In Vivo Crevicular Leukocyte Response to a Chemotactic Challenge: Inhibition by Experimental Diabetes

LORNE M. GOLUB,1*, GREGG A. NICOLL,1 VINCENT J. IACONO,2 AND NUNGA VARAM S. RAMAMURTHY1

Department of Oral Biology and Pathology1 and Department of Periodontics,2 State University of New York at Stony Brook, Stony Brook, New York 11794

Received 1 March 1982/Accepted 17 May 1982

Diabetes in rats inhibits the migration of neutrophils into the healing gingival crevice, an effect associated with impaired in vitro neutrophil chemotactic activity. We recently described the in vivo response of human and rat crevicular neutrophils to a chemotactic challenge and used this assay in the present study on streptozotocin-induced diabetic rats. Optimal concentrations of two chemotactic agents, casein (0.2 μl, 2 mg/ml) or N-formylmethionylleucylphenylalanine (0.2 μl, 10−5 M), were placed into the gingival crevices of control and diabetic rats (time zero) after the resting neutrophil count was measured. After a 15-min delay, the neutrophil counts and gingival crevicular fluid flow were assessed every 5 min for another 0.5 h. The control rats (n = 14) showed an increase in neutrophil counts which reached maximum levels 30 min after the N-formylmethionylleucylphenylalanine challenge ("peak" neutrophil response) and decreased dramatically 5 min later. Diabetes of 4 days (n = 4), 14 days (n = 8), and 20 days (n = 5) duration reduced the peak neutrophil response 45, 66, and 71%, respectively. Casein produced the same response as N-formylmethionylleucylphenylalanine in control rats. Uncontrolled diabetes of 20 days duration reduced the peak neutrophil response to casein by 83%; diabetics administered insulin on a daily basis showed a reduction of only 34%. The pattern of change in gingival crevicular fluid flow in response to chemoattractants paralleled the neutrophil response. The chemotactic activity of peritoneal neutrophils was assessed in vitro with the agarose gel technique and was found to be correlated (r = 0.84; P < 0.01) with the in vivo chemotactic response in the same rats. If the same in vivo defect is observed in humans with diabetes (or with other systemic diseases associated with leukocyte dysfunction), this test could be useful diagnostically to rapidly assess neutrophil chemotaxis in lieu of in vitro assays and to identify patients who are unusually susceptible to aggressive periodontal disease.

Numerous studies have confirmed the long-held view that diabetes mellitus influences the severity of periodontal disease (4, 5, 12, 14, 41). In fact, insulin-dependent diabetes increases the prevalence of periodontitis even in adolescents and can be associated with a pattern of alveolar bone loss reminiscent of that seen in localized juvenile periodontitis or periodontosis (8a). Basic investigations are now necessary to determine the pathological mechanism(s) responsible for the aggressive periodontal breakdown often seen in diabetics and, with this knowledge, to determine why there is an apparent variability in periodontal disease susceptibility among diabetic patients. In addition, the elucidation of diabetes-induced alterations in host responses may be relevant to the pathogenesis of periodontal disease in general. In this regard, diabetes has been shown to alter collagen metabolism in gingiva and bone (15, 18; M. Schneir, N. Ramamurthy, and L. Golub, J. Dent. Res. 60A:642, 1981) and to impair various functions of polymorphonuclear leukocytes (PMNLs) (36), including their ability to degrade collagen fibrils (33).

Assayed by standard in vitro techniques, impaired chemotactic activity of PMNLs has been observed in a number of clinical conditions, including localized juvenile periodontitis (8, 11), Down's syndrome (21), and Chediak-Higashi syndrome (10), in addition to diabetes mellitus (29, 31). Because this PMNL dysfunction appears to predispose humans (24) and animals (36) to severe forms of periodontitis, monitoring this defect in vivo could provide clinicians and researchers with an important diagnostic test. We recently described a new technique which measures the response of PMNLs in humans and rats to a chemotactic agent placed directly...
into the gingival crevice (17). In the current investigation, we determined (i) that this new in vivo technique could detect impaired PMNL function in the gingival crevice of diabetic rats and (ii) that the in vivo crevicular PMNL response correlated with chemotactic activity assayed with a standard in vitro technique.

MATERIALS AND METHODS

Preparation of animals. Four-month-old (350- to 400-g) male Sprague Dawley rats were made diabetic (D group) by injection of streptozotocin (70 mg per kg of body weight; The Upjohn Co., Kalamazoo, Mich.) into the tail vein after a 12-h fasting period (16). These animals were tested for the crevicular leukocyte response (see below) 4, 10 to 14, and 29 days after diabetes induction. Age-matched un.injected rats served as controls (C group). In a separate experiment, half of the D group animals (D+I group) were injected subcutaneously with 0.5 to 4.0 IU of insulin every day throughout the entire experimental period. In this experiment, the three groups of rats (C, D, and D+I) were tested for the crevicular leukocyte response in vivo and for PMNL chemotactic activity in vitro (see below) 3 weeks after the induction of experimental diabetes. The dosage of insulin was adjusted based on the amount of glucose excreted in the urine (monitored with Eli Lilly Test-tape; Eli Lilly & Co., Indianapolis, Ind.), on the urine volume (poluria), and on body weight changes. Blood glucose and glycosylated hemoglobin levels, the latter a measure of long-term blood glucose control, were assayed as described previously (33). Ketones in the blood were monitored with Keto-Diastix strips (Miles Laboratories, Inc., Elkhart, Ind.), and pH was measured with a Radiometer pH meter.

In vivo gingival crevicular response. The technique used to determine the in vivo gingival crevicular response was a slight modification of that described in detail by Golub and co-workers (17) for human subjects. Two gingival crevices, the distolabial crevices of the right and left maxillary incisors, were monitored for each rat during the assay. In brief, after each crevice was gently sprayed with water and air dried, it was washed with 10 μl of Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) delivered and aspirated with a Hamilton microsyringe (The Hamilton Co., Reno, Nev.). In one of the test crevices, leukocytes in the crevicular washes were counted with a hemacytometer before and at various times after the chemotactic agent was delivered, as described previously (17). In the other crevice, gingival crevicular fluid (GCF) was collected on filter paper strips (Periopath, Harco Electronics Ltd., Winnipeg, Manitoba, Canada) modified to fit the rat gingival crevice; the strips were cut with scissors to produce a point ( ). An electronic meter (Periotron, Harco Electronics), which was used to measure GCF volume, was calibrated with different volumes (0.05 to 0.5 μl) of sera from four C group rats (blood glucose of 93 ± 7 mg/dl) and four severely hyperglycemic D group rats (blood glucose of 687 ± 55 mg/dl) delivered onto the modified filter paper strips. The calibration curves were linear up to 0.5 μl and were identical for the C group and D group rats sera (serum volume versus Periotron score: r = 0.965 for the C group rats and r = 0.978 for the D group rats).

After base-line leukocyte counts and GCF readings were obtained, 0.2 μl of the chemotactic agent (see below) was delivered atraumatically onto the gingival margin with a Hamilton syringe. Casein or the peptide N-formylmethionylleucylphenylalanine (FMLP) was used as the chemoattractant. The optimal concentration of casein (2 mg/ml) in our in vivo assay had been determined previously (17). To determine the optimal concentration of the chemotactic peptide for the in vivo assay, 0.2 μl of solutions containing 10⁻⁴ to 10⁻³ M FMLP (Andrus Research Corp., Bethesda, Md.) was placed into the crevices of systemically normal C group rats, and the leukocyte counts and GCF flow were measured before and at different times after placing the chemoattractant. The concentration of FMLP that produced the greatest increase over baseline values was subsequently used to test the effect of diabetes on the in vivo crevicular response.

The dissolution of the poorly soluble FMLP in 0.9 M NaCl, at a final concentration of 10⁻³ M, was achieved by the dropwise addition of 2 N NaOH until pH 10 was reached. This stock solution was then diluted in saline to produce concentrations of 10⁻⁴, 10⁻³, and 10⁻⁶ M, all at pH 7.6. Both casein and FMLP preparations were chemotactically active in vitro (see below).

In vitro chemotactic activity of PMNLs. Peritoneal leukocytes were collected from eight C group, eight D group, and seven D+I group rats 3 weeks after the administration of streptozotocin, as described previously (37). The animals were injected intraperitoneally with 10 ml of 0.15% glycogen, and the exudate was collected 18 h later. The cells were collected, the erythrocytes were lysed, and PMNLs were separated by Ficoll-Hypaque gradient centrifugation (6), followed by suspension in Hanks balanced salt solution at a concentration of 25 x 10⁶ cells per ml.

The in vitro chemotactic assay used was a modification of the technique of Nelson et al. (32), as previously described (36). A series of three triplicate wells was punched into each agarose gel slice, and PMNL chemotaxis was assessed in triplicate for each rat. The middle well contained 10 μl of cell suspension (2.5 x 10⁵ PMNLs). The top well contained 10 μl of the chemoattractant casein (2 mg/ml), and the bottom well contained serum-free minimal essential medium modified for Spinner culture (GIBCO). In some experiments, FMLP (prepared as described above) in a concentration (10⁻⁶ M) used by others (30, 43) served as the chemoattractant. The slides were incubated at 37°C in a humid atmosphere containing 5% CO₂ in air for 8 h.

The cells were then fixed in methanol followed by Formalin and stained with Wright's solution, and the slides were examined under 40× magnification with a micrometer disk. The distance travelled by the PMNLs toward the chemoattractant (directed migration) and in the opposite direction (spontaneous migration) was measured as described previously (36).

RESULTS

In vivo crevicular response to FMLP. Placing 0.2 μl of 10⁻⁶ M FMLP into the gingival crevice produced no significant increase above base-line
values in either leukocyte count or GCF flow during the 45-min test procedure (Fig. 1). The three higher concentrations (10^{-5} M to 10^{-3} M) produced detectable increases over base-line values; however, 10^{-4} M FMLP was found to be optimal. The pattern of the response of GCF appeared to parallel that of the crevicular leukocytes, and both parameters reached maximum or peak values approximately 25 to 30 min after the chemotactic challenge was placed (Fig. 1, 2, and 3).

The C group and D group rats all showed relatively low crevicular leukocyte and GCF values at time zero (Fig. 2), before the FMLP was placed (base-line values [Fig. 2, insert]). With time, the C group rats showed a sharp increase in both parameters, which peaked or reached maximum values about 30 min after the crevicular challenge; the values decreased rapidly thereafter. Diabetes of only 4 days duration began to suppress the in vivo response of crevicular leukocytes and GCF. This defect became more pronounced with an increased duration of the diabetic condition and with an increased severity of the hyperglycemic state.

The blood glucose values for the groups of rats at the time that the crevicular response was tested were: C, 143 ± 41 mg/dl; D (4 days), 408 ± 75 mg/dl; D (10 to 14 days), 602 ± 36 mg/dl; D (20 days), 732 ± 154 mg/dl. Diabetes of 4, 10 to 14, and 20 days duration reduced the peak PMNL response 45, 66, and 71%, respectively (Fig. 2, insert).

After a 3-week duration of diabetes, no visual evidence of periodontal disease was noted. However, in other experiments at this time period, defleshed jaws of D group rats showed increased alveolar bone loss, compared with those of C group rats. Diabetes of 90 days duration resulted in localized areas of severe periodontitis, including abscess formation (N. S. Ramamurthy and L. M. Golub, unpublished data).

**In vivo crevicular response to casein.** As in the experiment with FMLP, the C group, D group, and D+I group rats showed relatively low leukocyte counts and GCF volumes at time zero (Fig. 3), as base-line values (Fig. 3, insert). The C group rats showed a sharp increase in both parameters, which reached maximum values about 25 min after the casein challenge and decreased sharply 5 to 10 min later. The D group rats showed little or no response to casein, whereas the D+I group rats showed a response similar in pattern to that seen in the C group rats (Fig. 3) although the peak height was somewhat lower. Once again, the leukocyte and GCF responses to the in vivo chemotactic challenge tended to parallel each other.

In this experiment, the blood from the C group rats showed a mean glucose concentration of 88 ± 4 mg/dl, HbA1 of 4.4 ± 0.5%, and negative results for ketones. The D group rats had markedly elevated values for glucose (504 ± 73 mg/dl), HbA1 (8.4 ± 2.1%), and ketones (10 to 15 mg/dl). Insulin treatment of the D group (D+I group) reduced these blood constituents (glucose of 114 ± 56 mg/dl; HbA1 = 4.9 ± 0.7%; and ketones of 0 to 5 mg/dl). The pH of freshly drawn blood was measured in a separate group of C and D rats. The ranges for the separate C group and D group rats were 7.50 to 7.75 (mean = 7.66) and 7.50 to 7.65 (mean = 7.61), respectively.

**In vitro PMNL chemotactic activity.** The in vitro chemotactic activity of PMNLs (from peritoneal exudates) from the same C group and D group rats used in the in vivo casein study (Fig. 3) is presented in Table 1. Uncontrolled diabetes produced a 69% reduction in the distance migrated toward the chemotactic agent casein, whereas the D+I group rats showed a 20% reduction in directed migration. Consistent with previous observations in rats (36) and humans (20, 28), diabetes produced little or no effect on the migration of PMNLs away from the chemotactant (distance B; spontaneous migration). For the D group animals, in vitro chemotactic activity, calculated as the chemotactic index or the chemotactic differential, was re-
duced by 57 and 82%, respectively, compared with controls. When the D group rats were treated with insulin, PMNL chemotactic activity was closer to control values.

**Relationship between in vivo crevicular response and PMNL chemotactic activity in vitro.** The in vitro chemotactic activity of PMNLs from C group and D group rats was correlated with the in vivo crevicular response in the same animals (Fig. 4). Casein (2 mg/ml) was the chemottractant used in both the in vitro and the in vivo assays. In vitro PMNL chemotactic activity, calculated as the chemotactic index (A/B), was positively correlated with both the in vivo crevicular leukocyte and the GCF responses (Fig. 4). Calculating in vitro chemotaxis as the chemotactic differential and correlating this parameter with both in vivo leukocyte and GCF responses resulted in nearly identical correlations (r = 0.84 and 0.64, respectively) compared to those calculated for the chemotactic index (Fig. 4).

In a separate experiment, 10^{-6} M FMLP was used as the chemoattractant in vitro. The in vitro chemotactic index of PMNLs with this agent was found to be equally positively correlated with either the in vivo crevicular leukocyte response (r = 0.76) or the GCF response (r = 0.73).

By use of linear regression analysis, all of the correlation coefficients calculated for the experiments with either casein or FMLP as the chemoattractant were found to be statistically significant (P < 0.01), with one exception. When the in vivo crevicular response of leukocytes or GCF to casein (or FMLP) was correlated with spontaneous migration measured in vitro for the same animals, no significant correlation was observed (r = 0.27; distance B versus in vivo peak response).

---

**FIG. 2.** Effect of diabetes of varying duration on the in vivo gingival crevicular response to FMLP. FMLP (10^{-6} M) was applied to the gingival crevice at time zero. Each value is the mean of 14 rats (C group), four rats (D group, 4 days), eight rats (D group, 10 to 14 days), or five rats (D group, 20 days) at each time period. (Inserts) Peak or maximum crevicular leukocyte (bottom) or GCF (top) response to FMLP in C group and D group rats. Each vertical bar represents the mean ± standard error of the base-line values or the peak values for the C group and D group rats independent of time. The number on top of each cross-hatched bar is the mean time (minutes) required to reach the peak response.
FIG. 3. In vivo gingival crevicular response to casein in C group, D group, and D+I group rats. Casein (2 mg/ml) was applied to the gingival crevice at time zero. Each value is the mean of seven to eight rats at each time period. (Inserts) Peak crevicular leukocyte (bottom) or GCF (top) response to casein in C group, D group, and D+I group rats. Each vertical bar represents the mean (± standard error) of the base-line values or the peak values for the C group, the D group, and the D+I group rats independent of time. The number on top of each cross-hatched bar is the mean time (minutes) required to reach the peak response.

DISCUSSION

Although the use of standard in vitro assays, including the Boyden chamber and agarose gel techniques, has been essential in the recent identification of a wide spectrum of clinical disorders involving impaired neutrophil chemotaxis, these observations may not be directly related to the activity of these cells in vivo (13). This reason, plus the technical problems inherent in the in vitro assays, led us to attempt to develop a technique which monitors PMNL chemotaxis in the gingival crevice in vivo (17). In the present study, we found that diabetes, a disease repeatedly shown to inhibit in vitro chemotaxis (20, 29, 31, 36), produced a marked suppression of the in vivo crevicular leukocyte and GCF responses to two chemotactic agents, casein and FMLP. Our current and earlier studies demonstrated (i) that the crevicular response was specific for chemoattractants since nonchemotactic protein or buffer challenges elicited no increase in leukocyte counts or GCF flow (17) and (ii) that the response of the crevicular leukocytes was specific for PMNLs, as indicated by the following. The differential leukocyte count for the crevicular washes before the chemotactic challenge was 85% lymphocytes and 10 to 15% PMNLs, reflecting the lymphocytotic nature of rat blood (1). The differential shifted to 90 to 95% PMNLs during the peak leukocyte response (data not shown), in agreement with our previously published results (17), indicating that only these cells respond to the in vivo chemotactic challenge.

The close correlation that we observed between the crevicular leukocyte and GCF re-
sponses in vivo (both responses were positively correlated with in vitro chemotaxis) was not surprising since this parallelism has been described by others in systemically normal humans (22) and diabetic dogs (25). In the latter study, diabetes suppressed the response of both parameters to plaque accumulation. In fact, hydrolytic enzymes such as those released by PMNLs can generate GCF flow by widening the intercellular spaces of the crevicular epithelium (41, 42).

However, the in vivo defect in the crevicular leukocyte response could reflect impeded migration through a pathologically thickened basement membrane, a major diabetic complication (37), rather than suppressed PMNL chemotaxis. We argue that our observations are due to the latter, rather than to the former diabetes-induced abnormality, because of the following.

(i) A decreased crevicular leukocyte response in vivo was detected as early as 4 days after diabetes was induced, too soon for significant changes to develop in this collagenous structure. Diabetes-induced basement membrane thickening occurs in a variety of tissues, including gingiva (38), and has been observed in diabetic rats as well as in diabetic humans. However, the accumulation of excessive basement membrane is a slow process (7), perhaps reflecting the slow turnover of collagen in this structure compared with other proteins (19), and would be expected to require many months in diabetic rats (35) or years in diabetic humans (33) to develop.

(ii) Diabetes increases the elastase activity in PMNLs (G. Nicoll, N. Ramamurthy, G. Gollapudi, and L. Golub, J. Dent. Res. 60A:344, 1981). This effect would favor basement membrane degradation (23) and would facilitate, rather than impede, leukocyte emigration.

(iii) We observed a strong positive relationship between the in vivo crevicular leukocyte response and in vitro chemotaxis by using a protocol in which both tests were run in each animal. No correlation was found between in vitro spontaneous migration and the in vivo crevicular response.

The PMNL disorder associated with the diabetic state was classified by Clark (9) as a cellular defect. This was based on the finding that nondiabetic relatives of diabetic patients (the former group having normal serum insulin and glucose levels) also exhibit impaired leukocyte chemotaxis (29), an observation recently confirmed by McMullen and co-workers (26). The basic mechanisms responsible for this cellular defect have not been conclusively established. Possible causes include an inhibition of the glycolytic pathway of the cell, abnormal...

![Graph](http://iai.asm.org/)  
**FIG. 4. Relationship between the in vivo and in vitro chemotaxis assays. Casein (2 mg/ml) was used as the chemoattractant in both assays. The correlation coefficients (r) were calculated by linear regression analysis.**

---

**TABLE 1. Effect of streptozotocin-induced diabetes on in vitro chemotactic activity of rat PMNLs**

<table>
<thead>
<tr>
<th>Experimental group (no. of rats)</th>
<th>Directed migration (A)</th>
<th>Spontaneous migration (B)</th>
<th>Chemotactic index (A/B)</th>
<th>Chemotactic differential (A - B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (8)</td>
<td>7.4 ± 0.9</td>
<td>1.8 ± 0.1</td>
<td>4.2 ± 0.5</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>D (8)</td>
<td>2.3 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>D+1 (7)</td>
<td>5.9 ± 1.3</td>
<td>2.1 ± 0.4</td>
<td>2.9 ± 0.5</td>
<td>3.8 ± 1.1</td>
</tr>
</tbody>
</table>

* Each value (number of squares; 1 square is 0.1 mm²) represents the mean ± standard error. PMNL activity was assayed in triplicate for each animal. The activity was determined by a modification of the agarose technique (32) described by Ramamurthy and co-workers (36).
cyclic nucleotide metabolism disrupting the organization of the microtubules and microfilaments of the leukocyte, a reduction in leukocyte membrane receptors, or some as yet unidentified mechanism (36). However, serum factors cannot be completely discounted (3) since glucose and insulin levels (which are both abnormal during diabetes) also affect leukocyte function (2, 20), even when added to the cells in vitro (31). Our data support this concept since we found a strong correlation between the development of elevated glucose levels and the in vivo leukocyte chemotactic defect. Moreover, insulin treatment of the D group rats reversed the PMNL chemotactic defect both in vivo and in vitro.

The importance of PMNL dysfunction as an abnormal host response in the oral tissues of diabetics is just beginning to be addressed. Ramamurthy and co-workers (36) found that diabetes inhibits the migration of PMNLs into the rat gingival crevice during the early inflammatory phase of wound healing. This in vivo defect is thought to contribute to the altered gingival collagen metabolism during inflammation (15) produced by the diabetic state (36). More recently, the clinical implications of these diabetic alterations have become somewhat clearer. Manouchehr-Pour and co-workers (24), in a study of systemically normal and diabetic patients, found that only the diabetics with severe periodontitis showed decreased PMNL chemotaxis. Diabetics with mild periodontal disease showed normal leukocyte function, as did systemically normal patients with mild or severe periodontitis. Consistent with these findings, McMullen and co-workers (26) demonstrated that patients with severe periodontitis who were related to diabetics (but who otherwise showed normal clinical laboratory values, including glycosylated hemoglobin levels) also exhibited decreased leukocyte chemotaxis. These studies imply that not all diabetics, only those (or their relatives) who show leukocyte dysfunctions, are predisposed to the development of severe periodontitis. They also reemphasize the complex nature and multifactorial etiology of periodontal disease(s).

It is of extreme interest that alterations in the crevicular microflora (27), like the PMNL dysfunction, can be detected within days after the onset of experimental diabetes. Although the cause-effect relationship between the leukocyte abnormality and changes in microbial population is unclear (27, 36, 39), both preceded by months the detection of severe periodontal breakdown (see above). If the same in vivo leukocyte defect is observed in humans with diabetes (or in other clinical conditions with associated in vitro leukocyte dysfunction), this test could be useful diagnostically (i) to rapidly assess PMNL chemotaxis in lieu of in vitro assays and (ii) to identify patients who are unusually susceptible to aggressive periodontal disease due to this abnormal host response.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE-03987 and DE-04296 from the National Institute of Dental Research.

We thank Audrey Nemiroff for excellent technical assistance and Nalini Manivannan and Harriet Sussman for their superb secretarial assistance.

LITERATURE CITED