Effects of oxidative stress on the virulence profile of *Prevotella intermedia* during experimental infection in gnotobiotic mice

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Prevotella intermedia is a component of the indigenous microbiota but is also responsible for anaerobic infections of the gastrointestinal tract and oral cavity. The aim of the present study was to investigate the influence of oxidative stress on the *in vivo* pathogenicity of *P. intermedia*. Germ-free mice were challenged intraperitoneally with parental (wt) or oxidative stress adapted (aero) strains. Bacterial virulence was evaluated by histopathology, hyperaemia and blood analysis [C-reactive protein (CRP), serum albumin and white blood cells (WBCs)], 3 and 10 days after challenge. CRP levels and WBC count were higher in animals challenged with the aero strain, and the albumin level was lower in this group, only 10 days after infection ($P < 0.05$). Body weight gain was significantly reduced whereas hyperaemia and ratios of spleen/organ weight were increased in animals challenged with the aero strain ($P < 0.05$). The liver of animals challenged with the aero strain showed hyperaemia, vasodilatation as well as an increase in the number of inflammatory cells and liver/organ weight ratio ($P < 0.05$). Similar, but more discrete, alterations were observed in the small intestine of animals challenged with the aero strain. Studies on stress responses of this putative pathogen may help to better understand the aggressive potential and virulence markers of anaerobic bacteria.

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INTRODUCTION

The anaerobic black-pigmented *Prevotella intermedia* is an important Gram-negative rod-shaped bacterium whose habitat is the strictly anaerobic environments of the gastrointestinal tract and gingival crevice, and is found as a component of the resident microbiota of these sites in humans and other animals (Shah & Gharbia, 1992). Since 1965, black-pigmented anaerobes have been considered key pathogens in mixed anaerobic infections (Socransky & Gibbons, 1965). In the oral cavity, these bacteria,

particularly *P. intermedia*, play an important role in the onset and subsequent development of the polymicrobial periodontal diseases (Rawlinson *et al.*, 1993; Socransky & Haffajee, 2005).

In response to tissue conditions and defence mechanisms found during an infective process, bacteria may produce several adaptive regulatory and metabolic activities that contribute to their pathogenicity (Sansone, 2001). During the initial stages of an infection progressing from the intestinal lumen to the internal body compartment, obligately anaerobic bacteria move from anaerobic to more oxygenated conditions. This oxidative stress is unavoidable and an adaptive response for survival may occur (Rocha *et al.*, 1996).

Abbreviations: aero strain, oxidative stress adapted strain; CRP, C-reactive protein; RBC, red blood cell; WBC, white blood cell; wt strain, parental strain.

Oxidative stress can be defined as a disturbance in the balance between oxidative substances and antioxidative substances, in favour of the former. Thus any condition that leads to increased levels of lethal reactive oxygen species or to a decrease in antioxidant compounds constitutes an oxidative stress (Zheng & Storz, 2000). According to the literature, anaerobic bacteria may have developed protective mechanisms against oxidative stress, which may be important adaptive factors during an infection. This adaptive response could be considered an important virulence factor allowing anaerobes to successfully resist not only new environmental conditions, but also defence mechanisms involving the host's immune oxidative burst (Zheng & Storz, 2000; Rocha *et al.*, 2000; Sansonetti, 2001; Imlay, 2003).

According to other authors (Diniz *et al.*, 2000; Sansonetti, 2001; Nilsen *et al.*, 2004), free radicals produced by chemical substances, radiation, metals and antimicrobial drugs may induce alterations in bacterial morphology by increased expression or inhibition of enzymes that initiate cell division. These morphological alterations may significantly alter physiological and biochemical characteristics of the bacteria, such as antigenicity, cell wall structure, surface adherence, susceptibility to host polymorphonuclear leukocytes, antimicrobial drug susceptibility and virulence patterns. Changes in these characteristics might increase or diminish the pathogenicity of a micro-organism (Ganusov, 2003). Nevertheless, the extent and consequences of these alterations on the virulence properties of pathogenic bacteria, in general, and *P. intermedia*, in particular, are unclear. Recent data showed that oxidative stress induced cellular and virulence alterations in *Fusobacterium nucleatum* (Silva *et al.*, 2005). In this way, the relationship between indigenous anaerobic bacteria and their hosts in health or in clinical infections is of increasing interest, especially concerning the ability of the bacteria to adapt to changing oxygen levels in the environment (Patschkowski *et al.*, 2000; Silva *et al.*, 2005).

Because there is little literature dealing with the adaptive defence mechanisms against oxidative stress in *P. intermedia* and their consequences on the host–bacteria relationships, the present study focused on investigating how adaptation to atmospheric oxygen exposure may interfere with the cellular morphology and virulence profile of *P. intermedia* during experimental infection in germ-free mice.

METHODS

Bacteria. *Prevotella intermedia* ATCC 25611 was used as wild-type (untreated) strain (wt strain). During experiments, all cultures were routinely incubated at 37 °C in an anaerobic chamber (Forma Scientific), containing an atmosphere of 85% N₂, 10% H₂, 5% CO₂. *Brucella* broth (BB) and *Brucella* agar (BA) (Difco), both supplemented with haemin (5 mg ml⁻¹), menadione (1 mg ml⁻¹) and yeast extract (5%), were used as culture media. Gram-stained smears were performed to confirm pure culture status as well as morphological changes in *P. intermedia* cells.

Adaptation of *P. intermedia* to oxidative stress by atmospheric oxygen exposure. A strain adapted to oxidative stress (oxygen treated: aero strain) was derived from the wt strain by serial atmospheric oxygen exposures, as follows. An overnight culture of *P. intermedia* wt strain was seeded as a 10% (v/v) inoculum in 300 ml BB and grown to the mid-exponential phase (OD₆₀₀=0.6). This culture was split into two 150 ml subcultures. One of the subcultures was kept in the anaerobic chamber as a control and the other was taken out of the chamber and kept under aerated conditions on a shaker (Cientec, Biosan) at 200 r.p.m. at 37 °C. Every 6 h, up to 72 h, samples from both subcultures were withdrawn; the OD₆₀₀ was measured on one aliquot and the other was plated onto BA after serial decimal dilutions, in triplicate, to count c.f.u. ml⁻¹, after 48 h anaerobic growth. The plate corresponding to the last point of atmospheric oxygen exposure at which viable cells could be recovered was used to reisolate a *P. intermedia* strain for the next experimental steps. The strain derived from the surviving colonies was submitted to four additional cycles of 12 h atmospheric oxygen exposure and the strain resulting from the fifth reisolation was considered as the one adapted to oxidative stress (aero strain).

Survival curve of the wt strain and the aero strain under atmospheric oxygen exposure. Two volumes of BB (300 ml each) were inoculated with 10% of an overnight culture (OD₆₀₀ initial=0.155) of *P. intermedia* wt strain or aero strain, respectively, and incubated in the anaerobic chamber at 37 °C for 12 h. Each culture was then split in two 150 ml subcultures which were treated as follows: one was kept inside the anaerobic chamber as a control and the other was removed and kept aerated at 37 °C, on a shaker at 200 r.p.m. Every 6 h, up to 96 h, samples from subcultures were withdrawn and evaluated for OD₆₀₀ and plated onto BA after serial decimal dilutions, in triplicate, for viable cell counts (c.f.u. ml⁻¹).

Animals. Germ-free NIH (Taconic) 21-day-old mice were used in this study. The animals were housed in flexible plastic isolators (Standard Safety Company) and handled according to established procedures (Pleasant, 1974). The animals were fed an autoclaved commercial diet for rodents (Nuvital, Curitiba, PR, Brazil), and the experiments were carried out in microisolators (UNO Roestvastaal). Controlled lighting (12 h light, 12 h dark) was used. All experimental procedures were carried out according to the standards set out in the 'Guide for the Care and Use of Laboratory Animals' (National Research Council, 1996) and approved by the Ethic Committee for Animal Experimentation of the Federal University of Minas Gerais (CETEA/UFGM – no. 117/04).

Experimental challenge. The germ-free mice were divided into three groups of 20 for intraperitoneal injection of: (i) 0.1 ml (10⁸ c.f.u. ml⁻¹) *P. intermedia* wt strain; (ii) 0.1 ml (10⁸ c.f.u. ml⁻¹) *P. intermedia* aero strain; (iii) 0.1 ml sterile culture medium (control group). Half of the animals in each group were killed by cervical dislocation on day 3 after challenge and the remaining mice on day 10. The strains for inoculation into the germ-free mice were grown anaerobically in BB for 24 h under anaerobic conditions at 37 °C.

Microbial counts in gnotobiotic groups. In an attempt to evaluate the germ-free or monoxenic status of the mice, faeces were obtained by anal stimulation before the challenge as well as before the necropsy. Slices of spleen, liver and intestine were also obtained at the necropsy. Faecal and tissue samples were weighed and immediately introduced into the anaerobic chamber. All these samples were diluted 100-fold in regenerated sterile buffered saline (pH 7.4) and homogenized by hand. Serial 10-fold dilutions were made and 0.1 ml aliquots were plated onto BA. The plates were incubated for 48 h in the anaerobic chamber at 37 °C to determine the viable counts [c.f.u. (g contents)⁻¹].

Blood analyses. Blood samples were taken from the retro-orbital plexus on days 3 ($n=10$ from each group) and 10 ($n=10$ from each group) after challenge. The samples were assayed for CRP, alkaline phosphatase, albumin, haemoglobin and haematocrit (packed cell volume), WBC and red blood cell (RBC) counts, and the blood was also cultured. The total WBC and RBC counts were performed with an automated system (KX Sysmex) and the other determination was made with a Cobas-Mira/Plus automated system (Roche Diagnostic Systems). For blood culture, 0.2 ml blood was inoculated into 1.8 ml BB and incubated anaerobically for 72 h at 37 °C.

Body and organ weight determination. Body weights were determined for each group of animals just before bacterial challenge and before the animals were killed (days 3 or 10) and the values were used to calculate the body weight gain and relative organ weight. After blood collection, the animals were killed by cervical dislocation and the entire small intestine, spleen and liver were excised, weighed and then stored for analyses. Relative organ weight was calculated as organ weight/body weight ratio.

Evaluation of tissue hyperaemia. The tissue-dissolved haemoglobin was measured by the Drabkin method (Drabkin & Austin, 1932). Parts of the small intestine, spleen and liver were homogenized in a sterile glass tissue-blender containing 3 ml Drabkin solution (Imbralab, Ribeirão Preto) and then centrifuged at 4000 g for 20 min. The supernatant was clarified by filtration through 0.45 μ m and 0.22 μ m Millipore membranes. The extracted haemoglobin was determined at A_{540} in a microplate spectrophotometer system

(Spectra Max Plus; Molecular Devices) and quantified using a curve ($r^2>0.99$) obtained for standard haemoglobin (Analisa Diagnostica LTDA) dissolved in Drabkin solution.

Histopathological evaluation. Spleen, liver and small intestine were initially evaluated macroscopically. Small intestine samples were identified as duodenum, proximal jejunum, distal jejunum and ileum, according to established procedures (Diniz *et al.*, 2003). Each segment was opened along the ante-mesenteric border, the contents were removed and the length was measured. The intestinal segments were processed as described previously (Arantes & Nogueira, 1997). Briefly, after pre-fixation in formaldehyde/PBS (1:10, pH 7.2) for 90–120 min, the segments were rolled up to form the so-called ‘Swiss roll’ and fixed for a further 18–20 h. The tissue fragments were dehydrated in alcohol solution using an automated tissue processor (Ti-tertek Autotechnicon Company) and then embedded in paraffin. Cross-sections of 4 μ m thickness were cut with a microtome (Spencer, # 820), stained with haematoxylin/eosin, coded and examined by a single pathologist, who was unaware of the experimental conditions of each group.

Statistical analysis. The data were compared by analysis of variance for collective analyses and Student–Newman–Keuls test for multiple comparisons. Statistical analysis was performed using the SigmaStat software (Scientific Software, version 1.0) with the level of significance set at $P<0.05$.

Fig. 1. Mean levels of some serum components in germ-free control and gnotobiotic mice intraperitoneally challenged with *Prevotella intermedia* ATCC 25611 wt strain or aero strain, 3 and 10 days after inoculation. (a) C-reactive protein; (b) albumin; (c) alkaline phosphatase. *Significantly different ($P < 0.05$).

RESULTS

Morphological and physiological characteristics of the aero strain

The efficiency of the adaptive process of *P. intermedia* to oxidative stress by exposure to atmospheric oxygen (aero strain) was demonstrated by the progressive increase in aerotolerance of *P. intermedia* ATCC 25611 (wt strain) observed after each successive cycle (survival time of 36, 42, 48, 54 and 60 h after cycle 1, 2, 3, 4 and 5, respectively).

Microscopy of Gram-stained smears showed significantly altered morphology in the aero strain with highly filamentous cell formation, when compared to the typical morphology of the *P. intermedia* wt strain.

To confirm the adapted phenotype, survival curves of the wt and aero strains under oxidative conditions were compared. Viable cells were no longer observed after 48 h exposure to atmospheric oxygen for the wt strain, whereas the aero strain was viable for up to 96 h under the same conditions.

Microbial counts in gnotobiotic mice and blood analyses

After inoculation of viable *P. intermedia* cells, no growth was

observed in cultures of liver, spleen, intestine, blood or faeces samples from any mouse group or challenge time.

Blood analyses showed significant alteration in the levels of serum CRP in the animals challenged with both the aero and wt strains, when compared with the control group ($P < 0.05$). Ten days after infection, the CRP values in the blood from animals challenged with the aero strain were significantly higher than those values recorded from the animals challenged with the wt strain (Fig. 1a). On day 10 after challenge, a significant decrease in albumin levels was observed in animals challenged with the aero strain when compared to controls and the animals challenged with the wt strain ($P < 0.05$) (Fig. 1b). Increased levels of alkaline phosphatase were observed only on day 3 of infection in both gnotobiotic groups in comparison to the control group (Fig. 1c).

For blood component counts, total peripheral leukocytes were always higher in animals challenged with the aero strain ($P < 0.05$) when compared to the other groups (Fig. 2a). Blood haemoglobin levels and haematocrit values were significantly lower in animals challenged with the aero strain when compared to control mice and animals challenged with wt strains ($P < 0.05$), 10 days after infection (Fig. 2b, c). Statistically significant alterations were not

Fig. 2. Mean levels of some blood components in germ-free control and gnotobiotic mice intraperitoneally challenged with *Prevotella intermedia* ATCC 25611 wt strain or aero strain, 3 and 10 days after inoculation. (a) WBCs; (b) haemoglobin; (c) haematocrit; (d) RBCs. *Significantly different ($P < 0.05$).

Fig. 3. Mean body weight gain and relative organ weight of germ-free control and gnotobiotic mice intraperitoneally challenged with *Prevotella intermedia* ATCC 25611 wt strain or aero strain, 3 and 10 days after inoculation. Body weight gain (a) and relative weight of the spleen (b), liver (c) and small intestine (d). *Significantly different ($P < 0.05$).

observed in erythrocyte counts between the three groups during the experiment (Fig. 2d).

Macroscopic evaluation of gnotobiotic mice

At the beginning of the experiment, the mean weight gain was significantly lower in both groups challenged with the bacteria than in the control group ($P < 0.05$), but was statistically similar between themselves. However, after 10 days, body weight gain was significantly lower for the group challenged with the aero strain (Fig. 3a). On day 3 of challenge, the spleens of mice that received the aero strain showed higher mean relative organ weights than the other groups ($P < 0.05$). By day 10, higher values were observed for the spleen from both challenged groups (Fig. 3b). Also, the mean relative organ weights of the liver and small intestine of animals challenged with the aero strain were higher than in the other two groups ($P < 0.05$) (Fig. 3c, d).

Hyperaemia in the tissues was significantly higher in the spleen, liver and small intestine of mice receiving the aero strain ($P < 0.05$) (Fig. 4).

Microscopic evaluation

Microscopy of tissues showed extensive lesions in the spleen and liver from animals challenged with the aero strain on days 3 and 10 after challenge. Few lesions or no alterations

were observed in mice challenged with the wt strain or in mice from the control group, respectively. Lesions in spleens from animals challenged with the aero strain were characterized by increased red pulp accompanied by haemorrhagic areas (Fig. 5). Hepatic lesions in the challenged mice were haemorrhagic areas with congested blood vessels and increased inflammatory cells (Fig. 6). No significant microscopic alterations were observed in the small intestine of the three groups of mice.

DISCUSSION

Results of the present study showed that the obligate anaerobe *P. intermedia* was able to develop an efficient adaptive response to oxidative stress conditions, which enabled it to resist exposure to molecular oxygen for up to 96 h. This adaptation, associated with morphological alterations of bacterial cells, influenced the virulence of the micro-organism as demonstrated by experimental infection in germ-free mice.

It is known that host–pathogen interactions may or may not result in damage to the host. Disease occurs when the host sustains sufficient damage to disturb homeostasis (Sansone, 2001; Ganusov, 2003). In this respect, damage is a term describing cell, tissue and organ injury. At the cellular level, this includes necrosis, apoptosis and

Fig. 4. Mean tissue-dissolved haemoglobin levels in germ-free control and gnotobiotic mice intraperitoneally challenged with *Prevotella intermedia* ATCC 25611 wt strain or aero strain, 3 and 10 days after inoculation. (a) Dissolved haemoglobin in the spleen; (b) dissolved haemoglobin in the liver; (c) dissolved haemoglobin in the small intestine. *Significantly different ($P < 0.05$).

malignant transformations whereas at the tissue level, granulomatous inflammation, chronic inflammation and tumour formation may occur. It is generally accepted that cytotoxicity or damage to host tissues is a component of microbial virulence (Casadevall & Pirofski, 1999). The murine model has been employed successfully in the study of microbial virulence, particularly concerning anaerobic bacteria, and the potential for abscess formation or tissue damage in animals experimentally challenged with one or more microbial lineages or species is usually evaluated (Baumgartner *et al.*, 1992). The likely contribution of these micro-organisms is sometimes difficult to determine in the presence of a broad and complex indigenous microbiota. The use of a gnotobiotic animal model with a simplified and controlled microbial status allows the *in vivo* observation of interrelationships between micro-organisms and the host. Furthermore, even discrete alterations in host–bacteria relationships can be observed because gnotobiotic animals are more susceptible to microbial virulence properties than conventional ones.

CRP belongs to a class of acute phase reactants related to the complement system, and its level rises dramatically during infectious and non-infectious inflammatory processes. In

full-term or pre-term human infants, there is good evidence to support the use of CRP measurements in conjunction with other established diagnostic tests (such as a WBC count with differential and blood culture) to establish or exclude the diagnosis of sepsis (Huntley & Kelly, 2005).

The role of polymorphonuclear cells in phagocytosis and consequent clearance of the intra-abdominal cavity has been shown by various authors (Dunn *et al.*, 1985; Shah & Gharbia, 1992). A similar clearance could explain the absence of *P. intermedia* in the cultured tissue from mice challenged with the wt strain or aero strain and such a hypothesis was reinforced by data on the CRP levels, peripheral leukocyte counts as well as by the presence of inflammatory infiltrate in the liver. Acute inflammation can damage endothelial cells and increase capillary permeability (Dunn *et al.*, 1985; Li *et al.*, 2000; Sansonetti, 2001), which could explain the expressive hyperaemia observed in the organs (spleen, liver and intestine) of challenged mice. Bacterial soluble antigens may enter the bloodstream, react with circulating specific antibodies to form a macromolecular complex and give rise to a variety of acute or chronic inflammatory reactions at the sites of deposition (Li *et al.*, 2000). Liver dysfunction is a frequent finding in the

Fig. 5. Histology of the spleen from germ-free mice 3 days after intraperitoneal challenge (experimental) or not (control) with *Prevotella intermedia* ATCC 25611 wt strain or aero strain. (a) Control group; (b) wt strain group; (c) aero strain group. Note the significantly increased red pulp (▲) with haemorrhagic areas in aero strain challenged mice and the discrete increase in RBCs (▲) in wt strain challenged mice, but no marked alteration of the lymphoid component (↑). Haematoxylin/eosin stained. Bar, 80 μ m.

Fig. 6. Histology of liver parenchyma from germ-free mice 3 days after intraperitoneal challenge (experimental) or not (control) with *Prevotella intermedia* ATCC 25611 wt strain or aero strain. (a) Control group; (b) wt strain group; (c) aero strain group. Note the significant increase in inflammatory cells (↑) with lipid degeneration and congestion (↑↑) in the aero strain challenged mice and the discrete increase in inflammatory cells in wt strain challenged mice. Haematoxylin/eosin stained. Bar, 80 μ m.

inflammation process and bacterial sepsis, and has been studied in experimental peritonitis in rats by measuring serum levels of alkaline phosphatase and albumin (Tung *et al.*, 2005). Alkaline phosphatase is an enzyme found throughout the body in humans, and is particularly produced by hepatic cells lining the small bile ducts (ductules). If the liver lesion is primarily of an obstructive nature (cholestatic), alkaline phosphatase will be the first and foremost enzymic increase (Fey & Gauldie, 1990; Tung

et al., 2005). Albumin is a hepatic protein involved in homeostasis of blood osmotic pressure. A decrease in albumin levels leads to problems in the abdomen (ascite) and in the lungs (pulmonary oedema) (Cabral *et al.*, 2001; Mello *et al.*, 2004; Orhue *et al.*, 2005). Similarly to human beings, alterations in albumin and alkaline phosphatase levels observed in animals challenged with the aero strain could be the results of inflammatory reactions (Fey & Gauldie, 1990; Cabral *et al.*, 2001).

Spleen hypertrophy could be related to the haemorrhagic process as well as to immunosuppression due to degeneration of lymphoid tissue (Casadevall & Pirofski, 1999; Papet *et al.*, 2003). These observations were confirmed by the increase in dissolved haemoglobin and the congestion of red pulp observed in the follicular area of the lymphoid component in the spleen of mice challenged with the aero strain. A decrease in haemoglobin levels and haematocrit were also noted in these animals.

Although significant morphological alterations were not observed in the intestines, higher dissolved haemoglobin levels were found in the organ in animals challenged with the aero strain. This could be due to the large extension of this organ resulting in alterations observed only biochemically but not histopathologically.

In conclusion, the higher cellular, tissue and systemic damage obtained in germ-free mice inoculated with the *P. intermedia* aero strain, associated with morphological alteration of bacterial cells, suggest that the adaptation to oxidative stress influences the virulence of the micro-organism. More studies on the many different cellular processes involved in the adaptation to changes in oxygen level during an infectious process could contribute to a better understanding of the aggressive potential and virulence markers of an anaerobic bacterium, as well as to the development of new therapeutic strategies for this important pathogenic micro-organism.

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