CYTOTOXIC ACTIVITY OF *BACTEROIDES GINGIVALIS* AND *BACTEROIDES ASACCHAROLYTICUS*

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SUMMARY. Culture filtrates of all eight strains of *Bacteroides gingivalis* and all five strains of *B. asaccharolyticus* were toxic for Vero cells. Cytotoxicity was in general greater with material from cultures of *B. gingivalis* than from *B. asaccharolyticus* but none of the culture filtrates from eight strains of *B. melaninogenicus* showed activity in this test. The toxic material was released during prolonged incubation and more detailed study of preparations from one strain indicated that it had a molecular weight of less than 3500 and was heat stable.

INTRODUCTION

The bacteroides of human origin that form black pigment are now allotted to one or other of three species. Representatives of *Bacteroides melaninogenicus* have been isolated from disease processes in the mouth (Spiegel *et al.*, 1979) and from other sites (Smith, 1975; Finegold, 1977). *B. gingivalis*, which is composed of mainly oral strains that were formerly assigned to *B. asaccharolyticus* (Coykendall, Kaczmarek and Slots, 1980; van Steenberg, Vlaanderen and de Graaff, 1981), seems to be very important in adult periodontitis (Slots, 1979; Spiegel *et al.*, 1979; Tanner *et al.*, 1979). The strains still classed as *B. asaccharolyticus* can be isolated from infections away from the mouth (Smith, 1975; Finegold, 1977). Little is known about the mechanisms of virulence of the black-pigmented bacteroides, although the action of collagenase was an early suggestion (Gibbons and MacDonald, 1961) and the organisms contain endotoxin (Hofstad, 1970). Eukaryotic cells maintained in culture have been useful indicators of cytotoxicity and this paper describes the results of testing material derived from representatives of three *Bacteroides* spp. on Vero cells, the continuous cell line derived from African green-monkey kidney cells.

MATERIALS AND METHODS

Bacterial strains. The micro-organisms used in this study were: *B. gingivalis* strains W83, W50 (Dr H. N. Shah, London Hospital Medical College, London), 376, 381, 382 (Dr S. S. Socransky, Forsyth Dental Center, Boston, USA), and H184, H185, H186 (our own isolates from patients with periodontitis); *B. asaccharolyticus* strains ATCC27067 and ATCC25260, and strains VPI4199, B536, B537 (Dr H. N. Shah); *B. melaninogenicus* subsp. *melaninogenicus* strains ATCC 25845, VPI9085, VPI9343, N334-71H (Dr H. N. Shah); *B. melaninogenicus* subsp. *intermedius* strains T588 (Dr H. N. Shah), ATCC25261, UJB 13a-c (Dr G. Sundqvist, University of Umea, Sweden), H187 (our own isolate from a patient with periodontitis); and *Escherichia coli* strain H10407 (Dr S. Falkow, University of Washington, Seattle). Strains were maintained by weekly subculture on blood-agar plates (Blood Agar Base No. 2; Oxoid, Basingstoke, Hants) containing horse blood (5% v/v) haemin (BDH, Poole, Dorset) 5 µg/ml and menadione (Merck, Darmstadt, Germany) 2 µg/ml. Cultures were grown in liquid BM medium (Shah *et al.*, 1976) as described previously (van Steenberg, de Soet and de Graaff, 1979) with and without glucose.

Received 1 Jun. 1981; accepted 28 Aug. 1981.

Preparation of culture filtrates. Cultures, usually incubated anaerobically for 5 days at 37°C, were then centrifuged at 3000 rpm for 15 min; the supernatant fluids were filter-sterilised through 0.45-µm membranes (Millipore). Dilutions of these materials were made in NaCl 8.0 g/L, KCl 0.2 g/L, Na₂HPO₄ 1.15 g/L, KH₂PO₄ 0.2 g/L at pH 7.35 (PBS). The following dilutions were tested: 1 in 5, 1 in 10, 1 in 20, 1 in 40.

Cell-culture assay of toxin. Vero cells (Flow Laboratories, PO Box 17, Irvine, Scotland) were grown as monolayers at 37°C for 1 week in closed bottles in Glasgow's modification of Eagle's medium with Na₂CO₃ 2.75 g/L but lacking glutamine (Flow Laboratories), and supplemented with fetal bovine serum (10% v/v) (Flow Laboratories), vancomycin 500 mg/L, gentamicin 20 mg/L and nystatin 5 × 10⁴ U/L (GME medium). Toxic activity was assayed, essentially, as described by Speirs, Stavric and Konowalchuk (1977) in plastic dishes (Falcon 3008) with 0.6 ml of Vero cell culture in each well. Almost confluent monolayers were used with 10⁵ Vero cells being seeded 2 days before use. Growth medium was then replaced by diluted culture filtrate or, in controls, by diluted BM medium. Cultures were then examined after incubation for 1–4 days at 37°C in an atmosphere containing CO₂ (5% v/v). The proportion of cells affected was estimated directly or after staining of methanol-fixed cells with Giemsa (10% v/v) in PBS for 10 min. The grading of the cytotoxic effects on the Vero cells was: +++ , more than 90% of Vero cells affected; ++ , 70–80% affected; + , 50–60% affected; ± , 30–40% affected; – , less than 20% affected.

RESULTS

Culture filtrates from all tested strains of *B. gingivalis* and *B. asaccharolyticus* caused a morphological change in Vero cells. The cytotoxic substance in filtrates from *B. gingivalis* strain W83 was studied in more detail. Vero cells grown for 1 or 2 days were used to study the effect; however, an almost complete monolayer was formed after 2 days and was easier to interpret. When a filtrate was diluted in GME medium, morphological changes of the Vero cells were observed but the cytotoxic effect was more obvious when PBS was used. Staining with Giemsa facilitated the observation of damaged cells, and this method was used for further studies.

In control cultures, only 5–10% of the Vero cells incubated with diluted sterile BM medium or with PBS alone were abnormal (fig. 1A). The effect of bacteroides culture filtrate

TABLE

Effect of diluted culture filtrates from B. gingivalis and B. asaccharolyticus strains on Vero tissue-culture cells after incubation for 2 days

Strain	Relative toxic effect* of culture filtrate diluted 1 in			
	5	10	20	40
<i>B. gingivalis</i>				
W83	+++	+++	+++	+++
W58	+++	+++	+++	+++
381	+++	+++	+++	+++
382	+++	+++	+++	+++
376	+++	+++	+++	+++
H184	+++	+++	+++	+++
H186	++	++	+	–
H185	+	+	+	±
<i>B. asaccharolyticus</i>				
ATCC25260	+++	+++	+	±
B536	+++	++	–	–
ATCC27067	++	+	±	–
VP14199	++	+	–	–
B537	+	+	+	–

* See *Materials and methods* for grading of toxic effect.

FIG. 1.—Vero-cell monolayer incubated: (A) for 48 h in BM medium (Shah *et al.*, 1976) diluted 1 in 5 in PBS (control). $\times 100$; (B) for 48 h in culture filtrate from *B. gingivalis* strain W83 grown for 5 days in BM medium. $\times 100$; (C) for 24 h in culture filtrate, diluted 1 in 5 in PBS and final pH 6.2, from *B. melaninogenicus* subsp. *intermedius* strain ATCC25261 grown in BM medium. $\times 100$.

on the cells could be detected after incubation for 16 h and increased during continued incubation for 2–4 days. The affected cells showed thick cell membranes, filamentous tendrils and rounding of the cell outline (fig. 1B). During prolonged incubation, more round cells were detected and these tended to float into the medium. Filtrates from all strains of *B. gingivalis* and *B. asaccharolyticus* caused an apparently similar morphological change in the Vero cells. In a 1 in 5 dilution, culture filtrates of most strains affected more than 70% of the Vero cells; when the culture filtrate was more dilute fewer cells were affected. The table shows that dilutions of filtrates from most strains of *B. gingivalis* had more toxic effect than

those of *B. asaccharolyticus*. In a positive control, the filtrate from *E. coli* strain H10407, which produces LT enterotoxin, caused morphological changes to the Vero cells similar to those of the *Bacteroides* filtrates.

To test the influence of the medium on the production of cytotoxic products, strain W83 was cultured in BM medium, Trypticase Soy Broth (BBL) and Thioglycollate Medium (Difco); all media were supplemented with haemin and menadione. With culture filtrates from cells grown in trypticase soy broth or thioglycollate medium, fewer Vero cells were affected; relatively more filamentous tendrils and less rounding of the cells were observed.

The cytotoxic activity of *B. gingivalis* strain W83 was not detected in the filtrate from bacteria grown overnight in BM but could be detected after incubation for 2 days. The amount of cytotoxic substance detectable in the supernate increased during incubation from 2 to 13 days (fig. 2). Dialysis of the filtrate against PBS showed that the cytotoxic substances could freely pass dialysis membranes permeable to molecules of mol. wt 12 000 and 3500; when 5 ml of filtrate was dialysed against 1 L of PBS, which was refreshed three times at 24-h intervals, cytotoxic activity was not detectable in the dialysate, whereas dialysis of 5 ml of filtrate against 45 ml of PBS resulted in the same cytotoxic activity in the dialysate and in the dialysing fluid. Heating of the filtrate for 15 or 25 min at 37, 50 or 70°C caused no decrease in cytotoxic activity, when tested in 1 in 5 or 1 in 10 dilution in PBS, and heating at 100°C for 10 or 25 min caused only a slight decrease. The cytotoxic activity of filtrates did not diminish on standing for at least 1 month at room temperature or at -20°C. The testing of cytotoxic

FIG. 2.—Morphological response of Vero cells to PBS dilutions of culture filtrate from *B. gingivalis* strain W83 grown in BM medium for 1 day (x—x), 2 days (■—■), 5 days (●—●) and 13 days (▲—▲).

activity was performed in aerobic conditions, suggesting that the cytotoxin is active in the presence of oxygen.

Dilutions of filtrates from strains of *B. melaninogenicus* subsp. *melaninogenicus* and *B. melaninogenicus* subsp. *intermedius* having a pH of less than 6.6, were found to cause an effect on the Vero cells, but different from that of *B. gingivalis* and *B. asaccharolyticus*. After incubation with *B. melaninogenicus* filtrates the cells were swollen and their outline vague; the cytoplasm and the nuclei stained less intensely, despite the fact that staining conditions were the same (fig 1C). This effect was not observed when the pH of the culture filtrate was brought to 7.35 with NaOH. Dilutions of unused BM medium acidified with HCl to a pH lower than 6.6 caused the same morphological changes of the Vero cells as did the culture filtrates of *B. melaninogenicus*.

DISCUSSION

Strains of *B. gingivalis* and *B. asaccharolyticus* produced a substance affecting Vero cells, and it could be detected in the filtrates of cultures grown for more than 1 day in BM medium. The morphological change caused by this cytotoxin was similar to the effect of culture filtrates of the LT enterotoxin of *E. coli* (Speirs *et al.*, 1977). As far as we know, the existence of this bacteroides cytotoxin has not been reported before. The effect of culture filtrates of *B. melaninogenicus* on Vero cells can be explained by the relatively low pH alone.

The strain of *B. gingivalis* strain W83 has been shown to be very virulent in guinea-pigs and mice (Kastelein *et al.*, 1981). The cytotoxin of this anaerobic bacterium was active in an aerobic atmosphere containing 5% CO₂; it was heat stable, had a mol. wt of less than 3500 and differed from the *E. coli* LT toxin, which is heat labile and has a mol. wt of more than 100 000 (Speirs *et al.*, 1977). The cytotoxin was detected in supernates of cultures grown into the late log phase or the stationary phase.

In general, filtrates from *B. gingivalis* strains were more cytotoxic than those of *B. asaccharolyticus*. This could of course be attributable to a higher concentration of the cytotoxin, differences in the cytotoxic substances or to the production of one or more additional cytotoxic substances by *B. gingivalis*.

Recent evidence suggests that *B. gingivalis* is more virulent than *B. melaninogenicus* and *B. asaccharolyticus* (Mayrand *et al.*, 1980; van Steenberg *et al.*, in press). The role of the cytotoxin in disease remains to be elucidated. It is possible that this cytotoxin, which is active *in vitro* against mammalian cells, had an effect *in vivo*, for instance in adult periodontitis, in which *B. gingivalis* is thought to be important (Slots, 1979). It is not known whether the effects of culture filtrates from *B. gingivalis* and *B. asaccharolyticus* are caused by one or more substances, and this is the subject of current investigations.

We are grateful to Miss J. L. den Boer for assistance with the tissue cultures.

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