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IN VITRO STUDY OF DOG NEUTROPHIL CHEMOTAXIS:
TECHNIQUE DEVELOPMENT AND
STUDY OF DOGS WITH DIABETES MELLITUS

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# IN VITRO STUDY OF DOG NEUTROPHIL CHEMOTAXIS: TECHNIQUE DEVELOPMENT AND STUDY OF DOGS WITH DIABETES MELLITUS

By

Julia Evans Stickle

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ABSTRACT

IN VITRO STUDY OF DOG NEUTROPHIL CHEMOTAXIS:
TECHNIQUE DEVELOPEMENT AND
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This research achieved several goals; assays of neutrophil function in other species were adapted for use with canine cells, canine cells were found unresponsive to formyl peptides, and the neutrophil responsiveness of diabetic dogs was evaluated.

Neutrophil response to stimulation was assessed by shape alteration and found to deviate from human cell response by a time dependent return to spherical in the presence of stimulant. Utilizing this technique it was found that dog neutrophils did not respond to formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). Binding studies with radiolabelled fMLP confirmed a lack of receptors for this compound on dog neutrophils.

Neutrophil adherence was evaluated by flow and gravity techniques and optimal test conditions were determined for whole blood and isolated granulocyte preparations. Assessment of neutrophil adherence in whole blood was found dependent on platelet activation by heparin at room temperature. Platelet activation was determined by increasing

particle counts (platelet clumps) and visual examination of the blood film.

Neutrophils behavior from dogs with juvenile onset diabetes mellitus which were well regulated with insulin was compared to cells from control dogs. Neutrophils from diabetic dogs changed shape readily but the return to spherical was inconsistently more rapid then normal dog cells. The source of this abnormality was not identified. Diabetic neutrophils produced increased amounts of thromboxane when stimulated. Neutrophil adherence in whole blood decreased with increased serum glucose concentration but was not different from normal cell adherence when isolated cells were evaluated. The decreased adherence in whole blood was considered the result of media factors and not dependent on altered neutrophil function. Cell migration (unstimulated and stimulated) was measured by passage through a filter and was not different from normal cell behavior.

Science becomes dangerous only when it imagines that it has reached its goal.

George Bernard Shaw

ACKNOWLEDGEMENTS

Many individuals have aided in this thesis and my developement. The graduate committee has been most supportive and helpful by sharing their expertise, experience, and time generously. More specifically Harold Tvedten has tirelessly reviewed manuscripts, advised, and given me the time to pursue and develope this research. Wayne Smith generously provided laboratory space and supplies allowing bench work start-up long before funding had been secured. But more significantly he was a major source of information and direction while fostering independent thought.

Numerous faculty and staff from Michigan State University have aided in my graduate studies. It is not possible to mention all individuals but this limitation does not deminish my appreciation for their assistence. The Department of Pathology provided laboratory space and some equipment which allowed work to continue after Dr. Smith's departure. Jimmy Hollers shared invaluable advise on laboratory techniques and research experience. Everyone associated with the clinical pathology section has been most supportive and helpful.

On the personnel front, "Aunt" Pat Virtue has provided consistent, loving, and dependable child care as well as giving me a genuine appreciation for the complications associated with diabetes mellitus.

Last, but not least, I owe appreciation to Russ and Sara who have tolerated me during this difficult period. They have had to endure my mental and/or physical abscence and have done it patiently and with good humor. Russ' loving support was also crutial in the completion of this work.

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# INTRODUCTION

This work deals with the laboratory evaluation of neutrophil chemotaxis. This area of study is just beginning to appear in the veterinary literature and holds great interest for the author. However, before worthy evaluation of neutrophil function in disease is possible it is mandatory for the testing techniques to be fully evaluated and understood for the species to be studied. The initial 2 experimental chapters therefore, represent technique developement and modification for the dog. The later chapter applies those techniques and a general assessment of chemotaxis (which had also been evaluated for use with dog cells) to a specific disease. A brief general literature review will be followed by chapters dealing with specific areas of inquiry, each will contain a more specific literature review and detailed discussion. It is the intent of this author that each of the later chapters stand as individual papers.

Before embarking on this work it is appropriate to review definitions of words and terms which will appear in this thesis. The following definitions are taken from Brecher (1976).

Locomotion: movement with displacement of the entire cell.

Motility: capacity to move actively.

Migration: active or passive movement of cells (usually a group or class of cells) from one location or organ to another.

Taxis: process of cell reacting to a stimulus by locomotion.

Positive: in direction of the stimulus.

Negative: away from stimulus.

Chemotaxis: taxis induced by chemical substance.

Phagocytosis: process by which extracellular solid particles are interiorized.

Capping: movement of selected portion of cell surface toward one pole.

Spreading: process of cell extending over increased surface of its support or another cell.

Protopod: front end of moving cell.

Uropod: tail end of moving cell.

Microvilli: short, fingerlike surface extensions.

Filopod: fine, long extensions.

Attachment filaments: cytoplasmic extension attached to support surface or other cells as a result of initial attachment and subsequent locomotion of cell, retraction of veil, etc. Also referred to as retraction filaments.

# CHAPTER 1

# NEUTROPHIL CHEMOTAXIS:
# A REVIEW OF THE LITERATURE

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## HISTORICAL PERSPECTIVE

The role of phagocyte mobilization in the inflammatory response was first reported in 1888 by Theodore Leber. The initial study by this ophthamologist involved movement of leukocytes into rabbit corneas. Metchnikoff (1887) expanded the appreciation of phagocytes by demonstrating their role in protecting the body against pyogenic infections. The theory of phagocytes as the sole defenders was soon modified as the interaction and cooperation of cellular and noncellular defense systems became apparent (Wright & Douglas, 1904). The interest in this discovery was intense and extended into the literature of the day as a major theme in George Bernard Shaw's play The Doctor's Dilemma (1911). After this auspicious beginning, study of phagocytes waned with most studies searching for the source(s) of stimulatory factors. As recently as 1960, Harris summarized the search for stimulatory factors by stating the only confirmed source was from bacteria. Progress was limited by a lack of in vitro techniques to study leukocyte migration and difficulty in obtaining relatively pure populations of cells.

A major technical advance in 1962 was the developement

by Boyden of a reliable method to quantitate cell movement. This technique utilized a double chamber system separated by a filter with small pores. Cell migration was quantitated by movement through the filter. Modifications of the original proceedure are still widely used in evaluating cell mobility.

Neutrophil isolation techniques were also improving with separation of total leukocytes by sedimenting agents (Boyum, 1964). Isolates of greater purity were possible with density gradient centrifugation (Boyum, 1968). This combination of advances has permitted a rapid expansion in the study of neutrophil function in the last two decades.

## CELL SHAPE AND ORIENTATION

Neutrophil movement was first described as involving altered cell morphology by Ramsey (1972b) and latter by Zigmond & Hirsch (1973). Generalized cell ruffling was identified (Gallin & Rosenthal,1974) and Ramsey described these as lamellipodia projecting in all directions with cell movement occurring only toward the lamellipodia nearest the stimulant. Retraction fibers at the rear of the cell were identified and attributed to lamellipodia which the cell had failed to fill (Ramsey, 1972b). Lichtman, et.al. (1976) later observed this alteration of shape to occur in suspended cells.

The role of microtubules in this shape alteration was

suggested by inhibition of migration by agents which reduce microtubule formation (Olmsted & Borisy, 1973). These findings, however, were variable depending on concentration and conditions (Klebanoff & Clark, 1978). Direct observation of microtubule assembly by electron microscopy has been reported (Goldstein, et.al., 1973 and Gallin & Rosenthal, 1974). Later electron microscopic studies demonstrated microtubules radiating from a prominent centriole and also mentioned prominent membrane associated microfilaments (Gallin, et.al., 1978). The role of microfilaments in the shape change response is primarily indirect (Klebanoff & Clark, 1978) but defective neutrophil chemotaxis has been described in a patient with dysfunctional leukocyte actomyosin (Boxer, et.al., 1974).

The shape of the cell is related to mobility with more elongate cells demonstrating faster movement (Keller, 1982). But bipolar cells are still capable of extending pseudopods in any direction when stimulated and indeed can reverse directions before a new lamellipodia has formed (Gerisch & Keller, 1981). This reorientation of the cell allows for modification and refinement of the neutrophil directional movement after initial activation (Zigmond, 1977). Previously oriented cells return to spherical when the stimulus is removed and the former polarity is not retained with restimulation (Zigmond, 1981). The site of directional control is unknown but does not reside in the nucleus (Keller &

Bessis, 1975).

Initial study of individual cell locomotion reported wide variations in the average speed of individual cells (Ramsey, 1972a). Later work, however, described fairly consistent movement of individual cells with alterations of population movement due to variable numbers of active cells (Keller, 1982). The effect of individual cell turning and reorientation was evaluated by Zigmond (1981). In this study, unstimulated cell turns generally deviated less then $20^{o}$ from the original course. When cells were maximally stimulated the interval between turns was prolonged with a resultant increase in the migratory rate observed.

ADHERENCE

The initial movement of neutrophils out of circulation and to an inflammatory site is dependent upon adherence to the vascular endothelial cells and substratum. The need for cell adherence for emigration was demonstrated by Ackerman, et.al. (1982) who showed that treatments which decrease neutrophil adherence also decreased cell accumulation into an inflammatory site. Excessive adherence, however, is also detrimental to cell mobility as Fehr and co-workers (1979) demonstrated. High doses of fMLP greatly enhanced adhesion with an associated decrease in cell mobility; it was proposed that this phenomena was the basis for neutrophil deactivation and also may be instrumental in cell trapping

at an inflammatory site. The role of adherence in neutrophil deactivation may also be related to the irreversible nature of in vitro stimulated adherence (Smith, Hollers, et.al., 1979). Keller and co-workers (1981b) confirmed enhanced neutrophil adherence will decreased migration. Enhanced neutrophil adherence has been associated with circulating neutropenia followed by a rebound neutrophilia (O'Flaherty, et.al., 1978) but circulating neutrophil numbers are influenced by many factors in addition to margination. The pattern of surface adherence may also relate to cell locomotion which can be influenced by surface treatment with various proteins (Keller, et.al.,1979).

Recent reports of patients with defective adherence have added greatly to the understanding of the crucial role of this function (Hayward, et.al., 1979, Crowley, et.al., 1980, Abramson, et.al., 1981 and Anderson, et.al., 1984). These similar works describe children with recurrent infections without neutrophil accumulation despite persistent leukocytosis. Defective neutrophil adherence and cell spreading has been documented in these children. Neutrophils responded to stimulation by changing shape but could not orient properly in a gradient or migrate toward the stimulus. A specific membrane protein (molecular weight around 127,000 daltons) was deficient in these patients.

Recent studies of neutrophil movement through a 3 dimensionalgel introduced a new perspective on the study of

cell function as cells do not adhere well tomaterials in which they are very capable of advancing through (Lackie & Brown, 1982). This finding is consistent with studies of compounds which interfere with surface adherence but do not influence migration through a filter (Ackerman, et.al., 1982). This cell behavior may represent migration through the tissues while surface adherence and subsequent movement may represent the initial emigration from the vessel.

# CHAPTER 2

# NEUTROPHIL FUNCTION IN THE DOG:

# SHAPE CHANGE AND RESPONSE TO SYNTHETIC TRIPEPTIDE

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## LITERATURE REVIEW

Neutrophil chemotaxis is, in part, dependent upon cell motility (Ramsey, 1974), which requires altered neutrophil shape (Bessis, 1973) in addition to other cell activities. The human neutrophil elongates and orients with the broad irregular surface toward the chemotactic stimulus and the narrower uropod at the posterior edge (Zigmond, 1977). Initial studies suggested that neutrophil polarity required surface contact (Ramsey, 1974); however, later work confirmed that cell shape was possible in solution (Lichtman, et.al., 1976). Assessment of cell shape has since been used to indicate cell responsiveness to stimuli without the requirement for other cell functions (Smith, et.al., 1979). The present work utilized this response in assessing the effect of N-formyl-methionyl-leucyl-phenylalanine (fMLP) on dog neutrophils.

Neutrophil chemotactic activity has been recovered from bacterial cultures (Keller & Sorkin, 1967 and Ward, et.al., 1968) with potent activity generated by Escherichia coli (Schiffman, et.al., 1974). Chemoattractant activity has also been defined for several N-formylmethionyl peptides

(Schiffman, Corcoran & Wahl, 1975). These compounds were later equated to chemotactic factors liberated from bacterial cultures (Marasco & Becker, 1982b) and fMLP determined as the most potent stimuli (Marasco, et.al., 1982a).

The advantages of fMLP over other chemotactic agents include easier and more precise quantitation, easier preparation and availability. Species, however, vary in responsiveness to fMLP. The presence of that response must be established for each species. Cell responsiveness to fMLP may be determined by binding of radiolabeled fMLP to neutrophils or by various measures of neutrophil function.

Studies of receptor numbers and kinetics utilizing tritiated fMLP (fML[$^3$H]P) have been completed on cells from human beings (Williams, et.al., 1977) and rabbits (Asawankumar, et.al., 1977). Cells from guinea pigs also responded chemotactically to N-formylmethionyl oligopeptides (Chenoweth, et.al., 1980). Equine neutrophils have a low number of receptors for N-formylmethionyl peptides and these stimulants induce lysosome release, but do not initiate chemotaxis (Snyderman & Pike, 1980). Although one study did report horse neutrophil chemotactic activity induced by high concentrations of fMLP (Zinkl & Brown, 1982), this finding may be secondary to liberation of leukocyte derived chemotactic factors (Klebanoff & Clark, 1978). Studies of the pig (Chenoweth, et.al., 1980) and cow (Gray, et.al.,

1982) did not identify receptors for N-formylmethionyl peptides on neutrophils from these species. The present work evaluated dog neutrophils for receptors to N-formylmethionyl peptides utilizing fML[$^3$H]P.

## MATERIALS AND METHODS

### Animals

Two healthy adult Golden Retrievers, 1 male and 1 female, and 1 male Samoyed were evaluated. The Samoyed, recovering from an *E. coli* septicemia (positive blood culture 4 days prior to sampling), was included because it may have altered receptors to fMLP. Therapy consisted of furosemide and IV penicillin in lactated Ringer's solution.

### Neutrophil Isolation

Citrate anticoagulated blood (5 ml) was diluted 2-fold with calcium free Dulbecco's phosphate-buffered saline solution (Ca-free PBSS), carefully layered over 4 ml Ficoll-diatrizoate sodium (Sigma Chemical Co., St. Louis, MO) (sp gr = 1.077) and centrifuged at 400 x g for 30 min. The upper layer of platelets and mononuclear cells and Ficoll-diatrizoate sodium were discarded and the remaining granulocytes and RBC resuspended in Ca-free PBSS. The granulocyte-RBC suspension was combined with 1.7 ml of 6% hetastarch (McGraw Laboratories, Irvine, CA) to enhance

gravity sedimentation of the RBC, which required 45 to 60 min. The granulocyte-rich supernatant was removed, washed with Ca-free PBSS, and centrifuged at 200 x g for 15 min. The resulting pellet was resuspended in Dulbecco's phosphate-buffered saline (PBSS) to a final concentration of $10^7$ cells/ml for shape-change assay or 5 x $10^7$ cells/ml for binding studies.

Visual examination with a phase microscope confirmed the presence of <5% nongranulocytic nucleated cells and no more than 10 RBC/granulocyte. Platelets were rarely encountered. Eosin exclusion determined cell viability between 95% and 98%.

Chemotactic Factors

Pooled normal dog serum was used as a known chemoattractant stimulus for the dog (Latimer, Crane & Prasse, 1981). A stock solution of $10^{-3}$M fMLP (Sigma Chemical Co., St. Louis, MO) in dimethyl sulfoxide was prepared every 30 days and aliquots for daily use were maintained at -70 C. Final concentration ($10^{-6}$ to $10^{-9}$M) of fMLP was attained by further dilution with PBSS.

Zymozan activated plasma (ZAP) was prepared by incubating 10 mg of zymozan/ml (ICN Nutritional Biochemicals Division, International Chemical and Nuclear Corp., Cleveland, OH) of fresh heparinized plasma (25 units/ml) at 37 C for 5 min. Zymozan was removed before aliquots were

stored at -70 C. Final stimulatory dose was 1% ZAP in the cell suspension.

Labeled fMLP (New England Nuclear, Boston, MA) at 0.167mM with 60 Ci/mM specific activity was stored at -70 C until diluted with PBSS to the final concentrations of 5 x $10^{-8}$, $10^{-7}$, 1.5 x $10^{7}$ and 3 x $10^{7}$M.

Human neutrophil isolation

As a control, citrated blood from 2 healthy female human volunteers was processed similar to dog samples for neutrophil isolation, however, RBC sedimentation required 30 to 40 min. The final concentration of cell suspension was 5 x $10^{7}$ cells/ml.

Neutrophil shape evaluation

Suspensions of 0.5 to 1.0 x $10^{6}$ cells in PBSS were incubated with pooled serum, ZAP or fMLP at 37 C. Cells were fixed for at least 1 hour with an equal volume of refrigerated 2% buffered glutaraldehyde (Sigma Chemical Co., St. Louis, MO) after 5, 10, 15, 30, 60, 90, 120 and 180 min. of incubation. Wet mounts of fixed cells were prepared with 100 cells classified according to shape with 100 X phase-contrast objective.

Binding studies

Formylmethionyl-peptide binding to dog neutrophils was assessed with fML[$^3$H]P. Ten million cells (0.2 ml of 5 x $10^7$ cells/ml suspension) were incubated for 10 min. at room temperature with 20 1 of fML[$^3$H]P in the final concentration (5 x $10^7$ cells/ml). Additional binding was terminated by the addition of 2 ml of cold PBSS. Neutrophils were collected by rapid filtration glass filters (Whatman, Inc., Clifton, NJ) and washed twice with 5 ml cold PBSS. Filters were dried for 24 hours at room temperature and suspended in 10 ml of insta-gel scintillation cocktail (Packard Instruments Co., Inc., Downers Grove, IL). Radioactivity was determined with scintillation counter (Beckman L57000) with a counting efficiency of 45%.

After cells had been incubated with labeled fMLP for 10 min., nonspecific binding was determined by addition of 20 1 of $10^{-4}$M unlabeled fMLP. Fifteen min. after this addition, the reaction was halted and samples processed as stated previously. Specific binding was defined as total cpm minus nonspecific cpm.

RESULTS

Neutrophil shape change

Glutaraldehyde-fixed dog neutrophils in suspension had various cell shapes observed in the humans, such as

spherical, ruffled, and bipolar with or without a uropod (Fig. 1). Studies with ZAP-stimulated dog cells (Fig. 2) demonstrated a dose response with more cells in the bipolar form for a longer time at the higher level of activation. Cell response to pooled healthy dog serum (Fig. 3) documented the rapid conformational change and gradual return to spherical, which was observed with dog neutrophils.

Comparison of stimuli (Table 1) indicated that unstimulated neutrophils consistently maintained the spherical configuration and cells exposed to a known chemotactic factor (pooled dog serum) readily elongated to the bipolar shape. Exposure to fMLP did not induce bipolar neutrophil shape after 10 min. of incubation; cells were also examined at 5 and 15 min. incubation with similar results.

The Samoyed, recovering from septicemia (positive blood culture for E. coli), did not have appreciably different results from the nonsepticemic dogs tested.

Binding studies

Human control cells had high binding of labeled fMLP (9,498 and 18,847 counts per minute); this binding activity was greatly reduced in the presence of excess unlabeled fMLP (3,755 and 3,613 counts per minute) and indicated binding specificity and reversibility (Fig. 4).

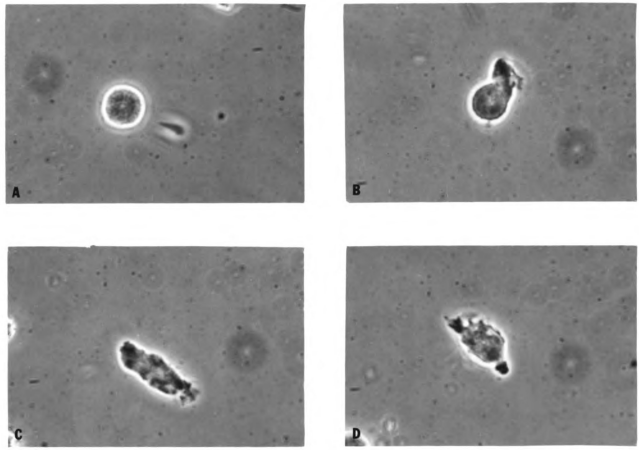


Figure 1. Stimulated and unstimulated neutrophil shapes: Unstimulated cells are spherical (A), stimulated cells appear ruffled (B), bipolar (C), or bipolar with uropod (D) depending on time and degree of stimulation.

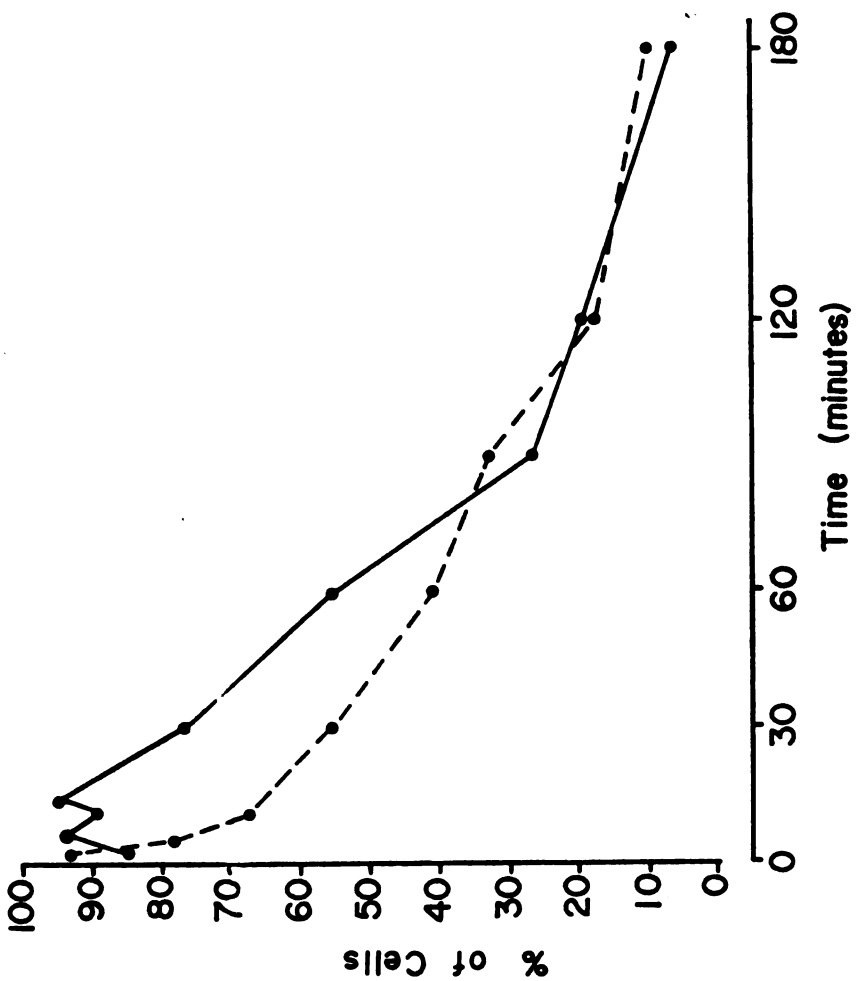


Figure 2. Proportion of neutrophil in bipolar conformation for 180 min. after stimulation, (——) mean of 13 samples stimulated with 1% ZAP, ( --- ) mean of 9 samples stimulated with 0.5% ZAP.

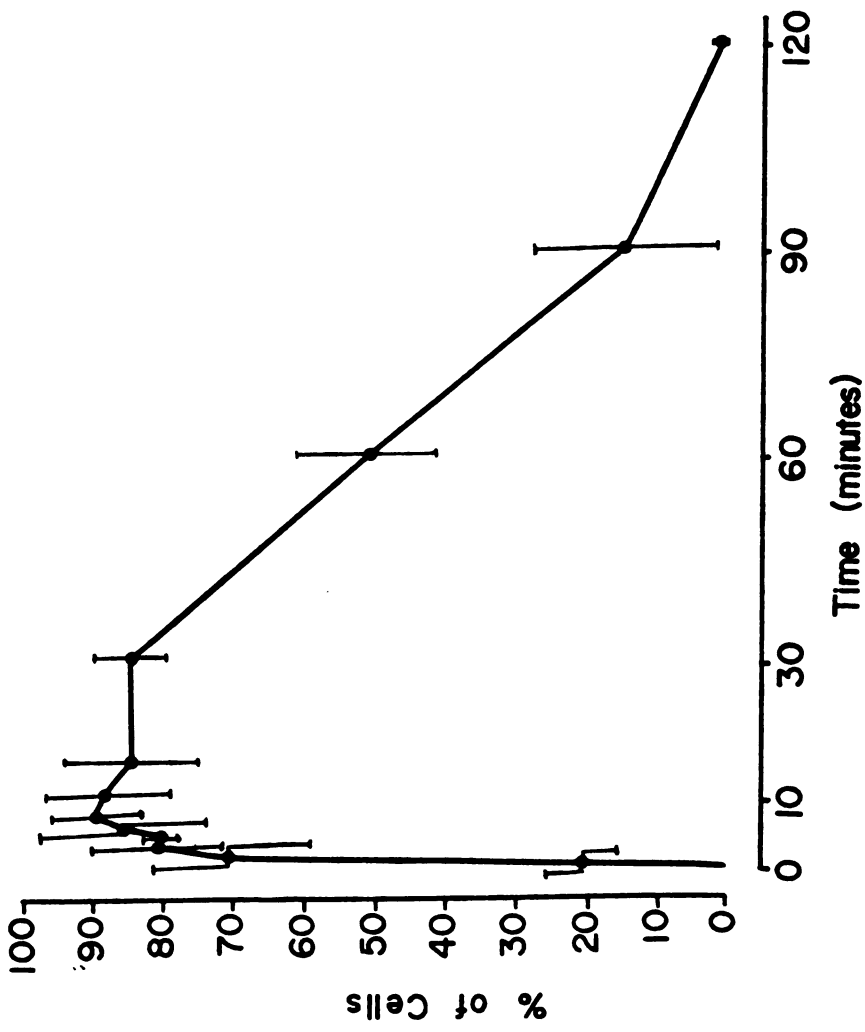


Figure 3. Proportion of neutrophils in bipolar conformation for 120 min. after stimulation with 2.5% pooled dog serum.

Table 1. Dog neutrophil bipolar shape formation with various stimulants; cells were incubated for 10 minutes prior to fixation.

| Stimulant | Stimulant Concentration | No. of Samples | % Bipolar Cells Mean ± SD |
|---|---|---|---|
| PBSS | 100% | 4 | 0.5 ± 1.0 |
| Pooled serum | 2.5% | 4 | 88 ± 3.6 |
| fMLP | $10^{-9}$M | 2 | 0 ± 0 |
| fMLP | $10^{-8}$M | 4 | 0.5 ± 1.0 |
| fMLP | $10^{-7}$M | 2 | 2.0 ± 1.4 |
| fMLP | $10^{-6}$M | 2 | 0.5 ± 0.7 |

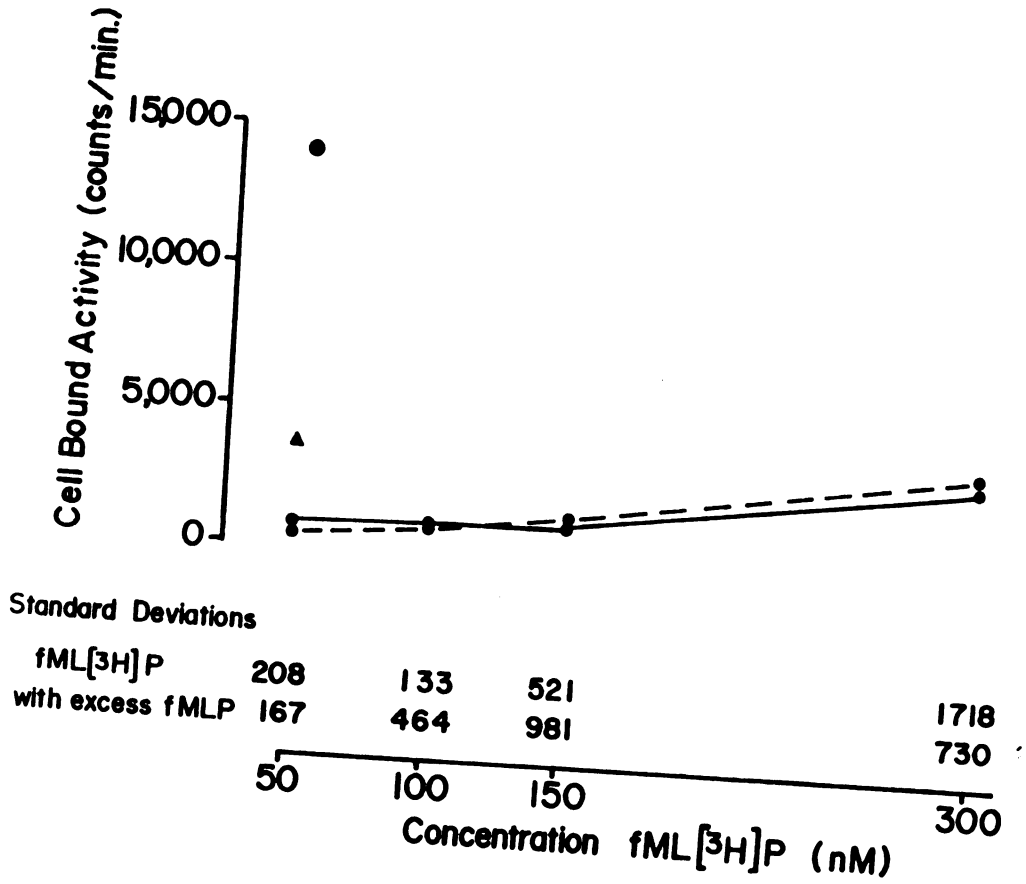


Figure 4. Neutrophil bound activity of fML[$^{3}$H]P. Mean of cells with 3 dogs ( —— ) and after the addition of excess unlabeled fMLP ( --- ). Mean of cells from 2 human beings (●) and after the addition of excess unlabeled fMLP (▲).

Dog cells had low cell-bound activity with similar results observed irrespective of the presence of excess unlabeled fMLP. A mild increase in cell-bound activity was observed with an increase in concentration of labeled compound. These results were consistent with the shape change studies that did not indicate cell response to fMLP.

## DISCUSSION

The data presented demonstrated that dog neutrophils adopt bipolar shape when activated by a known chemotactic factor. This conformational change was dose and time dependent with a greater cell activation at higher doses of chemotactic factor, which persisted for a longer time. The rapid occurrence of bipolar cells was consistent with observations in human cells (Smith, et.al., 1979). In the present study, the return to nonbipolar shape of cells with time was in conflict with one observation of human cells (Smith, et.al., 1979).

Dog neutrophil response to fMLP was not observed. Cells did not change shape and radiolabeled binding studies did not define specific binding for fMLP on dog neutrophils and suggested that receptors for this compound were lacking.

Lack of chemotactic responsiveness to N-formylmethionyl peptides has been reported for the pig (Chenoweth, et.al., 1980), horse (Snyderman & Pike, 1980), and cow (Gray, et.al., 1982). Pig neutrophils also did not respond to

purified bacterial chemotactic factor (Chenoweth, et.al., 1980) and cow cells did not react to cell-free media from E. coli (Carroll, Mueller & Panico, 1982).

Receptors to fMLP have been induced in vitro by treatment of human promyelocytic leukemia cells (HL60) with cyclic nucleotides (Chaplinski & Niedel, 1982). The possibility of in vivo induction of receptors was the basis for inclusion of 1 postsepticemic dog in the study. Sufficient time was allowed for cells influenced in maturation to reach the peripheral circulation (LoBue, 1970). Neutrophil shape change and binding studies were not different from nondiseased dogs tested, diminishing the possibility of in vivo induction of receptors; however, deactivation of the cells could not be excluded.

Lack of dog neutrophil response to fMLP stimulation was consistent with a lack of specific binding of labeled fMLP and suggested that dogs lack receptors for the formylated peptides. This conclusion was consistent with a study of chemotaxis (modified Boyden technique), aggregation, chemiluminescence, and superoxide generation of dog cells (Redl, et.al., 1983a), which also demonstrated a lack of response to fMLP. However, chemotaxis and aggregation were enhanced by a crude extract of E. coli culture indicating that an additional stimulatory compound may also be present.

# CHAPTER 3

# TECHNIQUES FOR THE STUDY OF DOG NEUTROPHIL ADHERENCE

# TECHNIQUES FOR THE STUDY OF DOG NEUTROPHIL ADHERENCE

## INTRODUCTION

In order to document or refute the role of neutrophil adhesion in various canine diseases the assays used in other species must be validated for and modified to the dog. Reports in the veterinary literature studied neutrophil function of bovine (Dorsey & Deyoe, 1982, Carroll, et.al., 1982 and Gray, et. al., 1982) porcine (Chenoweth, et.al., 1980, and Smith, et.al., 1981) equine (Snyderman & Pike, 1980 and Zinkl & Brown, 1982) and canine (Bowles, et.al., 1979, Kroese, et. al., 1981, Latimer, et.al., 1981, Latimer, et.al., 1982, Redl, et.al., 1983a and Latimer, et.al., 1984) enforcing the need to evaluate techniques for each species studied. This report examines 2 methods for determinating neutrophil adherence with emphasis on munipulations of the test system which influence the data obtained.

Two theoretical approaches to neutrophil adherence were investigated. Neutrophil adherence in a moving fluid environment was evaluated in a nylon wool column using whole blood and isolated granulocytes as samples. Neutrophil adherence in gravitational forces was examined with isolated granulocyte preparations.

# MATERIALS AND METHODS

## Animals

Clinically normal dogs of different breed, sex and age were used in this study. All animals were owned by and housed at the Veterinary Clinical Center, Michigan State University.

## Sample Collection and Preparation

All blood was collected by jugular venapuncture using syringe and 18 gauge needle. Samples for whole blood evaluation were immediately placed in sodium heparin (25 USP units/ml. blood), citrate (final concentration of 0.0105M), or potassium EDTA (1.5 mg./ml. blood) sample tubes (Becton-Dickinson, Rutherford, NJ 07070). Samples for granulocyte isolation were mixed with citrate (final concentration of 0.0105M) (Becton-Dickinson, Rutherford, NJ 07070). Sample tube vacuum was released before use.

## Isolation of Granulocytes

Blood for isolation of granulocytes was first diluted to 50% with calcium free phosphate buffered saline (Ca free PBS) and layered over Ficoll-Hypaque sp. gr. 1.077 (Sigma Chemical Co., St. Louis, MO 63178). The sample was centrifuged at 400xg for 30 minutes at 4$^{o}$C. The upper layers of plasma, mononuclear cells and platelets, and Ficoll-Hypaque

were removed and the remaining red blood cells and granulocytes resuspended in Ca free PBS. Gravity sedimentation of red blood cells was enhanced by the addition of 6% hetastarch (Travenol Laboritories, Inc., Deerfield IL 60015). Separation of red blood cells typically required 45 minutes at which time the granulocyte rich upper layer was removed, washed with Ca free PBS and centrifuged at 200xg for 15 minutes. The resultant pellet was resuspended in complete phosphate buffered saline (PBS) to a concentration of $10^7$ cells/ml (Model ZBI, Coulter Electronics, Inc., Hialeah, FL 33010).

Visual examination of the final granulocyte preparation confirmed greater than 95% neutrophils, less than 10 red blood cells/granulocyte, and essentially no platelets. Eosin exclusion determined greater than 97% cell viability.

Flow Assesment of Neutrophil Adherence

Nylon wool adherence assay has been previously reported (MacGregor et.al., 1974). Modifications for canine neutrophils included use of 1cc Tuberculin syringes and 18 gauge syringe needles (Becton-Dickinson, Rutherford, NJ 07070) with scrubbed nylon fiber (3 denier, 3.81 cm., type 200) (Fenwell Laboritories, Deerfield, IL 60015) at a density of 20mg. fiber/0.1ml. volume. All samples were run in triplicate and the data averaged.

White blood cell (Coulter Electronics, Inc., Hialeah FL

33010) and differential counts were determined on whole blood samples prior to exposure to the nylon wool column. A standard volume of blood was placed on the nylon wool column and allowed to pass through the column by gravitational force. The recovered sample was then evaluated for absolute neutrophil number. Adherence was the difference between original and final neutrophil numbers.

Nylon wool adherence assay with isolated neutrophils was similar to the whole blood technique except differential counts were not necessary. Modulation of the adherence level was accomplished by pretreatment of the nylon wool column with various concentrations of protein solution; 0.5ml. of protein solution were used followed by 1ml. PBS.

Gravitational Assesment of Neutrophil Adherence

Gravitational assesment of neutrophil adherence (Smith, et.al., 1979) utilized a Sykes-Moore chamber with two coverglasses. One coverglass was soaked in various concentrations of serum for 120 seconds and rinsed in PBS. This protein treatment modulated the degree of neutrophil adherence. The chamber was assembled with the treated coverglass on the bottom and filled with a suspension of $10^6$ cells/ml. The cells were allowed to settle for 400 seconds and cell density determined with an inverted phase microscope and 100x oil immersion lens; five fields were counted and averaged. The chamber was gently inverted and left

undisturbed for 400 seconds. During this interval the non-adherent cells settled to the untreated coverglass and tightly adhered to this surface. The chamber was then gently returned to the original orientation and the density of cells again determined. Neutrophil adherence was the difference between these two density counts.

## RESULTS

### Flow Assesment of Neutrophil Adherence, Whole Blood

Whole blood samples from 3 dogs were mixed with sodium heparin, citrate, or EDTA and passed through nylon wool columns containing 80, 100, or 120 mg. of nylon wool. Results (Figure 5) indicated increasing neutrophil adherence with greater amounts of nylon wool for the heparinized sample. Samples anticoagulated with either citrate or EDTA did not have increased adherence with greater surface area. Both citrate and EDTA function as anticoagulants by binding calcium and calcium is required for many neutrophil functions (Marasco et.al., 1980).

Incubation at room temperature enhanced neutrophil adherence. This was attributed to platelet activation and indirect effects on adherence. Figure 6 represents data from one representative dog of three dogs studied; a single sample was taken from the animal and alliquoted into five heparinized sample tubes. These sample tubes were then

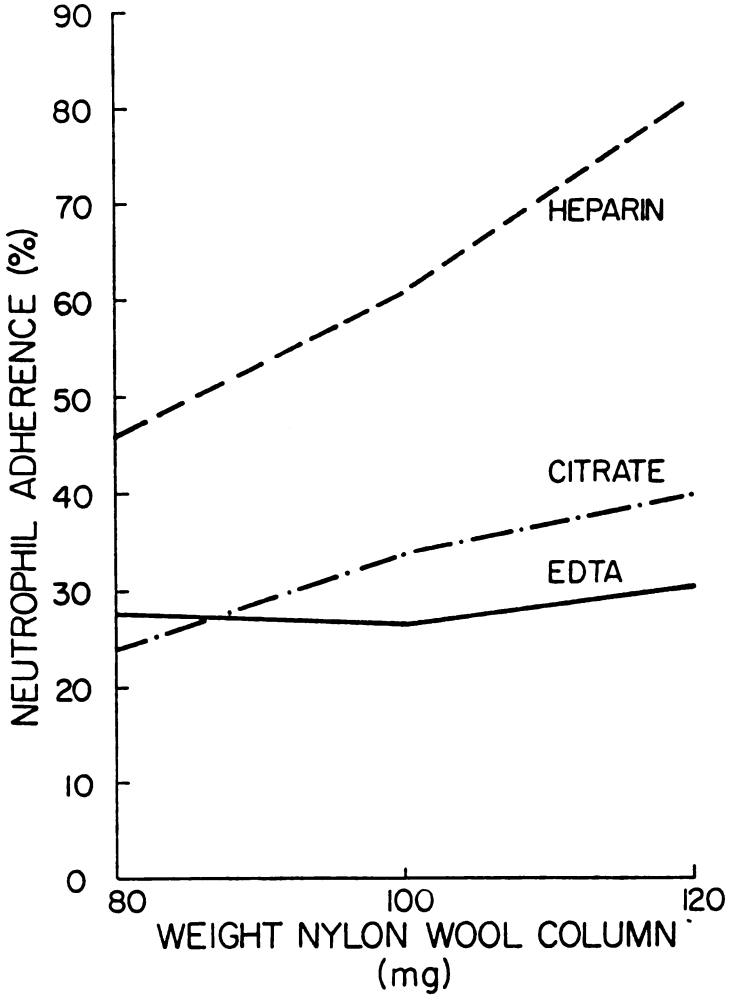


Figure 5. Whole blood wool adherence assay: Effect of nylon wool weight and anticoagulant on neutrophil adherence.

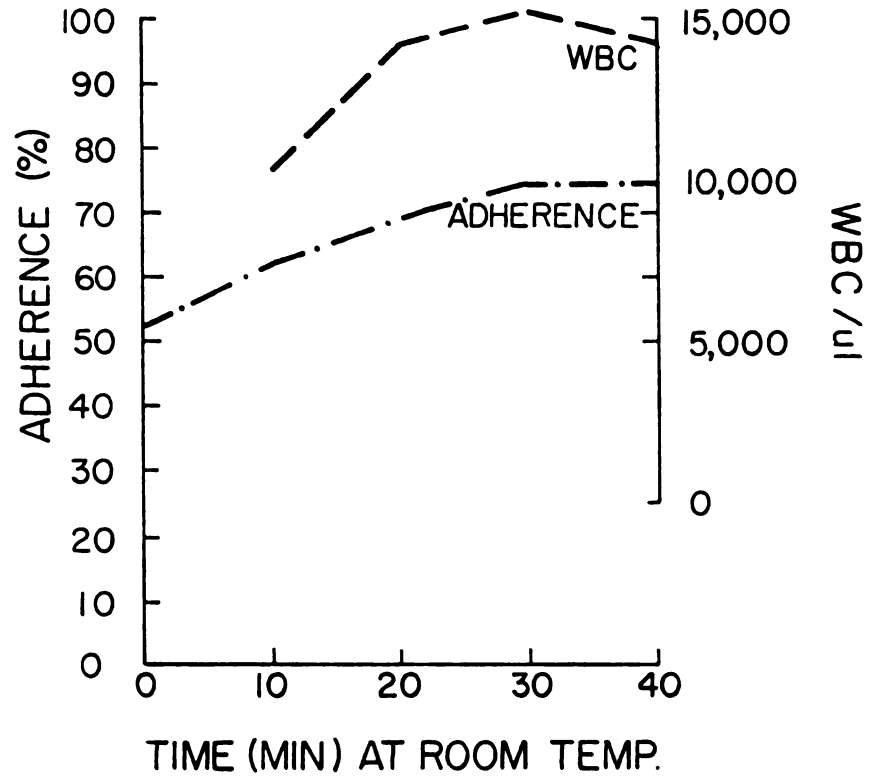


Figure 6. Whole blood nylon wool adherence assay: 5 samples from one dog incubated at room temperature for various times. Increased neutrophil adherence was accompanied by increased WBC count.

maintained at room temperature for 0, 10, 20, 30, and 40 minutes to allow various levels of activation of platelets. Samples were then evaluated for white blood cell count (Coulter Electronics, Inc., Hialeah FL 33010) and neutrophil adherence using an 80 mg. column of nylon wool. Slides made of each sample revealed increasing platelet clumps at the feathered edge of the smear, this was reflected in the increased white bllod cell count as the platelet clumps became large enough to be counted as particles. As these clumps began to enlarge and coalesce the total white cell count decreased. With the increased platelet clumping neutrophil adherence increased by approximately 20%.

Flow Assesment of Neutrophil Adherence,

Isolated Granulocytes

Isolated granulocytes were evaluated for adherence on columns containing 80mg. of nylon wool. Pretreatment of columns to modulate adherence was examined using different types and concentrations of albumin. Bovine albumin, Fraction V (Sigma Chemical Co., St. Louis MO 63178) and CRG (Armour Pharmaceuticals, Kankakee, IL 60901), were evaluated with cells from four dogs (Figure 7). The type of protein used did not alter adherence. Fraction V canine albumin (Sigma Chemical Co., St. Louis MO 63178) was also tested with cells from two dogs with similar results. Concentration of protein did influence adherence of canine neutro-

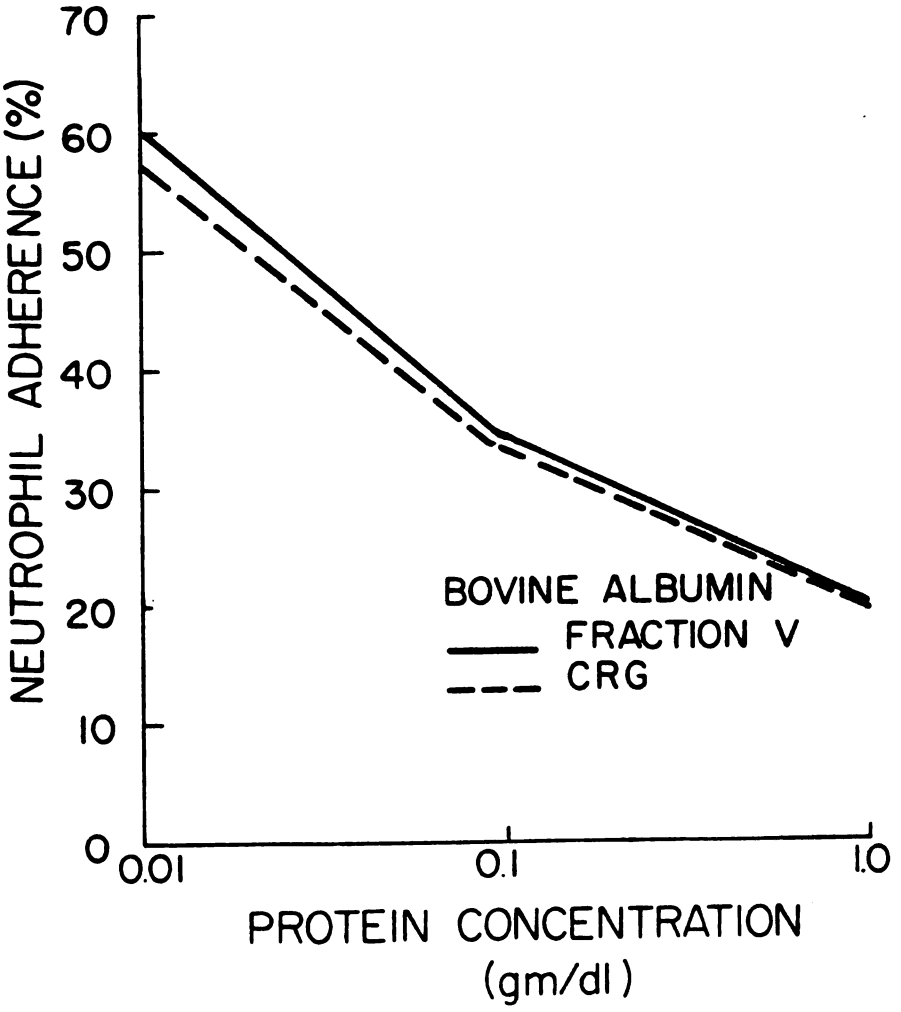


Figure 7. Isolated neutrophil nylon wool adherence assay: Effect of column pretreatment with albumin.

phils. Neutrophil adherence was increased when columns were treated with a lower concentration of protein.

Gravitational Assesment of Neutrophil Adherence

Isolated granulocyte preparations were used and the effects of protein coating of the test coverslip were evaluated (Figure 8). Concentrations of 0.5 and 1.0% serum were evaluated for six dogs and concentrations greater than 2% and no pretreatment were examined for two dogs. As previously noted for the flow techniques, greater concentration of protein pretreatment decreased neutrophil adherence.

DISCUSSION

The importance of neutrophil adherence is well summarized by the work of Fehr and Dahinden (1979) who studied the effect of increased adherence on chemotaxis. Excess levels of adherence inhibited neutrophil movement without a ffecting other cell functions. Conversely inadequate neutrophil adherence does not allow sufficient surface interaction for normal motility (Keller, 1981a). Therefore both increased and decreased adherence of neutrophils are of biologic significance. As demonstrated by this study the level of adherence in a test system can be established by munipulations of several variables. The adherence level of the test system may be set by the expected effect (i.e. if decreased adherence is anticipated the untreated adherence may be set

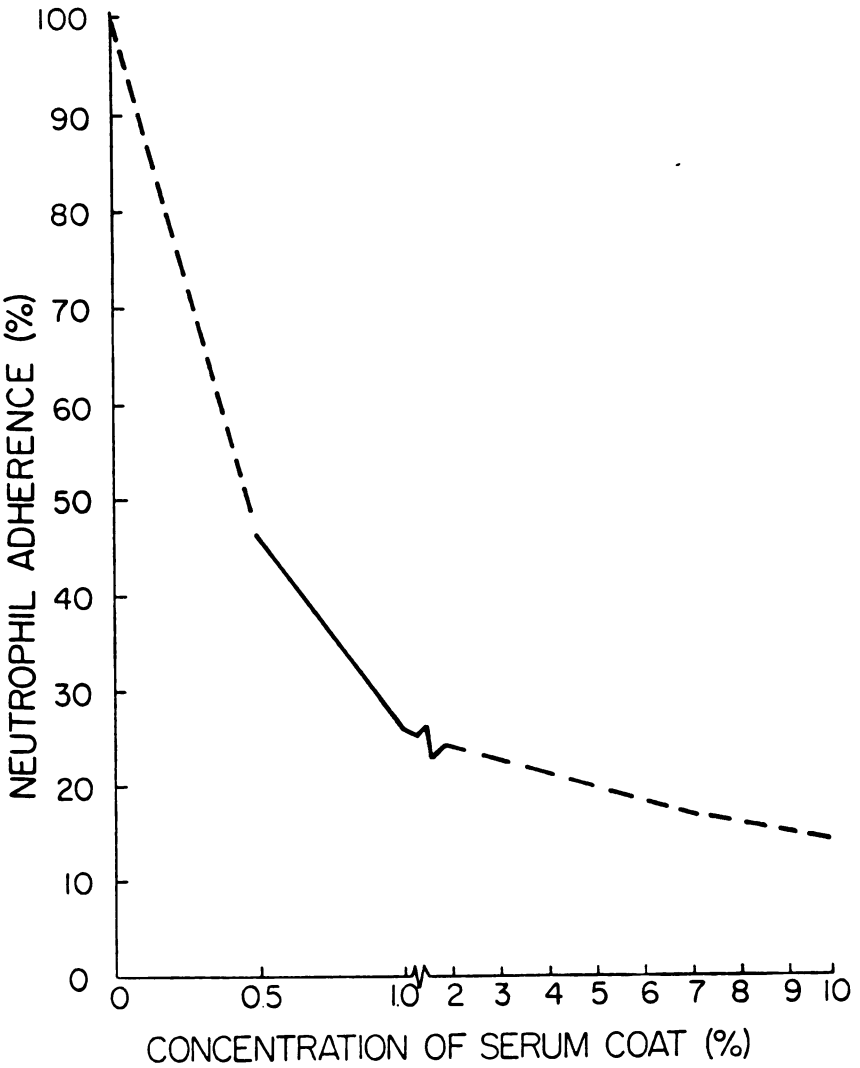


Figure 8. Gravity adherence assay: Effect of cover glass pretreatment with serum.

at a high level) or may be established in the mid-range to allow for detection of changes in either direction.

This study examined the use of nylon wool columns as a flowing medium technique for evaluating adherence. This method was selected because it is simple and convenient and is appearing in many veterinary reports. An alternative similar method utilizes a column of glass beads (Brandt, 1965). Both methods have similar variables including the surface area to which the neutrophils are exposed (Schneier et.al., 1977). For study of whole dog blood the mid-range of cell adherence occurred with 80 mg. of nylon wool. Once this volume of fiber was selected it was used for studies with isolated cells as well as whole blood.

An important finding of this research was that the use of calcium binding anticoagulants (citrate and EDTA) interfered with optimal adherence. The central role of calcium has been previously reported for rabbit neutrophils (Marasco et.al.,1980).

Modulation of cell adherence by proteins was demonstrated in both flow and gravitational methods when studing isolated neutrophils. Protein influence on neutrophil adherence was evaluated by Keller et.al. (1979). In this study both serum and various albumin solutions were evaluated with consistent results: greater amounts of protein surface treatment decreased neutrophil adherence. Again this variable was used to establish the level of cell adher-

ence; we recommended that nylon wool columns be pretreated with 0.01 gm./dl. albumin and coverglasses for the gravitational technique be treated with 0.5% serum. Interestingly the source or purity of albumin did not influence test results.

The role of platelet activation in modulating neutrophil adherence was clearly evident in the studies of heparinized whole blood held at room temperature for various intervals. Dog platelets are very sensitive to activation by heparin (Clemmons & Meyers, 1984) and this fact has allowed for demonstration of the influence these cells have on neutrophil adherence. Previous studies have demonstrated the effect of increased platelet numbers enhancing neutrophil adherence (Rasp,et.al., 1981) and the in vivo relationship of the cell's functions have been widely discussed. Control of platelet activation should be attempted by several methods; minimization of tissue fluid contamination, reduce turbulence of the blood, and placing samples immediately on ice for transport to the laboratory.

The study of neutrophil adherence has just begun with the influence of endogenous (Boxer, et.al., 1980, Lentnek et.al, 1976 and O'Flaherty et.al., 1978) and exogenous (MacGregor, 1977) compounds rapidly emerging. For further study to be beneficial it is crucial to understand the test systems used and the methods of modifying results.

# CHAPTER 4

# CANINE DIABETES MELLITUS:

# IN VITRO STUDY OF NEUTROPHIL CHEMOTAXIS

# CANINE DIABETES MELLITUS:
# IN VITRO STUDY OF NEUTROPHIL CHEMOTAXIS

## LITERATURE REVIEW

The association of diabetes mellitus with infectious processes has been entrenched in clinical medicine since the 3rd century A.D. The issue remains prominent in the literature (Rayfield, et.al., 1982). Detailed evaluation of neutrophil activities from diabetic patients appeared in the mid 1950's. The initial studies were done in vivo and demonstrated delayed appearance of neutrophils at an inflammatory site.

Cruickshank (1954) used rabbits with acute alloxan induced diabetes to study the effects of intradermal injection of Staphylococcus aureus and concluded that the acute inflammatory response was delayed but attributed this finding to shock and decreased tissue perfusion. The crucial role of tissue perfusion in the first 2 to 3 hours after infection was well defined by Miles and Niven (1950). Rabbits with acute alloxan induced diabetes were used by Sheldon and Bauer (1959) in the study of cutaneous mucormycosis. Histopathologic study of injection sites indicated delayed and reduced neutrophil influx and lack of fibro-

plasia but no difference in the appearance of large mononuclear cells when compared to control animals. Infection in diabetic animals was typically progressive and fatal whereas normal animals recovered after only limited tissue involvement. These authors assumed the neutrophils were less functional due to ever increasing numbers of organisms although work by Miles, Miles & Burke (1957) demonstrate the devastating effect of delayed appearance of phagocytes on coping with infectious situations. Histologic study of rats with acute alloxan induced diabetes with ketoacidosis also demonstrated decreased acute inflammation with delayed appearence of neutrophils and fibroplasia. Again large mononuclear cell appearence was not affected. The diabetic animals were unable to contain the infectious process with extensive fungal proliferation into adjacent soft tissue (Sheldon and Bauer, 1960). Exudative fluid volume from the peritoneal cavity of rats with alloxan induced diabetes appeared to be decreased in a study by Briscoe and Allison (1965) suggesting a chemotactic defect.

Placement of specially designed chambers over skin lesions allowed the study of human diabetics at various time intervals after injury. This study found a delayed and diminished appearence of granulocytes at the inflammatory site in acidotic diabetics when compared to normal and non-acidotic diabetic patients. Patients reevaluated after correction of ketoacidosis no longer demonstrated the abnor-

mality. High levels of ketone bodies were found in exudative fluid from poorly controlled diabetics and the authors suggest this feature was associated with increased susceptibility to infection (Perillie, Nolan & Finch, 1962). Work by Brayton, et.al. (1970) using chambers placed over abraded skin evaluated neutrophil chemotaxis in diabetic human patients; nonketoacidotic diabetics had a significant delay in neutrophil appearance during the first 2 to 4 hours with a more severe defect evident in patients with ketoacidosis.

The significance of serum alterations was studied utilizing rats with chronic alloxan induced diabetes without ketosis; mortality, lesion bacterial counts and histopathologic evaluation after induction of Type 25 pneumococcal pneumonia revealed decreased neutrophil appearance, increased bacterial growth and decreased phagocytosis in the diabetic animals. These workers felt that the defect resided in the serum and was derived from the high glucose concentration and primarily the increased osmotic pressure (Drachman, Root & Wood, 1966). The role of glycemia and acidosis in the acute inflammatory reaction was studied in ear chambers on normal rabbits. Hypertonic glucose was administered orally to induce hyperglycemia, hyperosmolality, and metabolic lactic acidosis in an otherwise normal animal. After treatment the inflammatory reaction was significantly decreased; this abnormality was evident when only hyperglycemia and hyperosmolality were present

hence decreasing the role of acidosis in inhibiting the inflammatory response (Ainsworth & Allison, 1970).

In vitro studies of neutrophil chemotaxis by assessing migration through a filter have also appeared in the literature with more variable results. Mowat and Baum (1971) reported decreased migration of diabetic neutrophils with increased glucose concentration in the incubation media having no effect on cell migration. Addition of insulin to the media gave diverse effects; insulin with phenol preservative decreased chemotactic activity at concentrations of greater than 100uU/ml. well preservative free insulin levels demonstrated an enhancing effect on neutrophil chemotactic activity. Similar results were reported by Miller and Baker (1972)with decreased cell migration seen inneutrophils from children with diabetes mellitus. These authors observed improved chemotaxis with the addition of low levels of insulin to the media but no effect at concentrations of greater then 10uU/ml.; the specific type of insulin used was not identified. Hill et. al. (1974) also report abnormal neutrophil chemotaxis for diabetic patients but were unable to relate the defect to duration of disease or degree of control. Incubation of cells with 100uU/ml. of low phenol insulin corrected the chemotactic defect. Evaluation of diabetics with periodontal disease revealed no difference between nondiabetic patients with variable severity of periodontal disease and diabetics with mild periodontal disease.

Decreased neutrophil chemotaxis was evident in patients with diabetes and severe periodontal disease (Manouchehr-Pour, et.al., 1981).

The finding of abnormal chemotaxsis of neutrophils from diabetics in the in vitro filter system is not consistent. Study of nonketotic juvenile and adult diabetics failed to demonstrate a defect in neutrophil chemotaxis (Fikrig, 1977). The source of this discrepency was proposed by Hamberg, et.al., (1976) when they studied both filter cell counts and cells that had passed through the filter but had failed to adhere to the bottom surface and were found free in the lower compartment. Comparison of normal versus diabetic response to plasma activated with antigen-antibody complexes for 5 minutes revealed a defect in the diabetic cells to adhere to the filter surface but equal numbers of cells had migrated through the membrane. Normal chemotaxis of diabetic neutrophils was also observed by Valerius and co-workers (1982) in their study of noninsulin and insulin dependent diabetics (some patients were ketoacidotic); patients were evaluated before and after initiation of insulin therapy. The methods employed accounted for possible poor adhesion of cells to the bottom surface of the menbrane.

Neutrophil adherence has been studied but all reports to date have used whole blood as the sample with adherence assessed by passage through a nylon wool column (see chapter

3 for discussion of adherence techniques). Streptozotocin induced diabetic rats were first found to have decreased adherence relative to normal controls (Stecher, et. al., 1977). Human diabetics were evaluated and adherence was decreased during poor regulation with reevaluation after more intensive insulin therapy demonstrating improved adherence, although normal levels were not obtained. The adherence level in this study was correlated with serum glucose concentrations and incubation of the samples with additional glucose induced decreased neutrophil adherence (Bagdade, Stewart & Walters, 1978). Study of noninsulin dependent diabetics also demonstrated an adherence defect. Patients successfully treated with tolazamide demonstrated improved neutrophil adherence while neutrophil adherence continued to decline in patients who did not respond to this therapy regime.

The ability of serum from diabetic patients to generate chemotactic activity has also been examined. Balch, et.al. (1963) reported increased hemolytic complement levels in serum from human patients with diabetes mellitus with no apparent correlation to clinical condition or infection. However, serum activation by antigen-antibody complexes was found deficient in juvenile diabetics by Miller & Baker (1972). Fikrig, et.al.(1977) found that the ability of sera from diabetic humans to generate chemotactic activity from zymosan activation of complement was not impaired. The level

of chemotactic activity generated was not correlated to duration of disease, treatment, or ketoacidosis. These findings were later confirmed by Manouchehr-Pour (1981). This discrepency of results may be related to mechanism of activation; antigen-antibody complex activation is via the classical cascade whereas zymosan stimulates the alternate pathway of complement.

Opsonic activity has also been examined with Bybee and Rogers (1964) reporting no phagocytic defect observed when normal cells were tested with serum from diabetic patients. This finding was later confirmed by Miller and Baker (1972) in their study of juvenile diabetics. However, this feature has not been consistent as decreased opsonic activity has been reported by Rayfield, et.al. (1978).

Neutrophil phagocytic activity has been examined by many groups using a variety of methods. Bybee and Rogers (1964) reported decreased ingestion of pathogenic Staphylococci by leukocytes from patients with diabetes and ketoacidosis. The observed defect was reversed with correction of the acidotic state. Study of peritoneal exudate neutrophils from rats with alloxan induced diabetes did not identify abnormal phagocytic activity (Briscoe and Allison, 1965). However, these animals were not ketoacidotic at the time of evaluation. Crosby and Allison (1966) found normal phagocytic activity of neutrophils from human diabetics without ketoacidosis, very high glucose concentrations in

the media did not influence phagocytic activity.

The influence of environmental conditions on phagocytosis has been addressed by Walters, Lessler and Stevenson (1971). These workers found no difference between normal and diabetic neutrophil phagocytic activity but when cells from the diabetic were incubated in hypertonic media the phagocytic activity decreased. This finding was disputed by a study of neutrophils from juvenile diabetics which demonstrated normal phagocytic function. The phagocytic function was unaffected by increased glucose concentration in the media. Decreased phagocytic activity of neutrophils derived from poorly controlled diabetic patients was correlated to serum glucose concentration in a study by Bagdade, Nielson and Bulger (1972). This defect was reversed when these patients were treated. Bagdade et.al.(1974) also found that serum from poorly controlled diabetic patients impaired phagocytic activity of normal cells but incubation of diabetic cells with normal serum did not fully correct the defect. Decreased phagocytic activity of neutrophils from nonketoacidodtic diabetics was reported by Tan, et.al. (1975), but these workers found no correlation of the phagocytic activity and diabetic serum or glucose concentration.

When phagocytic activity was evaluated at various incubation time intervals, Nolan, Beaty and Bagdade (1978) found decreased activity by the cells from diabetics at 20

minutes of incubation but normal response after 60 minutes. A recent study (Dziatkowiak, et.al., 1982) also reported no decrease in the phagocytic function of neutrophils from diabetic children but the incubation time was not indicated. The cause of a possible phagocytic defect was addressed by Subbaiah and Bagdade (1982) when they reported decreased conversion of lysolecithin to lecithin by stimulated diabetic neutrophils; increased lecithin is required for formation of the phagocytic vacuole with conversion dependent on the presense of insulin; the conversion defect was partially reversed by the addition of insulin.

Studies of neutrophil killing ability have primarily utilized bactericidal activity to assess this cell function. The initial report by Balch, et.al. (1963) described decreased killing activity of neutrophils against Staphylococcus albus and Escherichia coli for 33 and 18% of diabetic patients respectively. No correlation with ketone or glucose concentration was observed. Cells suspended in saline (no report of calcium or magnesium addition) were found to have no difference in killing ability between normal and diabetic humans (Briscoe and Allison, 1965). Further study by Crosby and Allison (1966) also failed to detect a difference in killing ability between neutrophils from normal and nonketoacidotic diabetic donors. Study of cells from "poorly controlled" but nonketotic diabetics disclosed decreased killing activity which was further

localized to serum from diabetics; normal cells incubated with diabetic serum had decreased killing function although evaluation of cells from diabetics with normal serum demonstrated only partial correction of the defect (Bagdade, et.al., 1974). Differentiation between abnormal killing and delayed phagocytosis has been attempted. Bagdade, Nielson & Bulger (1972) reported delayed killing of type 25 pneumococcus by whole blood from diabetics to be due to delayed phagocytosis rather than altered intracellular killing mechanisms. Tan, et.al. (1975) studied 31 diabetics and reported 3 with deficient killing and 3 with defective killing and phagocytosis. The patients with only defective killing did not have a history of increased infection but the 3 patients with the combined defect all had severe bacterial infections at the time of testing; 2 patients reverted to normal function with resolution of the infection. The separation of phagocytic and killing function was also considered by Dziatkowiak, Kowalska and Denys (1982) who reported normal engulfment but deficient killing by neutrophils from juvenile diabetics.

The relationship of improved control of the diabetes and neutrophil dysfunction has been examined. Rayfield, et.al. (1978) reported decreased bactericidal activity of whole blood from diabetic donors with less killing activity in samples from "poorly controlled" juvenile diabetics relative to "well controlled" diabetic patients. Neutrophils from

adult diabetics were evaluated for killing activity by Nolan, Beaty and Bagdade (1978) and found deficient with improved function noted after more successful treatment of the diabetes.

Evaluation of nitroblue tetrazolium dye reduction by neutrophils from patients with diabetes has been conflicting. Hill, et.al.(1974) studied juvenile diabetics and found no difference between activity of cells from normal and diabetic donors. However, Walters, Lessler & Stevenson (1971) reported decreased dye reduction by neutrophils from diabetic patients despite a very wide range for normal cells. Oxygen uptake was also examined in this study and diabetic neutrophils tended to have greater oxygen uptake with stimulated cells demonstrating a sharp early rise in oxygen consumption with an abnormally rapid decrease to nonphagocytic levels.

## MATERIALS AND METHODS

### Animals

The dogs were adult Golden Retrievers. Nine dogs with diabetes mellitus were 5 intact females and 4 males. These animals had juvenile onset insulin dependent diabetes and were well controlled at the time of study and were housed with the "Animal Models of Human Disease" colony at the Veterinary Clinical Center(VCC), Michigan State University (MSU). Insulin was administered twice daily at a schedule

appropriate for control of the individual animal.

Control dogs for this study were male and female Golden Retrievers. Some of these dogs were also housed in the Veterinary Clinical Center while other animals derived from the colony lines were housed in private homes. Some female dogs from private homes had been neutered.

Blood Samples

Venous blood samples were collected from the jugular vein between 8:30 and 9:00 A.M. before the diabetics had received their morning insulin and food. Samples for neutrophil isolation were collected into a syringe with an 18 gauge needle and sufficient 3.8% sodium citrate (Haemonetics Corp., Braintree, MA 85301) in the syringe to yield a final dilution of 1 part anticoagulant to 9 parts blood. Samples for whole blood evaluation of neutrophil adherence were taken with syringe and needle, placed in heparinized tubes (Vacutainers, Becton-Dickinson Co., Rutherford, NJ 07070), and immediately placed on ice to inhibit platelet activation.

Samples for glucose, triglyceride, and cholesterol determination were drawn into Vacutainers(Becton-Dickinson & Co., Rutherford, NJ 07070) without aniticoagulant. These samples were allowed to clot and the serum removed promptly and frozen for later evaluation. Samples for cortisol determination were drawn into evacuated blood collection

tubes (Becton-Dickinson Co., Rutherford, NJ 07070) which contained EDTA as the anticoagulant. These samples were centrifuged and the plasma removed within 15 minutes of sampling; plasma was then frozen for later study.

Neutrophil Isolation

Neutrophil isolation was accomplished as described in chapter 2.

Preparation of Neutrophil Stimulants

The stimulant was zymosan activated plasma (ZAP) prepared as previously described (chapter 2). Each experiment was performed using the same batch of ZAP which had been stored at -20$^{o}$C. until individual alliquots were used.

Neutrophil Shape Change Assay

Neutrophil shape change was assessed as described in chapter 2. The initial concentration of neutrophils used was 5 x $10^6$ cells/ml. with the final test containing 5 x $10^5$ cells/ml. Fixation of cells occurred after 5 & 15 or 10 minutes and after 30, 60 & 90 minutes of incubation.

Neutrophil Adherence Assay

The nylon wool adherence assay is described in chapter 3.

Whole blood adherence was evaluated with a column con-

taining 80mg. of nylon fiber packed to a final volume of 0.4ml. These columns were pretreated with 1ml. of complete phosphate buffered saline (PBS) to hydrate the column and produce a similar flow rate between the whole blood sample and isolated neutrophil preparation.

Isolated neutrophil adherence was evaluated with the same column size but the columns were pretreated with 0.5ml. of 0.01gm./dl. bovine serum albumin (BSA) (Fraction V, Sigma Chemical Co., St. Louis, MO 63178). This treatment was followed by 1ml. PBS to remove residual protien from the fluid phase.

Sample volume was 0.8ml. for both assays and the time required for sample movement through the column noted to detect major differences in the sample flow rate. Assay of both whole blood and isolated neutrophils was done in triplicate with the mean adherence value entered into the data.

Chemotaxis Assay

Neutrophil locomotion was evaluated by movement of cells through a fine pore filter. This method is a modification of the technique originally described by Boyden (1962).

Two plexiglass plates containing 30 wells were used to form double chambers seperatedby membranes between the 2 plates. Each well was 6mm. in diameter with the bottom chamber volume of 212ul. The bottom chamber was filled with

fluid, either 10% ZAP as the stimulant and 1% BSA in PBS as the control. A membrane with pore diameter of 3 microns (Schleicher and Schuell, Inc., Keene, NH 03431) was carefully layered over the fluid in the lower well without trapping air bubbles at the interface. The chamber assembled and the upper well then filled with 250 ul. of cell suspension consisting of $2 \times 10^6$ cells/ml. in 1% BSA. The plates were then incubated in 100% humidity at 37°C. for one hour. The apparatus was then cooled to 4°C. to halt cell migration until further processing was possible.

Membrane processing consisted of first fixing the membrane in absolute isopropanol (Aldrich Chemical Co., Milwaukee, WI 53233). Staining in hematoxalin (Gill's Formulation #3, Fisher Scientific Co., Fair Lawn, NJ 07410) was followed by destaining in a solution of 70% isopropanol and 1% hydrochloric acid. Stain color wasintensified by leaving the membranes in hard tap water over night.

The stained membranes were then dehydrated before clearing with double baths of xylenes (Sigma Chemical Co., St. Louis, MO 63178). Cleared membranes were mounted on microscope slides with Permount (Fisher Scientific Co., Fair Lawn, NJ 07410).

Cell migration was determined with a 100x oil immersion lens on a Zeiss Photomicroscope III. The plan of focus for the top of the membrane was determined and the fine focus calibration recorded. The plane of focus for the furthest

field into the membrane containing at least two nuclei in focus was then established and the difference used as the total migration distance into the membrane.

## Glucose, Triglycerides, & Cholesterol Determination

Serum values for glucose, triglycerides, and cholesterol were determined on a Flexigem Centrifigal Analyzer (Electro-Nucleonics, Inc., Fairfield, NJ 07006). All samples for a test were evaluated as a group to diminish possible variation. Glucose was measured by an endpoint reaction utilizing hexokinase specific degradation of glucose (Beckman Instruments, Inc., Fullerton, CA 92634). Triglycerides were also measured by an endpoint reaction initiated by glycerol liberation (Electro-Nucleonics, Inc., Fairfield, NJ 07006). Cholesterol determination was based on specific hydrolysis and oxidation of cholesterol esters (Electro-Nucleonics, Inc., Fairfield, NJ 07006).

## Cortisol Determination

Plasma cortisol levels were determined on all plasma samples at the same time. Methodology utilized was a commercially available radioimmunoassay kit (GammaCoat [$^{125}$I] Cortisol Radioimmunoassay Kit, Clinical Assays, Travenol Laboratories, Inc., Cambridge, MA 02139). This technique is routinely applied to dog samples in the Veterinary Clinical Endocrinology Laboratory, Michigan State

University.

## Prostacycline and Thromboxane Determination

Prostacycline and thromboxane levels were determined in the laboratory of Dr. Scott Walsh, Department of Physiology, Michigan State University (Walsh, et.al., 1984a and Walsh & Fenner, 1984b). Methodology employed was radioimmunoassay using dog specific antibodies (Seragen, Inc., Boston, MA) labelled with [$^3$H] (New England Nuclear, Boston, MA).

## Glycosylated Hemoglobin Assay

Samples for glycosylated hemoglobin determination were drawn 4 to 5 weeks prior to the study of neutrophil function and assayed at the University of New Mexico (Standefer & Eaton, 1983).

# EXPERIMENT I

## Introduction

In the spring of 1983 a diabetic dog from the MSU colony of diabetic dogs developed multiple infectious processes. Neutrophils from this dog were isolated and tested for shape change, adherence on a nylon wool column, and migration in a modified Boyden filter system. The results were compared with cell functions of a control dog from this colony run at the same time. Adherence and chemotactic

activity between the 2 dogs was essentially the same but the neutrophil shape change over time appeared quite different between the 2 animals. Cells from the diabetic dog responded differently than expected from previous experience. The diabetic dog cells had adopted the elongated conformation to the same extent as cells from normal dogs but had reverted to spherical shape much more quickly than expected. To determine if this alteration was consistently observed in neutrophils from diabetic dogs several dogs from both the MSU colony and client animals from the MSU Veterinary Clinical Center were evaluated over the remainder of the summer.

## Experimental Design

Diabetic dogs studied were from two sources; residents of the MSU colony of diabetic dogs were well regulated with insulin treatments at the time of evaluation and client dogs. The client owned dogs were not well regulated at the time of sampling either they had just been diagnosed as having diabetes or their current medical management was insufficient to control serum glucose concentration. Control animals were from the Animal Models of Human Disease kennel, dogs derived from the Golden Retrievers diabetic dogs and returned for breeding, or dogs housed in the VCC as teaching animals or blood donors.

The shape change assays were completed with the same

batch of ZAP which had been alliquotted into individual vials and frozen at -70°C. for later use. Isolated neutrophils at a standard comcentration of 5.2-5.3 x $10^6$cells/ml. were incubated with a final concentration of 0.5 & 1% ZAP at 37°C. for 5, 10, 15, 30, 60, & 90 minutes before fixation with 2% neutral buffered glutaraldehyde (Sigma Chemical Co., St. Louis, MO 63178). After fixation for at least 1 hour 100 cells were classified as to shape using a 100x oil immersion phase contrast lens (American Optical, Buffalo, NY 14215). Sixteen diabetic dogs were evaluated, 6 male and 4 female Golden Retreivers, and 6 female dogs of various types. Nine normal dogs were used as controls, 2 male and 4 female Golden Retreivers, 2 female English Pointers, and 1 male Boxer.

Glycosylated hemoglobin levels were also determined on all dogs as a long term indicator of the level of control of the diabetes mellitus attained in each animal.

## Results

Comparison of the data from the controls and diabetic dogs using split plot factorial analysis of variation failed to define a statistically significant difference in shape change between the populations (Figure 9). No difference was noted between control dogs and "poorly regulated" dogs when cell shape change was examined. But examination of data from only the Golden Retreiver colony of dogs (Figure

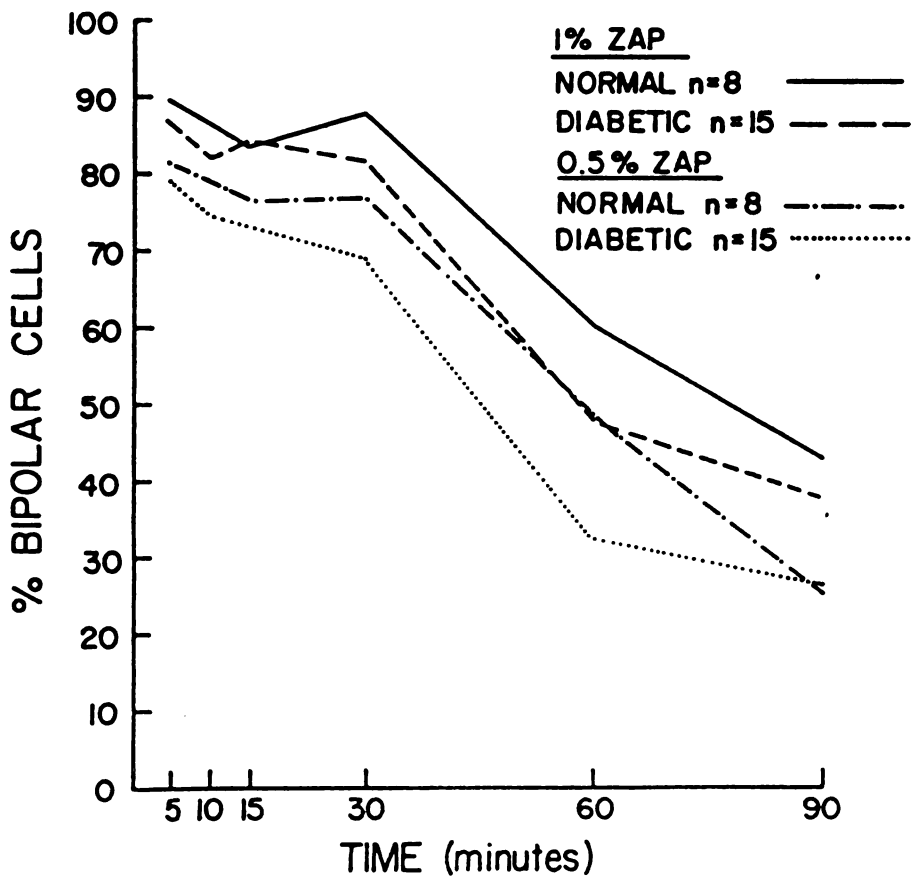


Figure 9. Experiment I: Neutrophil shape change kinetics, data from all dogs tested.

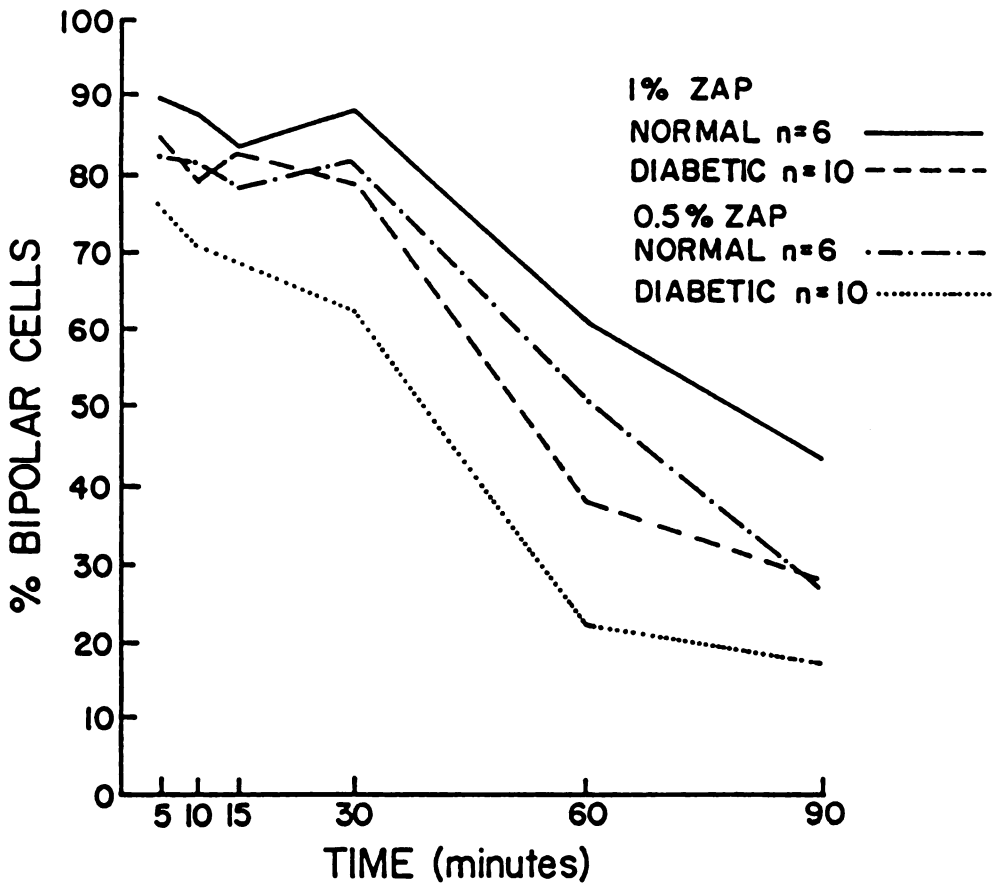


Figure 10. Experiment I: Neutrophil shape change kinetics, data from Golden Retrievers.

10) demonstrated a statistically significant difference ($p<0.005$) in the shape change time curve between normal and diabetic dogs.

Glycosylated hemoglobin data (Figure 11) were significantly different between all normal dogs and those with diabetes mellitus ($p<0.005$, Students t Test). Comparison of glycosylated hemoglobin levels with the % of cells in bipolar shape after 60 minutes of incubation with 1% ZAP yielded $r=0.3672$ (not significant). As glycosylated hemoglobin values increased the percent of cells remaining in the bipolar configuration increased.

## Discussion

Neutrophil shape change kinetics differed between normal and diabetic Golden Retreivers. The importance of this difference to cell migration was unknown as human cells when stimulated maintain the bipolar shape for 60 to 90 minutes. But, if the stimulant is removed human neutrophils will return to spherical (Smith, et.al., 1979). It was unclear why dog neutrophils gradually return to spherical and whether this characteristic is related to how the dog cells respond to a chemotactic gradient or interact with the environment. Further study was indicated and undertaken.

Glycosylated hemoglobin concentration was higher in the diseased population which was consistent with previous findings (Standefer & Eaton, 1983). The relationship of this

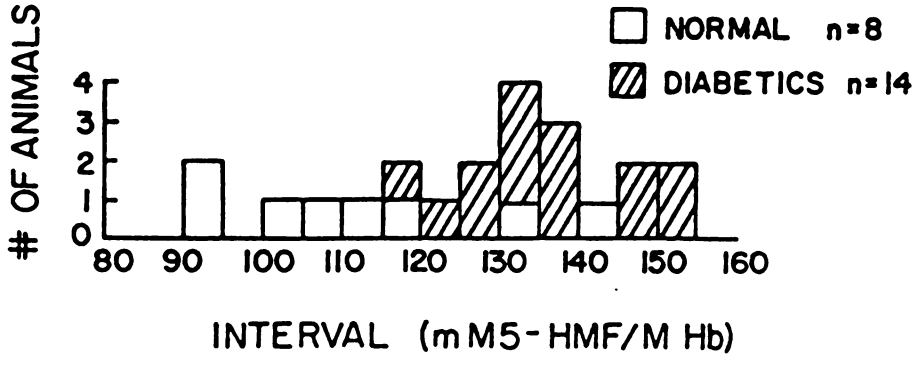


Figure 11. Experiment I: Glycosylated hemoglobins concentrations from all normal and diabetic dogs tested.

data to the shape change assay was not significant; it was anticipated that if a defect was detected in the diabetic population that that defect would either be unaffected by or increase in severity with poorer regulation of the glucose levels.

## EXPERIMENT II

### Introduction

The mechanism of the altered shape change was investigated in 2 general areas, examination of the interaction of the neutrophils and their environment and evaluation of intrinsic neutrophil functions.

Technique developement of the normal dog neutrophil shape change response suggested that the return to sherical form was accompanied by decreased stimulatory activity in the media. The difference in shape change response between normal and diabetic dogs then may have been related to a more rapid decrease in media chemotactic activity with incubation. Previous investigators suggested that serum glucose concentration (and osmolality) and/or insulin levels influenced neutrophil chemotaxis (Drachman, Root & Wood, 1966, Ainsworth & Allison, 1970, Mowat & Baum, 1971, Miller & Baker, 1972 and Hill, et.al., 1974).

The neutrophil's intrinsic "endurance" was evaluated by repeated stimulation; if the diabetic cells were not able to respond as long as normal cells this could relate to the

more rapid return to spherical form. The role of arachidonic acid metabolites as a second mediator of cell function was examined in cells from normal and diabetic dogs. This experiment was based on a recent paper which reported decreased generation of cyclooxigenase products by stimulated neutrophils from diabetic humans (Qvist & Larkin, 1983)

A more diversified study of chemotaxis in diabetic Golden Retriever dogs was also undertaken to identify other areas of altered behavior. The literature has been contradictory on chemotactic abnormalities. Studies of neutrophil adherence have uniformly reported decreased adherence in diabetic cells but have all used the nylon wool/whole blood test system.

Experimental Design

The influence of different glucose concentrations on the shape change assay was done on 5 female and 2 male diabetic and 3 female and 2 male normal Golden Retrievers. Neutrophils were isolated in the standard manner but then were incubated for 1 hour at 37$^{o}$C in isotonic media containing 3 or 6 times the standard glucose concentration (assay of standard media glucose was 96 mg/dl). Neutrophil response in hypertonic media with 3 or 6 times the concentration of standard media glucose was also studied. The shape change assay was then completed with cells fixed after

5, 15, 30, 60, and 90 minutes of incubation.

The influence of insulin in the media was evaluated on cells from 4 female and 3 male diabetic and 5 female and 2 male normal Golden Retrievers. After isolation, cells were incubated for 1 hour at 37°C in media without insulin or media with 10 or 100 uunits regular insulin/ml (Eli Lilly Co., Indianapolis, IN ). Shape change assay was then performed and cells fixed after 5, 15, 30, 60, and 90 minutes of incubation.

Isolated neutrophils from 5 female and 3 male normal and 4 female and 3 male diabetic dogs were evaluated for their influence on the stimulatory activity of the media. Cells were isolated in the routine manner and incubated in PBS with 1% ZAP for 10, 30, 60, or 90 minutes. After centrifugation, 0.9 ml of supernatant was removed to a new test tube and incubated with neutrophils from a Boxer dog for 10 minutes before fixation with glutaraldehyde and bipolar cell enumeration. The Boxer dog's cells were taken from either of 2 dogs (1 male and 1 female) from the Animal Models of Human Disease Colony at MSU. These cells were also evaluated for response to 1% ZAP which had not been previously exposed to cells. Other controls consisted of incubating the stimulant in either serum coated tubes or untreated tubes for 10, 30, 60, and 90 minutes and then evaluating their activity with Boxer cells.

Neutrophil response to repeat stimulation was also

evaluated. Neutrophils from 4 female and 3 male diabetic and 4 female and 2 male normal Golden Retrievers were exposed to 3 successive doses of 0.5% ZAP. Cells were fixed at 10 and 60 minutes after stimulation and 60 minutes elapsed between additions of ZAP.

Generation of arachidonic acid metabolites was assessed from both stimulated and unstimulated neutrophils. Neutrophils ($10^6$ cells/ml) were incubated in PBS or 1% ZAP for 60 or 90 minutes before centrifugation and removal of 0.8 ml. of supernatant. The supernatant was then frozen at -20°C prior to assay.

The study of chemotaxis and shape change related to other serum constituents derived data from 5 female and 4 male diabetic and 5 female and 4 male normal dogs. Citrated blood was drawn for neutrophil isolation with samples taken simultaneously for determination of glucose, triglyceride, cholesterol, and cortisol levels. Glycosylated hemoglobin samples had been taken from these animals 5 weeks prior to this study and the data is included as this value fluxtuate slowly. Thromboxane synthesis from stimulated and unstimulated neutrophils was evaluated at this time.

Adherence was studied on five female and 2 male diabetic and normal dogs (14 animals total). Three samples were taken at the same time, serum for glucose determination, citrated blood for neutrophil isolation, and chilled heparinized blood for whole blood adherence evaluation. All

proceedures utilized the methods previously stated.

Results

Evaluation of shape change kinetics was greatly impaired by an inability to consistently repeat the initial alteration (Table 2). Analysis of the data failed to identify alterations due to media composition, repeat stimulation or decreases in media stimulatory capacity (Appendix A).

Neutrophil movement during chemotactic stimulation was evaluated with the modified Boyden filter migration technique with mobility determined by the leading front technique (Table 3). Statistical analysis by the Student's t Test failed to define any significant difference between normal and diabetic dogs.

Neutrophil shape change over time (Figure 12) was evaluated with a split plot factorial analysis of variation, no differences were detected.

Serum glucose, triglycerides, cholesterol, cortisol concentrationdata from normal and diabetic dogs are presented in Table 4. Student's t Test was used to analyse these data with no signifcant difference in glucose (wide standard deviation for diabetic dogs) or triglyceride concentration noted. Plasma cortisol levels were not different between normal and diabetic males or male/female pooled data, but evaluation of data from only females yielded significant

Table 2. Statistical significance between normal and diabetic dog neutrophil shape change kinetics for several groups of data.

| Group | All Dogs | Female Only | Male Only |
|---|---|---|---|
| Experiment I | < 0.005 | -- | -- |
| Repeat Stimulation | NS | NS | NS |
| Variable Media | | | |
| PBS Only | -- | NS | NS |
| Isotonic Glucose | NS | NS | < 0.01 |
| Hypertonic Glucose | NS | NS | NS |
| Insulin | NS | < 0.005 | < 0.05 |
| PBS Only | -- | NS | NS |
| Supernatant Act. | NS | NS | NS |

Table 3. Filter migration distance in microns of unstimulated and stimulated neutrophils from normal and diabetic dogs.

| Test/Source | All Dogs n = 9 | Females Only n = 5 | Males Only n = 4 |
|---|---|---|---|
| | Mean ± 1 Standard Deviation | | |
| Unstimulated neutrophils | | | |
| Normal | 64.3±33.3 | 66.1±19.0 | 62.0±49.7 |
| Diabetic | 81.0±25.4 | 81.0±33.9 | 74.9±8.8 |
| Stimulated neutrophils | | | |
| Normal | 138.4±11.5 | 134.4±14.0 | 143.4±5.6 |
| Diabetic | 139.3±7.1 | 137.9±6.3 | 141.1±8.6 |

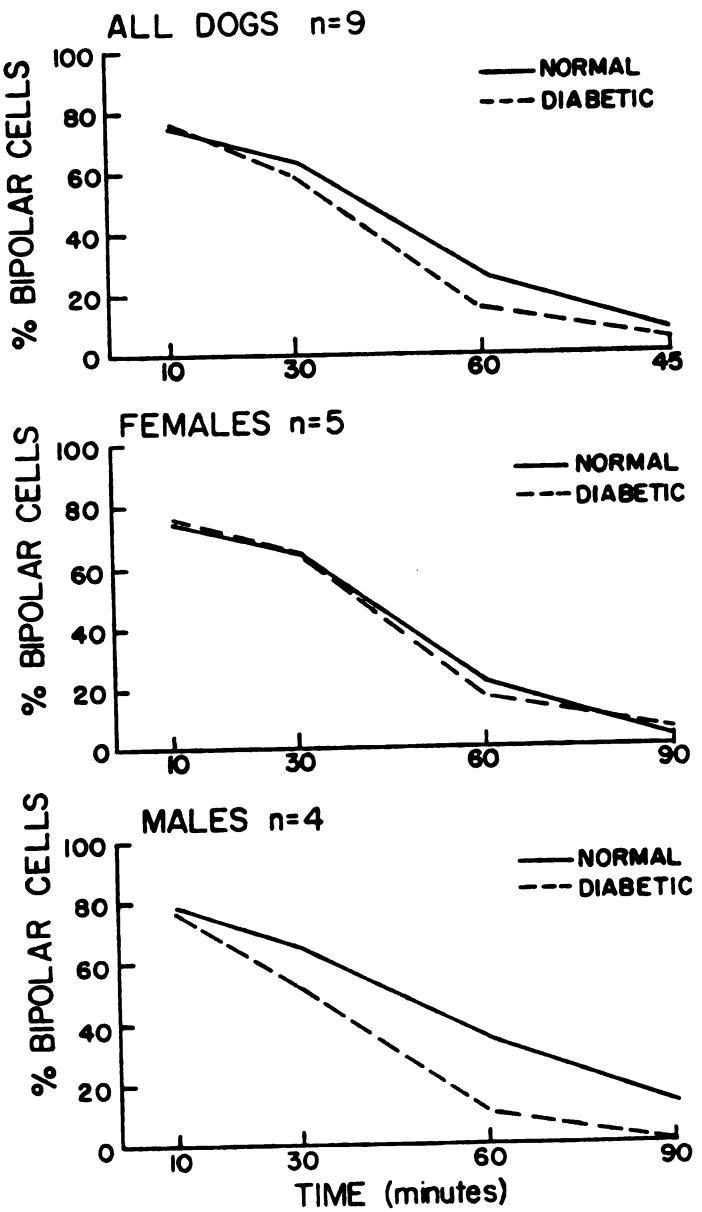


Figure 12. Neutrophil shape change kinetics of normal and diabetic Golden Retrievers.

Table 4. Serm concentrations of glucose, triglycerides, cholesterol, cortisol, and glycosylated hemoglobin from normal and diabetic dogs.

| Constituent/Source | All Dogs n = 9 | Females Only n = 5 | Males Only n = 4 |
|---|---|---|---|
| | Mean ± 1 Standard Deviation | | |
| Glucose (mg/dl) | | | |
| Normal | 91.4±12.7 | 95.4±12.2 | 86.5±13.1 |
| Diabetic | 181.2±145.9 | 170.0±120.1 | 196.0±192.2 |
| Triglycerides (mg/dl) | | | |
| Normal | 64.8±36.4 | 71.0±37.6 | 57.0±38.9 |
| Diabetic | 66.4±44.3 | 64.2±42.8 | 69.3±52.8 |
| Cholesterol (mg/dl) | | | |
| Normal | 204.0±62.7 | 248.4±40.2 | 148.5±30.8 |
| Diabetic | 305.7±81.8[c] | 339.4±58.3[d] | 263.5±95.1 |
| Cortisol (ngm/ml) | | | |
| Normal | 17.8±14.9 | 13.0±7.0 | 23.8±21.0 |
| Diabetic | 7.8±2.6[e] | 8.1±2.0 | 7.4±3.5 |
| Glycosylated Hemoglobin (m %) | | | |
| Normal | 2.8±0.5 | 2.7±0.5 | 3.0±0.6[b] |
| Diabetic | 5.1±1.8 | 5.3±1.3[a] | 4.9±2.3 |

[a] n = 4
[b] n = 3
[c] p <
[d] p < 0.05
[e] p < 0.01

difference ($p<0.01$). The 5 female diabetic dogs had lower plasma cortisol than their normal counterparts with females demonsrtating less variability within the data than was observed in the males. Serum cholesterol concentrations were higher in diabetic dogs with statistical significance demonstrated for all data ($p<0.05$) and data from females only ($p<0.05$).

Glycosylated hemoglobin values had been determined on samples taken 5 weeks prior to this study (Table 4).

Prostacycline and thromboxane production by resting and stimulated neutrophils was measured after 60 and 90 minutes of incubation. Diabetic neutrophils consistently produced more thromboxane then did neutrophils from normal dogs ($p<0.001$ all groups evaluated). A preliminary study suggested that prostacycline production was negligible and random samples from this study were consistent with that finding.

Neutrophil adherence data are presented in Table 5. No detectable difference in neutrophil adherence values was observed for either whole blood or isolated cell proceedures. Serum glucose was statistically different (Student's t Test) between all normal and diabetic dogs ($p<0.05$) and female dogs ($p<0.01$). No difference between males was detected but the sample size was small ($n=2$) and the diabetic dogs had a wide difference in the data obtained.

Figure 13 relates whole blood neutrophil adherence with

Table 5. Serum glucose and neutrophil adherence in whole blood and isolated granulocytes from normal and diabetic Golden Retrievers.

| Test/Source | All Dogs n = 7 | Females Only n = 5 | Males Only n = 2 |
|---|---|---|---|
| | Mean ± 1 Standard Deviation | | |
| Serum glucose (mg/dl) | | | |
| Normal | 87±6 | 87±6 | 86±6 |
| Diabetic | 296±134 | 334±116 | 202±169 |
| Whole blood neutrophil adherence (%) | | | |
| Normal | 41.8±22.5 | 42.7±25.5 | 39.5±20.5 |
| Diabetic | 50.3±17.4 | 58.1±12.1 | 30.6±12.2 |
| Isolated granulocyte adherence (%) | | | |
| Normal | 68.0±10.4 | 69.1±12.3 | 65.2±5.2 |
| Diabetic | 76.2±11.0 | 77.2±11.8 | 73.6±12.4 |

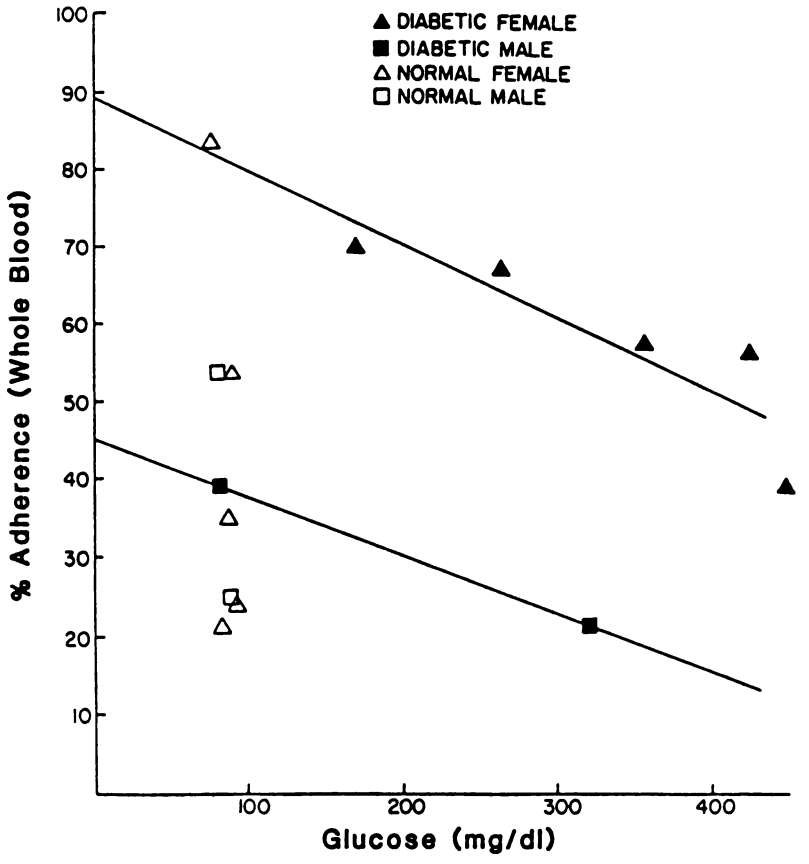


Figure 13. Serum glucose concentration and whole blood neutrophil adherence for normal and diabetic Golden Retrievers

data. Glycosylated hemoglobin levels are often more diagnostic of diabetes in the human because they are a reflection of average serum glucose concentration over an extended period; therefore it is appropriate that these data should be different when the fasting glucose is not significantly altered (Standefer & Eaton, 1983).

Chemotaxis was not significantly different between diabetic and normal dogs. Previous studies in humans have found no differnce in neutrophil chemotactic response in well regulated patients (Fikrig, 1977 and Valerius, et.al., 1982). A recent study in the veterinary literature also reports no decrease in chemotaxis of neutrophils from well orpoorlyregulateddiabeticdogs(Lattimer& Mahafetty,1984).

The shape change curve observed for all data was not significantly different between normal and diabetic dogs. Alterations in lipid metabolism are also well established for the diabetic and are reflected here by the significant difference in serum cholesterol concentrations ($p<0.05$ for all dogs and females only). Although serum triglyceride levels were not different in this study.

Resting plasma cortisol levels were not consistently different between groups (all dogs and males only) but a difference was detected when only females were examined ($p<0.01$). Although a difference was not consistently observed, the values for diabetics were generally lower then data from normal dogs; this is unexpected as diabetics often

have more active adrenal secretion (Ettinger, 1982). These data may not reflect adrenal secretion as well as an ACTH stimulation test would. Resting plasma cortisol levels were assayed here to determine if levels were sufficiently increased to interfere with liberation of membrane arahidonic acid and thus influence the metabolism of this crucial compound (Hirata, et.al., 1980). The inability to demonstrate a significant difference in cortisol and the fact that data from all dogs fell within the normal range for our laboratory (5-40 ngm/ml) reduces the potential impact on prostaglandin synthesis.

Thromboxane generation by resting and stimulated diabetic neutrophils was consistently above that observed in the normal dogs. This finding is in conflict with the initial report from human diabetic neutrophils (Qvist & Larkin, 1983). Further interpretation of this data is difficult due to the complexity and incomplete understanding of arachidonic acid metabolism. This study examined only 1 compound of many in this system. The increase in production of thromboxane may reflect increased mobilization of arachidonic acid to all end products or a shift in utilization away from lipoxigenase products to cyclooxigenase derivatives. Further studies may be useful.

Increased thromboxane production has been previously reported from diabetic platelets (Ziboh, et.al., 1979). Random platelet counts established that the platelet to

serum glucose values. A definite correlation of whole blood neutrophil adherence and serum glucose concentration for females was established ($r=0.883$, $p<0.05$). The correlation line slope for females was -0.093 with a Y intercept of 89.08. The correlation for data from the males was irrelevant due to the low number of samples ($n=2$) but the slope for the male data was -0.072 which is similar to the female data (Y intercept for males was 45.14). No other correlations were detected.

## Discussion

The inability to repeat the shape change kinetic alteration observed in Experiment I was unexplained. Without a reproducible abnormality present it became impossible to identify the mechanism of the change by manipulation of the abnormality. The inability to reproduce the initial finding may be the result of an initial inappropriate conclusion, subsequent changes in technique, or the sporatic appearence of this phenomena.

The lack of significant difference between diabetic and normal dog serum glucose concentration (chemotaxis study) reflects the wide range of data obtained for the diabetic animals. Also evident from these data is that most of the diabetic dogs are well regulated with insulin therapy. Glucose concentration was significantly different for females in the adherence study due to less variation in the

neutrophil ratio never exceded 1:5 in this study. Also human platelets did not produce thromboxane when exposed to zymosan activated serum (Goldstein, et.al., 1978).

The correlation between increasing serum glucose levels and decreasing neutrophil adherence for females is consistent with published studies on induced diabetes in rats (Stecher,et.al., 1977) and dogs (Latimer & Mahafetty, 1984) and in humans with naturally occurring disease (Bagdade, et.al., 1978 and Bagdade & Walters, 1980). The similar slope observed in the data from the males suggests a similar relationship exists in the male dog population but at a lower level of adherence. The lack of serum glucose influence on isolated neutrophil adherence suggests that the altered adherence is not on intrinsic quality of the neutrophil but somehow associated with the whole blood media.

The role of platelets in the whole blood adherence reaction has been described previously (Rasp, et.al., 1981) with alterations in platelet function well documented in the patient with diabetes (Mustard & Packham, 1984). The influence of platelet activation on whole blood neutrophil adherence has also been described in this work (Chapter 3) with increased adherence observed as platelet activation increases. In this experiment, samples for whole blood adherence assay were rapidly placed on ice to inhibit platelet activation by the heparin (Salzman, 1980), despite this precaution various levels of platelet clumping were observed

on whole blood smears. The degree of platelet activation could not be quantitated but did not appear to be influenced by the presence of diabetes. As reported in chapter 3 the samples incubated at room temperature formed platelet aggregates of increasing size and density and with greater incubation were surrounded by adherent neutrophils. This feature may represent aggregation enhancement (Redl, et.al., 1983b) or platelet satellitism (Payne, 1981, Poon, et.al., 1981 and Yoo, et. al., 1982).

Payne (1981) reported that rosette forming platelets were distinct from other platelets by an increased glycogen content. However, studies of leukocytes found decreased formation and storage of glycogen in cells from diabetics (Esmann, 1961, Stossel, et. al., 1970 and Goldstein & Curnow, 1980). Poon and co-workers (1981) described the presence of nonimmune glycoconjugates as the source of platelet/neutrophil interaction in their patient; perhaps this plasma factor activity is related to serum glucose concentration in the diabetic patients.

Spagnuolo and co-workers (1980) described thromboxane enhanced neutrophil adherence in a whole blood media and thrombin stimulated platelet enhancement of neutrophil adhesiveness. Platelet stimulation by thromboxane has been reported (Hamberg, et.al.,1975 and Malmsten, et.al., 1975) with a possible role for neutrophil thromboxane generation in the diabetic patient by activation of platelets in the

whole blood media with subsequent enhanced neutrophil adherence.

Increased serum cholesterol, observed in this experiment, is consistent with other reports from diabetics and may influence the lipid composition of membranes (vanOost, et. al., 1982 and Morita, et. al., 1983). But removal of neutrophils to PBS for the relatively short incubation and test period should not alter membrane composition appreciably and the effect of diabetes should be observed in adherence of isolated neutrophils also.

## SUMMARY

This thesis has dealt with 2 major areas; developement and validation of techniques for the in vitro study of canine neutrophil chemotaxis, and examination of chemotaxis of neutrophils from dogs with diabetes mellitus.

Major techniques that were modified for the dog were neutrophil shape change and adherence assays although other techniques required only minor modifications for application to dog cells. The shape change assay deviated from reports on human cells, dog neutrophils adopted a bipolar configuration, as with other species, but a return to spherical is observed in the continued presence the of stimulant. Utilizing this assay proceedure it was determined that the dog neutrophils did not respond to the synthetic tripeptide fMLP. Lack of fMLP receptors on canine neutrophils was documented by binding studies with radiolabelled fMLP. This finding is consistent with several other species (pig, cow, and horse) which also do not have a sufficient number of receptors to initiate chemotaxis.

Study of neutrophil adherence techniques established optimal experimental conditions for each assay. The influence of sample type (i.e. whole blood vs. isolated granulocyte preparation) was explored with the role of

platelet activation at room temperature readily apparent. The source or mechanism of this effect was not further defined.

Dogs with diabetes mellitus were studied by several techniques for evaluating chemotaxis. This work was undertaken when neutrophils of a diabetic dog had an altered shape change response. The abnormal shape change kinetics was confirmed on study of Golden Retrievers from the MSU colony of dogs with juvenile onset, insulin dependent diabetes. The altered shape kinetics were further investigated to discover the pathogenesis of this change. The initial abnormality could not be consistently reproduced. Neutrophil migration through filters and shape change kinetics were not significantly altered in the diabetic population. Neutrophil adherence in whole blood media was decreased with increased serum glucose concentration in the diabetic dogs. This finding is cocsistent with previous reports. Since the isolated neutrophil aderence was not effected by serum glucose and was not different from the normal dogs this variation in response may lead, directly or indirectly, to an explanation of the mechanism effecting diabetic neutrophil adherence in vivo. Also, evaluation of neutrophil production of thromboxane with stimulation were assessed with diabetic cells generating significantly greater amounts of thromboxane then normal cells.

# APPENDIX A

# EVALUATION OF NEUTROPHIL SHAPE CHANGE RESPONSE FOR NORMAL AND DIABETIC DOGS

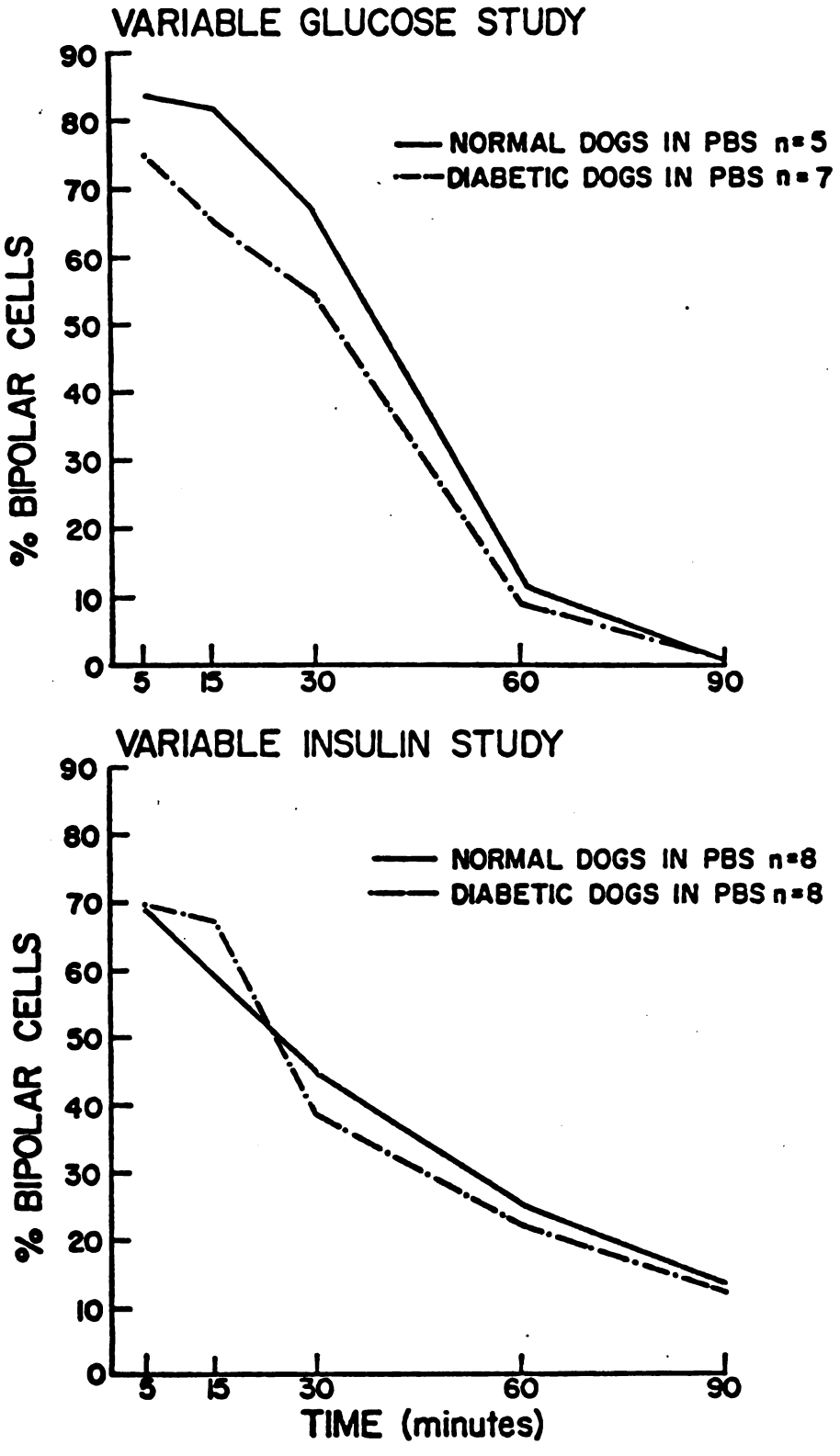


Figure 14. Neutrophil shape change kinetics; cells from normal and diabetic dogs in PBS. Data from study of media effects on shape change response.

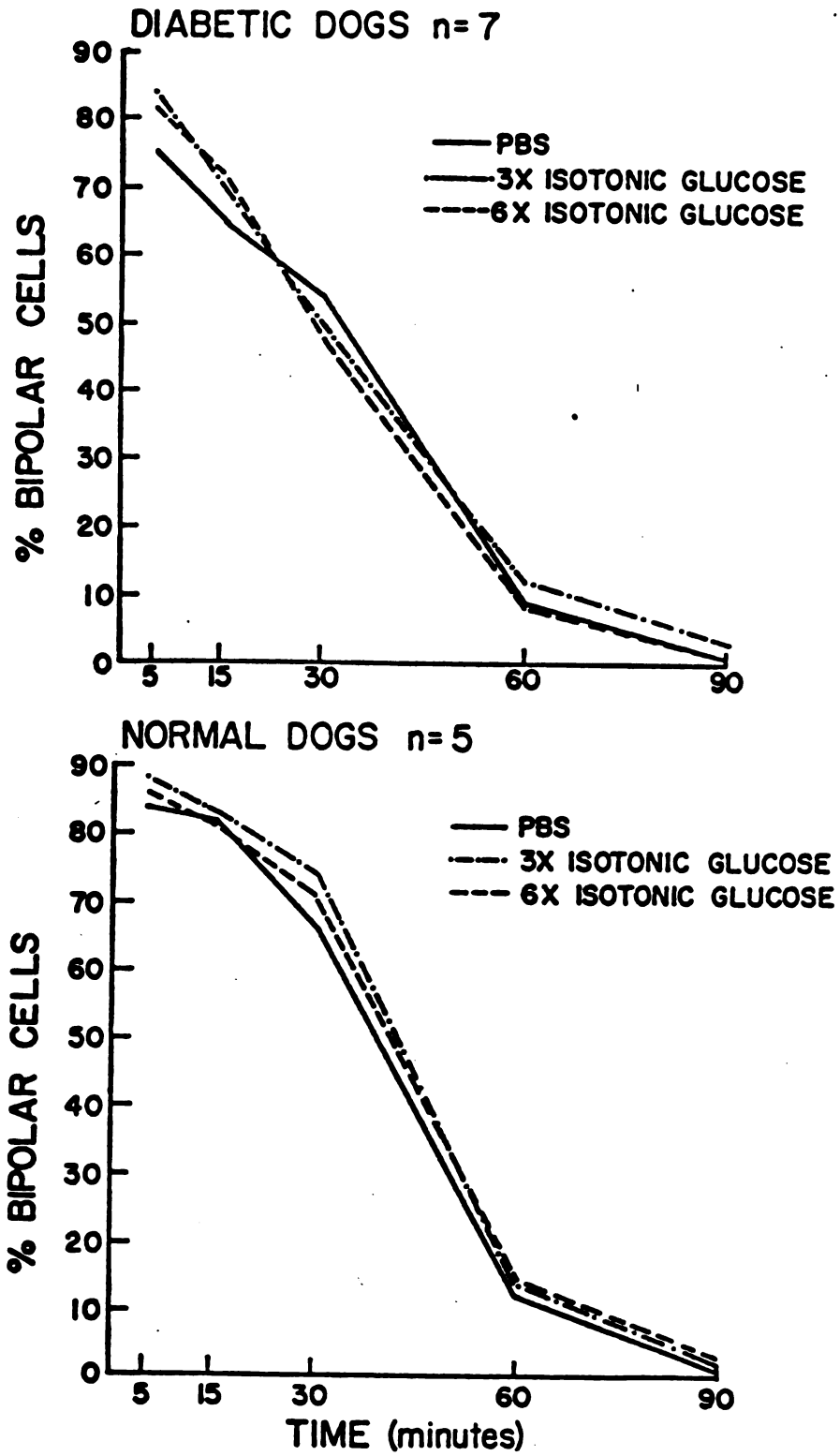


Figure 15. Effect of variable media isotonic glucose concentration on shape change kinetics for normal and diabetic dogs.

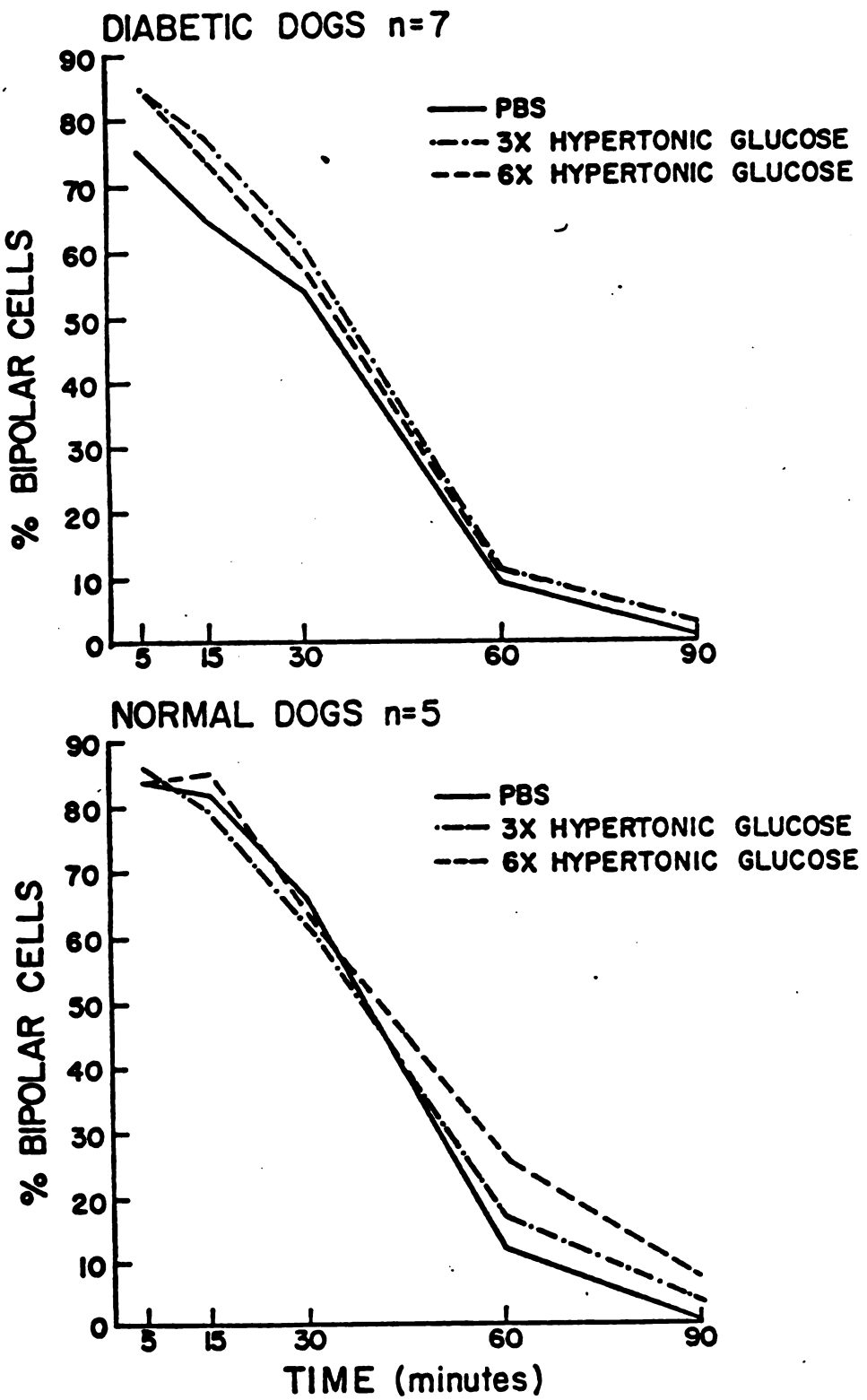


Figure 16. Effect of variable media hypertonic glucose concentration on shape change kinetics for normal and diabetic dogs.

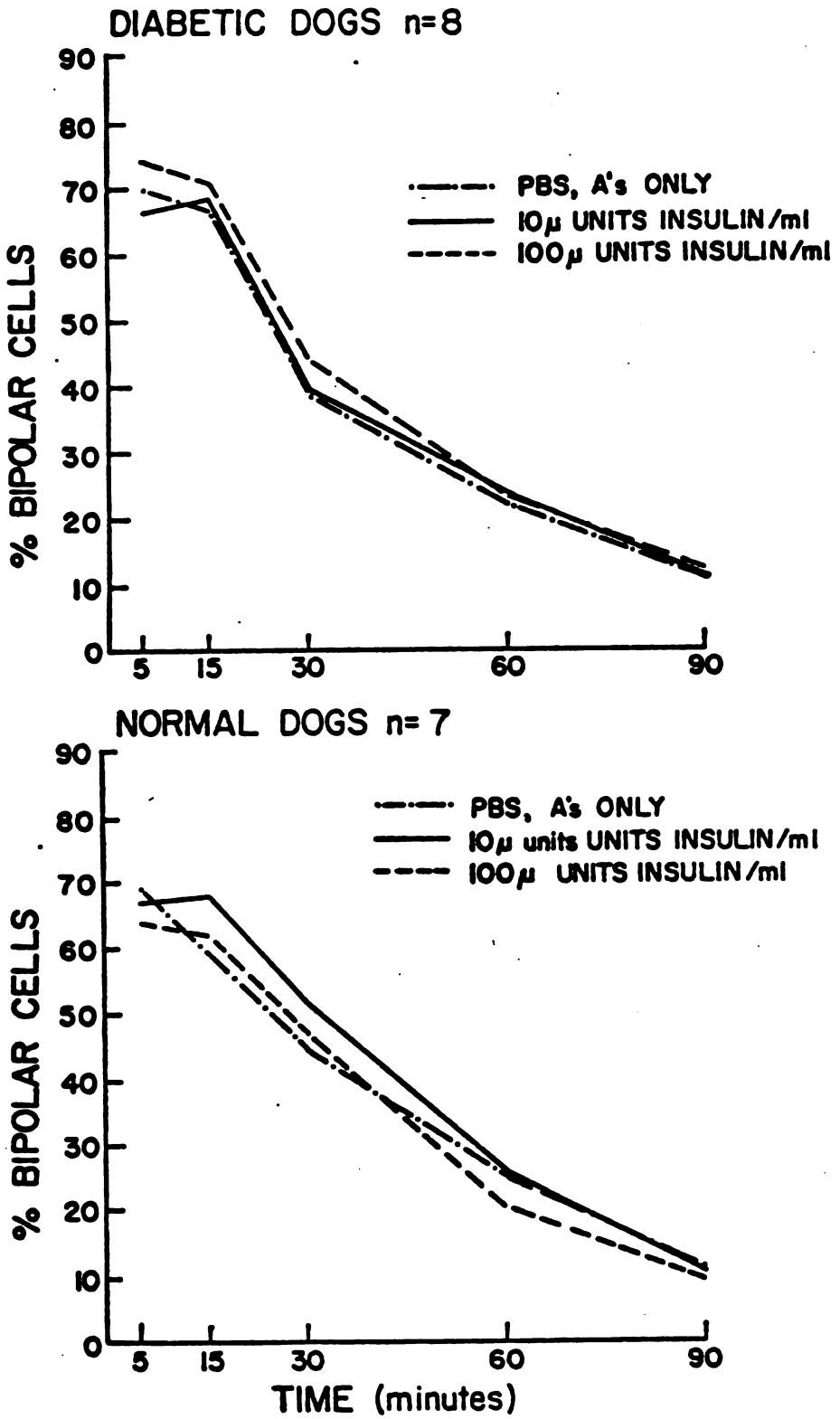


Figure 17. Effect of variable media insulin concentration on shape change kinetics for normal and diabetic dogs.

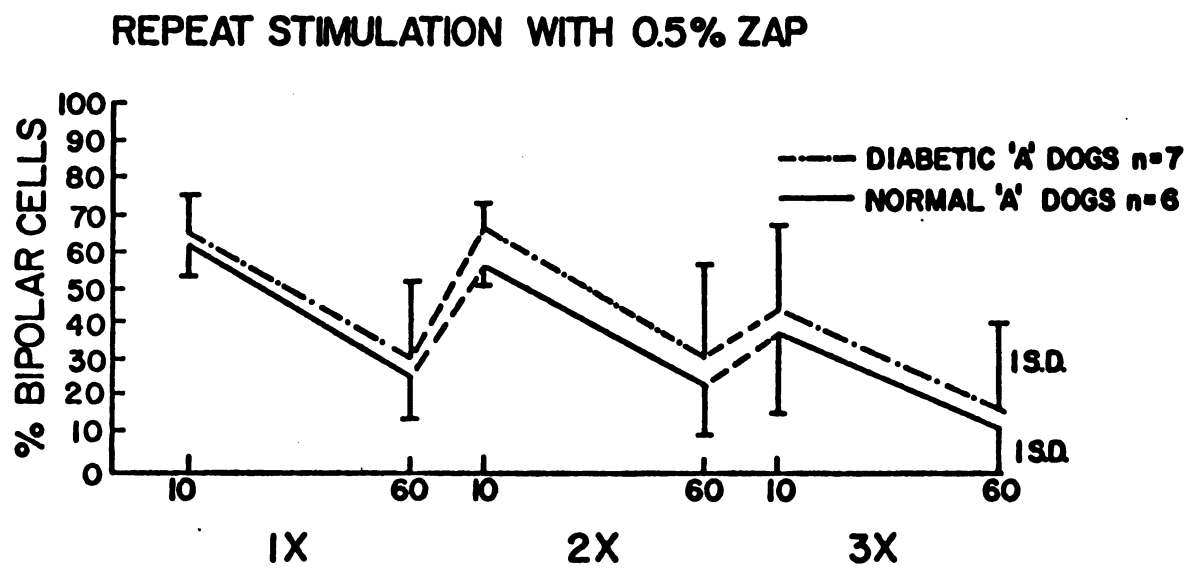


Figure 18. Effect of repeated stimulation on neutrophil shape change response for normal and diabetic dogs.

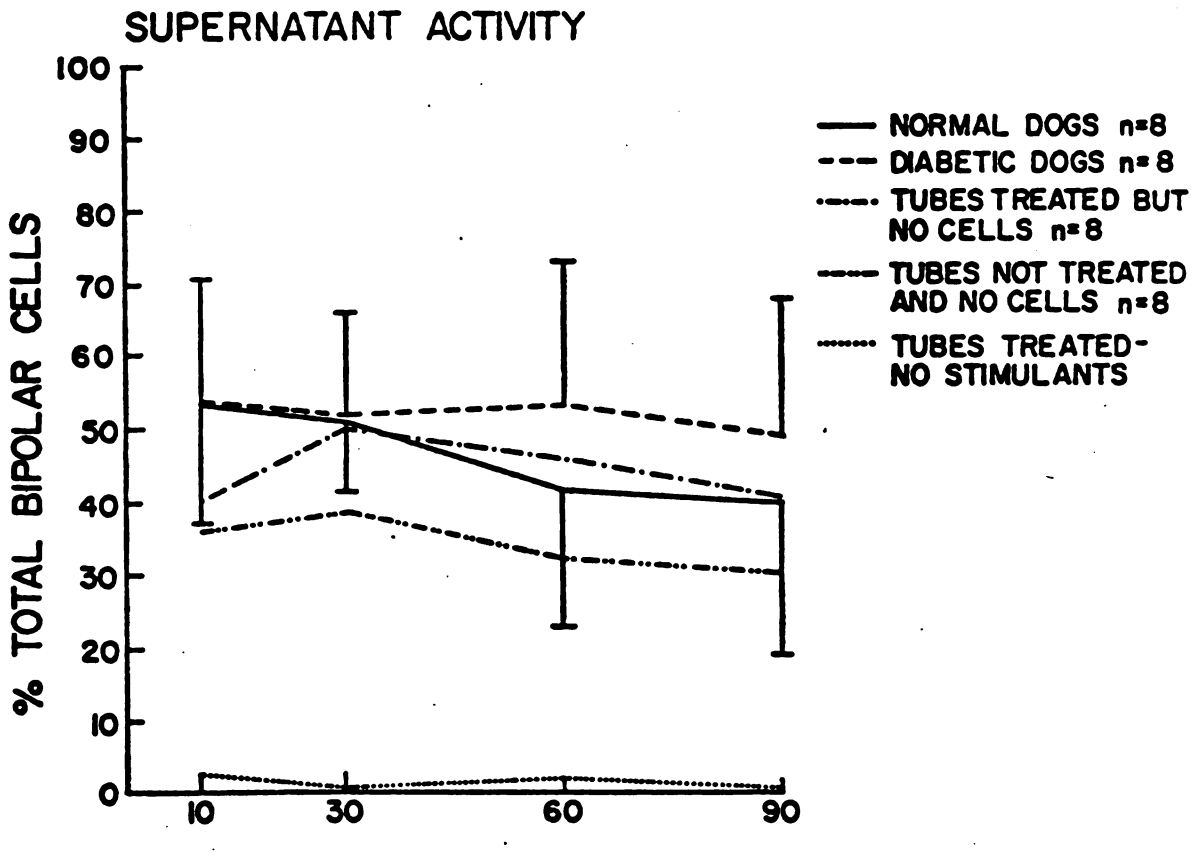


Figure 19. Residual stimulatory activity after incubation with normal or diabetic dogs' cells at 37° for 10, 30, 60 or 90 minutes.

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