DEGRADATION OF ALBUMIN, HAEMOPEXIN, HAPTOGLOBIN AND TRANSFERRIN, BY BLACK-PIGMENTED *BACTEROIDES* SPECIES

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SUMMARY. Strains of six black-pigmented *Bacteroides* species and one un-named strain were examined for their ability to degrade the plasma proteins albumin, haemopexin, haptoglobin and transferrin. Strains of *B. gingivalis* were most effective, degrading all four plasma proteins at different rates. Strains of *B. intermedius* and *B. asaccharolyticus* showed intermediate activities, degrading different individual plasma proteins; strains of *B. melaninogenicus*, *B. loeschei* and *B. denticola* were least active, degrading only haemopexin. These findings are discussed in relation to the availability in tissue fluids of iron for bacterial growth.

INTRODUCTION

Much of the body iron is unavailable, being bound in ferritin, haemosiderin, myoglobin and haemoglobin. Four plasma proteins—transferrin, haptoglobin, haemopexin and albumin—are involved in the transport of body iron and in preventing undue loss of iron through urinary excretion (Putnam, 1975). Transferrin is important in transporting iron to and among cells of the erythropoietic bone marrow, the reticuloendothelial system, spleen, liver, small intestine and muscle (Weinberg, 1978). Haptoglobin binds haemoglobin present as a result of haemolysis in the tissue fluid and transports it to the liver (Hershko, 1975; Jayle and Moretti, 1962). When tissue haptoglobin is depleted by an excess of haemoglobin, the latter is oxidised and dissociates into globin and haem. Haemopexin, which has a greater affinity than albumin for haem, binds and transports haem to the liver (Muller-Eberhard and Morgan, 1975).

Only a minor part of the body iron is associated with the transferrin of body fluids, and because of high association constants the concentration of free iron in body fluids is only 10^{-18} M (Bullen, 1981). However, most bacteria require about 10^{-6} M iron for growth, and so the iron available in the tissues is generally insufficient to support bacterial growth. Accordingly, to obtain iron in normal tissues, bacteria need iron-chelating agents with association constants similar to that of transferrin (Weinberg, 1978). Free haemoglobin and haem sometimes fulfil the bacterial requirement for iron (Bullen, Rogers and Griffiths, 1978; Eaton *et al.*, 1982), and some bacteria, such as black-pigmented *Bacteroides* and *Haemophilus* species, have an

absolute requirement for haem (Lwoff and Lwoff, 1937; Gibbons and Macdonald, 1960).

Because the availability of ionic iron or iron-containing compounds, such as haemoglobin and haem, may limit the growth of bacteria in tissues, albumin, haemopexin, haptoglobin and transferrin may play important roles in the host defence against bacterial infections by depriving tissue-invading bacteria of nutritional iron (Weinberg, 1978; Bezkorovainy, 1981; Bullen, 1981; Eaton *et al.*, 1982; van Asbeck and Verhoef, 1983). Thus, it is of interest that after acute infections and tissue lesions, the plasma levels of haemopexin and haptoglobins increase significantly (Aronsen *et al.*, 1972).

Pathogenic bacteria may overcome the iron-withholding capacity of the host by means of high-affinity systems for iron (Weinberg, 1978) or by degradation of iron-binding proteins. Thus, the black-pigmented *B. asaccharolyticus* strain NCTC9337 has been reported to degrade albumin, haptoglobin and transferrin (Werner and Müller, 1971).

This report describes the ability of strains of *Bacteroides* to degrade the plasma proteins involved in the transport and conservation of body iron.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Ten *Bacteroides* strains, the sources of which are given (table), were stored on plates of blood agar in an anaerobic box in an atmosphere of nitrogen with carbon dioxide (5% v/v) and hydrogen (10% v/v). The blood-agar medium, prepared as described by Holdeman, Cato and Moore (1977), contained defibrinated horse blood haemolysed by freezing and thawing. The bacterial suspending medium (MOPS), a modification of the medium of Neidhardt, Bloch and Smith (1974), contained 40 mM 3-N-morpholino-propanesulphonic acid, 4 mM tricine, 0.3 mM K₂SO₄, 0.5 μ M CaCl₂, 0.5 mM MgCl₂ and 50 mM NaCl; it was adjusted to pH 7.4 with KOH and sterilised by filtration.

Assay of proteolytic activity. Bacteria harvested from blood-agar plates after incubation for 2–5 days, depending on the growth rates, were suspended in MOPS to a concentration of c. 1.0 mg dry weight of bacteria (or 3×10^9 cfu)/ml. The proteolytic activity of bacterial cells was assayed in anaerobic conditions in a reaction mixture of pooled human serum from healthy adult donors (0.6 ml) and bacterial suspension (0.3 ml), incubated at 37°C for 48 h; from 0.1-ml portions taken at 0.5, 2, 4, 8, 24 and 48 h, bacteria were removed by centrifugation and the resultant supernates were stored at –80°C until their iron-binding proteins were analysed by “rocket” immunoelectrophoresis, as described by Laurell (1972). Samples (5 μ l) were applied to wells (2.5 mm) in agarose gels (HSA; Litex, Glostrup, Denmark) 1% w/v in tris-barbital buffer (pH 8.6). Electrophoresis was performed (2 V/cm for 20 h) in the same buffer. Gels were washed with saline (NaCl 0.85% w/v) to remove excess protein and stained with Coomassie Brilliant Blue R-250 0.1% w/v in a solvent of ethanol, acetic acid and water (50:10:40). Gels were decolourised in the same solvent. Antisera raised against albumin, haemopexin, haptoglobin and transferrin were commercial products (Dakopatts A/S, Copenhagen, Denmark).

RESULTS

B. gingivalis was the most effective of the black-pigmented *Bacteroides* species tested in degrading the plasma proteins albumin, haemopexin, haptoglobin and transferrin (table, fig. 1). Thus, within 2 h, strains of *B. gingivalis* had degraded haemopexin into fragments with different electrophoretic mobilities (table); haemopexin was not demonstrable in the reaction mixtures after 8 h (fig. 2). After incubation

TABLE

Capacity of black-pigmented Bacteroides species to degrade plasma proteins of human serum

Species and strain no.	Degradation* of				Source†	Site of isolation or case history
	albumin	haemopexin	haptoglobin	transferrin		
<i>B. gingivalis</i>						
W83	+(24)	+(2)‡	+(24)‡	+(24)	1	"Clinical specimen"
381	+(24)	+(2)‡	+(24)‡	+(24)	1	Subgingival plaque
<i>B. intermedius</i>						
NCTC9336	+(24)	—	—	+(24)	2	Vincent's gingivitis
UJB13-c	+(24)	—	—	+(24)	3	Infected dental-root canal
<i>B. asaccharolyticus</i>						
NCTC9337	+(48)	—	+(48)	+(24)	2	Infected haemorrhoids
VPI4198	—	—	+(2)	—	4	Empyema
<i>B. melaninogenicus</i>						
ATCC25845	—	+(24)‡	—	—	5	Human sputum
<i>B. loeschei</i>						
ATCC15930	—	+(24)‡	—	—	5	Gingival crevice
<i>B. denticola</i>						
NCDO2352	—	+(24)‡	—	—	6	Dental plaque
"Oral asaccharolytic"§						
Strain BN11a-f	—	—	+(48)	—	3	Infected dental-root canal

* Degradation of the protein was scored as: — = < 50% within 48 h; + = > 50% within the specified time (h).

† 1) T. J. M. van Steenberg, The Netherlands; 2) National Collection of Type Cultures, Colindale, UK; 3) Own isolate; 4) Virginia Polytechnic Institute, USA; 5) American Type Culture Collection; 6) National Collection of Dairy Organisms.

‡ Gross changes in electrophoretic mobility.

§ This strain is black-pigmented and asaccharolytic but has a DNA composition different from that of *B. asaccharolyticus* and *B. gingivalis* (van Steenberg, Vlaanderen and de Graaff, 1981).

for 2 days, most of the transferrin and albumin had disappeared, and haptoglobin had been split to fragments with different electrophoretic mobilities (table, fig. 1).

Though *B. intermedius* strains had degraded albumin and transferrin within 2 days, their breakdown of haemopexin and haptoglobin was insignificant (table, fig. 1). *B. asaccharolyticus* strain NCTC9337 had degraded transferrin within 2 days (table, fig. 1); strain VPI 4198, however, had degraded haptoglobin within 4 h (table, fig. 3). Although the strains of *B. melaninogenicus*, *B. loeschei* and *B. denticola* tested did not degrade albumin, haptoglobin or transferrin in 48 h, their action on haemopexin significantly altered its electrophoretic mobility (table, fig. 1).

DISCUSSION

Black-pigmented *Bacteroides* strains have long been thought to play an important role in the pathogenesis of polymicrobial infections (Hite, Locke and Hesseltine, 1949; Macdonald *et al.*, 1956; Socransky and Gibbons, 1965; Sundqvist *et al.*, 1979). Although the virulence factors of these bacteria have been sought for about 50 years, their pathogenic potential has been realised only recently. Significant advances in their taxonomy (Coykendall, Kaczmarek and Slots, 1980; Holdeman and Johnson, 1982; Johnson and Holdeman, 1983), helped demonstrate that some species give reproducible infections in animals (Kastelein *et al.*, 1981; van Steenberg *et al.*, 1982). Thus, strains of *B. gingivalis* were shown to produce rapidly spreading necrotic

FIG. 1.—“Rocket” immunoelectrophoresis of albumin, haptoglobin, haemopexin and transferrin from human serum incubated for 48 h at 37°C in anaerobic conditions with: *B. gingivalis* strain W83 (well 3), strain 381 (well 4); *B. intermedius* strain NCTC9336 (well 5), strain UJB13-c (well 6); *B. asaccharolyticus* strain NCTC9337 (well 7), strain VPI4198 (well 8); *B. melaninogenicus* strain ATCC25845 (well 9); *B. loeschei* strain ATCC15930 (well 10); *B. denticola* strain NCDO2352 (well 11); and the “oral asaccharolytic” strain BN11a-f (well 12). Well 1 contained untreated serum and wells 2 and 13 serum incubated for 48 h without bacteria. Before the assays, sera were diluted in tris-barbital buffer (pH 8.6): for albumin 1 in 250 and for haemopexin, haptoglobin and transferrin 1 in 10.

infection and those of *B. intermedius* to cause localised abscesses; strains of *B. melaninogenicus* and *B. asaccharolyticus*, however, are usually considered to have little virulence.

Black-pigmented *Bacteroides* species produce an array of lytic enzymes (Slots,

FIG. 2.—“Rocket” immunoelectrophoresis of haemopexin in human serum incubated with *B. gingivalis* strain W83 in anaerobic conditions. Well 1 contained untreated serum and wells 2–6 contained samples of reaction mixture after 0·5, 2, 4, 8 and 24 h, respectively. Before electrophoresis, samples were diluted (1 in 10) in tris-barbital buffer (pH 8·6).

FIG. 3.—“Rocket” immunoelectrophoresis of haptoglobin in human serum incubated with *B. asaccharolyticus* strain VPI4198 in anaerobic conditions. Wells 1 and 8 contained untreated serum and wells 2–7 contained samples of reaction mixture after 0·5, 2, 4, 8, 24 and 48 h, respectively. Before electrophoresis, samples were diluted (1 in 10) in tris-barbital buffer (pH 8·6).

1982) of which collagenase has attracted most attention (Gibbons and Macdonald, 1961; Kestenbaum, Massing and Weiss, 1964; Mayrand *et al.*, 1980). However, the capacity of some species to degrade various plasma proteins would seem to be a more important virulence factor because not only immunoglobins A1, A2 and G (Werner and Müller, 1971; Kilian, 1981) but also complement factor C3 (Sundqvist *et al.*, 1984) and proteinase inhibitors (Carlsson *et al.*, 1984; Herrmann, Carlsson and Sundqvist, *in press*) may be degraded. This ability may perturb significant parts of the host defence and may explain in part the interesting in-vitro finding that black-pigmented *Bacteroides* species inhibit not only their own phagocytosis and killing by polymorphonuclear leukocytes, but also that of other bacteria present concomitantly (Ingham *et al.*, 1977 and 1981; Tofte *et al.*, 1980; Jones and Gemmell, 1982; Namavar *et al.*, 1983).

The pathogenicity of many strains of black-pigmented *Bacteroides* species in polymicrobial infections often depends on the presence in the infective mixtures of

other bacteria (Macdonald, Socransky and Gibbons, 1963; Sundqvist *et al.*, 1979; Mayrand *et al.*, 1980), which themselves do not apparently possess any obvious virulence factors. However, many strains of black-pigmented *Bacteroides* species have an absolute nutritional requirement for vitamin K and haem (Lev, 1958; Macdonald *et al.*, 1963; Mayrand and McBride, 1980), and the major role of the other bacteria in polymicrobial infections may be to provide growth factors for the black-pigmented *Bacteroides* species.

If, indeed, the availability of iron or iron-containing compounds is growth-limiting for bacteria invading mammalian tissues, it may be that the presently demonstrated ability of strains of black-pigmented *Bacteroides* species to degrade haemoglobin-, haem- and iron-binding plasma proteins contributes significantly to their pathogenicity. Degradation of haemopexin and albumin by bacteria present in polymicrobial infections may avoid competition with these proteins for the haem available in tissues; again, by degradation of transferrin or haptoglobin, the host defence in the form of iron or haemoglobin limitation may be perturbed. The degradation of plasma proteins involved in iron metabolism may also impair the protection of tissues against iron-catalysed hydroxyl-radical production associated with activated polymorphonuclear leukocytes (Gutteridge *et al.*, 1981; Fantone and Ward, 1982; Winterbourn, 1983).

The significance of the present demonstration of bacterial degradation of the plasma proteins involved in iron metabolism cannot be assessed until further information is available about whether, for example, the various species of black-pigmented *Bacteroides* have similar haem requirements and whether other iron-containing compounds can satisfy that requirement. The effect of the black-pigmented *Bacteroides* species on the function of the plasma proteins involved in iron metabolism will also require further study, for it is probable that, in the present study, these proteins had lost the capacity to bind iron or iron-containing compounds long before their degradation was demonstrated immunologically.

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