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Polymorphonuclear Leukocyte Count Within Healing Wound Sites in Non-Diabetic and Diabetic Rat Oral Mucosa

James L. Discipio
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POLYMORPHONUCLEAR LEUKOCYTE COUNT
WITHIN HEALING WOUND SITES IN
NON-DIABETIC AND DIABETIC RAT
ORAL MUCOSA

BY
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A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
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DEDICATION

To my Mom and Dad, for their love, guidance and encouragement, enabling me to pursue and attain this goal. Their strong family ideals and dedication gave me the foundation and encouragement to accomplish this professional plateau.

To my wife Nancy, who has given her love and support during my professional education. Her strong will and devotion gave me the enthusiasm and strength to reach a little further.

I would also like to dedicate this thesis to my grandparents, my brothers, and their families for their continued love and support.
VITA

The author, James L. Discipio, is the son of Dr. Joseph V. Discipio and Darlene (Mrnka) Discipio. He was born March 2, 1955 in Berwyn, Illinois. He is married to Nancy (Mini) Discipio, and has two brothers, Joseph and Jeffrey.

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INTRODUCTION

Diabetes mellitus is often recognized as a metabolic disease that depresses the anti-bacterial defenses of the host. Many clinical investigators have reported that patients with uncontrolled diabetes are unusually susceptible to bacterial disease.

It appears that in the diabetic patient the function of leukocytes in inflammation is impaired. A decrease in glycolysis, inability of the diabetic leukocyte to phagocytize and a defect in leukotaxis can play a role in abnormal leukocyte function. These abnormalities of the leukocyte could contribute to increased infections in the uncontrolled diabetic patient.

The evidence that diabetes mellitus is related to functional disorders of neutrophils suggested the study of inflammation in streptozotocin induced diabetes mellitus. This study measures the neutrophilic infiltration in a simple mucosal wound in rats and the detection of nitroblue tetrazoleum reaction of blood neutrophils within oral tissue of non-diabetic and diabetic rats.

Since it has been shown that diabetics exhibit delayed healing when compared to non-diabetics, it is hoped that this investigation will assist in understanding the importance of polymorphonuclear leukocyte count during healing of the excised wound.
Diabetes mellitus is a hereditary or developmental disorder characterized by an abnormally high level of glucose in the blood. The basic defect is an absolute or relative lack of insulin which leads to abnormalities of carbohydrate, protein and fat metabolism.\(^1\)

Blood sugar is normally maintained in a range approximately 80 to 120 mg/dl.\(^2\) Values below this range reflect hypoglycemia, whereas values above the range reflect hyperglycemia. Diabetes mellitus is a disease in which a fluctuating and often persistent hyperglycemia results in a loss of glucose homeostasis. Since insulin helps to control the cellular uptake of glucose, diabetes mellitus has occasionally been defined as a relative or absolute insulin deficiency.

Insulin is normally produced by the beta cells within the pancreatic islets of Langerhans. Active insulin is made by progressive modification of preproinsulin. A rise in the concentration of blood glucose is a primary signal for secretion of insulin. The classic result of insulin action is a dramatic increase in the rate of transport of glucose into skeletal muscle and adipose tissue. Insulin also promotes the uptake of amino acids by skeletal muscles and increases protein synthesis. It accelerates lipid synthesis and inhibits lipolysis and gluconeogenesis.\(^3\) Lack of insulin may result from diminished production of insulin, faulty release of insulin, antibodies to insulin and/or abnormalities of the body's cells precluding normal action of insulin.\(^4\) Human insulin is a small protein
with a molecular weight of 5808 and is composed of two amino acid side chains, connected by disulfide bridges. An important effect of insulin is its ability to increase the rate of glucose transport through the membranes of most cells in the body. In the complete absence of insulin, the overall rate of glucose transport becomes only one fourth the normal value.\(^5\) The diabetic, therefore, has high levels of blood glucose with deficient cellular glucose. Because of cellular glucose deficiency, the diabetic relies on lipids for energy, with excessive production of acetylcoenzyme A and ketone bodies resulting in acidosis. Coenzyme A enhances the production of cholesterol, which, in turn, may be partially responsible for the increased frequency of severe atherosclerosis in diabetics.\(^6\)

The pathology of diabetes mellitus may be related to one or more of the following three major effects of insulin lack: (1) decreased utilization of glucose by the body cells, with a resultant increase in blood glucose concentration; (2) markedly increased mobilization of fats from storage areas, causing abnormal fat metabolism and especially deposition of lipids in vascular walls to cause atherosclerosis; and (3) depletion of protein in the tissues of the body.\(^5\)

Patients with diabetes mellitus exhibit polydipsia (excessive drinking), polyuria (excessive elimination of urine), polyphagia (excessive eating), loss of weight and asthemia (lack of energy) as the earliest symptoms of the disease. Polydipsia and polyuria are due to the hyperosmolality of the blood, which results from the increased glucose levels. Generalized weakness, increased tendency to infection and poor wound healing are also commonly present in the initial stages.\(^4\) These symptoms of
diabetes are related to excess blood glucose and also to increased breakdown of lipids, resulting in acidosis.

Diabetes may be experimentally produced in laboratory animals with injection of streptozotocin. Streptozotocin is an antibiotic extracted from *Streptomyces acromogenes*. In 1963, Rakieten, et al., reported that streptozotocin is diabetogenic, since its intravenous administration led to diabetes in dogs and rats. They found that the diabetes was attributed to damage of the pancreatic B-cells. Junod, et al., (1963) found in their study that streptozotocin is an effective cytotoxic agent for pancreatic B cells. They found that the action occurs early, having found histologic evidence after 1 hour, and intense necrosis strictly limited to B-cells evident at 7 hours.

Diabetes mellitus is often recognized as a metabolic disease that depresses the antibacterial defenses of the host. Many clinical investigators have reported that patients with uncontrolled diabetes are unusually susceptible to bacterial disease. Leukocytes differ from other body cells in that they have the capacity to migrate, contain few mitochondria and metabolize glucose primarily to lactic acid. As human diabetes mellitus is expressed in abnormalities of carbohydrate, lipid and protein metabolism, any resulting inefficient neutrophil bactericidal and/or leukotaxis mechanisms may be partly responsible for the increased susceptibility of patients with diabetes mellitus to infection.

The polymorphonuclear leukocytes are the most abundant of the leukocytes, constituting 55% to 65% of the total count. There are about 3,000 to 6,000 per cu. mm. or 20 to 30 billion in the circulation at any one
time. They are 10 to 12 microns in diameter and are easily recognized by their highly characteristic nucleus consisting of two or more lobules connected by narrow strands.  

The neutrophil is an end cell of myeloid differentiation and does not divide. The neutrophils arise in the bone marrow from a common ancestral stem cell, and after a series of divisions, undergo a maturation through a myeloblast, promyelocyte, metamyelocyte, band cell and mature PMN. After a short period in the blood (about 12 hours), the PMN's enter the tissues where they complete their life span of a few days.

With the maturation of the cells, there is an appearance of two distinct classes of granules: the primary (azurophilic) and the secondary or specific granules. The primary granules are electron-dense structures approximately 0.4 microns in diameter that appear early in maturation. They contain myeloperoxidase, arginine-rich basic (cationic) proteins, sulfated mucopolysaccharides, acid phosphatase and other acid hydrolases. The secondary or specific granules are not truly lysosomes; they are smaller and less dense than primary granules. They are rich in alkaline phosphate, lysozyme and aminopeptidase. As maturation proceeds, the secondary granules increase in number and appear to predominate. Both types of granules are important in the breakdown of ingested material and in the killing of microorganisms.

The life span of the neutrophils in the circulatory system is about seven hours. During times of tissue injury these cells can squeeze through the endothelial cells of the blood vessels by the process of diapedesis. Once the cells have entered the tissue spaces they move through the
tissues by ameboid movement. In the tissues, the neutrophils average a life span of eleven days.

The mature PMN is highly specialized for the performance of its primary function, the phagocytosis and destruction of microorganisms. Several functions are required in order for the neutrophil to perform its task of phagocytizing foreign matter. First, the cells must reach the site of the foreign configuration by chemotaxis. They must then ingest the foreign substance by phagocytosis. Finally, after a series of metabolic steps, they must destroy the foreign substance or inhibit the replication of the microorganism by microbial killing.

The killing of microorganisms is the primary purpose of the phagocytic system. The formation of an inflammatory exudate, composed primarily of neutrophils and monocytes, occurs rapidly in response to a local infection. Tissue damage initiates some form of stimulus which produces the following series of events. Neutrophils begin to marginate along the capillary and venule walls in the area of tissue damage and commence to diapedese between the endothelial cells lining the walls. The cells are held up for some time at the basement membrane and then move through this and on toward the damaged area to which they are attracted by a chemotactic stimulus.

Chemotaxis appears to be mediated by substances which directly influence neutrophils, such as bacterial products or certain components of complement (C₅, C₃ and the C₅₆₇ trimolecular complex) and by factors which interact with plasma to activate complement such as antigen-antibody complexes and lysosomal contents of phagocytes.
The trimolecular complex, \( C_{567} \), exists naturally in serum in an inactive form and can be activated by interaction in series of the other components of complement. The activated complex causes intense accumulation of polymorphonuclear leukocytes when injected intradermally into rabbits. The third component of complement can produce a fragment chemotactic for polymorphs when acted on by plasmin. There are also various chemotactic factors produced by bacteria such as \textit{Diplococcus pneumoniae}, \textit{Staph. albus}, \textit{Staph. areus} and \textit{Streptococcus faecalis}.

Once the polymorph has arrived at the inflammation site, it begins its function of phagocytizing foreign matter. The process of phagocytosis is a very intricate process. The phagocytosed material is not brought directly in contact with the cytoplasm. The cytoplasmic membrane invaginates and encloses the phagocytosed material within a membrane limited vacuole. Once the organism is taken up within the cell, the phagosome will come in contact with a lysosome and the two membranes will fuse. The lysosome will expel enzymes into the phagosome which are essential to the destruction of the bacterium. Before fusion with the phagosome the lysosome maintains a distance of 100 angstroms from the inner surface of the cell membrane. The collision of the lysosome with the phagosome appears to be a "random hit" phenomena.

Recognition by the cell that the particle is foreign is essential for phagocytosis to occur. The process of opsonization appears to play a role. Opsonins include immunoglobulins, certain components of the complement system and other plasma factors. The opsonin acts upon the particle to be phagocytized, not on the phagocyte.
Exocytosis appears to be less understood but the release of metabolically active and theoretically damaging materials into areas of exudate may contribute to tissue damage as well as being a chemotactic stimulus for more cells. A variety of secretory products are released from the cell as a result of exocytosis. Some of these enzymes include hyaluronidase, ribonuclease, lysozymes, histamine, leukocyte pyrogen and vitamin B$_{12}$ binding protein. The extent of these various enzymes remains to be investigated.

The metabolism of phagocytosis requires a modest expenditure of energy which is supplied by glycolysis and associated with increased glucose utilization and lactate production. Phagocytosis and lysosomal merger with the phagosome are followed by an energy burst derived primarily from hexose monophosphate shunt (HMS) activation. Associated with increased HMS activity is increased production of hydrogen peroxide which, in the presence of myeloperoxidase, a constituent primarily of lysosomes, provides a potent antibacterial mechanism for the leukocyte. The HMS is governed by the availability of NADP and the polymorph possesses several mechanisms for NADP regeneration. The most prominent pathway utilizes NADPH oxidase where H$_2$O$_2$ is an additional product of the reaction. The enzyme NADPH oxidase, gives rise to H$_2$O$_2$ and NAD, increasing the activity in phagocytosing cells. The H$_2$O$_2$ may also interact with reduced glutathione and glutathione peroxidase to give rise to oxidized glutathione which then may react with an NADPH linked glutathione reductase forming NADP for stimulation of the hexose monophosphate shunt-glycolytic pathway and reduced glutathione. The antibacterial activity
of the $\text{H}_2\text{O}_2$-myeloperoxidase system is enhanced by small amounts of halide co-factors which in the case of iodide result in iodination of bacterial proteins and, in the case of chloride result in decarboxylation and deamination of amino acids giving rise to toxic aldehydes.$^9$

Nitroblue tetrazolium (NBT) is an artificial electron acceptor which is reduced by the PMN during the phagocytosis-induced metabolic burst.$^{11}$ A quantitative NBT test is based on the metabolic activity of the phagocyte that occurs with particle uptake and intracellular digestion. The increased flow of protons thru the HMS shunt during phagocytosis and intracellular digestion is measured colorimetrically by the reduction of the nitroblue tetrazolium dye to a blue formazan pigment. The NBT test differentiates between the resting and phagocytosing states of neutrophils. The quantitative NBT test is markedly depressed in cases in which there is an impairment in metabolic activity of leukocytes, such as in chronic granulomatous disease.$^8$

Various factors suggest themselves as the cause of the lowered resistance to infection in the diabetic. These may be summarized as follows:

1) Increased sugar content of blood tissues.

2) Decreased activity of blood elements associated with resistance to infection: (a) subnormal activity of complement; (b) subnormal phagocytizing capacity of leukocytes; (c) subnormal bacteriostatic and bactericidal action of whole blood.

3) Inadequate functioning of fixed tissue cells.

4) Lowered capacity of tissues to react to antigenic stimuli.

5) Lowered state of general cellular nutrition.$^{12}$

Adherence of granulocytes may also play a role in susceptibility
to infections in diabetics. In studies of patients with poorly controlled diabetes, with fasting hyperglycemia but not ketoacidosis, the mean values for granulocyte adherence were significantly lower than for normal control cells.\textsuperscript{13}

No impairment of immunological competence has been detected in diabetic patients, and deficiencies in cellular defense have been demonstrated only in the presence of ketoacidosis, even though an insulin-correctable depression of glycolysis has been noted in the leukocytes of nonacidotic diabetics.\textsuperscript{14}

The susceptibility to infections in diabetics could also be due to a delay in the granulocyte response. Perillie\textsuperscript{15} et. al., (1962) showed that the early granulocyte phase of local cellular response was significantly delayed and diminished in patients with acidosis in comparison with normal and nonacidotic patients.

Esmann\textsuperscript{12} (1972) in his research on the diabetic leukocyte, concluded that evidence points towards glucose being freely permeable to the cell membrane. He placed special importance on the crowding effect, i.e. the decrease in metabolic rate observed by increasing the cell concentration, in this respect. Upon placing normal and diabetic cells in a bicarbonate buffer at the same low concentration to avoid the crowding effect, he found that the glycolysis of diabetic cells is decreased. Glucose-6-phosphate and fructose-6-phosphate accumulate in diabetes, suggesting a decreased activity of phosphofructokinase. The increase in glucose-6-phosphate concentration is probably responsible for a small decrease in glucose utilization and a slight increase in pentose cycle activity as well
as for an increase in the relative amount of glycogen synthetized. Insulin in vivo corrects the metabolic alterations.\textsuperscript{13}

Martin\textsuperscript{16} et. al., (1953) found that the cells of the diabetic subject have a significantly lower (p. 0.01) production of lactic acid with glucose and a significantly higher (p. 0.01) production with fructose. The utilization of glucose was significantly lower in the diabetic than in the control group (p. 0.02). There was a significant increase (p. 0.02) in utilization of glucose in the diabetic group (0.18\pm 0.04 micromole) on the addition of insulin.\textsuperscript{16} Martin and his associates indicated that the leukocyte of the diabetic subject responds to insulin by increasing lactate production. The effect of the metabolic defect noted in the leukocytes from subjects with diabetes mellitus may account for the alterations in susceptibility to disease. In an area of inflammation, the leukocyte of the diabetic subject might be unable to utilize the available glucose to produce bactericidal quantities of lactic acid.\textsuperscript{13}

Munroe and Shipp\textsuperscript{1} (1965) in their research on leukocytes and diabetes mellitus confirmed the results of others that the major fate of glucose in human leukocytes was lactic acid production. Glucose metabolism in leukocytes from diabetics differed in two respects. The per cent of glucose uptake oxidized in the pentose cycle was increased in leukocytes from all diabetics. In the insulin-treated diabetics glucose uptake and lactic acid formation were similar to controls. In contrast, glucose uptake and lactic acid formation were reduced in leukocytes from adult, latent, hypercholesteremic diabetics. Pyruvate and acetate oxidation was similar in diabetics and controls. Thus, in terms of leukocyte metabolism,
hypercholesteremia in the human diabetic was correlated with reduced glycolysis and not with reduced pentose cycle activity.\textsuperscript{1}

Dumm\textsuperscript{17} (1957) only found reduced glucose utilization in cells from patients with the most severe diabetes, and explained this finding by observing that plasma from diabetics was able to inhibit the glucose utilization of normal leukocytes.

Esmann\textsuperscript{18} (1964) in his experiment on the effect of cell concentration on the metabolism of normal and diabetic leukocytes in vitro, showed an overall decrease of glucose uptake, lactic acid production and glycogen synthesis in human diabetic leukocytes.

The marked increase in glycogen which occurs during the course of an inflammatory response in normal animals is due to the high glycogen content of the invading leukocytes. The glycogen content increases in the diabetic animal to an even greater extent than in the normals, due to the nature of the disease. Because the leukocytes from diabetic animals were found to have the same glycogen content as leukocytes from normal animals, it appears that, once leukocyte invasion begins, more cells enter the experimental site per unit time and that the duration of leukocyte invasion is increased, compared to nondiabetic animals.\textsuperscript{19}

Dumm\textsuperscript{17} (1957) found that although glucose utilization by leukocytes from diabetic humans was impaired, there was no reduction in lactate production.

Insulin binds to the surface of leukocytes. The effect of insulin in vitro on glucose utilization and glycolysis has been sought for, but with controversial results.
Martin\textsuperscript{16} (1953) found an increased glucose utilization and lactic acid formation with 0.1 U insulin/ml in diabetic, but not in normal cells. Esmann\textsuperscript{20} (1963) found a significant increase in glucose utilization, lactate and glycogen formation after 24 hours of treatment of diabetic patients with insulin.

The decreased rate of glycolysis in diabetic cells can be attributable to concentrations of essential enzymes. The diabetic leukocytes have high concentrations of glucose and fructose-6-phosphate, which are normalized upon treatment of the patients with insulin. This suggests a decreased activity of the phosphofructokinase reaction in uncontrolled diabetes and may explain the decreased rate of glycolysis found in diabetic cells.\textsuperscript{13} Esmann attributes the limited Kreb's cycle activity to the paucity of mitochondria in human polymorphonuclear leukocytes.

Marble\textsuperscript{21} (1952) hypothesized that the subnormal phagocytizing capacity of the leukocyte was one of the possible causes of reduced resistance of the diabetic to infection.

Wertman and Henney\textsuperscript{22} (1961) in their study on phagocytosis in diabetic leukocytes indicated that the animals with alloxan-induced hyperglycemia had an impaired defense mechanism. The rats with high concentrations of blood sugar had not only a lower percentage of active neutrophils but the activity of these cells was considerably reduced. The alloxan diabetic rat demonstrated an average of 45\% and 42\% active neutrophils and the rats with a normal sugar concentration an average of 91\%, 87\% and 88\%. The active neutrophil of the alloxan diabetic animal engulfed on the average 4 microorganisms per neutrophil as compared to 17 microorganisms per
neutrophil of the rat with a normal sugar concentration. It appeared evident that rats injected with sufficient alloxan to cause hyperglycemia were reduced in the capacity to phagocytize bacteria.\textsuperscript{13}

In 1974, Robertson and Polk, in their study on leukocyte malfunction showed that hyperglycemia alone led to decreased phagocytosis, decreased diapedesis and a decrease in intracellular killing of bacteria by PMN's. They theorized that an intrinsic defect in the PMN was a cause of the malfunction. They also found that there was no significant difference in immunoglobulins A, G and M on the surface of diabetic and normal PMN's; and that the metabolic pathways are altered in diabetic PMN's which may cause a malfunction in their ability to affect infectious organisms.

Bybee and Rogers\textsuperscript{23} (1964), in their study on the phagocytic activity of polymorphonuclear leukocytes obtained from patients with diabetes mellitus, suggest that PMN leukocytes obtained from nonacidotic diabetic patients exhibit normal phagocytic activity. In contrast, leukocytes obtained from diabetic patients in ketoacidosis showed suppression of their ability to ingest a pathogenic Staphylococcus. This abnormality was corrected when the acidosis was corrected. This quantitative decrease in phagocytic capacity appeared to be the result of an abnormality of the diabetic leukocyte.

Drachman\textsuperscript{24} et. al., (1966) showed that the phagocytic capabilities of polymorphonuclear leukocytes are affected by the high levels of glucose in the tissues of chronically diabetic rats. In the intact diabetic animal, not only did the histological and quantitative bacteriological findings suggest a depression of phagocytosis, but an impairment of in vivo
phagocytic activity was also demonstrated in a preformed inflammatory exudate. Also noted was that the principal defect in the diabetic animals resided in their serum rather than in their polymorphonuclear leukocytes. The depressive factor in the serum was identified as the abnormally high concentration of glucose.

Contrary work was shown by Miller and Baker (1972). Although hyperglycemia has been found to depress in vitro phagocytosis of pneumococci in diabetic rats and poorly controlled diabetic patients, no correlation between neutrophil function and blood glucose levels in juvenile diabetic patients was demonstrated.

It has been reported that leukocytes from rabbits with acute alloxan diabetes and acidosis had reduced bactericidal power for engulfed pneumococci. Richardson (1933) felt that the reduced bactericidal power of whole blood in his studies was directly related with the presence of acidosis.

The metabolic changes following phagocytosis have not been extensively investigated in diabetic leukocytes, but it has been reported that the mobilization of glycogen during phagocytosis is impaired in alloxan-diabetic rat cells. For the intracellular killing of bacteria, the formation of \( \text{H}_2\text{O}_2 \) in the pentose cycle is of importance. It is, therefore, essential that the normal increase in pentose cycle activity upon phagocytosis is not impaired in diabetic rat or human leukocytes.

Cantonzaro-Guimaraes (1968) noted that the diabetic animals exhibited an increased acute inflammatory cell population, primarily in the form of PMN's.
Leukocyte chemotaxis is the ability of the neutrophil to move uni-directionally toward an increasing chemotactic stimulus. Many investigators have demonstrated impaired leukocyte chemotaxis in diabetes mellitus. Steerman et al., (1971) described normal chemotaxis as a consequence of chemotactic factors derived from serum interacting with cells capable of responding to these factors. He described impairment of chemotaxis as the primary result of an intrinsic defect of polymorphonuclear leukocytes. These impairments of PMN function were demonstrated by an inability of the patient's PMN's to respond to chemotactic factors in 'activated' whole serum or in preparations of C5a. Since these abnormalities were not corrected by the addition of normal serum or plasma to the patient's cells, the deficiency in chemotaxis could not be accounted for solely by a deficiency of a humoral factor. Steerman and associates also theorized that since this patient had normal or elevated levels of haemolytic activity for total complement and the individual components of C1 through C5 and failed to show correction of PMN function with the addition of normal serum, that it is improbable that an abnormality of the complement system is responsible for his defects in PMN function.

Miller et al., (1973) in his research on leukocyte mobility and Lazy Leukocyte Syndrome (LLS), concluded that on the basis of current knowledge of the mechanisms involved in chemotaxis and random mobility, it seems possible that the basic defect in the familial disorder involves specific chemotactic receptor(s), while that of the LLS may involve a less specialized aspect of leukocyte integrity, perhaps a membrane defect.

Hill et al., (1974) in their study on impaired leukocyte
responsiveness in diabetes mellitus showed that leukocytes of patients with juvenile onset diabetes mellitus responded less actively than controls in unidirectional movement toward a chemotactic stimulus. Neither abnormalities of serum concentrations of cholesterol, triglycerides, glucose and creatine, nor the duration of the diabetic state had an apparent effect on the presence or severity of the leukotactic abnormality.

Ward and Becker (1970) have shown that leukotaxis is impaired when there is a depletion of intracellular potassium. They theorized that insulin, in the presence of glucose, may act upon enzyme systems resulting in an influx of potassium and enhancing the ability of leukocytes of diabetics to respond to a chemotaxis stimulus.

Goetzl and Austen (1974) found that several chemotactic factors increased both aerobic glycolysis and metabolism through the hexose monophosphate shunt. Their investigation indicates that suppression of both the HMPS and glycolysis inhibits chemotactic migration of polymorphonuclear leukocytes.

A study of leukocyte functions in juvenile diabetes mellitus by Miller and Baker (1972) found that chemotaxis of polymorphonuclear leukocytes from children with juvenile diabetes who had received insulin was not improved. They explained the deficiency in generation of chemotactic activity in that children might have increased amounts of circulating antigen-antibody complexes due to the production of anti-insulin antibodies. They theorized that these complexes might, then, activate the complement system, thus resulting in less available complement molecules for generation of chemotactic activity.
Mowar and Baum\textsuperscript{34} (1971) using an in vitro method of measuring the chemotaxis of polymorphonuclear leukocytes from peripheral blood calculated a chemotactic index. The mean chemotactic index in 31 patients with diabetes mellitus was significantly less (p. less than 0.0005) than that in 31 matched controls. They found that the defect in chemotaxis could not be correlated with plasma insulin, plasma glucose, serum CO\textsubscript{2} and blood urea nitrogen values or with any therapeutic agents. The defect in chemotaxis of the diabetic leukocytes was corrected by incubation of the cells with insulin. Insulin was found to be ineffective in the absence of glucose.

Golub\textsuperscript{35} et al., (1982) found in their study that diabetes in rats inhibits the migration of neutrophils into the healing gingival crevice. A decreased crevicular leukocyte response in vivo was detected as early as 4 days after diabetes was induced.

Manouchehr\textsuperscript{36} et. al., (1981) in their study on PMN chemotactic response in diabetic patients and periodontal disease demonstrated that diabetic patients and severe periodontitis have significant impairment of PMN chemotaxis. In contrast, diabetic patients and mild periodontal disease as well as nondiabetic subjects and either severe or mild periodontitis did not show such impairment. They indicated that the impairment of PMN chemotaxis may be important in the pathogenesis of severe periodontitis in diabetic patients.

It is commonly stated that patients with diabetes mellitus are more susceptible to infections. It appears that in the diabetic patient the function of leukocytes in inflammation is hampered. A decrease in
glycolysis, inability of the diabetic leukocyte to phagocytize and a defect in leukotaxis can play a role in abnormal leukocyte function; or a multifactorial problem may be evident. These abnormalities of the leukocyte could contribute to increased infections in the uncontrolled diabetic patient.

The evidence that diabetes mellitus is related to functional disorders of neutrophils suggested the study of inflammation in streptozotocin induced diabetes mellitus. This study measures the neutrophilic infiltration in a simple mucosal wound in rats and the detection of nitroblue tetrazolium reaction of blood neutrophils.
MATERIALS AND METHOD

Eight male Holtzman* rats, averaging 250 grams each in weight, were divided into two groups: Control group A and experimental group B. All animals were housed separately to help minimize stress and the possibility of cross infection. They were maintained on Purina Rat Chow** and water. The water was contained in a graduated bottle and was available ad libitum. The daily water consumption for each animal was recorded.

The control group A consisted of four rats which were not injected with streptozotocin.*** The experimental group B consisted of four rats which were intraperitoneally (I.P.) injected with streptozotocin (65 mg/kg in 1cc citrate buffer solution). The animals were anesthetized using diethyl ether to aid in injecting the streptozotocin.

The streptozotocin was weighed out (0.02 grams) and placed into each of four small dark bottles. A citrate buffer solution was prepared for addition to the streptozotocin. Two solutions were initially made. Solution A, consisted of 1.382 grams Na citrate diluted with 100 ml. NaCl 9.9%. In solution B, 1.114 grams of citric acid were diluted with 100 ml of 0.9% NaCl. Solution A was added to solution B until a pH of 4.5 was obtained.

A 1cc Tuberculin syringe was filled with the prepared citrate buffer solution and injected into the dark bottle containing 0.02 grams of

* Lock-Ericson Labs, Melrose Park, Illinois
** Scientific Small Animal Co., Arlington Heights, Illinois
*** Upjohn Co., Kalamazoo, Michigan
streptozotocin. The contents was then mixed thoroughly and 1cc of the mixture was withdrawn by syringe. The 1cc streptozotocin mixture was then injected I.P. into the anesthetized animal. The animal was then returned to its respective cage for recovery.

After seven days all the animals within group B were weighed and clinically evaluated for diabetes mellitus. The criteria recorded in evaluating the diabetic state of the experimental animals were: increased water consumption (the percent of consumption was estimated daily thru the use of the graduated water bottle), glucosuria (evaluated thru use of Tes-Tape* on a urine sample) and hyperglycemia (evaluated through use of Dextrostrip** on a blood sample). The above diagnostic tests were also made with control group A to insure that these animals were not diabetic.

Linear incisions 0.5 cm. in length were made in the palatal attached incisions 0.5 cm in length were made in the palatal attached gingivae and extended posteriorly to the left maxillary incisor in each rat. An American Standard 20/21 scalpel was utilized to make the incisions.

One member of each group was sacrificed with a lethal dose of diethyl ether at the following intervals: 3, 6, 12 and 24 hours postoperatively.

The surgical and corresponding non-surgical site of attached gingivae were removed by block dissection. The tissue samples were fixed in 10% neutral formalin for eight hours and prepared by conventional histologic techniques for evaluation. The specimens were washed in tap water overnight, dehydrated in ascending alcohols, cleared in xylene and embedded

* Lilly Co., Indianapolis, Indiana
** Ames Co., Elkhart, Indiana
in paraffin blocks. Ten sections approximately six microns in thickness were obtained from each specimen site and were stained with hematoxylin and eosin (H and E) in order to obtain a microscopically measureable comparison of the healing sites between groups A and B.

Blood samples were taken at the time of sacrifice and thin smears were prepared on slides and stained using NBT* procedure. 0.1 ml NBT solution (Reagent A') was transferred to a siliconized vial. 0.1 ml of well mixed heparinized blood was added to the vial and mixed gently by rolling the vial. The vial was incubated at 37°C for fifteen minutes then removed and let stand at room temperature an additional fifteen minutes. The heparinized blood-NBT mixture was transferred onto a clean glass slide and a smear was made. The slides were then stained with Wright stain.

Polymorphonuclear leukocyte (PMN) counts were made from each tissue specimen by counting the numbers of neutrophils within a 300 micron square area along the edge of the incised wound beneath the level of epithelium. The corresponding control tissue sections similarly were quantitatively evaluated and recorded.

The blood slides were examined and evaluated for positive or negative NBT activity within each group.

The sections from the gingival wounds were stained with H and E and were examined with an AO-10 Star** microscope at a 450X's magnification. The connective tissue in the lamina propria immediately adjacent to the incision was selected for counting PMN's. An ocular eyepiece with a 100

* NBT test, Sigma Co., St. Louis, Missouri
** AO Co., Buffalo, New York
micron square reticular was used as a guide in scanning the connective tissue. All counts were made within 300 microns parallel to the cut surface of the lamina propria. PMN's were identified and microscopic counts from nine 100 micron square fields were recorded. The mean per 100 micron square was calculated and recorded.

The NBT activity in the blood smears were recorded only according to the frequency of NBT positive or negative PMN's in each of the specimens, per 100 micron square field.
RESULTS

The four rats injected with streptozotocin were diagnosed as diabetic. Each diabetic animal demonstrated increased water consumption (Table 1), an elevated blood glucose level (Table 2) and glucosuria.

The average water consumption for the control group was approximately 8% of total water volume per day. The average water consumption for the diabetic group was approximately 12% of the total water volume per day.

Blood glucose levels for control and diabetic animals were determined using Dextrostix, expressed in mg. glucose/dl. blood. All control animals fell into the 90 mg. glucose/dl. blood range (Table 2). Three of the diabetic animals (3-D, 12-D and 24-D) had blood glucose levels of 175 mg. glucose/dl. blood, and one (6-D) had a blood glucose level of 130 mg. glucose/dl. blood (Table 2). The Tes-tape analysis of urine samples for the control group was normal. For the diabetic animals the urine sugar level was markedly elevated.

PMN counts for both groups can be found in Table 3 and Figures 1, 2 and 3. The average number of PMN's per 100 micron squared for the control group ranged from 19.3 PMN's per 100 micron squared for the 3 hour animal to the maximum number at 6 hours (27.8 PMN's per 100 micron square). In the diabetic group the 3 hour value was the lowest (11.0 PMN's per 100 microns squared) and the maximum value was reached at the 12 hour interval (26.0 PMN's per 100 micron squared).

As seen in Table 4, the NBT test of the blood smear was positive for
Figure 1 - Frequency distribution of polymorphonuclear leukocytes at the site of gingival incision wounds per 100 micron squared field in control group.
Figure 2 - Frequency distribution of polymorphonuclear leukocytes at the site of gingival incision wounds per 100 micron squared field in diabetic group.
Figure 3 - Frequency distribution of polymonuclear leukocytes at the site of gingival incision wounds per 100 micron squared field in control and diabetic animals.
the entire control and diabetic groups.

The findings in this experiment were not subjected to statistical analysis.
Table 1 - Daily water consumption for control and diabetic animals, ml. % consumed daily over a seven day period using a calibrated water bottle.

<table>
<thead>
<tr>
<th>Animal #</th>
<th>DAYS</th>
<th>Ave. day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1-Control</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>2-Control</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>3-Control</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>4-Control</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>5-Diabetic</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>6-Diabetic</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>7-Diabetic</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>8-Diabetic</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2 - Approximate blood glucose level in control and diabetic animals, using Dextrostix (mg. glucose/dl. blood).

<table>
<thead>
<tr>
<th>Animal #</th>
<th>mg. glucose/dl. blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Control</td>
<td>90</td>
</tr>
<tr>
<td>2-Control</td>
<td>90</td>
</tr>
<tr>
<td>3-Control</td>
<td>90</td>
</tr>
<tr>
<td>4-Control</td>
<td>90</td>
</tr>
<tr>
<td>5-Diabetic</td>
<td>175</td>
</tr>
<tr>
<td>6-Diabetic</td>
<td>130</td>
</tr>
<tr>
<td>7-Diabetic</td>
<td>175</td>
</tr>
<tr>
<td>8-Diabetic</td>
<td>175</td>
</tr>
</tbody>
</table>
Table 3 - Polymorphonuclear leukocytes in healing incision wounds of the gingivae in nine 100 micron squared fields.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>CONTROL</th>
<th>DIABETIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 Ave. = 19.3</td>
<td>7 Ave. = 11.0</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>6 hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 Ave. = 27.8</td>
<td>17 Ave. = 16.3</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>14</td>
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</tr>
<tr>
<td>29</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>12 hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 Ave. = 23.6</td>
<td>26 Ave. = 26.0</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>24 hr.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 Ave. = 16.6</td>
<td>21 Ave. = 18.4</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>13</td>
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</tr>
<tr>
<td>13</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>


Table 4 - Qualitative results of NBT test in blood smears - Control vs. Diabetic.

<table>
<thead>
<tr>
<th>HOUR</th>
<th>CONTROL</th>
<th>DIABETIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hr.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6 hr.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12 hr.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24 hr.</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
DISCUSSION

Four rats injected intraperitoneally with streptozotocin exhibited clinical signs of diabetes mellitus after seven days. Over this seven day period the daily water consumption for the diabetic rats significantly increased in comparison to the non-diabetic animals. The streptozotocin induced diabetic rats consumed an average of 12 ml of water daily while the non-diabetic rats consumed an average of 8 ml of water daily. This polydipsia was a result of the increase in blood glucose in the diabetic rats.

The diabetic rats exhibit marked increases of glucose in the blood and urine as measured with Tes-tape and Dextrostix, with this increase in glucose indicative of a diabetic state. The normal range for blood glucose is approximately 80-120 mg/dl. The control animals blood glucose level is 90 mg/dl and falls into the normal range. Three of the four experimental rats had blood glucose levels of 175 mg/dl, while the fourth rat had a blood glucose level of 130 mg/dl, all of which are above the normal range. Additional evidence of diabetes in the rats is the excessive glucose in the urine.

This study confirms the results of Rakieten, et. al., in 1963 that diabetes may be experimentally produced in laboratory rats by the intraperitoneal injection of streptozotocin, which causes pancreatic islet beta cell necrosis. The rats exhibited abnormally high blood and urine glucose levels, polydipsia, polyuria and polyphagia. This destruction of the pancreatic beta cells results in an increased blood glucose level due to a
lack of insulin.

The increase in water consumption by the diabetic rats in this study can be attributed to the elevated blood sugar levels. Guyton (1976) states that the elevated blood glucose has a dehydrating effect on the tissue cells. The elevated blood glucose has an osmotic diuretic effect on the kidney tubules, resulting in polyuria. Hence, the polydipsia is due to dehydration resulting from polyuria.

Infiltration of polymorphonuclear leukocytes into the healing wound of streptozotocin induced diabetic rats parallels those studies done by Hill, Perillie, Ward and Becker, and Robertson and Polk. When compared to the non-diabetic rats, the streptozotocin induced diabetic rats exhibit a delay in polymorphonuclear leukocyte response. This difference appears to manifest itself in the early stages of wound healing. There is a greater difference of PMN leukocyte response between the diabetic and non-diabetic rats in the 3 hour wound (an average difference of 8 PMN's per 300 X 300 m² area) and the 6 hour wound (an average difference of 11 PMN's per 300 X 300 m² area). At 3 and 6 hours the wounds in the non-diabetic animals had a greater infiltration of PMN's in the same corresponding wounds compared to the diabetic animals. However, in the 12 hour and 24 hour wound sites, there appears to be no significant difference in PMN leukocyte response between the diabetic and non-diabetic rats. Interestingly, this delay of PMN leukocyte response in the diabetic rats as seen in the early stages of wound healing is not evident as the wound healing process progresses. This early delay in PMN leukocyte response in the diabetic animal may be attributed to a variety of factors, none of which is conclusive.
Hill (et. al., 1973) in his study on impaired leukocyte responsiveness, suggests a deficient chemotactic stimulus may cause the delay in leukocyte response. If this is the case in this study, evidently the chemotactic stimulus, while inhibited at the early phases of wound healing in the diabetic rats, becomes restored as the wound healing phenomena progresses. Similarly, if the delay in PMN leukocytes response is attributed to deficient chemotactic factors, then an increase or pronounced expression of such chemotactic factors may account for the increase in PMN leukocyte response in the 12 hour and 24 hour wounds. An explanation for this change in neutrophil chemoattraction is suggested by Ward (1970), that upon destruction or death of the PMN at the site of inflammation, lysosomal factors are released which are chemotactic for PMN's. This may account for the increase in PMN response at the later stages of wound healing.

Perillie (et. al., 1962) shows that the early granulocyte phase of local cellular response is significantly delayed and diminished in patients with acidosis in comparison with normal and non-acidotic patients. In this study the delay in PMN leukocyte infiltration into the healing wounds of diabetic rats as compared to non-diabetic rats may also be due to acidosis. As the streptozotocin induced diabetic rats all show severe clinical signs of diabetes, the associated acidosis may in part be a factor for the early delay of PMN leukocytes at the healing wound site.

Ramamurthy and co-workers (1979) in their research found that diabetes inhibits the migration of PMN's into the rat gingival crevice during the early inflammatory phases of wound healing. This is consistent with the results in this study. The migration of PMN's in the diabetic animals is
inhibited during the early phases of wound healing. The PMN response dur­ing the later stages of wound healing in the diabetic animals appears to increase, more closely approximating the PMN response in the control ani­mals. This initial delay of PMN migration in the diabetic animals during the early stages of wound healing may be due to a number of factors. Ward and Becker in 1970, showed that leukotaxis is impaired when there is a de­pletion of potassium. They theorize that the decreased potassium concen­tration in the extracellular fluid of diabetics in the acidotic state, may inhibit enzyme systems reducing the ability of the leukocytes of diabetics to respond to a chemotaxis stimulus. However, their theory does not account for the correctable PMN response seen at the later stages of wound healing in this study.

Marble (1952), and Wertman and Henney (1961) in their studies hypothe­sized that the subnormal phagocytizing capacity of the leukocyte was one of the possible causes of reduced resistance of the diabetic to infection. Robertson and Polk (1974) showed that hyperglycemia alone led to decreased phagocytosis, decreased diapedesis and a decrease in intracellular killing of bacteria by PMN's. Drachman (et. al., 1966) showed that the phagocytic capabilities of PMN's are affected by the high levels of glucose in the tissues of chronically diabetic rats.

This study parallels the results reported by Miller and Baker. As there appears to be no observable defect of phagocytosis in the diabetic and non-diabetic rats as measured by the NBT test. Both diabetic and non-diabetic rats exhibited the reduction of nitroblue tetrazolium dye to a blue formazan pigment. Moreover, this positive NBT test for phagocytosis
was exemplified at both early and later stages of wound healing.
Eight male Sprague Dawley rats were experimentally made chemically diabetic by injecting streptozotocin. Seven days after the injection of the diabetogenic agent, the rats were clinically diagnosed as having diabetes mellitus. The diagnosis was confirmed by polydipsia, glycosuria, hyperglycemia and loss of weight.

The diabetic and non-diabetic animals were then subjected to a palatal incision wound and sacrificed at the following time intervals: 3, 6, 12 and 24 hours. The corresponding tissue sections were excised and fixed in 10% formalin and prepared according to conventional histologic technique utilizing hematoxylin and eosin staining procedures. Polymorphonuclear leukocyte (PMN) counts were made from each tissue specimen by counting the numbers of neutrophils within a 300 micron square area along the edge of the incised wound beneath the level of epithelium. The corresponding control tissue sections similarly were quantitatively evaluated and recorded.

Blood samples were taken at the time of sacrifice and thin smears were prepared and stained using NBT procedure. The blood slides were examined and evaluated for positive or negative NBT activity within each group.

The following conclusions can be drawn from this study:

1) Diabetes can be produced experimentally in rats thru the intraperitoneal injection of streptozotocin.

2) Rats made diabetic exhibit polydipsia, glycosuria, hyperglycemia and loss of weight.
3) There appears to be a delay in the response of PMN's to the site of wound healing within 3 and 6 hour post-injury diabetic rats.

4) The delay of PMN's in diabetic animals appears to correct itself within 12 to 24 hours post-injury.

5) There appears to be no defect in the diabetic or non-diabetic rats as to the ability of their PMN's to phagocytize.
BIBLIOGRAPHY


The thesis submitted by James L. Discipio has been read and approved by the following committee:

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Dr. Joseph Keene
Associate Professor, Periodontics, Loyola

Dr. Anthony Gargiulo
Professor, Periodontics, Loyola

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Oral Biology.

Feb 19, 1985  
Date

[Signature]

Director's Signature