HISTOPATHOLOGICAL CHANGES IN THE HIND FOOT OF THE MOUSE INDUCED BY BLACK-PIGMENTED *BACTEROIDES* STRAINS

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SUMMARY. The pathogenic potential of black-pigmented *Bacteroides* strains was studied in an animal model in which the effect on bone tissue could be determined. Bacteria suspended in agar were injected subcutaneously in the left hind paw of a mouse. After 3–5 days, *B. gingivalis* strain HG 66 had caused a massive infiltration with polymorphonuclear cells, destruction of the periosteum on the metatarsals and bone resorption by osteoclasts. After 7 days only a few osteoclasts remained and reactive bone formation was observed. In a comparative study with strains of several black-pigmented *Bacteroides* species, differences in bone resorbing potential were seen. *B. gingivalis* strains caused severe inflammation which resulted in bone resorption. Strains of *B. asaccharolyticus*, *B. endodontalis*, *B. intermedius*, *B. melaninogenicus* and *B. loeschei* caused less inflammation and less bone resorption. Killed bacteria or agar alone caused a relatively mild inflammation and no bone resorption.

INTRODUCTION

The advanced lesion in periodontitis is characterised by inflammation, epithelial proliferation, detachment of periodontal tissue, bone resorption and pocket formation (Page and Schroeder, 1981). The causes of osteoclastic bone resorption are not known. Resorption usually occurs in the proximity of inflammatory infiltrates but a close correlation between osteoclastic bone resorption and inflammatory-cell types has not yet been made. It is generally accepted that gram-negative anaerobic bacteria play an important role in the pathological process of periodontal diseases. Black-pigmented *Bacteroides* species, especially *B. gingivalis* and *B. intermedius*, are often isolated from deep pockets in adult periodontitis (Loesche *et al.*, 1981; Slots, 1982). To assess the virulence of black-pigmented *Bacteroides* strains we have used a mouse model (Kastelein *et al.*, 1981; van Steenberg *et al.*, 1982) in which single strains were injected subcutaneously and examined for their ability to cause inflammatory

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reactions. Because bone loss occurs in periodontal disease, we have now developed an animal model in which the effect of bacteria on bony tissues can be studied. Bacteria mixed with agar are injected into the hind foot of a mouse and the effect on the metatarsals is examined histologically.

MATERIALS AND METHODS

Bacteria. The *Bacteroides* strains used and their origins are listed in table I. Organisms were grown at 37°C in an anaerobic chamber in an atmosphere of N₂ 80%, H₂ 10% and CO₂ 10% (Coy's Manufacturing Co., Ann Arbor, M, USA) for 2 days in liquid medium (BM medium, Shah *et al.*, 1976), harvested by centrifugation (13 000 *g*, 20 min, 20°C) and resuspended in the supernatant fluid. The suspensions were standardised by optical density determination at 690 nm to a calculated OD of 15 (van Steenberg *et al.*, 1982). An equal volume of solidified agar 1% w/v in BM medium, after frequent passage through a syringe needle at 37°C, was added to the bacterial suspension. The average number of bacteria in the inocula was 2.5×10^{10} cfu/ml. To

TABLE I
Strains used in this study

Species	Strain designation	Source	Site of isolation
<i>B. gingivalis</i>	HG 66 (= W 83)	H. N. Shah*	"Clinical specimen"
	HG 76 (= W 50)	H. N. Shah	"Clinical specimen"
	HG 185	Own isolate	Periodontitis
<i>B. asaccharolyticus</i>	HG 111 (= ATCC 27067)	ATCC†	Human leg wound
	HG 124 (= B 537)	H. N. Shah	Leg lesion
<i>B. intermedius</i>	HG 65 (= T 588)	H. N. Shah	Dental plaque
	HG 189	Own isolate	Periodontitis
<i>B. melaninogenicus</i>	HG 73 (= VPI 9343)	H. N. Shah	Great toe
	HG 118 (= N 334-71 H)	H. N. Shah	Oral specimen
<i>B. loeschei</i>	HG 64 (= ATCC 15930)	H. N. Shah	Gingival crevice

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† ATCC, American Type Culture Collection, Rockville, MD, USA.

avoid contact with air, harvesting and preparation of the suspension were performed in strictly anaerobic conditions in the anaerobic chamber. Purity and viability of the suspensions were checked by examination of gram-stained smears and by aerobic and anaerobic culture on blood-agar plates. Suspensions of killed bacteria were prepared by heating for 30 min at 60°C.

Experimental design. To investigate the course of the infection, 12 male Swiss strain mice each of *c.* 30 g, were given subcutaneous injections in the upper side of the left hind foot of 50 µl of a suspension of *B. gingivalis* strain HG 66. On days 3, 5, 7 and 18 after injection, three mice were killed and tissues were prepared for histological examination.

Virulence of the different bacterial strains was compared by injecting suspensions of each strain into three mice as described, and killing the animals on the fourth day after injection. All strains were tested on two occasions.

Histological procedure. The hind feet were fixed in formalin 4% w/v in phosphate buffer and decalcified in Kristensen solution (formic acid 15.3% w/v and sodium formate 3.4% w/v). The feet were embedded in wax and were oriented in such a way that sections could be made perpendicular to the longitudinal axis of the feet. The phalanges were cut away and the region of the metatarsals was sectioned completely in transverse sections of 5 µm. One out of 10 sections was stained with haematoxylin and eosin and in this way 15–20 stained sections were obtained from each foot.

Evaluation of the histopathological lesions. All stained sections from each foot were examined

microscopically by one observer and assessed on a scale from — to + + + +. The following criteria were evaluated:

(1). The size of the inflammatory infiltrate at the site of injection: —, no infiltrate visible; +, a few inflammatory cells but no macroscopic swelling; ++, many inflammatory cells and macroscopic swelling <0.5 mm in diameter; + + +, heavy inflammatory infiltrate with macroscopic swelling of 0.5–1.5 mm; + + + +, heavy inflammatory infiltrate with macroscopic swelling >1.5 mm.

(2), (3) and (4). The number of osteoclasts, Howship's lacunae and small resorption spots. Large multinucleate cells were counted as osteoclasts; resorption pits in the bone, which were larger than an osteoclast, were counted as Howship's lacunae; and irregular pits in the bone, smaller than an osteoclast, were counted as small resorption spots. The total number present in histological sections from one foot was counted and values given are mean values for the three mice examined: —, 0; +, 1–50; ++, 50–100; + + +, 100–150; + + + +, >150.

(5) Destruction of the periosteum. The circumference of all metatarsals was measured on a computerised X–Y tablet, and the percentage of the periosteum which was heavily invaded or completely destroyed was determined. From one foot, all sections stained were examined and the values given were: —, <1%; +, 1–10%; ++, 10–20%; + + +, 20–30%; + + + +, >30%.

(6) Proliferation of the stratified squamous epithelium of the skin: —, no epithelial proliferation visible; +, one extra layer of cuboidal cells; ++, two extra layers of cuboidal cells; + + +, three extra layers of cuboidal cells; + + + +, more than three extra layers of cuboidal cells.

RESULTS

In an initial study, *B. gingivalis* strain HG 66 suspended either in BM medium or in agar 1% w/v in BM medium was injected into the hind feet of mice. Three to 5 days after injection, bacteria suspended in BM medium alone caused a slight inflammation. No destruction of the periosteum, signs of bone resorption, or proliferative response of the epithelium were observed. After 7 days no inflammation, reactive bone formation or epithelial response could be observed. Bacteria suspended in agar had a greater effect. Controls given injections of agar alone showed only slight inflammation with scarcely any sign of destruction of the periosteum and only a few osteoclasts were observed (fig. 1, table II).

Three days after injection of *B. gingivalis* with agar, severe inflammation developed with an enormous influx of polymorphonuclear cells. About 40% of the periosteum on the metatarsals was destroyed and there were signs of bone resorption by osteoclasts. After 4 days, inflammation was visible macroscopically as a swelling of the foot. This was the result of an influx of mainly polymorphonuclear cells. Few lymphocytes were seen and no oedema was observed. Concomitant with the inflammation, the periosteum was in part destroyed and bone resorption was evident. Bone resorption was recognised by the presence of osteoclasts and Howship's lacunae (fig. 2). The inflammation also resulted in a proliferative response of the epithelium of the skin. The normal squamous epithelium of the skin reacted after injection of the bacteria by producing a layer of cuboidal cells of up to seven cells thick. Five days after injection, osteoclastic bone resorption was observed but the osteoblasts on the margin of the bone were already activated and some spots of new bone formation were seen. On the seventh day, heavy reactive bone formation was seen (fig. 3) and was visible until 18 days after injection. There was a proliferative response of the epithelium at days three, five and seven after injection.

To compare the different black-pigmented *Bacteroides* strains in this model, the feet of mice were examined histologically 4 days after injection of bacteria. Differences

FIG. 1—Section of mouse foot given an injection of agar alone, showing the area of study ($\times 150$); M, metatarsal bone; E, epithelium of the skin; A, site of agar injection.

TABLE II
Histopathological examination of the hind foot, 4 days after injection of black-pigmented Bacteroides strains

Inoculum for injection	Histopathological changes*					
	1 Infil- trate	2 Osteo- clasts	3 Howship's lacunae	4 Small resorp- tion spots	5 Destruc- tion of perios- teum	6 Epithelial prolifer- ation
Uninoculated control	—	—	+	+	—	—
Agar control	—	+	+	+	+	—
<i>B. gingivalis</i> HG 66 (heat-killed)	+	+	+	+	+	+
<i>B. gingivalis</i> HG 66	++++	++++	++++	++++	++++	++++
HG 76	++++	++++	++++	++++	++++	++++
HG 185	++	+++	+++	+++	+++	++
<i>B. asaccharolyticus</i> HG 111	++	++	++	++	+++	+
HG 124	+	+	+	++	++	+
<i>B. intermedius</i> HG 65	++	++	+++	+++	+++	++
HG 189	++	++	+++	+++	++++	+++
<i>B. melaninogenicus</i> HG 73	+	+	++	+++	+++	+
HG 118	++	++	++	++	+++	++
<i>B. loeschei</i> HG 64	+	+	+	+	+	+

* See *Materials and methods* for scoring system.

FIG. 2—Section of a mouse foot 4 days after injection of *B. gingivalis* HG 66 suspended in agar ($\times 150$); I, inflammatory infiltrate; O, osteoclast in a Howship's lacuna; E, epithelial proliferation.

FIG. 3—Section of mouse foot 7 days after injection of *B. gingivalis* HG 66, showing reactive bone formation and inflammatory infiltrate ($\times 250$); M, metatarsal bone; I, inflammatory infiltrate; E, epithelial proliferation; R, reactive bone formation.

in response were observed between the strains used (table II). Heat-killed *B. gingivalis* produced little effect. Live *B. gingivalis* strains induced a far greater inflammatory response than the strains of the other species. This resulted in higher numbers of osteoclasts on the margins of the bone and greater destruction of the periosteum compared with the other species. *B. intermedius* strains HG 65 and HG 189, *B. asaccharolyticus* strain HG 111 and *B. melaninogenicus* strain HG 118 caused more moderate inflammation and less severe bone resorption as indicated by lower numbers of osteoclasts and Howship's lacunae on the margins of bone. *B. asaccharolyticus* HG 124, *B. melaninogenicus* HG 73 and *B. loeschei* HG 64 caused relatively mild inflammation with only a few signs of bone resorption by osteoclasts. The effects of these three strains were only slightly more than those of the agar control.

DISCUSSION

Black-pigmented *Bacteroides* species are commonly associated with the various types of periodontal diseases but their mere presence at the diseased sites is no proof of involvement in pathogenesis. However, demonstration of the potential of these bacteria to stimulate bone resorption would suggest involvement in periodontal disease but this has not been widely studied (Heijl *et al.*, 1980; Roeterink *et al.*, 1984). Studies on periodontal bone destruction in gnotobiotic and conventional animals have shown effects of several bacterial species (Page and Schroeder, 1982). *In vitro*, *Bacteroides* spp. have been tested for their ability to effect bone resorption from cultured fetal long bones and calvaria. Lipopolysaccharide from, among others, *B. fragilis* and *B. gingivalis*, has been shown to induce calcium release (Hausmann *et al.*, 1970; Sveen and Skaug, 1980; Hausmann *et al.*, 1982; Iino and Hopps, 1984).

The present study has shown that inflammation caused by *Bacteroides* spp. resulted in bone resorption as demonstrated by the presence of numerous osteoclasts and Howship's lacunae on the margins of the bones. Differences were also observed between species. *B. gingivalis* and *B. intermedius* strains, which form a significant part of the cultivable microflora from periodontal diseased sites (Slots, 1982), proved to be more virulent in regard to bone resorption than some strains of the other species. In other animal models, these bacteria also cause different types of infection (van Steenberg *et al.*, 1982; Roeterink *et al.*, 1984) but *B. gingivalis* seems to be the most virulent black-pigmented *Bacteroides* species.

Our results also show a correlation between the extent of the inflammation and the amount of bone resorption as can be seen from the induction of osteoclasts. This study did not reveal the mechanism of induction and formation of the osteoclasts. Possibly the inflammation triggers the induction of osteoclasts by the release of lymphokines such as Osteoclast Activating Factor (OAF) or the release of prostaglandins (Raisz *et al.*, 1975 and 1981). However, the role of these mediators in this model is unknown. Furthermore, *B. gingivalis* and *B. intermedius* possess several virulence factors, which could also have a direct effect (Slots and Genco, 1984). Finally, it is interesting to note that there was a marked proliferation of epithelial cells. Periodontal disease is characterised by epithelial proliferation which plays an important role in pocket formation. Again, the mechanism by which the inflammation caused this epithelial proliferation is not yet clear.

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