Bacteria recovered from dental pulp induce apoptosis of lymph node cells


INTRODUCTION

The interaction between infectious agents and the host defence system is the key element in determining health or disease. Accordingly, periapical bone destruction subsequent to dental pulp infections is thought to be caused by colonization by pathogenic bacteria and consequent triggering of host defence mechanisms (Kakehashi et al., 1965). Many questions concerning the mechanisms involved in the pathogenesis of periapical lesions have been answered. Still, there are many doubts that must be clarified, such as the capacity of dental pulp infection to promote apoptosis and the effects of apoptosis on the aetiopathogenesis of this disease. The strategies used by pathogens to activate pro-inflammatory cytokines represent one mechanism by which this bacterium mediates immunosuppression and inactivation of potentially responsive cells (Jewett et al., 2000). Here, we describe a reduction in the number of lymph node cells during experimental dental pulp infection in germ-free mice. The bacteria studied induced different levels of apoptotic cell death and the apoptosis-inducing molecules were heat- or paraformaldehyde-labile. Apoptosis of lymph node cells was dependent on the presence of the tumour necrosis factor (TNF) receptor p55 (TNFRp55).

METHODS

Animals. Germ-free Swiss/NIH mice (Gnotobiology Laboratory, ICB, UFMG, Belo Horizonte, Brazil) were used to perform the in vivo experiments (Ribeiro Sobrinho et al., 2001, 2002). Lymph node cells recovered from conventional Swiss/NIH mice (Gnotobiology Laboratory, ICB, UFMG) were used to perform the in vivo experiments (Ribeiro Sobrinho et al., 2001, 2002), while C57BL/6 mice (CEBIO, UFMG) and gene knockout mice for the p55 receptor for TNF (p55−/−); a kind gift from Dr Klaus Pfeffer, Institute of Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Germany) on a C57BL/6 background (Pfeffer et al., 1993) were used for the in vitro apoptosis assessments. Experimental protocols and care of animals were in accordance with the institutional requirements for humane treatment.

Bacteria. The bacteria used in this study were recovered from two patients with pulp necrosis treated at the Endodontic Clinic of the Dental School, UFMG (Lanna et al., 2001; Ribeiro Sobrinho et al., 2001). The bacteria were Gemella morbillorum from patient 1 and Bifidobacterium adolescents, F. nucleatum and Clostridium butyricum from patient 2. Samples were stored at −70°C and recovered, when necessary, in brain heart infusion (BHI) broth (Difco) supplemented and pre-reduced (BHI-S-PRAS), plated on to Petri dishes containing blood agar supplemented with haemin and menadione (Sutter et al., 1980) and incubated at 37°C inside an anaerobic chamber (Forma Scientific Co.).

Apoptosis is critical in the pathogenesis of several infectious diseases. The induction of apoptosis was assessed in mouse lymph node cells by four bacteria recovered from infected human dental pulp: Gemella morbillorum, Clostridium butyricum, Fusobacterium nucleatum and Bifidobacterium adolescentis. Smaller lymph nodes and smaller numbers of cells were observed after experimental dental pulp infection with C. butyricum, suggesting that this bacterium induces cell death. Apoptosis was evaluated by determination of cell ploidy and detection of DNA degradation in cells cultured with killed bacteria. Paraformaldehyde-killed C. butyricum and heat-killed G. morbillorum induced substantial cell death, while F. nucleatum and B. adolescentis induced cell death at lower levels. No bacterial preparations induced apoptosis in cells from mice genetically deficient for tumour necrosis factor receptor p55 (TNFRp55), implicating this receptor directly or indirectly as a mediator in the process. It was concluded that apoptosis may be induced during periapical lesions of pulpal origin.

Abbreviations: PI, propidium iodide; TNF, tumour necrosis factor.
Bacterial inoculation and its effect on murine lymphocytes. The bacterial inoculum was dripped into the germ-free mouse root canal using methodology previously described (Ribeiro Sobrinho et al., 2001). Before any surgical procedure, animals were anaesthetized intraperitoneally using pentobarbital (35 mg kg⁻¹) with fentanyl (25 µg kg⁻¹) (Cristalia). The inferior right incisor was selected for our studies. The tooth was trepanned with a steel file (33G Maillefer) in low rotation. The remaining pulp was removed using an exploring probe and the root canal system was dried with sterile absorbent paper tips. The inoculum was dripped into the instrumented root canal, with 1 ml syringes and 26G (13 x 4.5) needles (Injex Indústrias Cirúrgicas LTDA). Bacteria were inoculated separately or together (i.e. B. adolescentis, F. nucleatum and C. butyricum as found in patient 2). After inoculation, the orifice of the root canal was sealed with paraffin. Non-infected controls were also analysed. Mice were sacrificed on days 10 and 20 after inoculation. The submandibular lymph nodes adjacent to the tooth submitted to surgical procedures were removed and single-cell suspensions in RPMI 1640 (Sigma) were obtained. Since the number of cells recovered from the submandibular lymph nodes from individual animals was not enough to obtain a satisfactory number of culture wells to be stimulated, organs from different animals subjected to the same stimulus (n = 5) were pooled. Each experiment was repeated four times and the results pooled.

Bacterial preparations. Bacterial samples grown in BHI-S-PRAS for 48 h at 37 °C were adjusted to a concentration of 1 x 10⁹ – 5 x 10⁸ c.f.u. ml⁻¹, washed once with 4 ml sterile PBS (pH 7-0) and the pellet was resuspended in 4 ml PBS. The bacteria were either treated with 1 % sodium citrate, 0.005 % Triton X-100, 50 mM sodium iodide (PI) using a method described previously (Nicoletti et al., 1991). Viability of cells was tested on BHI agar. Live bacteria were exposed in vitro to paraformaldehyde (Jewett et al., 2000) or heat-killed (Ribeiro Sobrinho et al., 2002). Viability of cells was tested on BHI agar. Live bacteria were not used in vitro because they would grow in the cell culture and might deplete the medium of nutrients, thus affecting the response of mouse cells.

Cell cultures. Cells were cultured with bacterial stimuli as described previously (Ribeiro Sobrinho et al., 2002).

Detection of DNA fragmentation by gel electrophoresis. DNA isolation from 1 x 10⁸ pooled lymph node cells cultured in the presence or absence of bacterial stimuli was performed as described previously (Kurita-Ochiai et al., 1999). Samples were subjected to horizontal gel electrophoresis in a 1:5 % agarose gel containing 0.7 µg ethidium bromide ml⁻¹.

Statistical analysis of data. Statistical analysis was carried out using Student’s non-paired t-test. The null hypothesis was rejected when P < 0.05.

RESULTS AND DISCUSSION

Apoptosis is involved in many physiological processes including the immune response (Schwartzman & Cidlowski, 1993; King & Cidlowski, 1998). Inducers of apoptosis include TNF and bacterial products (Fraser & Evan, 1996; Nagata, 1997; Thompson, 1995; Weinrauch & Zychlinsky, 1999). Periapical diseases vary widely in severity among individuals, and it is reasonable to propose that this variability is due to differences in the composition of the microbiota as well as in the immune responses against the oral microbiota.

Bacteria (G. morbillorum from patient 1 and B. adolescentis, F. nucleatum and C. butyricum from patient 2) were inoculated into the root canals of germ-free mice. At 10 and 20 days after inoculation, the submandibular lymph nodes adjacent to the teeth submitted to surgical procedures were aseptically removed and the cells counted. At both time points, mice infected with C. butyricum alone or together with F. nucleatum and B. adolescentis presented smaller lymph nodes than the control group and the other mice infected with a single bacterial species (data not shown). Accordingly, smaller numbers of cells were recovered from lymph nodes from mice infected with C. butyricum (Fig. 1). One possible reason for the smaller lymph nodes after infection with C. butyricum was the triggering of cell death by the bacteria. Hence, we investigated whether apoptosis of lymph node cells would take place following exposure to the isolated bacteria. Lymph node cells from Swiss/NIH mice were exposed in vitro to paraformaldehyde-treated or heat-killed bacterial preparations. Addition of heat-killed

![Fig. 1. Number of cells recovered from submandibular lymph nodes from Swiss/NIH germ-free mice in the absence (open bars) or presence (filled bars) of infection on days 10 (a) and 20 (b) after inoculation. Experiments were performed independently. Lymph node cells from five mice were pooled. Each experiment (five mice per group per experiment) was repeated four times. Bars represent the means of individual experiments ± S.D. * Statistical differences between the mock-infected and infected groups (P < 0.05). Association, infection with B. adolescentis, F. nucleatum and C. butyricum.](ip:54.70.40.11)
G. morbillorum or paraformaldehyde-treated C. butyricum (the latter both when added alone and in combination with F. nucleatum and B. adolescentis) to lymph node cells from Swiss/NIH mice in culture induced high levels of apoptotic cell death, as determined by flow cytometry analysis of PI-stained cells (Fig. 2a). Paraformaldehyde-treated and heat-killed F. nucleatum and B. adolescentis induced similar levels of apoptotic cell death to the control cultures, as shown by the ratio of approximately 1 (Fig. 2a). In cultures where there was an increase in apoptotic cell death, we also found a parallel decrease in non-apoptotic cells (Fig. 3a). Interestingly, apoptosis induced by heat-killed C. butyricum or paraformaldehyde-killed G. morbillorum was marginal. Confirmation of apoptosis was obtained by DNA gel electrophoresis (Fig. 4) and Giemsa staining (data not shown). Heat-killed G. morbillorum and paraformaldehyde-killed F. nucleatum and C. butyricum induced DNA fragmentation of lymphocyte cells (Fig. 4). C. butyricum and G. morbillorum translocate to the draining lymph nodes at higher rates than other dental pulp bacteria studied (Ribeiro Sobrinho et al., 2001). It is possible that the apoptotic cell death observed here facilitates translocation to the draining lymph nodes by impairing host defences.

Numerous mechanisms are used by extracellular and intracellular pathogenic bacteria to induce apoptosis in host cells (Mahida et al., 1996; Morimoto & Bonavida, 1992) including pore-forming toxins, other toxins that express their enzymic activity in the host cytosol, effector proteins delivered directly into host cells by a highly specialized type III secretory system, superantigens and other modulators of host-cell death (Ikigai & Nakae, 1987; Stevens, 1997; Weinrauch & Zychlinsky, 1999). The best-characterized TNF receptor-like death receptors are CD95 (also called Fas or Apo1) and TNFRp55 (also called TNFR1 or CD120a) (Smith et al., 1994; Nagata, 1997). Thus, we investigated the role of TNFRp55 in the induction of apoptosis under our experimental conditions. Incubation of lymph node cells recovered from p55 gene knockout mice with heat-killed G. morbillorum and paraformaldehyde-killed C. butyricum induced significantly lower levels of cell death than those observed in Swiss/NIH cells (Figs 2 and 3). Thus, the dental pulp bacterial preparations used in this work triggered the process of cell death and involved TNFRp55. The mechanism by which G. morbillorum and C. butyricum induced apoptosis in our model is not completely understood: under our conditions, apoptosis was dependent on the presence of TNFRp55; however, whether our bacterial preparations were inducing TNF, which induces apoptosis, or whether a molecule derived from the bacteria was directly binding to the receptor, was not known. In addition, the role of TNFRp55 may be indirect. The bacterial apoptosis-inducing molecules present were paraformaldehyde-sensitive in G. morbillorum and heat-labile in C. butyricum. It is highly likely that TNF is being induced in these cultures, since bacteria are potent inducers of TNF production. Jewett et al. (2000) found that only paraformaldehyde-
Fig. 4. Gel electrophoresis of DNA from cells from C57BL/6 mice co-cultured with a bacteria:cell ratio of 1:1. DNA extracted from cells was loaded on to a 2 % agarose gel and submitted to electrophoresis. Lanes: 1, molecular size marker; 2, untreated cells; 3, cells co-cultured with paraformaldehyde-killed F. nucleatum; 4, cells co-cultured with paraformaldehyde-killed F. nucleatum; 5, cells co-cultured with paraformaldehyde-killed B. adolescentis; 6, cells co-cultured with heat-killed G. morbillorum. The results of one experiment of four performed are shown, two with lymph node cells from Swiss/NIH mice and two with lymph node cells from C57BL/6 mice, with identical results.

treated F. nucleatum was capable of inducing the death of human peripheral blood mononuclear cells, while this ability was lost when the bacterium was killed by heat treatment. The levels of apoptosis described by these authors were similar to the levels seen here, and were far below the levels of apoptosis induced by G. morbillorum and C. butyricum.

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