

Neutrophil migration, oxidative metabolism and adhesion in early onset periodontitis

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Abstract. The aim of this study was to evaluate neutrophil function in patients suffering from the generalized form of early onset periodontitis (EOP). We investigated neutrophil migration in vivo and neutrophil superoxide production and adhesion in response to a variety of compounds; neutrophils were isolated both from blood and a skin experimental exudate of 15 patients with EOP and of 15 sex- and age-matched normal control subjects. No difference was found in neutrophil migration in vivo ($71.2 \pm 16.4 \times 10^6$ and $68.8 \pm 10.7 \times 10^6$ PMN/cm²/24 h in patients affected by early onset periodontitis and normal subjects respectively) and in adhesion. The superoxide production in response to STZ and PMA was similar between the 2 groups, while superoxide production in response to fMLP was markedly lower in patients than in control subjects both in circulating neutrophils (5.6 ± 2.2 versus 10.4 ± 2.3 nmoles O₂⁻/10⁶ cells, $p < 0.0001$) and in exudate neutrophils (16.3 ± 4.3 versus 22.3 ± 4.7 nmoles O₂⁻/10⁶ cells, $p < 0.005$). In general, neutrophil function in patients suffering from early onset periodontitis does not differ from control subjects, suggesting that the overall defence function of these cells is normal. The only parameter that we have found to be different between the 2 groups is the low superoxide production after fMLP stimulation. The stimulus- and function-specificity of this defect in neutrophils from patients indicates the existence of a dysregulation of the signal transduction pathway distal to fMLP receptor and proximal to NADPH oxidase activation.

Key words: neutrophils; early onset periodontitis; superoxide production; adhesion; chemotaxis

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Polymorphonuclear leukocytes (PMN) are the first cells that migrate into tissues in response to invading pathogens. The accumulation of these cells at sites of inflammation is accompanied by modification of their activity (ability to release granule contents and toxic oxygen derivatives, adhesion capacity, etc.).

Previous investigations of human models, using as stimulant N-formyl-methionyl-leucyl-phenylalanine (fMLP), have shown that exudate PMNs are metabolically primed, being more responsive than blood PMNs (Van Epps & Garcia 1980, Briheim et al. 1988, Biasi et al. 1993a, Biasi et al. 1993b, Paty et al. 1990, Bellavite et al.

1994, Nurcombe et al. 1991). The increased activation of PMNs is relevant for non-specific immunological surveillance against microbial invaders and also because of the potentially harmful rôle played by these cells in pathologic processes such as adult respiratory distress syndrome, postischemic tissue disease, immunocomplex disease, etc. (Nurcombe et al. 1991, Ward et al. 1988, Weiss 1989, Follin et al. 1991). On the other hand, it has been also reported that the respiratory burst triggered by stimulants such as C5a, IL-8 (Follin et al. 1991) and TNF (Biasi et al. 1993a) is decreased in inflammatory PMN, suggesting that priming and desensitisation are factor-specific.

Generalized early onset periodontitis (EOP) is an inflammatory disease with severe periodontal destruction, occurring in the early twenties, teens or before. Recently a classification of periodontal diseases has been proposed by Ranney (Ranney 1993).

Some reports have evaluated PMN functions in patients affected by EOP, but the results are discordant. Some authors reported a defective chemotactic response to fMLP (Suzuki et al. 1984) and to complement-derived C5a (Cianciola et al. 1977, Genco et al. 1986). Contrary to the above mentioned studies, chemotaxis of EOP PMN has been reported to be normal (Kinane et al. 1989, Lariava et al. 1984) or in-

creased (Kinane et al. 1989, Repo et al. 1990). As far as the oxidative metabolism is concerned, an increase in the respiratory burst of EOP PMNs has been observed by two groups (Shapira et al. 1991, Leino et al. 1994). Furthermore, some authors reported increased PMN adhesion (Genco et al. 1980), while others have found this function to be diminished (Kinane et al. 1989, Zafiropoulos et al. 1988, Gutierrez et al. 1991).

Due to the complexity of the events involved, a clear picture of the cellular changes which are associated with exudation might come by evaluating, in the same experimental conditions, the effects of several PMN agonists that are known to interact at different levels with the stimulus-response coupling machinery. In the present study, we have investigated the metabolic responses and the adhesion of PMN which have been isolated from blood and from skin experimental exudate.

Materials and Methods

15 patients (6 male and 9 female, mean age 23.4 ± 5.4 years) and 15 control subjects were considered in this study. With the term of EOP we define a pathology that occurs in adolescents and young adults characterized by an early occurrence of periodontal attachment loss and a rapid progression of the destructive disease (Loe & Morrison, 1986). In addition we define the generalized form of EOP as a disease affecting 4 or more teeth of which at least 2 teeth were 2nd molars, premolars or cuspids (Loe & Brown, 1991). When a tooth showed an attachment loss ≥ 3 mm was considered as affected by periodontitis. According to these definitions the patients inclusion criteria were: a) age under 30 years; b) generalized form of EOP; c) absence of other genetic or acquired diseases. Controls were sex and age matched volunteers with clear medical histories.

During the 3 weeks before the evaluation, no subjects took non steroidal antiinflammatory drugs, corticosteroids or any other drug. All the patients and controls gave informed consent.

Reagents

The chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), zymosan and phorbol myristate acetate (PMA) were purchased from Sigma

Chemical Company, St Louis; cytochrome c from Boehringer, Mannheim, Germany; purified serum bovine albumin and human albumin from Behring Institut, Marburg, Germany. Percoll was from Pharmacia, Uppsala. Sterile 96-well microtiter plates with flat-bottomed wells (Linbro type) were from Flow Laboratories. Hank's balanced salt solution (HBSS) and reagents were of the highest purity available. Zymosan, opsonized with a pool of normal human sera (STZ), as described by Metcalf et al. (24), was stored in aliquots at -20°C . In order to avoid contamination, a possible cause of artifactual activation or priming of the cells, sterile apyrogenic solutions and disposable plasticware were used in all experiments, which were carried out, whenever possible, under a laminar flow hood. Reagents were prepared using pyrogen-free water or 0.9% NaCl solutions.

Cells preparation

Neutrophils were obtained from blood and from skin window exudates. Blood neutrophils were prepared from 40 ml of ethylene diamine tetraacetate-anticoagulated blood by centrifugation over Percoll gradients (Metcalf et al. 1986). Cells were suspended in HBSS containing 5 mM glucose and 0.2% human serum albumin (HGA) and kept at room temperature until use. A few minutes before use, $100\times$ concentrated solutions of CaCl_2 and MgSO_4 were added to the cell suspensions at the final concentration of 0.5 mM and 1 mM, respectively.

Exudate neutrophils ($>90\%$ neutrophils, $>95\%$ trypan-blue negative) were isolated according to the method described by Senn (Senn & Jungi 1975), with modifications (Biasi et al. 1993a). The volar surface of nondominant forearm was disinfected with ether and an abrasion of 1 cm^2 was obtained with a rotating sterile abrasive cylinder operated by a milling cutter (minidrill, Saint Julien en Genevois, France).

The abrasion did not cause bleeding as only the epidermis was removed and the wet, transuding surface of derma was exposed. A bell-shaped, sterile and disposable plastic skin chamber with circular adhesive base (FAR Italia, Verona, Italy) was put on the skin abrasion and fixed with a fenestrated sticking plaster. The chamber has on its top a 5 mm-wide hole equipped with a plug.

1 ml of autologous serum was then injected into the chamber and twenty-four hours later the exudate was collected by aspiration. The exudate cells were then centrifuged at 1200 rpm, washed twice with phosphate buffered saline (PBS) and finally suspended in HGA and kept at room temperature until use. Before use, the cell suspensions were supplemented with CaCl_2 and MgSO_4 as described above for blood cells.

Oxidative metabolism assay

Superoxide anion was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c (Babior et al. 1973) in a microplate assay system. Assays were performed in order to compare cells from blood and from exudate of the same subject and to compare cells from controls and patients with EOP in the same day and using the same microplate in matched replicate wells. The microplate assay for O_2^- production was performed according to previously published methods (Bellavite et al. 1992), with the following modifications. The wells were coated with fetal bovine serum, then were supplemented with $25\ \mu\text{l}$ of 0.6 mM cytochrome c in HGA containing 0.5 mM CaCl_2 and 1 mM MgSO_4 (HGACM) and with $25\ \mu\text{l}$ of the stimulants diluted in HGACM at a concentration exactly $4\times$ higher than that required in the assay. This was done because each agent is diluted $4\times$ in the final incubation mixture (see below). The plate was then brought to 37°C , and $50\ \mu\text{l}$ of the neutrophil suspensions (2×10^5 cells), pre warmed at 37°C , were added to each well using a multichannel pipette and the plates were incubated at 37°C for the desired time. Assays were done in triplicate for each experimental condition. At the time indicated, the plates were rapidly transferred into a microplate reader (Reader 400 SLT Labs Instruments) and the reduction of cytochrome c was measured at 550 nm using 540 nm as reference wavelength, and using 0.037 optical density units as standard for 1 nmole of reduced cytochrome c.

Adhesion assay

For adhesion measurements, the plates were transferred to an automatic washer (Easy Washer 2 SLT Labs Instruments) and subjected to two wash-

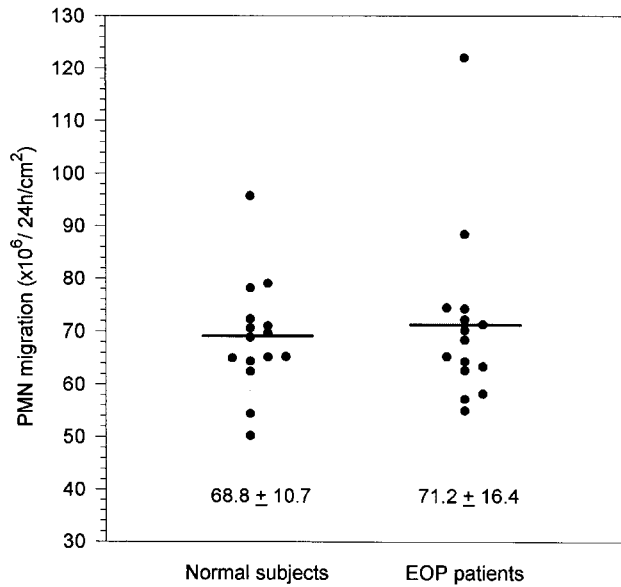


Fig. 1. Values of PMN migration in healthy normal subjects and in patients suffering from early onset periodontitis (EOP).

ing cycles with PBS at room temperature. Adherent cells were quantitated by measuring the membrane enzyme acid phosphatase and the percentage of ad-

hesion was calculated on the basis of a standard curve obtained with known numbers of PMNs (Bellavite et al. 1992).

Table 1. Superoxide production, under basal condition and in response to different stimuli, by PMN from blood (B) and from skin window exudate (SW) in healthy control subjects (n=15) and in patients suffering from periodontitis (n=15); values are nmoles O₂⁻/10⁶ PMN±standard deviation; incubations of resting cells, fMLP stimulated and PMA stimulated cells were carried out for 10 min; incubations of STZ stimulated cells were carried out for 40 min

Stimulant		Controls	Periodontitis
none	B	0.83±0.7	0.6±0.3
	SW	1.2±0.8	0.7±0.4
fMLP 10 ⁻⁷ M	B	10.4±2.3	5.6±2.2*
	SW	22.3±4.7	16.3±4.3 ⁺
STZ 0.1 mg/ml	B	7±1.7	7.1±2
	SW	7.2±1.8	7.5±1.6
PMA 10 ng/ml	B	22.3±6.4	21.2±3.7
	SW	21.9±6.2	19.7±5.1

* p<0.0001, ⁺p<0.005.

Table 2. Adhesion of PMN from blood (B) and from skin window exudate (SW) under basal condition and after different stimuli in control subjects (n=15) and in patients suffering from juvenile periodontitis (n=15); values are % of adherent PMN±standard deviation; incubations in all conditions were carried out for 40 min

Stimulant		Controls	EOP
None	B	10.4±6.3	9.4±3.4
	SW	27.6±12.8	23.5±7.74
fMLP 10 ⁻⁷ M	B	27.5±7.6	24.7±6.2
	SW	38.1±9.1	35.2±5.9
STZ 0.1 mg/ml	B	22.4±5.8	20.4±4.8
	SW	33.7±10.7	32.7±10.1
PMA 10 ng/ml	B	53.5±5.9	52.7±5.5
	SW	53.9±6	53.8±7.4

Statistics

The non-parametric test for unpaired data (Mann-Whitney test) was used for statistical analysis.

Results

Fig. 1 reports the data for the values of in vivo migration. The mean of the migration was 71.2±16.4×10⁶ and 68.8±10.7×10⁶ PMN/cm²/24 h in patients affected by EOP and normal subjects respectively. The difference between the mean migration in the two groups was not statistically significant.

Table 1 reports the data for superoxide production by circulating and migrated PMNs, unstimulated and after challenge with various stimuli (fMLP, STZ, PMA). Exudate cells of both patient and control groups showed an increased superoxide production, in response to fMLP, as compared with blood cells. This priming effect was not observed using STZ or PMA as stimulants. Circulating PMNs from patients showed markedly lower superoxide production after treatment with fMLP than normal subjects and also the exudate cells of patients had lower activity with respect to exudate cells of control subjects. The net increase of fMLP-triggered oxidative metabolism associated with migration into the inflammatory exudate (i.e. the priming effect) was similar in the two groups (10.7 and 11.9 in patients and controls respectively, n.s.). The other values of superoxide production in response to STZ and PMA were not different between the two groups.

Table 2 shows the results of adhesion of circulating and migrating PMNs unstimulated and after exposure to the above mentioned stimuli. Exudation caused a slight increase in the % of adherent (both unstimulated and stimulated) cells. There was no difference between patients and normal subjects.

Discussion

Previous studies on PMN function in patients with EOP have produced conflicting results. Some authors reported a defective chemotactic response (Suzuki et al. 1984, Cianciola et al. 1977, Genco et al. 1986). Others have reported normal (Kinane et al. 1989, Larjava et al. 1984) or increased chemotaxis (Kinane et al. 1989, Repo et al. 1990). It has been hypothesised that reduced chemotaxis is

due to decreased expression of fMLP receptors on the surface of PMN in EOP patients (Van Dyke et al. 1981).

Increase superoxide production by PMN has been reported in individuals with EOP (Shapira et al. 1991, Leino et al. 1994). Some authors observed an increase in the % of adherent PMNs (Genco et al. 1980), others, have found this function to be diminished (Kinane et al. 1989, Zafropoulos et al. 1988, Gutierrez et al. 1991). Similarly, many generalized EOP cases cannot be definitively associated with a specific PMN abnormality at present (Ranney 1993).

All the data concerning PMN functions have been obtained by *in vitro* experiment and, to our knowledge, no studies have been carried out *in vivo*. Moreover no study on oxidative metabolism and on adhesion have been evaluated simultaneously and in the same experimental conditions.

The skin window technique allows us to evaluate PMN migration *in vivo* and to obtain cells which have migrated from an inflammatory focus: they are characterized by the changes physiologically provoked by inflammation. Moreover our method permits the comparison of various functional parameters of circulating and migrating PMNs from the same subject, in a simultaneous assay. *In vivo* migration of PMNs was in the normal range in our patients, indicating that endogenous generation of chemotactic compounds, endothelial and tissue components involved in cell migration and PMN movement are normal.

In accordance with previous investigations from our and other laboratories (Van Epps & Garcia 1980, Briheim et al. 1988, Biassi et al. 1993a, Biassi et al. 1993b, Paty et al. 1990, Bellavite et al. 1994, Nurcombe et al. 1991), these results show that exudate PMNs are metabolically primed, being more responsive to fMLP stimulus than blood PMNs. Moreover, inflammatory cells show increased adherence in the absence of stimulants. This work shows that priming also occurs in patients with EOP. The net difference of oxidative response to fMLP between exudate and blood cells is similar in the two groups examined. This is in agreement with the normal chemotactic migration observed *in vivo* in patients suffering from EOP.

Our results do not perfectly agree with the literature data: in fact, in our

study, PMN migration and adhesion were similar in patients and in controls. PMN migration is a very complex phenomenon, based on both integrity of adhesive PMN function of cytoskeleton dynamics and the action of chemical substances; a skin window method permits the evaluation of this phenomenon in a global way, unlike *in vitro* techniques that study only chemotaxis. It is conceivable that *in vitro* evaluation of chemotaxis, that is carried out under specific conditions, might unmask specific defects that are not relevant for *in vivo* migration.

Our study demonstrated a lower superoxide production in patients affected by EOP than in controls by circulating PMNs after fMLP stimulus; we did not observe any difference by utilizing other stimuli. It is conceivable that some of the discrepancies regarding the activation of oxidative metabolism in PMNs of EOP patients might be due to the different methods of cell isolation. In this study Percoll gradients (one step method) were used, while earlier studies used the Ficoll procedure (two step method) and we have previously noticed that from time to time Ficoll can cause cell activation. Therefore, the PMN defect of patients seems to be both stimulus specific (fMLP versus STZ and PMA) and function-specific (superoxide production versus migration and adhesion). A defect of NADPH oxidase seems to be excluded, because the PMN response after PMA, that directly operates on protein kinase C, and to STZ, that operates through distinct receptors and transduction pathways, is similar in the patients and normal groups.

Some authors have demonstrated a diminished expression of fMLP receptors on the PMN membrane in patients with EOP (Van Dyke et al. 1981). We did not measure fMLP receptors in our study, but our data, showing that fMLP-stimulated adhesion and *in vivo* migration are normal, suggest that if a deficiency of chemotactic receptors is present, this is relevant only for some functions (oxidative metabolism) and not for others (adhesion and migration). According to our data, the stimulus- and function-specificity of blood PMN's defect in patients appears to be due to inhibition of some mechanism located in the signal transduction pathway between the fMLP receptor and NADPH oxidase.

The precise mechanism responsible

for these differences remains to be identified, but an hypothesis can be advanced on the basis of our previous findings using leukocyte activation inhibitors. We have demonstrated (Bellavite et al. 1992) that adenosine, a physiological nucleotide that is known to cause an increase in intracellular levels of cAMP (Cronstein et al. 1983, Cronstein et al. 1990), inhibits superoxide release induced by fMLP but not by PMA; moreover, physiological doses of adenosine (10^{-7} to 10^{-6} M) inhibit fMLP-induced superoxide release but not fMLP-induced adhesion. Finally, we have recently found (Carletto et al. 1997) that pentoxifylline, a cAMP regulating agent, inhibits superoxide production by blood cells much more effectively than superoxide production by exudate cells suggesting that the latter are more resistant to regulation. Taken together, these results strongly suggest that the disease-related defect of blood neutrophils described in this paper is due to impairment of the signal transduction pathways which are controlled by cAMP elevation, involving the activation of protein kinases and possibly the inhibition of phospholipase C (Della Bianca et al. 1986, Bellavite 1988, Iyengar 1996). It is worth noting that a similar stimulus-specific defect of superoxide production is present in PMNs of elderly subjects as compared with young subjects (Biassi et al. 1996).

In conclusion our study shows that PMN function in patients with EOP, including migration, priming, adhesion and activation by opsonized particles do not differ from control subjects, suggesting that the overall defence function of these cells is normal. The only parameter that we have found to be at marked variance between the two groups is low superoxide production after fMLP stimulus of blood cells. The specificity of this defect in PMN from patients could be a marker of either an increase of cellular cAMP or of an increase of sensitivity of cAMP-related cellular responses. Further studies are necessary to investigate the mechanism of the superoxide production defect in EOP.

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Zusammenfassung

Migration der Neutrophilen, Sauerstoffmetabolismus und Adhäsion bei früh einsetzender Parodontitis

Das Ziel dieser Studie war es die Neutrophilenfunktion bei Patienten, die an einer generalisierten Form der früh einsetzenden Parodontitis litten zu evaluieren. Die Migration der Neutrophilen wurde in-vivo untersucht und die Superoxidproduktion sowie Adhäsion der Neutrophilen als Reaktion auf verschiedene Substanzen wurde bestimmt. Die Neutrophilen wurden aus dem Blut gewonnen und ein experimentelles Hautexudat von 15 Patienten mit EOP sowie von 15 alters- und geschlechtsgleichen normalen Kontrollpersonen entnommen. In der in-vivo Migration und der Adhäsion der Neutrophilen wurde kein Unterschied festgestellt ($71.2 \pm 16.4 \times 10^6$ PMN/cm²/24 Stunden bei Patienten die befallen sind mit EOP und $68.8 \pm 10.7 \times 10^6$ entsprechend bei den normalen Kontrollpersonen). Die Superoxidproduktion als Reaktion auf STZ und PMA war bei den beiden Gruppen ähnlich, während die Superoxidproduktion als Reaktion auf fMLP bei den Patienten bedeutend niedriger lag. Dies betraf sowohl die zirkulierenden (5.6 ± 2.2 versus 10.4 ± 2.3 nmol O₂⁻/10⁶ Zellen, $p < 0.0001$) als auch die Neutrophilen aus dem Exudat (16.3 ± 4.3 versus 22.3 ± 4.7 nmol O₂⁻/10⁶ Zellen, $p < 0.005$). Im allgemeinen kann festgestellt werden, daß die Neutrophilenfunktion bei Patienten, die an einer früh einsetzenden Parodontitis leiden, sich nicht von den Kontrollpersonen unterscheidet. Dies läßt annehmen, daß die insgesamt Abwehrfunktion dieser Zellen normal ist. Als einziger Parameter, der sich zwischen den beiden Gruppen unterscheidet, wurde die niedrige Superoxidproduktion nach fMLP-Stimulierung herausgefunden. Die Stimulus- und Funktionsspezifität dieses Defekts der Neutrophilen dieser Patienten zeigt, daß Existenz einer Dysregulation des Signalübertragungsweges nach dem fMLP-Rezeptor und vor der NADPH-Oxidaseaktivierung.

Résumé

Migration de neutrophiles, métabolisme oxydatif et adhésion dans la parodontite précoce

Le but de cette étude a été d'évaluer la fonction neutrophile chez les patients souffrant de la forme généralisée de parodontite précoce (EOP). La migration des neutrophiles in vivo, la production de superoxyde des neutrophiles et l'adhésion en réponse à une variété de composants ont été analysés. Des neutrophiles ont été isolés du sang et d'un exsudat expérimental cutané chez quinze pa-

tients avec parodontite précoce et chez des sujets contrôles normaux du même âge et du même sexe. Aucune différence n'a été trouvée ni dans la migration neutrophile in vivo ($71.2 \pm 16.4 \times 10^6$ et $68.8 \pm 10.7 \times 10^6$ PMN/cm²/24 h chez les patients affectés par EOP et les contrôles) ni dans l'adhésion. La production de superoxyde en réponse à STZ et PMA était semblable dans les 2 groupes, tandis que la production de superoxyde en réponse au fMLP était nettement inférieure chez les patients que chez les sujets contrôles tant dans les neutrophiles circulant (5.6 ± 2.2 versus 10.4 ± 2.3 nmoles O₂⁻/10⁶ cellules, $p < 0.0001$) que dans les neutrophiles de l'exsudat (16.3 ± 4.3 versus 22.3 ± 4.7 nmoles O₂⁻/10⁶ cellules, $p < 0.005$). En général, la fonction neutrophile chez les patients EOP ne diffère pas de celle des sujets contrôles ce qui signifierait que la fonction de défense générale de ces cellules est normale. Le seul paramètre différent trouvé entre les 2 groupes est la plus faible production de superoxyde après stimulation fMLP. La spécificité stimulus et fonction de cette altération dans les neutrophiles des patients indique l'existence d'un mauvais fonctionnement du chemin du signal de transduction en distal du récepteur fMLP et en proximal de l'activation oxydase NADPH.

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