NEUTROPHIL CHEMOTAXIS IN IDDM: A COMPARISON OF TWO QUANTIFICATION METHODS AND THEIR RELATIONSHIP TO GLUCOSE METABOLISM

by

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AND THEIR RELATIONSHIP TO GLUCOSE METABOLISM

This thesis is submitted by Carl J. Gustke, D.D.S. and has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore in partial fulfillment of the requirements for the degree of Master of Science.

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I. INTRODUCTION

A. Statement of the Problem

Insulin-dependent diabetes mellitus (IDDM) is a disorder of glucose metabolism which has been associated with increased susceptibility to, and slower resolution of infections (Savin 1974, Rayfield et al. 1982). Periodontal disease is an infection of the supporting tissues of the teeth, resulting in loss of connective tissues and alveolar bone. Many investigations of the relationship between the two diseases have shown that in some IDDM patients, there is increased susceptibility to periodontal disease, which may be related to factors of glucose metabolism, duration of diabetes, decreased neutrophil function, and genetics. The purpose of this study is to investigate neutrophil chemotaxis of insulin-dependent diabetics, comparing two methods of quantification of neutrophil migration in a modified Boyden chamber assay.

B. Review of Related Literature

The autoimmune disease insulin-dependent diabetes mellitus long has been associated with increased susceptibility to periodontal disease (Stahl et al. 1952, Belting et al. 1964, Finestone & Boorujy 1967, Cohen et al. 1970, Sznajder et al. 1978, Cianciola et al. 1982, Hugoson et

al. 1989, Miller et al. 1992, Thorstensson et al., 1993). Insulin-dependent diabetic children and adolescents have displayed earlier deterioration of gingival and periodontal health than their non-diabetic peers (Bernick et al. 1975, Ringelberg et al. 1977, Rylander et al. 1986, DePommèreau et al. 1992, Pinson et al. 1995). Yet, this assertion has been controversial, because evidence exists both substantiating or failing to support the association of diabetes with periodontitis. (Badanes 1933, Mackenzie & Millard 1963, Hove & Stallard 1970, Ervasti et al. 1985, Tervonen & Knuuttila 1986, Hayden & Buckley 1989).

Studies of the Pima Indians have shown that non-insulin dependent diabetics (NIDDM) in this population are three times as likely to have attachment loss or radiographically evident alveolar bone loss (Emrich et al. 1991). A cross-sectional study of 1,426 subjects in Erie County, New York found diabetic subjects (IDDM/NIDDM) to have an odds ratio of 2.32 for periodontal attachment loss (Grossi et al. 1994). In one study (Tervonen & Oliver 1993), and a review of several studies (Oliver & Tervonen 1994), it was noted that attachment loss is greater in poorly-controlled diabetics with extensive calculus. However, these studies included both IDDM and NIDDM subjects, without making a distinction between types. Recently, studies have correlated attachment loss in NIDDM subjects with duration of diabetes (Cerda et al. 1994), and in IDDM, periodontal disease correlated with severe organ

complications (Karjalainen et al. 1994). Out of the conflicting reports, a consensus is emerging: Although many diabetics will not be affected, diabetes mellitus is a risk factor for periodontal disease.

Because of the conflicting reports of IDDM association with periodontitis, investigating host defense and genetic factors helps to explain why some groups exhibit more disease, while other groups have essentially the same health status as controls. A probable explanation for the association of increased severity of periodontitis and other infections with IDDM is the finding that neutrophil function is impaired in diabetic persons. Impaired neutrophil chemotaxis was the first defect to be demonstrated with combined IDDM and NIDDM subjects (Perillie et al. 1962, Mowat & Baum 1971), and then for patient samples that were exclusively IDDM (Hill et al. 1973, Molenaar et al. 1976, Tater et al. 1987). Reduced phagocytosis has been reported for diabetics with poor metabolic control (Bybee & Rogers 1964, Bagdade et al. 1974, Gin et al. 1984). Deficient neutrophil chemotaxis also has been reported in insulin-dependent diabetics with severe periodontal disease (Manouchehr-Pour et al. 1981), first-degree relatives of diabetics (Molenaar et al. 1976), and their relatives with periodontal disease (McMullen et al. 1981).

Early-onset forms of periodontitis (EOP) have been associated with neutrophil dysfunction. Rapidly progressive

periodontitis (Page et al. 1984) and localized juvenile periodontitis (LJP) (Clark et al. 1977, Van Dyke et al. 1980, 1982, Suzuki et al. 1984) are noted to have chemotaxis defects. Phagocytosis impairment in LJP has been reported (Suzuki 1984).

Various neutrophil structural and metabolic defects exist which may reduce their effectiveness. Neutrophils of diabetics have demonstrable deficiencies of respiratory burst/microbicidal activity (Tater et al. 1987, Wierusz-Wysocka et al. 1987) and Fcγ receptor expression (Tater et al. 1987). Neutrophils from EOP patients have been shown to have decreased numbers of binding sites to chemotactic agents C5a (Van Dyke et al. 1983) and FMLP (Van Dyke et al. 1981), decreased expression of cell surface glycoprotein GP110 (Van Dyke et al. 1987), depressed chemotactic response to leukotriene B4 (Offenbacher et al. 1987), decreased expression of cell surface receptor component CD11 (Page et al. 1987) and a defect in plasma membrane calcium channel activation (Daniel et al. 1993).

Severe, early-onset periodontitis is a noted feature of several neutrophil dysfunction syndromes. Chediak-Higashi Syndrome involves reduced granulocyte mobilization from bone marrow, impaired chemotaxis, with one effect being severe periodontitis which results in edentulism before adulthood (Charon et al. 1985). Lazy Leukocyte Syndrome is a defect of neutrophil locomotion (chemotaxis and random migration) with

onset of gingivitis by age two (Miller et al. 1971). Leukocyte Adhesion Deficiency (LAD) is a defect of the CD11/CD18 complex of adhesion molecules on the neutrophil surface (Anderson & Springer 1987, Waldrop et al. 1987, Katsuragi et al. 1994). LAD patients have frequent systemic infections, and lose all of their primary teeth by age three or four (Waldrop et al. 1987). Severe gingival inflammation, alveolar bone loss, and tooth loss also have been described in patients with Cyclic Neutropenia (Cohen & Morris 1961) and Familial Benign Chronic Neutropenia (Deasy et al. 1980; Kirstila et al. 1993).

The existence of these neutrophil defects in EOP, together with the markedly increased periodontitis susceptibility in neutrophil dysfunction syndromes, point to the neutrophil as a critical determinant of resistance to periodontitis (Genco et al. 1986). This has led to the recognition of neutrophils as being "especially important in defending against virulent bacteria such as those causing periodontal disease." (Genco 1992). As both LJP, and the concurrent chemotaxis impairment, tend to occur in families, a genetic basis is suggested not only for LJP, but for the neutrophil chemotaxis defect itself (Genco et al. 1986). This suggests a similar situation for IDDM: this is an autoimmune disease with a genetic basis, and therefore, genetics also may be involved in its neutrophil chemotaxis defect.

As IDDM is an autoimmune disease, specific human leukocyte (HLA) antigens have been associated with it. HLA-DR3 and DR4 were found to have respective odds ratios of 3.5 and 4.4 (Cavender et al. 1984). In an eight-year study, subjects positive for islet cell antibody (ICA) and heterozygous for DR3 and DR4 had a 70% risk for IDDM -- in contrast to ICA positive, DR3/4 negative subjects, whose risk was 37%. DR3/4 heterozygotes also were seen to progress to diabetes at a younger age (Deschamps et al. 1992). Furthermore, two HLA haplotypes in linkage disequilibrium with DR3 (HLA-B8 and B18) were observed to be associated with the age of onset of IDDM (Deschamps et al. 1988). Another study implicates maternally-inherited HLA-DR3 in IDDM risk (Deschamps et al. 1990). HLA-DQ alleles have been associated with decreased IDDM risk (Fletcher et al. 1988, Kockum et al. 1993). The associations of DR3 and DR4 with increased risk, and DQ with protection, have been observed in not only in whites, but also in black subjects (Fletcher et al. 1988).

HLA association with neutrophil chemotaxis has not been thoroughly examined, but an example of such a relationship has been demonstrated for HLA-B27, which is associated with an enhanced response of neutrophils in human subjects with *Yersinia* arthritis (Koivuranta-Vaara et al. 1984). Several studies in mice indicate that neutrophil responses to immunologic challenge are under genetic control. For example, Gervais et al. (1984) observed that *Listeria*-susceptible A/J

mice had greatly impaired neutrophil and macrophage mobilization compared to *Listeria*-resistant C57/BL10 mice. In another study, kaolin was injected into the peritoneal cavities of B10 and CBA mice. B10 mice responded with higher numbers of neutrophils in the peripheral blood, but fewer accumulated in the peritoneal cavity compared to CBA mice (Sluiter et al. 1987). In B10 mice, major histocompatibility complex $H\text{-}2^d$ haplotype has been noted to augment the neutrophil response to bacterial lipopolysaccharides (Marley et al. 1994). Therefore, neutrophil chemotaxis may be regulated by genes within the HLA complex, and may not necessarily be related to glucose metabolism.

C. Specific Aims

The specific aims of the project were as follows:

1. Determine the level of neutrophil chemotactic function in a sample of insulin-dependent diabetics by:
    a. Mean distal surface migration counts,
    b. Index of migrating/non-migrating cells.
2. Statistical analysis for correlations between chemotaxis methods, blood glucose, glycosylated hemoglobin levels and age.

II. MATERIALS AND METHODS

A. Study Design

This study was approved by the Human Assurance Committee of the Medical College of Georgia Institutional Review Board (Appendix A). The subjects for this investigation were insulin-dependent diabetics ages 10 - 21, who were patients at the Medical College of Georgia Pediatric Diabetes clinic. A total of 41 subjects were included, with the following race and gender characteristics: 11 white males, 12 black males, 9 white females, and 9 black females. Exclusion criteria were systemic illness (except IDDM), use of any medications in the past 24 hours (except insulin), long-term anti-inflammatory or immune suppressive drug use, smoking within the past 24 hours, pregnancy, and currently menstruating females. For chemotaxis assays, race and gender-matched controls with the same exclusion criteria were used in order to achieve a similar distribution. During routine clinic visits, parental consent and children's assent were obtained (see Appendix A). At the same visit, blood specimens (total volume 30-35 ml) were drawn to assess the following: capillary blood glucose, percent glycosylated hemoglobin, and neutrophil chemotaxis. Capillary blood glucose level was determined in the diabetes clinic, and percent glycolsylated hemoglobin values were determined by a

reference laboratory, then later retrieved from the patients' clinical records. The remainder of the sample (approximately 15 ml) was used to isolate neutrophils to assay chemotaxis activity.

B. Chemotaxis Assay (Detailed Method of Procedure, Appendix B)

For neutrophil separation, 5 ml of fresh, heparinized whole blood was pipetted gently over layers of Histopaque 1077 and Mono-Poly Resolving Medium in three 15 ml centrifugation tubes. After 30 minutes of centrifugation at 1200 RPM, the PMN band was evident between erythrocyte fraction and the mononuclear leukocytes. The neutrophil band was harvested, washed twice in PBS, and resuspended in Gey's GVB^{2+} media (GGVB) at 2.5 x 10^6 cells/ml. F-met-leu-phe (fMLP) was diluted to a concentration of 2 x 10^{-8}M in GGVB. 0.4 ml PMN suspension was placed in the upper well of the Boyden Chamber with simultaneous placement of 0.8 ml of fMLP in the lower well. The wells were separated by a nitrocellulose filter 114 μm thick with 5 μm pores. The loaded chambers were incubated for 2 hours at 37° in humidified air with 5% CO_2. The filters were removed, fixed for 10 minutes in neutral buffered formalin, then stained with Mayer's Hematoxylin. Neutrophils which migrated to the distal surface of each filter (triplicate set) were counted in three random, high-power (450X) fields. The level of chemotaxis (CTX) was expressed as the mean number of cells on the distal surfaces of the filters.

C. Chemotaxis Index

According to the protocol for the Boyden chamber assay, neutrophils were used at the same concentration (2.5×10^6 cells/ml) each time the assay was done. To compensate for possible undetected concentration variations (cells may be lysed during separaton procedures), and thereby to improve the comparability of assays performed on other days, a chemotaxis index for each patient and control was calculated according to a modification of the method of Baum et al. (1971). Five random fields on the bottom surface of the filter were counted, and the sum was divided by the total number of neutrophils in five random fields on the top (loaded) side of the filter. This ratio was multiplied by 100 for convenience.

$$\frac{\text{Sum of 5 fields bottom side of filter}}{\text{Sum of 5 fields top side of filter}} \times 100 = \text{Index}$$

D. Statistical Methods

Relationships between chemotaxis, chemotaxis index, blood glucose, glycosylated hemoglobin and age were determined by Pearson's Correlation Coefficient. Differences in these variables (except glycosylated hemoglobin) between diabetic and control groups were determined by a *t*-test. Differences between race/gender subgroups also were determined by a *t*-test, subsequent to analysis of variance.

III. RESULTS

A. Neutrophil Chemotaxis

Chemotaxis assays were characterized by high variability. The range of the simple chemotaxis determination (CTX) was 8.3 - 86.0 for diabetics, and 10.2 - 77.3 for the controls. The mean chemotaxis index (INDEX) ranged from 5.4 - 53.9 for diabetics, and 8.0 - 46.8 for controls. The age difference between the diabetics (mean 14.4 years) and controls (mean 27.6 years) was significant ($p<0.0001$). The mean chemotaxis index of the diabetics (22.02) was significantly less than the mean index of the controls (27.81, $p<0.0237$, Table I). No significant difference existed between diabetics and controls for the simple chemotaxis determination, CTX. When analysis was performed according to race/gender subgroups of all subjects, no significant differences were found between the groups for CTX or INDEX.

Table I

Neutrophil Chemotaxis

	Patient			Control			
	Mean	SD	SEM	Mean	SD	SEM	*p*
CTX	27.10	15.05	2.43	31.83	16.26	2.89	0.21
INDEX	22.02	9.94	1.61	27.81	10.82	1.91	0.02

CTX = chemotaxis (mean #cells per random field)
INDEX = chemotaxis index
mean = arithmetic mean of variable
SD = 1 standard deviation
SEM = standard error of the mean

1. Diabetics

Pearson correlation coefficients (r) for chemotaxis and glucose metabolism variables of diabetics are shown in Table II. Glucose metabolism variables were not correlated with neutrophil function variables. The two methods of chemotaxis determination, CTX and INDEX were strongly correlated ($r = 0.73$; $p<0.0001$). Additionally, CTX was weakly correlated with AGE ($r = 0.36$; $p<0.02$). No differences were observed between males and females for CTX and INDEX (Table III) and no racial difference existed for these variables (Table IV). None of the race/gender subgroups differed from the others in neutrophil function.

Table II

Correlations: Diabetics

Pearson Correlation Coefficients/Prob > |R| under Ho: Rho=0

	CTX	INDEX	CBG	HbA_1	AGE
CTX	1.0000 0.0	0.73429* 0.0001	-0.25011 0.1148	-0.10562 0.5110	0.35567* 0.0243
INDEX	0.73429* 0.0001	1.0000 0.0	-0.00719 0.9644	-0.05816 0.7180	0.17373 0.2837
CBG	-0.25011 0.1148	-0.00719 0.9644	1.0000 0.0	0.42637* 0.0054	-0.0846 0.6034
HbA_1	-0.10562 0.5110	-0.05816 0.7180	0.42637* 0.0054	1.0000 0.0	0.10865 0.5046
AGE	0.35567* 0.0243	0.17373 0.2837	-0.0846 0.6034	0.10865 0.5046	1.0000 0.0

*Statistically significant, *p* value shown

CTX = chemotaxis (mean #cells per random field)
CBG = capillary blood glucose
HbA_1 = glycosylated hemoglobin
INDEX = chemotaxis index
AGE = subject age in years

Table III

Neutrophil Function/Gender

		LS Mean CTX	*p*	LS Mean INDEX	*p*
Diabetics	Males	27.16	0.95	22.53	0.73
	Females	26.87		21.40	
Controls	Males	26.11	0.07	25.84	0.44
	Females	37.63		29.17	
Pooled	Males	27.07	0.27	23.93	0.78
	Females	31.23		24.65	

Table IV

Neutrophil Function/Race

		LS Mean CTX	*p*	LS Mean INDEX	*p*
Diabetics	Blacks	26.20	0.74	20.54	0.38
	Whites	27.82		23.39	
Controls	Blacks	29.51	0.45	25.40	0.33
	Whites	34.24		29.61	
Pooled	Blacks	27.45	0.37	22.25	0.12
	Whites	30.85		26.34	

2. Controls

Pearson correlation coefficients for chemotaxis and glucose metabolism variables of controls are shown in Table V. In contrast to the diabetics, glucose metabolism (CBG) was correlated with the neutrophil function variable CTX ($r = 0.52$; $p<0.01$). Once again, the two chemotaxis methods correlated well ($r = 0.66$; $p<0.0001$). Age did not show a relationship to neutrophil function, and glycosylated hemoglobin was not tested in the control subjects. As was noted with the diabetics, no differences in neutrophil function were found according to gender or race (Tables III and IV). Also, none of the race/gender subgroups differed from the others regarding CTX or INDEX.

Table V

Correlations: Controls

Pearson Correlation Coefficients/Prob > |R| under Ho: Rho=0

	CTX	INDEX	CBG	AGE
CTX	1.0000 0.0	0.66129* 0.0001	0.51581* 0.0118	-0.17257 0.3707
INDEX	0.66129* 0.0001	1.0000 0.0	0.31016 0.1498	-0.09599 0.6204
CBG	0.51581* 0.0118	0.310169 0.1498	1.0000 0.0	-0.20861 0.3395
AGE	-0.17257 0.3707	-0.09599 0.6204	-0.20861 0.3395	1.0000 0.0

*Statistically significant, *p* value shown

CTX = chemotaxis (mean #cells per random field)
CBG = capillary blood glucose
INDEX = chemotaxis index
AGE = subject age in years

B. Glucose Metabolism

A significant correlation existed between blood glucose (CBG) and glycosylated hemoglobin (HbA_1) for the diabetic group ($r = 0.43$; $p<0.0054$, Table II).

The following statistically significant findings are summarized on Table VI:

Mean CBG of the diabetics was 215.56, compared to 82.04 for the controls. This difference was highly significant ($p<0.0001$). White females were the only subgroup in which diabetic CBG was not significantly different from its matched control group.

Among the diabetic subjects, black females presented with higher CBG than black males ($p<0.02$) and white females ($p<0.005$). White females had significantly lower HbA_1 than black females ($p<0.02$) and black males ($p<0.004$). No such differences were observed in the control subjects. However, when all diabetic and control subjects were combined in race/gender subgroups, white females had significantly lower CBG than black females ($p<0.02$).

Table VI

Summary of Glucose Metabolism Results

Diabetic mean CBG 215.56; control mean CBG 82.04; $p<0.0001$

Diabetic subjects:

- Black female CBG higher than black males; $p<0.02$
- Black female CBG higher than white females; $p<0.005$
- White female HbA_1 lower than black females; $p<0.02$
- White female HbA_1 lower than black males; $p<0.004$

All subjects:

- White female CBG lower than black females; $p<0.02$

IV. DISCUSSION

The present study was undertaken to investigate neutrophil chemotaxis of diabetics by two quantification methods. Neutrophil chemotaxis was found to be impaired in diabetic subjects, when chemotaxis index was determined by the more precise method. The decreased neutrophil chemotaxis index observed in the diabetics agrees with the previous findings of Mowat and Baum (1971), Hill et al. (1974), and Molenaar et al. (1976), which used the method as for INDEX; and Tater et al. (1987), who used the under agarose assay system. However, this finding was contrary to that of Fikrig et al. (1977), who did not find decreased chemotaxis in insulin-dependent diabetics while using the an index method which subtractd random migration from chemotaxis.

A likely cause of the conflicting reports of chemotaxis function is the inherent variability in the assay technique. Chemotaxis also was found to be highly variable in this study. For example, INDEX of diabetics ranged from 5.4 - 53.9, and one standard deviation of the mean was 45%. This degree of variability is consistent with that reported in previous studies of diabetics, such as Hill et al. (1974), who reported the chemotactic index to range from 25 - 82, with a standard deviation approximately 30% of the mean. In the method

described by Baum et al. (1971), the average standard deviation was 17% of the mean for chemotaxis index. Molenaar et al. (1976) reported chemotaxis indices ranging from 39 - 140 for controls (mean=72), 13 - 64 for diabetics (mean=39), and 8 - 164 for first-degree relatives of diabetics (mean=55). In the study of Mowat and Baum (1971), the standard deviation of the mean chemotaxis index was 23% in the diabetic group. In the LJP investigation of Daniel et al. (1993), the mean and standard deviation of CTX for six subjects were 31.4 and 17.1 (54% of mean), respectively. This amount of variability is very similar to the findings for diabetics and controls in the present study (45% of mean for INDEX; 56% for CTX).

With the exception of the report of Daniel et al. (1993), precise determination of chemotaxis variability in LJP studies is more difficult. Many of these studies do not report individual chemotaxis indices (Suzuki et al. 1984), but may report them as percentage of a matched control subject (Clark et al. 1977, Agarwal et al. 1989, Kurihara et al. 1993), as depressed compared to a single control subject, (Van Dyke et al. 1982b, Van Dyke et al. 1987), or reporting group means with the standard error of the mean (SEM) rather than the standard deviations (Van Dyke et al. 1982a, Offenbacher et al. 1987, Van Dyke et al. 1987). SEM is a smaller number than

standard deviation, and may be misinterpreted. It does not directly represent the dispersion of the sample, but rather the accuracy of determining the true mean. In LJP studies, the SEM appears high. Additionally, using analysis of variance or a Student's *t*-test to compare the means of triplicate tests for single patient and control depends heavily on the assumption that there is a "normal" level of chemotaxis for healthy persons. Many LJP studies report this type of data analysis. With such great variability seen in so many studies, it is doubtful that this is a safe assumption. No analyses depending on this assumption were made in the present study.

At least part of the explanation for variability in the chemotaxis assay may be related to hormonal factors. Miyagi et al. (1992) studied the effects of testosterone, estradiol and progesterone on neutrophil chemotaxis both in vitro and in vivo. They noted that in vitro, estradiol reduced chemotaxis, while progesterone increased it. In vivo, chemotaxis and plasma progesterone levels were positively correlated, while estradiol and testosterone did not appear related to neutrophil function. In the present study, female subjects were excluded during menstruation, or if they were taking oral contraceptives. However, plasma estradiol levels peak at mid-cycle, immediately prior to ovulation, and progesterone levels peak at the end of the cycle, just prior to menstruation. This makes possible a large variation in the effects of

hormones for female subjects, and also suggests that a large variation in chemotaxis may be physiologically normal. Miyagi et al. (1992) did not report whether chemotaxis was different for males and females, and if one gender had greater variation. However, prior studies (Tater et al. 1987, Hill et al. 1974, and Baum et al. 1971) did not find gender correlations with neutrophil function. Likewise, no significant differences by gender were observed in the present study.

In addition, Schenkein et al. (1991) discussed the existence of racial variation in chemotaxis of periodontally healthy subjects and those with LJP. They reported healthy whites to have significantly higher neutrophil chemotaxis than healthy blacks and blacks with LJP. This study underscored the importance of race-matching controls. In the present study, no such racial difference in chemotaxis was found, using either the same chemotaxis method as Schenkein et al. (1991), or the method of Baum et al. (1971) However, the periodontal health status of these subjects is currently being investigated, and is presently unknown. The mean chemotaxis index is slightly lower in the present study than in some of the previous diabetes studies; those studies included mainly whites, while 50% of the diabetics in the present study are blacks.

In the present study, the age of the controls was significantly different from the diabetics. For this reason,

multiple regression analysis was not applied to the data. The importance of this problem is minimized, however, by the fact that age has failed to correlate with chemotaxis in previous studies (Hill et al. 1974, Baum et al. 1971, Molenaar et al. 1976). There was no age correlation found for the variable INDEX in this study, which is the variable of neutrophil function that significantly differed from diabetics to controls. In the present study, a weak correlation to age was found among the diabetics when using a chemotaxis quantification system from the periodontal literature (CTX), rather than that which was used in the diabetes literature (INDEX). This finding suggests that the chemotaxis impairment reported in studies may depend more on the method chosen for data analysis than on age. However, the two methods correlated well in the present study.

In agreement with the studies of Hill et al. (1974), Tater et al. (1987), Mowat & Baum (1971), and Manouchehr-Pour et al, (1981), blood glucose levels of diabetics were not correlated with chemotactic function, indicating that short-term metabolic control of IDDM did not affect chemotaxis. This finding was reinforced by the observation that black females, with higher CBG, and white females, with lower HbA_1, did not significantly differ in chemotaxis function. Interestingly, a correlation was found in the present study between CTX and blood glucose of the controls. But with such high variation in chemotaxis (10.2 - 77.3), and a low range of

blood glucose values (47 - 124) in the controls, this would suggest that low blood glucose could affect chemotaxis more than elevated blood glucose. The physiologic significance of this finding in IDDM is doubtful.

The LJP model has been utilized extensively to elucidate the biochemical and intracellular mechanisms responsible for impaired neutrophil chemotaxis. This has not been pursued in regard to the chemotaxis impairment of diabetics. Agarwal et al. (1989) reported lower free cytosolic calcium mobilization in LJP neutrophils. This was investigated in more detail by Daniel et al. (1993), noting that defective plasma membrane calcium channels were involved when the cytosolic free calcium rise in response to fMLP was less than normal neutrophils. This was suggested to negatively effect activation of enzymes necessary for normal neutrophil function. Signal transduction pathways involving diacylglycerol (DAG) (Tyagi et al. 1992) and protein kinase C (PKC) (Kurihara et al. 1993) have been implicated in the LJP chemotaxis impairment. It was proposed by Kurihara et al. (1993), and Van Dyke et al. (1993), that in chemotaxis impaired neutrophils, the high DAG levels induced chronic activation of PKC, which may then cause PKC downregulation by a feedback pathway. The cellular response (chemotaxis) would then be decreased. The IDDM neutrophil may have similar aberrant signal transduction related to its neutrophil function defects.

Alternatively, other pathways could be responsible. Dillon et al. (1987) observed increases for various inositol phosphate isomers in response to stimulation with chemoattractants (fMLP and LTB_4). Inositol phosphates have roles in the regulation of intracellular calcium and DAG levels, so anomalies of their metabolism also could alter neutrophil function. Additionally, insulin affects N-methyl transferase activity and changes cell membrane fluidity. Changes in membrane fluidity may influence calcium transport across the membrane, affecting neutrophil function i.e., chemotaxis. Nicotine has been demonstrated to decrease neutrophil superoxide production and lysosomal enzyme release, but not chemotaxis (Sasagawa et al. 1985, Pabst et al. 1995). However, Bridges et al. (1977) demonstrated that the water-soluble fraction of tobacco smoke (with factors in addition to nicotine) inhibited chemotaxis, unrelated to glucose catabolism by the neutrophil. A functional microtubule system is required for optimal chemotaxis (Valerius 1984) and so the possibility of cytoskeletal protein defects may be investigated in relation to the IDDM chemotaxis impairment. It would be of interest to investigate IDDM neutrophils for signal transduction defects similar to those found in LJP, and also to use the defective IDDM neutrophil as a model for investigation of neutrophil function.

The major finding of this study, impaired neutrophil chemotaxis of insulin-dependent diabetics, is not a novel

finding, and is not intended to be the endpoint for study of this patient sample. Yet, it does demonstrate that in a well-controlled study, in which race and gender of the controls was matched, that a neutrophil chemotaxis defect is present in a large group (n=41) of insulin-dependent diabetics. It further demonstrates that data obtained from assays with high inherent variability, such as that of neutrophil chemotaxis, require careful analysis and interpretation. The chemotaxis data gathered can now be used in ongoing investigation, to seek correlations of chemotaxis with class II HLA alleles, and with parameters of periodontal disease status.

V. SUMMARY

The goals of this investigation were: 1. to determine the level of chemotaxis function in a sample of IDDM patients and controls; 2. to determine metabolic control of IDDM at time of chemotaxis assessment; 3. to seek correlations between chemotaxis levels and metabolic control, and between chemotaxis methods.

Neutrophils were separated from blood of IDDM patients and a race/gender-matched control sample, and chemotaxis was assessed toward a gradient of fMLP in a modified Boyden chamber assay. Chemotaxis was expressed by one method frequently reported in the periodontal literature, and by another method often used in the medical literature. Glycosylated hemoglobin (for the diabetics only) and blood glucose levels were determined at the time of chemotaxis determination.

The results indicate that neutrophil chemotaxis is significantly impaired in insulin-dependent diabetics, that chemotaxis level of diabetics does not correlate with glucose control, and that chemotaxis may have only a weak age correlation. Chemotaxis was not shown to be affected by gender or race. The two chemotaxis methods correlated well.

These findings will be useful in the further evaluation of this IDDM patient sample to answer the following questions: Does the neutrophil chemotaxis defect of young IDDM patients render them more susceptible to periodontal disease? Is the chemotaxis defect related to genes that are associated with increased susceptibility to IDDM or periodontitis? In applying the findings of this study to those questions, the most significant meaning of this study will be found.

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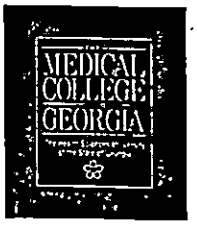
APPENDIX A

HAC Approval/Consent Forms



Human Assurance Committee
Institutional Review Board

June 13, 1994

Carl J. Gustke, D.D.S.
Oral Biology/Periodontics
Medical College of Georgia

RE: "Genetic Markers for Periodontitis and Diabetes: Association With Impaired Neutrophil Chemotaxis"

APPROVAL DATE: June 13, 1994
FILE NUMBER: 94-03-205

Dear Dr. Gustke:

The HUMAN ASSURANCE COMMITTEE has reviewed and approved the above referenced project in accordance with the DHHS policy and the institutional assurance on file with the DHHS.

The Committee would like to call your attention to the following obligations as Principal Investigator of this study. Under the terms of our approved Institutional Assurance to the Department of Health and Human Services, you must provide us with a progress report at the termination of the study, or at the annual anniversary date of this approval, whichever comes first. If the study will be continued beyond the initial year, an annual review by the HUMAN ASSURANCE COMMITTEE is required, with a progress report constituting an important part of the review. The Committee will notify you of the anniversary report by sending you an HAC-107 form for completion.

If VA patients or facilities are involved in this study, you must also have a letter of approval from the VA Research & Development Committee prior to involvement of VA patients or facilities.

Sincerely yours,

[redacted]

George S. Schuster, D.D.S., Ph.D.
Chairman
HUMAN ASSURANCE COMMITTEE

HUMAN ASSURANCE
APPROVED
INFORMED CONSENT
DATE: 6/13/94

Informed Consent Form - A

GENETIC MARKERS FOR PERIODONTITIS AND DIABETES:
ASSOCIATION WITH IMPAIRED NEUTROPHIL CHEMOTAXIS

Principal Investigator: Dr. Carl Gustke
Co-Investigators: Dr. Sidney Stein, Dr. William Hoffman

I have been invited to participate in a research study designed to identify the gene(s) responsible for defective white blood cell movement, increased infections and periodontal (gum) disease that occur in some diabetics. Identification of a gene that makes diabetics more susceptible to infections will help us to develop better treatments for persons affected. I understand that I (my child/ward) have been asked to participate because I have, or someone in my immediate family has insulin-dependent diabetes. I understand that I am one of approximately 40 persons to participate in this study.

In the study, I will be asked to provide a 6.6 tsp. (33 ml) blood sample. Strict aseptic procedures will be maintained during the drawing of blood from a vein in my arm to minimize the risk of infection. I understand that I will be informed if I have defective white blood cells, and that my doctor may use this information to help plan treatment if I ever have an illness or infection. I understand that this test will be free of charge, but I still am responsible for the normal costs of doctor visits.

I understand that participating in this study and donating blood may involve some risks. Since only a 6.6 tsp. (33 ml) sample of blood will be drawn (significantly less than the 500 ml unit obtained from normal blood donors), the likelihood of syncope (fainting) is minimal. Strict aseptic procedures will be followed to minimize the risk of infection. I understand that a bruise may form at the site where blood was drawn from my arm as a result of extravasation of blood. I understand that individual benefits will be minimal, and limited to a free diagnostic test that may help show my ability to fight infections.

I understand that my participation in this research study, and the research records specifically related to it, will be confidential, unless specifically required to be disclosed by state or federal law. Confidentiality will be maintained by using coded designations only. All samples will be collected under the supervision of Dr. William Hoffman, Department of Pediatric Endocrinology. I understand that my records will become part of the medical record, and that I will not be personally identified in any publication of the results of this study. I understand that the Medical College of Georgia assumes no obligation to pay any money or provide free medical care in case this project results in any harm to me.

I understand that Carl J. Gustke, D.D.S., who can be reached at (706) 721-2441, will answer any further questions I may have at any time concerning the study, the procedures, and any injuries that may appear related to the research. If I have any questions or concerns about the rights of research subjects, I may contact Dr. George Schuster at (706) 721-2991. In case of emergency, Dr. Gustke may be contacted at (706) 721-2441.

The risks and benefits to me, if I participate in this study have been explained. I have had the chance to ask questions and these have been answered.

Subject's Signature _____ Date _____

Witness' Signature _____

HUMAN ASSURANCE
APPROVED
INFORMED CONSENT
DATE: 6/13/94

Informed Consent Form - B

GENETIC MARKERS FOR PERIODONTITIS AND DIABETES:
ASSOCIATION WITH IMPAIRED NEUTROPHIL CHEMOTAXIS

Principal Investigator: Dr. Carl Gustke
Co-Investigators: Dr. Sidney Stein, Dr. William Hoffman

I have been invited to participate in a study that will determine white blood cell movement in diabetics and genetic associations with it. I understand that I have been chosen to participate because I do not have diabetes, and my white blood cells will be considered "normal" for comparison to diabetics. This will help to determine which diabetics have impaired white blood cell function. I understand that I am 1 of about 20 persons asked to donate blood for this reason in the study.

In the study, I will be asked to provide a 6.6 tsp. (33 ml) blood sample. I understand that participating in this study and donating blood may involve some risks. Since only a 6.6 tsp. (33 ml) sample of blood will be drawn (significantly less than the 500 ml unit obtained from normal blood donors), the likelihood of syncope (fainting) is minimal. Strict aseptic procedures will be followed to minimize the risk of infection. I understand that a bruise may form at the site where blood was drawn from my arm as a result of extravasation of blood. I understand that individual benefits will be minimal, and limited to a free diagnostic test that may help show my ability to fight infections.

I understand that my participation in this research study, and the research records specifically related to it, will be confidential, unless specifically required to be disclosed by state or federal law. Confidentiality will be maintained by using coded designations only. All samples will be collected under the supervision of Dr. William Hoffman, Department of Pediatric Endocrinology, or Dr. Carl Gustke, Department of Periodontics. I understand that I will not be personally identified in any publication of the results of this study. I understand that the Medical College of Georgia assumes no obligation to pay any money or provide free medical care in case this project results in any harm to me.

I understand that Carl J. Gustke, D.D.S., who can be reached at (706) 721-2441, will answer any further questions I may have at any time concerning the study, the procedures, and any injuries that may appear related to the research. If I have any questions or concerns about the rights of research subjects, I may contact Dr. George Schuster at (706) 721-2991. In case of emergency, Dr. Gustke may be contacted at (706) 721-2441.

My participation in this study is voluntary. I understand, however, that I may revoke my consent and withdraw from the study now or at any time in the future, without penalty or loss of care or other benefits to which I am otherwise entitled. I understand that I am to be informed if the study provides any new information that might affect my decision to participate so that I may decide whether to continue the study.

My participation in this study is voluntary. I understand, however, that I may revoke my consent and withdraw from the study now or at any time in the future, without penalty or loss of care or other benefits to which I am otherwise entitled. I understand that I am to be informed if the study provides any new information that might affect my decision to participate so that I may decide whether to continue the study.

The risks and benefits to me, if I participate in this study have been explained. I have had the chance to ask questions and these have been answered.

Subject's Signature _____ Date _____

Parent or Guardian's Signature* _____ Date _____

Witness' Signature _____

*The above individual verifies that he/she is the natural parent and/or legal guardian of _____ and as such has legal authority to consent to the study outlined above.

HUMAN ASSURANCE
APPROVED
INFORMED CONSENT
DATE 6/13/94

CHILDREN'S ASSENT

GENETIC MARKERS FOR PERIODONTITIS AND DIABETES:
ASSOCIATION WITH IMPAIRED NEUTROPHIL CHEMOTAXIS

Principal Investigator: Dr. Carl Gustke
Co-Investigators: Dr. Sidney Stein, Dr. William Hoffman

I am being asked to be in a research study, but before I decide to be in it, I am being given information and a chance to ask any questions I may have about it. The doctors in charge of this study are Dr. Hoffman and Dr. Gustke. They would like me to donate a 6.6 tsp. sample (33 ml) of my blood. The reason I am being asked to donate this sample is because I am diabetic. The doctors believe that some diabetic children have a defect in some of their blood cells which prevents them from moving towards an invading bacteria. Children are born with this defect in cell movement. It is controlled by something called a "gene." We inherit these genes from our parents. They can determine if I have a defective gene by looking at cells from my blood sample. This study may allow them to determine which gene causes the defect.

The drawing of blood will probably hurt some, but only for a short time. I do not have to be in this study and can decide not to be. Not being in this study will not upset anyone. The doctor will keep caring for me as he has done in the past. I can, and am expected to, let my parents, doctors, and nurses know about any unpleasant parts of the study or any bad effects following the collection of blood samples. The doctor will answer any questions I have at any time about the study.

My_____(parent/guardian) knows about this study and wants me to be a part of it.

I have read this document and it has been explained to me. I have had a chance to ask questions and they have been answered to my satisfaction. If I have any more questions I may call Dr. Carl Gustke DDS at (706) 721-2441. With this understanding, I hereby consent to participate in this study.

_____ _____
Subject's Signature Date

_____ _____
Parent or Guardian's Signature* Investigator

_____
Witness' Signature

*The above individual verifies that he/she is the natural parent and/or legal guardian of_____and as such has legal authority to consent to the study outlined above.

APPENDIX B

Method for Isolation of Neutrophils (Kalmar et al. 1988):

1. Place 3ml of Mono-Poly Resolving Medium (M-PRM) into a sterile 15ml polystyrene centrifuge tube. Carefully overlay 1ml Histopaque 1077.

2. Carefully layer 3.5-5.0 ml of fresh, heparinized, human whole blood onto medium. Maximum separation and functionality can be obtained if cells are separated within two hours of collection. Acceptable separation can be obtained for up to 6 hours, but functionality may decrease.

3. Centrifuge at 1200 RPM for 30 min in a swinging bucket rotor at room temperature. Some blood samples will not separate in that time. Continued spinning may be necessary for 15-30 min.

4. The following three fractions should be obtained when blood is separated with M-PRM and Histopaque 1077:
a. FR 1 (at the plasma-medium interface): MN band.
b. FR 2 (below the interface): PMN band.
c. FR 3: the red blood cell pellet.

5. With a sterile Pasteur Pipette, gently lower the tip to the FR 2 layer, withdraw and transfer the PMN band to a 50 ml centrifuge tube.

6. Wash the cells once with 25-50 ml PBS. Cells should not remain in contact with the separation medium for extended periods of time, as this could affect the cell viability.

7. If RBCs are present after the first wash, add 10 ml of COLD lysing buffer, gently resuspend the cells and allow them to stand for 6 min on ice. Bring up to 50 ml with PBS.

8. Centrifuge at 1200 RPM for 10 min at room temperature.

9. Pour off supernatant. Add 10 ml PBS and resuspend cells for counting. At this point, if the cells are to left standing for any length of time, they should be diluted to 5 x 10^6 with PBS.

Preparation of Responder Cells and fMLP (Van Dyke et al. 1979)

1. Isolate PMNs for chemotaxis as per protocol. Dilute to 2.5×10^6 cells/ml in Gey's GVB^{2+} media (GGVB), mix gently, but well. 0.45 ml is used for each chamber.

2. Prepare F-met-leu-phe in fresh GGVB. Use 3μl of frozen stock (0.66×10^{-4})/10 ml GGVB to yield a final concentration of 2×10^{-8}.

Preparation of Chambers:

1. Prepare modified Boyden chambers in triplicate, using 5μm, 13mm diameter Sartorious filters. Usually no more than 36-48 chambers should be run at one time.

2. Handle filters only with filter forceps on blotting paper. Number filters with high quality ink (Cross pen) on the underside of the filter (as it is packed in the shipping container). Place the numbered side down into the chamber. Number will indicate the bottom of the filter, the side that will be read under the microscope.

3. Screw the Delrin screw down firmly against the filter. Do not screw too lightly or screw will leak and prevent reaction; if too tight, filter will wrinkle.

Chamber Loading and Chemotaxis:

1. Slowly fill lower chamber with fMLP using 5.75 inch Pasteur pipet until liquid fills the chamber and convex meniscus forms. If air is introduced, discard the chamber.

2. IMMEDIATELY upon wetting the filter, add 0.4 ml cell suspension (with Pipet-Aid) to upper portion of screw. Insert pipet into hole in Delrin screw, carefully so as not to touch or penetrate the filter; slowly withdraw as chamber fills.

3. Fill triplicate chambers for each sample to be tested. Work carefully but quickly.

4. Incubate at 37°C in humidified air with 5% CO_2 for approximately two hours. End-point to be determined by experimentation.

Chamber Unloading:

1. After appropriate incubation, the filters are ready for fixation. Working quickly, unscrew and remove the screws (the reaction stops when the fluids mix). Do not aspirate the liquids. Lift the filters gently at the edge with a needle and remove carefully with filter forceps. The filters are quickly and gently dipped in absolute methanol one at a time and placed in a small Petri dish filled with Neutral Buffered Formalin. Place the filter bottom side up, one triplicate set per dish.

2. Fix the filters for a minimum of 10 minutes to overnight.

3. Following fixation, three filters (a triplicate set) are clipped to a glass microscope slide, bottom side up, secured with Millipore microclips, and are stained. Do not allow filters to dry before staining.

Staining and Mounting of Filters:

1. Slides are placed in a slide basket and into hematoxylin while basket is being filled. Counting from time of last slide addition, move basket in this sequence, draining briefly between each step:

2. a) Hematoxylin - 14 min.
   b) Fresh dHOH - dip
   c) 70% EtOH - 1 min.
   d) 95% EtOH - 2 min.
   e) 98% EtOH - 2 min.
   f) Isopropanol - 4 min.
   g) Xylene - 2 min.
   h) Xylene - 2-10 min.

3. Remove slides one at a time from xylene for mounting as follows:
   a) Label frosted end of a fresh slide with numbers of each filter of the triplicate set.
   b) Remove the clips with a forceps.
   c) With 9 in. Pasteur pipet, place a strip of Permount lengthwise on a fresh labelled slide.
   d) Carefully place each filter on the Permount bottom-side up--the numbered side.
   e) Place one or two drops of Permount on each filter.
   f) Place coverslip over filters, being careful not to trap air.
   g) Wait an hour or more, to allow some of the xylene to evaporate before counting PMNs. If they cannot be counted the same day, place in a desiccator overnight.

Counting the PMNs:

1. Using a grid eyepiece as an aid, count three random fields per filter, on the bottom surface of the filters, at 40X.
2. Compare chemotaxis with random migration to determine that chemotaxis has occurred.
3. Calculate the mean number of cells that migrated to the bottom of the filters for each assay, and calculate the standard deviation.
4. Tests that differ from the controls with a *t*-test $p<.05$ are considered to have significantly decreased chemotaxis.