the red cell membrane related to insulin deficiency probably have an important effect on red cell deformability and one would expect these to be more pronounced in type 1 diabetics. The association of red cell aggregation with VLDL, the lipoprotein which is most consistently raised in type 2 diabetes, may explain the increased aggregation in type 2 compared with type 1 diabetics.

Whole blood viscosity was increased in type 2 hypertensive diabetic patients, and because viscosity correlates with left ventricular mass (a determinant of cardiovascular complications) in non-diabetic hypertensive patients, this may help to explain the increased cardiovascular morbidity in diabetes. The results of the studies in red cell aggregation reported in type 2 hypertensive diabetic patients in the thesis also support the role of increased viscosity contributing to cardiovascular problems in this subgroup of hypertensive diabetic patients. It would be relevant to investigate in future studies the relationship between blood viscosity and cardiac hypertrophy in diabetic patients. Altered lipids and lipoproteins in type 2 patients may partly explain the abnormal rheology and the cause of the earlier onset of large vessel disease in diabetes as a whole.

Microvascular complications tend to be more prevalent in type 1 diabetic patients due to their longer duration of diabetes, and increased blood viscosity has been demonstrated in type 1 diabetics with microvascular disease in the viscosity studies described here. This finding is supported by the increased red cell aggregation which was found in type 1 diabetics with proliferative retinopathy. Alterations of red cell deformability in either microvascular or macrovascular disease have not been demonstrated in

any of the studies undertaken. However a reduction in red cell deformability although not related to specific problems in diabetes could in conjunction with microvascular abnormalities reduce blood flow to tissues and result in ischaemic damage to the retina, kidney, nerves or heart. The association between rheology and macrovascular complications such as ischaemic heart disease, peripheral vascular disease and cerebrovascular disease needs to be established in diabetic patients.

White cell deformability is an important determinant of blood flow in the microcirculation and because red cell deformability is reduced in diabetes, one may speculate that deformability of white cells would also be reduced. The preliminary study described here found no difference in white cell deformability between diabetic patients and healthy age matched controls, but only a small group of type 2 diabetics was examined. The technique therefore needs to be evaluated in a much larger sample group which should include both type 1 and type 2 diabetic patients, and this would allow any association of white cell deformability and vascular complications to be investigated.

A microvascular aetiology in conjunction with metabolic dysfunction is likely in diabetic peripheral neuropathy, but although blood rheology is undoubtably abnormal in affected diabetics, it appears not to be further increased in diabetic patients with neuropathy compared with those who have no evidence of neuropathy but are matched for other microvascular complications. Disturbance in microvessel structure may be a more important determinant of vascular related damage to peripheral nerves in diabetic patients.

This thesis presents evidence that diabetes mellitus is associated with abnormal blood rheology, and that there are differences in the determinants of blood viscosity between type 1 and type 2 diabetic patients. There was a measurable association between rheology and microvascular complications in type 1 diabetics and between rheology and hypertension in type 2 diabetics. The data was all cross-sectional, and to establish the true role of blood rheology in the complications of diabetes mellitus, it would be necessary to perform longterm prospective studies. Further research in the future is indicated to assess blood viscosity and red cell aggregation in particular, in hyperlipidaemic individuals with and without diabetes and to assess the effects of lipid-lowering agents on rheology. In hypertensive diabetics, early intervention with antihypertensive agents such as vasodilators may reduce blood viscosity and could prevent cardiac hypertrophy. The effect of antihypertensive agents on rheology therefore requires to be established.

Therapeutic agents which improve red cell deformability may have beneficial effects in diabetic patients, but they would require evaluation over a prolonged period of time to assess their potential benefits in vascular complications.

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