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Reduced Oxidative Function in Gingival Crevicular Neutrophils in Periodontal Disease

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Measurable amounts of viable and functional polymorphonuclear neutrophils (PMNs) are recovered from pooled washings of the gingival crevice of healthy individuals. In the present study, we have assessed the ability of the PMNs removed from single healthy or diseased pocket sites to mount an oxidative burst when challenged with phorbol myristate acetate (PMA) and compared these activities with each other and with those obtained with autologous peripheral-blood PMNs. The oxidative burst after PMA stimulation was evaluated by using methods developed for the flow cytometer. The results showed that the PMNs collected from untreated disease sites were minimally responsive to PMA when compared with peripheral-blood PMNs collected at the same time from the same individual. Thus, whereas the peripheral-blood PMNs exhibited significantly lower resting oxidative product formation and a 500% increase when stimulated with PMA, all gingival-crevicular PMNs exhibited significantly higher resting formation of oxidized products but only a 150% increase after PMA stimulation. PMNs obtained from a consistently healthy site had significantly higher resting production of oxidized products and were able to mount the greatest absolute increase in oxidized products after PMA stimulation when compared with PMNs collected from diseased sites. Mechanical debridement of these diseased sites, which both reduced the bacterial numbers and restored clinical health, resulted in the recovery of gingival-crevicular PMNs that exhibited an oxidative burst more typical of that observed in PMNs obtained from healthy gingival sites and from the peripheral blood. This suggested that the PMNs collected from the diseased sites either had been exhausted by the large numbers of bacteria present in these sites or had been specifically inhibited by these bacteria.

Measurable amounts of viable and functional polymorphonuclear neutrophils (PMNs) are recovered from pooled washings of the gingival crevice of healthy individuals (5, 8, 15). These gingival-crevicular PMNs (GC-PMNs) exhibit in vitro an impaired chemotactic activity to a variety of stimulants and reduced phagocytosis of *Candida albicans* when compared with autologous peripheral-blood PMNs (PB-PMNs) (5). The GC-PMNs are comparable to the PB-PMNs in their ability to generate superoxide radicals (5), suggesting that their oxidative killing mechanisms are functionally intact as they exit from the body into the healthy gingival sulcus. These findings indicate that the GC-PMNs are not as robust as PB-PMNs, particularly in regard to chemotaxis and phagocytosis. This could reflect that, as soon as they exited into the sulci, they encountered plaque bacteria and initiated some of their antibacterial functions, thereby making them less responsive to new challenges introduced subsequently during the in vitro testing.

In the present study we have assessed the ability of the GC-PMNs removed from single healthy or diseased pocket sites to mount an oxidative burst when challenged with phorbol myristate acetate (PMA) (2) and compared these activities with each other and with those obtained with autologous PB-PMNs. The oxidative burst after PMA stimulation was evaluated by using methods developed for the flow cytometer (3, 6). The results show that GC-PMNs collected from untreated disease sites are minimally responsive to PMA when compared with PB-PMNs collected at the same time from the same individual.

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MATERIALS AND METHODS

Clinical sampling sites. Both diseased and healthy sites were sampled. A diseased site had a probing depth of ≥5 mm, exhibited bleeding upon probing, had spirochetes averaging ≥30% of the microscopic count, and usually exhibited bone loss. A healthy site had a probing depth of ≤4 mm, did not exhibit bleeding upon probing, had spirochetes averaging ≤15% of the microscopic count, and did or did not exhibit prior bone loss. A chronically diseased site was a diseased site which had been treated, usually by mechanical debridement, with some clinical improvement, but which still bled upon probing. The chronic sites that we studied exhibited no loss of attachment for one or more years so that these lesions were not progressive. The probing depth, attachment level, gingival bleeding score, and plaque microscope count were obtained for each sample site.

Buffers and lysing solution. Phosphate-buffered saline (PBS) contained 0.147 M NaCl, 4.1 mM $Na_2HPO_4 \cdot 7H_2O$, 2.3 mM NaH_2PO_4, and 15 mM $NaNO_3$ (pH 7.4). PBS-gel is PBS which also contained 2 mM EDTA, 10 mM glucose, and 1% gelatin (Difco Laboratories, Detroit, Mich.) and was used for all cell incubations. Lysing solution consisted of 0.15 M NH_4Cl, 10 mM $NaHCO_3$, and 10 mM EDTA at pH 7.4.

Collection of PB-PMNs. A lancet was used to prick the finger so as to obtain 20 to 30 μl of blood for the analysis of PB-PMNs. Unpurified PB-PMNs were prepared by a modification of the method of Boyum (4) by layering this anticoagulated blood over an equal volume of Ficoll-Hypaque and allowing it to sediment at room temperature. This yielded an agglutinate of erythrocytes in the lower layers, an intermediate layer of the separation medium, and an upper layer of platelet- and leukocyte-rich plasma. This upper layer was

removed and gently centrifuged, and the resultant pellet, which consisted of leukocytes, was suspended in PBS-gel. The leukocytes were stained with trypan blue and Wright stain, and the number of PB-PMNs was determined.

Collection of GC-PMNs. GC-PMNs were collected from a single pocket site by rinsing the pocket with 10 μl of freshly prepared pyrogen-free PBS-gel. This rinsing procedure was repeated five times for each pocket, because this number of rinses depletes the pocket of available GC-PMNs (14). The five rinses were pooled and diluted to 200 μl with PBS-gel. This cell suspension (10 μl) was used for a cell count (hemacytometer) and determination of viability by using trypan blue exclusion. Thirty microliters was mixed with an equal volume of 5% human serum albumin and deposited on a glass slide by a cytocentrifuge and stained (Wright stain) to determine the differential count. The remaining suspension was centrifuged at 200 × *g* for 15 min, and the resultant PMN-enriched pellet was suspended in 20 μl of cold PBS-gel. The recovery of GC-PMNs was improved by using an Eppendorf pipette with 10-μl disposable tips as described by Thurre et al. (15) instead of the Hamilton syringe that was originally recommended (14).

Reagents. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) (Eastman Kodak Co., Rochester, N.Y.) was dissolved in absolute ethanol at a concentration of 5 mM and kept at 4°C for up to 4 weeks. 5′,6′-Carboxy-2′,7′-dichlorofluorescein diacetate (Molecular Probes, Junction City, Ore.) was dissolved under the same conditions. PMA (Consolidated Midland, Brewster, N.J.) was dissolved in dimethyl sulfoxide as a stock solution of 1 μM (2 mg/ml) and stored in 20-μl aliquots at −20°C.

Measurement of oxidative product. All PMN preparations were evaluated for the formation of an oxidative product before and after exposure to PMA, a potent PMN stimulant (2, 11). Attomolar concentrations of hydrogen peroxide can be detected by the formation of 2′,7′-dichlorofluorescein (DCF) from 2′,7′-dichlorofluorescin (DCFH), which is, in turn, a product of hydrolysis (deacetylation) of DCFH-DA (10). DCF is a product of oxidation by peroxide in the presence of peroxidase. This assay was adapted by Homan-Muller et al. (9) to measure hydrogen peroxide produced by phagocytizing neutrophils. More recently, Bass and colleagues (2, 3) reported on the detection, by flow cytometry, of DCF as an indicator of the activation of the respiratory burst by stimulated neutrophils and showed that this assay was not influenced by levels of cellular antioxidants such as glutathione, glutathione perioxidase, or catalase.

The calibration of actual amounts of DCF per cell was performed with a slight modification of the method described by Bass et al. (2, 3) in PB-PMNs and in GC-PMNs. In the case of the GC-PMNs the neutrophils were identified by light scatter characteristics and electronically "gated." To ensure that the cells contained in the gate were, in fact neutrophils, they were sorted onto a slide and their morphology was verified after Wright staining. In the experiments described, either PB-PMNs or GC-PMNs (10^6/ml) were incubated with the DCFH-DA fluorochrome (5 μM) for 15 min at 37°C. These loaded cells were then run through the flow cytometer (EPICS C or 753; EPICS Division, Coulter Electronics, Inc., Hialeah, Fla.) using an argon ion laser emitting 300 mW at 488 nm. Green fluorescence was measured by using a 525-nm band pass filter. Parameters collected included forward-angle light scatter, 90° light scatter, and green fluorescence. The PMNs were then stimulated with 100 ng of PMA per ml, and after incubation for 15 and 30 min a portion was removed and analyzed for fluorescence with the flow cytometer. The samples were coded so that the operator of the flow cytometer did not know the source of the GC-PMNs that were being processed.

RESULTS

The small numbers of PB-PMNs and GC-PMNs contained in our samples could be easily counted by the flow cytometer. Figure 1 shows the discriminatory powers of the flow cytometer in separating out neutrophils, epithelial cells, erythrocytes, and debris in the gingival washings. Each of these cell populations could be electronically gated, and therefore responses to various stimuli by discrete PMN populations could be monitored on a per-cell basis. A typical experiment showing the response of GC-PMNs and PB-PMNs to stimulation by PMA is displayed in Fig. 2.

The oxidative response in GC-PMNs and PB-PMNs obtained from a single individual over time is shown in Table 1. The PB-PMNs exhibited values expected on the basis of the literature (11, 13), whereas the values obtained from the GC-PMNs varied with the periodontal status of the sample site. Thus, GC-PMNs obtained from a consistently healthy site had significantly higher resting DCF levels and were able to mount the greatest absolute increase in DCF production after PMA stimulation when compared with the GC-PMNs from the chronically diseased site (bleeding upon sample collection) (Table 1). This variability between sites would be missed if the gingival washings had to be pooled, as is required by the insensitivity of other techniques (5, 14). It is of interest that, despite the differences in absolute terms between the healthy and diseased sites, the relative parameters, such as the ratio of resting to stimulated values and the percentage of increase, were similar for the GC-PMNs and markedly different from the PB-PMN values (Table 1).

Approximately 90% (range, 80 to 95%) of the GC-PMNs obtained from the diseased or healthy sites were viable, so

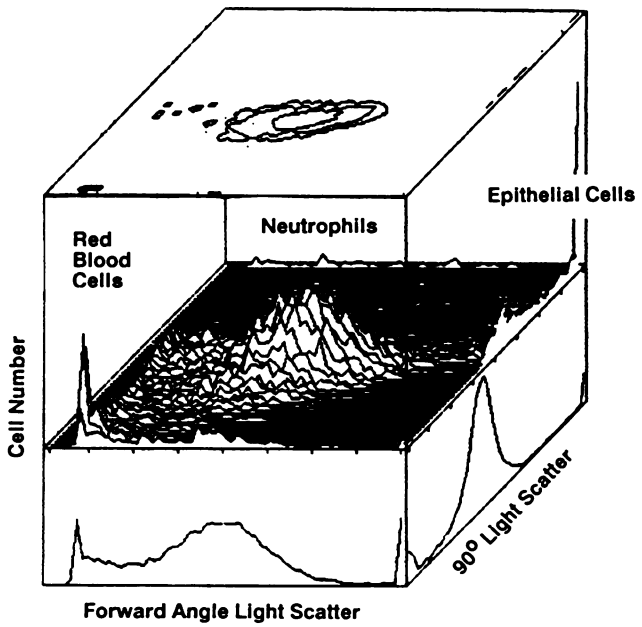

FIG. 1. Three-dimensional cube display of cells present in gingival-crevicular fluid. The central peak represents granulocytes (neutrophils). Epithelial cells are off scale (upper right), and erythrocytes are seen at the lower left corner. Approximately 5,000 cells were examined.

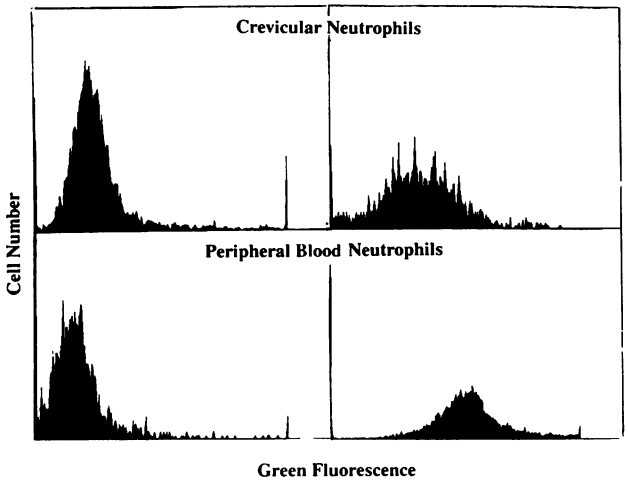

FIG. 2. Composite display of fluorescent histograms of light scatter-gated crevicular-cell suspensions previously loaded with 5 μM DCFH-DA. The histograms were obtained at rest (upper left frame) and after stimulation with PMA (100 ng/ml) and incubation at 37°C for 15 min (upper right frame). The scale of the upper right frame is 1:2. The lower frames display the fluorescent histograms of light scatter-gated blood neutrophils with resting cells in the lower left frame and PMA-stimulated cells in the lower right frame. The equation that describes the correlation between channel number and attomoles of DCF per cell is $y = -4.61 + 1.30\ x$. Results are the mean of 3,000 to 6,000 cells in each experiment.

that cytotoxicity did not seem to account for the functional differences in the GC-PMNs obtained from the two clinical entities. Direct cell counts of the washings indicated that there were from 10 to 30 times more GC-PMNs in washings obtained from diseased sites than in washings obtained from healthy sites. However, we did not rigorously determine the ratio of bacterial cells to GC-PMNs in the washings from either the diseased or the healthy sites.

We then examined the oxidative burst of GC-PMNs collected from patients undergoing periodontal treatment. GC-PMNs obtained from untreated diseased sites were essentially unresponsive to PMA stimulation (Table 2). Thus, the GC-PMNs obtained from 25 such sites in 12 patients showed an absolute increase of only 29 amol of DCF per PMN, whereas the PB-PMNs collected at the same time showed an absolute increase of 107 amol of DCF per PMN. This suggested that the oxidative burst of functionally intact PB-PMNs was either exhausted or altered when these cells were recovered from the gingival washings.

TABLE 2. Effect of periodontal treatment on ability of GC-PMNs to produce H_2O_2

PMN source (no. of sites)	H_2O_2 production (amol of DCF/PMN)		
	Resting	Stimulated	Difference
Periodontally diseased sites			
Untreated (25)	33	62[a]	29
Treated[b]			
Chronic (22)	72[a]	113	41
Healthy (19)	65[a]	125	60[a]
Periodontally healthy sites (3)[c]	99	176	77
Peripheral blood			
Periodontal patients (10)	43	150	107[a]
Healthy subjects (2)	36	197	161

[a] Values indicated are significantly different from all other values in column; Student's t test, $P < 0.05$. Applies only to periodontal patients.
[b] Teeth were scaled and root planed. Three to five weeks later there was (chronic) or was not (healthy) bleeding in these sites upon probing.
[c] Gingivitis index = 0.2.

In these and other patients, tooth surface debridement resulted in GC-PMNs that had significantly higher resting levels of oxidative product than did both PB-PMNs and GC-PMNs obtained from untreated sites (Table 2). However, when these GC-PMNs were stimulated with PMA, the PMNs from the chronic site mounted a feeble response relative to the response made by the PMNs from the successfully treated sites. Both responses were significantly lower than that observed with the PB-PMNs of these patients. For comparison purposes, GC-PMNs were collected from a periodontally healthy individual, i.e., a 32-year-old male with no pockets of >2 mm and a whole-mouth gingivitis score of 0.2 (which is quite low). In this individual, the resting levels of the oxidative product in the GC-PMNs were very high, and these cells were capable of mounting a vigorous response upon stimulation with PMA (Table 2).

We were interested in determining how quickly this inactivation of the oxidative burst of the PMNs could occur in a periodontal pocket. A previous study had indicated that after a crevice or pocket is rinsed, it takes about 60 min for the population of PMNs to return to prerinse levels (14). In one subject, we resampled a periodontally healthy site and a chronically diseased pocket after 60 min and compared the oxidative burst in these newly arrived GC-PMNs with that exhibited by the originally sampled GC-PMNs, whose duration in the pocket was of unknown age. The diseased and healthy sites were within one tooth of each other and would have a very similar source of PB-PMNs.

TABLE 1. Effect of clinical health of a pocket site on H_2O_2 production by GC-PMNs obtained from a single individual on multiple occasions

PMN source (no. of times sampled)	H_2O_2 production (amol of DCF/PMN)[a]			Resting/stimulated	% Increase
	Resting	Stimulated	Difference		
Healthy sites (11)[b]	69[c]	172	103	0.4	149
Chronic sites (9)[d]	44[c]	109[c]	65[e]	0.4	148
Peripheral blood (7)	25[c]	158	133[e]	0.16	532
ANOVA[f]	$P < 0.005$	$P < 0.08$	$P < 0.05$		

[a] For all resting versus stimulated values, $P < 0.005$.
[b] Two sites were sampled on multiple occasions.
[c] Value is significantly different from all other values in column; paired t test, $P < 0.05$.
[d] One site was sampled on multiple occasions.
[e] Values indicated are significantly different from each other; paired t test, $P < 0.05$.
[f] ANOVA, Analysis of variance.

TABLE 3. Effect of clinical status and length of time PMNs were in pocket environment on H_2O_2 production by GC-PMNs

Clinical status (*n*)	Length of time (h) PMNs were in pocket	H_2O_2 production (amol of DCF/PMN)		
		Resting	Stimulated[a]	Difference
Healthy[b] (8)	Unknown	62[c]	154[d]	91
	<1	84[c] $P < 0.005$	177[c] $P = 0.04$	93
Chronic[e] (6)	Unknown	49[f]	106	57
	<1	66[f] $P = 0.05$	142 $P = 0.08$	76

[a] Stimulated by PMA.
[b] Two sites were sampled on four different occasions over a 2-month period in a single individual. Healthy status indicates no bleeding with a probing depth of 3 mm.
[c,d,f] Values indicated with the same letter are significantly different from each other (paired *t* test).
[e] One site was sampled on six different occasions over a 3-month period. Chronic status indicates bleeding with a probing depth of 6 mm.

The GC-PMNs collected from the diseased site at zero time had a lower resting DCF production and exhibited a lower response to PMA than did the GC-PMNs collected from the healthy site at zero time (Table 3). The newly arriving GC-PMNs in both sites exhibited significantly higher resting DCF levels and were able to mount a more vigorous oxidative burst when stimulated with PMA, as compared with the GC-PMNs of unknown age that were collected at zero time (Table 3). The magnitude of the burst per PMN was greater in the PMNs collected from the healthy site.

DISCUSSION

These studies demonstrate that the flow cytometer can be used to count and to measure the functional activity of GC-PMNs obtained from a single gingival or periodontal site. The resolving power of the flow cytometer is such that PMNs can be separated from epithelial cells, erythrocytes, bacterial cells, and other debris that can be found in gingival-crevicular washings.

Large numbers of PMNs were attracted to the sites of periodontal inflammation, and these GC-PMNs were relatively sluggish upon stimulation with PMA. This appears to be a local environmental effect, because the PB-PMNs in these individuals were able to mount a normal oxidative burst when stimulated with PMA, as were GC-PMNs removed from treated sites in which the bacterial load had been reduced (Table 2). This suggests that local bacterial factors, represented either by the nonspecific overgrowth of all bacterial types or by the overgrowth of specific bacterial types, either had exhausted the capacity of the PMNs to mount an oxidative burst or had specifically inhibited this capacity.

Fewer GC-PMNs could be obtained from washings of healthy gingival sites, but these GC-PMNs at the time of sampling had elevated base-line levels of DCF production relative to PMNs isolated at the same time from the peripheral blood. This was emphasized by the kinetic studies in which the newly arriving GC-PMNs were highly active. In this sense it is incorrect to refer to these GC-PMNs as being in a resting state, because in reality they have contacted bacteria and are performing their normal antimicrobial functions, which include phagocytosis and elevated production of DCF.

The newly arriving GC-PMNs obtained from the chronic site, when stimulated by PMA, exhibited a sluggish response per cell as compared with the response observed in the newly arriving PMNs in the periodontally healthy site (Table 3). Even so, this response was more robust than that obtained with GC-PMNs of unknown age from the same diseased site. This suggested that length in residence in the pocket had affected the oxidative metabolism of at least some of the PMNs that were collected at zero time.

Whereas the oxidative burst is the main bacterial mechanism of the PMNs in the host (1, 11, 13), there is the possibility that there is not enough oxygen available in the periodontal pocket to effect this killing mechanism. In this regard, we have measured the pO_2 levels in pockets greater than 4 mm in depth and found 1 to 3% oxygen (12). This is comparable with pO_2 levels found at the gingival margin (7) and, as noted previously, there appeared to be no problem in superoxide production by PMNs obtained from the healthy gingival sulcus (5). If this is so, then the inability of pocket PMNs to exhibit a robust oxidative burst cannot be attributed to the anaerobic nature of the plaque ecosystem.

These data are novel and may be of clinical significance in regard to the pathogenesis of periodontal disease in any given tooth site. In health, the GC-PMNs have an elevated base-line oxidative metabolism relative to PB-PMNs, perhaps as a result of being recently chemotactically attracted to the site and being in the process of actively phagocytizing and killing plaque microbes. These GC-PMNs can mount a normal oxidative burst when stimulated by PMA, indicating that they still possess an excess oxidative capacity.

In diseased sites, the GC-PMNs did not exhibit such a high base-line oxidative metabolism (although their metabolism was still higher than that of the PB-PMNs) and displayed a minimal oxidative burst when stimulated by PMA. Newly arriving GC-PMNs in these diseased sites, however, appeared to be competent in regard to the oxidative burst, so that the incompetency noticed in GC-PMNs of unknown age probably reflected alterations of GC-PMN function that occurred locally in the periodontal pocket. Successful treatment of diseased sites resulted in restoration of competency in the GC-PMNs, again suggesting that the alteration of GC-PMN function is local and most likely related to the bacterial populations found in the subgingival plaques. Whether this is a nonspecific overgrowth which exhausts the GC-PMNs or whether the GC-PMNs are disabled by specific bacterial types remains to be demonstrated. At least one plaque organism, *Treponema denticola*, is capable of inhibiting (blocking) the oxidative burst of PB-PMNs that follows exposure to PMA (unpublished data).

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