Short communications

DIRECT EXAMINATION OF SUBGINGIVAL PLAQUE FROM A DISEASED PERIODONTAL SITE USING CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

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SUMMARY

Material taken directly from a periodontal site was investigated using immunofluorescence, acridine orange staining and confocal laser scanning microscopy (CLSM). Porphyromonas gingivalis was tracked by a specific polyclonal antibody and its pronounced occurrence in inflamed as compared to non-inflamed areas was demonstrated. Further accompanying microorganisms were counterstained with acridine orange which could provide information on the viability of individual cells. Optical sections by laser microscopy revealed the spatial arrangement of the investigated material. The combination of specific staining and CLSM allows a detailed microbiological investigation of clinical material obtained directly without cultivation.

KEY WORDS: subgingival plaque, periodontal disease, P. gingivalis, CLSM, polyclonal antiserum, immunofluorescence

Direct examination of subgingival plaque using CLSM
Periodontal disease is probably the most common chronic inflammatory disorder in adults and often leads to tooth loss (Williams, 1990). Among the nearly 300 different bacterial species found in subgingival sites, only some have a periodontopathic potential and can initiate disease when a critical population density is reached (Haffajee and Socransky, 1994). More particularly, Porphyromonas gingivalis, a gram negative anaerobic bacterium, is regarded as one of the important pathogens in periodontal disease (Slots and Rams, 1993). This bacterial species has several hydrolytic, toxic and adherence properties which can contribute to its virulence (Mayrand et al., 1991).

A unique approach is usually required for an accurate examination of clinical material from the site of an infection. This might be provided by a microscopy system involving confocal laser scanning, wherein the close relationship between microorganisms and the host would be depicted in the natural state. The confocal laser scanning system employs the xy and xz dimensions of microscopy to obtain a series of consecutive penetrative images. In fact, investigations of invasive potential of bacteria for tissue cells and catheter surfaces through a series of images have suggested a comparative penetration (Goldner et al., 1991, 1993; Gorman et al., 1993). Fluorescent oligonucleotide probes and immunofluorescent techniques (Gersdorf et al., 1993; Gonzalez-Abejas et al., 1995) and specific assessments of physiological or metabolic activity using fluorescent labels (Wells and Johnson, 1994; McFeters et al., 1995) are being used in conjunction with this scanning system (Amann et al., 1995). It seems feasible that these methods can also be applied to studies of subgingival plaque in periodontal disease.

This brief report concerning the oral bacteria present in the vicinity of the gingivae during periodontitis attempts to inspect the close relationship between suspected periodontopathogens and the host by using confocal laser scanning microscopy (CLSM). It signifies that clinical material has been taken directly as a sample for examination without culture so as to distinguish different characteristics with regard to the natural state of both bacteria and host cells.

Clinical material used in the present study was obtained from regular patients attending the dental clinic at the Université Laval (Quebec, Canada). The selected periodontitis patients had not received chemotherapy or periodontal therapy during a period of six months prior to sampling. Subgingival plaque was collected with a sterile curette from the most apical part of the site. One sample was obtained from a periodontal site with a probing depth of more than six mm, and which exhibited gingival inflammation and bled on probing. A second sample was obtained from a site with no clinical signs of periodontitis (probing depth less than three mm). Samples were immediately placed in 0.5 ml reduced transport fluid (Syed and Loesche, 1972), equilibrated for 2 h under an anaerobic atmosphere (N: H: CO2: 80: 10: 10) and kept at 4°C for observation.

For purpose of fluorescence and immunofluorescence staining (Schloetter et al., 1993), 1.5 ml reaction tubes were blocked at 37°C for 30 min with 3% bovine serum albumin in water (BSA, Boehringer, Ingelheim, Germany). The clinical sample (200 µl) was transferred to an unblocked reaction tube and centrifuged in a small table centrifuge (5000 x g for 5 min). The supernatant was discarded, the pellet was washed with 200 µl phosphate buffered saline (PBS: g/l: NaCl 8; K-HPO4 1.21; KH2PO4 0.34; pH 7.3) and resuspended in 200 µl wash solution (WS: 10% PBS, 0.5% BSA, 0.002% Tween 20). The suspension was transferred to the BSA-blocked reaction tube. For labelling with specific antibody, 20 µl of the primary antibody (anti-Porphyromonas gingivalis ATCC 33277 polyclonal antiserum raised by injection of whole cells in rabbits; 1:500 dilution) were added to the clinical sample resuspended in WS. The suspension was briefly vortexed to distribute the antiserum evenly and incubated at 37°C for 30 min. This material was then centrifuged (5000 x g for 3 min) and resuspended in 200 µl WS. The secondary antibody (5 µl; anti-rabbit IgG, 1:300 dilution, biotinylated species specific whole antibody; Amersham,
Braunschweig, Germany) was added, and the suspension was again vortexed and incubated for 45 min at 37°C, then centrifuged (5000 x g for 3 min) and resuspended in 200 µl WS. Streptavidin-texas red conjugated complex (10 µl: 1:100 dilution, Amersham) was added, and the suspension was again vortexed and incubated for 20 min at room temperature in the dark. Finally, the suspension was washed twice with 200 µl WS to eliminate background fluorescence, and then resuspended in 200 µl WS.

A drop of the suspension prepared for examination by fluorescence, whether labelled with antiserum to P. gingivalis or not, was transferred to a microscope slide coated with 2% agarose to immobilize bacterial cells. To counterstain total cells, acridine orange (Boehringer, Ingelheim, Germany) solution (7 mg in 50 ml PBS) was added to the slide for 1 min; afterwards the area was rinsed to remove excess stain. The slide was covered with a coverslip and maintained slightly moist with one or two drops of PBS, then observed under a confocal laser scanning microscope (LSM 410, Carl Zeiss, Jena, Germany) using dual laser excitation (488 and 543 nm) and a 100 x oil immersion lens (NA 1.3).

The periodontal material examined by fluorescence staining and by fluorescent antibody labelling is illustrated in Figures 1 and 2. The first figure depicts the clinical sample, obtained from a diseased periodontal site and stained with acridine orange (excitation wave length 488 nm; yellowish green indicative of viable cells, orangish red of non-viable cells). Figure 1a presents a projection of z-series (depth 7 µm). A variety of morphological types (rods, filaments, cocci) and associations of different bacteria and host cells (influxed) are observed in this sample. Some bacteria seem to be closely associated with host cells (inflammatory cells, fibroblasts) and even within these cells. The periodontal sample is thus a mixture of different bacterial types, viable and non-viable, and clearly indicative of ample potential for activity. Figure 1b shows a sagittal section (xz-scan, depth 55 µm) of the same periodontal sample, which reveals further details of the bacterial and host cell arrangement within the sample. The presence of small numbers of bacteria, both viable (green/yellowish-green fluorescence) and non-viable (red/orangish-red fluorescence) may be discerned in the vicinity of the host cells. Interestingly, recent reports have indicated the ability of some oral pathogens including P. gingivalis and Actinobacillus actinomycetemcomitans, to invade gingival epithelial cells (Sreenivasan et al., 1993; Lamont et al., 1995; Madianos et al., 1996). It was suggested that this may represent an important pathogenic mechanism in periodontal disease.

The second figure presents a z-series projection of a clinical sample (depth 10 µm), labelled with P. gingivalis fluorescent antibody...
conjugated with Texas red (excitation wave length 543 nm), followed by staining with acridine orange (excitation wave length 488 nm). While the material seen in Figure 2a is sampled from a diseased periodontal site, that for Figure 2b is taken from a so-called clinically normal (non-inflamed) area. The difference in the concentration of bacteria largely reflecting the labelling with *P. gingivalis* antibody (red fluorescence, masking the acridine orange label), between Figure 2a and b, is striking. Certainly, a considerable population of both bacteria and influxed host cells, the latter also noted in Figure 1, are present during periodontitis. Thus, the widely different views, depicted in Figure 2a and b, reveal that an infection may be in active process.

A way for observing the bacteria in relatively undisturbed clinical material, when interacting with host cells from the site of an infection, should be a definite asset. The prominence of certain types of organisms in the natural state in relation to disease may then be properly assessed. Further information on the investigated samples can be obtained by combining the immunological staining with in situ hybridization using fluorescent labelled oligonucleotide probes (Amann et al., 1995). In preliminary tests, using rRNA-targeted probes for major subgroups of gram-negative bacteria (Manz et al., 1992), we were able to discern the diversity of the bacterial flora of the clinical sample. Future use of confocal laser scanning microscopy should be expected to disclose the proximity to, involvement with and possible penetration of host tissue cells by periodontopathic bacteria following from this initial effort. Using specific antibodies, this technique may also allow examination of the behavior of selected periodontopathogens present in the complex microbial community found in the periodontal pockets.

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**REFERENCES**


