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Experimental oral chronic infection induced by actinomyces israelii and propionibacterium acnes

Infecção oral crônica experimental induzida por actinomyces israelii e propionibacterium acnes

Leandro C. Quirino[1], Rafael Tomaz Gomes[2], Sérgio M. Lima Jr., Felipe A. Braga, Jacques Robert Nicoli[3], Márcio M. Mattos[4] , Vagner Rodrigues Santos[5]

ABSTRACT

The purpose of this study was to evaluate an experimental animal model of oral chronic infection induced by A*ctinomyces israelii* and *Propionibacterium acnes* in mice. Swiss/NIH mice (n=100), 21 days of age, male and female were divided into two groups of 45 animals. *A. israelii* (n=45) and *P. acnes* (n=45) were inoculated in the anterior mandibular paraperiosteal periodontal tissue associated with sodium alginate gel particles. The animals were evaluated clinically and microscopically at 1, 3, 7, 15, 21, 30, and 45 days after inoculation. Actinomycotic and propioni lesions were induced in all animals. In control mice (n=10), no lesions were noted; however, differences in the clinical and histopathological evolutions of actinomycosis and propioni lesions were observed and are discussed in this study. Microorganisms entrapped in alginate gel provided a prolonged bacterial irritation, and chronic histopathologic features similar to those seen in human actinomycosis could be detected.

Uniterms: *Actinomyces. Propionibacterium acnes*. Experimental actinomycosis.

INTRODUCTION

Actinomyces spp and *Propionibacterium* spp form part of the normal human oral microbiota, but under certain circumstances they may become pathogenic[1-2]. Actinomycosis are sporadically occurring endogenous polymicrobial inflammatory processes in which fermentative actinomycetes of the genera *Actinomyces*, *Propionibacterium*, or *Bifidobacterium* act as the principal pathogens[3].

Injury to the oral mucosa allows the organism to penetrate the submucosal tissues[4]. Actinomycosis, a chronic, suppurative, granulomatous, and fibrosing disease, may subsequently develop[3, 5-7] and may be classified anatomically as cervicofacial, pulmonary, or ileo-caecal[6]. Clinically, this pathologic condition appears as a slowly-evolving induration in the mandibular-pre-auricular region, although it can occasionally be seen in the cervical and cranial skeleton. The lesion is often accompanied by fistular tracts to the skin that discharge typical sulfur granules[6].

Actinomyces israelii and *Propionibacterium acnes* can be isolated from various sites, including the oral mucosa, dental plaque, deep dental cavities, and periodontal pockets[8-11]. In general, however, it is difficult to induce experimental chronic inflammatory reactions such as those observed in human actinomycosis[12] and only a few published reports of experimental actinomycosis are available [13-17]. Recently, Asgor Moral et al.[16] and Sumita et al.[17] reported an animal model of chronic actinomycotic infection in the mouse peritoneum and cranium, respectively. These authors demonstrated that the use of an entrapping alginate gel is effective for the induction of chronic actinomycotic lesions. However, the peritoneum and cranium lesions are not satisfactory for an exact understanding of the clinical mandibular cervicofacial chronic lesion, since

[1] DDS, MSc. Laboratory of Microbiology and Biomaterials, School of Dentistry, UFMG, Brazil
[2] DDS, Laboratory of Microbiology and Biomaterials, School of Dentistry, UFMG, Brazil
[3] DDS, PhD. Laboratory of Microbial Ecology, Institute of Biological Sciences, UFMG, Brazil
[4] DDS, PhD. Laboratory of Pharmacology, School of Pharmacy, UFMG, Brazil
[5] DDS, PhD. Laboratory of Microbiology and Biomaterials, School of Dentistry, UFMG, Brazil.

the periodontal tissue is surrounded by bone that is very different from the peritoneum and cranium. The purpose of this work was to investigate and compare the infected lesions induced in mice by *Actinomyces israelii* and *Proprionibacterium acnes* using light microscopy, as well as clinical and cellular responses in an experimental oral model of actinomycotic and proprionic infection.

MATERIALS AND METHODS

Preparation of the inoculum and induction of the actinomycotic lesion

Wild strains of *A. israelii* (AIMIC01) and *P. acnes* (ATCC 33179), collected from oral lesions of patients attended to at the Stomatology Clinic, School of Dentistry, Universidade Federal de Minas Gerais (UFMG), identified morphologically and biochemically using the API-20A system (BioMérrieux, Marcy-l' Étoile, France), were grown in brain-heart infusion broth (Difco, USA), supplemented with 0.1% Hemin and 0.1% Menadione (Sigma, USA) at 37ºC for 6 days in an anaerobic globe box (Type Forma Scientific, Marietta, USA) with an atmosphere of 5% CO_2, 10% H_2, and 85% nitrogen. The bacteria were harvested by centrifugation, washed twice with sterile saline, and suspended in 1% solution of sodium alginate[16]. The suspension was agitated and then dropped into 0.07M $CaCl_2$ solution to form gel particles (0.8-1mm diameter) containing bacteria. The particles in the solution were stirred with a magnetic stirrer for 5 min to prevent their aggregation during curing. The particles were then collected in a stainless-steel wire net of 0.5 mm diameter mesh to eliminate smaller particles, washed twice with sterile saline, and suspended in 5 ml of sterile saline. The particles contained 7.5×10^6 bacteria/ml. 100 Swiss/NIH mice, 21 days of age, male and female (Ecology and Fisiology Microorganism Laboratory - UFMG - Brazil) were divided into two groups of 45 and were inoculated in the paraperiosteal periodontal tissue in the anterior mandibular area with *A. israelii* and *P. acnes*, respectively. Similarly, gel particles without bacteria were also prepared and injected into 10 mice, as a negative control. These experiments were approved by the Ethics Committee in Animal Experimentation (Nº 11/2002).

Clinical evaluation and Microscopy

Clinical evaluation and biopsy materials were performed at 1, 3, 7, 15, 21, 30, and 45 days after inoculation. Edema, hyperemia, abscess and/or fistula, and suppurative presence were analyzed during clinical observation. At each stage, the animals were anaesthetized by intraperitoneal injection of chloral hydrate (400mg/Kg) and perfused through the ascending aorta with physiological saline, followed by 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.4)[16]. The mandibular specimens, with lesions induced along the adjacent tissue, were removed and immersed overnight in the same flask before demineralization in a 10% nitric acid at 4ºC for 72 hours. The demineralized tissues were dehydrated through a graded series of ethanol and embedded in parafin. Parafin sections, of 5 μm in thickness, were stained with haematoxylin-eosin (HE), and Brown & Brenn[18]. The subjective criteria for the evaluation and classification of the tissue alterations were adapted, as previously described[19].

Bacteria identification from recovered induced lesions

The induced lesions were removed aseptically, homogenized in 1 ml of phosphate-buffered saline (pH 7.0) with a sterile glass homogenizer, and the dispersed samples diluted 10-fold (10^1-10^6) with the same buffered saline. Portions of 0.1 ml of the dispersed original solution and each dilution were cultured on brain-heart infusion blood agar plates supplemented with 0.1% Hemin and 0.1% Menadione (Sigma, USA) at 37ºC for 7 days in an anaerobic globe box (Type Forma Scientific, Marietta, USA) with an atmosphere of 5% CO_2, 10% H_2, and 85% nitrogen. [20] Bacterial genera and species were identified according to Bergey's manual[16, 21].

RESULTS

The viability and purity of cultured bacteria was tested by observing colony morphology, staining, and biochemical characteristics of the microorganisms collected at the moment before the animals' death. The collected microorganisms were compatible with *A. israelii* and *P. acnes*.

The excrement culture in tioglicolate broth, when compared to the culture of samples of the buccal injuries, did not display the presence of *A. israelii* and *P. acnes* in excrements, demonstrating that these microorganisms did not colonize the animals studied.

Clinical aspects

Actinomycotic (n=45) and proprioni (n=45) lesions were induced in all the studied animals. In control mice (n=10) no lesions were noted.

In actinomicotic infected mice, the skin was firm, elevated, and hair loss was noted, producing a red appearance. The abscess was observed after 45 days, and fistulas appeared seven days later. Fistulas remained until the sacrifice of the animals and presented various stages of the acute phase. The abscesses and fistulas, when located in the submandibular region, presented a crust and a region of underlying fibrosis, indicative of chronic abscess.

In proprionic infected mice, the skin was firm, elevated and had a red appearance. Acute abscesses were observed on the second day after inoculation and the appearance of fistulas was observed after the 4th day. Fistulas remained for a short duration, and, on the 15th day, no fistulas were observed. The acute inflammatory process did not return, and, after 30 days, clinical signs of inflammation were not observed. Injury remission was seen between the 14th and 30th days after inoculation.

Microscopy

After 1 day, all animals presented a mass of unstained amorphous material in the core of the lesion, in which bacteria were scattered, corresponding to the injected alginate gel containing *A. israelii* and *P. acnes*.

After 1 day of inoculation, *A. israelii* inoculated animals (n=45) were seen to be infiltrated by neutrophils and presented areas of necrosis; these alterations were not observed in adjacent soft tissues and the medular spaces of the alveolar bone. The Brown & Brenn-stained sections revealed that numerous Gram-positive bacteria were present in the alginate gel and in the neutrophils. In the control groups, numerous neutrophils were seen surrounding the alginate gel, although their number and aggregation were lower than in the experimental group. After 3-7 days, neutrophils were the predominant cells. In the Brown & Brenn-stained sections, Gram-positive bacteria appeared both in the alginate gel and in the masses of neutrophils. In the control group, there were fewer neutrophils surrounding the lesion, and, after 7 days, macrophage-like round cells with a rich cytoplasm could be observed. The alginate gel tended to disappear in the control, similarly to the previous stage. After 15-21 days, it could be observed that large foamy cells had emerged in the area and eosinophilic amorphous structures containing degenerate neutrophils could be observed invading the alginate gel islands. The cytoplasm of foamy cells contained clear and/or brown granules. Bacteria and neutrophils decreased in number. In the control group, no lesion was found between the muscle layer and periosteum. After 30 days, the lesion had become static. The number of bacteria decreased considerably, while the number of large foamy cells increased and invaded the alginate gel islands, taking the place of the neutrophils. A conspicuous collagenous capsule was seen around and in the alginate gel and surrounded the individual islands as well as the whole lesion. Bacteria were recognizable in the gel, but their number was considerably reduced. Plasma cells and lymphocytes were predominant. After 45 days, the number of neutrophils had decreased considerably. Foam cells remained, and the lesions became smaller than in the previous stages. A collagenous capsule separated the lesion and individual islands from the intact tissue. A few bacteria were recognizable in the gel.

One day after inoculation with *P. acnes* (n=45), infiltration by neutrophils and necrosis areas were observed. The Brown & Brenn-stained sections revealed that numerous Gram-positive bacteria were present in the alginate gel and in the neutrophils. In the control group, numerous neutrophils surrounded the alginate gel, although their number and aggregation were lower than in the experimental group. After 3-7 days, the predominant cellular elements were neutrophils. In the Brown & Brenn-stained sections, Gram-positive bacteria appeared both in the alginate gel and in the masses of neutrophils. In the control group, there were fewer neutrophils surrounding the lesion, and, after 7 days, macrophage-like round cells with a rich cytoplasm could be observed. The alginate gel tended to disappear in the control, similarly to the previous stage. After 15-21 days, the lesion was less developed. A few large foam cells had emerged in the area of eosinophilic amorphous structures containing degenerate neutrophils and invaded the alginate gel islands. Bacteria and neutrophils had decreased in number. In the control group, no lesion was found between the muscle layer and periosteum. After 30 days, the lesion had become static. Numbers of bacteria had decreased considerably, while a few foam cells had invaded the alginate gel islands, taking the place the few neutrophils. No collagenous capsules were observed surrounding the individual islands. After 45 days, the number of neutrophils and foam cells had decreased considerably. The tissue structure returned to normality with the disappearance of foam neutrophils and cells. No other signs of inflammation were observed

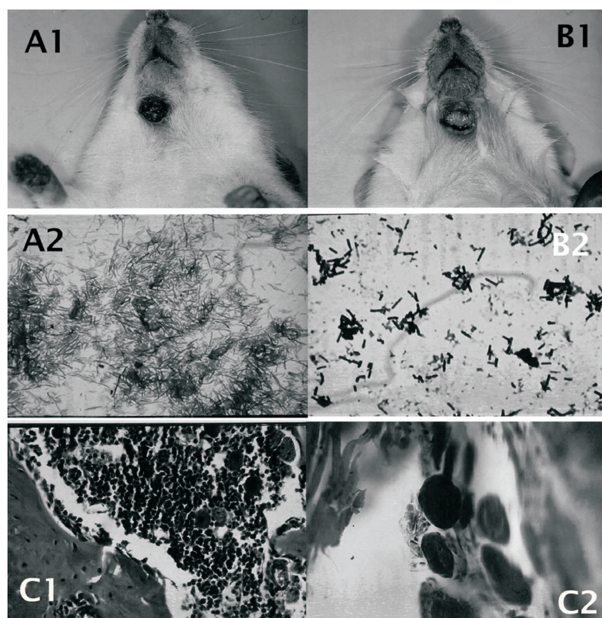

Figure 1 – Clinical aspects of actinomycotic (A1) and *Propionibacterium* (B1) lesions. Gram-staining microbiological aspects of Gram-positive filamentous *Actinomyces israelii* (A2) and Chinese shaped rods *Propionibacterium acnes* (B2). (C1) Haematoxylin and eosin staining sagital section of *Actinomyces* group - Magnification 250x. Aggregation of neutrophils is observed. Numerous neutrophils surround the alginate gel. (C2) Alginate gel is seen between the alveolar bone: *Propionibacterium* group. - Magnification 400x.

DISCUSSION

This study follows the experimental model considered by Asgor Moral et al.[16]; however *P. acnes* was added to the experiment. *P. acnes* is an autoctone bacterium of the mouth related to root canal infections and periodontal abscesses[9-11] and has been isolated from cervicofacial actinomycotic lesions[3]. *P. acnes* present low virulence, and its capacity to cause infections seems to depend on its association with other microorganisms[22]. In this study, *P. acnes* caused abscesses; however, the remission of infection presented a 30-day limited durability.

Moral Asgor et al.[16] did not observe any clinical signs/symptoms in the infected animals. In our study, acute abscesses were diagnosed after two days in *A. israelii* infected animals. Clinically, the abscesses remained until day 45 after inoculation in the animals carrying *A. israelli*, while in those with *P. acnes*, remission of the injuries occurred on day 14. This result demonstrates the high virulence of the wild sample of *A. israelii* when compared to *P. acnes* and to *A. israelli* (ATCC 10048) used by Asgor Moral et al.[16]. In this work, clinical features are very important to provide an exact understanding of actinomycotic periodontal chronic lesions. Abcesses and extra-buccal fistulas showed the chronic nature of the lesion after 30 days.

Numerous researchers have attempted to establish an animal model of periapical infection[23-25]; however, no studies regarding actinomycosis induced in the periodontal tissue of laboratory animals are available in the literature. Furthermore, authors have mainly demonstrated an acute inflammatory response that differs significantly in clinical pathology from that of cervicofacial actinomycosis, which is a chronic process. We demonstrate in this study that infections can be maintained for at least 45 days and that they can cause chronic inflammation in the periodontal tissue. Other authors demonstrated the presence of experimental chronic lesions at 120 days in the cranium[16]. The mandibular alveolar trabeculae and the surrounding soft tissues differ from those of the cranium. Moreover, we believe that the appearance of abscesses on day 2, which remain until day 45 in the animals with *A. israelii,* is related to the high virulence of the microorganism used. Lyophilized samples and long time culture of the samples can interfere with the biology of the microorganism and can delay the immune response of the host. Microorganisms were entrapped in alginate gel, thus bacterial irritation of the host immune system was prolonged.

Therefore, in this study the progression of the disease to the chronic stage was faster when compared to other reports[16-17]. In this experiment, actinomycotic lesions[12-17] had some histopathological features identical to those of clinical actinomycotic lesions[26-27], including periapical actinomycosis[5, 23, 28-29] and the so-called sulfur-granules by which this disease is diagnosed as actinomycosis.

The *Proprionibacterium* lesions induced in the periodontal tissue have not been reported before in the literature. In this study, *P. acnes* demonstrated aggressiveness, despite its low virulence, and the microorganism was able to induce similar injuries to those seen in actinomycosis, although to a lesser degree when compared to *A. israelii*.

The wild *A. israelii* used in this study represented a “rough” strain, which grows *in vitro* granulously and densely packed with intermessed filaments. Physically, this characteristic may represent a challenge for neutrophils and macrophage phagocytose[15], explaining the aggressiveness of the microorganism. This aggressiveness is represented by the abscess formation two days after inoculation, followed by chronic injury over a shorter time period.

Asgor Moral et al.[16] used the ATCC10048 strain, called the "smooth" strain.

Based on the present results and earlier studies [6, 13-14, 22, 30], it seems possible that *A. israelii*, and *P. acnes* by itself, can induce human actinomycotic lesions. However, the source of *A. israelii* in natural infections would most likely include mixtures of other organisms. Colonization of the oral cavity in germ-free rats by *A. israelii* and *P. acnes* is difficult and requires repeated inoculations[15].

CONCLUSION

The main feature of this animal model is the development of a persistent oral actinomycotic lesion analogous to chronic actinomycosis diagnosed in humans. Thus, it could be considered as a relevant animal model of clinical chronic cervicofacial pathosis, and might help us to understand the mechanisms of cervicofacial chronic inflammation.

RESUMO

O objetivo deste trabalho foi avaliar um modelo animal de infecção oral crônica induzida por *Actinomyces israelli* e *Propionibacterium acnes* em camundongos. Camundongos Swiss/NIH (n=100), de 21 dias de idade, machos e fêmeas, foram divididos em dois grupos de 45 animais. *A. israelli* (n=45) e *P. acnes* (n=45) foram inoculados no periodonto paraperiosteal da região anterior da mandíbula com partículas de gel de alginato com o respectivo microorganismo. Os animais foram avaliados clínica e microscopicamente 1, 3, 7, 15, 21, 30 e 45 dias após a inoculação. Lesões infecciosas foram induzidas em todos os animais. Nos animais controle (n=10) nenhuma lesão foi detectada. Diferenças na evolução clínica e histopatológica das lesões induzidas por *Actinomyces* e *Propionibacterium* foram observadas e são discutidas neste trabalho. Concluiu-se que microorganismos incluídos em gel de alginato produziram uma irritação bacteriana prolongada e características histopatológicas semelhantes àquelas detectadas em seres humanos puderam ser visualizadas. Sugere-se com este estudo que tal modelo animal pode ser utilizado como uma ferramenta auxiliar para o entendimento dos mecanismos patológicos envolvidos na inflamação crônica cérvico-facial.

Descritores: *Actinomyces israelii*, *Propionibacterium acnes*. Actinomicose.

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