行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※
※　　　　　　　　　　　　　　　　　　　　　　　　※
※　　　　以動物模型研究牙周病菌的混合感染　　　　※
※　　　　　　　　　　　　　　　　　　　　　　　　※
※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：¦**個別型計畫**　　□**整合型計畫**
計畫編號：NSC　89-2314-b-040-025
執行期間：　89　**年**　8　**月**　1　**日至**　90　**年**　7　**月**　31　**日**

計畫主持人：林育誼
共同主持人：錢佑

本成果報告包括以下應繳交之附件：
□**赴國外出差或研習心得報告一份**
□**赴大陸地區出差或研習心得報告一份**
□**出席國際學術會議心得報告及發表之論文各一份**
□**國際合作研究計畫國外研究報告書一份**

執行單位：中山醫學院口腔醫學研究所

中　華　民　國　九十　　年　十　　月　二十　　日

行政院國家科學委員會專題研究計畫成果報告
以動物模型研究牙周病菌的混合感染

Mixed infection of periodontal pathogens in an animal model

計畫編號：NSC 89-2314-b-040-025
執行期限：89 年 8 月 1 日至 90 年 7 月 31 日
主持人：　林育誼　中山醫學院口腔醫學研究所
共同主持人：　錢佑　中山醫學院微生物學研究所
計畫參與人員：黃漢欽 中山醫學院口腔醫學研究所
賴佑瑩 中山醫學院口腔醫學研究所

中文摘要

本研究利用動物模型 (Kinetic *in situ* Subcutaneous Chamber Model, KSCM) **觀察不同牙周病菌感染在此一模型所造成之病理變化，紀錄病變部位宿主免疫細胞種類及數目之變化，細菌數目和毒性表現之消長，以及抗體和發炎物質。***Pg* HG405 **單一感染在** KSCM **動物模型中造成局部發炎及膿瘍，相反的，***Bf* **或** *Fn* **細菌本身甚至無法存活於** KSCM **中。當** *Pg* **與** *Fn* **共同混合感染於此一動物模型時, 所需** *Pg* **之感染劑量可降至原劑量的十分之一。我們數據顯示此一** *Fn* **對** *Pg* **感染的協力作用** (synergism) **與細菌間凝集作用有關。另一方面，***Bf* **在** *Fn* **存在下亦能生存於此一動物模型並引致感染。***Fn* **對** *Bf* **的協力作用可被外來的營養素所取代。本研究指出當不同牙周病菌混合感染時，所需致病之劑量遠低於單一感染所需之劑量。**

關鍵詞：牙周病、協力作用、混合感染、動物模型

Abstract

A Kinetic *in situ* Subcutaneous Chamber Model (KSCM) was used in this study for the evaluation of the molecular, cellular and microbial events associated with the host-pathogen interactions. In these studies we have kinetically analyze the pathologic course of events leading to the local abscess formation that characterizes the clinically relevant scenario. In addition to the progress of macroscopical pathologies, the shift and reactions of host immune cell population and the expression of bacterial virulence were kinetically recorded. Pathogenic synergism between *Pg* and *Fn* has been demonstrated. Our data showed 1/10 original *Pg* infected dosage was capable of causing the same pathology in KSCM when co-inoculated with *Fn*. Both *Pg* and *Bf* produce trypsin-like enzyme and sialidase, which are thought to be involved with bacterial virulence and tissue destruction. The current study evaluated the outcomes of monoinfectious challenge with *Fn* or *Bf* in the KSCM animals and determined the influences of *Fn* inoculation on *Bf* infection, and synergy mechanism of this mixed infection. An exogenous nutrient supply is sufficient to replace the symbiotic effect of *Fn* on *Bf* indicating the synergism between these two bacteria is simply a nutritional dependence. We concluded that mixed infection needed far less bacterial dosage to induce infection in our animal model than monoinfection.

Keywords: animal model, bacterial synergism, *Bacteroides forsythus*, *Fusobacterium nucleatum*, mixed infection, periodontitis, *Porphyromonas gingivalis*

Introduction

Chronic periodontitis has been associated with gram negative anaerobic bacteria, among which *Porphyromonas*

gingivalis (*Pg*) ,*Fusobacterium nucleatum* (*Fn*), *Bacteroides forsythus* (*Bf*) are commonly encountered(1-4). Isolation of a single bacterial species from chronic abscesses is rather uncommon and when it occurs the bacterium frequently possesses a unique feature like an anti-phagocytic capsule, which allows it to survive the host response in this hostile, neutrophil flooded environment of the abscess(5-8).

Several mechanisms have been proposed, that may allow bacteria to survive in the hostile host environment. Some of them like the antiphagocytic capsule will allow only the bacterium having them to survive(4;9;10). Others, like complement and immunoglobulin degrading enzymes, may benefit also a bystanding partner. Many pathogenic *Pg* and *Bf* strains possess such proteases which are important in their ability to evade phagocytosis. This may potentially explain why other bacteria may associate with these strains in abscesses, but do these strains have a reason to associate with their partners such as *Fn*?

Induction of experimental abscesses in animals, using mildly pathogenic strains, requires relatively large inocula of $1 \times 10^9 - 1 \times 10^{10}$ viable bacteria. Including other bacteria in the inoculum has been shown to reduce the minimal infective dose. *Fusobacteria*, on the other hand, have been found to require additives like gastric mucin or agarose to allow them to reproducibly form abscesses in experimental animals. It is of interest that in both these examples it has been reported that when abscesses did form, the bacteria could be found in them in clumps or aggregates.

Coaggregation among oral bacterial strains including *Pg* and *Fn* has been extensively studied and is dependent on a plethora of bacterial adhesions which mediate this phenomenon. Interestingly, in an extensive study by Kolebrander *et al*, *Fn* was found to have many coaggregating partners(11-13), however this bacterium was the only coaggregating partner for *Porphyromonas gingivalis*. Most of these coaggregatioin studies were however aimed to elucidate the role of these adhesions in the complex events of dental plaque formation.

The present study was conducted to explore the possibility that such coaggregations may have yet another role in the pathogenicity of these bacteria: protecting the coaggregating pair from the host response.

Results

When equal numbers of *Pg* HG405 and *Fn* PK-1594 were mixed at room temperature, coaggregation occurred. The coaggregate was clearly seen but did not settle down immediately and could therefore be graded as 2+ by the Cisar scale. *Pg* A7436 and *Fn* PK-1594 on the other hand did not form any coaggregates (0 by Cisar's scale)

When examined microscopically, large aggregates could be seen in the first mixture, of *Pg* HG405 with *Fn* PK1594, with no free cells in the area between the aggregates, which corresponded with level (a) in the microscopic assay. With the second combination the cells were found in single cell dispersion, corresponding to level (d) in this assay.

When tested in the Vmax coaggregation assay a clear influence of the bacterial proportions on the rate of coaggregation could be demonstrated (figure1). The highest coaggregation rate was observed at *Pg* Hg405 to *Fn* PK1594 ratios of 3 to 1 and 2 to 1, while increasing the ratio in favor of the *porphyromonads* or decreasing it, in favor of the *Fusobacteria* lowered the rate of coaggregation (figure 1) The other bacterial pair did not coaggregate over the whole range of ratios from 10:1 through 1:10. (data not shown)

Galactose at 60 mM inhibited the formation of macroscopic coaggregates of *Pg* HG405 with *Fn* PK1594. This was also verified by microscopic examination that revealed uniform dispersion of the two bacterial strains with no aggregates formed (a) turned to (d).

The Vmax coaggregation assay also demonstrated that galactose at concentrations raging from 60 mM down to 0.6 mM effectively blocked the coaggregation of an optimal *Pg* to *Fn* ratio of 1:3 (figure 2)

Galactose at this concentration was also effective in fully dispersing previously formed coaggregates of these bacteria, to a level of single bacterial cell dispersion (data not shown).

Inoculation of the chamber with 1 x 10^9 *Pg* HG405, resulted in a successful colonization of the chambers that led to abscess formation in 100% of the animals and to sloughing of all the chambers on days 12-14 (table 1).

Inocula of 3 x 10^8 bacteria resulted in abscess formation and sloughing on days 12-14 in 60% of the chambers. On the other hand, inocula of 1 x 10^8 *Pg* HG405, uniformly failed to establish an infection: The bacteria could be detected in the chamber fluid on day 1 but by day 6 no bacteria could be detected any more in the fluid taken from any of the chambers (fig.3B). These chambers did not develop any significant thickening of the connective tissue around them and failed to develop an abscess or sloughing. In fact, macroscopically these chambers were undistinguishable from the control chambers, sham injected with sterile culture medium. This dose of 1 x 10^8 *Pg* HG405 was therefore defined as "sub-infective dose" and used as such in the following experiments.

Fn inocula of 1 x 10^9 bacteria successfully colonized 100% of the chambers. Viable bacteria could be detected in the chamber on day 1 (1.6 x 10^7 CFU/ml) (figure 3a) and their number gradually increased to 3.5 x 10^9 CFU/ml by day 6 and remained high through day 9 (5.4 x 10^8 CFU/ml) and day 15 (8 x 10^7 CFU/ml).

Macroscopic examination of the chambers revealed thickening of the connective tissue around the chamber by day 17. Swelling around the chamber appeared on day 20-21, however draining abscess formation or sloughing could not be seen until day 28-35. The content of this abscess was markedly different from that of the Pg containing abscesses: Rather than being liquid pus it was of highly viscose nature.

Adding 1 x 10^9 Fn PK 1594 to the chambers receiving 1 x 10^9 Pg HG405, dramatically changed the outcome of the infection. Rather than forming an abscess and sloughing on day 12-14, all the chamber containing the mixed infection developed an abscess and sloughed the chamber on days 4-5 (table 1)

Microscopic examination of the cytocentrifuge preparations revealed mixed inoculum of sub-infective dose of Pg HG405 with 1 x 10^9 Fn PK 1594. The presence of 1 x 10^9 *Fn* PK1594 in the inoculum uniformly changed the outcome of the inoculation with a sub-infective dose (1 x 10^8) of *Pg* HG405. Rather than clearing of the infection, the *Pg* successfully colonized the chambers. It could now be detected in the chamber fluid on day 1 in much higher numbers: 1.3 x 10^9 CFU/ml, as compared to 1.4 x 10^5 when injected alone (figure 3). Its numbers gradually increased and by day6, 2.4 x 10^{10} CFU/ml could be detected as compared to none in the control. The numbers remained high (1.4 x 10^{10} CFU/ml) on day 9. On day 9 swelling of the tissues surrounding the chamber was evident, followed by abscess formatioin and sloughing of 100% of the chambers on days 10-12 (table 2).

Reference

1. El-Bastawissi AY, Williams MA, Riley DE, Hitti J, Krieger JN. Amniotic fluid interleukin-6 and preterm delivery: a review. Obstetrics & Gynecology 2000;95:1056-1064.

2. Atkins GJ, Haynes DR, Geary SM, Loric M, Crotti TN, Findlay DM. Coordinated cytokine expression by stromal and hematopoietic cells during human osteoclast formation. Bone 2000;26:653-661.

3. Kong YY, Boyle WJ, Penninger JM. Osteoprotegerin ligand: a common link between osteoclastogenesis, lymph node formation and lymphocyte development. Immunology & Cell Biology 1999;77:188-193.

4. Ammann P, Bourrin S, Bonjour JP, Meyer JM, Rizzoli R. Protein undernutrition-induced bone loss is

associated with decreased IGF-I levels and estrogen deficiency. Journal of Bone & Mineral Research 2000;15:683-690.

5. Hentunen TA, Choi SJ, Boyce BF, et al. A murine model of inflammatory bone disease. Bone 2000;26:183-188.

6. Kong YY, Feige U, Sarosi I, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 1999;402:304-309.

7. Ebbesen EN, Thomsen JS, Mosekilde L. Nondestructive determination of iliac crest cancellous bone strength by pQCT. Bone 1997;21:535-540.

8. Turner CH. On Wolff's law of trabecular architecture. Journal of Biomechanics 1992;25:1-9.

9. Teitelbaum SL. Osteoclasts, integrins, and osteoporosis. Journal of Bone & Mineral Metabolism 2000;18:344-349.

10. Steffen MJ, Holt SC, Ebersole JL. Porphyromonas gingivalis induction of mediator and cytokine secretion by human gingival fibroblasts. Oral Microbiology & Immunology 2000;15:172-180.

11. Koide M, Okahashi N, Tanaka R, et al. Inhibition of experimental bone resorption and osteoclast formation and survival by 2-aminoethanesulphonic acid. Archives of Oral Biology 1999;44:711-719.

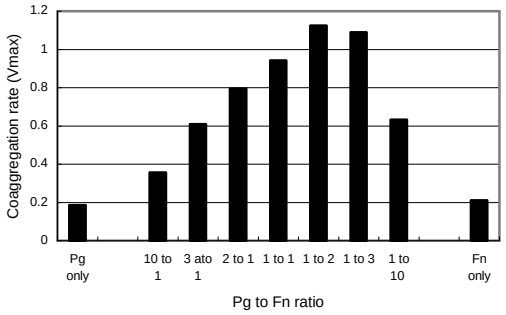
12. Kadono H, Kido J, Kataoka M, Yamauchi N, Nagata T. Inhibition of osteoblastic cell differentiation by lipopolysaccharide extract from Porphyromonas gingivalis. Infection & Immunity 1999;67:2841-2846.

13. Kong YY, Boyle WJ, Penninger JM. Osteoprotegerin ligand: a regulator of immune responses and bone physiology. Immunology Today 2000;21:495-502.

Figure and Table

Figure 1

Coaggregation of Pf HG405 with Fn PK-1594.



Effect of Pg to Fn ratio. Each bar represents a man Vmax value of 4 wells. Standard error < 10%.

Figure 2

Effect of galactose on the coaggregation of Pg HG405 and Fn PK-1594. Pg to Fn ratio =1 : 3. Each bar represents a mean of 4 wells. Sandard error < 10%.

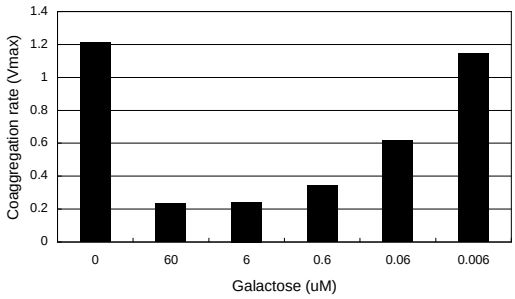


Figure 3

Viable bacteria in subcutaneous chambers inoculated with mixed infection of Pg HG405 with Fn PK-1594. The inoculum injected on day 0 consisted of either 1 x 10^9 Fn alone or 1 x 10^8 Pg alone or a mixture of both. (a) Fn in the chamber fluik, with or without Pg presence. (b) Pg in chamber fluid, with and without Fn presence. Mean CFU/ml ± SEM

Figure 3a

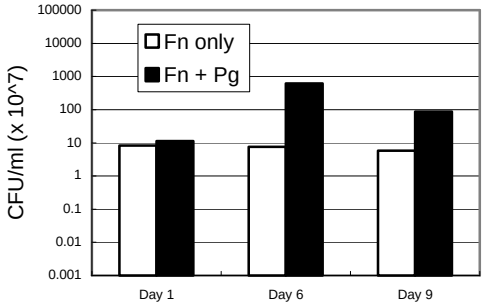


Figure 3b

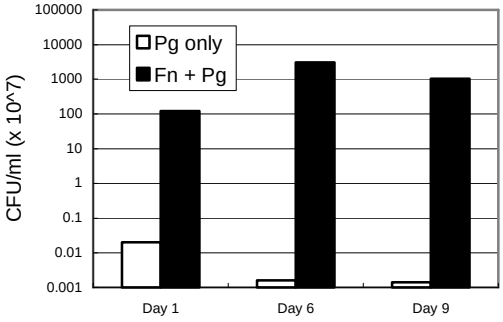


Figure 4

Effect of galactose on bacteria in subcutaneous chambers inoculated with mixed infection of Pg HG405 and Fn PK1594. (a) Fn in chamber fluid, Fn alone in the chamber. (b) Fn in the chamber fluid,

when Pg is present in chamber (c) Pg in chamber fluid, when Fn is present in the chamber. Mean CFU/ml ± SEM.

Figure 4a

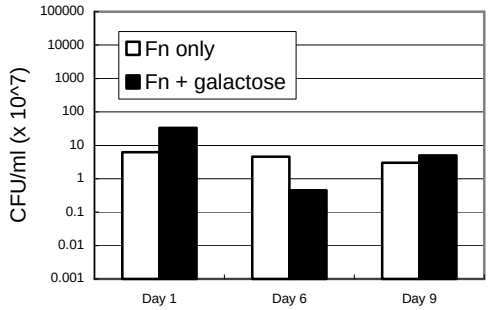


Figure 4b

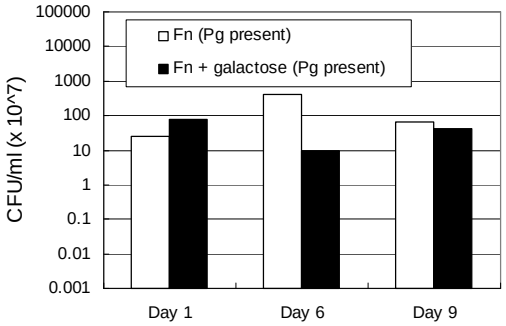


Figure 4 c

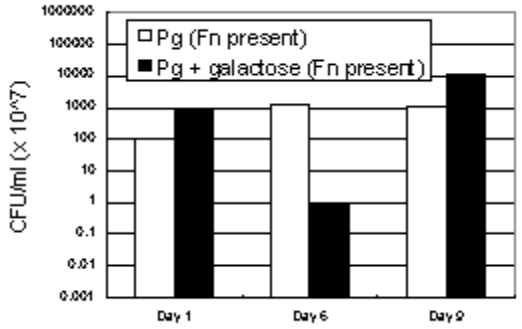


Table 1
Pathology induced by *Pg* monoinfection

Pg	**Lesion**	**Affected percentage**	**Duration of pathology**
1 x 10^9	Draining abscess	100%	12-14 d
3 x 10^8	Draining abscess	60%	12-14 d
	Infection cleared	40%	--
1 x 10^8	Infection cleared	100%	--

Table 2
Comparisons of *Pg* alone, *Fn* alone and mixed infection induced pathologies

Inocula	**Dosage**	**Lesion**	**Affected percentage**	**Duration of pathology**
Pg *Fn*	1 x 10^9 1 x 10^9	Draining abscess	100%	5-6 d
Pg *Fn*	1 x 10^8 1 x 10^9	Draining abscess	100%	12-14 d
Pg	1 x 10^8	Infection cleared	100%	--
Fn	1 x 10^9	Infection persisted	100%	>25 d