Identification of *Bacteroides* species from adult periodontal disease

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Summary. Samples from deep (4–7 mm) periodontal pockets were collected from 17 patients with adult-type periodontal disease and one with the juvenile form of the disease. They were streaked immediately on selective and non-selective media and incubated anaerobically for 96 h. There was a heavy growth of *Bacteroides* spp. from most samples and 10 representative colonies from each sample were sub-cultured for identification. In a total of 149 isolates from patients with adult-type disease, the commonest species were *B. oralis* (40), *B. asaccharolyticus* (35), *B. intermedius* (31), *B. fragilis* (12) and *B. ureolyticus* (10); *B. gingivalis* was not detected. The distribution of species was not distorted by multiple identical isolates from individual patients. There was a heavy growth of a single species, *B. ureolyticus*, from the patient with juvenile-type disease.

Introduction

Periodontal disease, a term that encompasses a range of inflammatory and destructive conditions of the tissues surrounding the teeth, is initiated by the overgrowth of bacteria in the gingival crevice (Socransky, 1977; Socransky *et al.*, 1982) causing tissue damage by a combination of direct action of toxic bacterial products that include endotoxins (Hausmann *et al.*, 1982) and hydrolytic enzymes such as collagenase and other proteolytic enzymes (Gibbons and MacDonald, 1961; Hardie, 1974), and stimulation of destructive host immunological responses (Genco, 1979). However, the role of particular bacterial species remains obscure, partly because of the complexity of the mixed microbial flora in the gingival crevice in health and disease (Moore *et al.*, 1982; Socransky *et al.*, 1982), and partly because there are several distinct forms of periodontal disease that affect patients in different age groups, progress in different ways, and probably have different microbial aetiologies (Schluger *et al.*, 1977; Socransky *et al.*, 1982).

The involvement of anaerobes has been clear since the early descriptions of the microflora of acute gingivitis by Plaut (1894) and Vincent (1896). Much interest has centred upon the roles of spirochaetes, fusobacteria and *Bacteroides* spp. of the melaninogenicus-oralis group. The spirochaetes

are associated particularly with acute necrotising ulcerative gingivitis (Loesche, 1976; Loesche and Laughon, 1982) whereas the fusobacteria and bacteroides may be more important in other forms of periodontal disease. Bacteroides and fusobacteria are constant members of the normal microbial flora of the healthy gingival crevice (Slots, 1977) where the commonest species identified are members of the non-pigmented *B. oralis* complex, the pigmented *B. melaninogenicus* and *B. intermedius*, and *Fusobacterium nucleatum* (Duerden, 1980a). There have been several reports that the pigmented *Bacteroides* spp. are more common in periodontal disease (Slots, 1982) and improved identification methods have shown that various species of pigmented *Bacteroides* are found in different forms of the disease. *B. intermedius* is commonly present in minimally inflamed advanced disease of long standing and in acute necrotising ulcerative gingivitis (Slots, 1982). *B. gingivalis*, an asaccharolytic species distinguished from *B. asaccharolyticus* in recent years (Coykendall *et al.*, 1980; Kaczmarek and Coykendall, 1980), is found principally in advanced and inflamed severe periodontitis, with major tissue and alveolar bone destruction, and in generalised juvenile periodontal disease (Loesche *et al.*, 1981). *B. melaninogenicus*, however, is common in people with healthy or diseased gingivae and has no specific association with periodontal disease (Slots, 1977 and 1982; Duerden, 1980a). The pigmented bacteroides secrete various toxic

products that can cause tissue damage (Gibbons and MacDonald, 1961; Socransky, 1970; Mayrand *et al.*, 1980). They also provoke potentially destructive host responses (Nisengard, 1977).

Anaerobes are not the only group of bacteria implicated in the pathogenesis of periodontal disease. *Actinobacillus (Haemophilus) actinomycetemcomitans* and *Capnocytophaga ochracea* may also play a part, especially in juvenile periodontal disease (Mandell and Socransky, 1981; Hammond and Stevens, 1982). However, the clinical and pathological features of the disease are characteristic of an anaerobe infection.

For the present investigation, methods developed in our laboratory for the isolation and identification of bacteroides and fusobacteria from the normal microbial flora of man and from clinical infections (Duerden, 1980*a* and *b*; Duerden *et al.*, 1976 and 1980; Rotimi *et al.*, 1980) were used to examine the anaerobic bacterial flora of deep gingival pockets in patients with periodontal disease, principally of the adult type, attending the Charles Clifford Dental Hospital, Sheffield.

Materials and methods

Patients

Samples were collected from 17 patients with adult-type periodontal disease and from one 17-year-old patient with the juvenile form of disease. The bacteriological studies were done only at the patient's first visit to the Department of Restorative Dentistry, Charles Clifford Dental Hospital, and patients were not accepted if they had received specific treatment for their periodontal disease, including antibiotics, during the preceding month.

Sampling

Superficial plaque and debris were removed from the area considered clinically to be the most severely affected and material from a deep periodontal pocket (4–7 mm in depth) was collected aseptically with a metal curette and seeded directly on to three plates of media for primary culture.

Primary cultures

The material from the periodontal pockets was plated immediately, in the clinic, on blood agar, BM agar (Williams *et al.*, 1975) as used by Holbrook *et al.* (1978) and BM agar made selective for *Bacteroides* spp. by the incorporation of kanamycin 75 mg/L and vancomycin 2.5 mg/L. All plates had been pre-reduced overnight in

an anaerobic jar with an atmosphere of H₂90%, CO₂10% at room temperature. The inoculum material was spread over a sector of the plate and streaked out in a standard manner to give a semi-quantitative assessment of growth as described by Rotimi and Duerden (1981). The inoculated plates were returned immediately to the Department of Medical Microbiology and placed in an anaerobic cabinet (Mark I Cabinet; Don Whitley Scientific, Shipley, W. Yorks) for incubation at 37°C in an atmosphere of H₂10%, N₂80%, CO₂10%. They were inspected after 48 h and up to five representative colonies of gram-negative anaerobic bacilli were selected and sub-cultured on to fresh BM agar. The primary plates were re-incubated for a further 48–72 h and another five colonies were then selected to give a total of 10 from each subject. Examples of each colony type were selected and the number made up to 10 with colonies selected in approximate proportions to their comparative numbers on the primary isolation plates. The density of growth of each colony-type was recorded on a scale from + (isolated colonies in the inoculum well only) to +++ (many colonies extending into the second and third sets of streaks). The selected isolates were checked for purity on BM agar incubated anaerobically and on blood agar incubated aerobically and then suspended in a freezing medium of Nutrient Broth No. 2 (Oxoid) with inactivated horse serum (Wellcome) 10% and glucose 1% and held at –70°C in a liquid nitrogen container until identification tests could be done.

Identification of isolates

The selected isolates were identified by a combined set of biochemical, tolerance, and antibiotic disk resistance tests (Duerden *et al.*, 1976 and 1980; Rotimi *et al.*, 1980) supported where appropriate by gas-liquid chromatography (GLC) of the fatty-acid end-products of metabolism. To distinguish between *B. asaccharolyticus* and *B. gingivalis*, all pigmented asaccharolytic strains were examined for: (i) fluorescence of colonies under ultraviolet illumination (Coykendall *et al.*, 1980); (ii) agglutination of sheep erythrocytes (Slots and Genco, 1979); and (iii) production of phenylacetic acid, as determined by GLC analysis of non-volatile fatty-acid products (Kaczmarek and Coykendall, 1980).

Results

Patients

Of the 18 patients studied, 17 had adult-type periodontal disease; these had a mean age of 40 years (range 23–63 years) and there were 6 male and 11 female patients. The youngest of the 17, a 23-year-old male had rapidly destructive periodontal disease. The other patient was a 17-year-old male with the juvenile form of periodontal disease.

Primary cultures

There was a heavy growth of gram-negative anaerobic bacilli from 16 samples, a light growth from one, and only four colonies were isolated from another. The latter specimen was heavily blood-stained and the atypical pattern of growth probably represented a problem of sampling. Of the 174 isolates selected for identification, four proved to be facultative CO₂-dependent strains and 11 failed to grow on repeated subculture; 159 isolates from the 18 patients were subjected to the identification procedure.

Identification of isolates

The primary cultures of the sample from the patient with juvenile periodontal disease were clearly different from all the others. There was a heavy growth of gram-negative anaerobes, but all were the same species, *B. ureolyticus*. The isolates were characteristic of the species—fine gram-negative bacilli that were strictly anaerobic, sensitive to metronidazole, formed spreading colonies that produced pitting or corroding of the agar surface, did not produce acid from carbohydrates and were urease-positive.

The 10 identical isolates from the one patient were removed from further analysis. One hundred and forty-nine isolates from the other 17 patients were identified (table I). The commonest species were *B. oralis* (40), *B. asaccharolyticus* (35) and *B. intermedius* (31). *B. fragilis* and *B. ureolyticus*

provided 12 and 10 isolates respectively but there were only single-figure isolations of other members of the melaninogenicus-oralis group, and of *Leptotrichia buccalis*. *Fusobacterium* spp. were not detected, although two isolates from a repeat specimen from one of the patients were identified as *F. nucleatum*. Despite a detailed examination, none of the pigmented asaccharolytic strains proved to be *B. gingivalis*.

The distribution of species was not distorted by multiple identical isolates from only one or two patients. Several species were isolated from each patient and the order of frequency of the species remained more or less the same when the number of patients from whom each species was isolated were counted (table II). *B. oralis* was isolated from 11 patients, *B. asaccharolyticus* from nine, *B. intermedius* and *B. ureolyticus* from eight and *B. fragilis* from seven.

Density of growth

Most species gave a ++ or +++ growth on primary culture from about half the specimens from which they were isolated. The exceptions were *B. fragilis*, *B. intermedius* and *B. oralis* which, when present, usually provided a heavy growth (table II).

Discussion

With the exception of the one patient with juvenile periodontal disease and one with rapidly progressive disease, the patients examined in this

Table I. Identities of 149 isolates of gram-negative anaerobic bacilli from 17 patients with adult-type periodontal disease

Species	Number of isolates*
Melaninogenicus-oralis group	
<i>B. oralis</i>	40
<i>B. intermedius</i>	31
<i>B. melaninogenicus</i>	7
<i>B. bivius</i>	4
<i>B. disiens</i>	2
Asaccharolytic group	
<i>B. asaccharolyticus</i>	35
<i>B. ureolyticus</i>	10
<i>B. levi</i>	2
Fragilis group	
<i>B. fragilis</i>	12
Fusiform group	
<i>Leptotrichia buccalis</i>	7

* Up to 10 isolates were selected from each patient for identification; see *Methods*.

Table II. Frequency of isolation and density of growth of gram-negative anaerobic bacilli from 17 patients with adult-type periodontal disease

Species	Number of patients with given species	
	Total	Heavy growth
Melaninogenicus-oralis group		
<i>B. oralis</i>	11	7
<i>B. intermedius</i>	8	6
<i>B. melaninogenicus</i>	4	1
<i>B. bivius</i>	3	0
<i>B. disiens</i>	1	0
Asaccharolytic group		
<i>B. asaccharolyticus</i>	9	4
<i>B. ureolyticus</i>	8	5
<i>B. levi</i>	2	1
Fragilis group		
<i>B. fragilis</i>	7	5
Fusiform group		
<i>Leptotrichia buccalis</i>	5	3

study were a clinically homogeneous group with adult-type periodontal disease. They were also bacteriologically similar. A heavy growth of gram-negative anaerobic bacilli was obtained from most patients and the general pattern of isolates was similar in all except the specimen from the patient with juvenile disease.

The species of *Bacteroides* isolated were a mixture of those usually present in the normal gingival flora (*B. oralis*, *B. intermedius*) and some that are not but are associated with necrotic and ulcerative lesions elsewhere in the body (*B. asaccharolyticus*, *B. ureolyticus*, *B. fragilis*) (Finegold, 1977; Duerden, 1984). Most interest in periodontal disease has centred upon the role of pigmented species (Slots, 1982) and the contribution of *B. oralis* is difficult to ascertain. Although a normal member of the gingival flora, it is not a harmless commensal at other sites in the head, neck and respiratory tract. It can be associated with soft-tissue abscesses of the head and neck, chronic otitis media and sinusitis, brain abscesses and lung abscesses (Finegold, 1977; Ingham *et al.*, 1977; Duerden, 1984) and in these conditions it clearly has the capacity, particularly in mixed infections, for causing considerable tissue damage. However, recent studies have indicated that "*B. oralis*" represents a group of non-pigmented species with distinct properties (Shah *et al.*, 1980; van Steenberg *et al.*, 1980) and further identification of the strains within this largest group of isolates may show specific associations with the disease state.

Identification of the pigmented isolates from our patients confirms that different species are associated with different groups of patients. *B. intermedius*, present only in small numbers in the normal gingival flora (Duerden, 1980a), was common in our patients. This distribution is similar to that described by Slots (1982). However, the species that has engendered most interest in recent years, *B. gingivalis* (Coykendall *et al.*, 1980; Kaczmarek and Coykendall, 1980), was not detected. This is probably because it is principally associated with a different group of patients—those with generalised juvenile periodontal disease or with advanced, destructive periodontitis (Loesche *et al.*, 1981). However, another pigmented member of the asaccharolytic groups of *Bacteroides*, *B. asaccharolyti-*

cus, was the second most common species isolated from our patients. This species is recognised as a significant pathogen in necrotic ulcers and abscesses. It is the second commonest species of *Bacteroides* isolated from clinical specimens (Duerden, 1980b) and is associated particularly with perianal and perineal abscesses and ulcers and genital ulcers (Masfari *et al.*, 1983), varicose and decubitus ulcers, and peripheral gangrene (Rissing *et al.*, 1974). It has considerable destructive potential but since the differentiation of *B. gingivalis* its contribution to periodontal disease has not been considered important. Its role in adult-type disease requires further investigation.

A non-pigmented asaccharolytic species, *B. ureolyticus*, was also found in significant numbers, but less commonly than *B. asaccharolyticus*, in the patients with adult-type disease. The most remarkable finding was the heavy "pure" growth (in terms of *Bacteroides* spp.) of this organism from the patient with juvenile periodontal disease. As with *B. asaccharolyticus*, this species is associated with destructive, ulcerative conditions and abscesses elsewhere in the body, particularly perineal and genital ulcers (Duerden *et al.*, 1982; Masfari *et al.*, 1985); it has also been implicated as a cause of non-specific urethritis (Fontaine *et al.*, 1984). It would appear to have significant pathogenic capacity. Both *B. asaccharolyticus* and *B. ureolyticus* are strongly proteolytic and produce a range of enzymes capable of breaking down tissue components.

Although *Bacteroides* spp. are present in the gingival pockets in large numbers, the evidence for their pathogenicity remains circumstantial. They may be specific pathogens or merely indicators of a pathological process in the gingival crevice. The finding of species that are not part of the normal gingival flora and that have established pathogenicity at other sites supports the hypothesis that they are important in the pathogenesis of the disease. Further detailed, longitudinal studies, by quantitative methods, of the gingival flora in patients with periodontal disease are needed to answer these questions.

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