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Immunology

Evaluation of chemokines and receptors in gnotobiotic root canal infection by *F. nucleatum* and *E. faecalis*

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Abstract: The present study aims to evaluate the longitudinal effects of induced experimental infections in gnotoxenic animals on the expression of inflammatory chemokines and their receptors in periradicular tissues. The null hypothesis tested was that *Enterococcus faecalis* and *Fusobacterium nucleatum* had no effect on CCR5, CCL5, CXCL10, CCL2/MCP-1, CXCR2 and CCR1 expression. Two groups of five animals (n = 5) aged between 8 and 12 weeks were used in this study. The animals were anaesthetized, and coronary access was performed in the first molar on the right and left sides. Microorganisms were inoculated into the left molar, and the right molar was sealed without contamination to function as a control. Animals were sacrificed 7 and 14 days after infection, and periapical tissues were collected. The cytokine mRNA expression levels were assessed using real-time PCR. The chemokine mRNA expression levels demonstrated that the experimental infection was capable of inducing increased chemokine expression on day 7 compared to that on day 14, except for CCR5 and CCL5, which showed no changes. The gnotoxenic animal model proved to be effective and allowed evaluation of the immune response against a known infection. Additionally, this study demonstrates that gene expression of chemokines and their receptors against the experimental infection preferentially prevailed during the initial phase of induction of the periradicular alteration (*i.e.*, on day 7 post-infection).

Keywords: Germ-free Life; Chemonikes; Periapical Diseases; Microbiology.

Introduction

Disruption of the integrity of mineralized tissues, enamel, and dentin promotes the entry of harmful elements to the pulp, which can lead to its inflammation and consequent tissue destruction[1]. The interaction between bacterial irritants and the host defence response results in the release of innumerable mediators that are capable of stimulating immune responses in the pulp and periapical region.[2,3,4,5] Previously, the cytokine profile expressed in the periapical tissues of gnotoxenic animals in response to root canal infections was demonstrated to depend on the bacterial challenge, because each species induced specific host immune responses.[6,7,8,9]

Among the mediators involved in pulpal and periradicular disorders, the number of chemokines is outstanding. Chemokines are members

of the family of cytokine regulatory proteins that have low molecular weights and stimulate leukocyte recruitment.[10,11,12] Moreover, chemokines and their receptors, such as CCL2/MCP-1, CCR/5, CXCL10, CCL5, CXCR2, and many others, are involved in many biological processes, including homeostasis and immune activation and regulation.[9,10,12] Chemokines act by binding to membrane receptors, which can bind to more than one chemokine.[13]

Many studies have been performed to elucidate the dynamics of the immune mechanisms that occur at the root apex of infection-bearing teeth by exposing the pulp to the oral microbiota.[4,5,9,14,15,16,17,18,19] To improve this model, researchers have induced experimental infections with a known microbiota in germ-free (GF) animals.[2,6,7,9,20]

The present study aims to evaluate the longitudinal effects of induced experimental infections in GF animals on the expression of chemokines and their receptors in the periradicular tissues of these animals. The null hypothesis tested was that *Enterococcus faecalis* and *Fusobacterium nucleatum* had no effect on CCR5, CCL5, CXCL10, CCL2/MCP-1, CXCR2 and CCR1 expression.

Methodology

Mice

Germ-free mice aged 4–8 weeks (Swiss/NIH, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil) were maintained in Trexler-type isolators (Class Biologically Clean, Madison, WI, USA). For the experimental procedures, the animals were transferred into microisolators (UNO Roestvastaal BV, Zevenaar, the Netherlands). All manipulations were performed under sterile conditions in a laminar flow hood (Veco, Campinas, Brazil). The animals were fed ad libitum. The animal ethics committee approved the experimental protocol (254/2013, CETEA/UFMG).

Microorganisms

The microorganisms inoculated in the root canal system (RCS) of the GF mice were the reference strains *F. nucleatum* (ATCC 10953) and *E. faecalis* (ATCC19433). The bacteria were maintained at -86 °C and recovered in broth-heart infusion medium (BHI) supplemented with yeast extract, hemin and menadione (BHI-SPRAS) (Difco, Detroit, USA). The microbial suspension with both strains was adjusted to approximately 10^7 CFU in 25 µL of the same medium (BHI-SPRAS, Difco) in which the species were grown. Incubations were performed at 37 °C in an anaerobic chamber containing 85% N_2, 10% H_2 and 5% CO_2 (Forma Scientific Company, Marietta, USA).

Experimental root canal infection

The experimental procedures were performed with the animals under general anaesthesia; the animals were anaesthetized using 100 mg kg^{-1} of ketamine hydrochloride (Dopalen, Division Vetbrands Animal Health, Jacareí, Brazil) and 10 mg kg^{-1} of xylazine (Anasedan, Agribrands do Brasil Ltda, Paulínia, Brazil). The pulpal chamber of the maxillary right first molar was accessed under an endodontic operative microscope (Alliance, São Paulo, SP, Brazil) with a one-fourth carbide bur (KG Sorensen, Barueri, Brazil) coupled to a controlled rotation handpiece (Driller, São Paulo, Brazil). The pulp chambers were opened until the orifices of the canals could be visualized and probed with a size 8 and 10 K-file (Dentsply, Maillefer, Ballaigues, Switzerland). The right molar was not inoculated and was used as a control. The left molar received inoculation of the microbial suspension, which was adjusted to approximately 10^7 CFU in 25 µL of the same medium (BHI-SPRAS, Difco) in which the species were grown. The bacterial suspensions were inoculated into the RCS using tuberculin syringes and needles. After inoculation, the teeth were sealed with Coltosol®.[21]

Sample preparation

The mice were sacrificed at 7 and 14 days after root canal inoculation. The periapical tissues surrounding the root apices and the bone subjected to surgery were aseptically removed, rinsed in phosphate-buffered saline, flash-frozen in a mixture of dry ice and ethanol and stored at -70°C. Total periapical tissues and bone RNA were isolated using the TRIzol reagent (Gibco/BRL Laboratories, Grand Island, USA). After the addition of TRIzol, chloroform was added, and the mixture was centrifuged at 12,000 × g at 4°C for 15 min. The aqueous phase was collected, and the

RNA was precipitated by the addition of isopropanol followed by centrifugation at 12 000 × g at 4°C for 10 min. The precipitated RNA was washed once with cold 75% ethanol, dried, dissolved in RNase-free water, incubated at 55°C for 10 min and then stored at -70°C.

Real-time PCR

Complementary DNA was synthesized using 2 mg of RNA by reverse transcription as previously described.[22] The standard PCR conditions were as follows: a holding stage of 95°C for 10 min; a cycling stage with 40 cycles of 95°C for 15 s and 60°C for 1 min; and a melting curve stage of 95 °C for 15 s, 60°C for 1 min, and 95°C for 15 s. The primer sequences used for analysis of CCR5, CCL5, CXCL10, CCL2/MCP-1, CXCR2 and CCR1 mRNA expression by quantitative real-time PCR are shown in Table. The real-time PCR was performed using the Step One Real-time PCR System (Applied Biosystems, Foster City, USA). The SYBR Green detection system (Applied Biosystems) was used to assay primer amplification. The housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) was also amplified and used to normalize the mRNA expression levels. All samples were run in duplicate in a 20-mL reaction volume with 1 mg of cDNA. The Sequence Detection Software, version v 2.0 (Applied Biosystems) was used to analyse the data after amplification. The results were obtained as threshold cycle (C_t) values, which represented the cycle number at which the fluorescence levels passed a fixed threshold. The expression levels were calculated using the $^{DD}C_t$ method. The C_t values are expressed as the mean of two independent measurements, and the mRNA expression levels for all samples are expressed as the ratio between the expression of the gene of interest and HPRT expression. All data were analysed using the SPSS 22 statistical program (SPSS Inc., Chicago, USA). Levene's test was used to assess the equality of variance of the data, whereas a t-test for independent samples was used to evaluate the significance of the differences observed between groups. Differences in mRNA expression levels were considered to be statistically significant when the p-value was < 0.05.

Table. Primer sequences.

Gene	Sense and antisense	Length (bp)
HPRT	5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′ 5′-GAT TCA ACT TGC CGT CAT CTT AGG C-3′	162
CXCL10	5′-CTC GCA AGG ACG GTC CGC TG-3′ 5′-CTC GCA AGG ACG GTC CGC TG-3′	193
CCL2	5′-AGG AAG ATC TCA GTG CAG AG-3′ 5′-AGT CTT CGG AGT TTG CCT TTG-3′	92
CCL5	5′-CGT GCC CAC ATC AAG GAG TA-3′ 5′-CAC ACA CTT GGC GGT TCT TTC-3′	91
CXCR2	5′-AGT GCC TGC CTC AAT GTC TCC A-3′ 5′-CCA GGA GCA AGG ACA GAC CCC-3′	249
CCR5	5′-CAA GAC ATT CCT GAT CGT GCA A-3′ 5′-TCC TAC CAA GCT GCA TAG AA-3′	129
CCR1	5′-TGC AGG TGA CTG AGG TGA TTG-3′ 5′-TGA AAC AGC TGC CGA AGG TAC-3′	108

HPRT: housekeeping gene hypoxanthine phosphoribosyltransferase; bp: base pairs of amplicon size.

Results

Real-time PCR analysis of the mRNA expression levels of the chemokines CCL5, CXCL10, and CCL2/MCP-1 and the chemokine receptors CCR5, CXCR2, and CCR1 was performed using samples from the periradicular tissues of axenic mice. No significant differences in CCR5 and CCL5 expression were found at either 7 or 14 days post-inoculation with a microbial association consisting of *F. nucleatum* and *E. faecalis* (Figure 1). However, the microbial association induced significantly increased gene expression at 7 to 14 days of the chemokines CXCL10, CCL2/MCP-1, CXCR2, and CCR1 (Figure 2). As expected, the absence of the microbial stimulus (*i.e.*, only the RCS instrumentation was performed in the control group) was unable to induce a change in gene expression of the evaluated chemokines at either 7 or 14 days.

Discussion

From the moment at which the organism is challenged by the microbiota, the inflammatory process begins in an attempt to limit or even eliminate the microbial invasion.[2,3,23,24] Several studies have

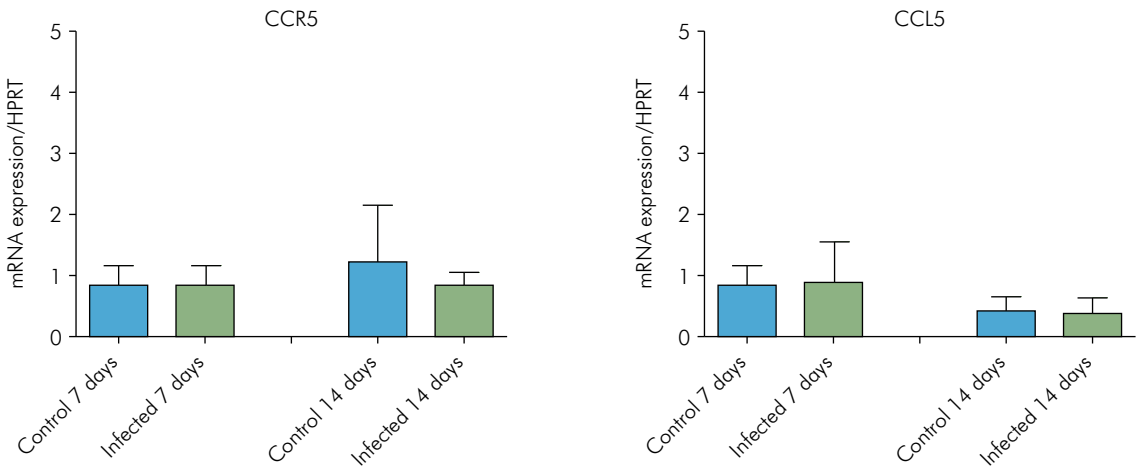

Figure 1. mRNA expression of the chemokines CCR5 and CCL5 analysed by real-time PCR in response to experimental infection in GF mice by microbial bi-association of *E. faecalis*, and *F. nucleatum*. The relative mRNA expression levels were quantified compared with those of the internal control (HPRT). The data were expressed as the mean ± standard error of two independent experiments with six mice per group. The bars represent the mean values of the samples recovered from the axenic mice, and the error bars represent the standard errors of the means.

shown that endodontic infections have a polymicrobial aetiology.[25,26,27,28] With an aim of analysing the effects of the microbiota on adjacent periradicular tissues, researchers have promoted exposure of the pulp to non-specific microbes in the oral cavity.[29,30,31] Conversely, the use of GF animals has enabled the induction of a known endodontic infection, which allows evaluation of its effects in a specific manner.[2,6,7,9,20] Moreover, gnotobiotic animals are good models for the study of periapical pathologies, because synergistic effects or antagonistic influences of the resident microbiota or its derivatives can be detected. These animal models are also instrumental in determining the true effect and identity of the aetiological agent of an infectious disease. [6,7,8,9,32]

The ability of bacteria to implant into specific sites in an organism is dependent on their concentrations, numbers, virulence, and host resistance.[33] In this study, two microorganisms prevalent in endodontic infections (*Enterococcus faecalis* (ATCC 19433), Gram-positive, and *Fusobacterium nucleatum* (ATCC 10953), Gram-negative, were selected to conduct an experimental infection in GF mice. Previously evaluated concentrations that were capable of inducing colonization by these microorganisms were used.[6,8,9,20] Among the factors that affect bacterial growth and RCS colonization, the potential for oxidation-reduction, microbial interactions, and available substrates is critical.[33] In this study, *E. faecalis* (facultative anaerobe) reduced the oxidation-reduction potential, thereby improving *F. nucleatum* growth.[20] Additionally, the strains were demonstrated to show no antagonism against each other.[34]

Periradicular tissues respond to endodontic infection by expressing a series of mediators that seek to limit infection within the RCS, which leads to the destruction of adjacent tissues via the release of soluble mediators derived from the host.[35] Researchers using a murine experimental model to analyse the development of periapical lesions demonstrated that an active phase occurred at 7 days, followed by a chronic phase from 14 days.[6,7,29,36,37] In agreement with these findings, the results of this study demonstrated greater CCL-2 gene expression during the initial phase of periapical lesion development. Similar results have been previously reported.[31] Conversely, in humans, the CCL2 and CCL-5 levels did not change after mechanical-chemical preparation and consequent reduction of the root canal microbial load.[38] CCL2/MCP-1 and CCL5 are a specialized group of cytokines that coordinate the movement of leukocytes into tissues.[11] These cytokines are involved in multiple biological processes, including organ development and homeostasis, angiogenesis, and activation and regulation of immunity.[39]

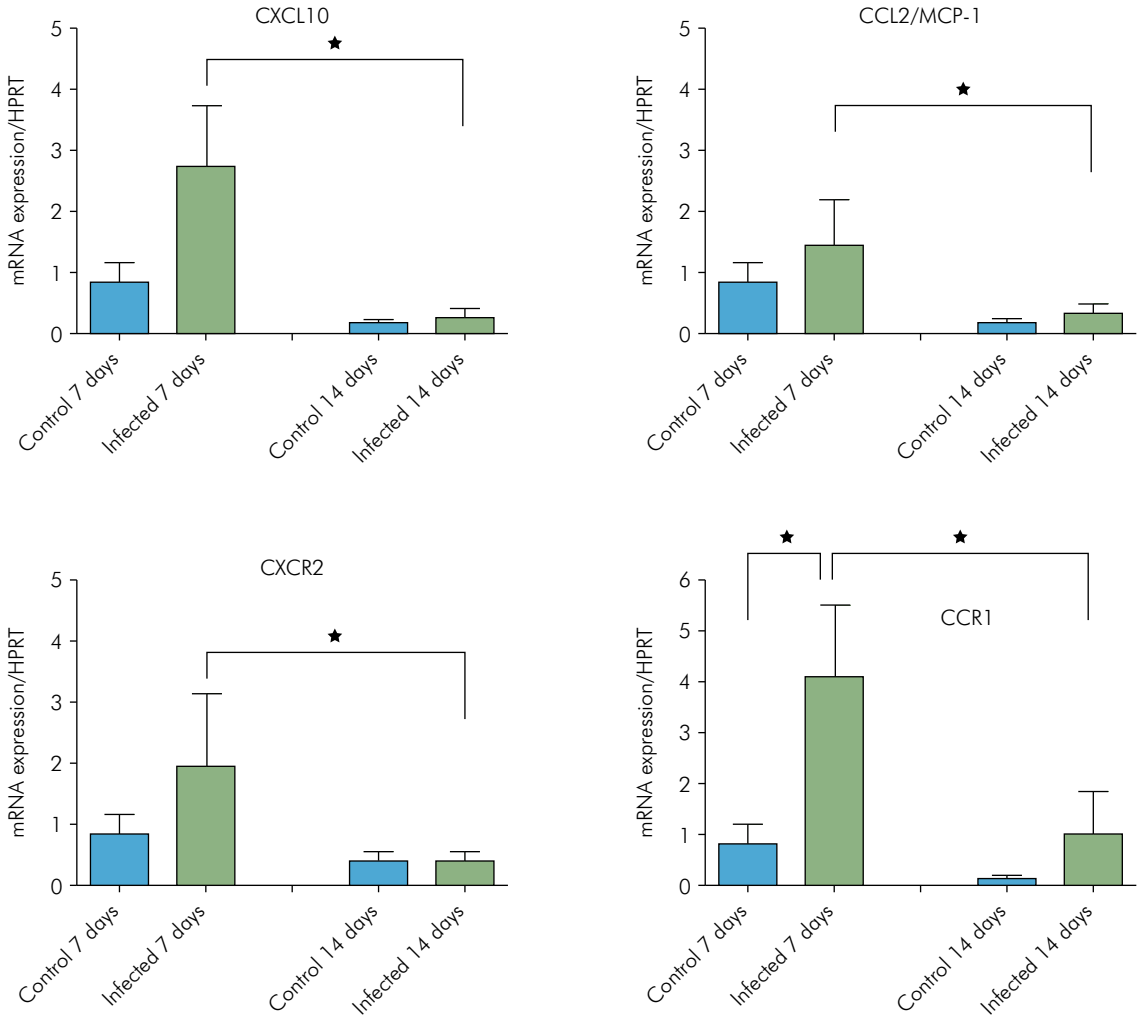

Figure 2. mRNA expression of the chemokines CXCL10, CCL2/MCP-1, CXCR2 and CCR1 analysed by real-time PCR in response to experimental infection in GF mice by microbial bi-association of *E. faecalis*, and *F. nucleatum*. The relative mRNA expression levels were quantified compared with those of the internal control (HPRT). The data were expressed as the mean ± standard error of two independent experiments with six mice per group. The bars represent the mean values of the samples recovered from the axenic mice, and the error bars represent the standard errors of the means. $P<0.05$ as determined by Student's t-test.

CXCL10 is a CXC family chemokine that is related to polymorphonuclear cell chemotaxis and angiogenesis.[10] In this study, higher CXCL10 expression was observed on day 7 of the 14-day study, demonstrating its involvement in the active phase of periradicular lesion development, as observed with CCL-2. Consistent with this finding, studies have also demonstrated elevated CXCL10 expression after stimulation of inflammatory cells in vitro.[19,40]

CCR5, CCR1, and CXCR2 are chemokine receptors. A receptor can bind to more than one chemokine due to redundancy of its activity.[13] Consistent with the higher chemokine expression observed on day 7 of the 14-day study, high CCR1 and CXCR2 levels were observed at the first evaluation period. Similar results for CXCR2 were also demonstrated in murine experimental non-specific endodontic infections.[18] In the present study, baseline CCR5 expression levels were observed in both the control and experimental groups during the two evaluated periods. Furthermore, knockout mice for the CCR5 receptor developed greater periapical lesions than wild-type mice.[17] In humans, results similar to those presented here were found, with no change in the

CCR5 expression levels after mechanical-chemical preparation of the RCS and consequent reduction of the microbial load.[38] Taken together, the results of this study demonstrate that the events in the apical area after *E. faecalis* and Fusobacterium nucleatum colonize the RCS interfere with the expression of chemokines and their receptors.

Conclusion

The gnotoxenic animal model proved to be effective for evaluation of the immune response against a known infection. Additionally, this study demonstrated that the gene expression of chemokines and their receptors against the experimental infection preferentially prevailed during the initial phase of induction of the periradicular alteration, confirming that the migration of cells to the surrounding tissues occurred in the presence of noxious agents within the RCS to promote much needed immunological surveillance.

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