ALTERED LYMPHOCYTE RHEOLOGY
IN NON-OBESE DIABETIC MICE

By

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ABSTRACT

Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

ALTERED LYMPHOCYTE RHEOLOGY IN NON-OBESE DIABETIC MICE

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Major Department: Biomedical Engineering

Understanding the rheological behavior of white blood cells (WBCs) is essential not only for the comprehension of microcirculatory flow dynamics, but also for the understanding of their functions and behaviors in health and disease. Rheological properties, together with the WBCs structural characteristics, determine the deformability of the cell, a characteristic which is crucial for the leukocytes to assure their role. In diabetic patients, the role of leukocyte dysfunction in the recurrence and increased frequency of infection has been suggested. Many immune disturbances are noted in the blood vessels of diabetics. Diabetic patients are more prone to vascular diseases of both small and large vessels: microangiopathy and atherosclerosis respectively. The overall aim of this work is to examine the specific rheological changes in lymphocytes from mice with diabetes in an effort to define the mechanisms of white blood cell dysfunction in this disease. In the present study, the rheological properties of passive lymphocytes from non-obese diabetic
(NOD) and healthy control mice are measured by micropipette manipulation. The state of activation of the lymphocytes, another important factor for cell flow and function is also quantified. We found that the passive lymphocytes from mildly diabetic NOD mice exhibit an altered rheology. The cortical tension to viscosity ratio was significantly higher in lymphocytes from mildly diabetic mice than from controls. Moreover, the diabetic mice’s lymphocytes had a significantly higher tendency toward spontaneous activation. This tendency was accentuated with the severity of diabetes, measured by the glucosuria. The significant increase in the number of activated lymphocytes is not to be neglected. Indeed, when leukocytes are activated they are less deformable, and their adhesion to the endothelium is promoted. These behaviors may play a role in the pathogenesis of vascular diseases generally found in diabetics.
CHAPTER 1
INTRODUCTION

Understanding the rheological behavior of white blood cells (WBCs) is essential not only for the comprehension of microcirculatory flow dynamics, but also for the understanding of their functions and behaviors in health and disease. Rheological properties, together with the WBCs structural characteristics, determine the deformability of the cell, especially during large deformations, such as those involved in their release from the bone marrow or their extravasion into the interstitium.

If the WBCs are by far outnumbered by the red blood cells (RBCs), the leukocytes however, by virtue of their larger volume and higher rigidity, have been shown to strongly influence microvascular blood flow.

In diabetic patients, the role of leukocyte dysfunction in the recurrence and increased frequency of infection has been suggested [32]. Many immune disturbances are noted in the blood vessels of diabetics. Diabetic patients are more prone to vascular diseases of both small and large vessels: microangiopathie and atherosclerosis respectively [34]. It is a severe condition since in diabetics, accelerated atherosclerosis, an inflammatory mediated occlusive disease of arteries [42], drives the vascular disease that results in strokes, heart attacks, renal failure, and limb loss.

Objectives

The overall objective is to find out if leukocytes from diabetic subjects exhibit different rheological properties than those from control donors. Our hypothesis is that diabetes is
characterized by an altered leukocyte rheology which could play a role in the increased frequency of vascular based pathology reported among diabetics.

Because of the large variation seen in the rheological data from our preliminary study with diabetic patients, a Non-Obese Diabetic (NOD) mouse model is used. In diabetic patients, other parameters than the presence of diabetes may affect the rheological properties of the leukocytes (e.g. hypertension, smoking habits, etc.). This can make the analysis of the results difficult since it would be hard to tell from which parameter the variations would come from without studying a numerous number of patients. The advantage of the NOD mouse animal model is that the mice can have the diabetes without having any other disease, moreover the smoking habit is no more an issue. For all these reasons, the NOD mouse animal model has been used.

**Plan of Action**

The rheological properties of the lymphocytes are characterized with the recovery experiment technique. Specifically, based on a liquid-drop model, we will calculate the rheological ratio $T_\theta/\mu$ for NOD and control mice’s lymphocytes.

The state of activation of the samples has also been investigated by counting the proportion of leukocytes activated. Activated leukocytes will be recognized anatomically by the presence of pseudopodia on their membrane. It is important to gather information relative to cell activation. Indeed the rheological properties that we measure concern only passive cells, whereas activated cells are known to be more rigid.

Finally, statistical analysis will be performed to evaluate if diabetic lymphocytes have impaired rheological properties.
CHAPTER 2
SIGNIFICANCE

Research has been aimed at clarifying the pathophysiology of atherosclerosis and vascular disorders with two main identifiable trends: one which considers abnormality in the vessel wall as the primary cause, the second which considers that vessel dysfunction is secondary to change in circulation of blood components. The two are intimately linked, because changes in physiology of vascular endothelial cells, for instance, depend on local flow forces and on interaction with blood leukocytes. The concept that we believe in is that changes in the rheological behavior of leukocytes can contribute to the genesis of vascular diseases, especially in diabetes.

The leukocytes can play their role in terms of their adhesion to the vascular endothelium, but also in terms of their capability to deform and flow through the vessels. Moreover, a central issue is cell activation, since it influences both the mechanical and adhesive properties of the cell.

Impaired WBCs rheology might significantly contribute to microcirculatory flow abnormalities jeopardizing O2 exchange in the terminal vascular bed. The pathogenesis of diabetic microangiopathy is still unclear, but there is general agreement that, in addition to vascular components, hemorheological disturbances can play a role in the impairment of microvascular flow. The major involvement of polymorphonuclear (PMN) cells in the genesis of angiopathy has been suggested by several teams [17,40]. In the development of diabetic retinopathy, capillary occlusion is an early event, and white blood cells have been shown to be involved [47]. Shroder et al [45] have shown that monocytes and granulocytes cause capillary obstruction, and endothelial cell damage, in a rat model of diabetic retinopathy.
In diabetes, leukocytes have been reported to be more activated [45], resulting in an increased rigidity of the cell. When increased PMN rigidity is associated with activation, the risk for microvascular injury may be enhanced. Indeed, activation of the cells promotes their adhesion to endothelium, thus adherent PMN may degranulate in situ, releasing various potent cytotoxic agents capable of significant local tissue destruction.

Whole blood viscosity and erythrocyte rheology in diabetes have prompted numerous studies. Much less is known, however, about the rheological behavior of leukocytes in diabetes. Hemorheological studies in diabetes have been recently carried out using filtration techniques. But the results, relative to alterations of blood cells rheological properties remain somewhat controversial. If the filtration technique gives a measurement of blood rheology, one cannot assess the respective roles played by adhesion to the filter and deformability of the cells in this experiment. Since cell adhesion has been shown to be increased in diabetes [8], and the activation of the WBCs could further increase this adhesion tendency, another approach to measure cell deformability needs to be used. The micropipette experiment technique offers the solution. With this method, individual WBCs are studied, and problems related to activated and/or adhesive cells are directly identified. Thus, the capacity of a passive WBC to deform is recorded, and analyzed, giving the rheological properties of the cell.
CHAPTER 3
BACKGROUND

Diabetes Mellitus

A) Diabetes Mellitus – A Still Devastating Disease

Diabetes is a serious disease whose prevalence is increasing in the United States. The symptoms and consequences of the disease have been well described, but an understanding of the pathophysiology is lacking. The following data pertain specifically to the United States of America:

- In 1992, 169,000 persons died from diabetes, or its complications with an overall cost of 98 billions dollars. In 1995, diabetes was the 7th leading cause of death listed on US death certificate.
- Today not only are there 10.3 millions of patients diagnosed with diabetes mellitus (DM) in the United States, but the number of undiagnosed cases is estimated at 5.4 millions.
- In general, people do not die directly from diabetes but from its complications or other diseases associated with DM.

Diabetics are at risk for:

- Amputation: more than ½ of lower limb amputations in United-States occur among people with diabetes.
- Blindness: diabetic retinopathy (18,000 new cases each year) is the leading cause of blindness.
- Kidney disease: Diabetes is the leading cause of end-stage renal disease. In 1995, 99,000 people underwent dialysis or kidney transplant, secondary to diabetic nephropathy.
- Heart disease: It is the first cause of diabetes-related deaths. Heart disease rates are about 2 to 4 times higher in people with diabetes as that of adults without diabetes.
- High blood pressure: 60% of people with diabetes have high blood pressure.
- Stroke: The rate of stroke is 2 to 4 times higher in people with diabetes.

**B) The Metabolism of Glucose**

The body relies on glucose as a primary source of fuel for organ functions. Therefore, an adequate supply of glucose is needed by the whole body at all times. The body gets the glucose it requires via the ingestion of food. After the food is enzymatically digested into nutrients, it is then absorbed into the bloodstream, where it is available for the body’s cells to use.

Cellular energy is derived directly from glucose. Carbohydrates are broken down and the energy released is stored as ATP (Adenosine Tri-Phosphate) molecules during a process called catabolism (Glycolysis and Citric acid cycle). If the amount of glucose entering cells is inadequate for their energy needs, cells manufacture ATP by an alternate pathway: they catabolize fats or proteins.

When the amount of glucose in the blood is more than needed by the cells, it is transformed into glycogen in a process called glycogenesis. The glycogen is then stored in the liver for further use. Later on, when the amount of glucose is inadequate to satisfy the metabolic needs of the cell, glycogen is broken apart into glucose via the glycogenolysis process, so that it can be made available and catabolized by the cell.

All the basic steps involved in the glucose metabolism are summarized in the figure 1.
1) Control of Glucose Metabolism

The complex mechanism that normally maintains homeostasis of blood glucose concentration consists of hormonal and neural mechanisms. At least five endocrine glands: pancreatic islets, anterior pituitary gland, adrenal cortex, adrenal medulla and thyroid gland and at least eight hormones secreted by these glands function as key parts of the glucose homeostatic mechanism. The figure 2 shows the main effects, and releasing sites of the hormones involved in glucose homeostasis.

The following section will focus on the pancreas and the main hormones it releases, namely: insulin and glucagon.
2) The Endocrine Part of the Pancreas.

The pancreas is an elongated gland (12 cm long) located in the abdomen that weighs up to 100 grams. The head of the gland lies in the beginning of the duodenum, with its body extending horizontally behind the stomach. The tissue of the pancreas is composed of both endocrine and exocrine tissues. The endocrine portion is made up of scattered, tiny islands of cells, called islets of Langerhans or pancreatic islets, which account for only 2% of the total mass of the pancreas. Each of the one to two million of pancreatic islets contains a combination of four types of endocrine cells. One type of pancreatic islet cell is the alpha cell, which secretes the hormone glucagon. Beta cells secrete the hormone insulin; delta cells secrete the hormone somatostatin, and F or PP cells secrete pancreatic polypeptide.

Glucagon, produced by the alpha cells increases blood glucose levels by stimulating the conversion of glycogen to glucose in liver cells. It also stimulates gluconeogenesis (transformation of fatty acids and amino acids into glucose) in hepatocytes. The glucose produced
via the breakdown of glycogen and by gluconeogenesis is released into the bloodstream, producing a hyperglycemic effect. Insulin, produced by beta cells promotes the movement of glucose, amino acids, and fatty acids out of the blood and into tissue cells. Hence, insulin tends to lower the blood concentrations of these substances and to promote their uptake and metabolism by tissue cells. Somatostatin inhibits the secretion of glucagon, insulin, and pancreatic polypeptide, and growth hormone. A simplified regulation of blood glucose levels, based only on insulin and glucagon is summarized in the figure 3.

Fig. 3. Blood glucose regulation.
Source [29] p 568

The presence of adequate amounts of insulin, and sufficient sensitivity to insulin are key to the entry of glucose into cells.
C) Diabetes Mellitus – An Overview

DM is a chronic disorder characterized by abnormalities in the metabolism of carbohydrate, protein and fat; it is often accompanied after a period of time by specific microvascular, macrovascular, and neuropathic complications. It is now recognized that diabetes mellitus encompasses a group of genetically and clinically heterogeneous disorders in which glucose intolerance is a common denominator. Thus, although DM affects the metabolism of all body fuels, its diagnosis depends upon identification of specific plasma glucose abnormalities.

At present, the term DM is applied to disorders characterized by fasting hyperglycemia or plasma glucose levels above defined limits during oral glucose tolerance testing.

In adults, the diagnostic of DM is restricted to those who have one of the following:

- A random plasma glucose level of 200mg/dL or greater, plus classic signs of diabetes such as polyuria\(^1\), polydipsia\(^2\), polyphagia\(^3\) and weight loss.
- A fasting plasma glucose level of 140mg/dL or greater on at least two occasions.
- A fasting plasma glucose level of less than 140mg/dL plus sustained elevated plasma glucose levels during at least two oral glucose tolerance tests.

Note that these criteria are slightly different for children and pregnant adults.

For comparison, the following table gives the fasting plasma glucose levels of subjects considered as healthy or in a impaired glucose tolerance state (data are for non pregnant adults only).

---

\(^1\) Because the blood glucose levels are supranormal, glucose spills over into the urine. Thus, increased glucose concentration in the urine causes an osmotic diuresis: the increase solute load draws water into the urine causing large volume of water to be lost, and this state is referred as polyuria.

\(^2\) The lost of water results in an excessive ongoing thirst, a state referred as polydipsia.
Table 1. Plasma glucose levels and corresponding classification

<table>
<thead>
<tr>
<th></th>
<th>Fasting plasma glucose level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological. (Healthy state)</td>
<td>&lt; 110 mg/dL</td>
</tr>
<tr>
<td>Subject with Impaired Glucose</td>
<td>&gt; 115 mg/dL but too low to satisfy the conditions required for</td>
</tr>
<tr>
<td>Tolerance (IGT)</td>
<td>Diabetes Mellitus diagnosis</td>
</tr>
</tbody>
</table>

Individuals who have plasma glucose levels that are higher than normal but lower than those with DM are considered to have impaired glucose tolerance (IGT). It has been shown that about 25% of patients with IGT eventually develop DM. There are three different mutually exclusive subclasses of DM, each of them having distinguishing characteristics:

- Type I or IDDM: Insulin-dependent diabetes mellitus, juvenile onset DM
- Type II or NIDDM: Non-Insulin-dependent diabetes mellitus, adult onset DM
- Other types.

1) Type I:

Patients with IDDM have severe insulinopenia and are prone to the development of ketoacidosis. Commonly, they are lean and have experienced recent weight loss. By definition these patients are dependent upon exogenous insulin to prevent ketoacidosis and death. Although IDDM may occur at any age, the major peak of onset occurs at about 11 years, and nearly all patients diagnosed before age 20 are of this type. Genetic factors are probably important because there is a clear association between IDDM and certain histocompatibility locus antigens (HLA) on chromosome 6. On the other hand, twin studies indicate that only 50% of identical twins of type I patients develops the disease. In some cases, IDDM may be an autoimmune disease, as suggested by the observation that the majority of patients have circulating islet cell antibodies at the time of diagnosis.

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3 Even if the blood sugar is high, the cells are deprived of glucose to burn as energy, resulting from an insufficient insulin state. Thus, people with diabetes suffer from intense and continuous hunger, a situation called polyphagia.
The development of IDDM can conceptually be divided into 6 stages, beginning with genetic susceptibility and ending with complete beta-cell destruction. The first stage is genetic susceptibility. Some genetically susceptible subjects have a hypothetical triggering event (stage 2), and active autoimmunity develops (stage 3). Initially, even patients with immunologic abnormalities have normal insulin secretion. During stage 4, immunologic abnormalities persist but glucose stimulated insulin secretion is progressively lost, despite normal blood sugar levels. In stage 5, when overt diabetes is recognized, some residual insulin secretion remains, but it is followed by complete beta-cell destruction (stage 6).

There are reasons to believe that T-lymphocytes are the major contributors to beta-cell destruction. Patients treated with cyclosporine (a drug blocking the activation of T-lymphocytes) showed an improvement in glucose homeostasis and no longer required treatment with insulin.

The treatment of IDDM involves:

- A proper diet, exercise
- Home blood glucose testing and insulin injections several times a day.

2) Type II:

Patients with NIDDM may have few or none of the classic symptoms of DM when first discovered. Although the symptomatology of NIDDM is less obvious than that of IDDM, this type of diabetes also is accompanied by vascular and neuropathic complications. In patients with NIDDM, insulin levels may be normal, mildly depressed, or elevated. Typically, insulin resistance (decreased tissue sensitivity or responsiveness to exogenous and endogenous insulin) is present. Unlike IDDM subjects, patients with NIDDM are not prone to development of ketoacidosis except during periods or conditions of stress, such as those caused by infections, trauma or surgery. This type of diabetes can occur at any age but usually, it is diagnosed after age 40, and is strongly associated with obesity. By definition, patients with NIDDM are not
dependent upon exogenous insulin for survival. Insulin may be required at times for control of transient, stress-induced hyperglycemia or hyperglycemia that persists in spite of other therapy. Intake of excessive calories leading to weight gain and obesity is probably an important factor in the pathogenesis of NIDDM. NIDDM is a heterogeneous disorder characterized by impaired beta cell function and diminished tissue sensitivity to insulin.

♦ Impaired Insulin Secretion

In normal subjects there are two phases of insulin release:

- An early phase that occurs within the first 10 minutes following glucose ingestion and that represents the release of insulin stored within the beta cells
- A later phase of insulin secretion that represents newly synthesized insulin

In individuals with IGT and fasting blood glucose levels of less than 115mg/dL (physiological), the total plasma insulin response following oral or intravenous glucose administration is normal or, more often elevated. Then, if for this type of individual, the fasting plasma glucose concentration exceeds 115mg/dL, the early phase of insulin secretion is lost or become markedly diminished, and the late phase of insulin secretion remains normal, or more often, is increased. The impairment in early insulin secretion has important physiological consequences. When the early surge of insulin release is inhibited, the portal vein insulin concentration remains low and hepatic glucose production is not suppressed. Continued endogenous output of glucose by the liver, supplemented by glucose entering the circulation via the gastrointestinal tract, leads to excessive hyperglycemia, which, in turn, leads to enhanced secretion of insulin during the hours following glucose ingestion. Eventually, the plasma glucose concentration will return to normal, but only at the expense of the resultant late hyperinsulinemia.

♦ Insulin Resistance

It is now well established that insulin resistance is present in the vast majority of individuals with IGT and in essentially all patients with NIDDM who have fasting blood glucose levels of
140mg/dL or greater. The severity of the insulin resistance is positively correlated with the elevation in fasting plasma glucose concentration.

In the most general sense, the action of insulin involves two processes:

- First, insulin binds to a specific receptor located on the cell surface.
- Second, this interaction activates a series of intracellular events, including enhanced glucose transport and stimulation of a variety of intracellular enzymatic pathways.

The primary cause of insulin resistance in patients with IGT or mild fasting hyperglycemia appears to be decreased insulin binding (insulin is the most important factor involved in the regulation of the number of its own receptors), whereas postbinding abnormalities (marked decrease in glucose transport, and other intracellular processes involved in glucose metabolism) are primarily responsible for insulin resistance in diabetic patients with significant fasting hyperglycemia (>160mg/dL).

The treatment of NIDDM consists of 3 modalities:

- Dietary modification
- Increased physical activity
- Pharmacologic intervention with either an oral hypoglycemic agent or insulin, or both

**D) Complications in Diabetes Mellitus**

The major tissues affected by diabetes (the retina, the kidney, and the nerves) are all freely permeable to glucose. Thus, increases in blood glucose concentration increase the intracellular accumulation of both glucose and its subsequent metabolic products.

Diabetic patients are twice as prone as nondiabetic individuals to die from coronary heart disease. It is prudent to recommend efforts to lower plasma lipid and glucose levels and to control
hypertension. The latter being particularly important in terms of its benefits for reducing risks of microvascular (retinopathy, nephropathy) and macrovascular (atherosclerosis) disease.

In the diabetic patient, accelerated atherosclerosis involving the coronary, cerebrovascular, and peripheral vessels occurs at an early age and with greater frequency than it does in nondiabetics patients.

The main complications of DM will now be discussed in detail.

1) Diabetic Retinopathy

More than 80% of all patients with diabetes have some form of retinopathy 15 years after diagnosis. Diabetic retinopathy does not cause visual symptoms until a fairly advanced stage has been reached, usually either macular edema or proliferative retinopathy.

There are 2 types of diabetic retinopathy:

- Background diabetic retinopathy, including a more advanced form called preproliferative diabetic retinopathy.
- Proliferative diabetic retinopathy.

♦ Background Diabetic Retinopathy (BDR)

The earliest stage of BDR, which first occurs about 5 to 7 years after diagnosis, is recognized during ophtalmoscopic examination of the retina by the detection of microaneurysms\(^4\) and retinal hemorrhages. This level of retinopathy should be expected in almost all patients with diabetes of 25 years duration. In many cases it does not progress. The most common causes of visual impairment in diabetic patients with background retinopathy are macular edema and hard exudates at or near the macula\(^5\). In these cases, leakage of fluid from abnormal vessels near the maculae disrupts the path of light to the maculae and results in loss of visual acuity.

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\(^4\) Section of a vessel that has become abnormally widened because of a weakening of the vessel wall.

\(^5\) Area of the retina which is off center.
The more advanced stage of BDR: preproliferative diabetic retinopathy is characterized as follow. The abnormal vessels, with increasingly severe retinopathy, can become occluded, leading to retinal ischemia with infarctions in the nerve layer of the retina, seen as soft exudates.

♦ Proliferative Diabetic Retinopathy (PDR)

The incidence of PDR among patients who have diabetes for 15 years or more is 30%. The final and most vision-threatening stage of diabetic retinopathy is characterized by neovascularization on the surface of the retina, sometimes extending to the posterior vitreous humor. This development of new vessels is a spontaneous response to ischemia. This condition is dangerous because such new vessels are prone to bleed, if bleeding into the preretinal space or vitreous occurs, the patient is likely to report floating objects or “floaters” in the field of vision. The patient who has a major retinal hemorrhage will experience a sudden and painless loss of vision. The current treatment for PDR is photocoagulation, which is used to stop neovascularization before recurrent hemorrhages into the vitreous cause irreparable damage.

2) Diabetic Renal Disease.

Diabetic nephropathy is a leading cause of end-stage renal disease, accounting for nearly one third of all new cases. The incidence of end stage renal disease is nearly 30% in IDDM patients, and ranges from 4 to 20% in NIDDM patients.

The incidence of diabetic renal disease 20 years after diagnosis in patients with NIDDM is about 5 to 10 %. Nephropathy is the diabetes-specific complication associated with the greatest mortality. Although a small fraction of patients with IDDM who have nephropathy may die of uremia, the majority die of concurrent cardiovascular disease, the risk of which is 30 to 40 times that in patients with IDDM who do not have nephropathy.
Usually, proteinuria\(^6\) is the first indication of renal disease. Thus the level of urinary albumin excretion assesses the severity of the diabetic renal disease. (The higher the level, the more severe the disease becomes). In DM, there are a number of conditions that worsen impairment of renal function, such as, hypertension, neurogenic bladder or the administration of nephrotoxic drugs. Therefore, the four approaches to the prevention of renal disease are control of hyperglycemia, treatment of hypertension, restriction of dietary protein, and avoidance of nephrotoxic dyes or drugs. It has also been shown that antihypertensive drugs such as angiotensin-converting-enzyme (ACE) inhibitors prevent the progression of renal disease both in patients with IDDM and in those with NIDDM, even in the absence of hypertension [23].

3) Diabetic Neuropathy

Diabetic neuropathy is one of the most common complications of diabetes mellitus and has a myriad of clinical presentations. Acute hyperglycemia decreases nerve function and chronic hyperglycemia is associated with the loss of myelinated and unmyelinated fibers. Reduced nerve conduction has also been shown in diabetes. A peripheral, symmetric sensorimotor neuropathy in a “stocking/glove” distribution is the most common form of diabetic neuropathy, although autonomic neuropathy can also affect the patients. Autonomic neuropathy can cause decreased gastric or intestinal motility, erectile dysfunction, bladder dysfunction, and impaired cardiac function. Whereas sensorimotor neuropathy is mainly characterized by a loss of sensation in the hands and especially in the feet.

4) Diabetic Foot Problem.

More than 50% of the nontraumatic amputations in the US occur in persons with diabetes. Foot lesions in people with DM are generally the result of peripheral neuropathy, peripheral vascular disease, superimposed infection, or, more usually, a combination of these complications. Usually foot lesions are found in feet that are insensate, deformed, or ischemic. Hyperglycemia is

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\(^6\) Abnormal presence of protein in the urine, due to a damaged glomerular membrane.
almost invariably associated with mild defects in nerve conduction, and the feet are highly susceptible to becoming insensitive. Neuropathy may also lead to a deformed foot secondary to tendon shortening, which leads to decreased mobility of toes. The combination of foot insensitivity and foot deformities allows undue stress on small areas of the foot and promotes the development of foot ulcers. Infection is a frequent complication of both vascular and neuropathic ulcers.

5) Metabolic Problems

The two major metabolic problems in patients with NIDDM are nonketotic hyperglycemic hyperosmolar coma and hypoglycemia.

♦ Nonketotic Hyperglycemic Hyperosmolar Coma

Nonketotic hyperglycemic hyperosmolar coma is the most common in older patients with NIDDM. It is life threatening, often fatal, and occurs spontaneously in persons with undiagnosed DM, or in known diabetic patients after long periods of uncontrolled hyperglycemia. The precipitating factors range from the use of potentially hyperglycemia-inducing agents, surgical procedures, or other associated acute or chronic diseases and conditions such as infection.

The major clinical features are:

- Severe hyperglycemia (>600mg/dL)
- Absence of or slight ketosis
- Plasma or serum hyperosmolality (>340mOsm/L)
- Profound dehydration

♦ Hypoglycemia

Occurring in both types of DM, hypoglycemia is often precipitated by excessive insulin administration. It can also occur when diabetic patients are exposed to potentially hypoglycemic agents, and when they engage in intensive exercise. The symptoms are the one indicative of a
cerebral response to inadequate blood glucose levels (coma, changes in behavior), as well as the adrenegic responses to low blood sugar (tachycardia⁷, increased sweating, and hunger). The plasma glucose level is usually less than 60mg/dL.

♦ Ketoacidosis.

In IDDM patients, insufficient levels of insulin causes the body to break down stored fats and proteins for energy needs. As fats are broken, the body converts these fats into ketones. If blood ketones levels rise too high, a life threatening condition called ketoacidosis can develop. Left untreated, ketoacidosis may results in severe acidosis, hyperglycemia and subsequent coma.

6) Atherosclerosis

Atherosclerotic occlusive vascular disease is the most common complication of diabetes especially in NIDDM patients [49]. The lesions of atherosclerosis represent a series of highly specific cellular and molecular responses that can have many characteristics of an inflammatory disease. These lesions occur principally in large and medium-sized arteries and can lead to ischemia of the heart, brain, or extremities, resulting in infarction, stroke or peripheral extremity ischemia. The arteries of diabetic patients accumulate plaque slowly, often over years. During this time, patients remain asymptomatic. Gradually the process accelerates until finally, vascular occlusion occurs and ischemia and tissue death results.

The Immune System

A) The Immune System – An Overview

The immune system is the system which is in charge of the defense of the body against infectious agents such as bacteria, viruses, toxins, and tumor cells. Its primary role is to destroy and eliminate these invading organisms and any toxic substances produced by them.

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⁷ Very rapid heart rhythm.
All the different mechanisms used by the immune system to ensure the integrity of the internal environment can be categorized into one of two major categories of immune mechanisms:

- Nonspecific immunity
- Specific immunity

The term nonspecific implies that these immune mechanisms do not act on only one or two specific invaders, but rather provide a more general defense by simply acting against anything recognized as not self. The specific immunity, on the other hand, involves mechanisms that recognize specific threatening agents (antigen) and respond by targeting their activity against these agents and these agents only. As we can see, the ability to distinguish foreign molecules from self molecules is a fundamental feature of the immune system.

The primary cells responsible for immunity are white blood cells (WBCs) known as leukocytes. They normally are found in large numbers ($2 \times 10^{12}$ in man) in the blood, the lymph and in specialized lymphoid tissues, such as thymus, lymph nodes, spleen, and appendix.

There are five types of leukocytes, classified according to the presence or absence of granules and the staining properties of their cytoplasm. Granulocytes, or polymorphonuclear leukocytes include the three WBCs that have large granules in their cytoplasm (basophils, neutrophils, eosinophils), whereas the remaining kind of WBCs are called agranulocytes, or mononuclear leukocytes (monocytes, lymphocytes) because they do not have granules in their cytoplasm and their nucleus contain only one lobe.
Table 2. The leukocytes and their characteristics

<table>
<thead>
<tr>
<th>Category</th>
<th>Type of immunity</th>
<th>Cell type</th>
<th>Picture</th>
<th>Description</th>
<th>proportion in WBCs population</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes - polymorphonuclear (PMN)</td>
<td>Non specific</td>
<td>Basophil</td>
<td><img src="image" alt="Basophil" /></td>
<td>11-14 µm in diameter; spherical shape; generally two-lobed nucleus</td>
<td>0.5 to 1%</td>
<td>Mobile and capable of diapedesis. Secrete heparin and histamine.</td>
</tr>
<tr>
<td>Granulocytes - polymorphonuclear (PMN)</td>
<td>Non specific</td>
<td>Neutrophil</td>
<td><img src="image" alt="Neutrophil" /></td>
<td>12-15 µm in diameter; spherical shape; multilobed nucleus; “coarse” appearance cytoplasm</td>
<td>65%</td>
<td>Highly mobile, and very active phagocytic cell that can migrate out of blood vessels and enter tissue spaces by diapedesis.</td>
</tr>
<tr>
<td>Granulocytes - polymorphonuclear (PMN)</td>
<td>Non specific</td>
<td>Eosinophil</td>
<td><img src="image" alt="Eosinophil" /></td>
<td>10-12 µm in diameter; spherical shape; generally two-lobed nucleus</td>
<td>2 to 5%</td>
<td>Their most important functions involve protection against infections caused by parasitic worms and involvement in allergic reactions.</td>
</tr>
<tr>
<td>Mononuclear - agranulocyte</td>
<td>Specific</td>
<td>Monocyte</td>
<td><img src="image" alt="Monocyte" /></td>
<td>12-17 µm in diameter; spherical shape; nucleus generally “horseshoe” shaped with convoluted surface; ample cytoplasm</td>
<td>4 to 7.5%</td>
<td>Mobile and highly phagocytic cell capable of engulfing large bacterial organisms and viral infected cells.</td>
</tr>
<tr>
<td>Mononuclear - agranulocyte</td>
<td>Specific</td>
<td>Lymphocyte</td>
<td><img src="image" alt="Lymphocyte" /></td>
<td>6-9 µm in diameter; spherical shape; round big (single lobe) nucleus</td>
<td>25%</td>
<td>Humoral defense. Secretes antibodies against specific antigens;</td>
</tr>
</tbody>
</table>

Note that the number of leukocytes per microliter of blood is of the order of seven thousands.

B) Life of the Leukocytes

Leukocytes develop from pluripotent hemopoietic stem cells, which give rise to all of the blood cells, including erythrocyte, leukocyte, and platelets. The stem cells are located primarily in the liver (in fetuses) and bone marrow (in adults).

Most of lymphocytes come from stem cells, which migrate from hemopoietic tissues via the blood to the thymus, where they proliferate and differentiate into lymphocytes. Then after being formed in the thymus, the lymphocytes migrate via the blood to the peripheral lymphoid tissue such as the lymph nodes, and spleen. It is in this peripheral lymphoid tissue that they react with foreign antigens.
The great majority of lymphocytes continuously circulate between the blood and lymph. They leave the bloodstream, squeezing out between specialized endothelial cells found in certain small veins, and enter various tissues, including lymph nodes. After percolating through a tissue, they accumulate in small lymphatic vessels, passing into larger vessel, the lymphocyte eventually enter the main lymphatic duct, which carries them back into the blood. This continuous recirculation presumably ensures that the appropriate lymphocytes will come into contact with antigen and serves to disperse the resulting activated cells to lymphoid tissues throughout the body. Most of the lymphocytes die within days or weeks after forming.

The granulocytes however, circulate in the blood for only a few hours before migrating into the connective tissue, where they reside somewhat longer. For example, neutrophils survive a few days after leaving the bloodstream and then die. Monocytes, by contrast, can persist as macrophages outside the bloodstream for months or perhaps even years.

The leukocytes can be found in vivo in two different states: passive and active state. In the passive state they show a spherical shape, and deform only in response to external applied stress such as plasma fluid stress or adhesive stress to endothelium. In the circulation, most leukocytes remain in the passive or resting state whereas they convert to the active state during emigration across the endothelium into the interstitium [28], migration in a chemotactic gradient [57], and phagocytosis [10]. Then, the active state is characterized by a spontaneous deformation of the cell which projects pseudopodia.

So far, it is obvious that the ability to deform is an essential feature for leukocytes. This ability is needed not only during the phagocytosis process, or the diapedesis: when the cells have to squeeze between endothelial cells to leave the bloodstream, but also when they flow into microcapillaries, and during their release from the bone marrow.
C) Structure of the Leukocytes

The structure of the WBCs is of importance, because the rheological properties of the leukocyte are a manifestation of the cell underlying structure and the organization of its structural component. Leukocytes are the only nucleated cells present in the blood. Basically it is composed of a lipid bilayer membrane which encloses the cytoplasm, and the nucleus. The lipid bilayer membrane is highly ruffled, giving an excess surface area to the leukocyte comparing to a sphere of the same volume. This feature permits deformation at constant volume. The cytoplasm is mainly a water-like fluid in which organelles can be found. Polymorphonuclear leukocytes have many granules in their cytoplasm. One of the components present in the cytoplasm, which is of interest, is the cytoskeleton. The cytoskeleton is a cytoplasmic network that maintains cell shape and effects cell motion. The molecular composition of the microfilamentous cytoskeleton in leukocytes is clearly described by Southwick and Stossel [48]. The cytoskeleton consists of:

- Microfilaments. (5-8nm thick, mainly composed of actin but also contains myosin, tropomyosin and α-actinin)
- Microtubules. (24nm in diameter with a hollow 15nm core, are composed of subunits of tubulin)
- Intermediate filaments. (fibrous polypeptides such as desmin)

Actin, an ubiquitous protein in all eukaryotic cells is the major component of the cytoskeleton. It is the most abundant structural protein in the human neutrophils [55]. It is a 43-kd globular protein that can condense to form F-actin. F-actin can then serve either a force-generating role by interacting with myosin, or a structural role through crosslinks formed with other structural proteins like actin-binding protein [22]. Actin appears to be largely confined to a thin cortical filamentous meshwork, and no actin-like structures are evident in the region between this cortex and the nucleus [46]. Microtubules, more numerous near the nucleus, radiate outward from the centrosome (near the nucleus) to the cell membrane in fine lace like threads [2].
As shown on the figure 4, Epi-fluorescence microscopy enables to visualize the distribution of microtubules within the cell.

**D) Leukocytes and Rheology.**

As it has been explained earlier, the ability of a leukocyte to deform and to flow in capillaries and/or to migrate in the tissue plays a critical role in many medically relevant conditions. This ability is essential to the cell in its response to diseases and infections (where the leukocytes have to deform, squeeze and flow through pores). This ability is determined by the cell’s rheological properties, which are a manifestation of its underlying structure and the organization of the structural component (F-actin, microtubules, intermediate filament, lipid bilayer) in cellular cytoplasm, nucleus and cortex. Since 1977, researchers have been trying to describe the rheological\(^8\) properties of leukocytes. Early studies involved the partial aspiration of neutrophils into a micropipette \([44]\), while later studies included the total aspiration of the cell into a pipette and/or its recovery after aspiration.

The neutrophils, which are the most abundant (in humans) and studied leukocyte, have been shown to exhibit viscoelastic properties. These viscoelastic properties are essentially liquid-like in character during low rates of deformation and elastic-like during high rates of deformation. Several models were used to characterize the cell behavior, including both liquid and solid

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\(^8\) In this section, the rheological properties concern only leukocytes in their passive state.
models [5,9,13,44]. None of these models is fully acceptable since they only reproduce the cell behavior for given ranges of deformation (high or low rates of deformation, given extent of deformation...). For instance, Evans and Kukan [13] considering the leukocyte as a liquid, noticed that as the rate of deformation increases, the cell viscosity is found to decrease. This has been speculated to be due to a shear-thinning effect [35].

When a cell is deformed into a micropipette and then expelled, it deforms spontaneously to recover its original geometry. This process called recovery is due to the rheological properties of the cell itself such as the cortical tension present in the cortex of the cell, or some elastic properties. It has been shown [51] that when the neutrophil is held inside the pipette for a short time (less than 5 seconds), and then expelled, it recovers faster than when held for a longer time. This behavior has been attributed to initial rapid recoil of the cell, when held for a short time. A correct rheological behavior can be obtained only if the major structural components are represented in the cell model. Kan et al. [18] found that in order to understand the rheological behavior of WBCs, it is essential to have information on the deformation of the cell nucleus. Tran-Son-Tay et al. [19] gave experimental evidence that the nucleus plays an important role in the deformation/recovery of the lymphocyte. In order to account for the nucleus, Kan et al. [18] developed the compound model to study the rheology of the lymphocytes. In this model the outer layer is the cortical region surrounding the second layer (the cytoplasm) and the third layer is the nucleus. The role of other cell structures on the rheological properties of the cell has been recently studied. Tsai et al [53], by breaking the microtubules of the cell with some chemicals (Colchicine, paclitaxel), have shown that the microtubules do not play an important role in the mechanical properties of the cell.

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9 Since it has a one-lobe almost spherical nucleus, the lymphocyte is the easiest leukocyte to modelize.
However, Tsai et al [52] and Ting-Beall et al [50] have shown, by disrupting the actin network with cytochalasin B and D, respectively, that actin plays a major role in the neutrophils rheologic behavior, especially in the cortex of the cell.

**Diabetes and Leukocytes Rheology**

The most common techniques used for studying diabetic blood rheology, are based on filtration (the parameters measured being the transit time through pores, the clogging particle on the filter membrane and the index of filtration). These techniques cannot differentiate if a longer transit time is due to the cell rheological properties or an adhesion of the cell to the filter membrane. On the other hand, these techniques are easy and quick to carry out, and moreover a high number of cells may be studied at the same time.

Most of the published studies concern whole blood, or erythrocyte suspensions, but not WBC sub-populations suspensions. Moreover NIDDM has been far more investigated that IDDM. Only a few studies on the rheology of mononuclear WBCs from diabetic patients have been reported. Kelly et al. [21] found that the mononuclear leukocytes of diabetic cats were less filterable than those of normal cats. Perego et al. [39] found a modest and statistically non-significant decrease in the flow rate of NIDDM mononuclear leukocyte passing through a filter. Some of the most recent studies include application of these filtration techniques to WBCs suspensions or PMNs suspensions.
The major findings are that RBC and WBC suspensions from diabetic patients show a longer transit time to pass through pores than control samples. Moreover, it has been observed that leukocytes sometimes clog the filter pores.

Table 3. Published Results on diabetic blood rheology.

<table>
<thead>
<tr>
<th>Research team</th>
<th>Lopresti et al. [25]</th>
<th>Mac Rury et al. [26]</th>
<th>Pecsvarady et al. [38]</th>
<th>Rong et al. [41]</th>
<th>Miyamoto et al. [31]</th>
<th>Ernst et al. [12]</th>
<th>Vernes et al. [54]</th>
<th>Perego et al. [39]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension type</td>
<td>PMN</td>
<td>PMN</td>
<td>PMN</td>
<td>PMN</td>
<td>PMN</td>
<td>PMN &amp; MN</td>
<td>PMN &amp; MN</td>
<td>PMN &amp; MN</td>
</tr>
<tr>
<td>RBC</td>
<td>no</td>
<td>no</td>
<td>+</td>
<td>+</td>
<td>no data</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RBC + WBC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WBC</td>
<td>no</td>
<td>no</td>
<td>+</td>
<td>+</td>
<td>no data</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RBC</td>
<td>no</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RBC + WBC</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WBC</td>
<td>no</td>
<td>no</td>
<td>no data</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(Filtration techniques were used by all of them)

As we can see in table 3, the results in terms of cell rigidity and clogging particle are controversial as well as the findings concerning the correlations between cell rigidity and clinical factors. To conclude, the decreased deformability of WBCs in diabetes is uncertain, since not only the results are controversial, but the techniques used are also unable to control the activation parameter, nor they can differentiate between diabetic leukocytes being less deformable, more adhesive, or both.

Two studies have used micropipette aspiration technique to characterize the deformability of PMN in NIDDM. By measuring a decrease in the aspirated PMN tongue length at constant pressure, both studies showed that the deformability of PMNs was less in NIDDM. Nevertheless, only cytoplasm is aspirated so the nucleus of the cell is not accounted for in the measurement.
These two studies let us think that the PMN deformability from NIDDM patients may be impaired, but it is difficult to be certain since only part of the cell is characterized.

To date, no study has looked at the rheological characteristics of diabetic cells, especially mononuclear cells from IDDM subjects. There is still a need to know if the leukocyte impairment in DM comes from a tendency toward activation and adhesion or from the rheological properties of the cell themselves.

On the comparison between the filtration and the micropipette techniques, Wautier et al [56] remind us that the micropipette manipulation is a complex and time-consuming methodology, but is also the only one that can allow to measure the structural viscoelasticity of individual cells.

The NOD Mouse Animal Model

As explained earlier, the main advantage of using an animal model resides in the fact that one can choose an animal which presents diabetes and only diabetes. Thus, while comparing between the diabetic animal population and the control population, the only difference between these two populations is the presence or absence of diabetes.

A) Presentation of the NOD Mice Strain

The animal model of diabetes that we are using is the Non-Obese Diabetic (NOD) mouse. In 1980, Makino et al. [27] discovered this strain of mouse, after spontaneous mutations arose from breeding noninbred ICR mice. For the first time, a large number of genetically identical NOD mice could be produced for large-scale studies. This type of mouse has been shown to develop IDDM.

It has been found that the NOD mouse has a genetic susceptibility to IDDM, with the major locus being found within the major histocompatibility (MHC) region on chromosome 17. In specific immunity, the MHC is the molecule that presents on the surface of antigen-presenting cells self and foreign antigen to activate lymphocyte T cells or promote self tolerance depending upon the nature of the antigen. The particularity about the NOD mice is that they present defects
in self tolerance in that these mice develop autoreactive lymphocyte T populations that destroy
the pancreatic beta cells in charge of the production of insulin.

At 5 weeks, the NOD mouse start to develop insulitis that is the infiltration of beta cell
destructive cells in the pancreas. At first, the insulin secretion is sufficient, until more and more
beta-cells get destroyed and the DM onset occurs (around the 20th week depending on the
mouse). Then, it takes 3 to 4 weeks for the mild hyperglycemia to become severe.

One must keep in mind that diabetes is not an obligatory consequence of insulitis
development. For example, for the female NOD mice we have been using, the diabetes incidence
is on the order of 90% by 10 months of age. That is the reason why, some of the mice we used did
not present any glucosuria at all.

To summarize: The mice we worked with can be sorted in 4 groups:

- NOD mice whose glucosuria is 100mg/dL, referred to as NOD100
- NOD mice whose glucosuria is over 2000mg/dL, referred to as NOD2000
- NOD mice whose glucosuria was 0mg/dL, referred to as NOD0
- B6xsv129 control mice, referred to as B6.

The NOD100 mice are the best models of IDDM patients, they are mildly diabetic. The
NOD2000 however, would represent untreated IDDM patients who do not take exogenous
insulin. The NOD0 presence can be explained by the fact that even if the NOD mouse strain is
genetically predisposed to DM, a portion of them will never develop the disease. Moreover, one
must keep in mind that some of these mice can still be very mildly diabetic: their glycemia can be
outside of the normal range but low enough not to excrete any glucose.

**B) Blood Composition: Human vs. Mouse**

One may wonder if there are any major differences in blood composition between human
and mouse. The table 4 gives some insight on this issue.
No data have been found concerning the B6xsv129 strain, yet the Parkes, ICR, and Yellow Ay strains give an idea of the blood counts values for typical strain considered as controls in many studies. However, one must be cautious, since the composition of mouse blood has been observed to be significantly influenced by genetic strain.

The overall picture is that the concentration of erythrocytes, and platelets seems to be higher in mouse blood. Concerning the leukocytes, the proportion of lymphocytes is higher in mouse than in humans, even if Kataoka et al have shown that it is decreased in 12 week old female NOD mice, as compared to ICR mice of the same sex and age.

Table 4. Comparative Blood Cell Counts in Human and Mouse.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Parkes</th>
<th>Regular</th>
<th>Yellow Ay</th>
<th>NOD</th>
<th>ICR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit</td>
<td>%</td>
<td>44.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC Count</td>
<td>M/µL</td>
<td>4.2 - 6.2</td>
<td>8.47</td>
<td>8.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WBC Count</td>
<td>K/µL</td>
<td>4.5 - 11.0</td>
<td>10</td>
<td>5.34</td>
<td>6.42</td>
<td>6.38</td>
</tr>
<tr>
<td>Platelet Count</td>
<td>K/µL</td>
<td>300</td>
<td>1339</td>
<td>1090</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differential WBCs count</td>
<td>%</td>
<td>65</td>
<td>11.6</td>
<td>14.3</td>
<td>25</td>
<td>84.2</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td>10</td>
<td>4.2</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other WBCs</td>
<td></td>
<td>4.2</td>
<td>3.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell diameter</td>
<td>µm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td>12.0 - 15.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td>6.0 - 9.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Source</td>
<td></td>
<td>a</td>
<td>b</td>
<td>b</td>
<td>c</td>
<td>d</td>
</tr>
</tbody>
</table>

Sources: a: From [3], b: From [43], c: From [20], d: lymphocyte diameter from the present study.
Comments: b: females, 4-6 months of age, blood collected by cardiac puncture, data collected from 130 samples, c: females, 3 months of age, data collected from 6 samples.

These trends are in agreement with our observation. In the mouse study, as opposed to the human study, we were unable to extract efficiently the neutrophils by density gradient centrifugation; moreover the numerous platelets present in the final cell suspension have also been a problem encountered specifically in the mouse study.
CHAPTER 4
MATERIALS AND METHODS

Blood Collection and Preparation.

The mice study has been performed with blood from NOD mice and B6xsv129 mice (common strain of laboratory mouse which is here used as controls). These mice are bred and taken care of at the Malcom Randall Veteran’s Affair Medical Center of the University of Florida.

Because the study is based on passive leukocytes, everything is done so that the WBCs do not become activated. For this purpose, the environment of the cells has to be as physiological as possible. The removal from their native plasma, the presence of anticoagulants, the resuspension in buffers, centrifugation, depletion of second messengers and other factors, are all associated with a shift in the state of cell activation [56]. That is why, attention has to be paid to each chemical, to all materials, which are going to be in contact with the suspension of cells throughout the process.

Moreover, if the blood has been withdrawn a few hours ago, the cells are expected to activate spontaneously. For that reason, the blood was withdrawn right before the experiment took place. The mice blood was withdrawn by cardiac puncture: while the mice is under anesthesia (Florane gas is used), its chest is surgically opened, the heart is punctured with a heparin flushed needle, and as much blood as possible is aspired into the syringe. Depending on the cases, we have been able to collect between 0.5 and 1.0mL of blood. The blood is then transferred into a 1.5mL Eppendorf centrifuge tube coated with 50 µL of Heparin (1000 units/1mL), and directly transported to the biorheology laboratory, where the leukocytes are separated
from the whole blood. The mice blood samples are not carried on ice since a study brought to our attention that rewarming blood increases integrin leukocyte expression [15].

As soon as the blood sample is received at the biorheology laboratory, it is diluted in an equal volume of suspending solution (Hank’s Balanced Salts Solution Ca$^{2+}$ and Mg$^{2+}$ free modified HBSS H9394, Sigma Chemical Co.). The batch has been specifically chosen from the manufacturer so that, its pH is 7.4 (blood pH is within the [7.35 - 7.45] range) and its osmolality is 280 mOsm/kg H$_2$O, which is within the physiological blood range (280-296mOsm/kg H$_2$O).

The severity of the diabetes of the NOD mice is assessed by measuring the concentration of glucose in their urine using reagent strips for urinalysis (Diastix, Bayer AG, Germany). Glucose is not present in the urine of healthy (non-diabetic) mice, however diabetes is accompanied with glucosuria.

**Preparation of the White Blood Cells.**

A) Separation of the White Blood Cells.

The whole blood contains 1 leukocyte for 1,000 erythrocytes. That is the reason why looking for the leukocytes in whole blood under microscope would be tedious and inefficient. The goal of the separation process is first to get rid of the erythrocyte and as many platelets as possible, but also to specifically extract the lymphocytes. The technique used is the one described by English and Andersen in 1974 [11]. The blood is diluted with equal volume of HBSS and carefully layered over Ficoll-Hypaque gradients (Histopaque 1077 and 1119 from Sigma chemical Co) having densities of 1.077 and 1.119, respectively at 25C. After 20 minutes centrifugation at 800g and room temperature, the cells are trapped between the layers of gradients as shown on the schematic below. Then, they are washed twice with 10x volume of HBSS, and the final cells are suspended in 50% autologous plasma/HBSS. The layer of granulocytes was not clearly visible while separating mice leukocytes, the PMN being mixed with the erythrocytes at the bottom of the tubes. This might be due to the fact that the fraction of neutrophils is smaller in mice than in
humans, and this protocol was originally designed for human blood. For that reason, and because of their relatively simple anatomy, only lymphocytes have been studied. Sterile 2mL microcentrifugation tubes (Eppendorf) were used for the separation process. The figure 6 shows the protocol used.

![Diagram of the protocol to separate human leukocytes from whole blood]

**Fig. 6.** Protocol to separate human leukocytes from whole blood

**B) Dye of the Nuclei.**

One can use an UV-excited, blue fluorescent dye (Hoechst 33342 or bisbenzimidazole) to dye the nucleus of leukocytes. It has been introduced in flow cytometric cell cycle analysis of living mammalian cells by Arndt-Jovin and Jovin [4]. It is a DNA-specific fluochrome when
bound to sequences of three A-T base pair in DNA. It requires exposure of cells to 5-10µM for at least 30 minutes.

![Figure 7](image_url)

**Fig. 7.** Picture of a cell with its dyed nucleus

During the experiment the dye is excited by an UV light source whenever needed. However, because of the quenching effect of the dye (the intensity of the light emitted by the dye decreases as the overall excitation time increases), cells are only illuminated for short periods of 1 second, and this, as often as possible.

However, at the end of the human study, it has been noticed that the leukocytes are more prone to adhere when the bisbenzimidazole dye is used. No quantitative data have been collected to support this observation. Nevertheless, in a try to increase the proportion of experiment which can be easily analyzed (no adhesion of the WBCs on the micropipette wall) the dye has not been used with the mice samples.

**The Micropipette Setup**

This section will focus on the main experimental setup, which includes the microscope, the micropipette with its positioning system, and the pressure setup (measurement and control).
Fig. 8. The micropipette setup

A) Fabrication of the Micropipette

The micropipette is the most important part of the design. Its geometry is of utmost importance, since the cell deformation ratio depends on it.
The micropipettes are formed from a 1mm outer diameter, and 0.5mm inner diameter, glass capillary tube (A-M Systems Inc, Everett, WA). Then the inner diameter is brought to a few microns enabling the cell to deform while flowing in.

For this purpose, the capillary tube is mounted on a pipette puller (Narishige PB-7, Tokyo, Japan). Mainly the tube is heated, and a load is applied on it, in order to decrease the diameter during the creep. By setting the temperature and the load, one can obtain a micropipette with either a flat or tapered geometry, and the wanted inner diameter. In our case, the 5µm pipettes are built in two steps.

The first one is a 4mm vertical creep of the pipette loaded with two weights at 86% of the maximal temperature of the heater 1. The second step is the heating of the center of the freshly made constriction with the same two weights at a temperature of 74% of the maximal temperature of the heater 2.

![Fig. 9. Picture of the micropipette puller.](image)

In order to have a flat and straight, a microforge is used (Narishige MF-83, Tokyo, Japan). The microforge consists of a horizontal dissecting microscope, a heated platinum wire on which a soda glass bead is located, and a horizontally mounted micromanipulator. The flat pipette tip is made by immersing the newly formed glass capillary into the molten glass bead, which has a
lower melting point than the glass capillary. The melted glass from the bead flows by capillarity into the pipette. Then the bead is allowed to cool. After a few seconds, the pipette is withdrawn and breaks by quick fracture to leave a flat tip. The pipettes have the tendency to fracture exactly at the point where the glass had stopped flowing into the pipette, resulting in flat-tipped pipettes.

The freshly made pipets are then stored in a closed jar to protect them from dust and particles. Right before use, the pipette is rinsed with HBSS containing albumin, using a micro-needle (Microfil MF34G, World Precision Instruments Inc), in order to reduce the likelihood of cell adhesion and to eliminate the presence of bubbles. Indeed, the governing equations of the cell flow are based on a free-slip boundary condition at the interface between the cell and the pipette.

To avoid occlusion by foreign particles and dust, the pipette is filled with the HBSS and albumin solution. If no drop is observed at the end of the pipette, it indicates an obstruction, and the pipette is discarded. Autologous plasma was not used to fill up the pipettes since the volume collected was too small for that purpose.

**B) The Pressure System**

This system connects the micropipette to a circuit containing a physiological solution. It allows aspiration and expulsion of cells in and out of the pipette, and also provides the pressure drop across the pipette as a function of time.

1) Adjustment of the Pressure

![Fig. 10. Micropipette linked to a reservoir](image)
If $P_c$ is the pressure in the chamber (in the cell suspension), and $P_r$ is the pressure in the reservoir, then from basic fluid dynamics, it is known that

- If $P_r < P_c$ then a cell will be aspirated into the micropipette.
- If $P_r = P_c$ then the system is at its equilibrium, no flow occurs.
- If $P_r > P_c$ then the cell will be blown away from the tip of the pipette.

Thus by setting the pressure difference, between the reservoir and the chamber, we can aspirate or expel cells into or from the micropipette. By lowering or rising the reservoir, one can respectively decrease or increase the pressure difference $\Delta P = P_r - P_c$.

Our setup is basically based on this principle, but instead of using one reservoir, 2 reservoirs are used. One is set to the equilibrium pressure $P_r$ (pressure of the chamber, easily recognized since no particles move) and is then not allowed to move. The other one is allowed to move in the z-direction to aspirate or expel the cells. Using two reservoirs, it is possible to switch quickly from an aspiration pressure to the equilibrium pressure, by clamping one of the two tubing lines.

Pressure differences of a few cm H$_2$O can be set quickly with a micrometer and with accuracy smaller than 0.1 cm H$_2$O. If pressure differences have to be set faster, or higher pressures have to be reached, then mouth suction is used. During the study, mouth suction has been the chosen solution to control the pressure.
2) Measurement of the Pressure

For data analysis and modelization it is very important to keep track of the pressure as a function of time. The aspiration pressure has to be constant and reflects the rate of deformation of the cell when it flows into the micropipette.

Differential pressure transducers (Validyne DP 45-14 and DP 15-30, Northridge, CA) are used for this task. One pressure transducer (DP45-14) allows us to measure pressure as small as 1 Pa, which corresponds to a column of 0.1mm of water. The DP15-30, on the other hand, measures higher pressure up to 50cm H₂O.

They are connected to the reference reservoir on one end, which is as the same pressure as the chamber. On the other end they are linked to the adjustable reservoir (same pressure as the one in the micropipette). The electrical signals delivered by the transducers are decoded via a carrier demodulator and the pressure measurements are then displayed on the screen.

C) The Fluorescent Video-Microscopy System

The experimental setup consists of a micropipette, a chamber mounted on a microscope, a water reservoir to control hydrostatic pressure at the tip of the micropipette, and a video system to videotape the experiments.

1) The Micropipette Micromanipulation

A three dimensional micromanipulator (MMN-1, Narishige, Tokyo, Japan) is used to position the pipette in the plan of focus, inside the chamber. An accuracy of a few microns is required to position the pipette closed to the cells of interest; thus, the fine motion is controlled by a hydraulic joystick (MMW-22D, Narishige, Tokyo, Japan).
2) The Chamber

The chamber is made of a 3mm-thick Plexiglas piece with a U-shaped opening. The latter is closed on the top and the bottom by 2 coverslips, and the side opening is left for the micropipette to be inserted. The volume of the thus created chamber is approximately 100µL.

![Schematic of the chamber.](image)

The cell suspension is poured into this chamber with a syringe, and the chamber is placed on the microscope stage. Thanks to the microscope stage, the chamber can be shifted in both the x and y directions and specific areas of the chamber can be visualized.

3) The Microscope

An Axiovert 100 (Zeiss, Germany) inverted microscope with 100x oil immersion objective and 10x eyepieces provides images with an overall magnification of 1000x. The microscope is also equipped with a fluorescent set: a variable intensity mercury arc lamp (HBO 100W Atto arc, Zeiss, Germany) can excite the dyed cell suspension with UV light so that the nuclei emit blue light.
The microscope enables images to be taken with a high sensitivity (0.02 lux) 3 chip video camera with real time digital image enhancement (CC4D Video camera system, ZVS-3C75DE, Zeiss, Germany). The images are displayed on a color TV monitor, and all experiments are videotaped (VCR AG1730, Panasonic).

**Experimental Procedures**

This section describes the time sequence of actions that has been followed during the mice study. Because activation is a major concern, it is important not to waste any time after the withdrawal of the blood. The chamber, pipettes, VCR, and microscope have to be ready before the cells are collected. Some actions are also performed during the centrifugation of the blood samples and the incubation of the cells in the dye (if used).

1. Pressure transducer calibration

   Once in a while, the pressure transducers are calibrated by using 2 vertical tubes and applying a known pressure difference. However, the zero of the pressure transducers is set before each experiment, by measuring the pressure difference between the 2 reservoirs after letting them reach the equilibrium.
2. Micropipette making

Pipettes with the desired inner diameters are made before the blood withdrawal and are stored in a clean container. It is not a good idea to build them days before, because they can get clogged or dusty. Several pipettes are prepared in case a pipette is clogged or breaks inside the chamber.

3. Cleaning

The microscope lenses, chambers, and coverslips need to be cleaned to obtain good quality images and to prevent activation of the cells. Note that in order to clean the lenses, 100% cotton swabs are used to avoid scratching.

4. Setting of the video equipment

The video equipment is switched on, a tape is inserted in the VCR and the title of the experiment is recorded on it. Also, if other users have worked with the microscope, it may be a good idea to check if the condenser light is well focused.

5. Making of the chambers

Clean cover-slips are stuck on both sides of the U-opening of the chamber. The chamber can then be flushed several times with buffer solution. When autologous plasma is available (human study only), the chamber is flushed with this media. After its making the chamber is stored in a location away from any dust.

6. Sample preparation

Right after the blood has been withdrawn, the WBCs are isolated, washed, and suspended in the buffer solution with or without dye. If dye is used, the cells are then incubated at room temperature for 30 minutes, in the dark.

7. Preparation of the micropipette

Once the cells are ready. A freshly made micropipette is filled with a diluted solution of albumin (HBSS) in order to remove all the bubbles from the pipette (autologous plasma has been used for the human study). It is important to check that there is no bubble inside the pipette, since
their presence could compromise the pressure control. If no drop appears at the tip of the pipette, the latter is discarded and the operation is renewed with a new pipette. After the pipette is seen to be bubble-free it is inserted in the pipette holder, and the connected reservoir is raised so that no air can be aspired.

8. Focusing of the sample

At this time only, the solution is poured into the chamber. Then we focus on the cells lying on the bottom of the chamber. The reason why the solution is poured in the chamber only after the pipette is ready is the following. Because of gravity, all the blood elements (WBCs, remaining platelets…) progressively fall down to the bottom of the chamber. The platelets by virtue of their lower weight need more time to fall than the WBCs. By waiting for everything to be ready before pouring the solution into the chamber, one can hope to be able to work on a few WBCs in an almost platelet-free environment. This can be important because in a platelet rich environment the pipette has a tendency to aspirate more easily the platelets than the WBCs and it has been noticed that when a platelet hits violently a leukocyte, the latter shifts toward its activation state.

9. Insertion of the micropipette into the chamber

Next, the pipette holder is placed into the micromanipulator. The pipette is then positioned in the plane of focus (on the bottom of the chamber, where the cells are). Care is taken in order not to break the pipette.

Fig. 15. Positioning of the micropipette over the lens
To do so, the pipette is naked eye positioned in the chamber, away from the bottom of the chamber. Then (fig. 14.1) the pipette is carefully moved back and forth over the lens, and down, until its shadow is visible under the microscope. Next (fig. 14.2) it is pulled toward the right and down toward the bottom of the chamber, so that its tip is focused in the middle of the field of view.

10. Setting of the equilibrium pressure reservoir

To do so, the pipette is placed closed to particles (cells, platelets...) suspended in the chamber and the reservoir is lowered or risen until there is no flow of the particles to or from the pipette.

11. Looking for an adequate cell.

The setup is ready, one can now look for a non-activated (no pseudopodia) circular (The model we are using assumes a spherical shape).

12. Experiment

After both the cell and the micropipette are focused on, the pipette is linked to the adjustable reservoir, by de-clamping it. The cell is aspirated into the pipette at a given aspiration pressure, while trying to maintain the focus, and to flash the cell repeatedly with the UV lamp (if dye is used). The pipette is switched to the equilibrium reservoir, to enable the completely aspirated cell to stay in the pipette. One more time the UV lamp is turned on to visualize the nucleus. After waiting the desired holding time, the cell is gently expelled out of the pipette, while trying to maintain the focus, to keep the cell in the field of view, and to flash it repeatedly with UV radiation. This way the cell is allowed to recover freely to its original shape.

13. Tape visualization

Experiments are recorded for further analysis. For recovery analysis, the cell must constantly be in focus from the beginning to the end of the recovery.

14. Image grabbing
Experiments of interest are grabbed onto a computer. One image is acquired from the VCR each time the length of the cell has visibly changed and the cell is well focused on. Thus, we can approximately get images every 5 seconds. The images are stored on a computer for further measurement.

15. Measurement

The only data we can get from this kind of experiment is the geometry of the cell as a function of time, that is to say, the way the cell deforms. Because the stress applied on the cell is well-characterized (pressure, geometry of the pipette) and because the deformation is observed, one can try to find the constitutive parameters of the cell: its elasticity and viscosity. The length of the cell is measured on each image ($L_{\text{cell}}$), as shown on the schematic below:

\[ 2 \times R_{\text{cell}} = D_0 \]

Fig. 16. Schematic of a lymphocyte and the dimensions measured.

16. Data analysis

Once we have the initial radii of the cell, and its length as a function of time, the aim is to find the constitutive characteristics of the cell. The schematic on figure 17 shows the timeline of an experiment from the cardiac puncture to its end. All mice experiments have been finished within four hours from blood withdrawal.
Results Extraction and Data Analysis.

After watching the experiments tape, the interesting experiments are selected and the images are transferred to a PC with an image grabbing board (Meteor, Matrox, Canada). The software used to grab the sequences of images is Grab and View (The Imaging Source, Charlotte, NC, USA). If the experiment went well in the sense that the cell stays in a passive state, and is focused on throughout the recovery, then it is analyzed.
On the other hand, if the experiment contains any problem, the nature of the problem (activation of the cell, cell out of focus, cell out of the field of view…) is recorded into a file so that the number of experimentally induced activation can be obtained.

Images are analyzed, and measurements are done with an image processing software which has been written under Matlab. Mainly the grabbed images are automatically loaded from their folder and the user can measure the cells lengths in a computer assisted way. As the image of the cell is displayed on the screen, the user points to several points belonging to the cell outer membrane. Then, the software draws a closed cubic spline under tension interpolating the points, which represents the cell membrane. The maximal length of the cell is searched and its axis is displayed so that the user can check its validity. The length is then converted from pixels to microns. The purpose of this code is double. First it diminishes the variability in the measurement due to human judgement. Then when the cell has a quasi-spherical shape, it is more efficient, for it is difficult to see naked eye the axis of the maximal length.

A) The Rheological Parameter $T_0 / \mu$.

The cell rheological properties are determined from the geometry of the cell. The ratio $T_0 / \mu$ is obtained by assuming a liquid drop model for the cell. It is well known that leukocytes behave as liquid drops under special conditions [14,51]. For example it has been shown that when the holding time is higher than 5s, leukocytes behave as liquid drops. It has recently been shown [19] that this liquid drop model is not correct but because of the lack of a comprehensive leukocyte model, recovery experiments are done with a holding time greater than 5s. Even though these values are only qualitative, rheological information about the cell can then be extracted and be used to assess difference between diabetic and normal cells.

The analysis of the recovery experiments uses the approach of Tran-Son-Tay et al. [51]. In this model, the cell interior is modeled as a uniform liquid with a constant apparent viscosity $\mu$; whereas the cell membrane is modeled as a shell under pre-stressed isotropic surface tension.
designated as cortical tension $T_0$. It has to be noticed that the nucleus is not accounted for in this model.

Given the reference state and the initial deformed state, the model tries to predict the dimensions of the cell (length) as a function of time. In order to be able to analyze a large number of cells of different size, its solution has been derived in a dimensionless form as shown:

\[
\frac{L}{D_o} = \frac{L_i}{D_o} + \tilde{A}t + B(t)^2 + C(t)^3
\]

Where $\tilde{t} = t / \left[ \mu / T_0 \right] R_0$

A, B, and C are constants function of $(L_i / D_0)$.

The dimensionless time $\tilde{t}$ is the time $t$ scaled with respect to the product of:

- The ratio of the viscosity over the cortical tension.
- The radius of the cell at its reference state.

---

![Fig. 19. Pictures of the recovery of a dyed lymphocyte](image)

Before aspiration, the free cell is at its reference state (picture 1) assumed to be a sphere of diameter $D_0$. Then, the pipette during the aspiration (picture 2) deforms it. When completely aspirated inside the pipette, the cell is at its initial deformed state, characterized by its length, $L_i$. This initial deformed state is assumed to be a sausage-like shape. Then, the cell is expelled (picture 3), and allowed to recover freely. During this process, the sausage-like shape constantly deforms to go back to its spherical reference state (pictures 4-13). This process can take various times, but the duration is in the order of a minute.
Theoretical values for the length of the cell can be calculated with respect to the
dimensionless time and compared to experimental values. A computer program is written to give
the best fit between the theoretical and experimental data, i.e., the optimal ratio $T_0/\mu$. This
Newtonian liquid drop model has been shown to be valid for large deformation, long holding
time, and low rate of deformation. Also, it has to be stressed that the following conditions have to
be satisfied: $(Di/D0)<3.5$ and $\dot{i}<3.5$. Here is an example of the curve fitting.

![Curve fitting of the experimental measurement of L/D0](image)

Fig. 20. Curve fitting of the experimental measurement of L/D0
These are the data concerning the recovery of a lymphocyte. $(Di/D0)=2.3$, the holding time was 6s, and the aspiration
pressure was 16cm H$_2$O. In this case the optimal ratio $T_0/\mu$ was found to be $20 \times 10^{-6}$ cm/s.

The ratio $T_0/\mu$ is defined as the rheological parameter and is an indicator of cell
deformability.

**B) Assessment of the State of Activation**

During the human study a high percentage of the leukocytes have been observed to be
activated. Only spherical, passive looking leukocytes were aspirated, but sometimes they became
activated either during the aspiration or during their recovery. This type of activation that is
probably the consequence of the application of a stress on the cell will be referred to as
mechanically-induced activation later in the text. mechanically-induced activation is to be
differentiated from spontaneous activation, which describes a leukocyte that becomes activated by itself with no known reason.

![Fig. 21. Picture of stress-induced activated leukocytes](image)

On the left: pseudopodia, on the right: irregular membrane

The spontaneous activation has also been quantified aside from the mechanically-induced activation. In order to quantify the percentage of leukocytes that become spontaneously activated, three hours after the blood withdrawal, the chamber is scanned and an important number of leukocytes are looked at. The state of activation is evaluated visually by looking at the cleanliness of the cell membrane, the activated cells being recognized by the formation of pseudopodia, or an excessively irregular membrane. The schematic in fig 22 shows which sections are looked at for spontaneous cell activation quantification. One can see that the cells on the right-hand side of the chamber are not counted in order not to count stress-induced activated cells within the spontaneously activated cells. Similarly, the lymphocytes on the sides of the chamber are not counted since they can sometimes be activated because of the presence of vacuum grease in these regions.
Fig. 22. Spontaneous Activation Quantification - Scanning.

- Cover Glass
- Area reserved for Recovery Experiments
- Scanning Pattern
CHAPTER 5
RESULTS

More than 300 recovery experiments have been performed with mouse blood. The average lymphocyte diameter has been found to be 8.42 ± 0.4 μm. The average deformation ratio, defined as the maximal deformed length of the cell divided by its original diameter was 1.8 ± 0.2, and the average holding time, time during which the deformed cell is kept in the micropipette before expulsion, was 16.5 ± 8.7 seconds.

The results are divided into two categories: rheological properties and state of activation of the lymphocytes. When cells are activated, they become more rigid and are more difficult to deform. Recovery experiments are performed only on non-activated lymphocytes. In this work, cells are defined as activated when formation of pseudopodia is seen on the cell’s surface.

Rheological Properties

One hundred recovery experiments have been analyzed. Out of the 100 lymphocytes for which the numerical model has been run, rheological parameters characterizing 86 lymphocytes have been kept as reliable. Indeed, for 14 of the analyzed lymphocytes, the model was unable to fit the cell behavior (Cf. appendix 3), that is why it would not have made sense to keep the rheological properties derived from the model.

In this section we want to compare controls with diabetic NOD mice in terms of rheological ratios. Since the NOD0 mice do not show any glucosuria, they have been included in the controls group, together with the B6 mice. A t-test has been performed and showed that the difference between the two populations is statistically significant (p<0.003). The data are presented in the figure 23. One may then want to look specifically at the values of the rheological ratios for each
mice group, in order to see if the rheological properties are dependent on the glucosuria level.

**Average Rheological Ratios for Diabetic and Non-Diabetic Mice**

![Graph showing average rheological ratios for diabetic and non-diabetic mice.](image)

Fig. 23. Average Rheological Ratios for Diabetic and Non-Diabetic Mice

The error bars represent the 95% confidence interval. n is the number of cells per group.

One can see on the figure 24 that the average ratios $T_0/\mu$ of the NOD100, NOD2000, NOD0, and B6 are 41.00, 24.57, 26.31, and 24.75 ($10^{-6}$ cm/s) respectively. The Tukey test is used to assess the significance of the differences seen between the four populations. The Tukey test has been chosen because it is an analysis of variance test that performs all pairwise multiple comparison. When comparing NOD100 with B6 mice, the difference of the means is 18.11 ($10^{-6}$ cm/s). This difference in rheological ratio is statistically different (Tukey test, p<0.05). That shows that the rheological properties of mildly diabetic NOD mice’s lymphocytes are altered: the ratio $T_0/\mu$ being higher for diabetic lymphocytes than for healthy lymphocytes.
Fig. 24. Average Rheological Ratios in Each Mice Group
The error bars represent the 95% confidence interval. n is the number of cells per group.

NOD0 and B6 cells have very comparable rheological properties and the difference is not statistically significant. This result is not surprising since the B6 strain is the control strain chosen for the diabetic NOD mice, and the NOD0 mice, who do not have glucosuria, are likely to be healthy mice.

Table 5. Pairwise multiple comparison of the rheological ratios

<table>
<thead>
<tr>
<th>Population Compared</th>
<th>Difference of Means ($10^{-6}$ cm/s)</th>
<th>Significance (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD100 vs. B6</td>
<td>18.11</td>
<td>Yes</td>
</tr>
<tr>
<td>NOD100 vs. NOD0</td>
<td>16.00</td>
<td>Yes</td>
</tr>
<tr>
<td>NOD0 vs. B6</td>
<td>2.11</td>
<td>No</td>
</tr>
<tr>
<td>NOD2000 vs. B6</td>
<td>0.09</td>
<td>No</td>
</tr>
<tr>
<td>NOD2000 vs. NOD0</td>
<td>2.20</td>
<td>No</td>
</tr>
<tr>
<td>NOD2000 vs. NOD100</td>
<td>18.20</td>
<td>Yes</td>
</tr>
</tbody>
</table>

This data correspond to the Tukey test
However, when one compares the severely diabetic mice NOD2000 with the B6 mice, it appears that the difference is insignificant. That means that no rheological properties change is observed between the severely diabetic mice and the healthy ones. The statistical results are summarized in the table 5.

A) Distribution of the Rheological Ratios

The rheological data show that diabetic and control lymphocytes have different properties. But one must keep in mind that while looking at histograms, one is looking at the average values of the ratios. With only histograms, one does not know if the values are equally distributed or if certain values are more represented than others (this information is hidden behind the average and the standard deviation). The distribution of the rheological ratios is shown on figure 25.

![Fig. 25. Distributions of the rheological ratios](image)

On the abscissa, 10 means $10 \leq \frac{T_o}{\mu} < 15$, 15 means $15 \leq \frac{T_o}{\mu} < 20$, and so on.

The ratios for the B6 mice cells are located in a narrow range; 62.5% of the ratios are between 15 and 30 cm/s. The NOD100 mice cells’ ratios are more spread, nevertheless 40.7% of their ratios fall between 35 and 50 cm/s. If we compare the B6 mice and the NOD100 mice’s
ratios’s distribution, it appears that they are radically different: where most of the B6 mice have their ratios, the NOD100 have a very small fraction of theirs, and vice versa. This representation of the data confirms that the two strains exhibit different lymphocytes’ rheological behaviors. In other respects, the NOD2000’s distribution closely matches the B6 controls’ one, this observation supports our findings that the NOD2000’s lymphocytes behave rheologically like the controls.

The NOD0 distribution is particularly interesting. On one hand it contains one peak in the 40 to 45 cm/s region just like the NOD100’s distribution does, but on the other hand 69.1% of the cells have ratios in the 15 to 30 cm/s region which is characteristic of the B6 controls. Since both diabetic and non-diabetic distribution characteristics seem to be represented in the NOD0 distribution, it confirms our assumption that some of the NOD0 mice can still be very mildly diabetic even if their glucosuria is null.

B) B6xsv129 vs. Human Lymphocyte

Rheological properties of mouse vs. human lymphocytes are presented in table 6. The lymphocytes from B6 mice have an average $T_0/\mu$ of 24.75, whereas the healthy human’s lymphocytes have an average ratio of 22.92.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Average $T_0/\mu \pm 95%$ CI Confidence Interval ($10^{-6}$ cm/s)</th>
<th>Number of samples n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Healthy Donors</td>
<td>22.92 ± 4.1</td>
<td>12</td>
</tr>
<tr>
<td>B6xsv129 Mice</td>
<td>24.75 ± 3.93</td>
<td>32</td>
</tr>
</tbody>
</table>

We can conclude that the order of magnitude of the rheological ratio is the same for both species.
C) On the Impaired Rheology of NOD Mice’s Lymphocytes

The difference found in terms of the ratio $T_0/\mu$ raises the question of the role played respectively by the surface tension $T_0$, and the viscosity $\mu$. Indeed, the increased of $T_0/\mu$ found in diabetic lymphocytes may be due either to an increase of $T_0$, a decrease of $\mu$, or both.

One way to find out if the difference in rheology observed represent an increase or a decrease in deformability is to compare the entry time of the lymphocyte inside the pipette, at a constant given pressure. The shorter the entry time is, the more deformable the cell is. From the data available we extracted the NOD100’s cells whose ratio is located in the 38 to 47 cm/s peak, and the B6’s cells whose ratio is within the 18 to 27 cm/s range (their distributions’ main peak). Then we try to match the cells whose aspiration pressure and deformation extent are similar, but it proved to be difficult to find reliable matches. Yet, the trend, even if insignificant, is that the entry times happen to be shorter in the NOD100 population.

Also we compared couples of cells from each population whose extent of deformation and holding time are similar. Since the aspiration pressure is not critical in this case, we were able to find few cells whose matching was good.

<table>
<thead>
<tr>
<th>Number of Samples n</th>
<th>$T_0/\mu$ $(10^{-6} \text{ cm/s})$</th>
<th>Recovery Duration (s)</th>
<th>Number of Samples n</th>
<th>$T_0/\mu$ $(10^{-6} \text{ cm/s})$</th>
<th>Recovery Duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>53</td>
<td>1</td>
<td>39</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>63.7</td>
<td>1</td>
<td>40</td>
<td>32</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>82</td>
<td>1</td>
<td>43</td>
<td>35</td>
</tr>
</tbody>
</table>

Each line represents B6 and NOD100 lymphocytes whose experimental conditions match in terms of extent of deformation and holding time.

Once again, it appears that the times needed by a NOD100 lymphocyte to recover its original shape is shorter than for a B6 lymphocyte. This observation confirms our findings. Indeed if
diabetic cells recover faster that can be due either to an increased cortical tension facilitating the recovery, or to a decreased viscosity (the viscosity being resisting the recovery).

With the present data, we cannot conclude about the respective roles played by the viscosity and the cortical tension in the increase in rheological ratio in diabetes. Aspiration experiments are needed to address this question.

**State of Activation**

As explained in chapter 4 (page 49), two different types of activation are quantified:

- **Mechanically-induced activation:** within the number of cells that have been aspirated inside the micropipette, the percentage of cells that become activated because of the stress involved in the aspiration process.
- **Spontaneous activation:** after an hour of recovery experiments, the central part of the chamber (where cells have not been aspirated) is scanned, and the percentage of cells in that area that are activated is referred to as the percentage of spontaneously activated cells.

**A) Spontaneous Activation**

The pie charts below are showing the respective numbers of spontaneously activated and passive lymphocytes in each mice group. The percentages of spontaneously activated lymphocytes in the B6, NOD0, NOD100, and NOD2000 groups are respectively 29%, 30%, 38%, and 46%. The trend is clear, the more diabetic the mice are, the higher the percentage of spontaneously activated lymphocytes is. It appears that when the mice are diabetic, their lymphocytes have a tendency to become activated spontaneously, and this tendency seems to be positively correlated to the severity of the diabetes.
Fig. 26. Proportions of Spontaneously Activated Lymphocytes
N is the number of mice per group. The darker or green pie pieces relate to activated cells, whereas the lighter or violet relate to passive cells.

This is an important finding since the state of activation of the lymphocyte has a great impact on its rheological and adhesive properties.

We want to investigate a contingency between proportion of activated cells and mice strain. To do so, we tested the hypothesis that the proportion of spontaneously activated lymphocytes is independent of the mice strain. Contingency tables has been made, and the test statistic $X^2$ has been computed and compared to the $\chi^2$ distribution.

<table>
<thead>
<tr>
<th></th>
<th>P</th>
<th>X^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 NOD100</td>
<td>0.025</td>
<td>6.44</td>
</tr>
<tr>
<td>B6 NOD2000</td>
<td>0.005</td>
<td>17.76</td>
</tr>
<tr>
<td>NOD100 NOD2000</td>
<td>0.1</td>
<td>3.52</td>
</tr>
<tr>
<td>B6 NOD0</td>
<td>1</td>
<td>0.0136</td>
</tr>
</tbody>
</table>

The level of significance is noted p. A value of one for p signifies independence between the nature of the strains present on the given row and their proportion of activated cells.

One can see that the data present sufficient evidence to indicate dependence between mice strain and state of spontaneous activation, while comparing B6 to NOD100, B6 to NOD2000, and NOD100 to NOD2000. The respective level of significance are 0.025, 0.005, and 0.1. However the data also show that while comparing B6 to NOD0, the proportion of activated cells is independent on mice strain.
B) Mechanically-Induced Activation

The results concerning the mechanically-induced activation are radically different from those on spontaneous activation.

![Pie charts showing proportions of mechanically activated lymphocytes](image)

Fig. 27. Proportions of Mechanically Activated Lymphocytes
N is the number of mice per group. The darker or green pie pieces relate to activated cells, whereas the lighter or violet relate to passive cells.

The B6 mice, the NOD0, and the NOD2000 all present a percentage of mechanically-induced activation around 27%, (29.2%, 27.0%, and 25.6% respectively) whereas a small proportion (12.6%) of the experiments carried on the NOD100’s lymphocytes activated them. This trend reminds us the trend observed for the rheological ratios, where the NOD2000 behaved as the controls, while the NOD100 were more deformable. These results are indeed consistent: the passive NOD100’s lymphocytes being more deformable, they flow more easily inside the pipette, and therefore are less likely to become activated during their deformation.

Table 9. Dependence of mechanically-induced activation on mice strain

<table>
<thead>
<tr>
<th></th>
<th>p</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 NOD0</td>
<td>1</td>
<td>0.061</td>
</tr>
<tr>
<td>B6 NOD2000</td>
<td>1</td>
<td>0.17</td>
</tr>
<tr>
<td>NOD100 B6</td>
<td>0.005</td>
<td>10.03</td>
</tr>
<tr>
<td>NOD100 NOD0</td>
<td>0.05</td>
<td>4.75</td>
</tr>
<tr>
<td>NOD100 NOD2000</td>
<td>0.05</td>
<td>4.09</td>
</tr>
</tbody>
</table>

The level of significance is noted p. A value of one for p signifies independence between the nature of the strains present on the given row and their proportion of activated cells.
Statistical analysis has been performed to look at the dependence of the proportion of activated cells on mice strain. The methodology which has been used is the same as for spontaneous activation.

One can observe that the only significant difference in the proportion of mechanically-induced activation is between the NOD100 and any other strain. In other words, the data shows that the proportion of experiments bringing the cell to its activated state is significantly lower in the NOD100 strain.
CHAPTER 6
CONCLUSION

Discussion

The majority of circulating leukocytes are in a relatively quiescent state referred to as passive state, while their immobilization on the vessel wall and directed migration into the tissues, phagocytosis, and degranulation require cell activation. To understand our findings one must keep in mind that the rheological properties that we measured concern passive state lymphocytes. Passive state lymphocytes represent only a sub-population of the overall leukocyte population. Since both the mechanical and the adhesive properties of the leukocytes depend strongly on the state of activation of the cells, parameters reflecting this status are essential for the interpretation of the results.

From the present data, we can draw two major conclusions regarding NOD mice:

• Mildly diabetic NOD mice: (glucosuria less than 100 mg/dL) possess lymphocytes with a significantly higher tendency toward spontaneous activation. The proportion of their circulating lymphocytes, which are in a passive state, exhibits a statistically significant increase of their cortical tension over viscosity value ratio, evidencing an altered rheology. However, these passive lymphocytes, showing an increased rheological ratio, are less prone to become activated when they undergo aspiration into the micropipette.

• If the NOD mice are not treated and become severely diabetic (glucosuria greater than 2000 mg/dL), their lymphocytes are then very prone to become spontaneously activated. The small proportion of lymphocytes, which are in a
passive state, exhibits a rheological behavior comparable to healthy lymphocytes. The fact still remains that a non-negligible proportion of their lymphocytes is in an activate state, characterized among others by a decreased deformability, and an increase tendency toward adhesion.

Our hypothesis is that the lymphocytes of NOD100 which exhibit a significantly high $T_0/\mu$ would, if the mouse is left untreated, become spontaneously activated. That would explain why, a higher proportion of lymphocytes are spontaneously activated in NOD2000 than in NOD100. The subpopulation of NOD2000 lymphocytes which are still passive, is however characterized by a normal rheology.

Our findings regarding increased spontaneous activation in severely diabetic NOD mice’s lymphocytes remind us of observations from teams studying other vascular diseases. Evaluation of blood samples from patients with a variety of vascular and inflammatory disorders showed that a subset of leukocytes may be in an activated state even though there are no apparent signals that call for their migration into selected tissues [1,6,7,33]. Grau et al. [16] has studied stroke using micropipette manipulation: they found that the rheological properties of non-activated neutrophils were unchanged, yet they show that the disease is associated with a significant enhancement of cell activation. The unchanged rheological behavior of severely diabetic passive lymphocytes should not hide their abnormal tendency toward spontaneous activation. This particularity of diabetic mouse cells to become spontaneously activated is not to be neglected. Activation of leukocytes in the circulation without focus to a specific inflammatory site may indeed cause microvascular blockade through mechanical and adhesive mechanisms.

Moreover this observation confirm the results of Vermes et al. [54] who found an increased clogging particle in filtration experiments with IDDM WBC suspensions.
As for the significant increase in $T_0/\mu$ of mildly diabetic mouse lymphocytes, it proves that these cells do not behave rheologically the same as controls. However, recovery experiment data are not sufficient to assess the role respectively played by the cortical tension and the viscosity.

Linderkamp et al. performed micropipette aspiration experiments on neutrophils from IDDM children [24]. By measuring the tongue lengths of partially aspirated neutrophils as a function of time, they showed that IDDM neutrophils flow in the micropipette slower than neutrophils from healthy donors. This means that diabetic cells are more viscous if the drop model holds.

Once the aspiration pressure overcomes the cortical tension, an increased viscosity would slow down the flow of the cell into the micropipette. If this observation apply to NOD mouse lymphocytes, our findings would indicate that not only the viscosity is abnormally elevated in NOD mice, but that the cortical tension is also increased to an even greater extent, making $T_0/\mu$ higher. Linderkamp et al. also counted more activated neutrophils in untreated IDDM children than in treated patients; this observation supports ours.

These results raise the following questions: How do these modifications in lymphocyte deformability and state of activation affect the clinical outcome of NOD mice? Can these results be related to IDDM patients?

**Future Work**

The major challenge common to assessment of any aspects of leukocyte function is the inherent reactivity of the cells: their behavior may shift significantly under experimental conditions. Isolation of the cells itself represents a stimulus. For that reason it is imperative to perform the experiments in a standard way, and to prevent as much as possible cell manipulation and stimulation.

Below, is a list of observations and suggestions that should be considered in order to improve our understanding of the rheological behavior of diabetic leukocytes.
1. Aspiration experiment at a constant aspiration pressure should be performed on NOD mouse leukocytes in order to assess the respective roles played by viscosity and cortical tension in the impaired rheological behavior of NOD mouse lymphocytes.

2. Leukocyte state of activation could be efficiently studied by measuring Nitroblue-tetrazolium reduction by leukocytes due to superoxide production [36,37].

3. Study on diabetic human leukocyte rheology should be done to find out if our results concerning diabetic hold. Efforts need to be made concerning the selection of the patients in a try to avoid as much as possible the presence of external factors influencing leukocyte rheology.

4. Experiments should be done with a XYZ stage in order to analyze a higher number of cells. Indeed, some of the experiments performed could not be analyzed because cells were not in focus throughout the experiment.
REFERENCES


BIOGRAPHICAL SKETCH

Nicolas Didier was born on May 8, 1977, in Paris, France. He received his “Diplôme d’ingénieur” in Biomedical Engineering from the “Université de Technologie de Compiègne”, France, in November 1999. His motivation to learn more and to get experience from a stay in a foreign country led him to come for graduate work at the University of Florida in the Biomedical Program in Fall 1998. He graduated with a Master of Science in Biomedical Engineering, in August 2000.