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# Extracellular neutrophil traps in periodontitis

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*Background and Objective:* Chronic periodontitis, the chronic inflammatory disease of the periodontium, is caused by bacteria and is characterized by an influx of neutrophils into the gingival crevice. Recently, a 'new' extracellular neutrophil defense mechanism – neutrophil extracellular traps – has been described. However, their role in periodontitis has not yet been investigated.

*Material and Methods:* Clinical examinations, transmission and scanning electron microscopy, as well as cytology and confocal laser-scanning microscopy, were employed to analyze gingiva biopsies and crevicular exudate from patients with chronic periodontitis.

*Results:* An abundance of neutrophil extracellular traps and some phagocytic neutrophils was found on the gingival pocket surface and in the purulent crevicular exudate. Finding neutrophil extracellular traps in the spontaneously effused purulent crevicular exudate clearly indicated that they are flushed from the pocket by the crevicular exudate. In cases of dispersal of subgingival plaque bacteria, their trapping by neutrophil extracellular traps in purulent crevicular exudate and on the gingival surface was demonstrated.

*Conclusion:* Trapping the crevicular bacteria prevents their adhesion to and invasion of the gingiva. The combination of neutrophil extracellular traps and crevicular exudate outflow appears to be a 'novel' defense mechanism for the clearance of crevicular bacteria in chronic periodontitis.

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Periodontitis is maintained by the microorganisms of the subgingival plaque (the biofilm coating the dental root; 1). As part of the response to bacterial challenge, the gingival tissues produce a serum transudate (2), called the gingival crevicular fluid, which collects around the tooth necks. Even in gingivitis, a marked increase is observed in the amount of gingival crevicular fluid (3). A strong, statistical correlation between the amount of gingival crevicular fluid and the extent of gingival inflammation has been established (3–7). The first important characteristic associated with gingival crevicular fluid flow is its flushing action, which causes substances present in the periodontal pocket to be rapidly washed out (3,8–10). As in other mucosal infections, the host response to the bacteria in chronic periodontitis is characterized by a mucosal efflux of polymorphonuclear neutrophils (11–13), which clinically appears as purulent exudate (13–17). Conceptually, purulent crevicular exudate can be viewed as a neutrophil-rich equivalent of gingival crevicular fluid. Suppuration (purulent exudate) is a frequent feature of periodontal inflammation, especially in cases of periodontitis. Indeed, an old name for periodontitis was pyorrhea alveolaris, which literally means a flowing of pus from the alveolus (13). The influx of polymorphonuclear neutrophils into the crevice is thought to be the first line of defense against plaque bacteria, whereby the function of polymorphonuclear neutrophils is believed to be exclusively phagocytotic (11,12). The interactions between polymorphonuclear neutrophils and crevicular bacteria are strongly affected by the following factors: (i) the bacteria form biofilm, a circumstance that considerably or completely obstructs the phagocytic and killing functions of neutrophils (18–21); (ii) the challenge of pocket epithelium by planktonic

bacteria (22) dispersed from the subgingival plaque, as dispersal is characteristic of biofilms (23–26); and (iii) the fact that polymorphonuclear neutrophil–bacteria interactions take place mainly outside the tissue, namely within the gingival crevice. Thus, defense mechanisms other than phagocytosis, particularly extracellular mechanisms, might play an important role in the periodontal pocket. Recently, a 'new' extracellular defense mechanism – neutrophil extracellular traps – has been described (27). Neutrophil extracellular traps are extracellular web-like fibers generated by activated polymorphonuclear neutrophils and are largely composed of nuclear constituents that disarm and kill bacteria extracellularly. Neutrophil extracellular traps have a DNA backbone, but also contain many bactericidal substances, such as histones, human neutrophil elastase, lysozyme, bactericidal permeability-increasing protein, human peptidoglycan-recognition protein S and other neutrophil proteins (27–29). Neutrophil extracellular traps bind gram-positive as well as gram-negative bacteria, immobilize them and thus prevent the colonization of new host surfaces. Neutrophil extracellular traps, being an extracellular mechanism that traps bacteria, might prevent bacterial dispersal within the crevice and play an important role in crevicular clearance. However, investigations concerning neutrophil extracellular traps in periodontitis have not yet been reported.

Therefore, the aim of this work was to examine whether crevicular neutrophil extracellular traps are involved in periodontitis.

## Material and methods

### Patient selection

Patients older than 35 years of age, with gingival redness and swelling, were selected from consecutively treated dental patients. The exclusion criteria were: necrotizing periodontal diseases; abscesses of the periodontium; periodontitis associated with endodontic lesions; systemic diseases; antibiotic therapy in the last 6 mo; treatment with steroids; and/or treatment with radiotherapy. The study protocol was approved by the local ethics committee, and informed written consent was obtained from each patient.

### Clinical examination

In all consecutively treated dental patients, orthopantomographs, questioning and clinical inspection of gingiva were routinely carried out. After collecting the purulent crevicular exudate, probing and further clinical examinations were performed in order to diagnose the periodontal disease according to the position paper of the American Academy of Periodontology (30).

### Collection of purulent crevicular exudate and supragingival plaque

Only patients with gingival redness and swelling, and with apparent purulent crevicular exudate outflow, were preselected. The teeth with apparent purulent crevicular exudate outflow were isolated using cotton rolls and the saliva was very gently dried with air. In 22 patients (mean age 52 years) at least one sample of nearly 0.5 μL of purulent crevicular exudate was collected up to 10 min after the drying process was carried out. A sample of 0.5 μL of purulent crevicular exudate was the necessary minimum for elaborating a microscopic sample. The purulent crevicular exudate was collected using a tiny loose pad, which consists of a few cotton fibers only, in order to avoid touching the dental root. In patients with sufficient outflow of purulent crevicular exudate, the cotton pad was immersed just minimally in the effused purulent crevicular exudate gathered around the tooth necks. In intervals lasting for up to 10 min, up to three samples were collected. In patients with a purulent crevicular exudate outflow of less than 0.5 μL, a clinical test to detect suppuration in periodontal inflammation (13) was employed to harvest a sufficient quantity of purulent crevicular exudate [i.e. gentle application of digital pressure (in a coronal direction) to the gingival surface]. After collection, the cotton pad containing purulent crevicular exudate was placed onto a cover slide coated with poly-D-lysine, immediately covered up with a second poly-D-lysine-coated cover slide and thereafter allowed to adhere for 5 min. By comparison, filter paper strips were completely inappropriate for transferring and adhering the crevicular exudate components to poly-D-lysine-coated slides. Subsequently, both slides were taken apart and instantly washed with saline. One slide was fixed immediately. The other slide (i.e. the control slide) was treated with 100 μL of 500 U/mL DNase (DNase I recombinant, grade I; Roche Diagnostics GmbH, Vienna, Austria) buffered with 50 mM Tris–HCl, pH 7.5, at 37°C for 30 min, and thereafter also fixed. Twenty-four samples (with the corresponding DNase-treated controls) were fixed with 4% paraformaldehyde (Merck, Hohenbrunn, Germany) in phosphate-buffered saline supplemented with 0.01% sodium azide for confocal laser-scanning microscopy and cytology analyses. Supragingival plaque samples were taken above the gingival margin in eight patients in the same manner; however, the dental roots were intentionally touched with the cotton fibers. The samples were adhered and fixed in the same way. Twelve samples of purulent crevicular exudate were fixed, using Karnovsky's fixative for 2 h at room temperature, for scanning electron microscopy analyses. All fixed samples were stored in the fixative at 4°C for up to 4 d until analysis.

### Biopsies

Twelve patients (mean age 56 years) with chronic periodontitis were selected for gingival biopsies. In all patients teeth extractions were indicated because of the necessity to improve the contour of the alveolar bone by reducing the bone prominences. Radiological examination revealed irregular bone resorption of more than 50% and probing depth exceeding 6 mm. Purulent crevicular exudate was observed in five of the patients during the first examination;

however, no attempt for registering the purulent crevicular exudate was made at the time-point of the biopsy harvest, which took place 1–3 wk later.

Immediately after extraction of the tooth, a piece of pocket epithelium, with dimensions of nearly 6 × 6 mm, was excised using a scalpel and instantly washed twice in 50 mL of saline, by moving it from side to side for 20 s, in order to remove all substances in contact with, but not attached to, the epithelial surface. If the excision took longer than 15 s, the biopsies were discarded because of the possibility of blood coagulation. The biopsies were divided into three pieces along the root axis. The first sample of each biopsy was processed for transmission electron microscopy. The second biopsy sample was incubated in 300 μL of 2000 U/mL of DNase at 37°C for 30 min, subsequently washed with 0.1 M sodium cacodylate buffer (pH 6.5) and then processed for scanning electron microscopy analysis. The third biopsy sample was directly processed for scanning electron microscopy analysis.

### Confocal laser-scanning microscopy analysis

The fixed samples of purulent crevicular exudate were washed with phosphate-buffered saline (pH 7.4) and blocked (0.5% normal goat serum, 0.5% bovine serum albumin and 0.5% Triton X-100 in phosphate-buffered saline). Subsequently, the samples were incubated with the primary antibody anti-human neutrophil elastase (BM382; Acris Antibodies GmbH, Hiddenhausen, Germany), as recommended by the manufacturer. A gentle washing step in phosphate-buffered saline was performed following incubation with each antibody and molecular probe. The primary antibody was detected using a secondary antibody coupled to fluorescein isothiocyanate (R1349F; Acris Antibodies GmbH). Finally, DNA was stained with the molecular probe propidium iodide (Sigma, Schnelldorf, Germany). The specimens were analyzed using a confocal laser-scanning microscope (Zeiss LSM 510 meta UV; Carl Zeiss GmbH, Vienna, Austria).

### Cytology

Twelve samples of purulent crevicular exudate (with the corresponding DNase-treated controls) and the supragingival plaque samples were stained with 0.1% toluidine blue and analyzed using a light microscope (Polyvar; Reichert-Jung, Vienna, Austria).

### Scanning electron microscopy analysis

Postfixation of the samples was performed with 1% osmium tetroxide (buffered at pH 6.5 with 0.1 M sodium cacodylate) for 2 h. The postfixed samples were dehydrated in an ascending series of ethyl alcohol, critical-point-dried and subsequently sputtered with gold (circa 5 nm). The specimens were examined in an environmental scanning electron microscope ESEM XL30 (FEI Company, PHILIPS, Eindhoven, the Netherlands) operating at 20 kV.

### Estimation of the bacterial count in samples of purulent crevicular exudate

The bacterial count was performed in an arbitrary microscopic field of 1000 $\mu m^2$ within each sample during the confocal laser-scanning microscopy or the cytological analyses. The bacterial count was categorized into three levels: (i) low (fewer than 10 bacteria); (ii) moderate (more than 10 but fewer than 100 bacteria); and (iii) high (more than 100 bacteria).

### Transmission electron microscopy analysis

The biopsies were stained using the ruthenium red–osmium tetroxide technique to enable the visualization of neutrophil extracellular traps (31) and bacterial glycocalyx (22,32). Briefly, the samples were fixed with 1.2% glutaraldehyde (buffered at pH 6.5 with 0.1 M sodium cacodylate) with addition of 0.05% ruthenium red for 2 h at room temperature. Postfixation was performed with 1% osmium tetroxide (buffered at pH 6.5 with 0.1 M sodium cacodylate) and 0.05% ruthenium red for 2 h at room temperature. The specimens were routinely embedded in Epon 812. Ultrathin sections were examined using a transmission electron microscope (LEO EM 910; LEO Elektronenmikroskopie Ltd, Oberkochen, Germany).

## Results

### Clinical examinations

Chronic periodontitis was diagnosed in all 22 patients from whom no less than 0.5 μL of purulent crevicular exudate had been collected as follows: a quantity (0.5 μL) of spontaneously effused purulent crevicular exudate, which was sufficient for microscopic analyses, could be collected from eight patients; whereas, in the other 14 patients, gentle digital pressure (in the coronal direction) on the gingival surface was applied in order to harvest a sufficient quantity of purulent crevicular exudate for microscopic analyses.

### Immunostaining, molecular probe, cytological and ultrastructural analyses of purulent crevicular exudate and pocket epithelium

Confocal laser-scanning microscopy, cytological and scanning electron microscopy analyses revealed that all crevicular exudate samples were thoroughly interwoven with neutrophil extracellular traps (Figs 1–3). The neutrophil extracellular traps formed a three-dimensional web, where only neutrophil extracellular traps threads were clearly distinguishable. Abundant polymorphonuclear neutrophils, bacteria and a few desquamated epithelial cells were entangled in the crevicular web of neutrophil extracellular traps, as shown by the 30-min DNase treatment, which resulted in the disintegration of neutrophil extracellular traps and the removal of nearly all bacteria and human cells. The count of entrapped bacteria largely varied (Fig. 4). Neutrophil extracellular traps were heavily loaded with entrapped

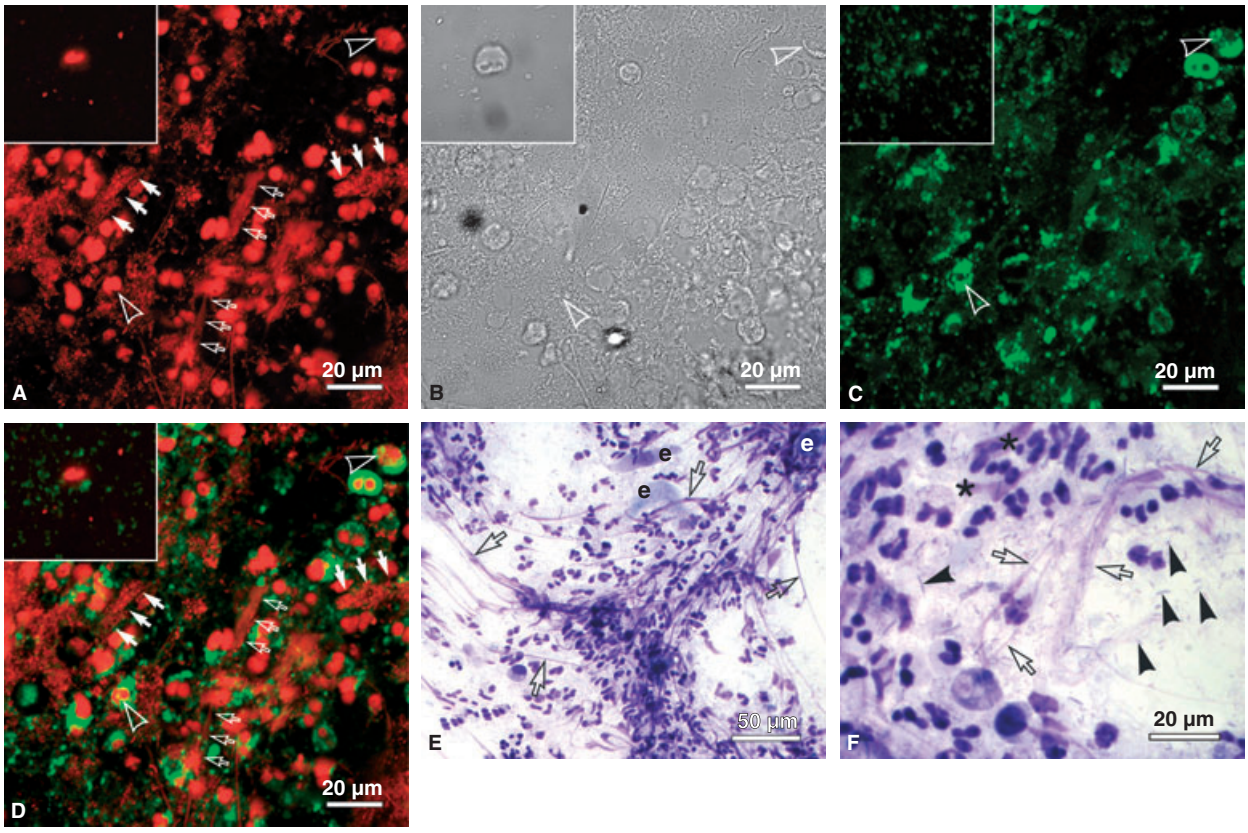


*Fig. 1.* Confocal laser-scanning microscopy optical slices of 1.7 µm. Crevicular exudate samples. Red, propidium iodide; green, human neutrophil elastase. (A) Propidium iodide channel. Propidium iodide-positive neutrophil extracellular traps threads with variable density fill almost the whole space between the nuclei. Solid white arrow triplets: neutrophil extracellular traps heavily loaded with entrapped bacteria. Open arrow triplets: neutrophil extracellular traps moderately loaded with bacteria. Open arrowheads: chromatin spreading. Inset: a DNase-treated sample. A few remnants, probably those of bacterial and neutrophil DNA, are evident. (B) Phase-contrast image. Some cell outlines are apparent. Multitudes of bacteria are distinguishable. Open arrowheads: the outlines of the neutrophil marked with the open arrowhead in (A) are not clearly distinguishable. Inset: only a few cellular elements are evident in the DNase-treated sample. (C) Human neutrophil elastase channel. In contrast to propidium iodide, which stains the neutrophil extracellular traps treads, human neutrophil elastase staining allows the globular domains of neutrophil extracellular traps to be visualized (27). Because the entire crevicular exudate is densely interlaced with neutrophil extracellular traps, the globular domains are superimposed and display the fibrillar appearance only faintly. Open arrowheads: human neutrophil elastase of neutrophils indicated with open arrowheads in (A). Inset: a DNase-treated sample. Some globular domains directly adhere to the poly-D-lysine layer. (D) Merged image of (A) and (C). Solid white arrow triplets: neutrophil extracellular traps heavily loaded with bacteria. Open arrow triplets: neutrophil extracellular traps moderately loaded with bacteria. Open arrowheads: an insignificant colocalization of human neutrophil elastase and spreading chromatin. Inset: a DNase-treated sample. (E) Crevicular exudate samples. Toluidine blue staining. All cellular elements are entrapped by neutrophil extracellular traps. e, desquamated epithelial cells; open arrows, neutrophil extracellular traps threads. (F) Crevicular exudate samples. Toluidine blue staining. Higher magnification. The entire crevicular exudate is interlaced with neutrophil extracellular traps of variable density. Many blurred neutrophil extracellular trap brachiations with entrapped bacteria are evident between the neutrophil extracellular trap threads. Black arrowheads indicate bacteria. Asterisks indicate spreading neutrophil extracellular traps that appear as blurred veils. Open arrows indicate neutrophil extracellular trap threads.

bacteria in nearly one-third of the cases (Figs 1–3). Most bacteria were solitary ones and only a few tiny clusters of a few bacteria were evident. By contrast, the samples of supragingival plaque exclusively showed sharply outlined compact biofilm fragments of at least 100 cells (results not shown). Cytological examinations at a higher magnification revealed that some polymorphonuclear neutrophils retained their phagocytic activity. Altogether, neutrophils were present in four different states in the crevice: (i) polymorphonuclear neutrophils without phagocytosed bacteria; (ii) polymorphonuclear neutrophils with phagocytosed bacteria; (iii) polymorphonuclear neutrophils in the late stage of neutrophil activation; and (iv) neutrophil extracellular traps. Depending on the number of dispersed crevicular bacteria, phagocytic or activated neutrophils prevailed. In cases of bacteria-loaded neutrophil extracellular traps, most polymorphonuclear neutrophils showed signs of being in the late stage of activation, characterized by homogenization of euchromatin and heterochromatin, nuclear swelling and DNA spreading (Fig. 2A). However, colocalization of human neutrophil elastase and DNA was observed only minimally in optical slices of 1.7 µm

and three-dimensionally, respectively (Fig. 2). The 30-min DNase-treatment completely disaggregated propidium iodide-positive webs (Fig. 1A, inset) and caused a distinct fading of the human neutrophil elastase staining, which also lost its web-like appearance (Fig. 1C, inset). Additionally a few cell remnants and bacteria were evident (Fig. 1A, B, D, insets). No differences between neutrophil extracellular traps of spontaneously effused purulent exudate and neutrophil extracellular traps harvested by gently applying digital pressure were observed. Scanning electron microscopy analysis correlated with the confocal laser-scanning microscopy findings. This revealed a very dense network with multitudes of entrapped bacteria (Fig. 3A). In fact, the entire crevicular content was interwoven into a common network of neutrophil extracellular traps (Fig. 3B). The 30-min DNase-treatment completely disaggregated the fibrillar network, resulting in the entrapped cells being washed away; however, myriads of granules with a predominant diameter of 25–50 nm, probably the globular domains of neutrophil extracellular traps, remained, as did a few cell remnants and bacteria directly adhering to the poly-D-lysine layer (Fig. 3A, inset). The neutrophil extracellular trap appearance on the pocket epithelium was very similar (Fig. 3C, D). The 30-min DNase treatment disaggregated neutrophil extracellular traps attached to the epithelium surface. Except for the alveolar margin of gingiva, many transmigrating polymorphonuclear neutrophils were observed within the pocket epithelium using transmission electron microscopy. Phagocytosed bacteria were apparent in polymorphonuclear neutrophils within the epithelium near to and on its surface. Multitudes of solitary bacteria adhered to the epithelial surface. Bacterial internalization and circumscribed biofilm-like bacterial aggregations on the epithelial surface were rarely observed. Spreading neutrophil extracellular traps and polymorphonuclear neutrophils (Fig. 5A), including the phagocytic neutrophils, were observed side by side on the epithelial surface.

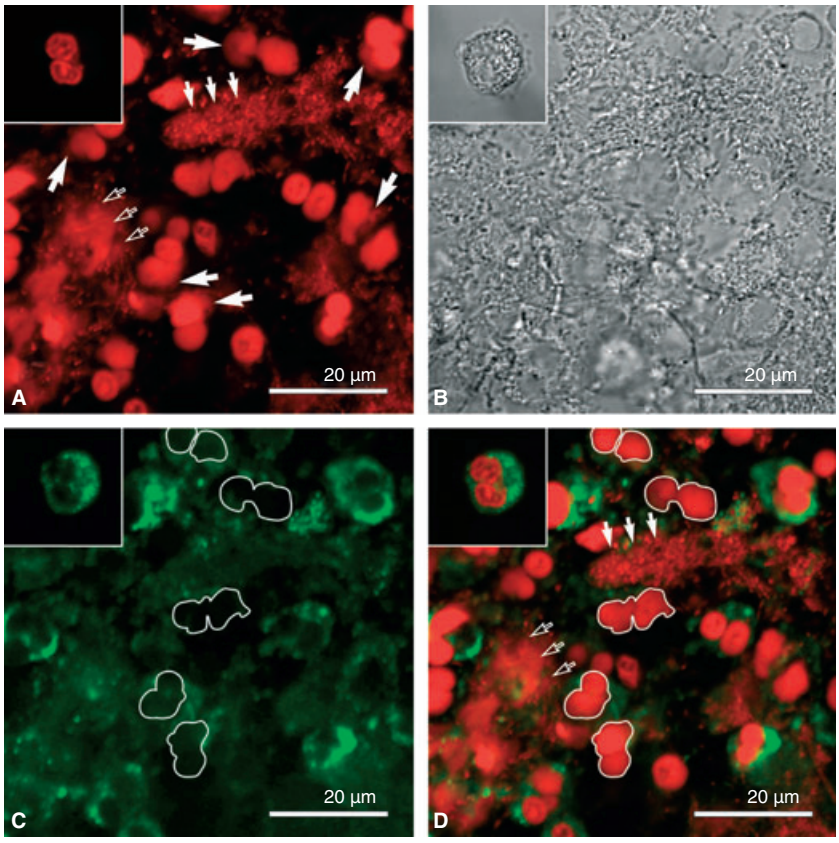


*Fig. 2.* Confocal laser-scanning microscopy optical slices of 1.7 µm. Crevicular exudate samples. Red, propidium iodide; green, human neutrophil elastase. (A) Propidium iodide channel. There is no difference between euchromatin and heterochromatin. Solid white arrows: some neutrophil nuclei are ballooned and begin spreading. The entire crevicular exudate is interlaced with neutrophil extracellular traps of variable density. Solid white arrow triplet: neutrophil extracellular traps heavily loaded with bacteria. Open arrow triplet: neutrophil extracellular traps moderately loaded with bacteria. Inset: an intact neutrophil from the same sample. The difference between euchromatin and heterochromatin is apparent. (B) Phase-contrast image. The cellular outlines are indistinct and cytoplasmic granules are not clearly distinguishable. Inset: the intact neutrophil from (A) shows sharp cellular outlines and distinguishable cytoplasmic granules. (C) Human neutrophil elastase channel. The human neutrophil elastase distribution differs considerably from that observed *in vitro* during the late stage of neutrophil activation, where a distinct colocalization of human neutrophil elastase and chromatin has been reported (38). Lines: outlines of the spreading nuclei from (A). Inset: human neutrophil elastase of the intact neutrophil from (A). (D) Merged image of (A) and (C). Lines: outlines of the spreading nuclei from (A). Solid wite arrow triplets: neutrophil extracellular traps heavily loaded with bacteria. Open arrow triplets: neutrophil extracellular traps moderately loaded with bacteria. Inset: the intact neutrophil from the same sample.

Higher magnifications revealed that the threads of neutrophil extracellular traps are just a superimposition of neutrophil extracellular trap fibers, with the characteristic dimensions, positioned closely abreast (Fig. 5B, C). Similarly to the scanning electron microscopy analysis, multitudes of bacteria entangled in the neutrophil extracellular traps were observed (Fig. 5D). Neutrophil extracellular traps also bound bacteria adhering to the gingiva (Fig. 5E) and this is likely to be the mechanism responsible for the attachment of neutrophil extracellular traps to the epithelial surface. Contacts between neutrophil extracellular traps and the epithelium were

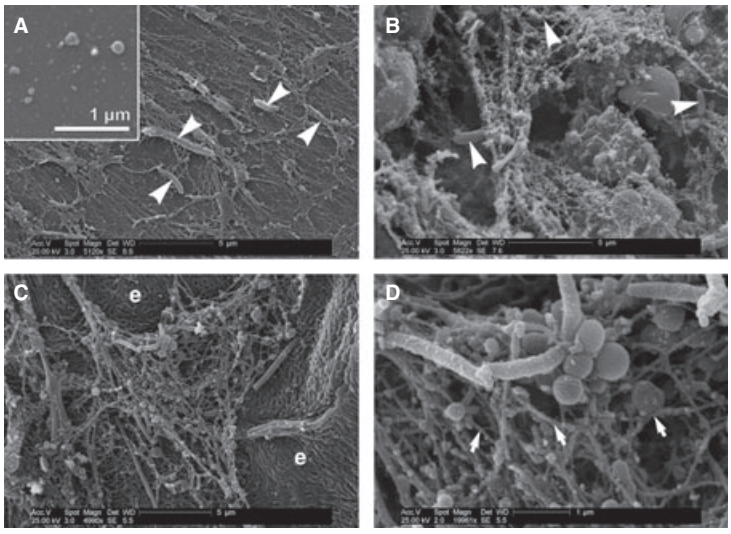


*Fig. 3*. Scanning electron microscopy. (A) Crevicular exudate samples. A dense network covers the entire slide surface. Many bacteria (solid white arrowheads) are entrapped by it. Inset: a DNase-treated sample. Only globular domains are evident. (B) The human and bacterial cells from the purulent crevicular exudate are entrapped by huge, three-dimensional neutrophil extracellular traps. One part of the neutrophil extracellular trap, with the characteristic web-like appearance, lies free on the sample surface. The solid white arrowheads indicate entrapped bacteria. (C) Pocket epithelium biopsies. Part of a neutrophil extracellular trap, with characteristic outlines and entrapped bacteria, lies on the epithelium surface. e, epithelial cell surface. (D) A detail from (C). A multitude of neutrophil extracellular trap fibers (small solid arrows) is evident. The thinnest fibers have a diameter of nearly 20 nm. An exact estimate of the fiber diameter is not possible because of the gold sputtering of the samples. Solitary bacteria entrapped by neutrophil extracellular traps predominated; tiny clusters of bacteria were infrequently observed.

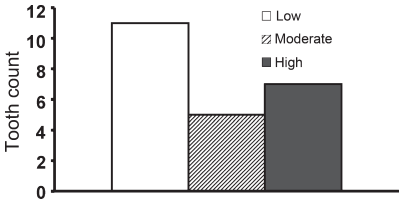


*Fig. 4*. Numbers of bacteria estimated in crevicular exudate samples from arbitrary microscopic fields of 1000 $\mu m^2$: low (fewer than 10 bacteria); moderate (more than 10 but fewer than 100 bacteria); and high (more than 100 bacteria). The large variation in the bacterial count indicates that the drastic increase of bacteria in crevice as a result of subgingival plaque dispersal is an intermittent phenomenon (i.e. the bacteria entrapped by neutrophil extracellular traps are flushed out by the crevicular exudate).

observed very infrequently. The interface of these contacts was too insignificant to assume adhesion between neutrophil extracellular traps and the epithelium. In most biopsy samples, neutrophil extracellular traps appeared to shield the gingiva from the crevicular planktonic bacteria (Fig. 5F).

## Discussion

In contrast to the supragingival plaque, which is repeatedly destroyed by mastication and/or toothbrushing, the biofilm coating the dental root, the so-called subgingival plaque, is situated within the crevice and hence is protected from mechanical disintegration. Consequently, the subgingival plaque might be considered as a persistent infection (33) because biofilms cannot be disintegrated by phagocytosis (18–21). Bacterial dispersal is a common biofilm strategy for the colonization of new surfaces (23–26). However, neither the dispersal of subgingival plaque bacteria, nor the fate of dispersed crevicular bacteria has been investigated until now. The existence of myriads of solitary bacteria in the crevice is difficult to explain by the multiplication of planktonic bacteria there, as a few planktonic bacteria could be (i) flushed out by the crevicular exudate outflow (3), (ii) phagocytosed, or (iii) harmed by complement activation (34) and other blood serum bactericides. By contrast, dispersal from the subgingival plaque can result in the production of a huge number of solitary bacteria in the crevice for a certain period of time. As demonstrated in this study, large quantities of dispersed bacteria in the crevice cannot be controlled by phagocytosis. As a result, there is a higher probability of bacterial adhesion to the pocket epithelium. As gingival crevicular fluid is a very hostile environment for dispersed bacteria, their only chance of remaining in the crevice is to adhere to the gingiva (22,32,35). Bacterial adhesion can trigger colonization of gingiva and even restricted formation of biofilm, but this is time-limited by the epithelial exfoliation (32). Another more promising bacterial fate is internalization of the adherent crevicular bacteria by gingiva (i.e. invasion) (22). However, the dispersed bacteria have to cross the crevice in order to reach the gingival surface and to adhere to it. On their way they encounter and are trapped by the neutrophil extracellular traps spreading from the gingival surface. Spreading crevicular neutrophil extracellular traps shield the epithelium and reduce the bacterial challenge to a large extent. The fact that neutrophil extracellular traps were found in purulent crevicular exudate effused from the periodontal pocket clearly indicates that neutrophil extracellular traps, including the entrapped bacteria, are flushed from the pocket by crevicular exudate outflow. Consequently, killing the entrapped crevicular bacteria may be of inferior significance because they are removed from the crevice by flushing. The immense quantity of entrapped bacteria suggests that the phagocytosis might play, compared with NETosis (36), a subordinate role in the elimination of huge quantities of dispersed crevicular bacteria. The high variation in the bacterial count in samples of purulent crevicular exudate suggests that the dispersal of crevicular

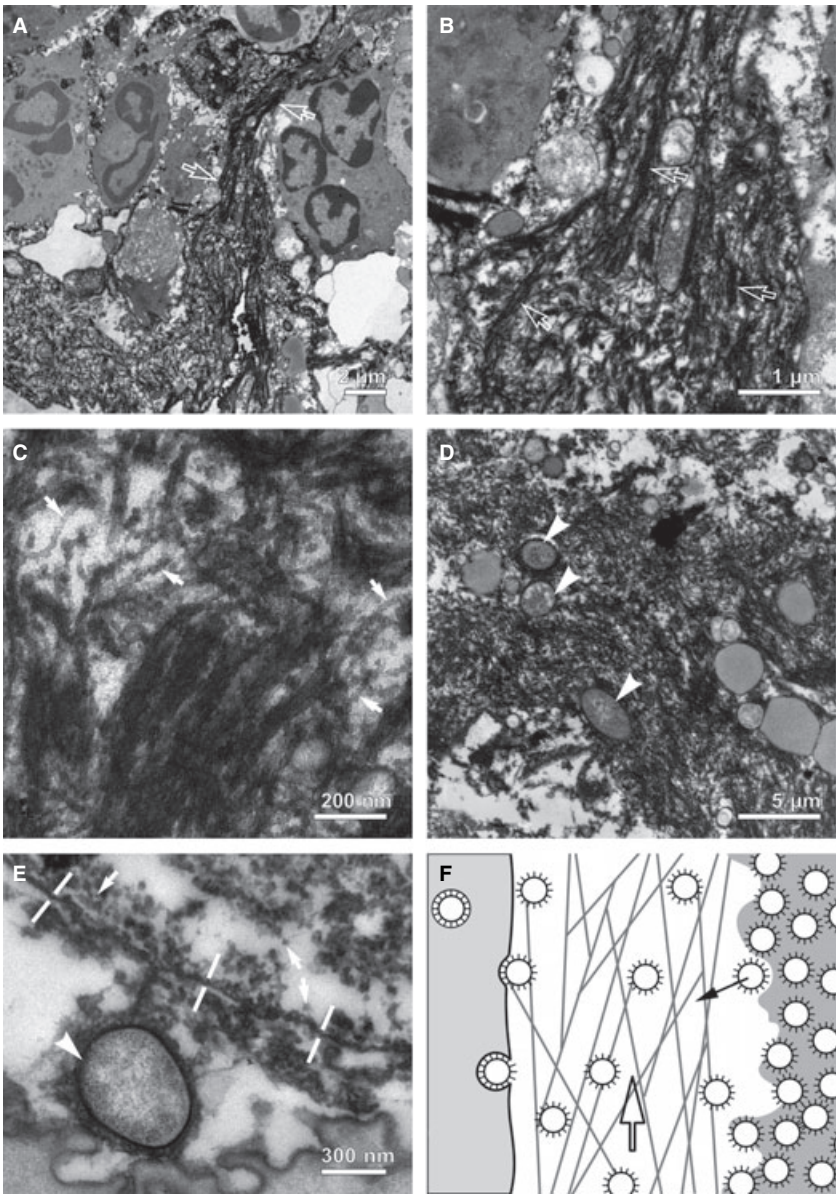


*Fig. 5*. Transmission electron microscopy. Pocket epithelium biopsies. (A) Overview of neutrophil extracellular traps on the pocket epithelium surface. The pronounced blackening is characteristic for neutrophil extracellular traps (31). The open arrows indicate neutrophil extracellular traps threads. (B) A detail of (A). The threads (open arrows) consist of individual fibers that run in parallel. (C) High magnification reveals that neutrophil extracellular traps consist of uniformly thick fibers (small solid arrows) with a diameter of nearly 15–17 nm. (D) Solitary bacteria (indicated by solid white arrowheads) entrapped by crevicular neutrophil extracellular traps. (E) A bacterium (solid white arrowhead) adherent to the epithelium surface is entrapped by neutrophil extracellular traps. A longitudinally running neutrophil extracellular trap fiber is delineated with white bars. Small solid white arrows: a multitude of cross-sectioned neutrophil extracellular trap fibers. (F) A scheme of a crevice. On the left: an epithelial cell with an adherent bacterium and bacteria in different stages of internalization. In the middle: periodontal crevice. Neutrophil extracellular traps build a 'firewall', protecting the gingiva from bacteria dispersing out of the subgingival plaque. The neutrophil extracellular traps, together with the entrapped bacteria, are continuously pushed into the oral cavity by the crevicular exudate outflow. Open white arrow: direction of the crevicular exudate outflow. On the right: subgingival plaque. Solid black arrow: a bacterium dispersing from the subgingival plaque.

bacterial is an intermittent phenomenon, as previously reported on some biofilms *in vitro* (26,37). Thus, the inability of the host to eliminate the biofilm through phagocytosis (18–21) appears to be partially compensated for by neutrophil extracellular traps, which control the dispersed bacteria in the crevice. The occurrence of phagocytic polymorphonuclear neutrophils beside neutrophil extracellular traps indicated that both innate immunity mechanisms complement each other in the crevice.

As demonstrated by transmission electron microscopy, NETosis (36) begins on the pocket epithelium surface. This suggests that the most probable candidates for activating NETosis in the crevice are the bacteria (29,38) and, in particular, lipopolysaccharide (27). The source of lipopolysaccharide in the crevice is the subgingival plaque, but lipopolysaccharide either penetrates gingival tissues only minimally or is rapidly cleared from the gingival tissue after penetration (39). Consequently, the crevicular NETosis may be initiated through the crevicular lipopolysaccharide after the transmigrating polymorphonuclear neutrophils reach the crevice. This may explain why no neutrophil extracellular traps were observed within the pocket epithelium. As neutrophil extracellular traps are also initiated by interleukin-8 (27,38), the crevicular NETosis may be indirectly induced through other activators, which induce interleukin-8 expression in epithelial cells and leukocytes.

Another very important question in periodontology is the possible damage of the periodontium by neutrophil enzymes, especially neutrophil elastase (2,12,40,41). Damage of the periodontal ligament *ex vivo*, by a commercially available soluble human neutrophil elastase, has been recently demonstrated (41). However, we observed epithelial damage only in areas heavily invaded by bacteria and mainly characterized by apoptosis, as previously reported (22). It has been suggested that the apoptosis observed in the pocket epithelium in periodontitis represents part of the epithelial clearance by exfoliation at sites of

chronic bacterial challenge in periodontitis (22,42). The vast majority of superficial gingival cells covered by neutrophil extracellular traps, including those colonized by bacteria, showed no damage. Soluble human neutrophil elastase has been suggested to trigger apoptosis in lung epithelium *in vitro* (43); however, the neutrophil extracellular trap neutrophil elastase is bound to the smooth stretches and globular domains (27). As a result, the neutrophil extracellular trap elastase barely comes into contact with the gingival epithelial cells, considerably attenuating the action of human neutrophil elastase upon the gingival epithelium. However, neutrophil extracellular traps contain multitudes of very potent proteolytic enzymes (27,38), so periodontal damage caused by neutrophil extracellular trap proteases cannot be excluded and requires further examination.

Despite the considerable bactericidal capability of neutrophil extracellular traps, some bacteria are able to resist them through the production of extracellular DNase. Extracellular DNase allows streptococci to escape killing in neutrophil extracellular traps (44,45) and is responsible for enhanced virulence in animal models (44–46). Many oral bacteria produce extracellular DNase; these include the group A streptococci, some *Fusobacterium* spp. (e.g. *Fusobacterium nucleatum*), some *Bacteroides* spp. (e.g. *Bacteroides melaninogenicus* and *Bacteroides oralis*), *Peptostreptococcus intermedius*, as well as *Peptostreptococcus anaerobius* (47–49). However, the continuous dilution and repulsion of the crevicular content by the crevicular exudate outflow may reduce the effect of the bacterial DNase insofar as an abundance of neutrophil extracellular traps was present in all gingival biopsies and samples of purulent crevicular exudate. Consequently, the crevicular exudate outflow might be an effective means for overbearing the bacterial DNase-mediated resistance to neutrophil extracellular traps in the crevice.

The combination of neutrophil extracellular traps and the crevicular exudate outflow appears to be an efficient clearance mechanism in periodontitis.

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