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MODULATION DE LA RÉPONSE INFLAMMATOIRE DE L'HÔTE PAR LES BACTÉRIES PARODONTOPATHOGÈNES : MÉCANISME DE PATHOGÉNICITÉ ET CIBLE THÉRAPEUTIQUE POTENTIELLE

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Résumé

Les parodontites sont des infections polymicrobiennes aux étiologies et pathogénies variées qui se traduisent par des destructions plus ou moins sévères des tissus parodontaux. Parmi plus de 500 espèces microbiennes dénombrées dans la plaque sous-gingivale, un complexe bactérien nommé complexe rouge, composé de *Porphyromonas gingivalis*, *Treponema denticola* et *Tannerella forsythia*, a été fortement associé aux lésions parodontales avancées. L'évolution des parodontites dépend à la fois de la virulence des bactéries parodontopathogènes et de l'intensité de la réponse immunologique de l'hôte. Certains patients atteints de parodontite chronique répondent d'une façon inadéquate au traitement classique de cette maladie consistant à éliminer la plaque bactérienne sous-gingivale. Par conséquent, des solutions thérapeutiques complémentaires visant à moduler la réponse de l'hôte ou à atténuer la virulence des bactéries parodontopathogènes pourraient être utiles pour traiter ces patients. Le but de cette étude était d'investiguer la capacité des bactéries parodontopathogènes à favoriser la réponse immunodestructrice de l'hôte ainsi que d'évaluer l'effet des constituants de la canneberge sur les mécanismes pathogéniques des parodontites. La capacité de *P. gingivalis* à moduler la production de médiateurs inflammatoires a été mise en évidence dans un modèle de co-culture de cellules épithéliales et de macrophages et dans un modèle *ex vivo* de sang complet. Nous avons aussi rapporté que les bactéries du complexe rouge agissaient de concert pour induire la production de cytokines, de prostaglandine E_2 (PGE_2) et de métalloprotéinase matricielle 9 (MMP-9). L'ensemble de ces résultats met en évidence le potentiel des bactéries parodontopathogènes à favoriser la réponse immunodestructrice de l'hôte. De plus, des effets synergiques entre l'hémoglobine et le lipopolysaccharide (LPS) de bactéries parodontopathogènes sur la réponse inflammatoire des macrophages ont été mis en évidence. Par ailleurs, la capacité d'une fraction de canneberge de haut poids moléculaire à diminuer la production de médiateurs inflammatoires (cytokines, chimiokines et PGE_2), de MMP-3 et de MMP-9 par des macrophages et des fibroblastes gingivaux stimulés avec le LPS de bactéries parodontopathogènes a été démontrée. De plus, cette fraction de canneberge s'est avérée capable d'inhiber l'activité de la MMP-3, de la MMP-9 et de l'élastase. Enfin, cette fraction a également démontré une capacité à inhiber la formation du biofilm et l'adhérence de *P. gingivalis* aux protéines humaines ainsi que les activités protéolytiques des bactéries du complexe rouge. L'ensemble de ces résultats suggère que les polyphénols de la canneberge pourraient contribuer à atténuer la réponse immunodestructrice de l'hôte et la virulence des bactéries parodontopathogènes, offrant ainsi des perspectives prometteuses pour le développement d'un traitement complémentaire pour les parodontites.

Abstract

Periodontitis are polymicrobial infections leading to destruction of the tooth supporting tissue. Among 500 bacterial species enumerated in the subgingival plaque, members of the red complex, which includes *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, are strongly related to more advanced periodontal lesions. Two major factors are involved in the pathogenesis of periodontitis, bacteria themselves and the host immune response to these periodontopathogens. Some patients with periodontitis do not respond correctly to conventional therapy consisting in the removal of the subgingival plaque. Consequently, adjunctive therapeutic solutions based on the attenuation of periodontopathogens virulence or of host response may be useful for periodontitis treatment. The aim of this study was to investigate the capacity of periodontopathogens to favor the host immunodestructive response and to evaluate the potential beneficial effects of cranberry constituents on the pathogenic mechanisms of periodontitis. The capacity of *P. gingivalis* to modulate inflammatory mediator production was characterized in a macrophage/epithelial cell co-culture model as well as in a whole blood model. We have also reported that bacteria of the red complex act in concert to induce the production of cytokines, prostaglandin E_2 (PGE_2) and matrix metalloproteinases 9 (MMP-9). Moreover, synergistic effects between hemoglobin and lipopolysaccharides (LPS) from periodontopathogens on the macrophage inflammatory response were demonstrated. In addition, the capacity of a high molecular weight cranberry fraction to reduce the production of inflammatory mediators (cytokines, chemokines and PGE_2) and MMPs (MMP-3 and MMP-9) by macrophages and fibroblasts stimulated with periodontopathogen LPS was shown. This fraction also inhibited the activity of MMP-3, MMP-9 and elastase. Finally, this cranberry fraction showed the capacity to inhibit adhesion and biofilm formation of *P. gingivalis* as well as proteolytic activities of bacteria of the red complex. These results suggest that cranberry polyphenols may contribute to limit the host immunodestructive response and periodontopathogen virulence, offering thus promising perspectives for the development of an adjunctive treatment for periodontitis.

Avant-propos

Je tiens à remercier sincèrement toutes les personnes qui ont apporté leur contribution à l'aboutissement de ce projet.

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Contributions des auteurs

Chapitre 1

Un article de revue de littérature est présenté dans le cadre de l'introduction (section 1.5). Cet article a été accepté dans la revue « Pathologie Biologie » sous la référence : Bodet, C., F. Chandad, and D. Grenier, Potentiel pathogénique de *Porphyromonas gingivalis*, *Treponema denticola* et *Tannerella forsythia*, le complexe bactérien rouge associé à la parodontite, *Pathol Biol (Paris)*, In press. L'article a été rédigé par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 2

Ce chapitre a été publié dans la revue « Microbes and Infection » sous la référence : Bodet, C., F. Chandad, and D. Grenier, Modulation of cytokine production by *Porphyromonas gingivalis* in a macrophage and epithelial cell co-culture model, *Microbes Infect,* 2005, 7(3):448-56. La dernière phase de la préparation des échantillons pour l'observation du modèle de co-culture en microscopie électronique à balayage a été réalisée par le Service de microscopie et histologie de l'Université Laval. Les expérimentations et la rédaction de cet article ont été effectuées par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 3

Ce chapitre a été publié dans la revue « Clinical and Experimental Immunology » sous la référence : Bodet, C., F. Chandad, and D. Grenier, *Porphyromonas gingivalis*-induced inflammatory mediator profile in an *ex vivo* human whole blood model, *Clin Exp Immunol*, 2006, 143(1):50-7. La collecte des échantillons de sang a été effectuée par Yannick Roussy et le dosage de certaines cytokines présentes dans les échantillons a été effectué par la compagnie « Upstate ; Lake Placid ». Les analyses statistiques ont été effectuées par le Service de statistiques de l'Université Laval. Les autres expérimentations et la rédaction de cet article ont été effectuées par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 4

Ce chapitre a été publié dans la revue « Microbes and Infection » sous la référence : Bodet, C., F. Chandad, and D. Grenier, Inflammatory responses of a macrophage/epithelial cell co-culture model to mono and mixed infections with *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, *Microbes Infect*, 2006, 8(1):27-35. Les expérimentations et la rédaction de cet article ont été effectuées par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 5

Ce chapitre a été soumis dans la revue « Journal of Dental Research » sous la référence : Bodet, C., F. Chandad, and D. Grenier, Synergistic effect of hemoglobin and periodontopathogen lipopolysaccharides on the inflammatory response of macrophages. Les LPS étaient disponibles au laboratoire. Les expérimentations et la rédaction de cet article ont été effectuées par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 6

Ce chapitre a été publié dans la revue « Journal of Dental Research » sous la référence : Bodet, C., F. Chandad, and D. Grenier, Anti-inflammatory activity of a high-molecular-weight cranberry fraction on macrophages stimulated by lipopolysaccharides from periodontopathogens, *J Dent Res*, 2006, 85(3):235-9. Les fractions de canneberge et les LPS étaient disponibles au laboratoire. L'analyse chimique de la fraction 1 a été réalisée par Robin Roderick (Ocean Spray Cranberries, Inc.) et les analyses statistiques ont été effectuées par le Service de statistiques de l'Université Laval. Les expérimentations et la rédaction de cet article ont été effectuées par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 7

Ce chapitre a été publié dans la revue « European Journal of Oral Sciences » sous la référence : Bodet, C., F. Chandad, and D. Grenier, Cranberry components inhibit IL-6, IL-8 and PGE_2 production by lipopolysaccharide-activated gingival fibroblasts, *Eur J Oral Sci*, 2007, 115(1):64-70. La fraction de canneberge et le LPS étaient disponibles au laboratoire. Les analyses des échantillons par une technique de « microarray » ont été effectuées par la compagnie « Kinex ; Vancouver » et les analyses statistiques ont été effectuées par le Service de statistiques de l'Université Laval. Les autres expérimentations et la rédaction de cet article ont été effectuées par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 8

Ce chapitre a été accepté dans la revue « Journal of Periodontal Research » sous la référence : Bodet, C., F. Chandad, and D. Grenier, Inhibition of host extracellular matrix destructive enzyme production and activity by a high molecular weight cranberry fraction, *J Periodontal Res*, 2007, 42(2):159-68. La fraction de canneberge et le LPS étaient disponibles au laboratoire. Les analyses de « microarray » ont été effectuées par la compagnie « Kinex ; Vancouver » et les analyses statistiques ont été effectuées par le Service de statistiques de l'Université Laval. Les autres expérimentations et la rédaction de cet article ont été effectuées par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 9

Ce chapitre a été publié dans la revue « Journal of Antimicrobial Chemotherapy » sous la référence : Bodet, C., M. Piché, F. Chandad, and D. Grenier, Inhibition of periodontopathogen-derived proteolytic enzymes by a high-molecular-weight fraction isolated from cranberry, *J Antimicrob Chemother*, 2006, 57(4):685-90. La fraction de canneberge était disponible au laboratoire. Les analyses statistiques ont été effectuées par le Service de statistiques de l'Université Laval. La majorité des expériences ont été réalisées par Marilou Piché sous la supervision du premier auteur, ce dernier ayant effectué certaines parties expérimentales (culture et préparation de bactéries, préparation de substrats, répétition de certaines expériences). La rédaction de cet article a été effectuée par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

Chapitre 10

Ce chapitre a été publié dans la revue « Journal of Antimicrobial Chemotherapy » sous la référence : Labrecque, J., C. Bodet, F. Chandad, and D. Grenier, Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of *Porphyromonas gingivalis, J Antimicrob Chemother*, 2006, 58(2):439-43. La fraction de canneberge était disponible au laboratoire. Les photos de microscopie électronique sur les biofilms de *P. gingivalis* ont été fournies par Fatiha Chandad. Les expériences sur la formation du biofilm et l'attachement de *P. gingivalis* aux surfaces ont été réalisées par Julie Labrecque. Le second auteur a réalisé les expériences sur la désorption du biofilm, la croissance et la viabilité de *P. gingivalis* et a participé à la rédaction du manuscrit. La rédaction de cet article a été effectuée par le directeur de recherche, Dr Daniel Grenier.

Appendice 1

Une revue de littérature est présentée en appendice. Cette revue a été soumise dans la revue « Critical Reviews in Food Sciences and Nutrition » sous la référence : Bodet, C., D. Grenier, F. Chandad, I. Ofek, D. Steinberg, and E.I. Weiss, Potential oral health benefits of cranberry. Les parties portant sur les constituants de la canneberge et la carie dentaire ont été écrites par le Dr Weiss, le Dr Steinberg et le Dr Ofek (Hebrew University-Hadassah, Jerusalem, Israel) alors que l'introduction et la partie sur la parodontite ont été écrites par le premier auteur sous la supervision du directeur de recherche, Dr Daniel Grenier, et de la co-directrice, Dre Fatiha Chandad.

À ma mère, ma grand-mère,
À Nelly,

« Savoir s'étonner à propos est le premier pas fait sur la route de la découverte »
Louis Pasteur

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Liste des abréviations

AP-1	Activator protein-1
APMA	*p*-aminophenylmercuric acetate
ATCC	American type culture collection
BHI	Brain heart infusion
BSA	Bovine serum albumin
COX2	Cyclo-oxygenase 2
DMEM	Dulbecco's modified Eagle's medium
DPP IV	Dipeptidyl peptidase IV
DTT	Dithiothreitol
EDTA	Acide éthylènediaminotétracétique
EGTA	Acide éthylèneglycoltétracétique
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fos	Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor
Hb	Hemoglobin
ICAM-1	Intracellular adhesion molecule 1
IFN-γ	Interferon gamma
IL	Interleukine
iNOS	Inducible nitric oxyde synthase
IP-10	IFN-γ-inducible protein 10
JNK	Jun N-terminus protein-serine kinases
Jun	Jun proto-oncogene-encoded AP-1 transcription factor
LPS	Lipopolysaccharide
LrrA	Leucine-rich repeat protein
MALDI-TOF	Matrix assisted laser desorption ionisation - time of flight
MAP	Mitogen-activated protein
MCP-1	Monocyte chemoattractant protein-1
MKK	MAP kinase protein-serine kinase
MMP	Matrix metalloproteinase
Mnk1	MAP kinase-interacting protein-serine kinase 1
MOPS	3-(N-morpholino) propanesulfonic acid
Msp	Major sheath protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NDM	Non-dialysable material
NO	Nitric oxyde
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
p38a MAPK	Mitogen-activated protein-serine kinase p38 alpha
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffer saline

PGE_2	Prostaglandin E_2
PMA	Phorbol myristic acid
Rac1	Ras-related C3 botulinum toxin substrate 1
RANTES	Regulated on activation normal T cell expressed and secreted
ROCK2	Rho-associated protein kinase 2
RPMI	Roswell park memorial institute
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDD	Subantimicrobial dose doxycycline
THB	Todd Hewitt broth
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor

CHAPITRE 1

INTRODUCTION

1.1. Écosystème buccal

La cavité buccale est un milieu chaud et humide offrant de nombreuses niches écologiques dans lesquelles cohabitent plusieurs centaines de micro-organismes commensaux. La microflore buccale résidente est composée de bactéries, de mycètes, de protozoaires et de virus dont l'abondance et la composition varient selon les individus et le niveau d'hygiène buccale. Cette microflore est majoritairement composée de bactéries appartenant à plus de 500 espèces différentes (Kroes *et al.*, 1999; Paster *et al.*, 2001). La bouche est un écosystème complexe où règne une grande compétition entres les micro-organismes qui y habitent (Darveau *et al.*, 1997). Des communautés microbiennes distinctes se retrouvent dans les différents types d'habitats de la cavité buccale incluant la langue, la muqueuse buccale, la plaque dentaire (sous-gingivale et supra-gingivale) et la salive. Cette microflore se constitue progressivement avec l'âge et varie suivant les déterminants écologiques et les facteurs immunologiques de l'hôte. La stabilité de cette microflore est due à l'équilibre des interactions complexes des micro-organismes entre-eux et des micro-organismes avec l'hôte. La complexité de cet écosystème implique une organisation structurale rigoureuse des bactéries (formation de biofilm) et des interactions bactériennes spécifiques et hiérarchisées notamment de type adhésives et nutritionnelles. Bien que la microflore buccale colonise l'ensemble de la bouche, la plaque dentaire constitue un habitat bactérien important de la cavité buccale.

1.2. Plaque dentaire

La plaque dentaire est une accumulation bactérienne à la surface de l'émail et du cément des dents à laquelle est associée entre autre des protéines de l'hôte et des résidus alimentaires. On estime qu'un milligramme de plaque contient environ 100 millions de bactéries. Ces bactéries se développent au sein d'une organisation complexe, le biofilm. Les biofilms dentaires sont très structurés et organisés fonctionnellement au sein d'une matrice extracellulaire composée de polysaccharides et de glycoprotéines dérivés de l'environnement et des bactéries elles-mêmes (Marsh, 2004). À l'intérieur du biofilm, il existe un système circulatoire qui permet l'apport en nutriments et l'évacuation des déchets métaboliques. Les biofilms abritent une grande variété d'espèces qui cohabitent grâce à de nombreuses interactions physiques, métaboliques et nutritionnelles et une signalisation moléculaire complexe (Marsh, 2005). Cette organisation en biofilm confère aux espèces microbiennes une plus grande résistance aux agents antimicrobiens et aux défenses de l'hôte ainsi qu'une virulence accrue (Marsh, 2004; Marsh, 2005). La colonisation de la surface dentaire s'effectue en étapes successives qui sont contrôlées par les conditions environnementales locales, la composition de la plaque variant selon les sites anatomiques d'une dent. Deux types de plaque dentaire existent en fonction de leur localisation anatomique par rapport à la gencive : la plaque supra-gingivale et la plaque sous-gingivale.

1.2.1. Plaque supra-gingivale

L'environnement supra-gingival est aérobie et baigné par la salive. Le premier stade de formation de la plaque supra-gingivale consiste en un dépôt de glycoprotéines salivaires sur les surfaces des tissus, nommé pellicule exogène acquise, qui permet l'adhésion des micro-organismes à la surface de la dent. La colonisation microbienne va s'organiser suivant des critères écologiques qui déterminent une répartition des espèces en colonisateurs primaires et secondaires. Parmi les espèces pionnières, on retrouve essentiellement des streptocoques (*Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus salivarius* et *Streptococcus sanguinis*) et des actinomycètes. Le métabolisme des espèces

pionnières modifie les conditions locales permettant la colonisation par des colonisateurs secondaires, notamment des bactéries anaérobies strictes (*Fusobacterium* spp. et *Prevotella* spp.). La composition de la flore supra-gingivale va influencer grandement la composition de la plaque sous-gingivale.

1.2.2. Plaque sous-gingivale

Les bactéries de la plaque supra-gingivale colonisent également le sillon gingival même si la composition de cette plaque est très différente (Ximenez-Fyvie *et al.*, 2000). Contrairement à l'environnement supra-gingival, l'environnement sous-gingival est baigné par le fluide créviculaire et possède une faible teneur en oxygène favorisant ainsi le développement d'espèces bactériennes anaérobies. En effet, la plaque sous-gingivale se compose essentiellement de bactéries anaérobies strictes incluant les espèces *Porphyromonas gingivalis*, *Fusobacterium* spp., *Prevotella* spp. et *Actinomyces* spp. Plus de 500 espèces microbiennes différentes ont été dénombrées dans la plaque bactérienne sous-gingivale formant ainsi une véritable niche écologique (Kroes *et al.*, 1999; Paster *et al.*, 2001). Les bactéries de la plaque sous-gingivale colonisent à la fois la surface dentaire et la surface des cellules épithéliales gingivales. Certaines espèces bactériennes se développant au sein de la plaque sous-gingivale sont impliquées dans l'étiopathogenèse des maladies affectant le parodonte (Socransky *et al.*, 1998).

1.3. Parodonte

Le parodonte est l'organe qui regroupe les tissus de soutien de la dent (Figure 1.1). Il est constitué de quatre tissus : la gencive, le ligament parodontal, le cément et l'os alvéolaire.

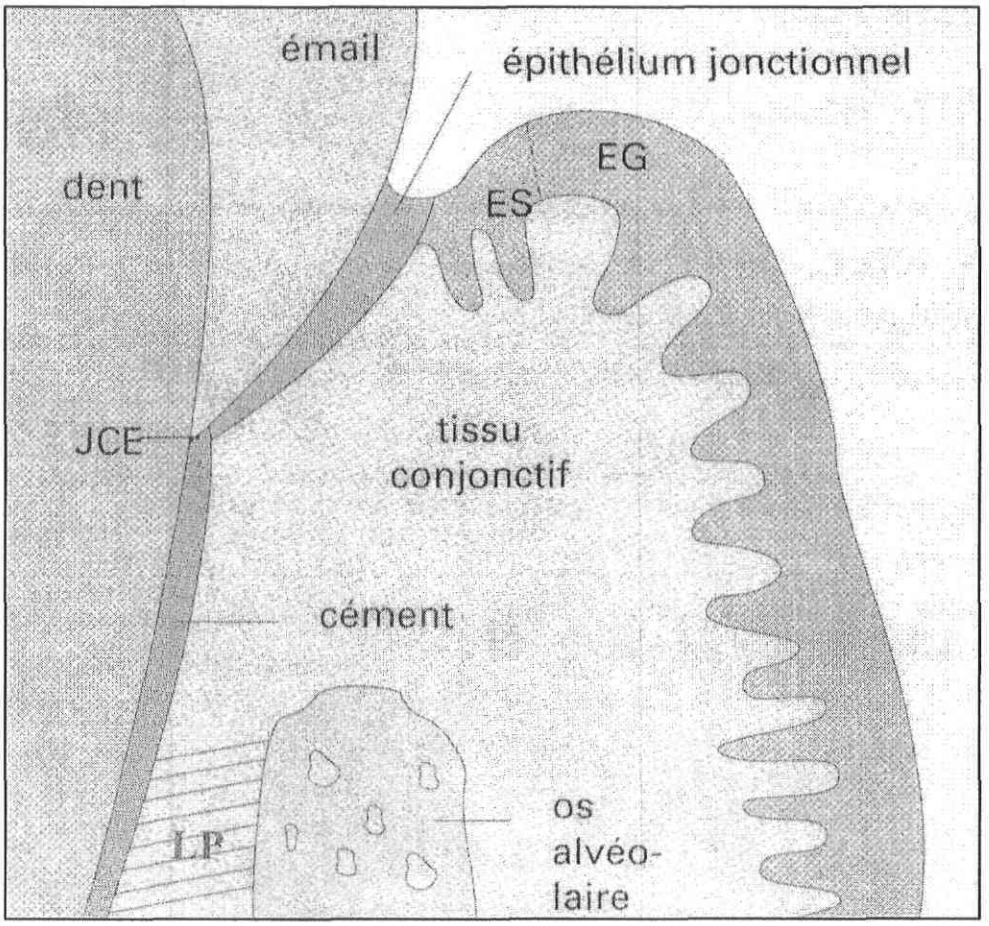

Figure 1.1. Schéma du parodonte. EG : épithélium gingival ; ES : épithélium sulculaire ; JCE : jonction cément-émail ; LP : ligament parodontal. Figure adaptée d'après : Brostoff J. Scadding G.K. Male D. Roitt I.V. Immunologie clinique. DeBoeck Université.

1.3.1. Gencive

La gencive est composée d'épithéliums et de tissu conjonctif. Le tissu conjonctif gingival assure le soutien, la cohésion des tissus et la protection du parodonte profond (os, cément et ligament). Il est composé de cellules (fibroblastes et leucocytes), de fibres de collagène, d'éléments vasculaires et nerveux et de substances fondamentales constituées principalement de glycoprotéines et de polysaccharides. Le tissu conjonctif gingival est recouvert d'épithéliums. On distingue trois types d'épithéliums différents dans la gencive : l'épithélium gingival, l'épithélium sulculaire et l'épithélium de jonction. L'épithélium gingival, composé de cellules épithéliales kératinisées, est situé sur la face externe de la gencive. L'épithélium sulculaire est squameux, non kératinisé et représente la partie interne de l'épithélium gingival non attaché à la surface dentaire. L'épithélium de jonction, composé de cellules épithéliales squameuses non kératinisées, est interposé entre la dent et le tissu conjonctif gingival et assure la sertissure du parodonte à la dent. La gencive est attachée à la surface dentaire au niveau de l'épithélium de jonction et est séparée de la dent par le sillon gingival dans sa partie apicale. Le sillon gingival, l'espace situé entre la dent et la gencive, a une profondeur variant de 0.5 à 3 mm chez le sujet sain mais qui peut atteindre 8 à 9 mm dans le cas d'une pathologie. Le sillon gingival est rempli de fluide créviculaire, un exsudat sérique ayant transité par l'épithélium de jonction.

1.3.2 Ligament parodontal

Le ligament parodontal ou desmodonte est un tissu conjonctif dense qui s'étend entre la partie interne de l'os alvéolaire et le cément. Il permet notamment d'amortir les forces auxquelles la dent est soumise et participe à la cicatrisation des tissus parodontaux. Le ligament parodontal contient différents types cellulaires incluant des fibroblastes, des cellules endothéliales, des ostéoclastes, des débris épithéliaux de Malassez et des cémentoblastes.

1.3.3. Cément

Le cément est un tissu conjonctif minéralisé de type osseux non vascularisé qui recouvre la racine dentaire et permet l'ancrage des fibres du ligament parodontal. Le cément est composé essentiellement d'hydroxyapatite, de fibres de collagène et de protéines telle que la chondroïtine sulfate ou la fibronectine. Le cément se présente sous deux formes principales : le cément acellulaire composé de fibres et le cément cellulaire contenant les cémentoblastes.

1.3.4. Os alvéolaire

L'os alvéolaire est un tissu calcifié qui s'organise autour des alvéoles qui ancrent la dent. Les alvéoles dentaires sont formées d'os compact et entre elles se trouve l'os spongieux. L'os alvéolaire présente des perforations à travers lesquelles s'insèrent les vaisseaux sanguins et les fibres nerveuses. Le tissu osseux possède la capacité de se remodeler par une succession équilibrée de résorption et de formation osseuse. Les ostéoblastes sont impliqués dans la synthèse, la formation et le maintien de la structure osseuse tandis que les ostéoclastes assurent la lyse et le remodelage de l'os alvéolaire.

1.3.5. Cellules non-résidentes présentes dans le parodonte

Outre les différents types cellulaires énumérés précédemment, le parodonte est infiltré par des leucocytes présents aussi bien dans le sillon gingival qu'au sein des tissus parodontaux. Ces leucocytes participent à la défense des tissus parodontaux contre les agents infectieux et au maintien de l'homéostasie entre les bactéries de la plaque dentaire et l'intégrité des tissus parodontaux. Parmi les différents types de leucocytes présents dans le parodonte, on retrouve essentiellement des neutrophiles, des monocytes/macrophages et des lymphocytes B et T (Schroeder and Listgarten, 1997). L'abondance et la composition des leucocytes au sein des tissus parodontaux et dans le fluide créviculaire varient fortement

suivant les individus et l'état de santé du parodonte (Kornman *et al.*, 1997; Delima and Van Dyke, 2003; Berglundh and Donati, 2005).

1.4. Maladies parodontales

1.4.1. Définition et classification

Les maladies parodontales sont des affections bucco-dentaires qui touchent le parodonte. Elles sont initiées par une agression bactérienne et provoquent l'apparition d'une réaction inflammatoire. Les maladies parodontales se subdivisent en deux catégories : la gingivite et la parodontite. Lorsque l'inflammation se limite à la portion marginale du parodonte et qu'elle est réversible, le terme gingivite est utilisé. La parodontite réfère quant à elle à une destruction irréversible de l'ensemble des tissus de soutien de la dent, incluant les tissus parodontaux profonds (ligament parodontal, cément et os alvéolaire). On estime qu'environ 35% des adultes de plus de 30 ans aux Etats-Unis sont atteints de parodontites (Albandar *et al.*, 1999). Plus spécifiquement, 22% des individus sont atteints de la forme légère de la maladie alors que 13% souffrent d'une forme modérée à sévère. Au Québec, la moitié de la population âgée de 35 à 44 ans montre des signes évidents de parodontites (Brodeur *et al.*, 1998). La Direction de la santé de Montréal-Centre estime qu'environ un adulte québécois sur cinq souffrira un jour d'une parodontite sévère pouvant impliquer la perte de dents. A l'échelle mondiale, la prévalence et la sévérité des maladies parodontales tendent à augmenter avec l'âge. Les parodontites sont des infections polymicrobiennes aux étiologies et pathogénies variées qui se traduisent par une destruction plus ou moins sévère des tissus parodontaux. Selon la classification de l'Académie américaine de parodontologie réalisée en 1999, trois grands sous-groupes de parodontites sont maintenant reconnus : les parodontites chroniques, les parodontites agressives et les parodontites en tant que manifestations de maladies systémiques.

1.4.2. Étiopathogénie des maladies parodontales

La plaque dentaire est associée depuis longtemps aux maladies parodontales. En 1966, une corrélation directe entre le degré d'accumulation de plaque dentaire et la sévérité de la gingivite a été établie (Theilade *et al.*, 1966). Depuis le dix-neuvième siècle, l'hypothèse de la plaque non-spécifique selon laquelle les maladies parodontales étaient simplement causées par l'augmentation de la quantité de plaque dentaire avait été proposée (Miller, 1890; Theilade, 1986). Or dans les années 1970, cette hypothèse était remise en question par l'établissement de la théorie de la plaque spécifique associant la présence d'une flore spécifique aux maladies parodontales (Loesche and Syed, 1978). Cette dernière hypothèse est aujourd'hui supportée par le fait que seulement une dizaine d'espèces ont été associées aux parodontites parmi plusieurs centaines d'espèces présentes dans la plaque sous-gingivale (Haffajee and Socransky, 1994). Les maladies parodontales peuvent être considérées comme des infections opportunistes. Bien que la présence de bactéries parodontopathogènes soit essentielle à l'initiation de la parodontite, celle-ci n'est pas suffisante pour faire progresser la maladie. La réponse immunitaire de l'hôte, en réponse à l'agression bactérienne, est un facteur déterminant dans l'évolution des parodontites (Okada and Murakami, 1998; Kinane and Lappin, 2001). En effet, les maladies parodontales sont aujourd'hui considérées comme des pathologies infectieuses pour lesquelles la réponse immunitaire de l'hôte exagérée face à l'infection entraîne l'apparition d'un phénomène inflammatoire chronique.

1.4.3. Rôle des bactéries

Les parodontites sont des infections polymicrobiennes. Très peu d'espèces bactériennes retrouvées dans la plaque dentaire sont considérées comme pathogènes. Parmi plus de 500 espèces présentes dans la cavité buccale (Paster *et al.*, 2001), seulement une dizaine d'espèces ont été associées à la parodontite, incluant *P. gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigrescens*, *Campylobacter rectus* et

Peptostreptococcus micros (Haffajee and Socransky, 1994; Socransky *et al.*, 1998). Ces bactéries, nommées parodontopathogènes, possèdent de nombreux facteurs de virulence (O'Brien-Simpson *et al.*, 2004). Ces espèces sont souvent présentes dans la plaque dentaire de sujets sains et peuvent être considérées comme faisant partie de la microflore indigène (Moore and Moore, 1994). Cependant leur proportion dans la plaque sous-gingivale augmente significativement chez les individus atteints de parodontites (Socransky *et al.*, 1991; van Winkelhoff *et al.*, 2002). Les facteurs écologiques contribuent au déséquilibre au sein de la communauté microbienne résidente de la plaque favorisant ainsi la croissance d'espèces potentiellement pathogènes qui étaient initialement présentes en minorité (Marsh, 1994). Les interactions positives et négatives entre les bactéries parodontopathogènes influencent la concentration des espèces dans les lésions parodontales et agissent ainsi sur la progression des parodontites (Grenier and Mayrand, 2000). De plus, des facteurs environnementaux locaux pourraient modifier la composition de la plaque dentaire en affectant la réponse de l'hôte (Clark and Loe, 1993). D'autres facteurs peuvent induire un déséquilibre de la flore buccale normale, notamment une diminution de la proportion de bactéries bénéfiques ou une augmentation d'un apport nutritionnel exogène favorisant une espèce donnée au sein de l'écosystème (Grenier and Mayrand, 2000). Les parodontites résulteraient ainsi d'un déséquilibre écologique (Grenier and Mayrand, 2000).

Parmi les parodontopathogènes, *A. actinomycetemcomitans* et *P. gingivalis* sont reconnus comme des agents étiologiques majeurs des parodontites. *A. actinomycetemcomitans* est associé à la parodontite agressive localisée alors que *P. gingivalis* est considéré comme l'agent étiologique majeur des parodontites chroniques chez l'adulte (Haffajee and Socransky, 1994; Slots, 1999; Henderson *et al.*, 2002). Les parodontites sont néanmoins reconnues comme des infections mixtes. Une étude qui a tenté de définir les communautés bactériennes associées aux parodontites a mis en évidence des complexes bactériens qui ont pu être associés avec un état sain ou un état pathologique du parodonte (Socransky *et al.*, 1998). Le complexe rouge, composé de *P. gingivalis*, *T. denticola* et *T. forsythia*, a été fortement associé aux mesures cliniques des parodontites et aux lésions parodontales les plus avancées (Socransky *et al.*, 1998).

1.5. Potentiel pathogénique de *Porphyromonas gingivalis*, *Treponema denticola* et *Tannerella forsythia*, le complexe bactérien rouge associé à la parodontite

1.5.1. Résumé

Les parodontites sont des infections mixtes qui sont à l'origine de la destruction des tissus de soutien de la dent, incluant le ligament parodontal et l'os alvéolaire. Parmi plus de 500 espèces bactériennes présentes dans la cavité buccale, un complexe bactérien nommé « complexe rouge » et composé de *Porphyromonas gingivalis*, *Treponema denticola* et *Tannerella forsythia* a été fortement associé aux lésions parodontales avancées. Bien que les bactéries parodontopathogènes soient le facteur étiologique primaire des parodontites, la destruction des tissus mous et durs résulte essentiellement de la réponse immunitaire de l'hôte face à l'agression bactérienne. Les espèces du complexe rouge sont des bactéries anaérobies à Gram négatif exprimant de nombreux facteurs de virulence qui leur permettent de coloniser l'espace sous-gingival, de perturber le système de défense de l'hôte, d'envahir et de détruire les tissus parodontaux ou encore de promouvoir la réponse immunodestructrice de l'hôte. Cet article passe en revue les connaissances actuelles des mécanismes pathogéniques des bactéries du complexe rouge contribuant aux destructions tissulaires et osseuses observées au cours des parodontites.

1.5.2. Abstract

Periodontitis are mixed bacterial infections leading to destruction of tooth-supporting tissues, including periodontal ligament and alveolar bone. Among over 500 bacterial species living in the oral cavity, a bacterial complex named "red complex" and made of *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* has been strongly related to advanced periodontal lesions. While periodontopathogenic bacteria are the primary etiologic factor of periodontitis, tissue destruction essentially results from

the host immune response to the bacterial challenge. Members of the red complex are Gram negative anaerobic bacteria expressing numerous virulence factors allowing bacteria to colonize the subgingival sites, to disturb the host defense system, to invade and destroy periodontal tissue as well as to promote the immunodestructive host response. This article reviews current knowledge of the pathogenic mechanisms of bacteria of the red complex leading to tissue and alveolar bone destruction observed during periodontitis.

1.5.3. Introduction

Les maladies parodontales sont des infections mixtes, impliquant un groupe spécifique de bactéries anaérobies à Gram négatif, qui sont à l'origine de la destruction des tissus de soutien de la dent, incluant le ligament parodontal et l'os alvéolaire. Ces maladies sont déclenchées par une agression polymicrobienne qui provoquent l'apparition d'une réaction inflammatoire. Les maladies parodontales constituent l'une des maladies inflammatoires chroniques les plus communes parmi la population adulte ; la parodontite chronique représentant la forme la plus courante et la principale cause de la perte de dent chez l'adulte. Les parodontites se caractérisent notamment par la formation d'une poche parodontale, une perte d'attache épithéliale, un saignement au sondage et dans certains cas une résorption de l'os alvéolaire. Bien que les bactéries parodontopathogènes soient le facteur étiologique primaire des parodontites, la destruction des tissus mous et durs résulte essentiellement de la réponse immunitaire de l'hôte face à l'agression bactérienne. Cette dernière se traduit par une forte production de cytokines pro-inflammatoires (interleukine (IL)-1β, IL-6, IL-8 et le facteur tumoral de nécrose alpha (TNF-α)), de prostaglandine E_2 et de métalloprotéinases matricielles (MMP-2, MMP-3, MMP-8 et MMP-9) qui est fortement associée à la destruction des tissus parodontaux [1-3]. L'évolution des parodontites dépend donc à la fois de la virulence des bactéries impliquées et du niveau de la réponse immuno-inflammatoire de l'hôte. Par ailleurs, l'usage du tabac et divers facteurs systémiques comme le stress, certains traitements médicamenteux, des déséquilibres endocriniens ou des dysfonctions leucocytaires, contribuent à la progression des parodontites en rendant les tissus parodontaux plus vulnérables aux effets des facteurs locaux [4].

La cavité buccale constitue l'un des écosystèmes microbiens les plus complexes de l'organisme humain. Les parodontites résultent d'un déséquilibre écologique au sein de la communauté microbienne résidente de la plaque dentaire favorisant ainsi la croissance d'espèces pathogènes initialement présentes en faible proportion [5]. Parmi plus de 500 espèces bactériennes présentes dans la cavité buccale [6], un complexe bactérien nommé « complexe rouge » et composé de *Porphyromonas gingivalis*, *Treponema denticola* et *Tannerella forsythia*, a été fortement associé aux mesures cliniques des parodontites et plus particulièrement avec les lésions parodontales avancées [7]. *P. gingivalis*, *T. denticola* et *T. forsythia* sont des bactéries anaérobies à Gram négatif exprimant de nombreux facteurs de virulence qui leur permettent de coloniser l'espace sous-gingival, de perturber le système de défense de l'hôte, d'envahir et de détruire les tissus parodontaux ou encore de promouvoir la réponse immunodestructrice de l'hôte [8]. Cet article passe en revue les connaissances actuelles des mécanismes pathogéniques des bactéries du complexe rouge contribuant aux destructions tissulaires et osseuses observées au cours des parodontites.

1.5.4. Relations écologiques entre les bactéries du complexe rouge

P. gingivalis, *T. denticola* et *T. forsythia* sont des bactéries fréquemment détectées simultanément dans les sites montrant une perte d'attache clinique importante, et leur proportion relative dans la plaque sous-gingivale augmente significativement chez les patients atteints de parodontites [7, 9-11]. Cette coexistence suggère donc que des relations écologiques existent entre ces trois espèces. Une forte association entre *P. gingivalis* et *T. forsythia* a été démontrée dans la plaque sous-gingivale prélevée dans des poches parodontales de différentes profondeurs [12]. De plus, *T. forsythia* et *P. gingivalis* sont fréquemment isolées dans les sites de parodontite chronique en phase active de destruction [13-15]. Ces deux espèces ont été également associées aux parodontites débutantes suggérant ainsi un rôle prépondérant de ces bactéries dans le déclenchement de la maladie [16]. Simonson et al. [17] ont rapporté une relation synergique entre *P. gingivalis* et *T. denticola* dans des échantillons de plaque sous-gingivale provenant d'individus de différents groupes ethniques atteints de parodontite sévère suggérant que *T. denticola* nécessite la présence de *P. gingivalis* pour s'établir. Les espèces du complexe rouge

adhèrent fortement entres elles via des interactions spécifiques [18-21] (Figure 1.2) et peuvent également se lier à d'autres bactéries commensales retrouvées au sein du biofilm dentaire [19, 22, 23]. Il a été montré *in vitro* que *P. gingivalis* et *T. denticola* agissaient en synergie pour produire un biofilm [24, 25]. Ce phénomène est supporté par des observations *in vivo* indiquant que *P. gingivalis* et *T. denticola* se retrouvent physiquement liés au sein de la plaque sous-gingivale [26]. Cette capacité d'adhérence bactérienne hétérotypique peut par ailleurs permettre le transport de *P. gingivalis* et *T. forsythia* par *T. denticola*, la seule espèce motile du complexe rouge, via un mécanisme de « piggyback ». Ces bactéries pourraient ainsi coloniser les zones profondes de la poche parodontale représentant un environnement plus propice à leur croissance notamment à cause de la concentration d'oxygène plus faible. L'adhésion interbactérienne peut également faciliter les interactions nutritionnelles entre les bactéries du complexe rouge. Il a été démontré qu'un extrait cellulaire de *T. forsythia* stimule la croissance de *P. gingivalis* dans un milieu nutritionnel pauvre [27]. De plus, certaines protéines secrétées par *P. gingivalis* stimulent la croissance de *T. denticola* [28]. Une relation de mutualisme entre *P. gingivalis* et *T. denticola* fondée sur l'échange d'acides organiques a également été caractérisée [29]. La prolifération des espèces du complexe rouge est donc supportée par des interactions d'adhérence et nutritionnelles qui participent probablement au développement d'une plaque sous-gingivale pathogénique chez les sujets souffrant d'une maladie parodontale.

1.5.5. Effets synergiques lors d'infections expérimentales chez l'animal

Des effets synergiques de virulence dans des modèles animaux entre les espèces bactériennes du complexe rouge ont été rapportés. Comparée à des monoinfections, une infection mixte, causée par *P. gingivalis* et *T. denticola*, provoque une réponse inflammatoire excessive et un taux de mortalité plus élevé dans un modèle de pneumonie chez la souris [30]. Il a également été rapporté que *T. denticola* augmentait la virulence de *P. gingivalis* dans un modèle murin [31]. Plus précisément, l'addition de *T. denticola* à l'inoculum bactérien accroît la capacité de destruction tissulaire de *P. gingivalis* et réduit sa dose nécessaire pour causer une infection létale. Des interactions synergiques lors d'infections expérimentales chez l'animal entre *P. gingivalis* et *T. forsythia* ont aussi été

rapportées. Dans un modèle d'abcès chez le lapin, les souches de *T. forsythia* étaient plus virulentes et invasives lorsqu'elles étaient inoculées en association avec *P. gingivalis* [32]. Enfin, *P. gingivalis* et *T. forsythia* ont montré des effets synergiques lors de la formation d'abcès chez la souris [33]. Ces résultats supportent l'existence d'interactions spécifiques *in vivo* entre les bactéries du complexe rouge résultant en un pouvoir pathogène plus élevé lors d'une infection polymicrobienne.

1.5.6. Adhésion et invasion tissulaire

L'adhésion aux surfaces dentaires et aux muqueuses buccales constitue une étape cruciale à la colonisation des sites sous-gingivaux et à la survie des bactéries dans un environnement constamment soumis aux flux de la salive et du fluide créviculaire. Les bactéries du complexe rouge possèdent des molécules et des structures (adhésines, lipopolysaccharides, fimbriae) leur permettant de s'attacher à diverses surfaces et d'interagir avec l'hôte. Les adhésines, des protéines ou des glycoprotéines impliquées dans l'adhésion bactérienne, sont présentes sur la membrane externe mais peuvent également être associées aux fimbriaes [34]. De plus, deux cystéines protéases présentes à la surface de *P. gingivalis*, l'Arg-gingipaïne A et la Lys-gingipaïne, contiennent jusqu'à quatre domaines hémagglutinines/adhésines fortement impliqués dans l'adhésion de cette bactérie [35]. En plus de leur capacité d'adhérence interbactérienne hétérotypique décrite précédemment, *P. gingivalis*, *T. denticola* et *T. forsythia* possèdent de nombreuses adhésines leur permettant d'adhérer à différents types de cellules, notamment les cellules épithéliales, les fibroblastes, les érythrocytes et les leucocytes, ainsi qu'à de nombreuses protéines salivaires, du fluide créviculaire et de la matrice extracellulaire [18, 22, 23, 36-39]. Les fimbriaes et les hémagglutinines jouent un rôle crucial dans l'adhésion de *P. gingivalis* aux cellules de l'hôte [40-42]. *P. gingivalis* exprime au moins cinq hémagglutinines différentes (HagA, HagB, HagC, HagD et HagE) sur sa surface cellulaire qui sont impliquées dans la liaison de cet organisme aux récepteurs des cellules humaines (essentiellement des oligosaccharides) [23]. Chez *T. denticola*, l'adhésion aux cellules de l'hôte et aux protéines de la matrice extracellulaire est associée aux protéines LrrA (leucine-rich repeat protein) et Msp (major sheath (or surface) protein) [18, 36, 38]. La couche S de

T. forsythia est quant à elle impliquée dans l'adhésion de cette bactérie aux surfaces cellulaires [37]. De plus, les protéases de *T. denticola* et également de *P. gingivalis* peuvent favoriser l'adhérence des parodontopathogènes aux tissus en exposant des sites d'adhésion bactériens masqués (cryptitopes) à la surface des cellules eucaryotes [43].

L'adhésion constitue l'étape initiale de l'invasion bactérienne des tissus et des cellules humaines. *In vitro*, *P. gingivalis* et *T. forsythia* adhèrent à la surface des cellules épithéliales et pénètrent dans le cytoplasme [37, 44]. Cette adhérence bactérienne est à l'origine d'un dialogue moléculaire entre les bactéries et les cellules eucaryotes. En effet, l'adhésion de *P. gingivalis* aux cellules épithéliales, d'une part mène à l'activation de différentes voies de signalisation cellulaire chez la cellule hôte incluant l'activation des MAP kinases, la phosphorylation de protéines, l'augmentation du flux calcique et la réorganisation du cytosquelette [44-46], et d'autre part modifie l'expression de gènes chez la bactérie [47, 48]. L'adhésion de *P. gingivalis* aux cellules épithéliales induit la formation d'une invagination de la membrane plasmique qui entoure la bactérie conduisant à son internalisation [46, 49]. *P. gingivalis* possède la capacité de se multiplier à l'intérieur des cellules épithéliales [44, 49]. Yilmaz et al. [50] ont récemment montré que *P. gingivalis* pouvait se propager d'une cellule épithéliale à une autre sans passer par l'espace extracellulaire, assurant ainsi sa dissémination dans les tissus parodontaux en restant protégé de la réponse de l'hôte. L'invasion des cellules épithéliales par *P. gingivalis* empêcherait la migration des neutrophiles à travers l'épithélium en inhibant notamment la production d'IL-8 et d'ICAM-1 (intracellular adhesion molecule 1) [51]. Par ailleurs, plusieurs études suggèrent que *P. gingivalis* et *T. denticola* peuvent migrer à travers la membrane basale et pénétrer profondément dans les tissus parodontaux [52-55]. Ces résultats obtenus *in vitro* sont supportés par les observations *in vivo*. En effet, *P. gingivalis*, *T. denticola* et *T. forsythia* ont été observés à l'intérieur des cellules épithéliales buccales chez des sujets sains [56, 57]. Les bactéries intracellulaires et les cellules envahies sont en majorité viables soulignant la complexité des interactions entre cette flore polymicrobienne et les cellules épithéliales buccales [58]. Chez les patients atteints de parodontites, les bactéries du complexe rouge se retrouvent également associées aux cellules épithéliales

[59]. *T. forsythia* semble être la bactérie la plus commune à la surface et à l'intérieur des cellules épithéliales issues de poches parodontales [60].

Par ailleurs, *P. gingivalis* et *T. denticola* produisent des vésicules à partir de leur membrane externe [61, 62]. Ces vésicules membranaires contiennent les principaux facteurs de virulence connus de ces bactéries et possèdent une très petite taille (environ 50 nm de diamètre) qui leur permet de diffuser très facilement à l'intérieur des tissus parodontaux. Ces vésicules assurent donc une dissémination des facteurs de virulence bactériens dans des zones inaccessibles aux cellules entières, fragilisant ainsi l'intégrité du parodonte. Cette propriété facilite l'invasion et la destruction des tissus parodontaux par les bactéries parodontopathogènes.

L'invasion tissulaire et l'internalisation intracellulaire des bactéries du complexe rouge sont fortement impliquées dans leur pouvoir pathogénique. En effet, ces bactéries vont pouvoir exercer des effets délétères sur les tissus profond du parodonte perturbant ainsi l'homéostasie tissulaire.

1.5.7. Apoptose

Le phénomène d'apoptose, caractérisé notamment par l'activation des caspases, a récemment été impliqué dans la destruction des tissus parodontaux [63]. En effet, l'activation des caspases 3 et 7 est considérablement augmentée dans les tissus gingivaux de patients atteints de parodontites [63]. Les bactéries du complexe rouge pourraient jouer un rôle dans l'induction de l'apoptose observée au cours des parodontites. En effet, *P. gingivalis* et la protéine Sip (immunosuppressive protein) de *T. denticola* ont la capacité d'induire l'apoptose chez les lymphocytes T [64, 65]. *P. gingivalis* peut également induire l'apoptose chez les cellules épithéliales gingivales en augmentant l'expression du Fas ligand et en activant les caspases 3 et 8 [66]. De plus, les lipoprotéines de *T. forsythia* induisent l'apoptose chez différentes lignées cellulaires humaines [67]. L'induction de l'apoptose par les bactéries du complexe rouge peut contribuer à l'altération des tissus et à

la réduction du nombre de leucocytes dans la poche parodontale favorisant ainsi la progression des parodontites.

1.5.8. Activités protéolytiques des bactéries du complexe rouge

P. gingivalis, *T. denticola* et *T. forsythia* possèdent un fort pouvoir protéolytique qui joue de multiples rôles dans la pathogenèse des parodontites. En effet, les protéases produites par ces bactéries sont impliquées dans l'adhérence, la nutrition et la dissémination des bactéries mais participent également à la perturbation des défenses de l'hôte et à la destruction des tissus parodontaux [68]. *P. gingivalis* est une des bactéries parodontopathogènes possédant le plus fort pouvoir protéolytique résultant en grande partie de l'activité des gingipaïnes, trois cystéines protéases fortement impliquées dans la virulence de cet organisme [35]. L'activité des gingipaïnes est positivement corrélée avec les pertes d'attache observées chez les patients atteints de parodontites chroniques [69]. Par ailleurs, la destruction des tissus parodontaux a été également corrélée avec la quantité de bactéries du complexe rouge présente dans la poche parodontale et l'intensité de leurs activités protéolytiques [70, 71].

1.5.8.1. Rôle dans l'acquisition de nutriments

Les bactéries du complexe rouge sont asaccharolytiques et requièrent pour leur croissance des peptides et des acides aminés. L'arsenal protéolytique des bactéries du complexe rouge va leur permettre de satisfaire leurs besoins nutritifs à partir d'une grande variété de substrats, notamment des protéines tissulaires et plasmatiques, pour assurer leur prolifération dans la poche parodontale [8]. Les bactéries du complexe rouge expriment des hémagglutinines et des activités hémolytiques leur permettant de se lier aux érythrocytes puis de les lyser, libérant ainsi de l'hémoglobine, une source de fer essentielle à la croissance des parodontopathogènes [72-74]. Les gingipaïnes de *P. gingivalis* augmentent la perméabilité vasculaire en activant le système kallikréine-kinine et perturbent la coagulation sanguine en dégradant le fibrinogène, la fibrine et le facteur X [35]. Ces

processus favorisent la production du fluide créviculaire et le saignement des sites de parodontites fournissant ainsi une source de nutriments propice à la croissance et à la virulence des bactéries parodontopathogènes. Par ailleurs, certains déchets métaboliques produits par ces bactéries sont toxiques pour les cellules de l'hôte [75, 76].

1.5.8.2. Rôle dans l'évasion des défenses de l'hôte

Les protéases des bactéries parodontopathogènes, particulièrement les gingipaïnes, contribuent au contournement des défenses immunitaires de l'hôte favorisant ainsi l'établissement de ces bactéries. En effet, les gingipaïnes de *P. gingivalis* et la dentilisine de *T. denticola* possèdent la capacité de dégrader les immunoglobulines, certains récepteurs membranaires, les protéines du complément, les cytokines pro-inflammatoires, incluant l'IL-1β, le TNF-α et l'IL-6, et les chimiokines notamment l'IL-8 et le RANTES [35, 77-80]. La dégradation des cytokines par *P. gingivalis* peut être massive et dépend à la fois de la concentration bactérienne et de la souche utilisée [79]. Cette dégradation résulte en une forte perturbation des réactions inflammatoires locales et une rupture du gradient de chimiokines à proximité de la plaque sous-gingivale ayant pour effet d'inhiber l'afflux des leucocytes au site de l'infection. De plus, *P. gingivalis* et *T. denticola* dégradent les immunoglobulines (IgA, IgG et IgM) [80, 81] leur permettant ainsi d'échapper à l'opsonisation par les cellules phagocytaires et à l'action du système du complément. *P. gingivalis* atténue également l'effet bactéricide du complément en clivant la protéine C3 [81] ou en inactivant le récepteur de la protéine du complément C5a présent à la surface des neutrophiles [82]. La destruction de ce dernier récepteur, jouant un rôle majeur dans le chimiotactisme des neutrophiles au site de l'infection, résulte en une perturbation des fonctions phagocytaires. D'autres récepteurs cellulaires, incluant le CD14 (récepteur des LPS) et le récepteur de l'IL-6 (IL-6R) sont clivés sous l'action des gingipaïnes [83, 84] altérant ainsi les réactions inflammatoires locales induites par le LPS et l'IL-6. *P. gingivalis* et *T. denticola* mettent donc en œuvre différentes stratégies permettant de perturber les réponses immunes. L'évasion des défenses immunitaires de l'hôte par les bactéries parodontopathogènes est un facteur déterminant de leur potentiel pathogénique, de leur prolifération et de leur survie à long terme.

1.5.8.3. Rôle dans la destruction des tissus parodontaux

Les protéases des bactéries du complexe rouge sont aussi impliquées dans la destruction tissulaire observée au cours des parodontites. *P. gingivalis* possède des activités collagénases dégradant le collagène de type I, le constituant majeur du tissu conjonctif gingival [85, 86]. Les gingipaïnes de *P. gingivalis* et la dentilisine de *T. denticola* dégradent de nombreuses composantes de la membrane basale et de la matrice extracellulaire, incluant le collagène de type IV, la laminine et la fibronectine [23, 35, 52] suggérant que ces enzymes jouent un rôle dans la destruction des tissus parodontaux. Les gingipaïnes peuvent par ailleurs cliver le syndecane-1 de la surface des cellules épithéliales gingivales, un co-récepteur de divers facteurs de croissance et de molécules de la matrice extracellulaire impliqué notamment dans l'adhésion des cellules à la matrice extracellulaire [87]. Ce phénomène pourrait jouer un rôle important dans l'invasion bactérienne des tissus parodontaux. Les protéinases de *P. gingivalis* et *T. denticola* ont également la capacité de dégrader les jonctions intercellulaires des cellules épithéliales [55, 88], affectant la perméabilité de la barrière épithéliale ainsi que l'intégrité des tissus parodontaux. Par ailleurs, *P. gingivalis* et *T. denticola* dégradent de nombreux inhibiteurs de protéases de l'hôte (α1-antitrypsine, antichymotrypsine, α2-macroglobuline, antithrombine III, antiplasmine et cystatine C) qui ont pour fonction de réguler l'activité de nombreuses enzymes protéolytiques de la réponse inflammatoire [89]. *P. gingivalis* peut également activer le plasminogène en plasmine [89], une enzyme impliquée à la fois dans la dégradation de protéines de la matrice extracellulaire (fibronectine et laminine) et dans l'activation des formes latentes de métalloprotéinases matricielles (MMPs). L'activation du plasminogène et la dégradation des inhibiteurs de protéases de l'hôte peut résulter en une destruction non-contrôlée des tissus parodontaux et interférer avec la réparation des tissus détruits. Ces différents mécanismes soulignent la capacité des bactéries du complexe rouge d'affecter l'homéostasie des tissus parodontaux.

La sécrétion par les bactéries du complexe rouge d'enzymes protéolytiques permet donc de dégrader les structures cellulaires et de contourner les systèmes de défenses de l'hôte. Toutefois, la destruction tissulaire observée au cours des parodontites résulte d'un

processus complexe qui fait intervenir d'autres types d'interactions hôte-bactéries. En effet, la stimulation des cellules de l'hôte par les facteurs de virulence microbiens engendre l'expression d'enzymes hydrolytiques et la sécrétion de médiateurs inflammatoires (cytokines, prostaglandines) permettant d'activer diverses voies de dégradation.

1.5.9. Stimulation de la réponse de l'hôte par les bactéries du complexe rouge

Les parodontites se caractérisent par une forte production de médiateurs inflammatoires par les cellules de l'hôte, un phénomène associé aux destructions parodontales [2]. Histologiquement, les parodontites se traduisent par une accumulation de cellules inflammatoires (macrophages, neutrophiles et lymphocytes) dans les tissus parodontaux. Ces cellules en association avec les cellules résidentes du parodonte (fibroblastes et cellules épithéliales) génèrent une production excessive de cytokines, de prostaglandines et de MMPs en réponse à la stimulation continue par les bactéries parodontopathogènes [1, 8, 23, 38].

1.5.9.1. Cytokines

La destruction tissulaire observée au cours des parodontites est liée à un processus inflammatoire chronique du parodonte impliquant de nombreuses cytokines notamment l'IL-1β, l'IL-6, l'IL-8 et le TNF-α [2]. Ces cytokines pro-inflammatoires sont présentes en forte quantité dans le fluide créviculaire et les tissus parodontaux des patients atteints de parodontites et possèdent des rôles physiologiques étroitement liés à l'étiologie de ces maladies [2]. Par exemple, une production locale excessive d'IL-1β dans le parodonte pourrait jouer un rôle clé dans la pathogenèse des parodontites [2]. En effet, l'IL-1β exerce des effets pléiotropiques sur de nombreuses lignées cellulaires en induisant la production de récepteurs cellulaires, d'autres cytokines, de MMPs, de prostaglandine E_2 et d'activateurs du plasminogène favorisant ainsi le processus inflammatoire et la résorption osseuse. L'IL-1β est produite sous la forme d'un précurseur inactif (pro-IL-1β) qui devient biologiquement actif après un clivage par des enzymes de l'hôte. *T. denticola* possède la

capacité d'activer la pro-IL-1β suggérant que cette bactérie exerce ainsi un rôle proinflammatoire dans les parodontites [90]. De plus, *P. gingivalis*, *T. denticola* et *T. forsythia*, seuls ou en combinaisons, induisent la sécrétion d'IL-1β dans un modèle de co-culture de cellules épithéliales et de macrophages [78]. Bien que des infections polymicrobiennes ne résultent pas en une production massive d'IL-1β par le modèle de co-culture en comparaison à des infections simples, ces trois espèces bactériennes agissent de concert pour stimuler la sécrétion de cette cytokine pro-inflammatoire suggérant leur capacité à induire diverses voies de destruction parodontale. De nombreuses études ont rapporté la production de cytokines pro-inflammatoires (incluant l'IL-1β, l'IL-6 et le TNF-α) et de chimiokines (notamment l'IL-8) par différentes lignées cellulaires suite à des stimulations par les espèces du complexe rouge ou certaines de leurs composantes [8, 23, 38, 54, 67, 78, 79]. Ces bactéries pourraient ainsi favoriser la réponse immunodestructrice de l'hôte.

1.5.9.2. Prostaglandine E_2

La prostaglandine E_2 (PGE_2) est reconnue comme un puissant inducteur de la résorption osseuse qui joue un rôle déterminant dans la destruction de l'os alvéolaire et les pertes d'attache de la dent observées chez les patients atteints de parodontites [91]. Une forte quantité de PGE_2 a été mise en évidence dans le fluide créviculaire de poches parodontales co-infectées par les trois espèces du complexe rouge [92]. *In vitro*, *P. gingivalis*, *T. denticola* et *T. forsythia* induisent la sécrétion de PGE_2 dans différents modèles de co-culture [78, 93]. L'augmentation de la production de PGE_2 a été impliquée dans la formation d'ostéoclastes induite par *P. gingivalis* et *T. denticola* [93]. Ces données mettent en évidence le potentiel des bactéries du complexe rouge à induire la résorption de l'os alvéolaire. Par ailleurs, les prostaglandines E_2 peuvent contribuer à la dégradation des tissus parodontaux en stimulant l'expression de MMPs.

1.5.9.3. Métalloprotéinases matricielles

Les MMPs jouent un rôle critique dans les destructions tissulaires et osseuses observées au cours des parodontites [3]. Ces protéinases constituent une famille d'endopeptidases à zinc capables de dégrader la quasi-totalité des composantes de la matrice extracellulaire, incluant le collagène, la laminine et la fibronectine. La majorité des types cellulaires présents dans le parodonte, notamment les fibroblastes, les cellules épithéliales, les macrophages et les neutrophiles, peuvent synthétiser des MMPs [94]. La production de MMPs est contrôlée par de nombreux médiateurs intercellulaires notamment des cytokines, des métabolites de l'acide arachidonique ou des facteurs de croissance [3, 94]. Les MMPs sont sécrétées sous une forme latente et deviennent actives après un clivage protéolytique, impliquant la plasmine ou d'autres enzymes, ou un changement de conformation induit entre autres par les agents oxydants [94]. Leurs activités sont par ailleurs strictement contrôlées par des inhibiteurs tissulaires spécifiques (TIMPs pour Tissue Inhibitor of MetalloProteinases) [94]. Chez le sujet sain, les MMPs participent à l'homéostasie du tissu conjonctif gingival qui est en constant remaniement. Par contre, dans des conditions pathologiques, la surexpression et l'activité accrue des MMPs peuvent entraîner des lésions tissulaires. Les MMPs, notamment la MMP-2, la MMP-3, la MMP-8 et la MMP-9, ont été associées à la destruction des tissus parodontaux [3, 94]. Une forte corrélation entre la sévérité de la maladie parodontale et le taux de MMPs présent dans le fluide créviculaire et les tissus parodontaux a été établie [3, 95]. La destruction tissulaire chez les patients atteints de parodontite implique un déséquilibre entre les quantités de MMPs actives et de TIMPs [95, 96]. Les bactéries du complexe rouge contribuent probablement à ce déséquilibre par différents mécanismes. En effet, *P. gingivalis*, *T. denticola* et *T. forsythia* stimulent la production de MMPs par les cellules de l'hôte [78, 97, 98]. Des infections simples et mixtes d'un modèle de co-culture de cellules épithéliales et de macrophages par les bactéries du complexe rouge induisent une forte sécrétion de MMP-9 [78]. De plus, *P. gingivalis* et *T. denticola* activent les formes latentes de MMPs par un clivage protéolytique [97, 99]. *P. gingivalis* possède également la capacité de dégrader le TIMP-1 [97, 100]. Ces propriétés des bactéries du complexe rouge contribuent probablement à l'augmentation du niveau de MMPs actives observée au cours des

parodontites. Ce phénomène pourrait favoriser une dégradation du collagène gingival, une destruction du ligament parodontal et une résorption de l'os alvéolaire [94].

1.5.10. Implication des bactéries du complexe rouge dans les maladies systémiques

Des bactériémies sont fréquemment observées à la suite des procédures dentaires (extraction de dent, chirurgie parodontale, traitement endodontique, détartrage radiculaire) mais également après la mastication d'aliments ou le brossage des dents [101]. De plus, la présence d'infections buccales chroniques telles les parodontites représente une condition qui favorise également l'entrée de bactéries dans le tissu conjonctif et dans la circulation sanguine. Cette propagation des bactéries buccales dans la circulation sanguine pourrait entraîner des maladies systémiques. En effet, les maladies parodontales ont été associées avec diverses désordres systémiques, notamment certaines pathologies cardiovasculaires et respiratoires, des endocardites infectieuses et des accouchements prématurés [101]. Une prévalence plus élevée des bactéries du complexe rouge a été détectée dans les poches parodontales chez les mères d'enfants prématurés [102]. Récemment, *P. gingivalis*, *T. denticola* et *T. forsythia* ont été détectés dans les lésions athérosclérotiques suggérant que ces bactéries se disséminent dans la circulation sanguine et se localisent dans les plaques d'athéromes [103, 104]. Une relation directe de cause à effet entre la présence de ces bactéries et le développement de l'athérosclérose n'a pas été établie mais les propriétés d'invasion et de stimulation de la réponse immuno-inflammatoire de l'hôte par ces bactéries pourraient contribuer au développement de cette maladie [105, 106]. En effet, des études ont mis en évidence que *P. gingivalis* peut notamment induire les lésions athérosclérotiques dans un modèle de souris, déclencher les évènements inflammatoires impliqués dans l'initiation de l'athérosclérose ou encore induire l'agrégation des plaquettes et la formation de macrophages spumeux [106]. De plus, les interactions de *P. gingivalis* et *T. forsythia* avec les récepteurs Toll induisent des mécanismes impliqués dans le développement de l'athérosclérose [105]. Ainsi les bactéries du complexe rouge, particulièrement *P. gingivalis*, pourraient contribuer directement ou indirectement à l'athérosclérose. *P. gingivalis* a également été détecté dans le fluide cérébrospinal d'une patiente atteinte d'un abcès au cerveau [107] et il a été avancé que cette bactérie pourait être impliqué dans le

développement de certaines pathologies pulmonaires, notamment des pneumonies, des abcès du poumon ou des broncho-pneumopathies chroniques obstructives [30, 108].

1.5.11. Conclusions

L'amélioration de la compréhension de l'étiopathogenèse des maladies parodontales résulte de la convergence de la recherche fondamentale et clinique. Ainsi, les espèces bactériennes impliquées et leur rôle dans le développement de ces maladies sont de mieux en mieux caractérisé. Les données accumulées établissent clairement le rôle des espèces du complexe rouge dans l'initiation et le développement des parodontites. Ces bactéries possèdent le potentiel pathogénique pour déclencher et diriger les séquences d'événements responsables des destructions tissulaires et osseuses observées au cours des maladies parodontales (Tableau 1.1). Pourtant, les mécanismes moléculaires des interactions microbiennes et du synergisme entre *P. gingivalis*, *T. denticola* et *T. forsythia* restent peu connus. Une meilleure compréhension des infections polymicrobiennes engendrées par ces bactéries nécessite des études utilisant notamment des combinaisons d'espèces parodontopathogènes. Par ailleurs, des études récentes suggèrent que les parodontites doivent être considérées comme un facteur de risque important pour le développement des maladies systémiques. Certains patients atteints de parodontite chronique répondent mal au traitement classique de cette maladie consistant à retirer la plaque bactérienne sous-gingivale. Ainsi, de nouvelles stratégies thérapeutiques fondées sur la modulation de la réponse de l'hôte sont en cours de développement pour soigner les parodontites. D'autres approches visant à atténuer la virulence des bactéries parodontopathogènes, notamment des espèces du complexe rouge, sont envisagées et pourraient être utiles particulièrement chez les individus possédant un risque élevé de développer une forme sévère de la maladie.

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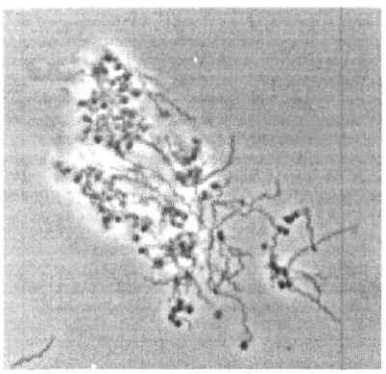

Figure 1.2. Culture mixte de *P. gingivalis* et *T. denticola*, observée par microscopie en contraste de phase, montrant *P. gingivalis* (cocco-bacille) attaché à la surface de *T. denticola* (spirochète).

Tableau 1.1. Principaux mécanismes pathogéniques des trois espèces bactériennes du complexe rouge : *P. gingivalis*, *T. denticola* et *T. forsythia.*

Colonisation du site	Evasion des défenses de l'hôte	Destruction tissulaire
Adhésion tissulaire et interbactérienne ➢ Fimbriae, adhésines, hémagglutinines ➢ Exposition de cryptitopes par les protéases **Nutrition** ➢ Acquisition d'acides aminés via l'action des protéases ➢ Acquisition de fer via les hémagglutinines et les hémolysines ➢ Interactions nutritionnelles (commensalisme et mutualisme) ➢ Augmentation de la perméabilité vasculaire et perturbation de la coagulation sanguine par les gingipaïnes	**Perturbation de la réponse inflammatoire** ➢ Dégradation des cytokines ➢ Dégradation des récepteurs de surface cellulaire **Résistance à la phagocytose** ➢ Dégradation des chimiokines ➢ Dégradation des immunoglobulines ➢ Dégradation des protéines du complément ➢ Dégradation du récepteur C5a	**Invasion des tissus de l'hôte** **Induction de l'apoptose** **Dégradation protéolytique des composantes tissulaires** **Induction de la production de cytokines, de PGE_2 et de MMPs** **Activation du plasminogène et des formes latentes de MMPs** **Dégradation d'inhibiteurs de protéases tissulaires** **Production de produits métaboliques toxiques et de vésicules membranaires**

1.6. Cibles thérapeutiques pour le traitement des maladies parodontales

Le développement des connaissances sur les mécanismes pathogéniques des parodontites a permis une meilleure compréhension du déclenchement et de la progression de ces maladies. Ainsi diverses cibles thérapeutiques potentielles ont été identifiées et plusieurs approches différentes sont envisagées afin de développer de nouveaux traitements pour les parodontites. En effet, certains patients atteints de parodontite chronique répondent mal à la thérapie conventionnelle de cette maladie consistant à éliminer la plaque bactérienne sous-gingivale par détartrage et surfaçage radiculaire. Des traitements complémentaires ont donc un intérêt particulièrement pour les personnes possédant un risque élevé de développer une forme sévère de parodontites, comprenant les diabétiques, les fumeurs et les individus présentant des prédispositions génétiques (Kornman, 1999). Deux types de stratégies sont envisagés, celles ciblant la réponse de l'hôte et celles ciblant les bactéries parodontopathogènes.

1.6.1. Modulation de la croissance, de l'adhérence et des activités protéolytiques des bactéries parodontopathogènes

Les bactéries parodontopathogènes de la plaque sous-gingivale sont le facteur étiologique primaire des maladies parodontales. L'élimination de ces bactéries est donc une approche thérapeutique privilégiée pour traiter les parodontites. En complément des pratiques courantes visant à éliminer mécaniquement la plaque bactérienne sous-gingivale, des agents antimicrobiens peuvent être utilisés dans certains cas. L'usage d'antibiotiques est néanmoins controversé à cuse de son efficacité clinique mitigée et de l'apparition de micro-organismes résistants (Venezia and Shapira, 2003; Bonito *et al.*, 2005). Des agents sans effets antibiotiques ciblant l'inhibition de l'adhésion des bactéries parodontopathogènes ou la formation du biofilm constituent une alternative thérapeutique intéressante pour la prévention et le traitement des maladies parodontales (Baehni and Takeuchi, 2003). Par ailleurs, basé sur les évidences de l'implication des protéases bactériennes dans la pathogénicité des parodontites, l'utilisation d'agents inhibant les activités protéolytiques

des bactéries parodontopathogènes a été suggérée comme une nouvelle approche pour le traitement des parodontites (Kadowaki *et al.*, 2004; Okamoto *et al.*, 2004). Les agents ciblant l'adhésion, la formation du biofilm ou les activités protéolytiques des bactéries parodontopathogènes pourraient ainsi constituer une alternative thérapeutique à l'usage d'antibiotiques.

1.6.2. Modulation de l'activité des cytokines

Les cytokines ont été fortement associées à la destruction des tissus parodontaux et à la progression des parodontites (Birkedal-Hansen, 1993a; Okada and Murakami, 1998). L'augmentation de l'expression de l'IL-1β et du TNF-α dans les tissus parodontaux enflammés et les fortes concentrations de ces cytokines retrouvées dans le fluide créviculaire de patients atteints de parodontites suggèrent que ces médiateurs inflammatoires jouent un rôle important dans le développement des parodontites (Graves and Cochran, 2003). L'utilisation de récepteurs solubles pour l'IL-1β et le TNF-α, qui inhibent par compétition l'action de ces cytokines, a été testée dans un modèle de parodontite expérimentale chez un primate (*Macaca fascicularis*). L'injection locale de ces récepteurs chez le macaque permet de réduire l'infiltration des cellules inflammatoires à proximité de l'os alvéolaire, et d'inhiber tant la formation des ostéoclastes que la résorption osseuse (Assuma *et al.*, 1998; Delima *et al.*, 2002; Oates *et al.*, 2002). Ces résultats suggèrent que les thérapies ciblant les cytokines pourraient être une approche bénéfique pour limiter la progression des maladies parodontales.

1.6.3. Modulation des métabolites de l'acide arachidonique

Les métabolites de l'acide arachidonique représentent une famille de médiateurs inflammatoires qui inclut les prostaglandines, la prostacycline et les thromboxanes générés par les cyclo-oxygénases ainsi que les leukotriènes générés par les lipo-oxigénases. De nombreuses études cliniques ont corrélé le niveau des métabolites de l'acide arachidonique

dans les tissus parodontaux ou dans le fluide créviculaire avec la sévérité des parodontites (Dewhirst *et al.*, 1983; Offenbacher *et al.*, 1993; Tsai *et al.*, 1998; Eberhard *et al.*, 2000; Emingil *et al.*, 2001). Les agents anti-inflammatoires non stéroïdiens (NSAIDs) qui empêchent la synthèse des métabolites de l'acide arachidonique en inhibant les cyclo-oxygénases (Fitzgerald and Patrono, 2001) sont envisagés comme traitement complémentaire des parodontites. Plusieurs études ont rapporté des effets bénéfiques des NSAIDs, notamment une diminution des destructions tissulaires et osseuses dans des modèles animaux de parodontites expérimentales (Kornman *et al.*, 1990; Paquette *et al.*, 1997; Holzhausen *et al.*, 2002; Gurgel *et al.*, 2004). Les NSAIDs améliorent également la résolution de l'inflammation gingivale dans des modèles de gingivites expérimentales chez l'homme (Johnson *et al.*, 1990; Heasman *et al.*, 1994; Jones *et al.*, 1999). Les NSAIDs exercent néanmoins des effets secondaires, tels que des ulcères gastriques (Hawkey, 1993) ou des dysfonctions rénales (Lindsley and Warady, 1990), ce qui rend leur usage systémique délicat pour le traitement d'une maladie chronique comme les parodontites. Par ailleurs, d'autres métabolites de l'acide arachidonique, les lipoxines et les 15-épi-lipoxines, ont été impliqués dans la modulation de la migration des leucocytes et la résolution d'un processus inflammatoire (Kantarci and Van Dyke, 2003). Il a été mis en évidence dans un modèle de parodontite induite par ligature chez le lapin qu'une application topique de 15-épi-lipoxine A4 ou la surexpression de lipoxines chez des lapins transgéniques réduisait la résorption osseuse et l'inflammation gingivale induites par *P. gingivalis* ou la ligature seule (Serhan *et al.*, 2003). Les lipoxines possèdent donc un potentiel comme nouvelle stratégie thérapeutique pour le traitement des parodontites.

1.6.4. Modulation de l'activité de l'oxyde nitrique

L'oxyde nitrique (NO) est un médiateur de nombreux processus biologiques, notamment le métabolisme osseux, la régulation de la contraction/dilatation vasculaire et les fonctions immunes, qui ont été impliqués dans les destructions tissulaires et osseuses observées au cours des parodontites (Ugar-Cankal and Ozmeric, 2006). Il a été rapporté que la synthèse du NO augmente dans les tissus gingivaux enflammés (Matejka *et al.*, 1999).

L'expression d'une enzyme impliquée dans la synthèse du NO, l'oxyde nitrique synthase inductible (iNOS), est plus forte dans les tissus gingivaux de patients atteints de parodontites chroniques que chez les sujets sains (Kendall *et al.*, 2000; Lappin *et al.*, 2000; Hirose *et al.*, 2001). Les bactéries buccales seraient à l'origine de la production de NO dans les tissus parodontaux (Lohinai *et al.*, 2001). Le NO peut induire la production et l'activation des MMPs, un phénomène qui pourrait participer à la destruction des tissus parodontaux (Brennan *et al.*, 2003). Les voies impliquées dans la synthèse du NO peuvent donc être considérées comme des cibles thérapeutiques potentielles pour le traitement des maladies parodontales. D'ailleurs, des approches différentes sont envisagées pour moduler l'activité du NO. Récemment, il a été démontré que des flavonoïdes inhibaient la production de NO et l'expression de l'iNOS chez des macrophages et des fibroblastes gingivaux stimulés par des LPS de bactéries parodontopathogènes (Gutierrez-Venegas *et al.*, 2006; Houde *et al.*, 2006). Il a été aussi rapporté que l'inhibition de l'iNOS réduisait la résorption osseuse dans un modèle de parodontite induite par ligature chez le rat (Lohinai *et al.*, 1998; Leitao *et al.*, 2005). De plus, l'inhibition de l'enzyme PARP (poly ADP-ribose polymerase), qui est activée par le NO, réduit les lésions osseuses induites par ligature chez le rat (Lohinai *et al.*, 2003). Ces résultats suggèrent que la modulation de l'activité du NO dans les tissus parodontaux pourrait être bénéfique pour prévenir ou traiter les parodontites.

1.6.5. Modulation de l'activité des MMPs

Comme mentionné précédemment, les MMPs ont été associées aux destructions parodontales (Birkedal-Hansen, 1993b; Reynolds and Meikle, 1997). La modulation de leur activité a donc été envisagée pour traiter les parodontites (Ryan *et al.*, 1996). Les tétracyclines, que ce soit sous une forme non-modifiée comme la doxycycline ou sous une forme modifiée chimiquement ne possédant pas d'activité antibiotique, sont connues pour inhiber l'activité des MMPs, notamment les collagénases (Vernillo *et al.*, 1994). Il a également été rapporté que ces substances possèdent la capacité d'inhiber les activités protéolytiques des bactéries parodontopathogènes (Grenier *et al.*, 2002). L'utilisation de la doxycycline à dose sous-antimicrobienne (SDD; subantimicrobial dose doxycycline) pour

le traitement des pathologies parodontales a récemment été approuvée par la « Food and Drug Administration » aux Etats-Unis d'Amérique. L'usage de la doxycycline à dose sous-antimicrobienne permet de prévenir les effets secondaires indésirables liés à un usage à long terme tels que les perturbations gastro-intestinales ou l'apparition de bactéries résistantes (Walker *et al.*, 2000). L'utilisation de la SDD comme traitement complémentaire des parodontites a montré des effets bénéfiques dans plusieurs études cliniques. En effet, ce traitement permet d'inhiber les activités collagénases dans les tissus parodontaux et le fluide créviculaire, d'augmenter l'efficacité du détartrage et surfaçage radiculaire (réduction de profondeur de poche parodontale et gain d'attache clinique) et de réduire la progression des parodontites (Caton *et al.*, 2000; Golub *et al.*, 2001; Novak *et al.*, 2002; Preshaw *et al.*, 2004b). L'inhibition de l'activité des MMPs au sein des tissus parodontaux semble donc une approche efficace comme traitement complémentaire des parodontites.

1.6.6. Modulation de la résorption osseuse

Puisque les parodontites se traduisent par une résorption osseuse, des applications thérapeutiques visant à inhiber ce processus sont envisagées pour traiter les parodontites. Les bisphosphonates, qui sont couramment utilisés dans le traitement de l'ostéoporose, inhibent l'activité des ostéoclastes (Rogers *et al.*, 2000). Les études sur les effets des bisphosphonates ont montré que ces molécules pouvaient réduire la résorption osseuse associée aux parodontites dans des modèles animaux et chez l'homme bien que des données contradictoires ont été rapportées (Salvi and Lang, 2005). En effet, l'efficacité clinique des bisphosphonates est parfois difficile à apprécier car bien que la densité osseuse semble augmentée dans certains cas, les paramètres cliniques comme la profondeur des poches et l'inflammation gingivale restent inchangés en comparaison du groupe placebo (Brunsvold *et al.*, 1992; Reddy *et al.*, 1995). Les bisphosphonates pourraient même amplifier le processus inflammatoire *in vivo* en stimulant la production d'IL-1 et d'IL-6 (Adami *et al.*, 1987; Schweitzer *et al.*, 1995). Des études supplémentaires sont donc requises pour évaluer l'efficacité de cette approche thérapeutique.

1.7. Description du projet de recherche

Les destructions tissulaires et osseuses observées au cours des parodontites résultent d'un processus complexe opérant selon deux voies principales : i) la sécrétion par les microorganismes d'enzymes protéolytiques qui dégradent les structures cellulaires et permettent de contourner les défenses de l'hôte, et ii) la stimulation des cellules de l'hôte par les facteurs microbiens (LPS, toxines, vésicules membranaires, enzymes), qui engendre directement l'expression d'enzymes hydrolytiques et la sécrétion de cytokines permettant d'activer diverses voies de dégradation. Le processus de destruction implique donc différents facteurs, notamment la virulence des bactéries parodontopathogènes et le niveau de la réponse immuno-inflammatoire de l'hôte (Kornman *et al.*, 1997; Reynolds and Meikle, 1997; Okada and Murakami, 1998; O'Brien-Simpson *et al.*, 2004; Holt and Ebersole, 2005). De nombreuses études ont montré que les bactéries parodontopathogènes ou leurs facteurs de virulence induisaient une modulation de la production de médiateurs inflammatoires et de MMPs par les cellules de l'hôte (Ogawa *et al.*, 1994; Fravalo *et al.*, 1996; Rosen *et al.*, 1999; Sandros *et al.*, 2000; Steffen *et al.*, 2000; Sfakianakis *et al.*, 2001; Belibasakis *et al.*, 2005). Cependant, la faiblesse de ces études est due au fait que les stimulations ont été réalisées sur des lignées cellulaires prises individuellement et que ces stimulations n'ont pas été effectuées avec des combinaisons de bactéries parodontopathogènes. En effet, les parodontites sont reconnues comme des infections mixtes et de nombreux types cellulaires présents dans le parodonte participent à la réponse immuno-inflammatoire de l'hôte (Haffajee and Socransky, 1994; Kornman *et al.*, 1997; Socransky *et al.*, 1998). Afin de prendre en considération les interactions entre les différents types de cellules de l'hôte ainsi que les interactions entre les bactéries parodontopathogènes, cette étude a utilisé des modèles pluricellulaires et testé l'effet de combinaisons de bactéries parodontopathogènes. De plus, une corrélation entre la concentration d'hémoglobine dans les tissus gingivaux et les phases actives des parodontites ayant été suggérée (Hanioka *et al.*, 1989; Hanioka *et al.*, 1990; Hanioka *et al.*, 1991), l'effet de l'hémoglobine sur la réponse inflammatoire induite par les LPS de bactéries parodontopathogènes a été investigué. Par ailleurs, dans le but de développer des

solutions thérapeutiques complémentaires pour les patients atteints de parodontites chroniques répondant mal au traitement classique de cette maladie et/ou possédant un risque élevé de développer une forme sévère de parodontites, le potentiel de fractions de canneberge à inhiber certains mécanismes pathogéniques des parodontites a été évalué.

Dans le cadre de ce projet de recherche, trois hypothèses de travail ont été définies :

- Les bactéries parodontopathogènes, et plus particulièrement *P. gingivalis*, induisent une forte production de médiateurs inflammatoires chez différentes lignées cellulaires; un phénomène contribuant à la réponse immunodestructrice de l'hôte.
- L'augmentation de la concentration d'hémoglobine dans les tissus gingivaux a un effet sur la réponse de l'hôte.
- Les polyphénols de la canneberge (voir appendice 1) ont des effets potentiellement bénéfiques pour la santé parodontale en inhibant certains mécanismes pathogéniques impliqués dans l'initiation et le développement des parodontites.

Afin de vérifier ces hypothèses, quatre objectifs ont été définis :

1. Caractériser l'interaction hôte-*P. gingivalis* par l'utilisation d'un modèle de cellules épithéliales et de macrophages en co-culture et d'un modèle *ex vivo* de sang complet.
2. Évaluer un effet synergique causé par une combinaison de bactéries parodontopathogènes lors d'une stimulation des cellules de l'hôte.
3. Caractériser l'effet de l'hémoglobine sur la production de médiateurs inflammatoires induite par le LPS des bactéries parodontopathogènes chez les macrophages.
4. Évaluer les effets bénéfiques potentiels des polyphénols de la canneberge sur *P. gingivalis* ainsi que sur les enzymes protéolytiques et les médiateurs inflammatoires impliqués dans la pathogenèse des parodontites.

Les résultats de l'objectif 1 sont détaillés dans les chapitres 2 et 3, ceux de l'objectif 2 sont présentés dans le chapitre 4. L'objectif 3 constitue l'objet du chapitre 5 et l'objectif 4 celui des chapitres 6 à 10.

Dans le chapitre 2, un modèle de co-culture de cellules épithéliales et de macrophages a été développé afin de caractériser la production de cytokines pro-inflammatoires induite par *P. gingivalis*. L'effet des activités protéolytiques de cet agent pathogène, notamment leur implication dans la dégradation des cytokines sécrétées, a été évalué.

Dans le chapitre 3, l'induction par *P. gingivalis* de la production de médiateurs inflammatoires dans un modèle *ex vivo* de sang complet a été caractérisée. La capacité de différentes souches de *P. gingivalis* à stimuler la sécrétion de nombreux médiateurs inflammatoires (cytokines, chimiokines et prostaglandines) dans une population mixte de leucocytes a été testée. L'activité des protéases de *P. gingivalis* en présence de sérum a également été évaluée.

Dans le chapitre 4, le modèle de co-culture de cellules épithéliales et de macrophages a été infecté par les cellules entières et les LPS de *P. gingivalis*, *T. denticola* et *T. forsythia* pris individuellement ou en combinaison. L'effet de ces stimulations sur la sécrétion de cytokines et chimiokines pro-inflammatoires, de PGE_2 et de MMP-9 a été caractérisé. La dégradation de la chimiokine RANTES par *T. denticola* a également été mise en évidence.

Dans le chapitre 5, l'effet de l'hémoglobine sur la production de médiateurs inflammatoires induite par le LPS des bactéries parodontopathogènes chez les macrophages a été investigué. La sécrétion de cytokines et chimiokines par les macrophages stimulés par différentes concentrations d'hémoglobine et le LPS de *P. gingivalis* et *F. nucleatum* a été mesurée. De plus, l'effet de l'hémoglobine sur la liaison des LPS aux macrophages a été caractérisé.

Dans le chapitre 6, différentes fractions de la canneberge ont été testées afin de déterminer leur capacité d'inhibition de la production de cytokines induite par les LPS des bactéries parodontopathogènes, incluant *A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *T. denticola* et *T. forsythia*. Une fraction de haut poids moléculaire obtenue par dialyse du jus concentré et nommée NDM (non dialysable material) ainsi que du jus concentré lyophilisé ont été utilisés.

Dans le chapitre 7, l'effet de la fraction NDM sur les fibroblastes gingivaux a été caractérisé. La sécrétion de médiateurs inflammatoires induite par le LPS d'*A. actinomycetemcomitans* a été mesurée en présence ou non de la fraction NDM. Des analyses de « microarray » ont été effectuées afin d'investiguer les voies de signalisation cellulaires affectées par la fraction NDM.

Dans le chapitre 8, l'effet de la fraction NDM sur la production de MMPs induite par le LPS d'*A. actinomycetemcomitans* chez les macrophages et les fibroblastes gingivaux a été évalué. Des analyses de « microarray » ont été effectuées pour caractériser l'effet de la fraction NDM sur les voies de signalisation cellulaire impliquées dans la production des MMPs. Le potentiel d'inhibition de la fraction NDM sur l'activité de la MMP-3, de la MMP-9 et de l'élastase a également été mesurée.

Dans le chapitre 9, l'activité des protéases des bactéries du complexe rouge en présence de la fraction NDM a été caractérisée. L'effet de la fraction NDM sur la dégradation de la transférine et du collagène de type I par *P. gingivalis* a été aussi mesurée.

Dans le chapitre 10, l'effet de la fraction NDM sur la formation d'un biofilm par *P. gingivalis* ainsi que sur la croissance et la viabilité de cette bactérie a été testé. L'adhérence de *P. gingivalis* au collagène de type I, au fibrinogène et aux protéines du sérum humain a été mesuré en présence ou non de la fraction NDM.

CHAPITRE 2

Modulation of cytokine production by *Porphyromonas gingivalis* in a macrophage and epithelial cell co-culture model

2.1. Résumé

Les cellules épithéliales et les macrophages jouent un rôle majeur dans la réponse de l'hôte envers *Porphyromonas gingivalis*, un agent étiologique majeur des parodontites chroniques. La sécrétion de fortes quantités de cytokines par ces cellules contribue à la destruction des tissus parodontaux. Afin d'investiguer les interactions entre *P. gingivalis* et ces deux lignées cellulaires majeures, la production d'IL-1β, d'IL-6, d'IL-8, de TNF-α et de RANTES par un modèle *in vitro* de cellules épithéliales et de macrophages en co-culture stimulé par différentes souches de *P. gingivalis* a été caractérisée. *P. gingivalis* a induit la sécrétion de cytokines pro-inflammatoires (IL-1β et IL-6) et de chimiokines (IL-8 et RANTES) par le modèle de co-culture. Les réponses aux infections par *P. gingivalis* ont été influencées par le ratio macrophages / cellules épithéliales présent dans le modèle de co-culture. De plus, le niveau de sécrétion de ces médiateurs inflammatoires était dépendant de la souche bactérienne et de la dose infectieuse utilisées. Les résultats obtenus avec un mutant déficient pour les gènes codant les gingipaïnes ou avec un pré-traitement des bactéries par un inhibiteur de cystéine protéases ont suggéré que les quantités de cytokines sécrétées par le modèle de co-culture sont sous-estimée à cause d' une dégradation protéolytique importante. Cette étude suggère que *P. gingivalis* peut moduler la production de médiateurs inflammatoires, ce qui pourrait contribuer à la progression des parodontites.

2.2. Abstract

Epithelial cells and macrophages play a major role in the host response to *Porphyromonas gingivalis*, a major etiologic agent of chronic periodontitis. Secretion of high levels of cytokines by these cells is believed to contribute to periodontal tissue destruction. To investigate the interactions between *P. gingivalis* and these two major cell types, we characterized the production of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α) and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) by an *in vitro* co-culture model composed of epithelial-like transformed cells (HeLa cell line) and macrophage-like cells (phorbol myristic acid-differentiated U937 cell line) following a challenge with different strains of *P. gingivalis*. *P. gingivalis* cells stimulated the secretion of pro-inflammatory cytokines (IL-1β and IL-6) and chemokines (IL-8 and RANTES) in the co-culture model. Responses to *P. gingivalis* infection were influenced by the macrophage/epithelial cell ratios of the cultures. In addition, the level of secretion of these inflammatory mediators was dependent on the bacterial strain and the multiplicity of infection (MOI) used. The use of a gingipain-deficient mutant of *P. gingivalis* or the addition of a cysteine protease inhibitor suggested that the level of cytokines secreted by the co-culture model was underestimated due to an extensive proteolytic degradation. This study showed that *P. gingivalis* can modulate the levels of inflammatory mediators, which may contribute to the progression of periodontitis.

2.3. Introduction

Periodontal diseases are complex, multifactorial, polymicrobial infections characterized by the destruction of tooth supporting tissues. While human subgingival plaque harbors some 500 bacterial species [1] evidence points to *Porphyromonas gingivalis*, a Gram-negative anaerobic bacterium, as the major etiologic agent of chronic periodontitis [2]. *P. gingivalis* produces a broad range of virulence factors, including three

cysteine proteinases (gingipains) that apparently contribute to periodontal tissue destruction either directly or indirectly by modulating the host inflammatory response [3-5]. More specifically, Arg-gingipains (RgpA and RgpB) and Lys-gingipain (Kgp) cysteine proteinases are the main endopeptidases produced by *P. gingivalis* and can be cell bound or secreted [5]. The incidence and rate of progression of periodontitis involve complex interactions between periodontopathic bacteria and cells of the host immune system. Indeed, the continuous and high secretion of various cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α), by host cells following stimulation by periodontopathogens is thought to modulate periodontal tissue destruction [6, 7].

Epithelial cells are an important mechanical barrier to periodontopathogens and act as sensors during microbial infections [8]. They offer an interactive interface for subgingival bacterial plaque, generating and transmitting signals between bacteria and the adjacent and underlying immune cells of the periodontal tissues. It is now believed that epithelial cells form an integral part of the innate immune system and take an active role in the inflammatory response [9, 10]. They notably have the capacity to secrete inflammatory mediators in response to bacterial stimulation [9, 10]. Monocytes and macrophages, which are found in higher numbers in active periodontal lesions than in inactive sites [11], are key members of the innate immune system and play a critical role in the host response during chronic infections [12]. Mononuclear leucocytes, especially monocytes, are mostly found within gingival connective tissues and are a major part of the inflammatory infiltrate [13]. Given the multifunctional roles of monocytes, macrophages and epithelial cells, they are likely important in the initiation and maintenance of inflammatory processes and the alveolar bone loss observed in adult periodontitis [14]. Interactions between macrophages and the epithelium are one of the pathways involved in regulating local immune mechanisms [15]. In this study, we developed an *in vitro* macrophage/epithelial cell co-culture model to investigate the interactions between *P. gingivalis* and two major immune cell types. Such a model may offer the advantage of providing a more comprehensive view of the cytokine response mediated by this periodontopathogen. Co-cultures with different macrophage/epithelial cell ratios were stimulated with various concentrations of *P.*

gingivalis strains and were analyzed for cytokine production. The impact of *P. gingivalis* gingipain cysteine proteinases on cytokine levels was also investigated.

2.4. Materials and methods

2.4.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *P. gingivalis* ATCC 33277, ATCC 53977 and KDP128. KDP128, which is a gingipain null mutant (*rgpA*, *rgpB* and *kgp*) of ATCC 33277, was kindly provided by K. Nakayama (Nagasaki University, Japan) and constructed using suicide plasmids [16, 17]. Bacteria were grown in Todd Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 0.001% hemin and 0.0001% vitamin K. Bacterial cultures were incubated at 37°C under anaerobic conditions (N_2:H_2:CO_2/80:10:10).

2.4.2. Epithelial cell and monocyte cultures

Epithelial-like transformed cells (HeLa cell line) were maintained as frozen stocks and cultured as monolayers in a 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine (DMEM; HyClone Laboratories, Logan, UT, USA), 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO, USA) and 100 µg/mL of penicillin-streptomycin (DMEM-10% FBS). U937 cells (ATCC CRL-1593.2; monoblastic leukemia cell line) were maintained in a 5% CO_2 atmosphere at 37°C in 25 cm^2 tissue culture flasks by twice weekly splitting of cells in Roswell Park Memorial Institute 1640 medium (RPMI; HyClone Laboratories) supplemented with 10% (v/v) heat-inactivated FBS and 100 µg/mL of penicillin-streptomycin (RPMI-10% FBS). Monocytes (2 x 10^5 cells/ml) were incubated in RPMI-10% FBS containing 10 ng/ml of phorbol myristic acid (PMA; Sigma Chemical. Co.) for 48 h to induce differentiation into adherent macrophage-like cells as previously reported [18]. PMA is known to induce the appearance of cell characteristics consistent with mature

macrophages [19]. Following the PMA treatment, the medium was removed by aspiration and replaced with fresh medium. The differentiated cells were incubated for an additional 24 h prior to use.

2.4.3. Macrophage-epithelial cell co-culture model

Adherent macrophages were harvested by scraping and suspended in 15 ml of RPMI-10% FBS. The suspension was centrifuged at 200 x *g* for 8 min. The cells were washed once in RPMI-10% FBS and suspended in DMEM-1% FBS at a density of 1 x 10^6 cells/ml as determined using a hemacytometer. Epithelial cells were suspended by gentle trypsinization with a 0.05% Trypsin-EDTA solution (Gibco-BRL, Grand Island, NY, USA), washed once in DMEM-10% FBS and suspended at a density of 1 x 10^6 cells/ml in DMEM-1% FBS. Mixtures with macrophage/epithelial cell ratios of 1:100 and 1:5 were prepared and cultured in 6-well plates (2 x 10^6 cells/well in 2 ml) at 37°C in a 5% CO_2 atmosphere for 3 h to allow adhesion of the cells prior to infection.

2.4.4. Infection of the co-culture model

Bacteria were grown to the early stationary phase (24 h) at 37°C in an anaerobic chamber. They were harvested by centrifugation at 11,000 x *g* for 10 min and suspended in DMEM-1% FBS at a concentration of 1 x 10^{10} cells/ml as determined using a Petroff-Hausser counting chamber. The bacterial suspensions were added to the macrophage/epithelial cell co-cultures at a multiplicity of infection (MOI) of 50 and 500 bacteria per mammalian cell. The infected co-cultures were incubated at 37°C in a 5% CO_2 atmosphere. Samples of culture medium were removed at 24 h post-infection, centrifuged at 11,000 x *g* for 10 min to eliminate the bacteria and stored at –20°C until used. Control cultures were incubated in the absence of bacteria. All infections were carried out in triplicate.

2.4.5. Treatment of *P. gingivalis* with protease inhibitor

To investigate the impact of *P. gingivalis* gingipain cysteine proteinases on cytokine determination in the co-culture model, *P. gingivalis* cells were pretreated with a cysteine proteinase inhibitor. *P. gingivalis* ATCC 33277 and ATCC 53977 cells were harvested by centrifugation (11,000 x *g*), suspended in PBS containing 1 mM Nα-*p*-tosyl-L-lysine chloromethylketone (TLCK; Sigma Chemical Co.) and incubated for 1 h at 37°C. The cells were then washed once in PBS and resuspended in DMEM-1% FBS to a final concentration of 1 x 10^{10} cells/ml. Infections of the co-culture model were performed as described above.
2.6. Cell viability.

2.4.6. Cell viability

Viability of macrophages and epithelial cells was measured by Trypan Blue exclusion. A 100 µl aliquot of cell suspension was mixed with an equal volume of 0.4% Trypan Blue solution (Gibco-BRL) and viable cells were counted with a hemacytometer using a light microscope.

2.4.7. Determination of cytokine production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify IL-1β, IL-6, IL-8, TNF-α and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) in cell-free culture supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (model 680; BioRad Laboratories, Mississauga, ON, Canada) with a wavelength correction set at 550 nm. Cytokine concentrations were determined in triplicate using a standard curve prepared for each assay.

2.4.8. Determination of cytokine degradation

A possible cytokine degradation mediated by *P. gingivalis* occurring during infection of the co-culture model was investigated. Supernatants of an unstimulated 1:5 co-culture (incubated during 30 h) were harvested prior to adding the bacteria at a concentration equivalent to that used for the infection. After a 24 h incubation, the residual cytokine concentrations in the supernatants were determined by ELISA and compared to the basal levels of a control incubated (24 h) in the absence of bacteria.

2.4.9. Determination of protease activities

Washed whole *P. gingivalis* cells (24 h culture) were incubated with equal volumes of synthetic chromogenic substrates (1 mM in distilled water) and 10 mM dithiothreitol as a reducing agent. Substrates used were: glycyl-proline-*p*-nitroanilide (specific for dipeptidyl peptidase IV), benzoyl-arginine-*p*-nitroanilide (specific for Arg-gingipains A and B), and N-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide (specific for Lys-gingipain). The assay mixtures were incubated 4 h at 37°C. Cells were removed by centrifugation and hydrolysis of the chromogenic substrates was determined by measuring the absorbance at 405 nm.

2.4.10. Scanning electron microscopy

The co-culture model was cultivated (uninfected and infected with *P. gingivalis*) during 24 h in a 6 well-plate as previously described except that glass coverslips were placed into wells. Cells were fixed 24 h at 4°C with 2.5% glutaraldehyde, 4% paraformaldehyde and 2 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer (pH 7.3). Samples were dehydrated through a graded series of ethanol, critical point dried, gold sputtered and examined by a JEOL JSM6360LV scanning electron microscope.

2.4.11. Statistical analyses

Statistical analyses were performed by the Student's t test for paired values and data were considered significant at a p value of < 0.05.

2.5. Results

2.5.1. Effect of *P. gingivalis* on IL-6 production by the co-culture model

The ability of three strains of *P. gingivalis* to modulate the secretion of IL-6 in a macrophage/epithelial cell co-culture model was first examined. Since no data were available on the *in vivo* ratio of macrophages to epithelial cells in the periodontium, two ratios were defined and tested. The 1:100 ratio was considered to correspond to a relative healthy state while the 1:5 ratio was considered to reflect an inflamed periodontium. There were no significant differences in the basal levels of cytokines secreted by cells grown in co-culture and cells cultivated individually (data not shown). During the stimulation of the co-culture model, the macrophages and epithelial cells remained adherent and retained their normal morphological characteristics (Fig. 2.1). Scanning electron microscopy of the infected model showed the presence of micro-colonies of *P. gingivalis* mostly attached to epithelial cells (Fig. 2.1C)

No significant induction of IL-6 secretion was observed in response to a challenge by *P. gingivalis* (ATCC 33277, ATCC 53977 and KDP128) at an MOI of 50 and a co-culture ratio of 1:100 except in the case of TLCK-treated ATCC 53977 cells, which induced a significant increase in IL-6 secretion (Fig. 2.2A). Surprisingly, no IL-6 was detected in the supernatants following stimulation of the co-culture model (at either ratio) by *P. gingivalis* ATCC 33277 at an MOI of 500 (Figs. 2.2A and 2.2B). However, *P. gingivalis* ATCC 33277 cells preincubated with the cysteine protease inhibitor TLCK at an MOI of 50 slightly stimulated IL-6 secretion by a 1:5 co-culture. At a MOI of 500, both TLCK-treated ATCC 33277 and the gingipain-null mutant KDP128 induced an increase in

IL-6 secretion. These results suggest that the absence of IL-6 in the supernatant of the co-culture infected with a high MOI is likely due to the gingipains produced by *P. gingivalis* ATCC 33277. Compared with the unstimulated co-culture control, no significant difference in the amount of secreted IL-6 was observed following stimulation of the 1:5 co-culture by ATCC 53977 (Fig. 2.2B). This strain, which is virulent in an animal model [20], thus had a lower capacity than ATCC 33277 to modulate IL-6 secretion.

To exclude the possibility that cell toxicity due to the *P. gingivalis* infection of the co-cultures might have been responsible for the lack of IL-6 in the culture supernatants or the decrease in IL-6 levels, the viability of the cells making up the co-culture model was evaluated by Trypan blue exclusion. No obvious cytotoxic effects following infections of the co-culture model with *P. gingivalis* at either MOI were detected, and cell viability was $\geq$ 96% of the controls in all experiments (data not shown). A previous study also reported that *P. gingivalis* (10^6 to 10^{11} bacteria/ml) did not affect epithelial cell viability after a 24 h incubation [21].

2.5.2. Effect of *P. gingivalis* on TNF-α and IL-1β production by the co-culture model

No detectable TNF-α was secreted by the co-culture model in any of the experimental conditions tested (data not shown). IL-1β was detected only at a co-culture ratio of 1:5 (Fig. 2.3), suggesting that a greater density of macrophages is required to produce detectable levels of IL-1β in the model. Low amounts of IL-1β were detected in the co-culture supernatants except when the model was stimulated with TLCK-treated *P. gingivalis* ATCC 33277 cells at an MOI of 500, which caused a significant increase in IL-1β secretion. These data suggest that *P. gingivalis* ATCC 33277 was able to induce a strong IL-1β response in the co-culture model and that the secreted cytokines were degraded by *P. gingivalis* proteases. TLCK-treated *P. gingivalis* ATCC 53977 did not induce higher levels of IL-1β secretion than the untreated bacteria.

2.5.3. Effect of *P. gingivalis* on RANTES production by the co-culture model

Although the chemokine RANTES has recently been associated with periodontitis [22], RANTES production by human cell lines in response to a *P. gingivalis* challenge is poorly documented. RANTES concentrations in the supernatants of the co-culture model following stimulation with the *P. gingivalis* strains is reported in Figure 2.4. Stimulation of the co-culture model by either TLCK-treated *P. gingivalis* ATCC 33277 or the gingipain-null mutant KDP128 at an MOI of 50 induced an increase in RANTES secretion by co-cultures at both ratios whereas the stimulatory effect at an MOI of 500 was only observed with a co-culture ratio of 1:5. In contrast, stimulation by *P. gingivalis* ATCC 53977 caused a significant decrease in RANTES concentrations under all experimental conditions. In addition, no residual RANTES was detected in the supernatant of the co-culture model following stimulation by ATCC 33277 at an MOI of 500. However, this effect was not observed when the gingipain-deficient strain KDP128 was used. Much higher concentrations of RANTES were detected in the supernatants of co-cultures stimulated with ATCC 33277 or ATCC 53977 preincubated with 1 mM TLCK than with untreated bacterial cells. These results suggest that *P. gingivalis* gingipains contribute to the decrease in RANTES concentrations in the supernatant.

2.5.4. Effect of *P. gingivalis* on IL-8 production by the co-culture model

When a co-culture at a ratio of 1:100 was used, *P. gingivalis* ATCC 33277 induced a strong IL-8 response at an MOI of 50 whereas no detectable IL-8 was found at an MOI of 500 (Fig. 2.5A). However, when the co-culture was stimulated with TLCK-treated ATCC 33277 or the gingipain-null mutant KDP128, IL-8 concentrations in the supernatants were much higher than that induced by ATCC 33277 at an MOI of 500 but much lower than that induced by ATCC 33277 at an MOI of 50. This suggests that proteases of ATCC 33277 were responsible in part for the induction of IL-8 secretion at a low MOI whereas the same enzymes were involved in the degradation of IL-8 at a high MOI. No modulation of IL-8 concentrations was observed when the 1:100 co-culture model was stimulated with strain

ATCC 53977. However, when ATCC 53977 was pretreated with 1 mM TLCK, IL-8 secretion was higher than that observed with untreated bacteria and the control. Stimulation of the 1:5 co-culture model at an MOI of 50 induced an increase in IL-8 secretion under all conditions (Fig. 2.5B). ATCC 33277 induced a stronger IL-8 response that ATCC 53977. Furthermore, no significant difference was observed between TLCK-treated and untreated ATCC 33277 and ATCC 53977. However, when ATCC 33277 was used at an MOI of 500, no accumulation of IL-8 was observed whereas IL-8 concentrations increased under all the other conditions tested. These results suggest that IL-8 degradation by ATCC 33277 is responsible for the lack of IL-8 accumulation in the co-culture supernatant and that ATCC 33277 has a higher capacity to degrade IL-8 than ATCC 53977.

2.5.5. Proteolytic degradation of IL-6, IL-8 and RANTES by *P. gingivalis*

Since no IL-6, IL-8 or RANTES was detected in the supernatants of the co-culture model following a challenge with *P. gingivalis* at an MOI of 500, we assessed the capacity of cells of ATCC 33277 and ATCC 53977 (treated or not with 1 mM of TLCK) to degrade IL-6, IL-8 and RANTES. Bacteria at a concentration equivalent to that used to stimulate the co-culture model were incubated for 24 h at 37°C with cell-free supernatant containing basal levels of cytokines. A bacterial concentration of 5×10^7 cells per ml corresponded to an MOI of 50 whereas 5×10^8 cells per ml corresponded to an MOI of 500. As reported in Table 2.1, cells of ATCC 33277 showed a strong capacity to degrade IL-6, IL-8 and RANTES. Partial degradation of the cytokines was observed at a low bacterial concentration while the cytokines were completely hydrolyzed at the high bacterial concentration. The degradation of IL-6, IL-8 and RANTES was significant ($p < 0.05$) even at the low bacterial concentration. Pretreatment of ATCC 33277 with 1 mM of TLCK reduced the degradation of IL-6 and IL-8 but had a much lower effect on the degradation of RANTES. Unlike ATCC 33277, ATCC 53977 only slightly degraded IL-6 and IL-8, even at the high bacterial concentration. In addition, no significant differences in IL-6 and IL-8 degradation were observed between TLCK-treated and untreated bacteria. However, pre-existing RANTES was completely degraded by ATCC 53977 cells at both bacterial concentrations. The ability of ATCC 53977 to degrade RANTES was partially inhibited by

preincubating the bacterial cells with 1 mM of TLCK, suggesting that proteases are responsible for the degradation. These results support the hypothesis that *P. gingivalis* possesses a strong capacity to degrade cytokines, although the capacity differs among strains. Assessment of cell-associated protease activity in ATCC 33277 and ATCC 53977 have revealed that these strains have different proteolytic capacities. While Arg-gingipain activities are similar between the two strains, the strain ATCC 53977 possesses a much higher (2-fold) Lys-gingipain activity than strain ATCC 33277, whereas this latter has a higher (1.7-fold) dipeptidyl peptidase IV activity than strain ATCC 53977 (data not shown). Lastly, no significant Arg- and Lys-gingipain activities were detected when bacteria were treated with 1 mM TLCK.

2.6. Discussion

A number of studies have reported that *P. gingivalis* modulates the expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) by gingival epithelial cells [23-25]. In addition, several reports have indicated that specific components of *P. gingivalis* including the fimbriae and LPS induce the production of pro-inflammatory cytokines, in particular IL-1, IL-6, IL-8 and TNF-α, by human and murine monocyte and macrophage lines [26-31]. However, all these studies used individual cell lines. In the present study, we characterized the production of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) and chemokines (IL-8 and RANTES) in a macrophage/epithelial cell co-culture model at two different cell ratios (1:100 and 1:5) in response to challenges by different strains of *P. gingivalis*. This model offers the advantage of characterizing the production of cytokines by macrophages and epithelial cells that are stimulated by *P. gingivalis* while taking into consideration the interactions that may occur between these two cell types. However, one should not exclude the possibility that the use of immortalized cells may not reflect exactly the *in vivo* situation.

Various effects of *P. gingivalis* on the production of IL-8 by epithelial cells have been previously reported. Darveau *et al.* [23] showed that *P. gingivalis* disrupts the ability of gingival epithelial cells to produce IL-8. Attenuation of IL-8 production by KB cells

following *P. gingivalis* challenge has also been reported by Madianos *et al.* [32]. A study by Huang *et al.* [33] demonstrated that *P. gingivalis* increases IL-8 mRNA levels and decreases IL-8 protein levels. In contrast, other studies have reported that *P. gingivalis* (cell surface components or whole cells) induces a strong IL-8 response in oral epithelial cells [24, 34]. In addition, the stimulation of macrophages by *P. gingivalis* LPS has been shown to induce increased IL-8 secretion [26]. Our study using a macrophage/epithelial cell co-culture model showed that strain ATCC 33277 induced a strong IL-8 response at a lower MOI and that no IL-8 was detected in co-culture supernatants at a higher MOI. However, the IL-8 did not disappear when *P. gingivalis* was pretreated with the cysteine proteinase inhibitor TLCK, which results in a complete neutralization of gingipain activities. The decrease in IL-8 levels was much less pronounced when *P. gingivalis* ATCC 53977 was used to infect the model. This indicates that *P. gingivalis* enhances IL-8 secretion by a macrophage/epithelial cell co-culture but that IL-8 levels decrease because of extensive proteolytic degradation. The intensity of this effect depends on the strain and bacterial concentration used. This may explain the discrepancies found in the literature concerning the effect of *P. gingivalis* on IL-8 production.

High levels of RANTES have been detected in gingival crevicular fluid and inflamed gingival tissue adjacent to periodontal pockets of adult patients with periodontitis, and have lead the authors to suggest that RANTES is important in the initiation and progression of periodontitis [22, 35]. The effect of *P. gingivalis* on RANTES production is poorly understood. An increase in RANTES secretion by the macrophage/epithelial cell co-culture model following *P. gingivalis* challenge was only observed with the gingipain-null strain KDP128 and TLCK-treated ATCC 33277, suggesting that *P. gingivalis* is able to degrade RANTES even at a low bacterial concentration. This was confirmed by the fact that the RANTES initially present in the supernatant of an unstimulated co-culture disappeared following the addition of *P. gingivalis*. *P. gingivalis* thus induces RANTES secretion but this effect is masked by the subsequent proteolytic degradation of the chemokine.

IL-1β in the supernatant of the co-culture was only detected at a 1:5 ratio. This was especially evident when TLCK-treated *P. gingivalis* ATCC 33277 was used to infect the co-culture, indicating that IL-1β may be highly sensitive to the proteolytic activity of *P. gingivalis*. However, only a small amount of IL-1β was detected following stimulation with the gingipain-null mutant. This suggests that proteases other than gingipains may be involved in the degradation of IL-1β or that the previously reported pleiotropic effects of the gingipain mutation [36] may result in a lower capacity of *P. gingivalis* to induce IL-1β secretion.

The phenomenon of cytokine degradation may result in an underestimation of the inflammatory response mediated by *P. gingivalis* and may explain, at least in part, the divergence in the scientific literature concerning the modulation of cytokine production by *P. gingivalis*. Major proinflammatory cytokines including interferon-γ (IFN-γ) [37], IL-1β [38], TNF-α [39], IL-6 [40], IL-8 [41] and IL-12 [42] have been reported to be degraded by *P. gingivalis* cysteine proteinases. These studies used recombinant cytokines and purified proteases or bacterial fractions. Using a different approach, we confirmed the susceptibility of IL-6 and IL-8 and we report for the first time the proteolytic inactivation of RANTES by *P. gingivalis*. The strains of *P. gingivalis* tested showed different proteolytic capacities, which may explain the variations observed in degradation of IL-6, IL-8 and RANTES. Based on our results, the proteolysis of cytokines is an important factor to take into account when conducting studies on the production of cytokines following a *P. gingivalis* challenge. In our model, we do not exclude the possibility that gingipains may degrade cell surface receptors on the macrophage receiving signals from bacterial surface. Such a phenomenon may contribute to reduction of cytokine production. Interestingly, it has been reported that gingipains provoke a proteolysis of IL-6 receptor [43] and a loss of receptor CD14 [44-46] from the surface of monocytes, macrophages and fibroblasts leading to lipopolysaccharide hyporesponsiveness. It is also possible that *P. gingivalis* and its proteases could be indirectly involved in cytokine degradation through inductions of host derived matrix-metalloproteinases production [47] or activation of latent forms of matrix-

metalloproteinases [48]. Indeed, these proteinases have been reported to have the capacity to mediate cytokine proteolysis [49].

IL-6 plays an important role in regulating the immune response to periodontal pathogens and is notably responsible for the differentiation of osteoclasts and activated B cells into immunoglobulin-secreting plasma cells. Degradation of IL-6 by *P. gingivalis* may thus contribute to the perturbation of the inflammatory response and promote the progression of periodontitis. IL-8 and RANTES are potent chemokines that direct the migration of neutrophils, eosinophils, monocytes and T_H1 cells to sites of infection [50]. Our results provide evidence that *P. gingivalis* has the potential to modulate chemokine levels in periodontal tissue. On the one hand, *P. gingivalis* induces increased RANTES and IL-8 secretion under certain conditions. On the other hand, it possesses the capacity to degrade these mediators. Chemokine inactivation at the local level may disturb the immune response by interfering with the recruitment of monocytes, lymphocytes T and neutrophils, which play a critical role in maintaining periodontal health by defending against bacterial infections. The biological significance of such degradation may be important during the course of periodontitis. Indeed, proteolysis of cytokines by *P. gingivalis* may deregulate the complex host cytokine network responsible for periodontal health and the maintenance of both innate and acquired immunity.

In our experiments, the cytokine degradation was bacterial dose dependent. It suggests that degradation of cytokines may occur especially near the bacterial plaque, where protease concentrations are high. In gingival tissue, where bacterial and protease concentrations are low, the degradation of cytokine may be a minor phenomenon allowing the development of an intense inflammatory reaction in response to periodontopathogen stimulations. Such a phenomenon may result in down-modulation of pro-inflammatory reaction in the area proximal to the periodontal plaque whereas simultaneously, at more distal sites high level of inflammatory cytokines would enhance chronic inflammation leading to the progressive tissue destruction. This hypothesis is consistent with the fact that IL-6 concentration in gingival crevicular fluid of unresolved sites following phase therapy was low whereas gingival tissue concentration was high [51]. Periodontitis are

characterized by active and inactive phases of disease progression. We have observed that the modulation of the inflammatory response was dependent of bacterial concentration and macrophage/epithelial cells ratio. For instance, we observed that more IL-1β, IL-8 and RANTES was secreted by the 1:5 co-culture model than the 1:100 model. This suggests that the accumulation of macrophages in periodontal tissue may enhance the production of pro-inflammatory IL-1β, IL-8 and RANTES, all of which contribute to tissue destruction. This supports the concept that the modulation of inflammatory response by *P. gingivalis* depend of the local conditions of complex microenvironments occurring differently according to the stage of disease progression.

In summary, our study showed that *P. gingivalis* can stimulate the secretion of pro-inflammatory cytokines (IL-1β and IL-6) and chemokines (IL-8 and RANTES) in a macrophage/epithelial cell co-culture model. The increase in detectable cytokines was down-regulated by an extensive proteolytic degradation of these mediators. These results illustrate that *P. gingivalis* can modulate the concentrations of inflammatory mediators involved in periodontitis and that the intensity of the modulatory effect is strain-dependent.

2.7. Acknowledgments

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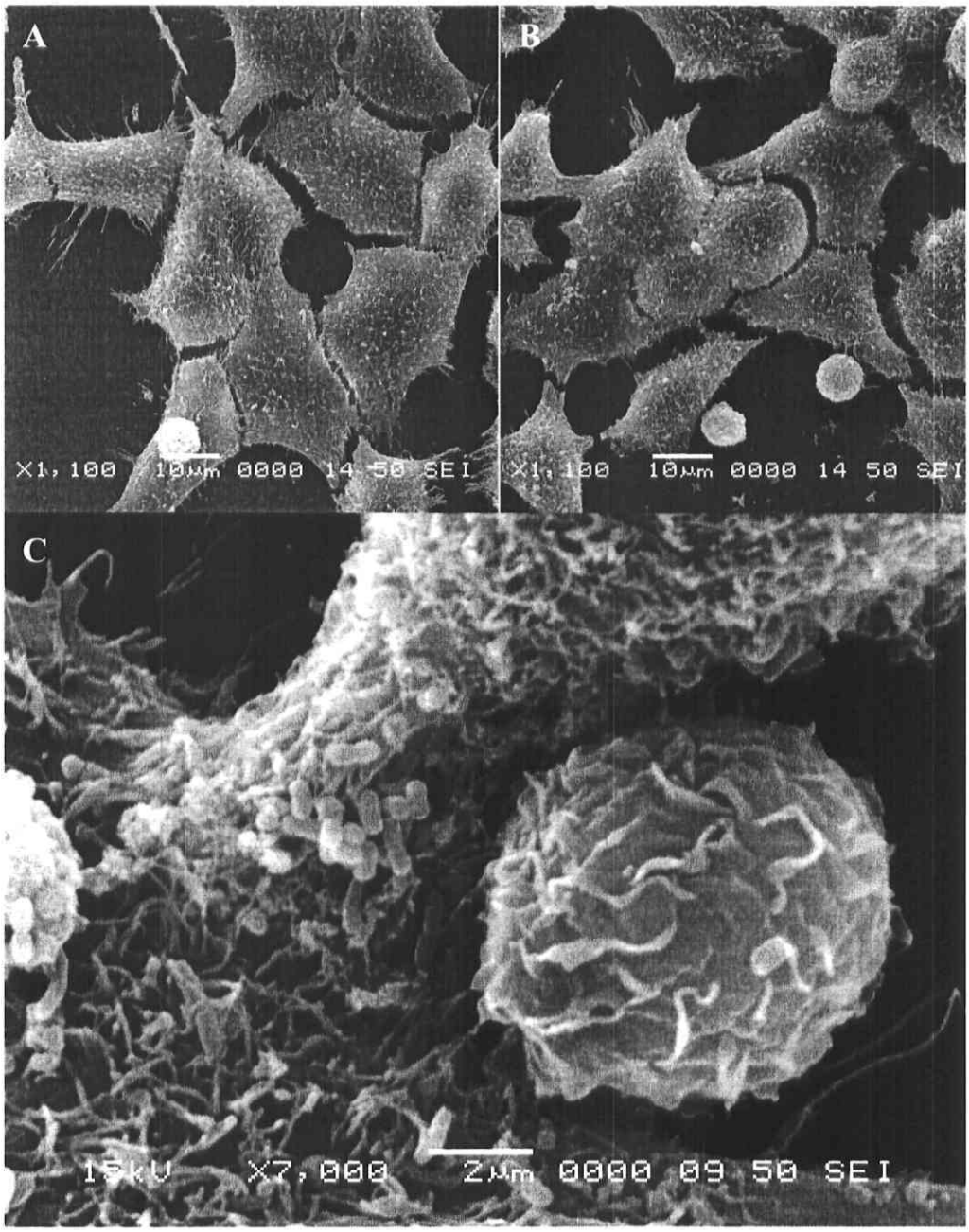

Figure 2.1. Scanning electron microscopy of the macrophage and epithelial cell co-culture model at a ratio of 1:5. Unstimulated co-culture control observed at a magnification of 1,100X (A), co-culture model infected with *P. gingivalis* ATCC 53977 for 24 h at MOI of 500 observed at a magnification of 1,100X (B) and 7,000X (C).

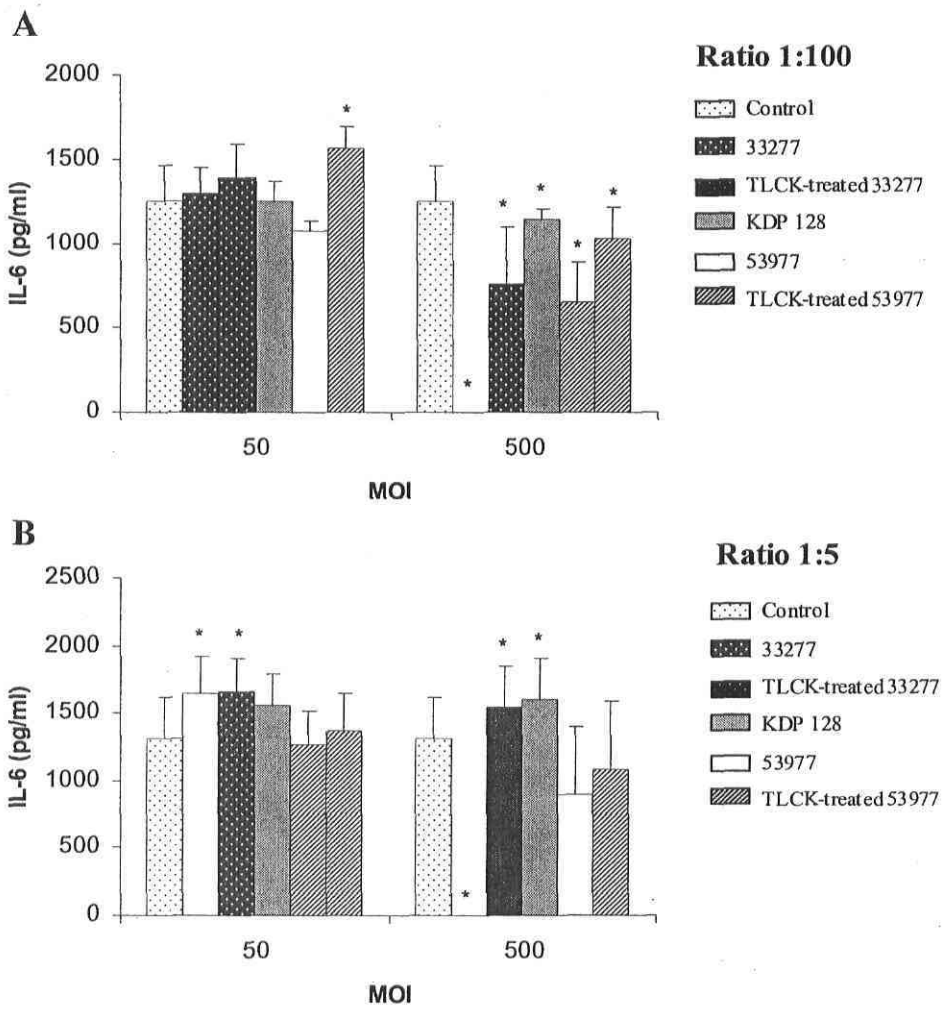

Figure 2.2. IL-6 secretion by a macrophage/epithelial cell co-culture at a ratio of 1:100 (A) and 1:5 (B) following *P. gingivalis* challenge. The co-culture was infected with *P. gingivalis* ATCC 33277, ATCC 33277 pretreated with 1 mM TLCK, KDP128, ATCC 53977 or ATCC 53977 pretreated with 1 mM TLCK for 24 h at MOIs of 50 and 500. IL-6 secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three separate experiments. *, *P* value of < 0.05 compared to uninfected control.

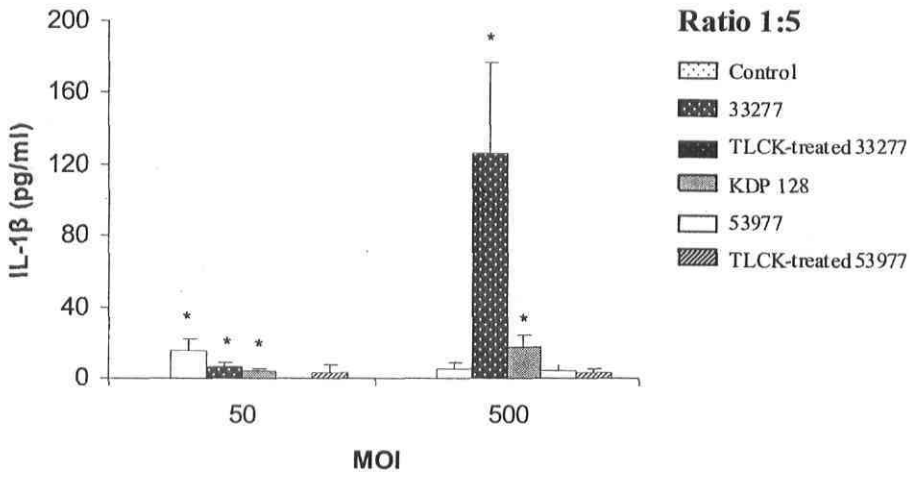

Figure 2.3. IL-1β secretion by a macrophage/epithelial cell co-culture at a ratio of 1:5 following *P. gingivalis* challenge. The co-culture was infected with *P. gingivalis* ATCC 33277, ATCC 33277 pretreated with 1 mM TLCK, KDP128, ATCC 53977 or ATCC 53977 pretreated with 1 mM TLCK for 24 h at MOIs of 50 and 500. IL-1β secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three separate experiments. *, *P* value of < 0.05 compared to uninfected control.

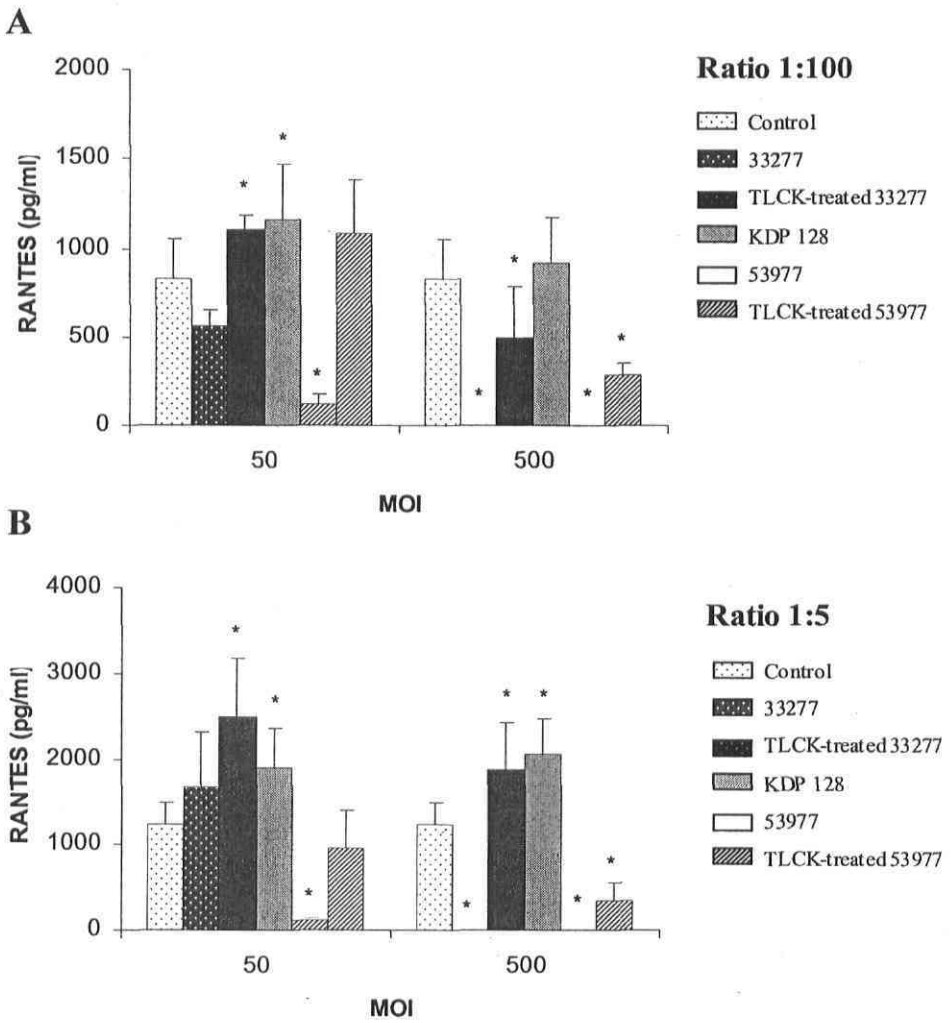

Figure 2.4. RANTES secretion by a macrophage/epithelial cell co-culture at a ratio of 1:100 (A) and 1:5 (B) following *P. gingivalis* challenge. The co-culture was infected with *P. gingivalis* ATCC 33277, ATCC 33277 pretreated with 1 mM TLCK, KDP128, ATCC 53977 or ATCC 53977 pretreated with 1 mM TLCK for 24 h at MOIs of 50 and 500. RANTES secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three separate experiments. *, *P* value of < 0.05 compared to uninfected control.

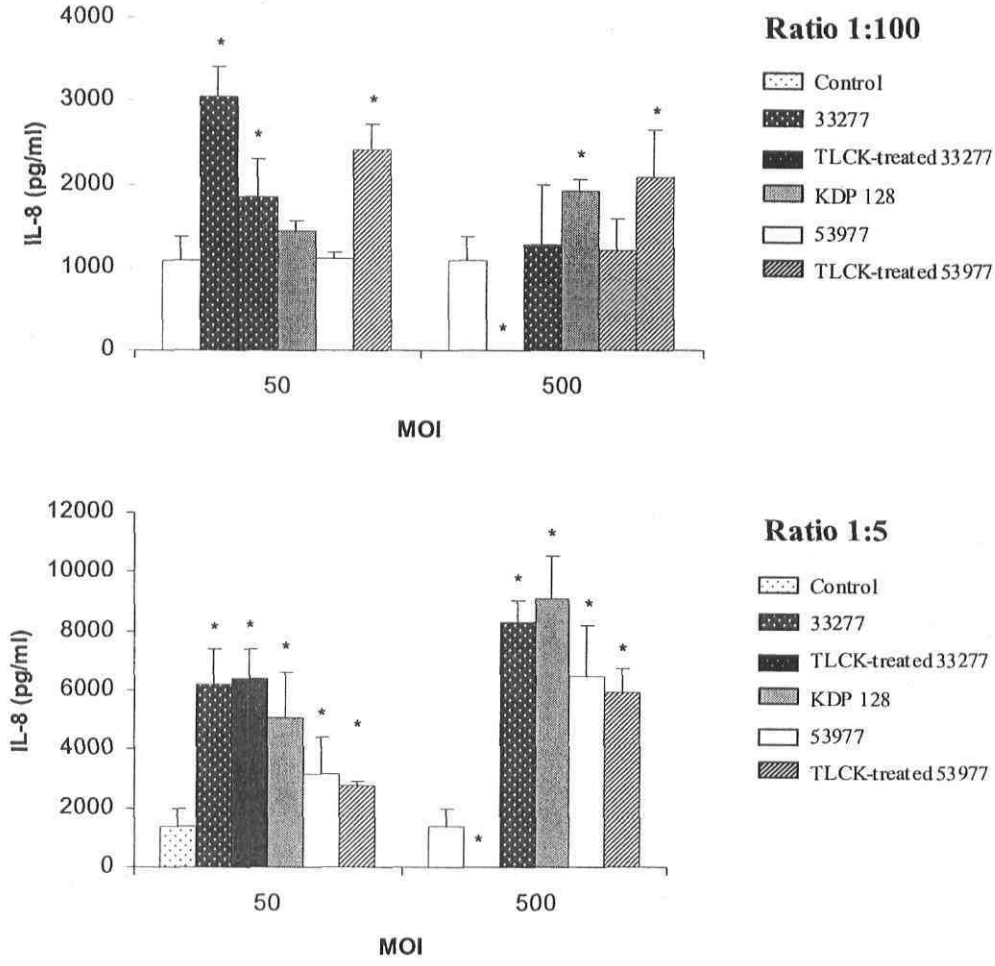

Figure 2.5. IL-8 secretion by a macrophage/epithelial cell co-culture at a ratio of 1:100 (A) and 1:5 (B) following *P. gingivalis* challenge. The co-culture was infected with *P. gingivalis* ATCC 33277, ATCC 33277 pretreated with 1 mM TLCK, KDP128, ATCC 53977 or ATCC 53977 pretreated with 1 mM TLCK for 24 h at MOIs of 50 and 500. IL-8 secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three separate experiments. *, P value of < 0.05 compared to uninfected control.

Table 2.1. Degradation of pre-existing IL-6, IL-8 and RANTES by *P. gingivalis* ATCC 33277, ATCC 33277 pretreated with 1 mM of TLCK, ATCC 53977 and ATCC 53977 pretreated with 1 mM of TLCK after a 24 h incubation. The amounts of IL-6, IL-8 and RANTES were assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three separate experiments.

Bacteria added to the co-culture supernatant	Amount of cytokine detected (pg/ml)		
	IL-6	IL-8	RANTES
None	1636 ± 136	1678 ± 95	1806 ± 164
P. gingivalis ATCC 33277			
5.10^7cells/ml	1070 ± 126	1365 ± 83	406 ± 85
5.10^8 cells/ml	0 ± 0	0 ± 0	0 ± 0
TLCK-treated *P. gingivalis* ATCC 33277			
5.10^7cells/ml	1568 ± 88	1351 ± 121	1334 ± 369
5.10^8 cells/ml	1567 ± 92	1227 ± 32	360 ± 39
P. gingivalis ATCC 53977			
5.10^7cells/ml	1505 ± 127	1453 ± 128	0 ± 0
5.10^8 cells/ml	1524 ± 153	1482 ± 164	0 ± 0
TLCK-treated *P. gingivalis* ATCC 53977			
5.10^7cells/ml	1580 ± 39	1302 ± 116	1030 ± 61
5.10^8 cells/ml	1516 ± 143	1604 ± 175	177 ± 68

CHAPITRE 3

Porphyromonas gingivalis-induced inflammatory mediator profile in an *ex vivo* human whole blood model

3.1. Résumé

Les parodontites se caractérisent par une accumulation de cellules inflammatoires dans les tissus parodontaux et les poches parodontales. Les leucocytes jouent un rôle majeur dans la réponse de l'hôte à *Porphyromonas gingivalis*, un agent étiologique majeur des parodontites chroniques. La sécrétion de fortes quantités de médiateurs inflammatoires contribue à la destruction des tissus parodontaux. Le but de cette étude est d'investiguer la réponse inflammatoire d'un modèle *ex vivo* de sang complet stimulé par *P. gingivalis*. La production d'IL-1β, d'IL-4, d'IL-5, d'IL-6, d'IL-8, d'IL-10, d'IL-12p70, d'IL-13, de TNF-α, d'IFN-γ, d'IP-10, de MCP-1, de RANTES et de PGE_2 a été dosée par ELISA. *P. gingivalis* a induit la sécrétion de cytokines pro-inflammatoires (IL-1β, TNF-α, IL-6 et IFN-γ), de chimiokines (IL-8, RANTES et MCP-1) et de PGE_2 dans le modèle *ex vivo* de sang complet. Les quantités sécrétées étaient dépendantes de la souche bactérienne et de la dose infectieuse utilisées. Bien que le profil de médiateurs induits était le même entre les six sujets sains, une forte variabilité interindividuelle des niveaux de médiateurs sécrétés a été observée. Cette étude supporte l'hypothèse que *P. gingivalis*, en induisant de fortes quantités de médiateurs inflammatoires dans une population mixte de leucocytes, pourrait contribuer à la progression des parodontites.

3.2. Abstract

Periodontitis is characterized by an accumulation of inflammatory cells in periodontal tissue and subgingival sites. Leukocytes play a major role in the host response to *Porphyromonas gingivalis*, a major etiologic agent of chronic periodontitis. Secretion of high levels of inflammatory mediators, including cytokines and prostaglandins, by leukocytes is believed to contribute to periodontal tissue destruction. The aim of this study was to investigate the inflammatory response of an *ex vivo* whole blood model to *P. gingivalis* stimulation. The production of interleukin-1 beta (IL-1β), IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IFN-γ-inducible protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), Regulated on Activation Normal T cell Expressed and Secreted (RANTES), and prostaglandin E_2 (PGE_2) were quantified by enzyme-linked immunosorbent assays. *P. gingivalis* induced the secretion of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ, the chemokines IL-8, RANTES and MCP-1, and the inflammatory mediator PGE_2 in an *ex vivo* human whole blood model. The secretion levels were dependent on the strain and the infectious dose used. While the mediator profiles were comparable between six healthy subjects, a high inter-individual variability in the levels of secreted mediators was observed. This study supports the view that *P. gingivalis*, by inducing high levels of inflammatory mediators from a mixed leukocyte population, can contribute to the progression of periodontitis.

3.3. Introduction

Periodontitis is a multifactorial polymicrobial infection characterized by a destructive inflammatory process affecting the tooth supporting tissues and resulting in periodontal pocket formation, alveolar bone resorption, and eventually tooth loss. While human subgingival plaque harbors some 500 bacterial species [1], evidence points to *Porphyromonas gingivalis*, a Gram-negative anaerobic bacterium, as the major etiologic

agent of chronic periodontitis [2]. *P. gingivalis* produces a broad range of virulence factors, including cysteine proteinases, lipopolysaccharides (LPS) and fimbriae (FimA), which contribute to the host inflammatory response. Variations of FimA have been associated with pathogenesis of *P. gingivalis*. Indeed, a majority of periodontitis patients harbor type II *fimA* strains, followed by type IV *fimA* strains while type I *fimA* strains are mainly isolated from periodontal healthy subjects [3, 4]. The local host response to periodontopathogens and their products includes the recruitment of polymorphonuclear neutrophils and macrophages and the subsequent release of inflammatory mediators, cytokines, and matrix metalloproteinases, which are thought to play crucial roles in the pathogenesis of periodontal disease. Indeed, the continuous, high secretion of various cytokines and inflammatory mediators, including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α), and prostaglandin E_2 (PGE_2) by host cells following stimulation by periodontopathogens can modulate periodontal tissue destruction [5, 6].

While all types of blood cells are present in normal gingival connective tissue [7], an accumulation of inflammatory cells is observed during periodontitis [8]. Moreover, the number of leukocytes migrating to the gingival sulcus and periodontal pockets increases during the progression of inflammation [9, 10]. While most neutrophils recruited into the gingival tissues migrate to the gingival epithelium and sulcus, the majority of the mononuclear cells, including monocytes/macrophages, T lymphocytes, and B cells persist in the connective tissue and form the local cell infiltrate [11]. *P. gingivalis* has been shown to penetrate an engineered human oral mucosa *in vitro* [12] and has been observed within gingival tissues *in vivo* [13, 14], indicating that it may reach deeper structures of connective tissues. In addition, *P. gingivalis* can actively invade endothelial cells [15], thus supporting the view that the well-vascularized connective tissues of the periodontium may allow *P. gingivalis* to enter the bloodstream. This indicates that *P. gingivalis* may encounter a heterogeneous leukocyte population depending on where it is in periodontal tissue. In order to take into consideration the interactions between different immune cell types, this study used an *ex vivo* human whole blood model to characterize the inflammatory response to *P. gingivalis* stimulation. This model has the advantage of providing a comprehensive view of

the cytokine and PGE_2 responses mediated by this periodontopathogen. In addition, the *ex vivo* whole blood model reduces the confounding factors that may be associated with the isolation procedures, such as activation of isolated cells, and the risk of contamination with biological stimulants such as lipopolysaccharides. Moreover, whole blood represents a more physiologic environment for investigating the production of inflammatory mediators in response to challenges by bacterial cells or their components since cellular interactions are preserved in the presence of various plasma proteins (soluble CD14, LPS-binding proteins, hormones, soluble cytokine receptors, etc.). In this study, the *ex vivo* human whole blood model was infected with different strains of *P. gingivalis* at various concentrations and analyzed for pro-inflammatory cytokine, chemokine, and PGE_2 production.

3.4. Materials and methods

3.4.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *P. gingivalis* ATCC 33277, ATCC 53977, and W83. The bacteria were grown in Todd Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 0.001% hemin and 0.0001% vitamin K. The cultures were incubated at 37°C under anaerobic conditions (N_2:H_2:CO_2/80:10:10).

3.4.2. Whole blood collection

Samples of venous blood were collected from the antecubital vein of six healthy subjects using the Vacutainer™ system and sterile endotoxin-free blood collection tubes containing 150 IU sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA). Informed consent was obtained from all donors prior to the experiments, and the protocol was approved by the ethics committee of Université Laval. Healthy subjects were free of any clinical symptoms of infections, were non-smokers, and had clinically healthy gingiva with no periodontal pockets $\geq$ 3 mm in depth. Hematologic analyses of whole blood samples

performed at the Centre Hospitalier de l'Université Laval (Quebec City, Canada) showed that all the subjects had normal leukocyte counts. Whole blood samples were diluted 1:3 with RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) and divided into 6-ml aliquots in 6-well plates.

3.4.3. Whole blood stimulation

Bacteria were grown to the early stationary phase (24 h) at 37°C under anaerobiosis. The cells were harvested by centrifugation at 11000 *g* for 10 min and suspended in RPMI-1640 medium to a concentration of 1 x 10^9 bacteria/ml as determined using a Petroff-Hausser counting chamber. The bacterial suspensions were added to the diluted whole blood samples to obtain a final concentration of 10^6 and 10^7 bacteria per ml. The infected whole blood samples were incubated at 37°C in a 5% CO_2 humidified atmosphere with occasional gentle shaking. After 6 h, the samples were centrifuged at 2000 *g* for 5 min. The supernatants were collected, centrifuged at 11000 *g* for 10 min to eliminate bacteria, and stored at –20°C until used. Control blood was incubated in the absence of bacteria.

3.4.4. Determination of bacterial viability

Infected whole blood cultures were harvested at times 0 and 6 h. The first time point (0 h) was considered as the 100% viability control. The blood cells were lysed by the addition of sterile distilled water. After 3 min, the bacteria were sedimented at 11000 *g* for 10 min and resuspended in THB. The bacterial suspensions were 10 fold serially diluted (10^{-1}-10^{-8}) in THB culture medium. The bacterial cultures were incubated at 37°C under anaerobic conditions (N_2:H_2:CO_2/80:10:10). The absence of bacterial growth after four days of incubation was an indication that no viable bacteria were present in the suspension.

3.4.5. Determination of cytokine production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify IL-1β, IL-6, IL-8, TNF-α, and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) in cell-free blood supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (model 680; BioRad Laboratories, Mississauga, ON, Canada) with the wavelength correction set at 550 nm. Cytokine concentrations were determined in triplicate using a standard curve prepared for each assay. The sensitivities of the commercial ELISA kits were 31.2 pg/ml for IL-8, 15.6 pg/ml for RANTES and TNF-α, 9.3 pg/ml for IL-6, and 3.9 pg/ml for IL-1β. The concentrations of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10), interleukin-13 (IL-13), interleukin-12 (IL-12p70), interferon gamma (IFN-γ), IFN-γ-inducible protein 10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1) in the cell-free blood supernatants were determined by the Upstate testing service using Luminex® multiplex technology (Upstate, Lake Placid, NY, USA).

3.4.6. Determination of PGE_2 production

A competitive enzyme immunoassay (EIA) was performed on the cell-free blood supernatants according the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance at 415 nm was read using a microplate reader, and PGE_2 concentrations were determined in triplicate. The detection level of the PGE_2 kit was 35 pg/ml.

3.4.7. Degradation of human serum albumin by *P. gingivalis*

Degradation of albumin was monitored to determine whether *P. gingivalis* proteinases are active in the model. Following the 6 h incubation period, uninfected and

infected (10^7 bacteria per ml) whole blood supernatants were diluted 1:50 in PBS. The degradation of the albumin was monitored by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli [16]. The proteins were transferred to a nitrocellulose membrane and reacted with an alkaline phosphatase-conjugated rabbit polyclonal anti-albumin antibody (IgG fraction, 1:10,000 dilution) for 2 h. Albumin and albumin-related fragments were detected using nitroblue tetrazolium and 5-bromo-chloro-3-indolyl-phosphate in N-N-dimethyl-formamide diluted in 100 mM Tris-HCl (pH 8.5).

3.4.8. Statistical analysis

Differences between whole blood infections (bacterial strains and infectious doses) were determined using a two-way randomized block design analysis of variance. Statistical analyses of differences between the cytokine levels of uninfected and infected blood were performed using the Student's t paired test. Interactions between variables were studied using Pearson's correlation. Data were considered significant at a p value < 0.05.

3.5. Results

3.5.1. Influence of strains on the *P. gingivalis*-induced cytokine response in human whole blood

Preliminary experiments were first carried out with the blood of only one individual to establish the conditions for determining the capacity of *P. gingivalis* to stimulate cytokine production in the whole blood model (data not shown). Human whole blood was stimulated with *P. gingivalis* ATCC 33277 at infectious doses ranging from $1x10^4$ to $5x10^8$ bacteria/ml for 6 and 24 h. The whole blood supernatants were then analyzed for IL-1β, IL-6, IL-8, TNF-α, and RANTES production. When the whole blood was challenged with a *P. gingivalis* concentration of 10^4 or 10^5 bacteria/ml, only a minor cytokine response was

detected. The highest IL-1β, TNF-α, and IL-6 responses were observed after a 6 h incubation at an infectious dose of 10^7 cells/ml. A higher infectious dose or a 24 h incubation period resulted in increased levels of IL-8 and RANTES whereas the concentrations of the other cytokines were not affected or decreased. Based on the preliminary analysis, an infectious dose of 10^6 and 10^7 bacteria/ml and an incubation period of 6 h were selected for further experiments.

Whole blood from six healthy volunteers was challenged with three strains of *P. gingivalis* (ATCC 33277, ATCC 53977, and W83) at an infectious dose of 10^6 or 10^7 bacteria/ml (Table 3.1). The viability of *P. gingivalis* in whole blood after a 6 h incubation period was estimated at approximately 10%. To determine whether *P. gingivalis* proteinases were active in the whole blood despite the presence of various plasma proteinase inhibitors, the degradation of albumin, which is the most abundant serum protein, was studied by SDS-PAGE/Western immunoblotting. The albumin was poorly degraded by *P. gingivalis* during the incubation period, suggesting that the proteinases of this bacterium were only weakly active in the whole blood model (data not shown). The amounts of cytokine produced in the model under the various infective conditions were evaluated using a two-way randomized block design analysis of variance. In all cases, the interactions between the two variables (strain and dose) were not significant ($p > 0.05$). Furthermore, for all analyses the homogeneity of variance and the normality assumptions were met. All the *P. gingivalis* strains tested induced a significant increase in IL-1β, IL-6, TNF-α, IL-8, and RANTES levels. These cytokine and chemokine responses were bacterial dose-dependent ($p < 0.05$). There were significant differences ($p < 0.05$) in IL-8 and RANTES responses between *P. gingivalis* strains. *P. gingivalis* ATCC 53977 induced a higher chemokine response than *P. gingivalis* ATCC 33277 and W83. IL-1β and TNF-α responses were also dependent on the *P. gingivalis* strain used ($p < 0.05$) with *P. gingivalis* ATCC 33277 inducing lower levels of IL-1β and TNF-α secretion than *P. gingivalis* ATCC 53977 and *P. gingivalis* W83. In addition, *P. gingivalis* ATCC 53977 was the most potent inducer of TNF-α production. While all the combinations of cytokines tested were

positively correlated, the strongest correlations were observed for IL-1β/TNF-α ($r = 0.91$) and IL-6/IL-8 ($r = 0.88$).

3.5.2. Detailed profiles of cytokine and PGE_2 production induced by *P. gingivalis* ATCC 33277 in human whole blood

The inflammatory mediator profile induced by *P. gingivalis* in the *ex vivo* human whole blood model was characterized in more detail. Whole blood from six healthy volunteers was stimulated by *P. gingivalis* ATCC 33277 and analyzed for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-12p70, IFN-γ, TNF-α, and PGE_2 production. As shown in Table 3.2, additional mediators induced by *P. gingivalis* were identified. *P. gingivalis* induced a significant increase ($p < 0.05$) in IL-1β, TNF-α, IL-6, IFN-γ, and PGE_2 secretion. While IL-10 secretion was enhanced in some subjects by *P. gingivalis*, there was no significant induction of this cytokine for any of the subjects tested. A high inter-individual variability in the amounts of inflammatory mediator secreted following the *P. gingivalis* challenge was observed for all the mediators tested. The level of TNF-α secreted was positively correlated with that of IL-1β ($r = 0.94$) and IL-6 ($r = 0.90$).

3.5.3. Detailed profile of chemokine production induced by *P. gingivalis* ATCC 33277 in human whole blood

The detailed chemokine response induced by *P. gingivalis* ATCC 33277 in human blood from the six healthy volunteers is presented in Table 3.3. *P. gingivalis* induced a significant ($p < 0.05$) increase in IL-8, RANTES, and MCP-1 secretion by the leukocytes in the whole blood from all six subjects. While *P. gingivalis* induced a large increase in IP-10 levels in some blood samples (2, 4, and 5), there was no statistically significant induction of IP-10 in the study population. There was a high inter-individual variability of the amounts of chemokine secreted. The strongest correlations between cytokines were observed for IL-6/IL-8 ($r = 0.94$) while the level of MCP-1 was strongly positively correlated with that of IL-1β ($r = 0.92$), TNF-α ($r = 0.93$), and IL-6 ($r = 0.89$).

3.6. Discussion

P. gingivalis interacts with various types of leukocyte that may differ depending on where it is in the periodontal tissues and the phase of the disease process. In order to characterize the overall inflammatory response of mixed leukocyte populations to *P. gingivalis*, whole blood from six subjects was stimulated by *P. gingivalis* and analyzed for cytokine and PGE_2 production. The advantage of the *ex vivo* whole blood model is that it contains all the relevant cell populations that are likely to come in contact with periodontopathogens during periodontitis. In addition, it takes into consideration the complex cell/cell interactions that occur *in vivo*. This model is highly relevant for studying periodontitis since the gingival crevicular fluid, which bathes the periodontal pocket, is derived from gingival capillary beds and contains resident and emigrating inflammatory cells.

IL-1β and TNF-α are determinants of the progression of periodontitis [17]. Local inhibition of these two mediators in periodontal tissues significantly reduces the inflammatory response and bone loss in ligature-induced periodontitis in monkeys [18]. *P. gingivalis* showed a strong capacity to induce IL-1β and TNF-α secretion by the mixed leukocyte populations in whole blood, suggesting that it can activate various periodontal tissue destruction pathways mediated by these cytokines. We observed that the levels of IL-1β and TNF-α produced by the whole blood model following *P. gingivalis* challenge were strongly correlated. Stashenko et *al.* also reported a highly significant correlation between the levels of IL-1β and TNF-α in the periodontal tissues of diseased sites, suggesting the coordinated expression of these two mediators [19]. IL-6 plays an important role in regulating the immune response to periodontal pathogens and is notably responsible for the differentiation of osteoclasts and activated B cells into immunoglobulin-secreting plasma cells. IL-6 levels increase in the diseased gingiva of patients with periodontitis compared to periodontally healthy subjects [20]. In the whole blood model used in this study, IL-6 secretion was highly up-regulated by all the *P. gingivalis* challenges, suggesting that this bacterium likely plays a determinant role in IL-6 induction during periodontitis. In addition,

IL-6 participates in the recruitment of leukocytes to sites of inflammation by increasing the local production of chemokines such as MCP-1 and IL-8 [21]. This was confirmed by our data, which showed that IL-6 levels were highly positively correlated with MCP-1 and IL-8 levels.

Selective chemokine-mediated recruitment of different cell types to the gingival tissue is potentially involved in the immunopathogenesis of periodontitis. IL-8, RANTES, MCP-1, and IP-10 are chemokines found in diseased periodontal tissues [22, 23]. MCP-1 is a potent chemoattractant for monocytes and macrophages [24] whereas IP-10 attracts monocytes and activated T lymphocytes to inflammatory foci [25]. IL-8 and RANTES are involved in the recruitment of neutrophils, eosinophils, monocytes, and TH_1 cells to infected sites [26]. RANTES is also a chemotactic factor for osteoclasts [27]. Our results showed that *P. gingivalis* possesses a strong capacity to induce IL-8, RANTES, and MCP-1 production by a mixed leukocyte population. This suggests that *P. gingivalis* can trigger a dense infiltration of various inflammatory cells in periodontal tissue through the induction of these chemokines, which in turn may induce a strong inflammatory reaction at local sites, resulting in the destruction of tissue and alveolar bone. PGE_2 is a potent stimulator of bone resorption [28] and is associated with attachment loss [29]. The secretion of this inflammatory mediator by the whole blood model was enhanced following *P. gingivalis* challenges, suggesting that *P. gingivalis* may cause alveolar bone resorption by stimulating PGE_2 production by leukocytes.

Among the large array of virulence factors produced by *P. gingivalis*, fimbriae (Fim A), which are filamentous structures on the cell surface, are important stimulators of an inflammatory response [30, 31]. There is a close relationship between *P. gingivalis* clones with specific *fimA* fimbriae and periodontitis. Most patients with periodontitis harbor type II *fimA* strains, while type I *fimA* strains are mainly isolated from periodontal healthy subjects [3, 4]. Sugano *et al.* [32] recently reported that a *P. gingivalis* strain with type II *fimA* fimbriae induced statistically high levels of IL-1β, IL-8, IL-12, and TNF-α mRNA in the U937 macrophage-like human cell line whereas a *P. gingivalis* strain with type I *fimA* fimbriae caused no significant induction of these cytokines. Interestingly, in a mouse

abscess model, type II *fimA P. gingivalis* induced greater inflammatory changes than type I *fimA P. gingivalis* [33]. In the study reported here, the ability of three distinct phenotypes of *P. gingivalis* strains, classified as type I *fimA* (ATCC 33277), type II *fimA* (ATCC 53977), and type IV *fimA* (W83), to induce cytokine production in the whole blood model was investigated. *P. gingivalis* ATCC 53977, which produces type II *fimA* fimbriae, induced significantly higher levels of IL-8, MCP-1, and TNF-α than the other strains tested. Type II *fimA P. gingivalis* ATCC 53977 also induced higher amounts of IL-1β than type I *fimA P. gingivalis* ATCC 33277. These results are in agreement with previous studies [32, 33] suggesting that type II *fimA P. gingivalis* strains are more potent inducers of cytokine production by leukocytes than type I *fimA P. gingivalis* strains. However, phenotypic variations other than the type of fimbriae may exist between the *P. gingivalis* strains used and it was recently suggested that FimA may not play a prominent role in the innate immune response to *P. gingivalis* [34]. One should not exclude the possibility that the strains of *P. gingivalis* used in our study may respond in a different way if whole blood is obtained from patients with active periodontitis.

While the cytokine profiles induced by *P. gingivalis* in the whole blood from the six subjects were similar, we noted a high inter-individual variability in the levels of cytokines secreted. This could be related, at least in part, to cytokine gene polymorphisms. While reports of genetic polymorphisms associated with periodontitis are increasing, further studies are required to clearly determine the genetic basis of periodontitis [35]. The inter-individual variability in cytokine levels observed may also be the consequence of a more or less recent microbial challenge or sub-infectious state in certain subjects, resulting in leukocyte pre-activation. These quantitative differences in inter-subject cytokine responses could explain, at least in part, the various degrees of susceptibility to periodontal infections. Not everyone is equally susceptible to periodontitis [36] and susceptibility also varies greatly between individuals who harbor the same pathogenic microflora [37]. More and more evidence is pointing to the host response to a bacterial challenge as a major determinant of periodontitis susceptibility. In this regard, the whole blood model appears promising for studying host responses to periodontopathogens in different subject

categories and may provide interesting data for characterizing variations in the immune response of periodontitis patients.

Transient bacteremias occur from periodontal infection, surgical dental procedures [38], periodontal probing [39], and mastication [40]. Numerous studies suggested that periodontopathogens, such as *P. gingivalis*, may be involved in the initiation and progression of artherosclerosis and subsequent coronary disease [41]. This was supported by identification of *P. gingivalis* in artherosclerotic plaques [42, 43] and in aortic tissue [44]. In addition, *P. gingivalis* infection accelerates artherosclerosis in animal model [45, 46]. Both systemic and localized inflammation in the arteries contribute to the initiation and progression of artherosclerosis [47]. As reported here, *P. gingivalis* infection stimulates circulating immune cells to produce inflammatory mediators, such as IL-1β, TNF-α, IL-6, IFN-γ, MCP-1 and PGE_2, which are known to be implicated in artherosclerosis [41, 47, 48]. This suggest that *P. gingivalis* may contribute to the development of artherosclerosis.

In summary, our study showed that *P. gingivalis* induces the secretion of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, and IFN-γ, the chemokines IL-8, RANTES, and MCP-1, and the inflammatory mediator PGE_2 in the *ex vivo* human whole blood model. The level of secretion of these inflammatory mediators was dependent on the bacterial strain and the infectious dose used. This study supports the view that *P. gingivalis* can contribute to the progression of periodontitis by inducing the production of high levels of inflammatory mediators from a mixed leukocyte population.

3.7. Acknowledgements

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Table 3.1. Secretion of IL-8, RANTES, TNF-α, IL-1β, and IL-6 in human whole blood from six healthy individuals following stimulation with *P. gingivalis* ATCC 33277, *P. gingivalis* ATCC 53977, and *P. gingivalis* W83 at final concentration of 10^6 or 10^7 bacteria/ml for 6 h. The cytokine concentrations in the supernatants of whole blood cultures were assessed by ELISA. Control whole blood samples were incubated without bacteria. The data are the means ± standard deviations (SD) of triplicate assays of all six blood samples.

Bacterial strain added to the whole blood	Amount of cytokine detected (pg/ml)				
	IL-8	RANTES	TNF-α	IL-1β	IL-6
None	ND	2234 ± 2214	ND	ND	ND
P. gingivalis ATCC 33277					
10^6cells/ml	4810 ± 5821	4634 ± 3705	3455 ± 2171	2653 ± 1989	4345 ± 4041
10^7cells/ml	11675 ± 7886	5720 ± 3767	7316 ± 2493	6570 ± 3549	8024 ± 2951
P. gingivalis ATCC 53977					
10^6cells/ml	8281 ± 8293	5276 ± 3544	6528 ± 3460	5332 ± 3981	5675 ± 3519
10^7cells/ml	11773 ± 7740	6544 ± 3835	10091 ± 2389	8943 ± 3180	7801 ± 3210
P. gingivalis W 83					
10^6cells/ml	5445 ± 5885	4218 ± 3982	5142 ± 2467	4921 ± 3726	5389 ± 3849
10^7cells/ml	10919 ± 6881	5831 ± 3262	8412 ± 2886	8024 ± 3229	7531 ± 3361

ND: Not detected (below the detection level)

Table 3.2. Secretion of IL-1β, TNF-α, IL-6, IFN-γ, IL-10 and PGE_2 in human whole blood from six healthy individuals following stimulation with 10^7 cells/ml of *P. gingivalis* ATCC 33277 for 6 h. The cytokine concentrations in the supernatants of the whole blood cultures were assessed by ELISA. PGE_2 concentration was assessed by EIA. Control whole blood samples were incubated without bacteria. The data are the means ± SD of triplicate assays.

Individual	*P. gingivalis* ATCC 33277 (cells/ml)	PGE_2 (pg/ml)	Amount of cytokine secreted (pg/ml)				
			IL-1β	TNF-α	IL-6	IFN-γ	IL-10
1	0	ND	ND	ND	ND	77 ± 22	37 ± 18
	10^7	133 ± 11	1478 ± 39	3656 ± 233	6005 ± 137	115 ± 26	110 ± 8
2	0	ND	ND	ND	ND	143 ± 20	36 ± 15
	10^7	67 ± 8	4334 ± 234	5616 ± 330	5779 ± 293	304 ± 21	44 ± 11
3	0	ND	ND	ND	ND	65 ± 3	43 ± 9
	10^7	311 ± 23	7688 ± 621	9377 ± 276	10556 ± 154	132 ± 23	307 ± 16
4	0	ND	ND	ND	ND	60 ± 6	29 ± 1
	10^7	96 ± 13	10416 ± 316	7189 ± 413	5651 ± 179	195 ± 43	77 ± 8
5	0	ND	ND	ND	ND	68 ± 13	26 ± 2
	10^7	84 ± 7	10325 ± 223	10557 ± 201	7435 ± 115	2239 ± 71	49 ± 7
6	0	ND	ND	ND	ND	102 ± 5	41 ± 13
	10^7	263 ± 16	5180 ± 79	7502 ± 162	12721 ± 146	208 ± 35	287 ± 31
Mean	**0**	**ND**	**ND**	**ND**	**ND**	**85 ± 31**	**35 ± 6**
	10^7	**159 ± 102***	**6570 ± 3549***	**7316 ± 2493***	**8024 ± 2951***	**532 ± 838***	**145 ± 119**

*: $p < 0.05$ compared to an uninfected control

ND: Not detected (below the detection level)

Table 3.3. Secretion of IL-8, RANTES, MCP-1, and IP-10 in human whole blood from six healthy individuals following stimulation with 10^7 bacteria/ml of *P. gingivalis* ATCC 33277 for 6 h. The chemokine concentrations in the supernatants of whole blood cultures were assessed by ELISA. Control whole blood samples were incubated without bacteria. The data are the means ± SD of triplicate assays.

Individual	*P. gingivalis* ATCC 33277 (cells/ml)	Amount of chemokine secreted (pg/ml)			
		IL-8	RANTES	MCP-1	IP-10
1	0	ND	565 ± 49	73 ± 5	443 ± 31
	10^7	6628 ± 408	2848 ± 180	487 ± 15	1395 ± 25
2	0	ND	437 ± 57	97 ± 28	425 ± 50
	10^7	2845 ± 123	3201 ± 101	1616 ± 206	15257 ± 316
3	0	ND	6207 ± 290	60 ± 13	447 ± 38
	10^7	20981 ± 441	12544 ± 237	3432 ± 211	1025 ± 73
4	0	ND	1278 ± 79	86 ± 6	181 ± 24
	10^7	9864 ± 248	3986 ± 194	3002 ± 439	9150 ± 217
5	0	ND	3383 ± 201	44 ± 8	187 ± 27
	10^7	7891 ± 322	7708 ± 192	2323 ± 135	12431 ± 227
6	0	ND	1535 ± 43	103 ± 20	339 ± 31
	10^7	21842 ± 565	4036 ± 141	2855 ± 872	950 ± 231
Mean	**0**	**ND**	**2234 ± 2214**	**77 ± 22**	**337 ± 124**
	10^7	**11675 ± 7886***	**5720 ± 3767***	**2285 ± 1080***	**6701 ± 6410**

*: $p < 0.05$ compared to an uninfected control
ND: Not detected (below the detection level)

CHAPITRE 4

Inflammatory responses of a macrophage/epithelial cell co-culture model to mono and mixed infections with *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*

4.1. Résumé

De nombreuses données désignent *Porphyromonas gingivalis*, *Treponema denticola* et *Tannerella forsythia* comme trois agents étiologiques majeures des parodontites chroniques. Les cellules épithéliales et les macrophages jouent un rôle de premier plan dans la réponse de l'hôte face à une agression par les bactéries parodontopathogènes. La sécrétion de médiateurs inflammatoires et de MMPs par ces cellules contribue à la destruction des tissus parodontaux. Le but de cette étude est de caractériser la réponse inflammatoire d'un modèle *in vitro* de macrophages et de cellules épithéliales en co-culture stimulé par les cellules entières ou les LPS de *P. gingivalis*, *T. denticola* et *T. forsythia*, pris individuellement ou en combinaison. Il a été observé que des infections simples ou mixtes du modèle de co-culture ont induit la sécrétion d'IL-1β ,d'IL-6, d'IL-8, de PGE_2 et de MMP-9. *P. gingivalis* et *T. forsythia* ont induit une augmentation de la sécrétion de RANTES alors que *T. denticola* seul ou en combinaison provoque une diminution des quantités de RANTES produites. Toute les stimulations réalisées avec les LPS ont induit la sécrétion de chimiokines, de MMP-9 et de PGE_2. Aucun effet synergique n'a été observé sur la production de médiateurs inflammatoires et de MMP-9 induite par les mélanges de

bactéries ou de LPS. Cette étude supporte l'hypothèse que *P. gingivalis*, *T. denticola* et *T. forsythia* pourraient induire de fortes quantités de médiateurs pro-inflammatoires et de MMP-9 dans les lésions parodontales, contribuant ainsi à la progression des parodontites.

4.2. Abstract

Accumulated evidence points to *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* as three major etiologic agents of chronic periodontitis. Epithelial cells and macrophages play a major role in the host response to periodontopathogens, and the secretion of inflammatory mediators and matrix metalloproteinases (MMPs) by these host cells is believed to contribute to periodontal tissue destruction. The aim of this study was to investigate the inflammatory response of a macrophage/epithelial cell co-culture model following mono or mixed infections with the above three periodontopathogens. An *in vitro* co-culture model composed of epithelial-like transformed cells (HeLa cell line) and macrophage-like cells (phorbol myristic acid-differentiated U937 monocytic cell line) was challenged with whole cells or lipopolysaccharides (LPS) of *P. gingivalis*, *T. denticola*, and *T. forsythia*, individually and in combination. Following stimulation, the production of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α), Regulated on Activation Normal T cell Expressed and Secreted (RANTES), prostaglandin E_2 (PGE_2), and MMP-9 were quantified by enzyme-linked immunoassays. We observed that mono or mixed infections of the co-culture model induced the secretion of IL-1β, IL-6, IL-8, PGE_2, and MMP-9. *P. gingivalis* and *T. forsythia* induced an increase in RANTES secretion whereas *T. denticola* alone or in combination resulted in a significant decrease in RANTES levels. All LPS challenges induced an increase in chemokine, MMP-9, and PGE_2 production. No synergistic effect on the production of cytokines, chemokines, PGE_2, and MMP-9 was observed for any of the bacterial or LPS mixtures tested. This study supports the view that *P. gingivalis*, *T. denticola*, and *T. forsythia* may induce high levels of pro-inflammatory mediators and MMP-9 in periodontal lesions, thus contributing to the progression of periodontitis.

4.3. Introduction

Periodontal diseases are polymicrobial infections that lead to the destruction of tooth supporting tissue. The gingival sulcus is home to a complex microbial ecosystem [1, 2]. While human subgingival plaque harbors over 500 bacterial species (cultivable and non-cultivable), only a few are involved in the initiation and progression of periodontal disease [3, 4]. Specific bacterial species and bacterial complexes occur more frequently in diseased sites while others are associated with healthy or stable periodontal tissues. The red complex, which includes *Porphyromonas gingivalis*, *Tannerella forsythia* (formerly *Bacteroides forsythus*), and *Treponema denticola*, is strongly related to clinical measures of periodontitis, particularly pocket depth and bleeding on probing [5, 6]. These three anaerobic Gram-negative bacterial species produce a broad array of virulence factors that allow them to colonize subgingival sites, resist host defenses, and cause tissue destruction [7-9]. Studies have shown that these bacteria are able to invade primary gingival epithelial cells and transformed cell lines [10-12] as well as oral epithelial cells studied *in vivo* [13]. The incidence and rate of progression of periodontitis involve complex interactions between periodontopathic bacteria as well as between periodontopathic bacteria and host immune cells. These interactions lead to host cell secretion of various cytokines, including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF-α), as well as prostaglandin E_2 (PGE_2) and matrix metalloproteinases (MMPs), which modulate periodontal tissue destruction [14-16].

Epithelial cells are a major physical barrier to periodontopathogens and act as sensors during microbial infections [17], generating and transmitting signals between bacteria and the adjacent and underlying immune cells of the periodontal tissues. They form an integral part of the innate immune system and actively participate in the inflammatory response of local gingival tissues [18, 19]. Monocytes and macrophages are also key members of the innate immune system and are present in higher numbers in active periodontal lesions than in inactive sites [20]. Considering the multifunctional roles of monocytes/macrophages and epithelial cells, they likely play an important role in the

initiation and maintenance of the inflammatory processes and alveolar bone loss observed in chronic periodontitis [21]. Furthermore, contact with epithelial cells modulates the phenotype of human macrophages [22], a phenomenon that may influence their response to bacterial challenges. We recently developed an *in vitro* macrophage/epithelial cell co-culture model and reported that cytokine production is modulated following stimulation with different strains of *P. gingivalis* [23]. In the present study, we used the co-culture model to investigate the effects of polymicrobial infections on the inflammatory responses. More specifically, the macrophage/epithelial cell co-culture model was used to evaluate the production of IL-1β, IL-6, IL-8, TNF-α, Regulated on Activation Normal T cell Expressed and Secreted (RANTES), PGE_2, and MMP-9 induced by mono or mixed infections with whole bacterial cells of the red complex (*P. gingivalis*, *T. denticola*, and *T. forsythia*) or their lipopolysaccharides (LPS). This model offers the advantage of providing a more comprehensive view of the cytokine response mediated by these major periodontopathogens.

4.4. Material and methods

4.4.1. Bacterial strains and growth conditions

The bacterial strains used were *P. gingivalis* ATCC 33277 and ATCC 53977, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037. The *P. gingivalis* strains were grown in Todd-Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 0.001% hemin and 0.0001% vitamin K. *T. denticola* was grown in oral spirochete medium as previously described [24]. *T. forsythia* was grown in Brain Heart Infusion broth (BHI; BBL Microbiology Systems) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO, USA) and 0.001% N-acetyl muramic acid (Sigma). The bacterial cultures were incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 24 h (*P. gingivalis*) or 4 days (*T. denticola* and *T. forsythia*).

4.4.2. LPS preparation

LPS were isolated from *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037 using the protocol described by Darveau and Hancock [25], which is based on the protein digestion of a whole cell extract by proteinase K, and successive solubilization and precipitation steps. The LPS preparations were freeze dried and stored at –20°C. The amount of contaminating protein was evaluated using a protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as a control and was less than 0.001% in all LPS preparation. *T. denticola* possesses a lipooligosaccharide (although it will be called LPS in the present paper) which have distinct properties from the classical LPS of *Bacteroides* group [26].

4.4.3. Epithelial cell and monocyte cultures

Epithelial-like transformed cells (HeLa cell line) were maintained as frozen stocks and cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine (HyClone Laboratories, Logan, UT, USA), 10% heat-inactivated FBS (DMEM-FBS), and 100 µg/mL penicillin-streptomycin in a 5% CO_2 atmosphere at 37°C. U937 cells (ATCC CRL-1593.2), a monoblastic leukemia cell line [27], were cultured in RPMI-1640 medium (HyClone) supplemented with 10% heat-inactivated FBS (RPMI-FBS) and 100 µg/mL penicillin-streptomycin in a 5% CO_2 atmosphere at 37°C. The U937 cells were maintained in 25 cm^2 tissue culture flasks by twice weekly splitting. Monocytes (2 x 10^5 cells/ml) were incubated with RPMI-FBS containing 10 ng/ml phorbol myristic acid (PMA; Sigma) for 48 h to induce differentiation into adherent macrophage-like cells. This treatment has been reported to induce the appearance of the characteristics consistent with mature macrophages [28]. Following the PMA treatment, the medium was removed by aspiration and replaced with fresh medium. The differentiated cells were then incubated for an additional 24 h prior to use.

4.4.4. Macrophage/epithelial cell co-culture model

The co-culture model was prepared as previously described [23]. Briefly, adherent macrophages were harvested by scraping and were suspended in RPMI-FBS. The suspension was centrifuged at 200 x *g* for 8 min, the cells were washed once in RPMI-FBS and suspended in DMEM with 1% heat-inactivated FBS at a density of 1 x 10^6 cells/ml as determined using a hemocytometer. Epithelial cells were harvested by gentle trypsinization (0.05% trypsin-EDTA; Gibco-BRL, Grand Island, NY, USA), washed once in DMEM-FBS, and suspended at a density of 1 x 10^6 cells/ml in DMEM with 1% heat-inactivated FBS. Mixtures of macrophage/epithelial cells at a ratio of 1:5 were prepared. The mixtures were cultured in 6-well plates (2 x 10^6 cells/well in 2 ml) for 3 h at 37°C in a 5% CO_2 atmosphere to allow the cells to adhere prior to initiating the infections.

4.4.5. Infection of the co-culture model

Bacteria were harvested by centrifugation at 11,000 x *g* for 10 min and suspended in DMEM at a concentration of 1 x 10^{10} cells per ml as determined using a Petroff-Hausser counting chamber. Polymicrobial inocula were prepared by mixing equal volumes of bacterial suspensions and were incubated for 15 min at 37°C prior to initiating the infection. The mixtures tested were *P. gingivalis*/*T. denticola*, *P. gingivalis*/*T. forsythia*, *T. denticola*/*T. forsythia*, and *P. gingivalis*/*T. denticola*/*T. forsythia*. The effects of individual bacterial species were also tested. The bacterial suspensions were added to the macrophage/epithelial cell co-culture at multiplicity of infection (MOI) of 50 bacteria per mammalian cell and incubated at 37°C in 5% CO_2. At this MOI, only minor proteolytic degradation of cytokines by *P. gingivalis* occurs [23]. Samples of culture medium were removed 24 h post-infection, centrifuged at 11,000 x *g* for 10 min to remove the bacteria, and supernatants were stored at –20°C until used. Co-cultures without bacteria were used as controls.

4.4.6. LPS stimulation of the co-culture model

LPS mixtures were prepared by mixing equal volumes of LPS solutions of 1 μg/ml. LPS from *P. gingivalis*, *T. denticola*, and *T. forsythia* were added alone or in combination to the macrophage/epithelial cell co-culture at final concentration of 1 μg/ml. After a 24 h stimulation period (37°C in 5% CO_2), samples of culture medium were removed and supernatants were stored at –20°C until used. Co-cultures without LPS were used as controls.

4.4.7. Cell viability

Macrophage and epithelial cell viability was measured by Trypan Blue exclusion. A 100 μl aliquot of cell suspension was mixed with an equal volume of 0.4% Trypan Blue solution (Gibco, Grand Island, NY, USA) and viable cells were counted using a hemocytometer.

4.4.8. Cytokine and MMP-9 production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify levels of IL-1β, IL-6, IL-8, TNF-α, RANTES, and MMP-9 in the cell-free culture supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (model 680, Bio-Rad) with a wavelength correction set at 550 nm. Cytokine and MMP-9 concentrations were determined in triplicate using a standard curve prepared for each assay. The sensitivities of the commercial ELISA kits were 31.2 pg/ml for IL-8, 15.6 pg/ml for RANTES and TNF-α, 9.3 pg/ml for IL-6, 3.9 pg/ml for IL-1β, and 0.31 ng/ml for MMP-9.

4.4.9. PGE_2 production

A competitive enzyme immunoassay (EIA) was performed on the supernatant fluids according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance at 415 nm was read using a microplate reader, and PGE_2 concentrations were determined in triplicate.

4.4.10. RANTES degradation

Bacteria, at the same concentrations as those used in the infection assays, were added to the supernatant of a 30-h unstimulated macrophage/epithelial cell co-culture containing basal level of RANTES. After a 24 h incubation, the RANTES concentrations in the untreated and treated supernatants were determined by ELISA as described previously.

4.4.11. Statistical analyses

The Student's t test for paired values was used and data were considered significant at P value of < 0.05.

4.5. Results

4.5.1 Secretion of cytokines by the co-culture model following stimulation by bacterial species of the red complex

The capacity of bacterial species of the red complex (*P. gingivalis*, *T. denticola*, and *T. forsythia*) to modulate cytokine secretion in a macrophage/epithelial cell co-culture model was examined. Following the stimulation by the red complex, the macrophages and epithelial cells remained adherent and retained their normal morphological characteristics.

In all experiments, Trypan blue exclusion revealed that the viability of cells in the co-culture model was > 96% (data not shown). TNF-α secretion by the co-culture model was not detected in any of the experimental conditions tested. A significant increase in IL-6 secretion was detected following stimulation with *P. gingivalis* alone or in combination with *T. forsythia*, *P. gingivalis* alone induced higher level of IL-6 than combined with *T. forsythia* (Fig. 4.1A). A strong IL-8 response was induced by all three bacterial species, whether alone or in combination (Fig. 4.1B). The strongest response was obtained following stimulation by *P. gingivalis*. Infections with *P. gingivalis/T. denticola* or *P. gingivalis/T. denticola/T. forsythia* induced similar levels of IL-8 than monoinfection with *P. gingivalis*, despite that the number of *P. gingivalis* cells was lowered 2- and 3-fold respectively. IL-1β secretion was induced by all three bacterial species, whether alone or in combination (Fig. 4.1C). *P. gingivalis* alone or in combination with *T. forsythia* was the most potent inducer of IL-1β. Mono or mixed infections by *P. gingivalis* and *T. forsythia* of the co-culture model induced an increase in RANTES secretion whereas infections by *T. denticola* alone or in combination resulted in a significant decrease in RANTES levels (Fig. 4.1D). Assessment of proteolytic degradation of RANTES showed that this chemokine is highly susceptible to degradation by *T. denticola* proteases and to a lesser extent by *P. gingivalis* proteases (Fig. 4.2).

When the co-culture model was infected (mono and mixed infections) using a second strain of *P. gingivalis* (ATCC 53977), similar results were obtained, with the exception of RANTES, for which low levels were detected (data not shown). *P. gingivalis* ATCC 53977 possesses a higher capacity than *P. gingivalis* ATCC 33277 to degrade RANTES as previously reported [23] and this property may be at least in part responsible for the decrease in the RANTES concentration.

4.5.2. Secretion of cytokines by the co-culture model following stimulation by LPS

There was no significant stimulation of IL-1β, TNF-α, and IL-6 secretion by LPS, whether alone or in combination (data not shown). However, RANTES secretion was

slightly stimulated by all bacterial LPS challenges (Fig. 4.3A). LPS from bacteria of the red complex, whether alone or in combination, all stimulated significant IL-8 secretion (Fig. 4.3B). *T. forsythia* LPS alone or in combination with *P. gingivalis* LPS or *T. denticola* LPS induced the highest levels of IL-8 secretion.

4.5.3. Secretion of MMP-9 by the co-culture model

While all the bacteria and LPS, whether alone or in combination, resulted in increased MMP-9 secretion (Fig. 4.4), the strongest response was observed with *T. denticola* cells (Fig. 4.4A) and a combination of *P. gingivalis* LPS and *T. forsythia* LPS (Fig. 4.4B) .

4.5.4. Secretion of PGE_2 by the co-culture model

While stimulations of the co-culture model with all the bacteria and LPS, whether alone or in combination, induced an increase of PGE_2 production by the co-culture model (Fig. 4.5), the strongest response was observed with whole cells of *P. gingivalis* alone or in combination with *T. forsythia* (Fig. 4.5A) and with *P. gingivalis* LPS alone or in combination with *T. denticola* LPS (Fig. 4.5B).

4.6. Discussion

P. gingivalis, *T. denticola*, and *T. forsythia* are frequently detected together in periodontal sites [5, 6, 29, 30]. This coexistence suggests that an ecological relationship exists between these bacterial species. Binding interactions between these three species have been reported [31-33] and may contribute to the establishment of the periodontopathogenic plaque. A recent report indicated that cell extracts from *T. forsythia* stimulate the growth of *P. gingivalis* [34] while a mutual nutritional relationship between *P. gingivalis* and *T. denticola* has been characterized [35, 36]. Mixed infections with *P.*

gingivalis and *T. denticola* cause an excessive inflammatory response in a mouse pneumonia model compared to monoinfections [37]. Kesavalu *et al.* reported that *T. denticola* significantly enhances the virulence of *P. gingivalis* in a murine model [38]. Synergistic interactions between *P. gingivalis* and *T. forsythia* have also been reported [39, 40]. In a rabbit abscess model, *T. forsythia* strains are highly virulent and invasive when combined with *P. gingivalis* [39]. In addition, *P. gingivalis* and *T. forsythia* have a synergistic effect on abscess formation in mice [40]. To our knowledge, no studies have addressed the impact of bacterial interactions on human inflammatory responses such as the production of cytokines, matrix metalloproteinases, and prostaglandins.

A number of studies have reported that periodontopathogenic bacteria modulate the expression of inflammatory mediators or MMPs by gingival epithelial cells or macrophages [7, 14, 21]. However, these studies generally used individual cell lines stimulated by individual bacterial species. In the present study, we characterized the production of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), chemokines (IL-8 and RANTES), PGE_2, and MMP-9 in a macrophage/epithelial cell co-culture model in response to mono and mixed stimulations by whole cells of three major periodontopathogens (*P. gingivalis*, *T. denticola*, and *T. forsythia*) also known as members of the red complex [5], and their LPS. In a previous study using this model, two macrophage/epithelial cell ratios were tested [23]. The 1:100 ratio was considered to correspond to a relatively healthy state while the 1:5 ratio was considered to correspond to an inflamed periodontium. In this study, the 1:5 ratio was selected to investigate the inflammatory response induced by polymicrobial infections because it resulted in the highest cytokine responses.

We report here the capacity of *P. gingivalis*, *T. denticola*, and *T. forsythia*, alone or in combination, to induce IL-1β secretion by a macrophage/epithelial cell co-culture model. The release of IL-1β stimulates the production of secondary mediators, including chemokines and prostaglandins, which amplifies the inflammatory response, the induction of connective tissue-degrading enzymes, and osteoclastic bone resorption [41]. The stimulation of IL-1β secretion by members of the red complex observed in this study may

be an important mechanism that contributes to the activation of various periodontal tissue destruction pathways. However, IL-1β secretion was not observed following LPS challenges. This can be explained by the presence on the bacterial species of the red complex of various cell-associated proteins which can participate to the host cell response [9]. In addition, cell response to membrane-bound LPS can differ from that of soluble LPS by the fact that lipid A is exposed to immune cells in the extracted form [42].

IL-8 and RANTES are involved in the recruitment of neutrophils, eosinophils, monocytes, and TH_1 cells to infected sites [43]. We observed that all combinations of bacteria and LPS induced a significant increase in IL-8 secretion by the co-culture model. This phenomenon may favor the accumulation of leukocytes at inflamed sites and thus amplify the host inflammatory response in periodontal tissues. High levels of RANTES have been detected in gingival crevicular fluid and inflamed gingival tissue adjacent to periodontal pockets of patients with periodontitis, suggesting that RANTES is an important mediator in the initiation and progression of periodontitis [44-46]. However, little is known about the effect of periodontopathogens on the production of RANTES by host cells. A stimulation of RANTES secretion by the co-culture model was observed following challenges with LPS from periodontopathogens. However, only whole cells of *P. gingivalis* and *T. forsythia* alone or in combination induced an increase in RANTES secretion. The stimulation of RANTES secretion mediated by periodontopathogens may contribute to amplifying the host response by recruiting inflammatory cells into active diseased sites and inducing the release of other cell mediators. On the other hand, *T. denticola*, alone or in combination with other periodontopathogens, decreased the amount of RANTES detected in the culture supernatant of the co-culture. It is well known that proteolytic enzymes produced by *P. gingivalis* can degrade cytokines and chemokines [23, 47, 48]. *T. denticola* also secretes proteinases, including trypsin-like and chymotrypsin-like activities, which have been suggested to contribute to the degradation of cytokines [49, 50]. In the present study, the incubation of RANTES with *T. denticola* resulted in a significant decrease in the levels of this chemokine. This suggests that *T. denticola* possesses the capacity to degrade RANTES and that the low levels of RANTES observed in the co-culture model following a challenge by *T. denticola* were likely caused by a massive proteolytic degradation of

RANTES by this bacterium. This phenomenon may thus result in an underestimation of the inflammatory responses mediated by bacteria of the red complex in *in vitro* models. *In vivo*, the ability of periodontopathogens to degrade cytokines and chemokines may play an important role in their pathogenicity by disrupting the host inflammatory response. For instance, the disruption of the chemokine gradient near periodontopathogenic plaque may reduce phagocytic leukocytes afflux and favor overgrowth of periodontopathogens.

MMPs are involved in connective tissue remodeling and may play a significant role in the degradation of the collagenous structure of periodontal tissue, a critical outcome of periodontal disease. Periodontal tissue destruction has been associated with high levels of active MMP-9 in gingival crevicular fluid [51]. This metalloproteinase is also highly expressed in the inflamed gingival tissues of patients with periodontitis [52, 53]. Stimulations of the co-culture model by bacterial species of the red complex or their LPS induced a significant increase in MMP-9 secretion. This suggests that these periodontopathogens contribute indirectly to gingival extracellular matrix degradation by stimulating the secretion of host MMPs. Prostaglandins found within periodontal tissue have been suggested to play a role in the progression of periodontal disease [54]. PGE_2 is a potent stimulator of bone resorption [55] and is associated with attachment loss [56]. High levels of PGE_2 have been detected in the gingival crevicular fluid of periodontitis sites co-infected with the three bacterial species of the red complex [57]. These *in vivo* observations were supported by our results, which showed that these bacteria or their LPS induce an increase in PGE_2 secretion by the macrophage/epithelial cell co-culture model. This stimulation of prostaglandin secretion by bacterial species of the red complex may thus contribute to periodontal tissue destruction and be an important factor in their pathogenicity.

In summary, we observed that *P. gingivalis*, *T. denticola*, and *T. forsythia* stimulate the secretion of pro-inflammatory cytokines (IL-1β, IL-6), chemokines (IL-8, RANTES), PGE_2, and MMP-9 in a macrophage/epithelial cell co-culture model. This indicates that these periodontopathogens have a strong potential for activating host-mediated destructive processes. No synergistic effects on cytokine, chemokine, PGE_2, or MMP-9 production

were observed for the bacterial mixtures compared to monoinfections by individual bacterial species. This study supports the view that bacterial species of the red complex act in concert to increase the levels of pro-inflammatory mediators and MMP-9 in periodontal tissues, a phenomenon that may significantly contribute to the progression of periodontitis.

4.7. Acknowledgments

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4.8. References

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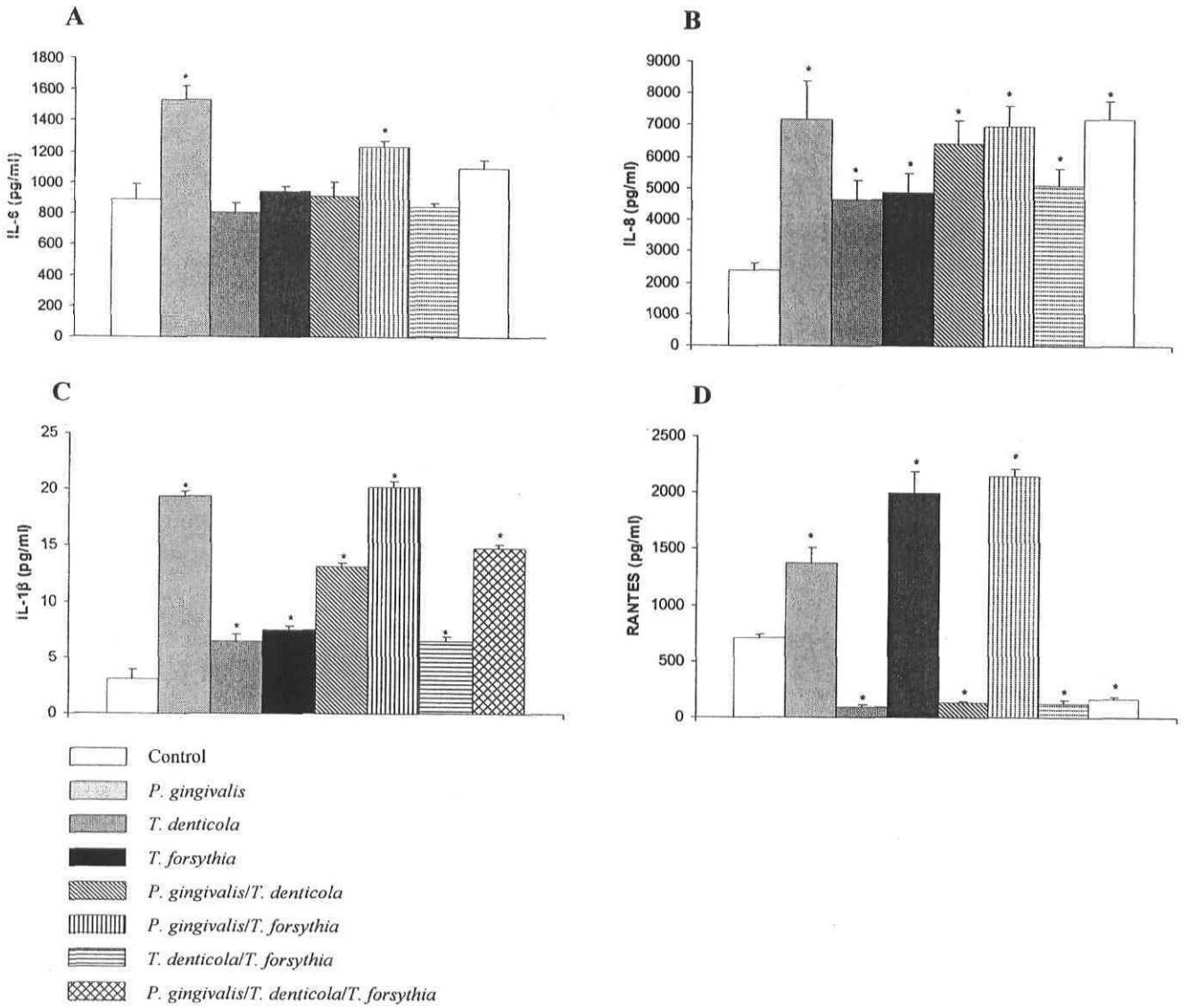

Figure 4.1. Secretion of IL-6 (A), IL-8 (B), IL-1β (C), and RANTES (D) by a macrophage/epithelial cell co-culture following mono or mixed infections with whole cells of bacterial species of the red complex (*P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037) for 24 h at a MOI of 50. The bacterial combinations tested were *P. gingivalis/T. denticola*, *P. gingivalis/T. forsythia*, *T. denticola/T. forsythia* and *P. gingivalis/T. denticola/T. forsythia*. Cytokine secretion was assessed by ELISA. The data are the means ± standard deviations (S.D.) of triplicate assays. *, $P < 0.05$ compared to uninfected control.

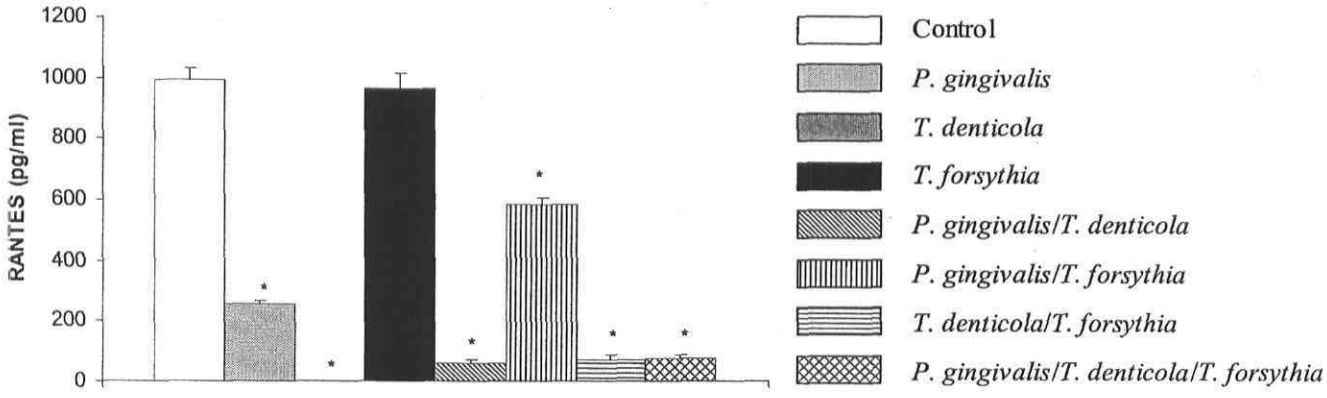

Figure 4.2. Degradation of RANTES by *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037, alone and in combination. The bacterial mixtures tested were *P. gingivalis/T. denticola*, *P. gingivalis/T. forsythia*, *T. denticola/T. forsythia* and *P. gingivalis/T. denticola/T. forsythia*. A bacterial concentration equivalent to that used for stimulation of the co-culture model was incubated for 24 h with a cell-free supernatant containing basal level of RANTES. The RANTES concentrations were assessed by ELISA. The data are the means ± S.D. of triplicate assays. *, $P < 0.05$ compared to uninfected control.

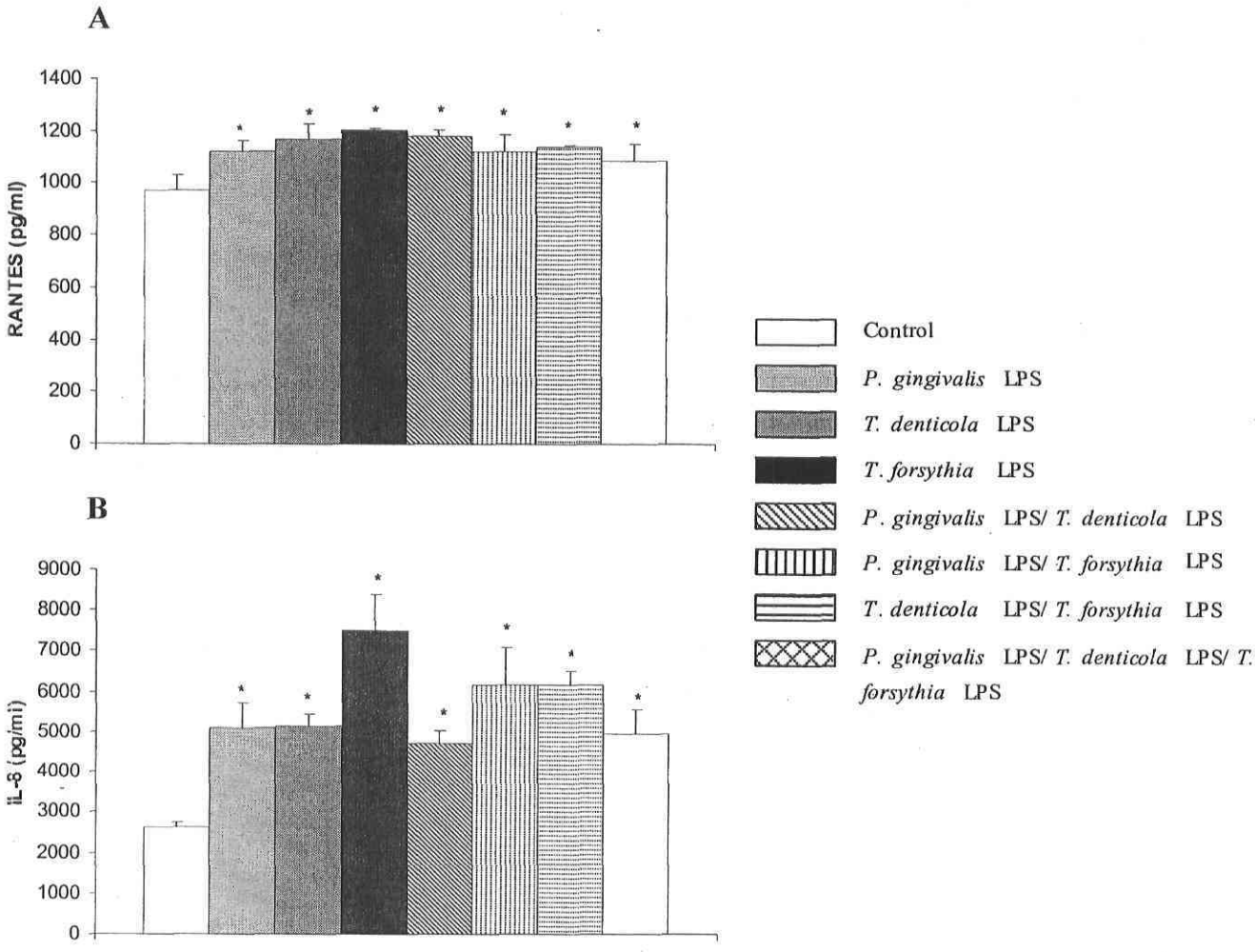

Figure 4.3. Secretion of RANTES (A) and IL-8 (B) by a macrophage/epithelial cell co-culture model following stimulation by LPS (1 μg/ml) from *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037 for 24 h. The LPS combinations tested were *P. gingivalis*/*T. denticola*, *P. gingivalis*/*T. forsythia*, *T. denticola*/*T. forsythia*, and *P. gingivalis*/*T. denticola*/*T. forsythia*. Cytokine secretion was assessed by ELISA. The data are the means ± S.D. of triplicate assays. *, $P < 0.05$ compared to an uninfected control.

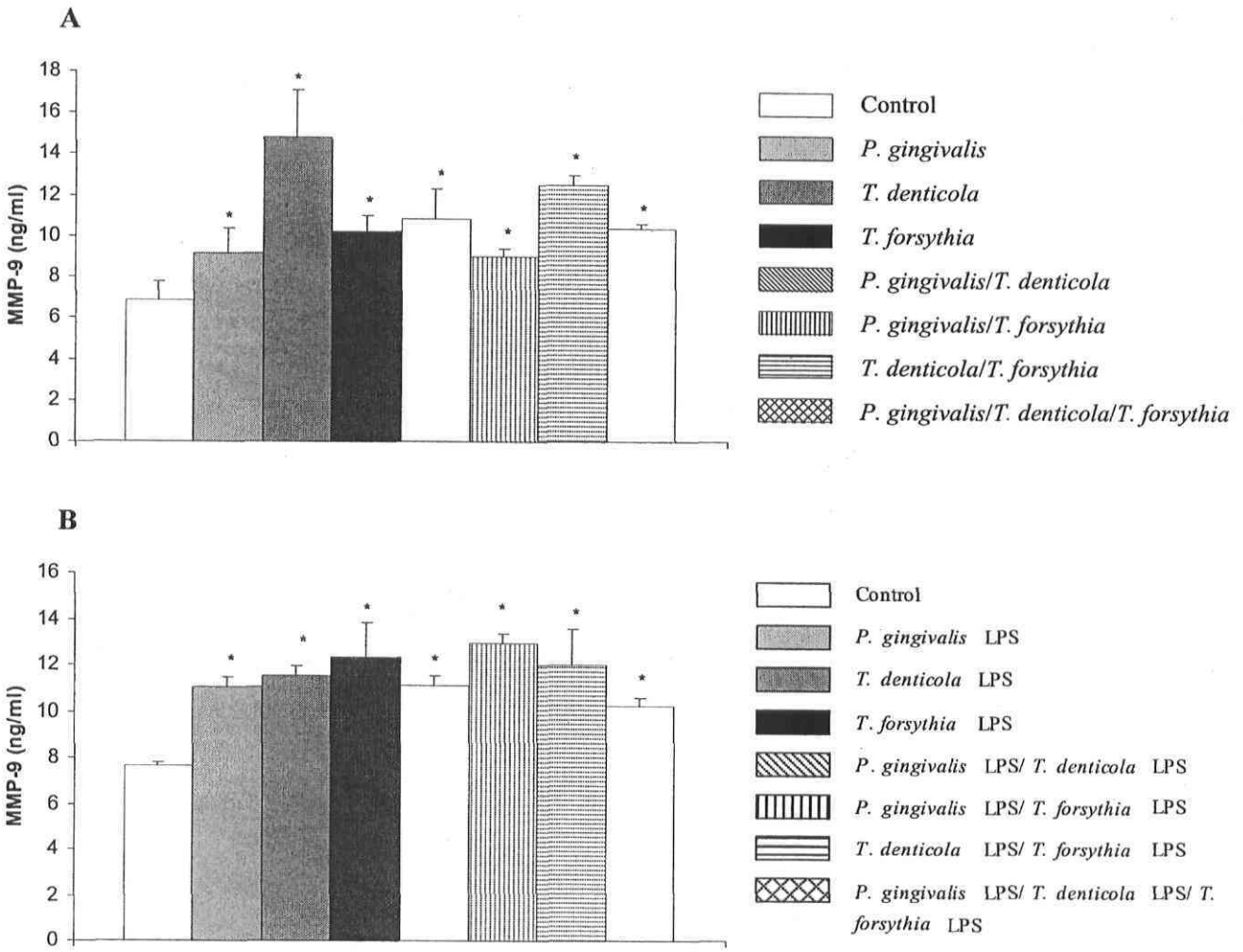

Figure 4.4. Secretion of MMP-9 by a macrophage/epithelial cell co-culture following mono or mixed infections with whole cells of bacterial species of the red complex (*P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037) for 24 h at a MOI of 50 (A) or their LPS at final concentration of 1 μg/ml (B). The bacterial and LPS combinations tested were *P. gingivalis/T. denticola*, *P. gingivalis/T. forsythia*, *T. denticola/T. forsythia* and *P. gingivalis/T. denticola/T. forsythia*. MMP-9 concentrations were assessed by ELISA. The data are the means ± S.D. of triplicate assays. *, $P < 0.05$ compared to an uninfected control.

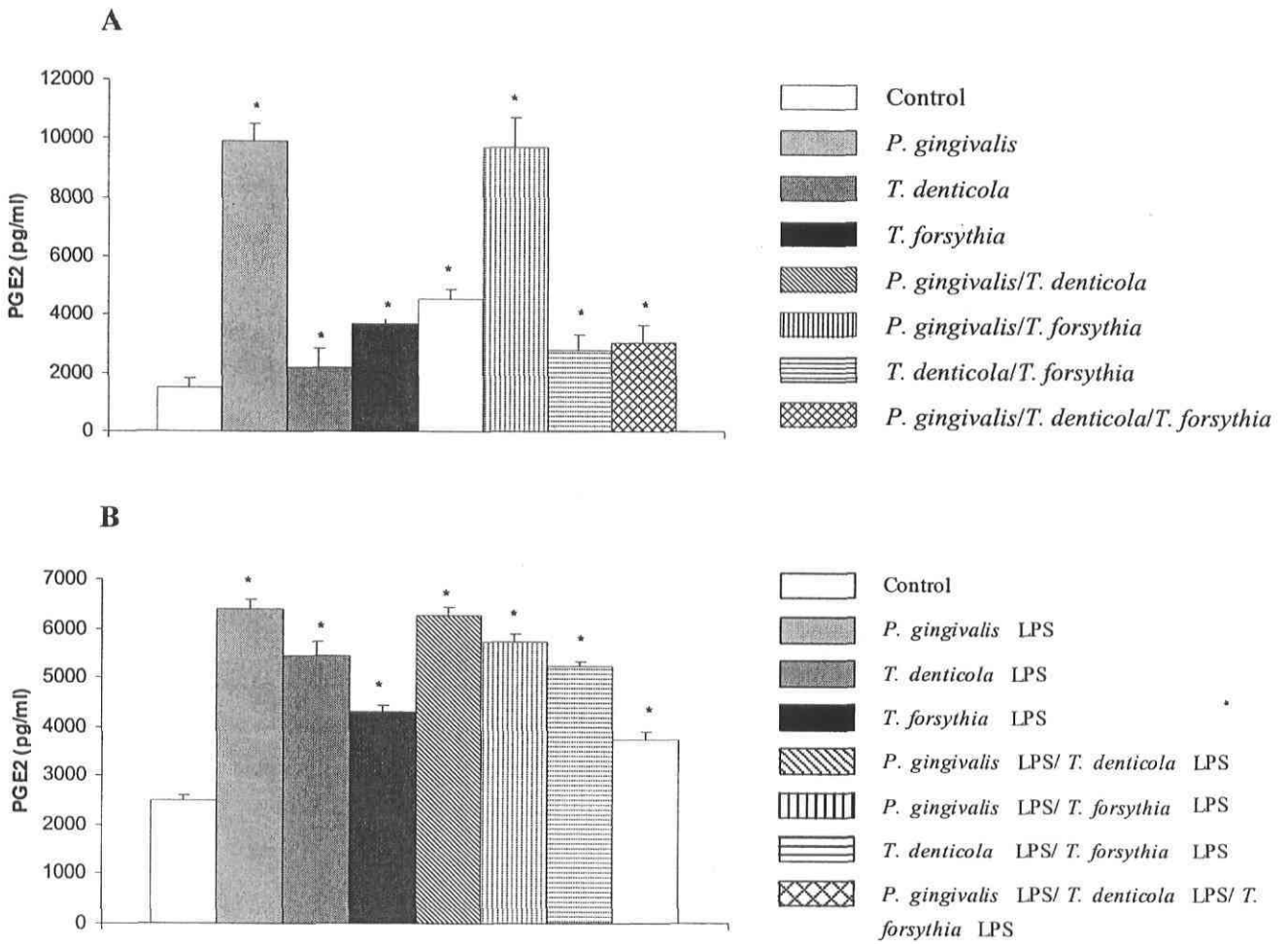

Figure 4.5. Secretion of PGE_2 by a macrophage/epithelial cell co-culture following mono or mixed infections by *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037 for 24 h at a MOI of 50 (A) or their LPS at final concentration of 1 μg/ml (B). The bacterial or LPS combinations tested were *P. gingivalis/T. denticola*, *P. gingivalis/T. forsythia*, *T. denticola/T. forsythia*, and *P. gingivalis/T. denticola/T. forsythia*. PGE_2 concentrations were assessed by ELISA. The data are the means ± S.D. of triplicate assays. *, $P < 0.05$ compared to an uninfected control.

CHAPITRE 5

Synergistic effect of hemoglobin and periodontopathogen lipopolysaccharides on the inflammatory response of macrophages

5.1. Résumé

Les parodontites sont des maladies inflammatoires chroniques affectant les tissus de soutien de la dent. La forte production continue de cytokines par les cellules de l'hôte, incluant les macrophages, qui est provoquée par les bactéries parodontopathogènes et leur LPS participe à la destruction des tissus de soutien de la dent. Les phénomènes de rupture vasculaire et de saignement se produisant au cours des parodontites augmentent probablement la concentration d'hémoglobine dans le fluide créviculaire. Le but de cette étude est de caractériser l'effet de l'hémoglobine sur la réponse inflammatoire de macrophages humains stimulés par le LPS de bactéries parodontopathogènes. La production d'IL-1β, d'IL-6, d'IL-8, de TNF-α et de RANTES induite par les LPS de *Porphyromonas gingivalis* et *Fusobacterium nucleatum* chez les macrophages en présence ou non d'hémoglobine a été caractérisée par ELISA. L'effet de l'hémoglobine sur la liaison du LPS à la surface des macrophages a été investigué en utilisant des ^{3}H-LPS. L'hémoglobine a agi en synergie avec le LPS des bactéries parodontopathogènes pour augmenter la production d'IL-1β, d'IL-6, d'IL-8 et de TNF-α par les macrophages. L'hémoglobine a augmenté aussi la liaison du LPS aux macrophages. Cette étude suggère que l'hémoglobine contribue à augmenter les quantités des médiateurs pro-inflammatoires

dans les sites de parodontites en agissant en synergie avec le LPS des bactéries parodontopathogènes, favorisant ainsi la progression des parodontites.

5.2. Abstract

Periodontitis is a chronic inflammatory disease affecting the tooth-supporting tissue. Continuous, high cytokine production by host cells, including macrophages, triggered by periodontopathogens and more specifically by lipopolysaccharides (LPS) is thought to be responsible for periodontal tissue destruction. Vascular disruption and bleeding during periodontitis likely increases the levels of hemoglobin in gingival crevicular fluid. The aim of this study was to investigate the effect of hemoglobin on the inflammatory response of human macrophages stimulated with LPS isolated from periodontopathogens. The production of interleukin-1 beta (IL-1β), IL-6, IL-8, tumor necrosis factor alpha (TNF-α) and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) by macrophage-like cells (phorbol myristic acid-differentiated U937 monocytic cell line) following challenges with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* LPS in the presence or absence of human hemoglobin was analyzed by ELISA. The effect of hemoglobin on LPS-binding to macrophages was evaluated using ^{3}H-LPS. Hemoglobin and LPS from periodontopathogens acted in synergy to stimulate the production of high levels of IL-1β, IL-6, IL-8 and TNF-α by macrophages. Hemoglobin also enhanced LPS-binding to macrophages. This study suggests that hemoglobin contributes to increasing the levels of pro-inflammatory mediators in periodontal sites by acting in synergy with LPS from periodontopathogens thus favoring the progression of periodontitis.

5.3. Introduction

Periodontitis is a multifactorial polymicrobial infection characterized by a destructive inflammatory process affecting the tooth-supporting tissues and resulting in periodontal pocket formation, alveolar bone resorption and eventually tooth loss.

Porphyromonas gingivalis and *Fusobacterium nucleatum* are major periodontopathogens [1] and their numbers in subgingival plaque increase significantly during the active phase of periodontitis [2]. The host response to these bacterial species and their products is a critical determinant in the initiation and progression of periodontitis. More specifically, lipopolysaccharides (LPS) from Gram-negative bacteria are potent inducers of pro-inflammatory mediators and can initiate a number of host-mediated destructive processes. Excessive, continuous production of cytokines, including interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor alpha (TNF-α) in inflamed periodontal tissues is responsible for disease progression and periodontal tissue destruction [3].

The periodontal tissue of patients affected with periodontitis is highly infiltrated with inflammatory cells. Macrophages make up an important proportion of such cells in diseased tissues and play an essential role in the host response to periodontopathogens [3]. A clinical characteristic of periodontitis is vascular disruption and bleeding. Hemoglobin accounts for 95% of the protein content of erythrocytes and is present in both plasma and gingival crevicular fluid. It has been reported that the amount of hemoglobin β-chain in whole saliva from subjects who bleed after gentle probing and who have pocket depths ≥ 4 mm is at least seven-fold higher than in whole saliva from healthy subjects [4]. Several studies have revealed a close relationship between bleeding on probing and inflamed gingival tissue [5, 6]. A correlation between hemoglobin concentration in gingival tissue and the active phase of periodontitis has also been suggested. Indeed, the hemoglobin concentration increases rapidly over the first seven days following ligature-induced experimental periodontitis in dogs [7] as well as in human gingiva with increasing inflammation [8]. Interestingly, the increased hemoglobin concentration in human gingiva is restored to normal levels when the inflammation is resolved [9].

Hemoglobin interacts with LPS resulting in an intensification of the biological activity of LPS in the chromogenic *Limulus* amebocyte lysate test [10]. Hemoglobin also enhances the lethal toxicity of LPS in animal models [11, 12]. In addition, hemoglobin stimulates the release of proinflammatory cytokines from leukocytes in whole blood [13]

and enhances TNF-α production in human monocytes [14]. The aim of the present study was to investigate the effect of hemoglobin on the periodontopathogen-LPS-induced inflammatory response by human macrophages. More specifically, the production of IL-1β, IL-6, IL-8, TNF-α, and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) by macrophages stimulated with *P. gingivalis* and *F. nucleatum* LPS in the presence of increasing concentrations of hemoglobin was quantified by ELISA. The effect of hemoglobin on LPS binding to macrophages was also evaluated.

5.4. Materials and methods

5.4.1. Bacterial strains, growth conditions and LPS preparation

P. gingivalis ATCC 33277 and *F. nucleatum* subsp. *nucleatum* ATCC 25586 (hereafter referred to as *F. nucleatum*) were grown in Todd Hewitt Broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with hemin (10 μg/ml) and vitamin K (1 μg/ml) at 37°C for 24 h under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2). LPS were isolated by the method of Darveau and Hancock [15], which is based on protein digestion of a whole cell extract by proteinase K and successive solubilization and precipitation steps. The LPS preparations were freeze-dried and stored at –20°C. The concentration of contaminating proteins in the preparations was evaluated using a protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as a control and was less than 0.001%.

5.4.2. Radiolabeling of LPS

The method used to prepare ^{3}H-LPS was a modification of the procedure previously described by Rokita and Menzel [16]. Stock solutions of *F. nucleatum* and *P. gingivalis* LPS (1 mg/ml) were prepared in 100 mM carbonate buffer (pH 9) containing 100 mM NaCl. The LPS solutions were vortexed in a glass tube for 5 min prior to adding a solution

of ^{3}H-acetic anhydride in toluene (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) to obtain a final concentration of 1.25 µCi/µl (2.4 µM). The LPS solutions were vortexed vigorously for a further 5 min and incubated at room temperature for 1 h with gentle agitation. The LPS solutions were then diluted 1:4 by adding three volumes of 100 mM phosphate-buffered saline (pH 7.2) (PBS). Seven successive dialyses (10 h at 4°C) against PBS (4 l) were performed until less than 50 dpm/100 µl of buffer were detected in the dialysate. Radioactive counts and specific activity (µCi/mg LPS) of the ^{3}H-LPS preparations were determined using EcoLite scintillation liquid (ICN, Costa Mesa, CA, USA) and a multi-purpose scintillation counter (Beckman Coulter, Fullerton, CA, USA). Sodium azide (0.1%) was added to the ^{3}H-LPS preparations, which were then stored at –20°C. The radiolabeling procedure produced ^{3}H-LPS preparations with a specific activity of 4 µCi/mg for *F. nucleatum* and 23.5 µCi/mg for *P. gingivalis*. Radiolabeled LPS were analyzed by denaturing electrophoresis on a 12.5% polyacrylamide gel. The gels were treated with an Entensify solution (NEN Life Science Products, Boston, MA, USA) and exposed on a Kodak BioMax MS Film at –80°C for one week.

5.4.3. Monocyte and macrophage cultures

U937 cells (ATCC CRL-1593.2), a monoblastic leukemia cell line, were cultivated at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (RPMI-FBS) and 100 µg/mL of penicillin-streptomycin. Monocytes (2 x 10^5 cells/ml) were incubated in RPMI-FBS containing 10 ng/ml of phorbol myristic acid (PMA; Sigma) for 48 h to induce differentiation into adherent macrophage-like cells. Following the PMA treatment, the medium was replaced with fresh medium and the differentiated cells were incubated for an additional 24 h prior to use. Adherent macrophages were suspended in RPMI-FBS and centrifuged at 300 x *g* for 5 min. They were washed and suspended in RPMI with 1% heat-inactivated FBS at a density of 1 x 10^6 cells/ml and incubated in 6-well plates (2 x 10^6 cells/well in 2 ml) at 37°C in a 5% CO_2 atmosphere for 2 h prior stimulation.

5.4.4. Macrophage stimulation

The macrophages were stimulated with *P. gingivalis* LPS or *F. nucleatum* LPS at final concentrations of 0.1, 1 and 10 μg/ml in the presence or absence of hemoglobin at final concentrations of 10, 50 and 100 μg/ml. After a 24 h incubation (37°C in 5% CO_2), the culture medium supernatants were collected and stored at –20°C until used. Cells incubated in culture medium with or without hemoglobin, but not stimulated with LPS, were used as controls.

5.4.5. Cell viability

Macrophage viability was evaluated using an MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Briefly, macrophages were incubated in 96-well plates (1×10^5 cells/well in 100 μl) at 37°C in a 5% CO_2 atmosphere for 2 h prior to stimulation with LPS and/or hemoglobin as previously described. After 24 h, the cells were incubated with MTT for 4 h and the insoluble formazan dye was solubilized overnight at 37°C. The absorbance was read at 550 nm using a microplate reader with the wavelength correction set at 650 nm.

5.4.6. Determination of cytokine production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify IL-1β, IL-6, IL-8, TNF-α and RANTES concentrations in the cell-free culture supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader with the wavelength correction set at 550 nm. The sensitivities of the commercial ELISA kits were 31.2 pg/ml for IL-8, 15.6 pg/ml for RANTES and TNF-α, 9.3 pg/ml for IL-6 and 3.9 pg/ml for IL-1β.

5.4.7. ^{3}H-LPS binding to hemoglobin-, BSA- and gelatin-coated microplates

The wells of 96-well plates were coated overnight with 100 µl of hemoglobin (Hb;1 mg/ml), bovine serum albumin (BSA; 1 mg/ml) or gelatin (1 mg/ml). The wells were washed three times with PBS and ^{3}H-LPS was added to the protein-coated wells at a final concentration of 10, 1 or 0.1 µg/ml. After a 2 h incubation, the wells were washed three times with PBS. The wells were detached from the microtiter plate, and bound ^{3}H-LPS was measured in triplicate wells using a multi-purpose scintillation counter.

5.4.8. Effect of hemoglobin on ^{3}H-LPS binding to macrophages

Macrophages were cultured in 6-well plates as previously described. They were incubated with ^{3}H-LPS from *P. gingivalis* and *F. nucleatum* at final concentrations of 10 and 1 µg/ml in the presence or absence of hemoglobin (10, 50 and 100 µg/ml). After a 24 h incubation, the macrophages were suspended in PBS and washed three times. The amount of ^{3}H-LPS bound to the macrophages was measured in triplicate assays using a multi-purpose scintillation counter.

5.4.9. Statistical analyses

Statistical analyses were performed using the Student's t test for paired values. To determine the synergistic effect of LPS-Hb on cytokine production, cytokine levels induced by hemoglobin alone were added to the levels induced by LPS alone and compared to levels induced by LPS in presence of hemoglobin. The data were considered significant at $p < 0.05$.

5.5. Results

5.5.1. Effect of hemoglobin on LPS-induced cytokine production by macrophages

To evaluate the effect of hemoglobin on LPS-induced pro-inflammatory cytokine production, macrophages were stimulated with *P. gingivalis* and *F. nucleatum* LPS (0.1, 1 and 10 μg/ml) in the presence or absence of hemoglobin (10, 50 and 100 μg/ml). The effect of LPS and hemoglobin on macrophage viability was evaluated using an MTT test. A hemoglobin concentration of 100 μg/ml caused a 23% reduction in macrophage viability whereas concentrations of 50 μg/ml and 10 μg/ml resulted in 16% and 7% reductions in macrophage viability, respectively (data not shown). A high concentration of hemoglobin (500 μg/ml) was very cytotoxic for macrophages, decreasing cell viability to 20%. In addition, the treatment with both hemoglobin and LPS did not result in a further decrease in cell viability compared to the treatment with hemoglobin alone. Stimulation of macrophages with hemoglobin alone induced the secretion of IL-1β, TNF-α, IL-6 and IL-8 (Figs. 5.1, 5.2, 5.3 and 5.4). This effect was concentration-dependent and IL-8 was the most strongly induced.

A synergistic effect of both *P. gingivalis* and *F. nucleatum* LPS and hemoglobin on IL-1β secretion was observed with hemoglobin at concentrations of 50 and 100 μg/ml (Fig. 5.1). At a hemoglobin concentration of 100 μg/ml, the synergistic effect was observed at all concentrations of both LPS. On the other hand, while a synergistic effect was observed at all *F. nucleatum* LPS concentrations in combination with a hemoglobin concentration of 50 μg/ml, a significant ($p < 0.05$) enhancement of LPS-induced IL-1β secretion was only observed at a high concentration of *P. gingivalis* LPS (10 μg/ml). Hemoglobin (50 and 100 μg/ml) and LPS at all concentrations tested acted in synergy to enhance TNF-α production by macrophages (Fig. 5.2). A synergistic effect between hemoglobin at all concentrations tested and LPS were also observed for IL-6 secretion (Fig. 5.3). More specifically, a significant effect was observed at a high concentration (10 μg/ml) of *P. gingivalis* LPS and at a lower concentration of *F. nucleatum* LPS (1 and 10 μg/ml). Hemoglobin also enhanced

LPS-induced IL-8 secretion by macrophages (Fig. 5.4). At *P. gingivalis* LPS concentrations of 0.1 and 1 μg/ml, the synergistic effect on IL-8 secretion was only observed at a high hemoglobin concentration (100 μg/ml) (Fig. 5.4A). At a higher *P. gingivalis* LPS concentration (10 μg/ml), a significant effect was observed at all the hemoglobin concentrations tested. A synergistic effect on IL-8 production by macrophages was observed at a low concentration of *F. nucleatum* LPS (0.1 μg/ml) at all the hemoglobin concentrations tested. At a high concentration of *F. nucleatum* LPS (10 μg/ml), a significant synergistic effect was only observed with a hemoglobin concentration of 10 μg/ml. While hemoglobin enhanced RANTES secretion, it had no significant synergistic effect in the presence of LPS (data not shown).

5.5.2. Effect of hemoglobin on LPS binding to macrophages

P. gingivalis and *F. nucleatum* ^{3}H-LPS preparations were analyzed by SDS-PAGE autoradiography and revealed smooth and rough morphotype profiles, respectively (data not shown). The capacity of hemoglobin to bind to periodontopathogen LPS was investigated by incubating *P. gingivalis* and *F. nucleatum* ^{3}H-LPS in protein-coated microplate wells. *P. gingivalis* and *F. nucleatum* ^{3}H-LPS bound to hemoglobin-coated wells in similar quantities than to BSA- and gelatin-coated wells (data not shown).

The effect of hemoglobin on ^{3}H-LPS-binding to macrophages was then characterized (Fig. 5.5). Hemoglobin enhanced *P. gingivalis* and *F. nucleatum* LPS-binding to macrophages. At a low hemoglobin concentration (10 μg/ml), the binding to macrophages of *P. gingivalis* LPS at a concentration of 10 μg/ml and *F. nucleatum* LPS at a concentration of 1 μg/ml was significantly enhanced ($p < 0.05$). The binding of *P. gingivalis* and *F. nucleatum* LPS at all concentrations was also significantly enhanced in presence of hemoglobin at 50 and 100 μg/ml. This effect was observed in the presence or absence of serum, which contains proteins that participate in the interaction of LPS with cells, including LPS-binding protein (LBP) and soluble CD14 (sCD14) (data not shown).

5.6. Discussion

Strong cytokine production by host cells contributes to the progression of periodontitis. The periodontal tissue destruction typical of this disease is accompanied by gingival vascular disruption and bleeding. Several studies have supported a close relationship between bleeding on probing and inflammatory reactions of the gingiva [5, 6] as well as between hemoglobin concentration in gingival tissues and gingival inflammation [7-9]. We investigated here the effect of hemoglobin on periodontopathogen LPS-induced cytokine secretion by macrophages.

Hemoglobin induced IL-1β, TNF-α, IL-6, IL-8 and RANTES production by macrophages. This is consistent with previously reported data regarding the proinflammatory properties of hemoglobin [13, 14]. Indeed, free hemoglobin enhances TNF-α production in isolated human monocytes [14] and stimulates the release of IL-6, IL-8 and TNF-α by a mixed leukocyte population from whole blood [13]. This suggests that the increased hemoglobin concentration observed in inflamed gingiva [8] may participate in the inflammatory process by stimulating the secretion of pro-inflammatory cytokines by leukocytes. In addition, we observed that high concentrations of hemoglobin had a cytotoxic effect on macrophages. This is in agreement with Yadav *et al.* [17], who showed that hemoglobin can mediate cytotoxicity and apoptosis and shares some of the bioactivities ascribed to TNF-α.

Hemoglobin increased periodontopathogen LPS immunostimulatory activity. Indeed, the secretion of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) and a chemokine (IL-8) by macrophages following stimulation with *P. gingivalis* and *F. nucleatum* LPS was strongly enhanced in the presence of hemoglobin. Similar effects of hemoglobin on LPS-induced cytokine secretion by macrophages was also observed with *Actinobacillus actinomycetemcomitans* LPS, suggesting that the synergistic effect is not specific to one type of periodontopathogen LPS (data not shown). In addition, the effect of hemoglobin on cytokine production by macrophages following stimulation with whole *P.*

gingivalis cells was also investigated and no significant effect was observed, suggesting that the synergistic effect occurs only with detached LPS (data not shown). The synergistic effect between hemoglobin and LPS leading to enhanced production of inflammatory mediators associated with periodontitis progression may play an important role in the development of this disease. On the one hand, gingival crevicular levels of endotoxins have been correlated with gingival inflammation [18] and are higher in periodontitis sites [19]. On the other hand, the concentration of hemoglobin increases in human gingiva with increasing inflammation [8]. This suggests that increasing levels of these factors in combination may play a role in the continuous, high expression of inflammatory mediators by host cells in inflamed gingiva, thus contributing to the progression of periodontitis.

Hemoglobin enhances the toxicity or lethality of LPS in animal models [11, 12]. This effect has been attributed in part to the capacity of hemoglobin to increase the TNF-α response of cultured macrophages and mice to LPS [12]. Another study reported that hemoglobin enhances TNF-α synthesis by LPS-stimulated macrophages at least 1,000-fold [20]. We also observed that the TNF-α response of macrophages to periodontopathogen LPS was strongly enhanced by hemoglobin. On the other hand, Yang *et al.* [21] reported that LPS binding to globin (hemoglobin minus heme) antagonizes the action of LPS on cultured macrophages and in mice. They speculated that the heme moiety of hemoglobin is likely involved in the effects of hemoglobin-LPS interactions. In our study, we found that *P. gingivalis* and *F. nucleatum* LPS bound to hemoglobin, although it also attached to gelatin and BSA. *A. actinomycetemcomitans* LPS has been reported to have a strong capacity to bind hemoglobin [22]. The binding of hemoglobin to *Escherichia coli* and *Proteus mirabilis* LPS has previously been reported, and it has been proposed that this binding increases the biological activity of LPS by promoting the disaggregation of LPS complexes [10], which can result in better accessibility of the binding and recognition groups of LPS to target structures such as serum and membrane proteins. Another study reported that hemoglobin induces conformational changes in the lipid A moiety of LPS, which results in an increase in LPS-induced cytokine induction [23]. In addition, it has been suggested that the intercalation of a hemoglobin-LPS complex in the cell membrane may act as a cell activator at the sites of signaling proteins such as Toll-like receptors [23, 24]. It

is likely that many of these proposed mechanisms contribute to the synergistic effect between hemoglobin and LPS on the inflammatory response, but further studies are needed to clarify this issue.

Hemoglobin also enhances LPS binding to human endothelial cells [25]. This has been attributed to the disaggregation of LPS by hemoglobin. In the present study, we showed that hemoglobin enhanced the binding of periodontopathogen LPS to human macrophages. This capacity of hemoglobin is likely involved in the enhanced cytokine response to LPS observed in presence of hemoglobin. Gorbenko [26] reported that hemoglobin penetrates into the phospholipid layer of model membranes. An intercalation of hemoglobin-LPS complexes in phospholipid liposomes corresponding to the composition of human macrophages has been also reported [24]. This may also lead to the increased binding of LPS to macrophages observed in the presence of hemoglobin. As suggested by Jürgens et *al.* [23], intramembranous hemoglobin-LPS complexes may exert strong mechanical stress at the site of signal-transducing proteins such as the Toll-like receptors or ion channels, leading to conformational changes of these proteins with subsequent triggering of the signal cascade.

The average concentration of hemoglobin in plasma is between 10 and 40 μg/ml [27]. The protein composition and concentration of gingival crevicular fluid bathing the periodontal pocket is derived from gingival capillary beds and is similar to that of serum [28]. The vascular disruption and bleeding that occurs during periodontitis likely increases the levels of free hemoglobin in gingival crevicular fluid. We observed a synergistic effect in a limited number of conditions with a hemoglobin concentration of 10 μg/ml whereas we observed the effect in the majority of the conditions tested with a higher hemoglobin concentration (50 and 100 μg/ml). This provides support for the idea that a synergistic effect between LPS and hemoglobin occurs most especially in pathological conditions, such as inflamed periodontal tissues where there are higher hemoglobin concentrations. In addition, *P. gingivalis* and *F. nucleatum* possess both hemagglutinating and hemolytic activities, conferring on them the ability to agglutinate and lyse red blood cells [29, 30]. This suggests that periodontopathogens may contribute to enhancing the release of

hemoglobin into gingival crevicular fluid and periodontal tissue during the frequent bleeding stages characterizing periodontitis. Hemoglobin is a source of iron for periodontopathogens and may modulate their growth and virulence [30]. Higher levels of hemoglobin may thus contribute to promoting bacterial growth and the subsequent release of LPS as well as to increasing the host inflammatory response.

Our findings suggest that hemoglobin enhances the binding of LPS to macrophages thus increasing cytokine production. This suggests that hemoglobin contributes to enhancing the levels of pro-inflammatory mediators in periodontal sites by acting in synergy with LPS from periodontopathogens, a phenomenon that may contribute significantly to the progression and severity of periodontitis.

5.7. Acknowledgements

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5.8. References

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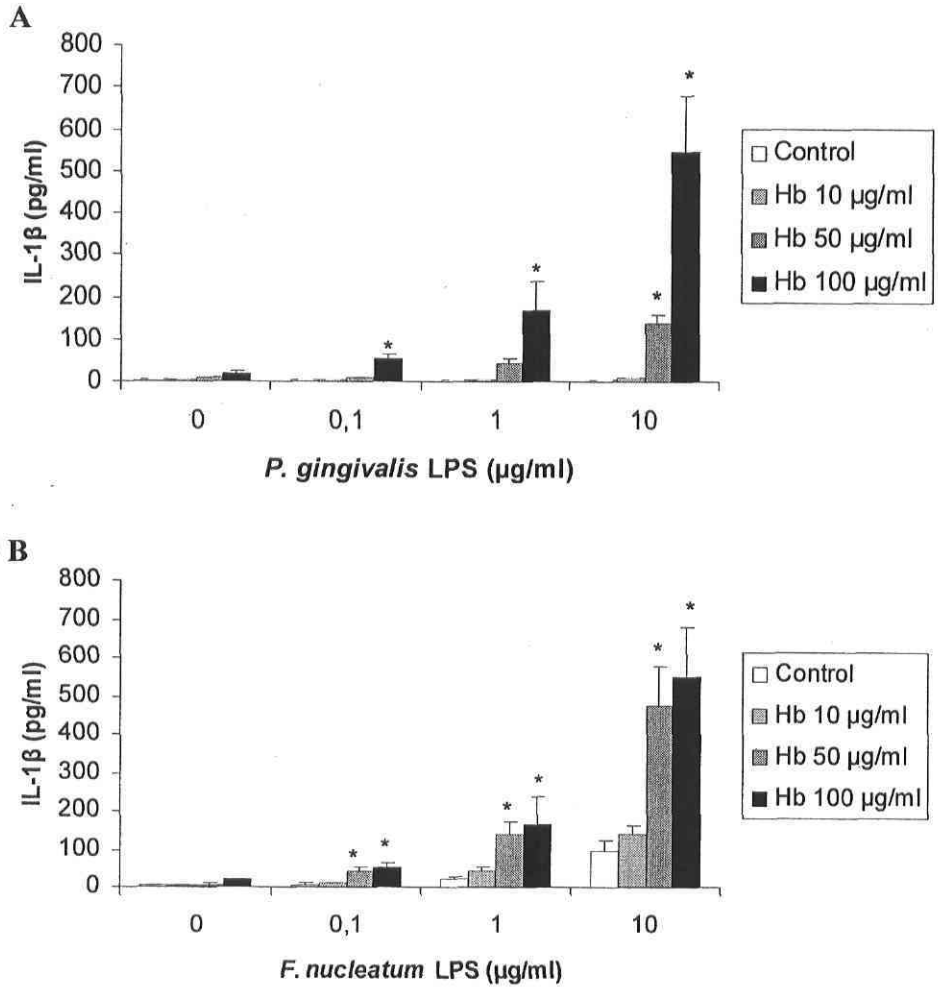

Figure 5.1. Secretion of IL-1β by macrophages stimulated with LPS from *P. gingivalis* (A) or *F. nucleatum* (B) in presence or absence of hemoglobin (10, 50, 100 µg/ml) for 24 h. Cytokine secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays. * $p<0.05$.

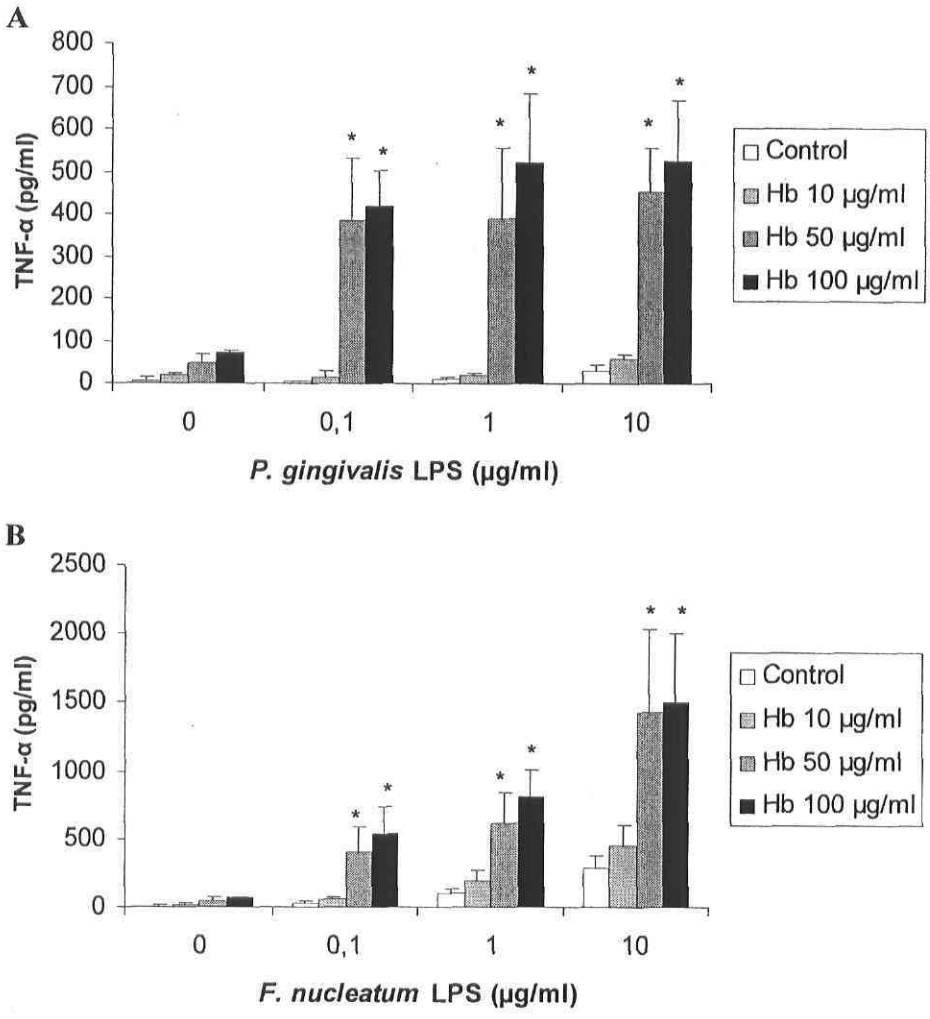

Figure 5.2. Secretion of TNF-α by macrophages stimulated with LPS from *P. gingivalis* (A) or *F. nucleatum* (B) in presence or absence of hemoglobin (10, 50, 100 µg/ml) for 24 h. Cytokine secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays. * $p<0.05$.

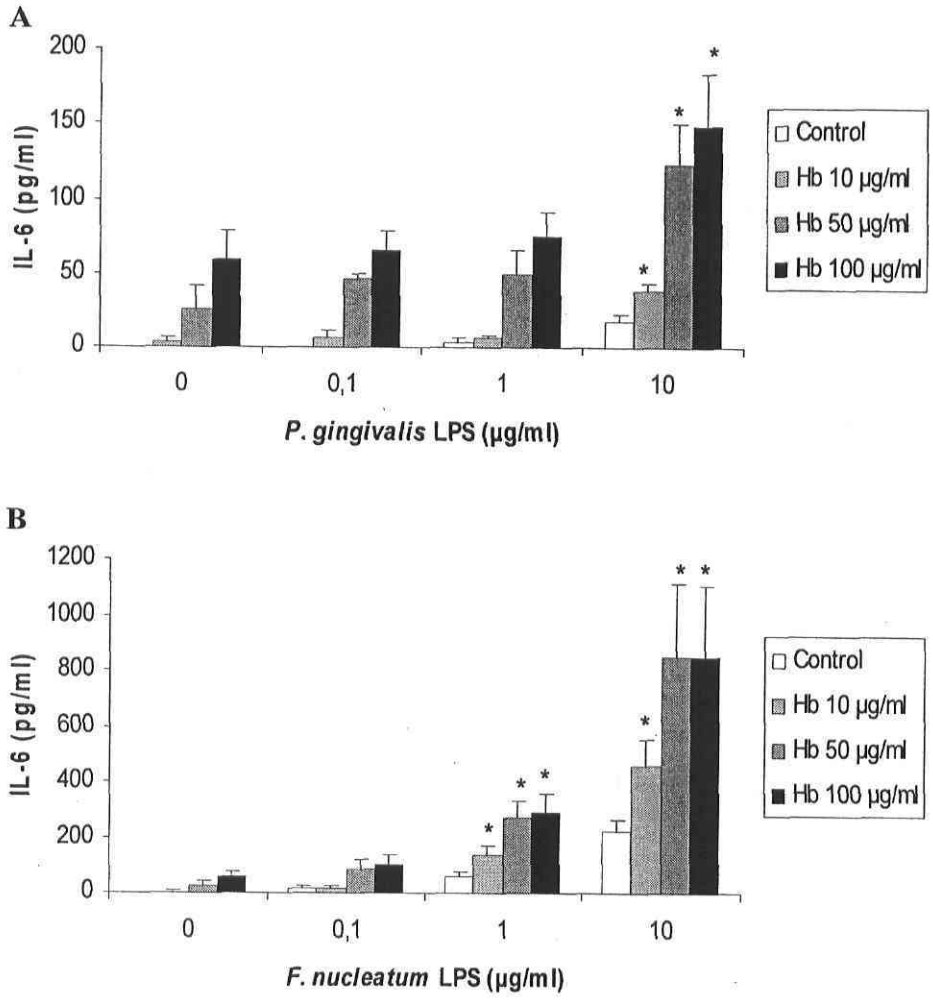

Figure 5.3. Secretion of IL-6 by macrophages stimulated with LPS from *P. gingivalis* (A) or *F. nucleatum* (B) in presence or absence of hemoglobin (10, 50, 100 µg/ml) for 24 h. Cytokine secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays. * $p<0.05$.

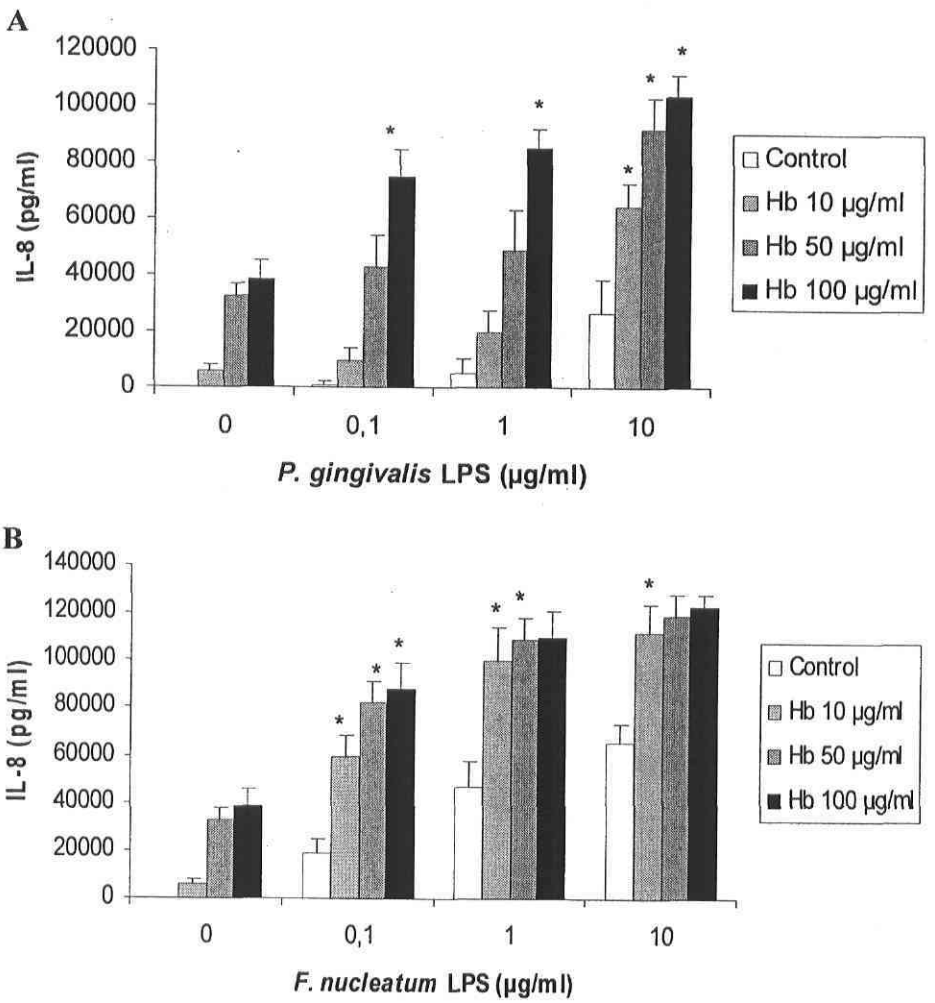

Figure 5.4. Secretion of IL-8 by macrophages stimulated with LPS from *P. gingivalis* (A) or *F. nucleatum* (B) in presence or absence of hemoglobin (10, 50, 100 µg/ml) for 24 h. Cytokine secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays. * $p<0.05$.

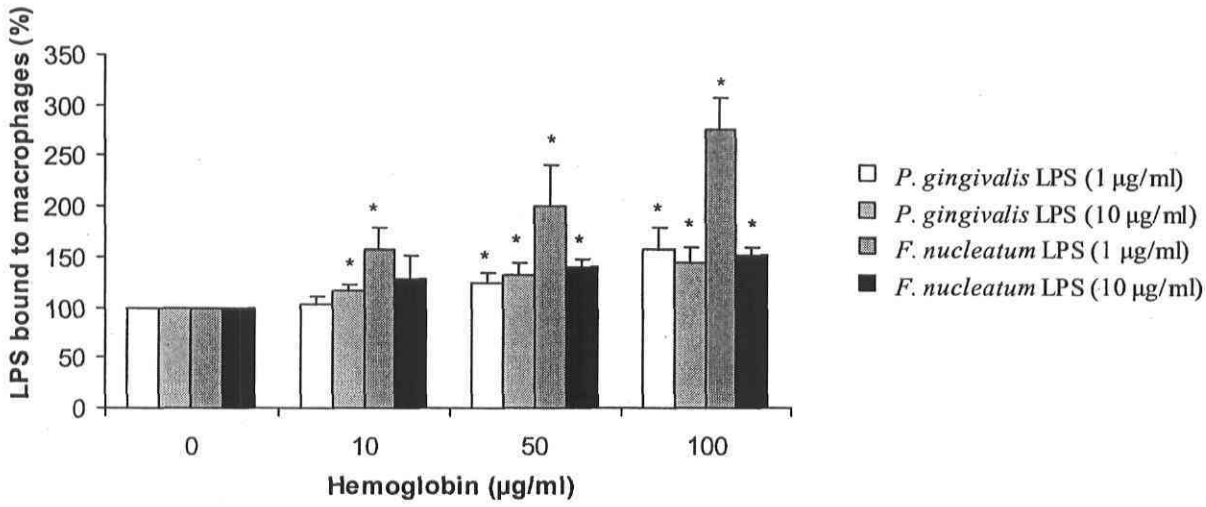

Figure 5.5. Effect of hemoglobin on LPS-binding to macrophages. ^{3}H-LPS from *P. gingivalis* and *F. nucleatum* were added to macrophages at a final concentration of 1 or 10 µg/ml in presence or absence of hemoglobin (10, 50, 100 µg/ml). After 24 h, the quantity of ^{3}H-LPS bound to macrophages was determined using a multi-purpose scintillation counter. A value of 100% was assigned to the amount of LPS bound in the absence of hemoglobin. *$p<0.05$.

CHAPITRE 6

Anti-inflammatory activity of a high-molecular-weight cranberry fraction on macrophages stimulated by lipopolysaccharides from periodontopathogens

6.1. Résumé

Les parodontites sont des maladies inflammatoires chroniques affectant les tissus parodontaux. La forte production continue de cytokines par les cellules de l'hôte stimulées par les bactéries parodontopathogènes est connue pour être responsable de la destruction des tissus de soutien de la dent. Les macrophages jouent un rôle critique dans la réponse inflammatoire de l'hôte aux parodontopathogènes. Le but de cette étude est de caractériser l'effet d'une fraction non dialysable préparée à partir du jus de canneberge concentré sur la production de cytokines pro-inflammatoires induite par les LPS d'*Actinobacillus actinomycetemcomitans*, de *Fusobacterium nucleatum*, de *Porphyromonas gingivalis*, de *Treponema denticola*, de *Tannerella forsythia* et d'*Escherichia coli*. La production d'IL-1β, d'IL-6, d'IL-8, de TNF-α et de RANTES par les macrophages traités par la fraction de canneberge avant d'être stimulés par les LPS a été évaluée par ELISA. Les résultats indiquent clairement que la fraction de canneberge est un puissant inhibiteur de la production de cytokines pro-inflammatoires et de chimiokines induite par les LPS. Cela suggère que les constituants de canneberge pourraient offrir des perspectives pour le développement d'une nouvelle approche thérapeutique pour la prévention et le traitement des parodontites.

6.2. Abstract

Periodontitis is a chronic inflammatory disease affecting oral tissues. The continuous, high production of cytokines by host cells triggered by periodontopathogens is thought to be responsible for the destruction of tooth-supporting tissues. Macrophages play a critical role in this host inflammatory response to periodontopathogens. The aim of this study was to investigate the effect of non-dialysable material prepared from cranberry juice concentrate on the pro-inflammatory cytokine response of macrophages induced by lipopolysaccharides (LPS) from *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum* subsp. *nucleatum*, *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, and *Escherichia coli*. Interleukin-1 beta (IL-1β), IL-6, IL-8, tumor necrosis factor alpha (TNF-α), and Regulated on Activation Normal T cell Expressed and Secreted (RANTES) production by macrophages treated with the cranberry fraction prior to LPS stimulation was evaluated by ELISA. Our results clearly indicate that the cranberry fraction was a potent inhibitor of the pro-inflammatory cytokine and chemokine responses induced by LPS. This suggests that cranberry constituents may offer perspectives for the development of a new therapeutic approach for the prevention and treatment of periodontitis.

6.3. Introduction

Periodontal diseases are a group of inflammatory disorders that lead to the destruction of tooth-supporting tissues and are caused by a specific group of Gram-negative anaerobic bacteria, including *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, (Haffajee and Socransky, 1994). The host response to these bacteria and their products is a critical determinant in the initiation and progression of periodontitis. More specifically, lipopolysaccharides (LPS) of Gram negative bacteria are potent inducer of pro-inflammatory mediators and can initiate a number of host-mediated destructive processes (Henderson *et al.*, 1996). Monocytes and macrophages, which are found in higher

numbers in active periodontal lesions than in inactive sites (Zappa *et al.*, 1991), play an important role in the host inflammatory response to periodontopathogens (Kornman *et al.*, 1997). The continuous, high secretion of various cytokines, including interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) by host cells following stimulation by periodontopathogens, modulates periodontal tissue destruction (Okada and Murakami, 1998). Active compounds endowed with a capacity to modulate the host inflammatory response are now receiving considerable attention as they may be potential new therapeutic agents for the treatment of periodontal diseases (Paquette and Williams, 2000).

The cranberry is a native North American fruit with various beneficial properties for human health such as the inhibition of human cancer cell line proliferation (Ferguson *et al.*, 2004; Seeram *et al.*, 2004) and the prevention of adherence of urinary tract infectious agents (Raz *et al.*, 2004). In the area of dental research, it has been reported that a high molecular weight fraction prepared from cranberry juice inhibits the coaggregation of many oral bacteria (Weiss *et al.*, 1998) and affects dental biofilm formation (Steinberg *et al.*, 2004; Yamanaka *et al.*, 2004). In addition, this cranberry fraction reduces mutans streptococci levels in saliva, inhibits *in vitro* adhesion of *Streptococcus sobrinus* to hydroxyapatite (Weiss *et al.*, 2004), and promotes *S. sobrinus* desorption from artificial biofilms (Steinberg *et al.*, 2005).

In this study, we hypothesized that cranberry may have a beneficial effect for periodontitis by exerting an anti-inflammatory effect. Therefore, we investigated the effect of a high-molecular-weight cranberry fraction prepared from juice concentrate on the production by macrophages of pro-inflammatory cytokines and chemokines associated with periodontitis. More specifically, the cytokine and chemokine responses of macrophages were induced by LPS prepared from *Escherichia coli* and from the major periodontopathogens: *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum* subsp. *nucleatum*, *P. gingivalis*, *T. denticola*, and *T. forsythia*.

6.4. Material & Methods

6.4.1. Cranberry fraction

Concentrated juice from the American cranberry *Vaccinium macrocarpon* was kindly provided by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA, U.S.A.). The juice was exhaustively dialyzed (5 days) in 14,000 MW cut-off dialysis bags at 4°C against distilled water and then lyophilized. The non-dialysable material was considered as fraction 1. Undialyzed concentrated juice was also lyophilized and represented fraction 2. The cranberry powders were dissolved in distilled water prior to use. Chemical analyses of fraction 1 was realized by Robin Roderick (Ocean Spray Cranberries, Inc.) and revealed that this fraction is devoided of sugars and acids and contains 0.35 % of anthocyanins (0.055 % of cyanidin-3-galactoside, 0.003 % of cyanidin-3-glucoside, 0.069 % of cyanidin-3-arabinoside, 0.116 % of peonidin-3-galactoside, 0.016 % of peonidin-3-glucoside and 0.086 % of peonidin-3-arabinoside) and 65.1% of proanthocyanidins. In addition, commercial epigallocatechin gallate (EGCG; Sigma Chemical Co., St. Louis, MO, U.S.A.), a polyphenol isolated from green tea, was used as a positive control (Yang *et al.*, 1998).

6.4.2. LPS preparation

A. actinomycetemcomitans ATCC 29522, *F. nucleatum* subsp. *nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037 were grown in their appropriate culture media (Grenier, 1996). LPS were isolated from these bacterial strains as previously reported (Darveau and Hancock, 1983). This method is based on protein digestion of a whole cell extract by proteinase K and successive solubilization and precipitation steps. The LPS preparations were freeze dried and kept at –20°C. The amount of contaminating protein was evaluated using a protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as a control and was less than 0.001% in all LPS preparations. *T. denticola* possesses a lipooligosaccharide

(although it will be called LPS in the present paper) which have rather distinct properties from the classical LPS of *Bacteroides* group (Schultz *et al.*, 1998). A standard LPS preparation from *E. coli* O55:B5 (Sigma Chemical Co.) was also used.

6.4.3. Monocyte and macrophage cultures

U937 cells (ATCC CRL-1593.2), a monoblastic leukemia cell line, were cultivated at 37°C in a 5% CO_2 atmosphere in RPMI-1640 medium (HyClone Laboratories, Logan, UT, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (RPMI-FBS) and 100 µg/mL of penicillin-streptomycin. Monocytes (2 x 10^5 cells/ml) were incubated in RPMI-FBS containing 10 ng/ml of phorbol myristic acid (PMA; Sigma) for 48 h to induce differentiation into adherent macrophage-like cells as previously reported (Rovera *et al.*, 1979). Following the PMA treatment, the medium was replaced with fresh medium and the differentiated cells were incubated for an additional 24 h prior to use. Adherent macrophages were suspended in RPMI-FBS and centrifuged at 200 x *g* for 8 min. They were washed and suspended in RPMI with 1% heat-inactivated FBS at a density of 1 x 10^6 cells/ml and seeded in a 6 well-plate (2 x 10^6 cells/well in 2 ml) at 37°C in a 5% CO_2 atmosphere.

6.4.4. Treatment of macrophages

The macrophages were treated with increasing concentrations of fraction 1, fraction 2, and EGCG, ranging from 10 to 50 µg/ml and incubated at 37°C in 5% CO_2 for 2 h before stimulation with LPS at a final concentration of 1 µg/ml. After a 24 h incubation (37°C in 5% CO_2), the culture medium supernatants were collected and stored at –20°C until used. Cells incubated in culture medium with or without cranberry fraction or EGCG, but not stimulated with LPS, were used as controls.

6.4.5. Cell viability

Macrophage viability was evaluated by 0.2 % Trypan Blue staining. Cell viability of macrophages was also evaluated by a MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

6.4.6. Determination of cytokine production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, U.S.A.) were used to quantify IL-1β, IL-6, IL-8, TNF-α, and RANTES concentrations in the cell-free culture supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read in a microplate reader with the wavelength correction set at 550 nm.

6.4.7. Statistical analyses

Two-way analyses of variance were performed to compare the means of the different conditions. Differences were declared significant at the 0.05 level (*P* value). Protected Fisher least significant difference were used for pair wise comparisons.

6.5. Results

6.5.1. Effect of cranberry fractions on LPS-induced pro-inflammatory cytokine production

To investigate the effect of cranberry fractions on pro-inflammatory cytokine production, macrophages were treated with lyophilized non-dialysable material of cranberry juice concentrate (fraction 1) or lyophilized cranberry juice concentrate (fraction 2) prior to stimulation with the LPS of major periodontopathogens. To exclude the possibility that cell toxicity due to the cranberry fractions might have been responsible for a decrease in cytokine levels, the viability of the macrophages was evaluated by a MTT test and Trypan blue exclusion. No obvious cytotoxic effects following treatments of macrophages with both fractions were detected and cell viability was $\geq$ 94% of the untreated controls in all experiments (data not shown).

For IL-1β, TNF-α and IL-6, the interaction between the two factors LPS and cranberry was significant ($P < 0.05$) and the results of pair wise comparisons were presented. The TNF-α and IL-6 responses of the macrophages stimulated by LPS from *A. actinomycetemcomitans* were significantly reduced by the treatments with fraction 1 (25 and 50 μg/ml) and EGCG (10 μg/ml) (Figs. 6.1A and 6.1B). This effect was not observed when the macrophages were treated with fraction 2. Among the others LPS tested, the LPS from *F. nucleatum* subsp. *nucleatum* and *E. coli* induced TNF-α response whereas an IL-6 response was induced only by the LPS of *F. nucleatum* subsp. *nucleatum* (Table 6.1). Fraction 1 at a final concentration of 50 μg/ml inhibited the TNF-α and IL-6 responses of macrophages induced by the LPS of *F. nucleatum* subsp. *nucleatum* as well as the TNF-α response induced by the LPS of *E. coli*.

Antagonist effects of fraction 1 on *A. actinomycetemcomitans* LPS-induced IL-1β release were observed (Fig. 6.1C). At low concentrations (10 and 25 μg/ml), fraction 1 and

the LPS of *A. actinomycetemcomitans* showed a synergistic effect on IL-1β production. However, at a concentration of 50 μg/ml, fraction 1 caused a significant reduction of LPS-induced IL-1β secretion by macrophages that was comparable to that obtained with EGCG. This concentration-dependent effect on IL-1β production was also observed when macrophages were stimulated with the LPS of *F. nucleatum* subsp. *nucleatum* (Table 6.1). The treatment of macrophages with 10 μg/ml fraction 1 induced an increase of *F. nucleatum* subsp. *nucleatum* LPS-stimulated IL-1β secretion whereas the treatment with a higher concentration (50 μg/ml) significantly reduced the amount of IL-1β secreted. Treatment of the macrophages with fraction 1 (10 and 50 μg/ml) without LPS stimulation had no effect on the basal level of IL-1β (data not shown). Only a weak IL-1β secretion was observed following stimulations of macrophages with the LPS of *P. gingivalis*, *T. denticola*, or *T. forsythia*, and fraction 1 showed no significant effect (Table 6.1).

6.5.2. Effect of cranberry fractions on LPS-induced chemokine production

For IL-8, the interaction between LPS and cranberry was not significant and the effects of cranberry fraction 1 were analyzed without discrimination of LPS source. LPS of *A. actinomycetemcomitans* induced an higher IL-8 response than the other LPS tested ($P < 0.05$). Fraction 1 (50 μg/ml) significantly reduced the IL-8 response of macrophages stimulated with LPS (Fig. 6.2A and Table 6.1). Fraction 2 and EGCG had no effect on IL-8 production by LPS-stimulated macrophages.

For RANTES, the interaction between LPS and cranberry was significant and the results of pair wise comparisons were used. Fraction 1 and EGCG significantly reduced the RANTES response induced by the LPS of *A. actinomycetemcomitans* at all the concentrations tested (Fig. 6.2B). Fraction 1 also inhibited the RANTES response induced by the LPS of *F. nucleatum*, *P. gingivalis*, *T. forsythia*, and *E. coli* at all the concentrations tested (Table 6.1). A concentration of 50 μg/ml of fraction 1 was necessary to cause a significant decrease in the RANTES response induced by the LPS of *T. denticola* (Table 6.1).

6.6. Discussion

The host inflammatory response to periodontopathogens is considered a major factor causing the local tissue destruction observed in periodontitis. Macrophages participate in the host response induced by periodontopathogens and are the principal target for LPS. To determine whether cranberry extracts can interfere with LPS signaling and reduce the production of pro-inflammatory molecules, we stimulated cranberry-fraction-treated macrophages with the LPS from major periodontopathogens and from *E. coli*. The cranberry non-dialysable material significantly reduced LPS-induced pro-inflammatory cytokine and chemokine production.

Cytokines, more particularly IL-1β, are potential markers of the progression and severity of periodontitis as well as indicators of an appropriate response to treatment (Hou *et al.*, 1995). It has been reported that cytokine synthesis inhibitors can reduce bone resorption in experimental periodontitis in rats (Lima *et al.*, 2004). Moreover, local inhibition of both IL-1 and TNF production in periodontal tissues significantly inhibits the inflammatory response and bone loss in ligature-induced periodontitis in monkeys (Assuma *et al.*, 1998). This suggests that local inhibition of cytokines may be a successful approach for inhibiting bone resorption in periodontitis. In this study, we showed that treating macrophages with the non-dialysable material of cranberry juice can inhibit LPS-induced IL-1β, TNF-α, and IL-6 production and may thus contribute to reducing the impact of cytokine-mediated host destructive processes in periodontitis.

IL-8 and RANTES are potent chemokines that direct the migration of neutrophils, eosinophils, monocytes, and T_H1 cells to sites of infection (Luster, 1998). Stimulation of chemokine production by periodontopathogens favors the accumulation of leukocytes during active inflammation, which contributes to periodontal tissue destruction. Interestingly, periodontal therapy reduces cell numbers in the infiltrate and the levels of IL-8 and RANTES, suggesting a relationship between these chemokines and periodontal status (Gamonal *et al.*, 2001). The non-dialysable material of cranberry juice reduced LPS-

induced IL-8 and RANTES production by macrophages. In the context of the development of novel therapeutic strategies targeting the control of periodontal inflammatory reactions, these results suggest that this cranberry fraction may help reduce the influx of inflammatory cells at disease sites.

The lyophilized cranberry juice did not show any capacity to inhibit LPS-induced cytokine production by macrophages. This is likely related to the fact that active compounds were concentrated in the non-dialysable material of cranberry juice. One such group of compounds that showed a 125-fold enrichment was the proanthocyanidins (data not shown). EGCG, the green tea polyphenol used as positive control, also showed an anti-inflammatory activity. This is in agreement with the previously reported capacity of EGCG to inhibit LPS-induced TNF-α production by mouse macrophages (Yang *et al.*, 1998). Previous studies have revealed that some plant flavonoids may inhibit the expression of inflammation-related proteins/enzymes by suppressing activation of transcription factors such as nuclear transcription factor-κB and activator protein-1 (Kim *et al.*, 2004). Future studies will investigate the cellular mechanisms by which cranberry constituents modulate cytokine expression.

Therapeutic agents that modulate host inflammatory mediators have shown promise for managing adult periodontitis and may be highly useful for individuals with a substantially increased risk for periodontitis (Kornman, 1999). In addition to the previously recognized inhibitory effect of the cranberry non-dialysable material fraction on the aggregation of oral bacteria and dental biofilm formation (Steinberg *et al.*, 2004; Weiss *et al.*, 1998), we showed that this fraction was a potent inhibitor of the pro-inflammatory cytokine and chemokine responses induced by periodontopathogens and *E. coli*. This provides promising perspectives for the development of novel host-modulating therapies for adjunctive treatments of periodontitis or others inflammatory diseases using the high molecular weight constituents from cranberries.

6.7. Acknowledgments

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6.8. References

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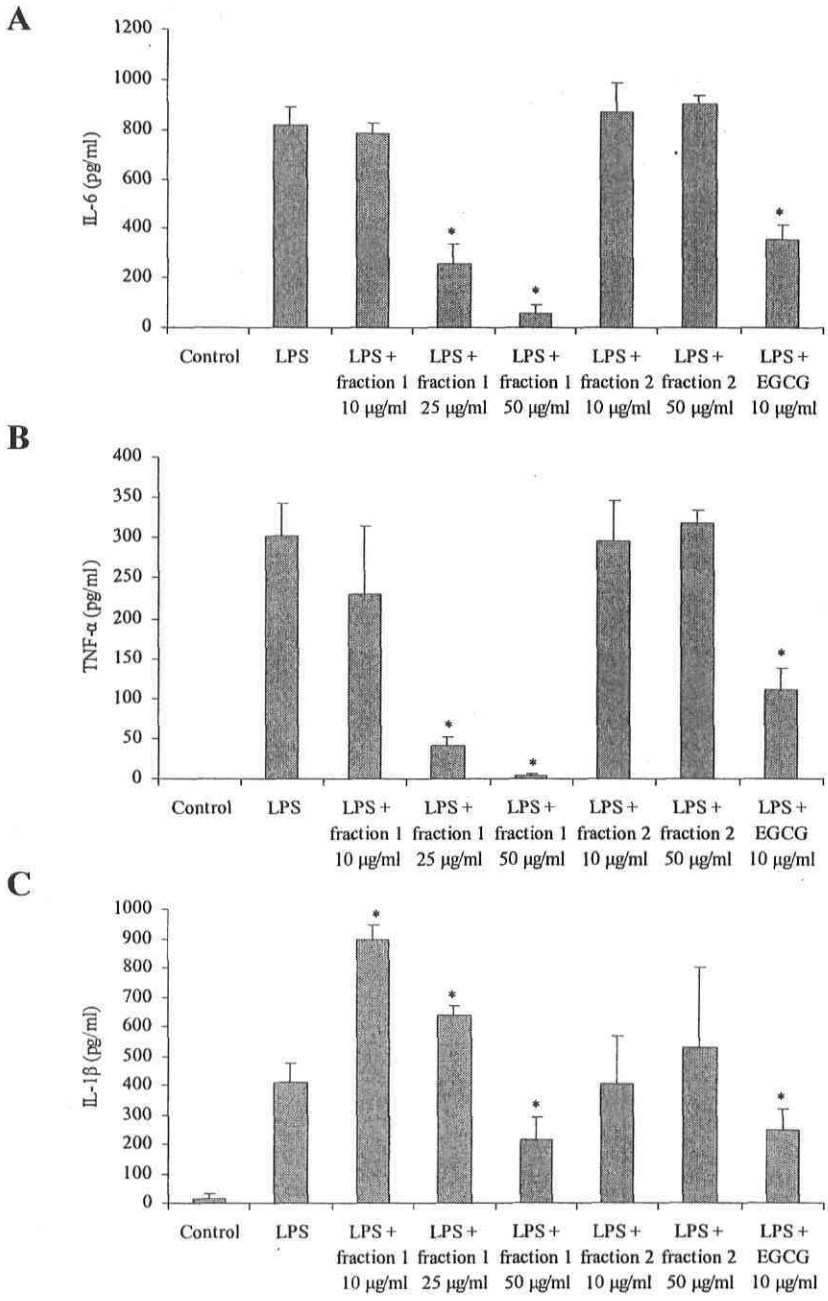

Figure 6.1. Effect of treating macrophages with fraction 1 (non-dialysable material of cranberry juice), fraction 2 (cranberry juice), and EGCG on the secretion of IL-6 (A), TNF-α (B) and IL-1β (C) induced by LPS (1 μg/ml) of *A. actinomycetemcomitans* ATCC 29522 for 24 h. Macrophages were treated with cranberry fractions, or EGCG for 2 h prior to LPS stimulation. Cytokine secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three independent experiments. *, *P* value of < 0.05 compared to untreated control.

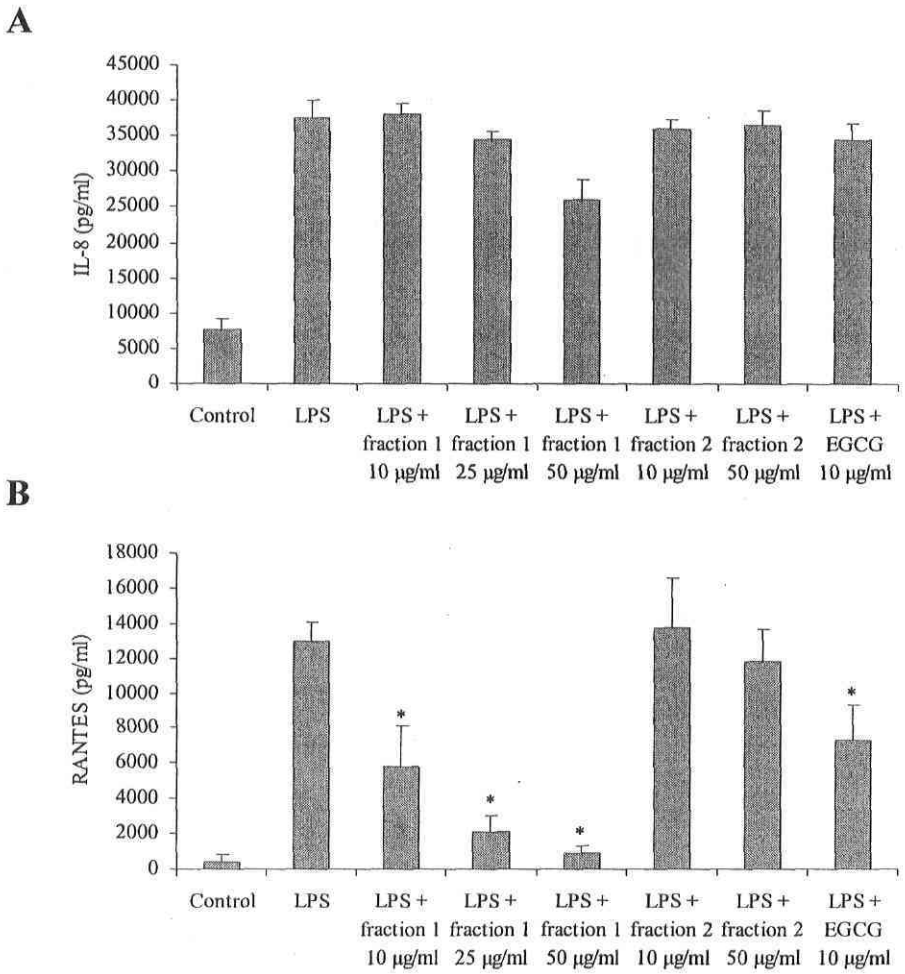

Figure 6.2. Effect of treating macrophages with fraction 1 (non-dialysable material of cranberry juice), fraction 2 (cranberry juice), and EGCG on the secretion of IL-8 (A) and RANTES (B) induced by LPS (1 µg/ml) of *A. actinomycetemcomitans* ATCC 29522 for 24 h. Macrophages were treated with cranberry fractions, or EGCG for 2 h before LPS stimulation. Cytokine secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three independent experiments. *, P value of < 0.05 compared to untreated control.

Table 6.1. Effect of the cranberry non-dialysable material (fraction 1) on the secretion by macrophages of IL-1β, TNF-α, IL-6, IL-8, and RANTES induced by LPS (1 μg/ml) of *F. nucleatum* subsp. *nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, *T. forsythia* ATCC 43037 and *E. coli* O55:B5 for 24 h. Macrophages were treated with fraction 1 (10 and 50 μg/ml) for 2 h prior to LPS stimulation. Cytokine secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three independent experiments. *, *P* value of < 0.05 compared to untreated control. ND: not detected.

LPS (1 μg/ml)	Fraction 1 Treatment	IL-1β (pg/ml)	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-8 (pg/ml)	RANTES (pg/ml)
F. nucleatum subsp.	None	322 ± 5	120 ± 7	403 ± 17	30,763 ± 3,275	12,909 ± 619
nucleatum	10 μg/ml	563 ± 17*	79 ± 30	336 ± 61	28,714 ± 3,230	4,927 ± 695*
ATCC 25586	50 μg/ml	186 ± 43*	7 ± 5*	5 ± 6*	12,858 ± 4,214*	359 ± 147*
P. gingivalis	None	39 ± 13	ND	ND	11,241 ± 3,095	5,185 ± 1,875
ATCC 33277	10 μg/ml	28 ± 14	ND	ND	12,296 ± 3,950	1,893 ± 827*
	50 μg/ml	48 ± 29	ND	ND	4,514 ± 2,203*	728 ± 205*
T. denticola	None	25 ± 12	ND	ND	10,423 ± 3,519	3,055 ± 1,627
ATCC 35405	10 μg/ml	34 ± 25	ND	ND	12,183 ± 5,342	1,492 ± 331*
	50 μg/ml	38 ± 15	ND	ND	8,215 ± 4,721*	359 ± 147*
T. forsythia	None	37 ± 7	ND	ND	12,326 ± 5,323	5,714 ± 1,169
ATCC 43037	10 μg/ml	45 ± 14	ND	ND	13,237 ± 5,446	1,719 ± 719*
	50 μg/ml	47 ± 18	ND	ND	4,641 ± 2,257*	716 ± 200*
E. coli	None	ND	32 ± 7	ND	10,351 ± 922	7,358 ± 770
O55:B5	10 μg/ml	ND	14 ± 9	ND	10,156 ± 1,599	3,886 ± 870*
	50 μg/ml	ND	4 ± 3*	ND	3,912 ± 237*	655 ± 43*

CHAPITRE 7

Cranberry components inhibit IL-6, IL-8 and PGE_2 production by lipopolysaccharide-activated gingival fibroblasts

7.1. Résumé

Les parodontites sont des maladies inflammatoires chroniques affectant les tissus de soutien de la dent. Les fibroblastes gingivaux sont les cellules les plus abondantes dans les tissus parodontaux et ils participent activement à la réponse inflammatoire de l'hôte aux bactéries parodontopathogènes, qui est impliquée dans la destruction des tissus au cours des parodontites. Le but de cette étude est d'investiguer l'effet d'une fraction de canneberge enrichie en proanthocyanidines, obtenue à partir du jus de canneberge concentré, sur la réponse inflammatoire induite par le LPS d'*Actinobacillus actinomycetemcomitans* chez les fibroblastes gingivaux. La production d'IL-1β, d'IL-6 et de PGE_2 par les fibroblastes traités par la fraction de canneberge avant d'être stimulés par le LPS a été évaluée par ELISA. Les modifications de l'expression et de la phosphorylation de protéines de signalisation intracellulaires induites par le LPS d'*A. actinomycetemcomitans* et la fraction de canneberge chez les fibroblastes gingivaux ont été caractérisées par une technique de « microarray ». La production d'IL-1β, d'IL-6 et de PGE_2 induite par le LPS chez les fibroblastes a été inhibée par la fraction de canneberge. Cette fraction a inhibé plusieurs protéines de signalisations intracellulaires chez les fibroblastes gingivaux, un phénomène qui pourrait conduire à une inhibition de l'activité du facteur transcriptionnel AP-1. Les constituants de la canneberge ont réduit aussi l'expression de la cyclo-oxygénase 2. Cette

étude suggère que le jus de canneberge contient des molécules intéressantes pour le développement d'une nouvelle stratégie thérapeutique basée sur la modulation de la réponse de l'hôte pour le traitement complémentaire des parodontites.

7.2. Abstract

Periodontitis is a chronic inflammatory disease affecting the tooth supporting tissues. Gingival fibroblasts are the most abundant cells in periodontal tissues and participate actively in the host inflammatory response to periodontopathogens, which is known to mediate local tissue destruction in periodontitis. The aim of this study was to investigate the effect of a proanthocyanidin-enriched cranberry fraction prepared from cranberry juice concentrate on inflammatory mediator production by gingival fibroblasts stimulated by the lipopolysaccharide (LPS) of *Actinobacillus actinomycetemcomitans*. Interleukin (IL)-6, IL-8, and prostaglandin E_2 (PGE_2) production by fibroblasts treated with the cranberry fraction and stimulated by *A. actinomycetemcomitans* LPS was evaluated by enzyme-linked immunosorbent assay. Changes induced by *A. actinomycetemcomitans* LPS and the cranberry fraction in expression and phosphorylation state of fibroblast intracellular signaling proteins were characterized by antibody microarrays. The LPS-induced IL-6, IL-8 and PGE_2 responses of gingival fibroblasts were inhibited by treatments with the cranberry fraction. This fraction was found to inhibit fibroblast intracellular signaling proteins, a phenomenon that may lead to a downregulation of activating protein-1 activity. Cranberry components reduced also cyclo-oxygenase 2 expression. This study suggests that cranberry juice contains molecules with interesting properties for the development of new host-modulating therapeutic strategies for adjunctive treatment of periodontitis.

7.3. Introduction

Periodontitis is an inflammatory disease of the tooth supporting tissues. The disease is initiated by an overgrowth of specific Gram negative anaerobic bacteria that leads to gingival connective tissue destruction and irreversible alveolar bone resorption. The continuous high secretion of various cytokines including interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) as well as of prostaglandin E_2 (PGE_2) by host cells following stimulation with periodontopathogens and their products, is a critical determinant of periodontal tissue destruction (1, 2). Lipopolysaccharides (LPS) of Gram negative bacteria are potent inducers of pro-inflammatory mediators and can initiate a number of host-mediated destructive processes (3). In particular, the LPS of *Actinobacillus actinomycetemcomitans*, a major etiological agent in localized aggressive periodontitis (4, 5), is thought to be involved in the processes of alveolar bone loss and connective tissue degradation (6, 7). One target of LPS is the gingival fibroblasts, which play an important role in the remodeling of periodontal soft tissues. Gingival fibroblasts are a major constituent of gingival connective tissue and actively participate in inflammatory events in periodontal disease (8). Regulation of fibroblast inflammatory reactions has been suggested to be one of the ways to prevent/control periodontitis progression (9). Recently, active compounds endowed with a capacity to modulate the host inflammatory response have received considerable attention as they may represent potential new therapeutic agents for treatment of periodontal diseases (10).

Cranberries (*Vaccinium macrocarpon*), a native North American polyphenolic rich fruit, exhibit various beneficial properties for human health (11-13). Previously, we reported that a high molecular weight fraction prepared from cranberry juice concentrate inhibits LPS-induced pro-inflammatory cytokine and chemokine production by human macrophages (14). The aim of this study was to investigate the effect of the high-molecular-weight cranberry fraction on production of inflammatory mediators (IL-6, IL-8 and PGE_2) by human gingival fibroblasts stimulated with *A. actinomycetemcomitans* LPS.

7.4. Materials and methods

7.4.1. Preparation of cranberry high-molecular-weight fraction

Concentrated juice (Ocean Spray Cranberries, Lakeville-Middleboro, MA, USA) from the American cranberry *Vaccinium macrocarpon* was exhaustively dialysed in 14 000 MW cut-off dialysis bags at 4°C against distilled water and lyophilized. This non-dialysable material named NDM was dissolved in distilled water prior to use. Chemical analyses of NDM was realized by Robin Roderick (Ocean Spray Cranberries) and revealed that this fraction is devoided of sugars and acids and contains 0.35% of anthocyanins (0.055% of cyanidin-3-galactoside, 0.003% of cyanidin-3-glucoside, 0.069% of cyanidin-3-arabinoside, 0.116% of peonidin-3-galactoside, 0.016% of peonidin-3-glucoside and 0.086% of peonidin-3-arabinoside) and 65.1% of proanthocyanidins. The proanthocyanidins showed a 125-fold enrichment in the NDM fraction as compared to undialized lyophilized concentrated cranberry juice.

7.4.2. Preparation of LPS

A. actinomycetemcomitans ATCC 29522 was grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 1% yeast extract. The bacterial cultures were incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 2 days. LPS were isolated as previously reported (15). This method is based on protein digestion of a whole cell extract by proteinase K and successive solubilization and precipitation steps. The LPS preparation was freeze dried and kept at –20°C. The amount of contaminating protein in the LPS preparation was evaluated using a protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as a control and was less than 0.001%.

7.4.3. Fibroblast culture conditions

Human gingival fibroblasts HGF-1 (ATCC CRL-2014) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated FBS (DMEM-FBS) and 100 µg/mL penicillin-streptomycin. Fibroblasts were seeded at a concentration of 25 x 10^3 cells/cm^2 in 24 well-plate and cultured in DMEM-FBS during 24 h at 37°C in a 5% CO_2 atmosphere to allow cells adhesion prior experiments. The medium was replaced by DMEM with 1% heat-inactivated FBS prior fibroblast treatments.

7.4.4. Treatment of fibroblasts

Fibroblasts were treated with increasing concentrations of NDM (0, 10, 25 or 50 µg/ml) and incubated at 37°C in 5% CO_2 for 2 h prior to stimulation with LPS at a final concentration of 1 µg/ml. After 24 h of incubation (37°C in 5% CO_2), culture medium supernatants were removed and stored at -20°C until use. Cells incubated in culture medium with or without NDM but not stimulated with LPS were used as controls.

7.4.5. Cell viability

Fibroblast viability was evaluated by a MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, which measures the mitochondrial reduction of MTT to formazan, according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Firstly, fibroblasts were seeded at a concentration of 25 x 10^3 cells/cm^2 in a 96-well-plate and cultured in DMEM-FBS during 24 h at 37°C in a 5% CO_2 atmosphere prior to treatment with NDM and stimulation with LPS as previously described. In a second experiment, fibroblasts were seeded at a concentration of 15 x 10^3 cells/cm^2 in a 96-well-plate, treated with NDM (0, 10, 25 or 50 µg/ml) and LPS (0, 1 or 5 µg/ml) and cultured in

DMEM-FBS during 5 days at 37°C in a 5% CO_2. After stimulation, cells were incubated with MTT for 4 h and the insoluble formazan dye was solubilized overnight at 37°C before measurement of the absorbance at 550 nm with wavelength correction set at 650 nm using a microplate reader.

7.4.6. Determination of cytokine production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify IL-1β, TNF-α, IL-6, IL-8, and RANTES concentrations in the cell-free culture supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read in a microplate reader with the wavelength correction set at 550 nm. The sensitivities of the commercial ELISA kits were 31.2 pg/ml for IL-8, 15.6 pg/ml for RANTES and TNF-α, 9.3 pg/ml for IL-6 and 3.9 pg/ml for IL-1β. Cytokine concentrations were determined in triplicate.

7.4.7. Determination of PGE_2 production

A competitive enzyme immunoassay was performed on the above supernatant fluids according the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). The absorbance at 415 nm was read using a microplate reader, and PGE_2 concentrations were determined in triplicate. The sensitivities of the assay was 15 pg/ml.

7.4.8. Antibody microarray analyses

Changes induced by the *A. actinomycetemcomitans* LPS and the NDM cranberry fraction in the expression and phosphorylation state of fibroblast signaling proteins were characterized by the Kinex™ Antibody Microarray service (Kinexus, Vancouver, BC, Canada). This antibody microarray tracks over 600 different cell signaling proteins in duplicate for more than 250 different phospho-sites, 240 protein kinases and 110 other cell

signaling proteins in two samples. In the initial screen, fibroblasts were treated with 1 μg/ml of *A. actinomycetemcomitans* LPS during 3 h and compared with unstimulated cells as control. In the second screen, fibroblasts were treated with 50 μg/ml of the cranberry fraction NDM and incubated at 37°C in 5% CO_2 for 2 h before stimulation with LPS at a final concentration of 1 μg/ml during 3 h. Fibroblasts stimulated with 1 μg/ml of LPS during 3 h were used as a control. After the incubation period, cell lysates were prepared according to the manufacturer's protocol (Kinexus). In brief, cells were washed twice with ice-cold PBS and homogenized at 4°C in a buffer containing 20 mM MOPS (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate (pH 7.2), 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin, 0.5% Triton X-100, and 1 mM DTT. Cells were broken by sonification on ice and cell lysates were centrifuged at 136,000 × *g* for 30 min at 4°C in a LE-80K ultracentrifuge (Beckman, Mississauga, ON, Canada). Protein concentrations of the cell lysate supernatant fractions were estimated by using the Bradford assay (Bio-Rad Laboratories) and adjusted to a concentration of 2 mg/ml. Kinex™ antibody microarray analyses of the supernatants were performed by Kinexus.

7.4.9. Statistical analyses

Analyses of variance were performed to compare the means of the different conditions. Data were transformed by square-root to respect the homogeneity of variance and the normality assumptions. Differences were considered significant at the P value $\leq$ 0.05. Protected Fisher least significant differences were used for pair-wise comparisons.

7.5. Results

To investigate the effect of cranberry components on production of pro-inflammatory mediators by gingival fibroblasts, cells were treated with the NDM fraction prepared from cranberry juice concentrate prior to stimulation by the LPS of *A.*

actinomycetemcomitans. No obvious cytotoxic effects following a 24 h treatment of fibroblasts with cranberry NDM were detected by a MTT test thus indicating that a decrease in inflammatory mediator production may not be related to cell toxicity (data not shown). In addition, the presence of NDM (50 μg/ml) significantly inhibited the decrease of cell viability induced by a 5-day treatment with *A. actinomycetemcomitans* LPS at final concentration of 1 and 5 μg/ml (Figure 7.1). No difference was observed in the growth of fibroblasts following NDM treatment suggesting that the NDM fraction protects cells from the decrease in MTT reducing activity induced by LPS.

Treatment of the unstimulated fibroblasts with the NDM fraction showed no effect on the basal level of inflammatory mediators secreted by these cells (data not shown). Although supernatants of *A. actinomycetemcomitans* LPS-stimulated fibroblasts were analyzed for IL-6, IL-8, RANTES, and PGE_2 production, only IL-6, IL-8 and PGE_2 responses were observed. The chemokine IL-8 response of fibroblasts stimulated by LPS of *A. actinomycetemcomitans* was significantly reduced by treatment with cranberry NDM at all the concentrations tested (Fig. 7.2). NDM at a final concentration of 50 μg/ml completely inhibited the IL-8 response of fibroblasts induced by LPS whereas a 72% inhibition was noted at a concentration of 10 μg/ml. Cranberry NDM at final concentrations of 25 and 50 μg/ml also significantly inhibited the LPS-induced IL-6 response of fibroblasts (Fig. 7.3). The PGE_2 response of fibroblasts induced by *A. actinomycetemcomitans* LPS was also significantly reduced by cranberry NDM treatments (25 and 50 μg/ml) (Fig. 7.4). At these concentrations, the PGE_2 levels secreted by NDM-treated fibroblasts were similar to the basal PGE_2 level secreted by unstimulated fibroblasts.

Changes induced by the *A. actinomycetemcomitans* LPS and the cranberry NDM fraction in the expression and phosphorylation state of fibroblast signaling proteins were characterized by antibody microarrays. Among the various changes induced by these two compounds, we selected only those for which the expression or phosphorylation state of proteins were enhanced by the *A. actinomycetemcomitans* LPS and decreased by the presence of cranberry NDM fraction during LPS treatment (Table 7.1). On the one hand, the expression of MAP kinase protein-serine kinase (MKK) 6, Fos-c FBJ murine

osteosarcoma oncoprotein-related transcription factor (Fos) and cyclo-oxygenase 2 (COX2) was enhanced by respectively 59%, 48% and 26% following treatment with *A. actinomycetemcomitans* LPS. The LPS treatment enhanced also strongly the phosphorylation state of Jun proto-oncogene-encoded AP1 transcription factor (Jun) (+ 96%), MKK3 (+ 84%) and MAP kinase-interacting protein-serine kinase 1 (Mnk1) (+60%). On the other hand, the cranberry NDM fraction inhibited strongly the phosphorylation of Jun N-terminus protein-serine kinases (JNK), Jun and Ras-related C3 botulinum toxin substrate 1 (Rac1) by respectively 115%, 85% and 54%. NDM reduced also the phosphorylation of Fos, MKK3 and Mnk1 by about 30 %. In addition, the expression of COX2, MKK6 and Fos were inhibited following NDM treatment by respectively 57%, 41% and 23%.

7.6. Discussion

The host inflammatory response to periodontopathogens, which results in a strong release of inflammatory mediators by host cells, is known to mediate local tissue destruction in periodontitis. Indeed, the presence of high levels of inflammatory mediators into periodontal tissues and gingival crevicular fluid of periodontitis patients has been extensively reported and their etiological correlation with periodontitis has been well demonstrated (1, 2). Consequently, it is logical to consider therapeutic approaches that modulate the host response, in addition to antibacterial approaches in the management of chronic periodontitis. Gingival fibroblasts are the most abundant cells in periodontal tissue and respond to bacterial stimuli by releasing prostaglandins, cytokines, and chemokines, which can directly or indirectly contribute to periodontal tissue destruction. Regulation of gingival fibroblast inflammatory reactions may be one of the ways to prevent and control periodontal disease (9). To demonstrate whether cranberry components can reduce the production of pro-inflammatory mediators, fibroblasts were stimulated with LPS of a major periodontopathogen following treatment with the cranberry NDM fraction.

One pathway implicated in periodontal disease pathogenesis involves the synthesis and release of arachidonic acid metabolites within periodontal tissue. PGE_2, an arachidonic acid metabolite with well-known proinflammatory and immunomodulatory effects, is thought to be involved in periodontal tissue destruction, due to its role as a potent stimulator of bone resorption and association with attachment loss (16). PGE_2 levels are elevated in inflamed gingival tissues (17, 18) and are higher in gingival crevicular fluid of periodontitis sites as compared to healthy sites (19). Gingival fibroblasts secrete PGE_2 in response to proinflammatory cytokines (20), periodontopathogens (21) and LPS (22) and therefore contribute to PGE_2-mediated destructive process in periodontal disease. In addition, COX2 expression, the enzyme involved in the PGE_2 production, is significantly upregulated in inflamed periodontal tissue as well as in gingival fibroblasts stimulated with cytokine and periodontopathogens (18). Interestingly, systemic non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit prostaglandin synthesis, have been shown to reduce alveolar bone loss in periodontitis (23). Cranberry NDM treatments of fibroblasts inhibited the PGE_2 response induced by LPS. This is likely related to the inhibition of COX2 expression by the cranberry NDM fraction. These suggest that cranberry NDM may reduce the adverse effects of PGE_2 on bone and connective tissue homeostasis in periodontitis patients.

Another pathway contributing to periodontal disease pathogenesis involves the secretion by host cells of high levels of pro-inflammatory cytokines and chemokines within periodontal tissue. IL-8 is a potent chemokine that directs the migration of polymorphonuclear leukocytes, monocytes and macrophages to the site of infection. Increased levels of IL-8 are found in the gingival crevicular fluid of inflamed periodontal sites compared with healthy sites (24-26). Periodontal therapy reduces immune cell numbers in the infiltrate and the levels of IL-8, suggesting a relationship between this chemokine and periodontal status (27). In addition, human gingival fibroblasts taken from diseased sites of chronic periodontitis patients produce greater amounts of IL-8 in vitro than cells from healthy sites (28). Cranberry NDM fraction exhibited the capacity to inhibit LPS-induced IL-8 production by fibroblasts. This result suggests that cranberry NDM may contribute to reduce the afflux of inflammatory cells at diseased sites. IL-6, a multifunctional cytokine, plays an important role in regulating the immune response during

periodontal disease. IL-6 expression was found to be higher at sites of periodontal inflammation and closely related to clinical severity of periodontitis (29). In addition, its level increase in the diseased gingiva of patients with periodontitis compared to periodontally healthy subjects (30). It has also been reported that gingival fibroblasts isolated from diseased tissue produce a larger amount of IL-6, both constitutively and after induction, than those isolated from healthy tissue (31). IL-6 promotes bone resorption (32) and acts as a potent inducer of osteoclast formation *in vitro* (33) suggesting that IL-6 can contribute to the bone resorption associated with periodontitis. In this study, we showed that cranberry NDM treatment of fibroblasts can reduce LPS-induced IL-6 production. This suggests that cranberry components may contribute to reduce the impact of host destructive processes mediated by IL-6 occurring in periodontitis.

Antibody microarray analyses showed that the cranberry NDM fraction inhibits the phosphorylation state and expression of some gingival fibroblast intracellular signaling proteins induced by the *A. actinomycetemcomitans* LPS. The results suggest that NDM fraction can act by reducing the activator protein-1 (AP-1) activity. Indeed, AP-1 complexes are heterodimers of proteins of two proto-oncogene families (Jun and Fos) which are prominently involved in the transcriptional regulation of many proinflammatory mediators such as IL-6, IL-8 and PGE_2 (34-37). LPS from *Porphyromonas gingivalis*, a major periodontopathogen, induces AP-1 expression in human gingival fibroblasts (38). Our results suggest that *A. actinomycetemcomitans* LPS induces also the AP-1 expression in gingival fibroblasts. Transcriptional activity and protein stability of Jun is increased by phosphorylation of serine 63 by JNK (39). In this study, the phosphorylation of Jun on serine 63 was strongly reduced by NDM, probably a consequence of the decreased level of phosphorylated form of JNK observed following NDM treatment. This may contribute to the inhibition of inflammatory mediators gene transcription. We also observed that treatment of fibroblasts with NDM reduced the expression of Fos induced by *A. actinomycetemcomitans* LPS. Fos expression is important for AP-1 activity, because Jun/Fos heterodimers are more stable than Jun/Jun homodimers (40), thus leading to a more stable AP-1 complex. The reduction of Fos expression induced by NDM decreases the Jun/Fos heterodimers formation and participates probably to reduce the AP-1 activity. It

was reported that AP-1 and nuclear factor-κB cooperatively regulate the synthesis of IL-6, IL-8 and PGE_2 in human gingival fibroblasts (35). All these data support that AP-1 activity inhibition by cranberry NDM could reduce inflammatory mediator production in gingival connective tissue of periodontitis patients. We observed also that MKK3 phosphorylation and MKK6 expression, two dual specificity kinases implicated in the release of IL-6 and IL-8 by fibroblasts (41) were inhibited by the cranberry NDM fraction. In addition, it has been demonstrated that the activation of Rac1 is a major pathway involved in TNF-α secretion following LPS challenge in macrophages and suggested that targeting Rac1 could be a useful therapeutic strategy for attenuating the proinflammatory cytokine response (42). These suggest that inhibition of Rac1 by NDM may be implicated in the reduction of cytokine production. Finally, it was shown that Mnk1 inhibitor blocked soluble IL-1β and IL-8 production (43) and that Mnk1 is implicated in cytokine production representing a potential new target for anti-inflammatory therapy (44). NDM induced a decrease of Mnk1 phosphorylation, a phenomenon that may contribute to the reduction of cytokine production observed following NDM treatment. The NDM cranberry fraction seems to affect the phosphorylation and expression of various intracellular proteins which are implicated in cytokine production. Our results strongly suggest that the NDM cranberry fraction may act notably via a dowregulation of AP-1 activity leading to inhibition of inflammatory mediator production.

Polyphenols have been reported to confer protection against toxicity induced by various compounds (45-47). Our results suggest that NDM can protect gingival fibroblasts from the LPS-induced cytotoxicity. Nevertheless, we do not exclude that the increase in MTT reduction observed here may be related to an increase in mitochondrial respiratory activity as previously reported by Bernhard *et al.*, which showed that polyphenolic compound can increase MTT-reducing activity without an increase in the number of living cells (48).

Modulation of host inflammatory mediators is a promising therapeutic approach for the management of adult periodontitis, particularly for individuals with increased risk for periodontitis (49). In addition to the previously recognized anti-biofilm effects of the

cranberry NDM fraction on oral bacteria (50-53), we have recently reported that this fraction is a potent inhibitor of the pro-inflammatory cytokine and chemokine responses of macrophages induced by LPS of periodontopathogens (14) as well as the matrix metalloproteinase production and activity (54). In this study, we showed that NDM can also inhibit the IL-6, IL-8 and PGE_2 response of gingival fibroblasts, the major cell population in periodontal tissue. This suggests that cranberry NDM by attenuating the inflammatory response of two major cell types may have a beneficial effect on slowing periodontal disease progression in combination with conventional therapy. In this regard, the cranberry NDM fraction offers promising perspectives as a host modulatory agent for the development of novel therapies for adjunctive treatment of periodontitis.

7.7. Acknowledgements

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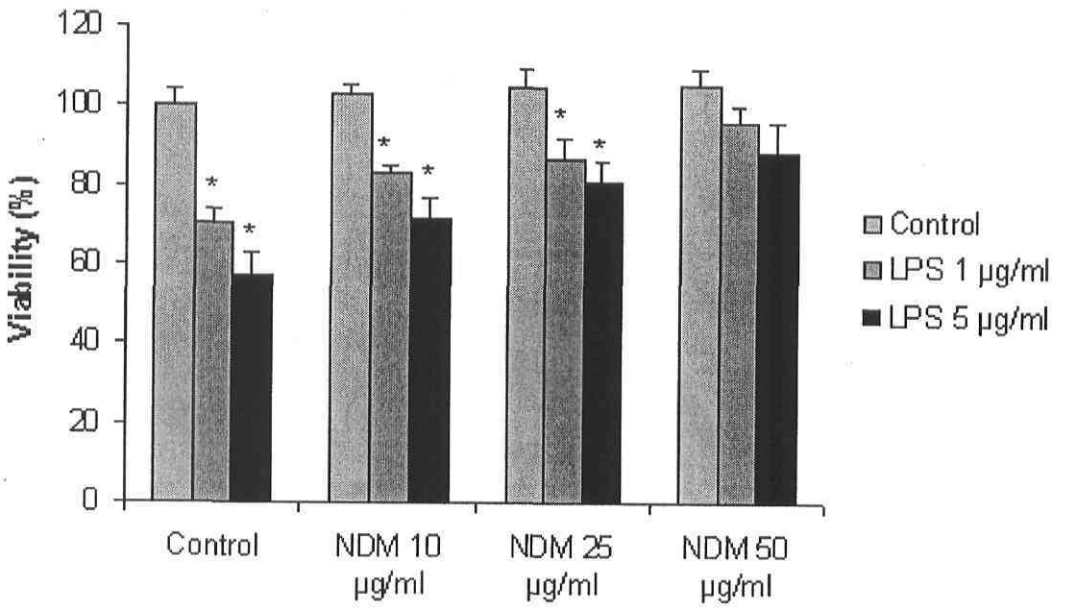

Figure 7.1. Effect of treating gingival fibroblasts with LPS and cranberry NDM on the cell viability. Fibroblasts were treated with NDM (10, 25 or 50 µg/ml) and LPS (1 or 5 µg/ml) of *A. actinomycetemcomitans* ATCC 29522 for 5 days. Cells incubated without NDM but stimulated or not with LPS were used as control. Cell viability was assessed by a MTT test. *, *P* value of < 0.05 compared to untreated control.

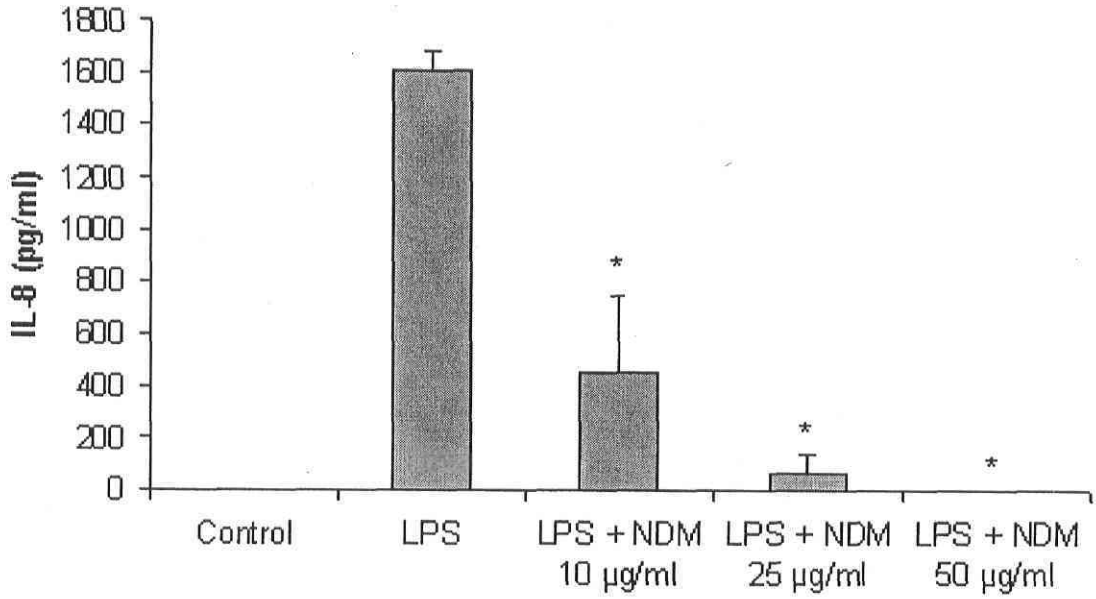

Figure 7.2. Effect of treating gingival fibroblasts with cranberry NDM on the secretion of IL-8 induced by stimulation with LPS (1 µg/ml) of *A. actinomycetemcomitans* ATCC 29522 for 24 h. Fibroblasts were treated with NDM (10, 25 or 50 µg/ml) for 2 h prior to LPS stimulation. Untreated cells were used as control. IL-8 secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three independent experiments. *, *P* value of < 0.05 compared to untreated control.

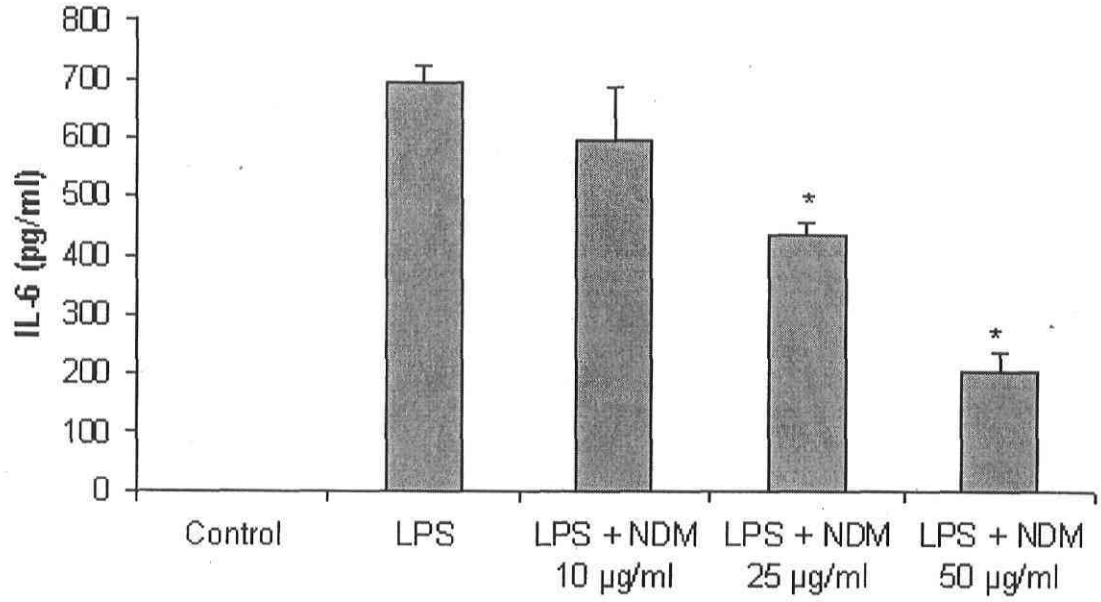

Figure 7.3. Effect of treating gingival fibroblasts with cranberry NDM on the secretion of IL-6 induced by stimulation with LPS (1 µg/ml) of *A. actinomycetemcomitans* ATCC 29522 for 24 h. Fibroblasts were treated with NDM (10, 25 or 50 µg/ml) for 2 h prior to LPS stimulation. Untreated cells were used as control. IL-6 secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three independent experiments. *, P value of < 0.05 compared to untreated control.

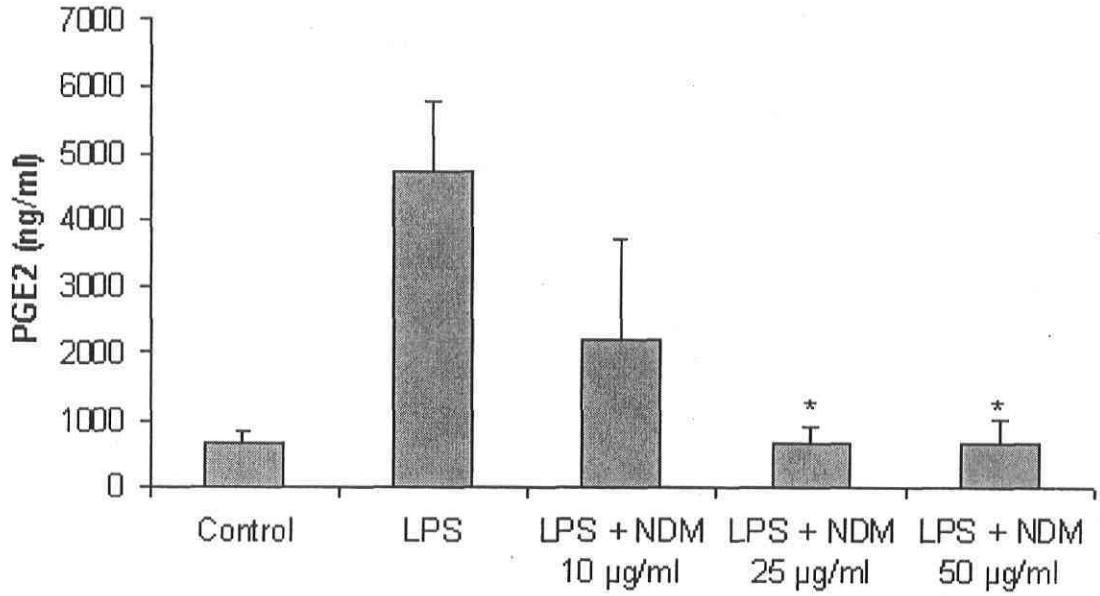

Figure 7.4. Effect of treating gingival fibroblasts with cranberry NDM on the secretion of PGE_2 induced by stimulation with LPS (1 µg/ml) of *A. actinomycetemcomitans* ATCC 29522 for 24 h. Fibroblasts were treated with NDM (10, 25 or 50 µg/ml) for 2 h prior to LPS stimulation. Untreated cells were used as control. PGE_2 secretion was assessed by EIA. The data are the means ± standard deviations of triplicate assays for three independent experiments. *, *P* value of < 0.05 compared to untreated control.

Protein			Signal (% change from control)	
Full name	Abbreviation	Phospho Site(s)	Unstimulated vs LPS	LPS vs LPS + NDM
Cyclo-oxygenase 2 (prostaglandin G/H synthase 2 precursor)	COX2	Pan-specific	26	-57
Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor	Fos	Pan-specific	48	- 23
Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor	Fos	T232	5	- 28
Jun N-terminus protein-serine kinases (stress-activated protein kinase (SAPK)) 1/2/3	JNK	T183+Y185	21	- 115
Jun proto-oncogene-encoded AP1 transcription factor	Jun	S63	96	- 85
MAP kinase protein-serine kinase 3	MKK3	S189/S207	84	- 36
MAP kinase protein-serine kinase 6	MKK6	Pan-specific	59	- 41
MAP kinase-interacting protein-serine kinase 1	Mnk1	T209+T214	60	-33
Ras-related C3 botulinum toxin substrate 1	Rac1	S71	14	-54

Table 7.1. Changes induced by the *A. actinomycetemcomitans* LPS and the cranberry NDM fraction in the expression and phosphorylation state of gingival fibroblasts intracellular signaling proteins. Two antibody microarrays were performed to screen these changes. Firstly, unstimulated fibroblasts were used as control and compared to fibroblasts stimulated with *A. actinomycetemcomitans* LPS (1 μg/ml) during 3 h. Secondly, fibroblasts stimulated with *A. actinomycetemcomitans* LPS (1 μg/ml) during 3 h were used as control and compared to fibroblasts treated with the cranberry NDM cranberry fraction (50 μg/ml) during 2 h prior to stimulation with *A. actinomycetemcomitans* LPS (1 μg/ml) during 3 h.

CHAPITRE 8

Inhibition of host extracellular matrix destructive enzyme production and activity by a high molecular weight cranberry fraction

8.1. Résumé

Les parodontites sont des maladies inflammatoires chroniques, initiées par un groupe spécifique de bactéries à Gram négatif, qui conduit à la destruction des tissus conjonctifs de la gencive. Les enzymes protéolytiques, notamment les MMPs et l'élastase, produites par les cellules de l'hôte en réponse aux bactéries parodontopathogènes ou à leurs produits jouent un rôle majeur dans la destruction des tissus gingivaux. Le but de cette étude est d'investiguer l'effet d'une fraction de canneberge de haut poids moléculaire sur l'activité de la MMP-3, de la MMP-9 et de l'élastase ainsi que sur la production de MMPs induite par le LPS d'*Actinobacillus actinomycetemcomitans* chez les macrophages et les fibroblastes gingivaux. La production de MMPs par ces cellules a été mesurée par ELISA et l'activité de la MMP-3, de la MMP-9 et de l'élastase en présence de la fraction de canneberge a été évaluée en utilisant des substrats colorimétriques et fluorescents. Les modifications de l'expression et de la phosphorylation de protéines de signalisation intracellulaire induites par le LPS d'*A. actinomycetemcomitans* et la fraction de canneberge chez les fibroblastes gingivaux ont été caractérisées par une technique de « microarray ». La production de MMP-3 et de MMP-9 induite par le LPS chez les macrophages et les fibroblastes gingivaux a été inhibée par la fraction de canneberge. Cette fraction a inhibé

l'expression et l'activité de plusieurs protéines de signalisation intracellulaire chez les fibroblastes gingivaux, un phénomène qui pourrait conduire à une inhibition de l'activité du facteur transcriptionnel AP-1. L'activité de la MMP-3, de la MMP-9 et de l'élastase a été inhibée efficacement par la fraction de canneberge même à faible concentration. Ces résultats suggèrent que les constituants de la canneberge offrent des perspectives prometteuses pour le développement d'un traitement complémentaire pour les parodontites fondé sur la modulation de la réponse de l'hôte.

8.2. Abstract

Background and objective: Periodontal diseases are a group of inflammatory disorders initiated by specific Gram negative bacteria, and leading to connective tissue destruction. Proteolytic enzymes, including matrix metalloproteinases (MMPs) and elastase, produced by resident and inflammatory cells in response to periodontopathogens and their products play a major role in gingival tissue destruction. The aim of this study was to investigate the effect of a high-molecular-weight fraction prepared from cranberry juice concentrate on MMP-3, MMP-9 and elastase activities as well as on MMP production by human cells stimulated with lipopolysaccharide of *Actinobacillus actinomycetemcomitans*.

Methods: MMP-3 and MMP-9 production by gingival fibroblasts and macrophages treated with the cranberry fraction followed by lipopolysaccharide (LPS) stimulation was measured by ELISA. MMP-3, MMP-9 and elastase activities in the presence of the cranberry fraction were evaluated using colorimetric or fluorogenic substrates. The changes in expression and phosphorylation state of fibroblast intracellular signaling proteins induced by *A. actinomycetemcomitans* LPS and the cranberry fraction were characterized by antibody microarrays.

Results: The LPS-induced MMP-3 and MMP-9 responses of fibroblasts and macrophages were inhibited dose-dependently by the cranberry fraction. This fraction was found to inhibit fibroblast intracellular signaling proteins, a phenomenon that may lead to a downregulation of activating protein-1 activity. MMP-3, MMP-9 and elastase activities

were also efficiently inhibited by the cranberry fraction even when used at low concentrations.

Conclusion: These results suggest that cranberry compounds offer promising perspectives for the development of novel host modulating strategies for an adjunctive treatment of periodontitis.

8.3. Introduction

Periodontal disease are a group of inflammatory disorders leading to tooth supporting tissue destruction, which are initiated by a group of Gram negative anaerobic bacteria. Among these bacteria, *Actinobacillus actinomycetemcomitans* has been implicated as an etiological agent of localized agressive periodontitis (1, 2). The host response to these bacteria and their products, such as lipopolysaccharide (LPS), is a critical determinant for the initiation and progression of periodontitis. The LPS of *A. actinomycetemcomitans* has been proposed to contribute to alveolar bone loss and connective tissue degradation in periodontal disease (3, 4). It is also one of the most potent inducer of inflammatory mediator production among LPS of major periodontopathogens (5). The continuous and high secretion of matrix metalloproteinases (MMPs), such as MMP-2, MMP-3, MMP-8 and MMP-9, by host cells following stimulation by periodontopathogens is known to contribute to periodontal tissue destruction (6, 7). Indeed, MMP levels and their activities are significantly increased in both gingival tissue and gingival crevicular fluid of periodontitis subjects (8, 9). MMPs are produced by the major cell types found in human periodontal tissue, including neutrophils, macrophages and fibroblasts (6, 7). Inhibition of MMP production and activity in periodontitis patients may contribute to reduce periodontal tissue destruction. Interestingly, the use of subantimicrobial doses of doxycycline, which downregulate MMP activity, is indicated as an adjunctive treatment for periodontitis and confers clinical benefits to patients with periodontitis (10). In addition, elastase activity is increased in gingival crevicular fluid from periodontitis sites (11) and has been suggested as an indicator of periodontal disease progression (12). Recently, active compounds endowed with a capacity to modulate the host inflammatory response have received considerable

attention as they may represent potential new therapeutic agents for treatment of periodontal disease (13).

Polyphenolic compounds are able to inhibit both MMP expression and activity and have been proposed as new therapeutics molecules in various diseases (14-17). The cranberry is a polyphenolic rich berry fruit exhibiting several beneficial properties for human health such as inhibition of human cancer cell line proliferation (18, 19), reduction of dental biofilm formation (20, 21), and prevention of urinary tract colonization by infectious agents (22). Previously, we reported the anti-inflammatory activity of a high-molecular-weigh fraction prepared from cranberry juice concentrate (5). The aim of this study was to investigate the effect of cranberry on MMP activity and expression as well as on elastase activity. More specifically, the capacity of a high-molecular-weight cranberry fraction to inhibit the MMP-3 and MMP-9 production by macrophages and gingival fibroblasts following stimulation with LPS from *A. actinomycetemcomitans* was examined. In addition, the changes in expression and phosphorylation state of fibroblast intracellular signaling proteins induced by *A. actinomycetemcomitans* LPS and the cranberry fraction were characterized by antibody microarrays. The effect of this cranberry fraction on activity of host extracellular matrix degradative enzymes MMP-3, MMP-9 and elastase was also investigated.

8.4. Materials and methods

8.4.1. Cranberry fraction

Concentrated juice from the American cranberry *Vaccinium macrocarpon* was obtained from Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA). The juice was exhaustively dialysed in 14 000 MW cut-off dialysis bags at 4°C against distilled water and lyophilized. This non-dialysable material was named NDM. This cranberry powder was dissolved in distilled water. Chemical analyses of NDM were realized by Robin Roderick (Ocean Spray Cranberries, Inc.) and revealed that this fraction is devoided of sugars and

acids and contains 0.35 % of anthocyanins (0.055 % of cyanidin-3-galactoside, 0.003 % of cyanidin-3-glucoside, 0.069 % of cyanidin-3-arabinoside, 0.116 % of peonidin-3-galactoside, 0.016 % of peonidin-3-glucoside and 0.086 % of peonidin-3-arabinoside) and 65.1% of proanthocyanidins.

8.4.2. LPS preparation

A. actinomycetemcomitans ATCC 29522 (serotype b) was grown in Todd-Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 1% yeast extract. The bacterial culture was incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 2 days. LPS were isolated by the method of Darveau and Hancock (23), which is based on protein digestion of a whole cell extract by proteinase K and successive solubilization and precipitation steps. The LPS preparation was freeze dried and kept at –20°C. The presence of contaminant proteins in the LPS preparation was evaluated using a protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as a control and was less than 0.001%.

8.4.3. Monocyte and macrophage cultures

U937 cells (ATCC CRL-1593.2), a human monoblastic leukemia cell line, were cultivated at 37°C in an atmosphere of 5% CO_2 in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (RPMI-FBS) and 100 µg/mL penicillin-streptomycin. Monocytes (2 x 10^5 cells/ml) were incubated with RPMI-FBS containing 10 ng/ml of phorbol myristic acid (PMA; Sigma) for 48 h to induce differentiation into adherent macrophage-like cells as previously reported (24). Following PMA treatment, the medium was replaced with fresh medium and differentiated cells were incubated for an additional 24 h prior to use. Adherent macrophages were suspended in RPMI-FBS and centrifuged at 200 x *g* for 8 min. Cells were washed, suspended in RPMI with 1% heat-inactivated FBS at a density of 1 x 10^6 cells/ml and seeded in a 6 well-plate (2 x 10^6 cells/well in 2 ml) at 37°C in a 5% CO_2

atmosphere prior to be treated with NDM and stimulated with *A. actinomycetemcomitans* LPS.

8.4.4. Fibroblast cultures

Human gingival fibroblasts HGF-1 (ATCC CRL-2014) were cultured in an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine (HyClone Laboratories, Logan, UT, USA) supplemented with 10% heat-inactivated FBS (DMEM-FBS) and 100 µg/mL penicillin-streptomycin. Cells used in all experiments were between passage 5 and 10. For the experiments, fibroblasts were seeded at a concentration of 25 x 10^3 cells/cm^2 in 24 well-plate and cultured in DMEM-FBS during 24 h at 37°C in a 5% CO_2 atmosphere. The medium were replaced by DMEM with 1% heat-inactivated FBS prior the fibroblast treatments.

8.4.5. Treatments of fibroblasts and macrophages

Fibroblasts and macrophages were treated with the cranberry NDM fraction at 10, 25, and 50 µg/ml, and incubated at 37°C in 5% CO_2 for 2 h before stimulation with LPS at a final concentration of 1 µg/ml. After 24 h of incubation, conditioned media were removed and stored at -20°C until use. Cells incubated in culture medium with or without the cranberry fraction but not stimulated with LPS were used as controls.

8.4.6. Cell viability

The viability of NDM-treated fibroblasts and macrophages was evaluated by an MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

8.4.7. MMP-3 and MMP-9 production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify MMP-3 and MMP-9 in the conditioned media according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (Bio-Rad Laboratories) with a wavelength correction set at 550 nm. MMP-3 and MMP-9 concentrations were determined in triplicate using a standard curve prepared for each assay. These assays determine both the active and latent forms of the MMP. The sensitivities of the commercial ELISA kits were 0.156 ng/ml for MMP-3 and 0.313 ng/ml for MMP-9.

8.4.8. Antibody microarray analyses

Changes induced by the *A. actinomycetemcomitans* LPS and the NDM cranberry fraction in the expression and phosphorylation state of fibroblast signaling proteins were characterized by the Kinex™ Antibody Microarray service (Kinexus, Vancouver, Canada). This antibody microarray tracks over 600 different cell signaling proteins in duplicate for more than 250 different phospho-sites, 240 protein kinases and 110 other cell signaling proteins in two samples. In the initial screen, fibroblasts were treated with 1 μg/ml of *A. actinomycetemcomitans* LPS during 3 h and compared with unstimulated cells as control. In the second screen, fibroblasts were treated with 50 μg/ml of the cranberry fraction NDM and incubated at 37°C in 5% CO_2 for 2 h before stimulation with LPS at a final concentration of 1 μg/ml during 3 h. Fibroblasts stimulated with 1 μg/ml of LPS during 3 h were used as a control. After the incubation period, cell lysates were prepared according to the manufacturer's protocol (Kinexus). In brief, cells were washed twice with ice-cold PBS and homogenized at 4°C in a buffer containing 20 mM MOPS (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM β-glycerophosphate (pH 7.2), 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin, 0.5% Triton X-100, and 1 mM DTT. Cells were broken by sonification on ice and cell lysates were centrifuged (LE-80K ultracentrifuge; Beckman, Mississauga, ON, Canada) at 36,000 × *g* for 30 min at 4°C.

Protein concentrations of the cell lysates supernatant fractions were estimated by using the Bradford assay (Bio-Rad Laboratories) and adjusted to a concentration of 2 mg/ml. Kinex™ antibody microarray analyses of the supernatants were performed by Kinexus.

8.4.9. Inhibition of MMP-3 and MMP-9 activities

Human recombinant MMP-3 and MMP-9 were used (Calbiochem, San Diego, CA, USA). MMPs were prepared at a concentration of 20 µg/ml in 5 mM Tris-HCl, pH 7.5, containing 0.1 mM $CaCl_2$, 0.005% Brij 35, and 10% glycerol. MMPs were activated in 0.5 mM *p*-aminophenylmercuric acetate (APMA) in 50 mM Tris-HCl, pH 7.5 by incubation at 37°C of 1 h for MMP-9 and overnight for MMP-3. Activated MMPs were diluted at a final concentration of 1 µg/ml for MMP-3 and 10 µg/ml for MMP-9 in the reaction buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2 % Brij 35) in the presence or absence of increasing concentrations of cranberry NDM (10, 25, 50, 75, 100 and 150 µg/ml) or the metalloproteinase inhibitor ilomastat (25 µM; Calbiochem). MMPs were incubated at 25°C during 15 min prior to add the appropriate fluorogenic substrates. MMP-9 activity was quantified with the fluorogenic substrate fluorescein isothiocyanate-labeled DQ™ gelatin (Molecular Probes, Eugene, OR, USA) and MMP-3 activity was quantified with the fluorogenic substrate 6-(7-Nitro-benzo[1,2,5]oxadiazol-4-ylamino)-hexanoyl-Arg-Pro-Lys-Pro-Leu-Ala-Nva-Trp-Lys(7-dimethylaminocoumarin-4-yl)-NH_2 (Bachem Bioscience Inc., King of Prussia, PA, USA). The assay mixtures were incubated 2 h at 37°C in the dark with the substrate at a final concentration of 50 µg/ml. The fluorescence was measured by means of a fluorometer (VersaFluor model; Bio-Rad Laboratories) at excitation and emission wavelengths of 490 nm and 520 nm for MMP-9, and 360 nm and 460 nm for MMP-3. Fluorescent substrates alone or with NDM were used as control.

8.4.10. Inhibition of elastase activity

The capacity of the cranberry fraction to inhibit human neutrophil elastase (Calbiochem) was assessed. The assay mixtures contained 2 µl elastase (50 µg/ml), 25 µl elastase colorimetric substrate I (4 mM; Calbiochem) and 73 µl reaction buffer (100 mM Tris-HCl, 500 mM NaCl, pH 7.5) with or without cranberry fraction at various final concentrations (10, 25, 50, 75, 100 and 150 µg/ml). Assay mixtures were incubated in 96-well-plate at 37°C for 1 h. Hydrolysis of the chromogenic substrate was determined by measuring the absorbance at 415 nm using a microplate reader (BioRad Laboratories). Chromogenic substrate alone or with NDM was used as control.

8.4.11. Statistical analyses

Data are expressed as the means ± standard deviations of three independent experiments. Analyses of variance were performed to compare the means of the different conditions. Differences were considered significant at a P value < 0.05. Protected Fisher least significant difference and Student's t test were used for pair wise comparisons.

8.5. Results

8.5.1. Effect of cranberry NDM on MMP-3 and MMP-9 production by macrophages and fibroblasts

To investigate the effect of cranberry compounds on production of MMP-3 and MMP-9 by gingival fibroblasts and macrophages, cells were treated with the NDM fraction prepared from cranberry juice concentrate prior to stimulation with the LPS of *A. actinomycetemcomitans*. This LPS showed a strong capacity to induce MMP production by fibroblasts (Fig. 8.1) and macrophages (Fig. 8.2). Although, supernatants of LPS-stimulated fibroblasts were analyzed for MMP-3 and MMP-9 production, only an MMP-3 response

was observed. The MMP-3 response of fibroblasts stimulated with LPS of *A. actinomycetemcomitans* was significantly reduced ($P < 0.05$) by the treatments with NDM at all concentrations tested (Fig. 8.1). A 100% inhibition of the MMP-3 production induced by LPS was observed at an NDM concentration of 25 µg/ml. At 50 µg/ml of NDM, the amount of MMP-3 secreted by fibroblasts was below the basal level produced by unstimulated fibroblasts.

The MMP-3 and MMP-9 responses of macrophages stimulated with LPS of *A. actinomycetemcomitans* were significantly reduced by the treatments with NDM at a final concentration of 25 and 50 µg/ml (Fig. 8.2A and 8.2B). NDM at a final concentration of 50 µg/ml completely inhibited the LPS-induced MMP-3 and MMP-9 responses of macrophages. No obvious cytotoxic effects following treatments of fibroblasts and macrophages with NDM were detected by an MTT test indicating that the decrease in MMP levels is not related to cell toxicity (data not shown).

8.5.2. Effect of cranberry NDM on expression and phosphorylation state of fibroblast intracellular signaling proteins

Changes induced by the *A. actinomycetemcomitans* LPS and the cranberry NDM fraction in the expression and phosphorylation state of fibroblast signaling proteins were characterized by antibody microarrays. Among the various changes induced by these two compounds, we selected only those for which the expression or phosphorylation of proteins were enhanced by the *A. actinomycetemcomitans* LPS and decreased by the presence of cranberry NDM fraction during the LPS treatment (Table 8.1). On the one hand, the expression of MAP kinase protein-serine kinase (MKK) 6 and Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor (Fos) was enhanced by respectively 59% and 48% following treatment with the *A. actinomycetemcomitans* LPS. This LPS enhanced also strongly the phosphorylation state of Jun proto-oncogene-encoded AP1 transcription factor (Jun) (+ 96%) and MKK 3/6 (+ 84%). On the other hand, the cranberry NDM fraction inhibited strongly the phosphorylation of Jun N-terminus protein-serine

kinases (JNK), Jun and Ras-related C3 botulinum toxin substrate 1 (Rac1) by respectively 115%, 85% and 54%. NDM reduced also the phosphorylation of MKK3/6 and Fos by about 30 %. In addition, the expression of Rho-associated protein kinase 2 (ROCK2), MKK6 and Fos were inhibited following NDM treatment by respectively 73%, 41% and 23%.

8.5.3. Effect of cranberry NDM on human MMP-3, MMP-9 and elastase activities

The effect of the cranberry NDM fraction on MMP-3, MMP-9 and elastase activities was also investigated. MMP-3 and MMP-9 activities were significantly inhibited by NDM at all concentrations tested (Fig. 8.3A and 8.3B). An NDM concentration as low as 10 μg/ml significantly reduced by about 40% the MMP-3 activity and 50% the MMP-9 activity. A concentration of 100 μg/ml of NDM totally inhibited the MMP-9 activity. The cranberry NDM fraction has revealed to be an MMP inhibitor as potent as ilomastat at 25 μM (a broad-spectrum MMP inhibitor) used as positive control. Elastase activity was also significantly inhibited by NDM at all concentrations tested (Fig 8.4). A 50% inhibition was observed with NDM at a concentration of 10 μg/ml. The strongest inhibition (75%) was obtained with an NDM concentration of 75 μg/ml.

8.6. Discussion

The connective tissue of the periodontium is composed of fibrous proteins (collagen, elastin) and non-fibrous glycoproteins (laminin, fibronectin, proteoglycan), both of which are degraded during pathological conditions such as periodontitis. Accumulated evidence point to host-derived MMPs, produced by both infiltrating and resident cells of the periodontium, as key destructive enzymes in periodontal disease (25). Excessive MMP activity is a hallmark of human periodontal disease, leading to loss of gingival collagen, degradation of periodontal ligament and resorption of alveolar bone. Inhibition of MMP activities may thus be considered as an effective therapeutic approach for periodontitis patients. Gingival fibroblasts are the most abundant resident cells in periodontal tissue and can be considered as a major source of MMPs into periodontal diseased tissue (26).

Macrophages, which constitute an important part of the inflammatory infiltrate in active periodontal lesions (27), are also potent MMP producers in gingival connective tissue from periodontitis patients (26). The significant role of MMP-3 and MMP-9 in the initial destruction of periodontal extracellular matrix macromolecules has been previously reported (7). Elastase, a protease found at high levels in periodontitis patients (11), has also the capacity to degrade matrix constituents. To demonstrate the potential of cranberry compounds to inhibit both MMP-3 and MMP-9 production, fibroblasts and macrophages were stimulated with LPS of *A. actinomycetemcomitans* following treatment with the cranberry NDM fraction. The effect of NDM on the activity of these MMPs as well as on the elastase activity was also tested.

We observed that LPS of *A. actinomycetemcomitans* act as potent inducer of MMP-3 and MMP-9 production by macrophages and fibroblasts suggesting that it may participate in gingival tissue destruction observed in periodontal disease. MMP-3 (stromelysin-1) and MMP-9 (gelatinase B) can degrade a wide range of extracellular matrix proteins such as gelatin, elastin, proteoglycan, laminin, fibronectin, type II, III, IV, V, VII, IX and X collagens (7, 28) and fibrin (29). In addition, these MMPs cleave α1-proteinase inhibitor, tumor necrosis factor-α (TNF-α) precursor and interleukin-1β (IL-1β) (28, 30). Furthermore, it was reported that MMP-3 is an activator of latent pro-MMP-1, -8 and -9 (31-33), and an inactivator of plasminogen activator inhibitor I (34). Periodontal tissue destruction has been also associated with high levels of MMP-3 and MMP-9 in gingival tissue (35-38) and gingival crevicular fluid (39, 40). MMP-3 levels in gingival crevicular fluid has been proposed as a prognostic factor of attachment loss in established peridontitis sites (39) and is reduced after periodontal therapy (41). It was also suggested that the active form of MMP-9 could be a marker for the clinical severity of periodontal disease (37). All these data supports that MMP-3 and MMP-9 can contribute to destruction of connective tissues in periodontitis. In this study, the cranberry NDM fraction showed a strong capacity to inhibit LPS-induced MMP-3 and MMP-9 production by both macrophages and fibroblasts suggesting that cranberry compounds may limit the overexpression of these MMPs in periodontitis patients. NDM was also shown to be a potent inhibitor of MMP-3

and MMP-9 activity and may thus contribute to limit MMP-3 and MMP-9-mediated destructive processes occurring in periodontal disease.

The expression of MMPs is regulated by a variety of factors, including cytokines, growth factors, chemical agents and physical stresses (42). It has been reported that inflammatory cytokines increase MMP secretion by human gingival fibroblasts (43, 44). Prostanglandin-E_2 (PGE_2) also modulates MMP-3 expression by gingival fibroblasts. Ruwanpura *et al.* (45) reported that IL-1β-induced MMP-3 production was downregulated by PGE_2 in human fibroblasts from healthy gingiva and upregulated by PGE_2 in fibroblasts from periodontally diseased tissue. In addition, pro-inflammatory cytokines, such as TNF-α and IL-1β, selectively up-regulate the macrophage expression of MMP-9 (46). Previously, we reported that the cranberry NDM fraction inhibits inflammatory cytokine production by LPS-stimulated fibroblasts and macrophages (5) and PGE_2 production by LPS-stimulated fibroblasts (submitted). Therefore, the inhibition of MMP production by NDM observed here can be related in part to anti-inflammatory properties of this cranberry fraction. It has been demonstrated that MMP inhibitors reduce alveolar bone loss in experimental periodontal disease (47). In addition, the efficiency of MMP inhibitors in chronic periodontitis patients is enhanced when combined with a non-steroidal anti-inflammatory drug (48). This suggests that therapeutic strategies based on inhibition of diverse periodontal destruction pathways could be a successful approach for periodontitis treatment. The cranberry NDM inhibits MMP production and activities as well as pro-inflammatory mediator production and thus by interfering with some of the destructive host mechanisms may have beneficial effects in slowing the disease progression.

The results of the antibody microarray analyses showed that the cranberry NDM fraction inhibits the phosphorylation and expression of some fibroblast intracellular signaling proteins induced by the *A. actinomycetemcomitans* LPS. These results suggest that NDM fraction can act by reducing the activator protein-1 (AP-1) activity leading to the inhibition of MMP production. AP-1 complexes are heterodimers of proteins of two proto-oncogene families (Jun and Fos) which regulate MMP gene expression (49). Indeed, AP-1 binding site is found in the promoter region of inducible MMP-3 and MMP-9 genes (49).

Transcriptional activity and protein stability of Jun is increased by phosphorylation of serine 63 by JNK (50). In this study, the phosphorylation of Jun on serine 63 was strongly reduced by NDM suggesting that this may result in an inhibition of MMP gene transcription. The reduction of Jun phosphorylation induced by NDM was probably a consequence of the decreased level of phosphorylated form of JNK. Treatment of fibroblasts with *A. actinomycetemcomitans* LPS resulted to an increased production of Fos expression that is important for AP-1 activity, because Jun/Fos heterodimers are more stable than Jun/Jun homodimers (51), thus leading to a more stable AP1 complex. The reduction of Fos expression induced by NDM decreases the Jun/Fos heterodimers formation and participates probably to reduce also the AP-1 activity. We also observed that MKK3/6 phosphorylation and MKK6 expression, two dual specificity kinases implicated in the p38 pathway activation, were inhibited by NDM. p38 pathway contributes also to the AP-1 activity (50) as well as to MMPs gene expression (49) suggesting that inhibition of these two major activators of the p38 pathway by NDM can participate to the inhibition of MMP production. In addition, Rac1/Cdc42 activates p38 MAPK cascades and can promote gene transcriptional changes (52). This upstream signaling molecule, that stimulates also the JNK and Jun transcriptional activities, play a critical role in controlling the JNK signaling pathway (53, 54). This suggests that inhibition of this Rho GTPase by NDM may be implicated in the reduction of MMP production by affecting AP-1 activity through both JNK and p38 pathway. Finally, it was reported that ROCK inhibition leads to a reduction in MMP-9 mRNA levels likely by acting at the transcriptional level, possibly as a result of reduced transcription factor binding (55). NDM induced an important decrease of ROCK expression, a phenomenon that may contribute to the reduction of the MMP expression observed following NDM treatment. The NDM cranberry fraction seems to affect the phosphorylation and expression of various intracellular proteins which are implicated in MMP production. Our results strongly suggest that this cranberry fraction may act notably via a dowregulation of AP-1 activity leading to inhibition of MMP production. However, additional studies are required to clearly identify the exact mechanism of action of the cranberry NDM fraction.

Excessive production and/or activity of MMPs is widely recognized as potential therapeutic target in a number of host disorders such as periodontal disease, arthritis, and cancer. Consequently, there are intense interest in development of MMP inhibitors for therapeutic application in many human diseases. For instance, modulation of MMP production and/or activity by several naturally occurring substances, such as flavonoids, green tea polyphenols and curcumin, represents novel potential therapeutics options for cancer (14). In addition, MMP inhibition can be considered as a promising approach in periodontal disease treatment. There are various potential strategies to reduce levels of MMPs, including blocking production of MMPs, blocking activation of the proenzyme, blocking activity of the enzyme, and increasing inhibitor production. This study suggests that cranberry compounds by inhibiting both production and activity of MMPs offer promising perspectives to develop novel and innovative therapies for the treatment of pathologic conditions characterized by excessive MMP activity.

Neutrophil elastase is a serine protease, which degrades a wide variety of extracellular matrix proteins, including elastin, collagen, proteoglycan, fibronectin and laminin (56, 57). Elastase has been observed in gingival crevicular fluid of periodontitis patients at elevated levels (11, 58) and is reduced after periodontal therapy (59). It was also reported that elastase remaining in periodontal tissue may impair the regenerative response initiated by periodontal ligament cells (60) and the gingival fibroblast-mediated host defense (61). In this study, we showed that NDM inhibited elastase activity suggesting that cranberry components may reduce the adverse effect of high elastase activity on peridontal tissue.

One hypothesis regarding periodontal disease pathogenesis is that host cells stimulated directly or indirectly by components of the dental biofilm secrete proteinases, such as MMPs and elastase, which are associated with altered connective tissue remodeling and alveolar bone resorption. Consequently, it is logical to consider therapeutic approaches based on host-proteinase inhibition for managing adult periodontitis. Host modulators may be highly useful for individuals with a substantially increased risk for periodontitis (62). In this study, a cranberry NDM fraction exhibited a high capacity to inhibit MMP-3, MMP-9

and elastase activities as well as MMP-3 and MMP-9 production by macrophages and gingival fibroblasts. This suggests that cranberry compounds may contribute to limit extracellular matrix degradation and others pathologic processes implicating these enzymes. Therefore, the local application of cranberry NDM or NDM-derived molecules in diseased sites may offer perspectives for the development of novel adjunctive treatment for periodontal disease.

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8.8. References

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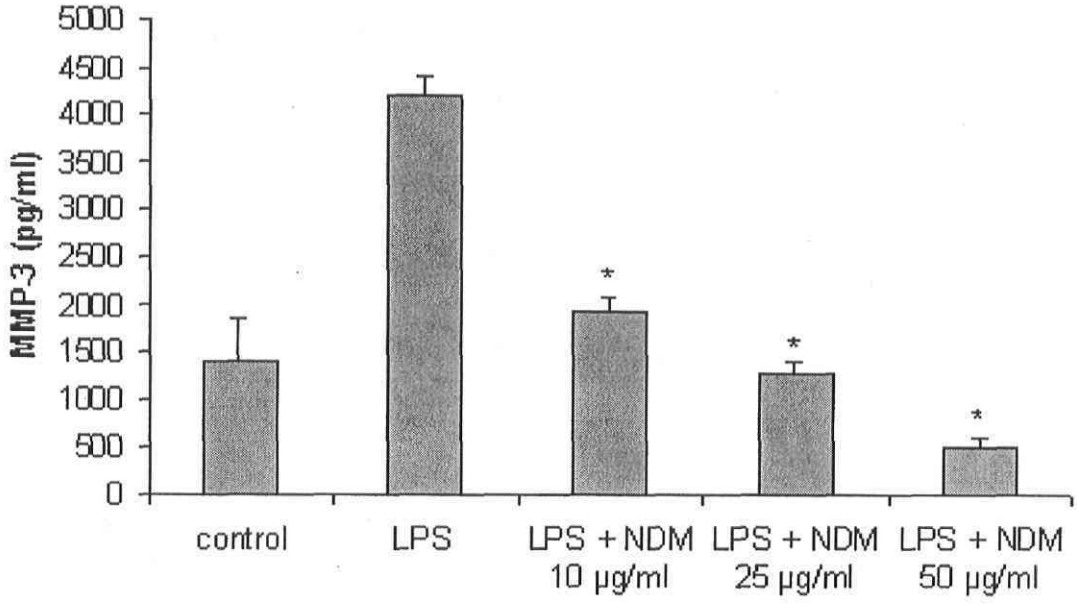

Figure 8.1. Effect of cranberry NDM fraction on MMP-3 production by human gingival fibroblasts stimulated with LPS (1 µg/ml) of *A. actinomycetemcomitans* ATCC 29522. Fibroblasts were treated with the NDM fraction for 2 h prior to LPS stimulation for 24 h. MMP-3 secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three independent experiments. *, *P* value of < 0.05 compared to untreated control.

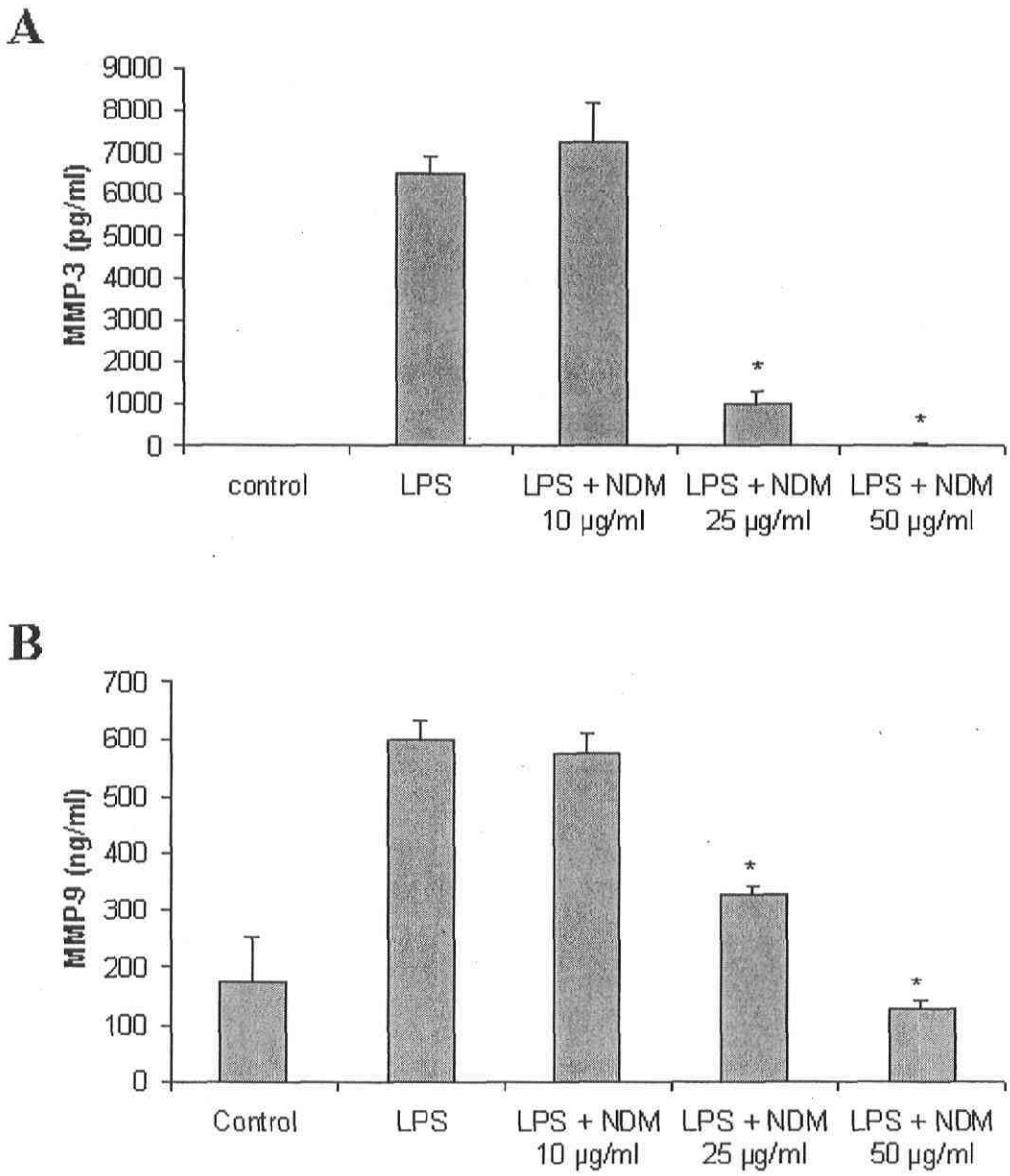

Figure 8.2. Effect of cranberry NDM fraction on MMP-3 (A) and MMP-9 (B) production by human macrophages stimulated with LPS (1 µg/ml) of *A. actinomycetemcomitans* ATCC 29522. Macrophages were treated with the NDM fraction for 2 h prior to LPS stimulation for 24 h. MMP secretion was assessed by ELISA. The data are the means ± standard deviations of triplicate assays for three independent experiments. *, *P* value of < 0.05 compared to untreated control.

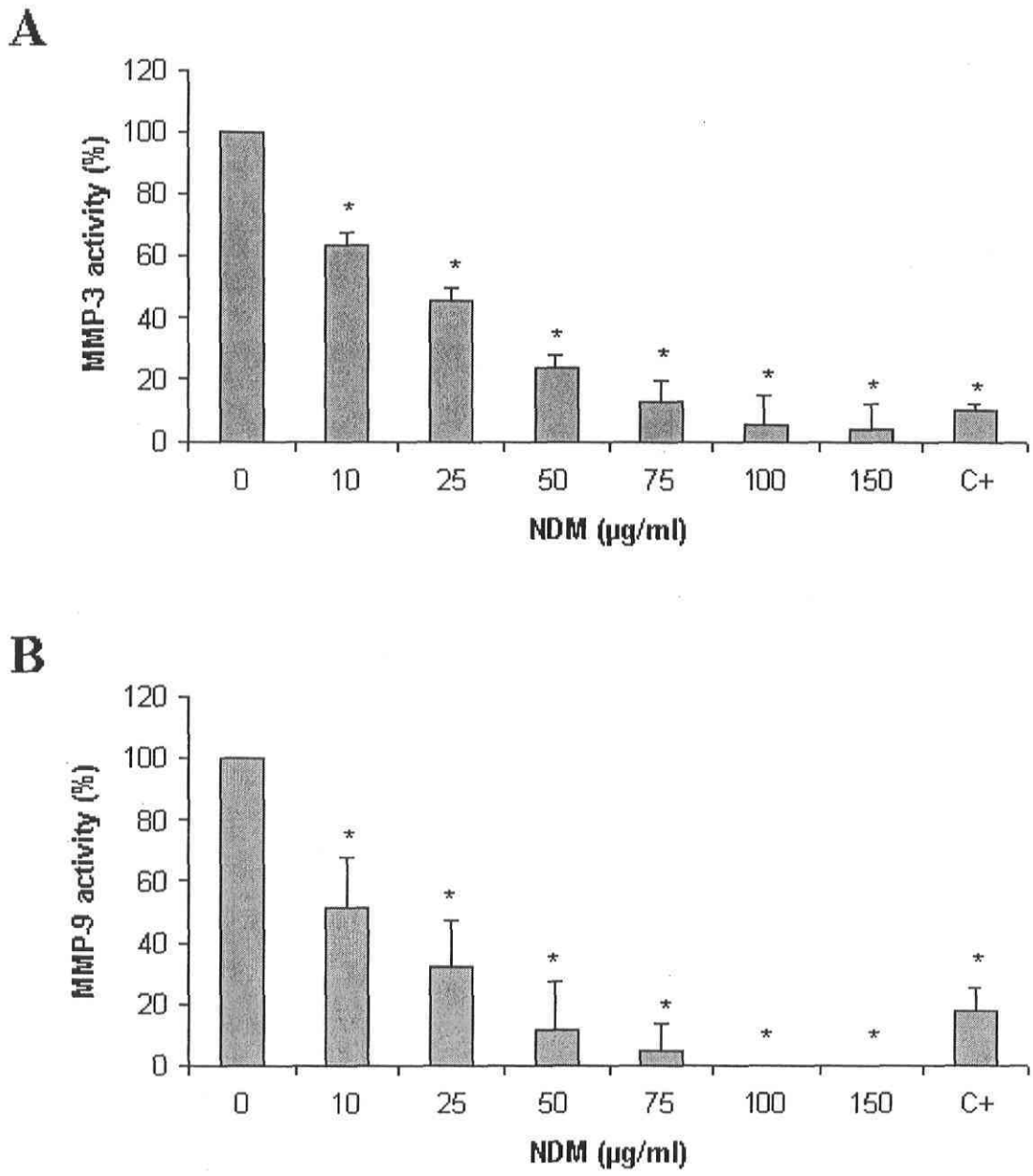

Figure 8.3. Effect of cranberry NDM fraction on MMP-3 (A) and MMP-9 (B) activities. MMPs were incubated 15 min with or without NDM (10, 25, 50, 75, 100 and 150 µg/ml) or 25 µM ilomastat (C+) prior to adding fluorogenic substrates. MMP activity was mesured after 2 h incubation at 37°C with a fluorometer. The data are the means ± standard deviations of three independent experiments. * $P < 0.05$ between NDM of various concentrations and control without NDM.

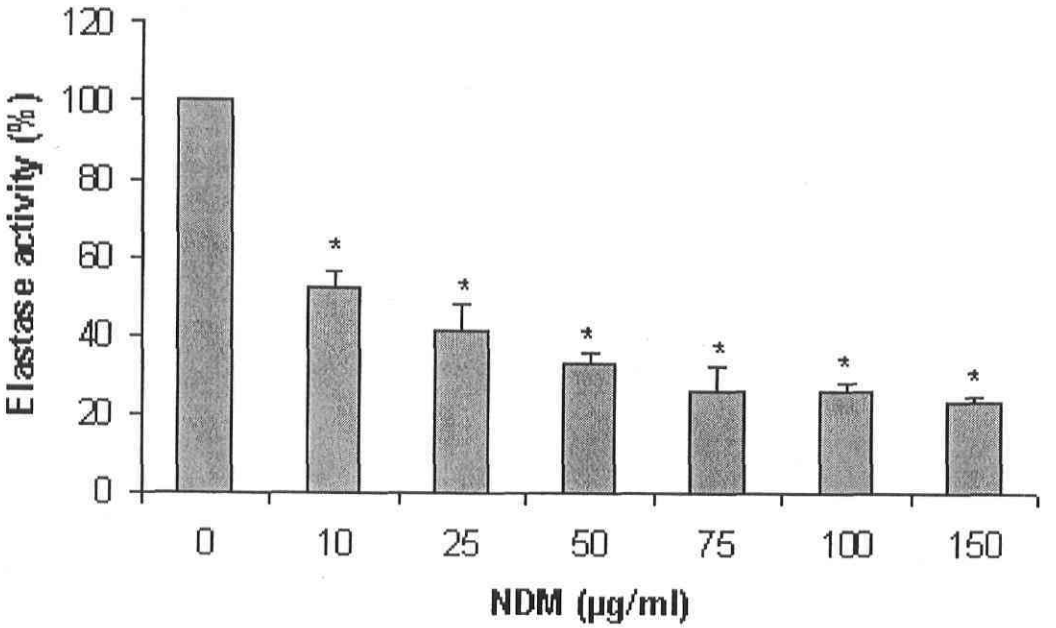

Figure 8.4. Effect of cranberry NDM fraction on elastase activity. Elastase was incubated 15 min with or without NDM (10, 25, 50, 75, 100 and 150 µg/ml) prior to adding the chromogenic substrate. Elastase activity was mesured after 1 h incubation at 37°C with a spectrophotometer. The data are the means ± standard deviations of triplicate assays for three independent experiments. * $P < 0.05$ between NDM of various concentrations and control without NDM.

Table 8.1. Changes induced by the *A. actinomycetemcomitans* LPS and the cranberry NDM fraction in the expression and phosphorylation state of gingival fibroblast intracellular signaling proteins. Two antibody microarrays were performed to screen these changes. Firstly, unstimulated fibroblasts were used as control and compared to fibroblasts stimulated with *A. actinomycetemcomitans* LPS (1 μg/ml) during 3 h. Secondly, fibroblasts stimulated with *A. actinomycetemcomitans* LPS (1 μg/ml) during 3 h were used as control and compared to fibroblasts treated with the cranberry NDM cranberry fraction (50 μg/ml) during 2 h prior to stimulation with *A. actinomycetemcomitans* LPS (1 μg/ml) during 3 h.

Protein			Signal (% change from control)	
Full name	Abbreviation	Phospho Site(s)	Unstimulated vs LPS	LPS vs LPS + NDM
Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor	Fos	Pan-specific	48	- 23
Fos-c FBJ murine osteosarcoma oncoprotein-related transcription factor	Fos	T232	5	- 28
Jun N-terminus protein-serine kinases (stress-activated protein kinase (SAPK)) 1/2/3	JNK	T183+Y185	21	- 115
Jun proto-oncogene-encoded AP1 transcription factor	Jun	S63	96	- 85
MAP kinase protein-serine kinase 3/6	MKK3/6	S189/S207	84	- 36
MAP kinase protein-serine kinase 6	MKK6	Pan-specific	59	- 41
Ras-related C3 botulinum toxin substrate 1	Rac1/cdc42	S71	14	-54
Rho-associated protein kinase 2	ROCK2	Pan-specific	40	-73

CHAPITRE 9

Inhibition of periodontopathogen-derived proteolytic enzymes by a high-molecular-weight fraction isolated from cranberry

9.1 Résumé

Porphyromonas gingivalis, *Treponema denticola* et *Tannerella forsythia* sont trois agents étiologiques majeurs des parodontites chroniques. Les fortes activités protéolytiques de ces bactéries sont essentielles à leur survie puisque leur énergie est obtenue de peptides et d'acides aminés issus de la dégradation de protéines. De plus, les protéases sont un facteur important contribuant à la destruction des tissus parodontaux par une variété de mécanismes incluant la dégradation directe des tissus et la modulation de la réponse inflammatoire de l'hôte. Le but de cette étude est de déterminer l'effet d'une fraction de canneberge non dialysable (NDM) obtenue à partir du jus concentré sur les activités protéolytiques de *P. gingivalis*, *T. denticola* et *T. forsythia*. L'effet du NDM sur l'activité des gingipaïnes et de la dipeptidyl peptidase IV de *P. gingivalis*, l'activité trypsine de *T. forsythia* et l'activité chymotrypsine de *T. denticola* a été évalué en utilisant des peptides synthétiques chromogéniques. De plus, la dégradation du collagène de type I et de la transférine marqués à la fluorescéine a été évaluée par fluorométrie. Le NDM a inhibé les activités protéolytiques de *P. gingivalis*, *T. denticola* et *T. forsythia* ainsi que la dégradation du collagène de type I et de la transférine par *P. gingivalis*. Ces résultats suggèrent que le NDM possède le potentiel pour réduire la prolifération de *P. gingivalis*, *T. denticola* et *T. forsythia* dans les poches parodontales ainsi que les processus de destruction tissulaire impliquant leurs protéinases au cours des parodontites.

9.2. Abstract

Background: *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* are three major etiologic agents of chronic periodontitis. The strong proteolytic activities of these bacteria are critical to their survival since their energy source is obtained from peptides and amino acids derived from proteins. In addition, proteases are important factors contributing to periodontal tissue destruction by a variety of mechanisms, including direct tissue degradation and modulation of host inflammatory responses.

Objectives: The aim of this study was to investigate the effect of non-dialysable material (NDM) prepared from cranberry juice concentrate on the proteolytic activities of *P. gingivalis*, *T. forsythia* and *T. denticola*.

Methods: The effect of NDM on gingipain and dipeptidyl peptidase IV activities of *P. gingivalis*, trypsin-like activity of *T. forsythia* and chymotrypsin-like activity of *T. denticola* was evaluated using synthetic chromogenic peptides. In addition, the capacity of *P. gingivalis* to degrade fluorescein-labeled type I collagen and fluorescein-labeled transferrin in the presence of NDM was evaluated by fluorometry.

Results: NDM dose-dependently inhibited the proteinases of *P. gingivalis*, *T. forsythia* and *T. denticola* as well as collagen type I and transferrin degradation by *P. gingivalis*.

Conclusions: These results suggest that NDM has the potential to reduce either the proliferation of *P. gingivalis*, *T. forsythia* and *T. denticola* in periodontal pockets or their proteinase-mediated destructive process occurring in periodontitis.

9.3. Introduction

Periodontal diseases are multifactorial infections caused by a specific group of Gram-negative anaerobic bacteria leading to destruction of the tooth supporting tissue, including the alveolar bone and the periodontal ligament. Two major factors contribute to the pathogenesis of periodontitis. Firstly, periodontopathogens cause direct damage to periodontal tissue through the secretion of toxic products. Secondly, the host response to

periodontopathogens, which results in release of inflammatory mediators (pro-inflammatory cytokines, matrix metalloproteinases and prostanoids), is also involved in the progression of periodontitis. Specific bacterial species and bacterial complexes occur more frequently in diseased sites while others are associated with healthy or stable periodontal tissues.[1] Among the suspected periodontopathogens, the red complex, which includes *Porphyromonas gingivalis*, *Tannerella forsythia* (formerly *Bacteroides forsythus*), and *Treponema denticola*, is strongly related to clinical measures of periodontitis, particularly pocket depth and bleeding on probing.[1,2] These three major periodontopathogens produce a broad array of virulence factors, such as proteolytic enzymes, that allow them to colonize subgingival sites, resist host defenses, and cause tissue destruction.[3-5] The strong proteolytic activities exhibited by bacteria of the red complex are an important factor contributing to periodontal tissue destruction through a variety of mechanisms, including direct tissue degradation and modulation of host inflammatory responses.[4-8] Inhibitors of periodontopathogen proteinases have been proposed to reduce the bacterial pathogenicity and are considered potentially new therapeutics agents for periodontal diseases.[9-11]

The cranberry is a polyphenolic rich fruit exhibiting various beneficial properties for the human health, notably by interfering with microbial pathogens. Indeed, cranberry juice fractions were found to inhibit the adhesion to host cells of bacteria such as *Escherichia coli*[12] and *Helicobacter pylori*[13] and the coaggregation of many oral bacteria.[14] Cranberry juice compounds were also reported to promote *Streptococcus sobrinus* desorption from artificial biofilms[15] and to affect influenza virus adhesion and infectivity.[16] In addition, it was reported that a high-molecular-weight fraction of cranberry juice inhibits glucosyltransferase and fructosyltransferase activities of oral bacteria.[17] Previously, we reported that this fraction exhibits anti-inflammatory properties.[18] In this study, we investigated the capacity of the high-molecular-weight cranberry fraction to inhibit the proteolytic activities of three major periodontopathogens: *P. gingivalis*, *T. forsythia* and *T. denticola*.

9.4. Material and methods

9.4.1. Cranberry fraction

Juice concentrate from the American cranberry *Vaccinium macrocarpon* was kindly provided by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA, U.S.A.). The juice was exhaustively dialyzed (5 days) in 14,000 MW cut-off dialysis bags at 4°C against distilled water and then lyophilized. This non-dialysable material was named NDM. The cranberry powder was dissolved in distilled water prior to use. Chemical analysis of NDM was realized by Robin Roderick (Ocean Spray Cranberries, Inc.) and revealed that this fraction is devoided of sugars and acids and contains 0.35 % of anthocyanins (0.055 % of cyanidin-3-galactoside, 0.003 % of cyanidin-3-glucoside, 0.069 % of cyanidin-3-arabinoside, 0.116 % of peonidin-3-galactoside, 0.016 % of peonidin-3-glucoside and 0.086 % of peonidin-3-arabinoside) and 65.1% of proanthocyanidins.

9.4.2. Bacterial strains and growth conditions

The bacterial strains used were *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037. *P. gingivalis* was grown in Todd-Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with hemin (10 μg/mL) and vitamin K (1 μg/mL). *T. denticola* was grown in oral spirochete medium as previously described.[19] *T. forsythia* was grown in Brain Heart Infusion broth (BHI; BBL Microbiology Systems) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO, USA) and 0.001% N-acetyl muramic acid (10 μg/mL; Sigma). The bacterial cultures were incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 24 h (*P. gingivalis*) or 4 days (*T. denticola* and *T. forsythia*).

9.4.3. Hydrolysis of synthetic chromogenic peptides

Bacterial cells were harvested by centrifugation (10,000 x g for 10 min), washed, and suspended in 50 mM PBS (pH 7.4) to an optical density at 660 nm ($OD_{660\ nm}$) of 2 for *P. gingivalis* Arg-gingipains A and B activities and of 1 for *P. gingivalis* Lys-gingipain and dipeptidyl peptidase IV (DPP IV) activities, *T. forsythia* trypsin-like activity and *T. denticola* chymotrypsin-like activity. Bacterial cells were incubated with PBS with or without NDM at various final concentrations (10, 25, 50, 75, 100 and 150 μg/mL), synthetic chromogenic peptides (2 mM in distilled water) and 10 mM dithiothreitol as a reducing agent. The substrates used were: glycyl-proline-*p*-nitroanilide (specific for *P. gingivalis* DPP IV; Bachem Bioscience Inc., King of Prussia, PA, USA), benzoyl-arginine-*p*-nitroanilide (specific for *P. gingivalis* Arg-gingipains A and B and *T. forsythia* trypsin-like activity; Sigma), N-*p*-tosyl-glycine-proline-lysine-*p*-nitroanilide (specific for *P. gingivalis* Lys-gingipain; Sigma), and *N*-succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (specific for *T. denticola* chymotrypsin-like activity; Sigma). The assay mixtures were incubated for 1 h at 37°C. Cells were removed by centrifugation (10,000 x g for 10 min) and hydrolysis of the chromogenic substrates was determined by measuring the absorbance at 405 nm of the supernatants using a microplate reader (model 680; BioRad Laboratories, Mississauga, ON, Canada). Chromogenic substrates alone or with NDM were used as control.

9.4.4. Degradation of fluorescein-labeled transferrin and type I collagen

The capacity of the NDM cranberry fraction to inhibit the degradation of fluorescein-labeled human transferrin (Molecular Probes, Eugene, OR, USA) by *P. gingivalis* was assessed. 100 μL of bacterial cells suspended in 50 mM PBS to an $OD_{660\ nm}$ of 2 were mixed with 67 μL dithiothreitol (30 mM), 20 μL fluorescein-labeled transferin (1 mg/mL) and 13 μL PBS with or without the cranberry NDM fraction at a final concentration ranging from 0 to 150 μg/mL. Assay mixtures were incubated at 37°C for 3 h in the dark prior to removing cells by centrifugation (10,000 x g for 10 min). The

fluorescence of the supernatant was measured using a fluorometer (VersaFluor model; Bio-Rad Laboratories) at excitation and emission wavelengths of 490 nm and 520 nm, respectively. Fluorescein-labeled human transferrin alone or with NDM were used as control. The effect of the cranberry NDM fraction on the degradation of fluorescein-labeled type I collagen by *P. gingivalis* was also evaluated. The reaction mixtures contained 20 µL of bacterial cells (OD_{660}=1.5 in PBS), 10 µL of self-quenched bovine skin type I collagen labeled with fluorescein (Molecular Probes; 1 mg/mL), 137 µL of PBS with or whitout the cranberry fraction, and 33 µL of 30 mM dithiothreitol. The reaction mixtures were incubated for 2 h in the dark at 25°C. Cells were then removed by centrifugation (10,000 × *g* for 10 min) and the fluorescence of the supernatants was measured using a fluorometer at excitation and emission wavelengths of 490 and 520 nm, respectively. Fluorescein-labeled type I collagen alone or with NDM were used as control.

9.4.5. Statistical analyses

Each experiment was repeated three times. Data are expressed as the means ± standard deviations. Statistical analysis was performed by one-way analysis of variance (ANOVA) for repeated measurements followed by Dunnett's test. A difference was considered significant if $p < 0.05$.

9.5. Results

9.5.1. Effect of the cranberry NDM fraction on proteolytic activities of *P. gingivalis*, *T. denticola* and *T. forsythia*

The effect of the cranberry NDM fraction on proteolytic activities of three major periodontopathogens was investigated. NDM efficiently inhibited the proteolytic activities of *P. gingivalis* (Figure 9.1). Arg-gingipain and DDP IV activities were significantly reduced ($p< 0.05$) by NDM at concentrations ranging from 50 to 150 µg/mL (Figure 9.1A

and 9.1C). These activities were reduced by 50 % at an NDM concentration of 75 µg/mL for Arg-gingipain and of 150 µg/mL for DDP IV. Lys-gingipain was significantly inhibited by a lower concentration of NDM, a 50% inhibition being obtained with 25 µg/mL (Figure 9.1 B). *T. denticola* chymotrypsin-like and *T. forsythia* trypsin-like activities were also affected by NDM (Figure 9.2). The chymotrypsin-like activity of *T. denticola* was highly sensitive to NDM at all concentrations tested (Figure 9.2A). Indeed, the lowest NDM concentration of 10 µg/mL caused approximately 30% of inhibition of this proteolytic activity while a 60% inhibition was obtained with a NDM concentration of 25 µg/mL. NDM at concentrations of 100 and 150 µg/mL significantly inhibited the trypsin-like activity of *T. forsythia* (Figure 9.2B).

9.5.2. Effect of the cranberry NDM fraction on degradation of collagen type I and transferrin by *P. gingivalis*

The capacity of *P. gingivalis* to degrade type I collagen and transferrin was affected by NDM from cranberry (Figure 9.3). NDM at a concentration of 50 µg/mL significantly reduced the collagenase activity of *P. gingivalis* by 30 % (Figure 9.3A). At a high concentration of NDM (100 µg/mL), the type I collagen degradation was completely inhibited. The degradation of transferrin by *P. gingivalis* was also significantly reduced by a NDM concentration of 50 µg/mL (Figure 9.3B). A 95 % inhibition was obtained with NDM at concentration of 150 µg/mL.

9.6. Discussion

Accumulated evidences point to the members of the red complex, *P. gingivalis*, *T. denticola* and *T. forsythia*, as three major etiologic agent of chronic periodontitis.[1,2,5] These bacteria produce proteases that are able to degrade a broad range of connective tissue proteins and to disrupt host defense mechanisms.[5,8] In addition, these proteases are likely critical for their survival since most periodontopathogens derive their energy from protein sources. Therefore, it has been proposed that proteases produced by *P. gingivalis*, *T.*

forsythia and *T. denticola* may participate in the destructive process occurring in periodontitis.[8] As suggested by various studies,[9-11,20] agents that inhibit protease activities of periodontopathogens may be potentially useful for the prevention or treatment of periodontal disease. The aim of this study was to investigate the effect of a cranberry high-molecular-weight fraction on proteolytic activities exhibited by three major periodontopathogens.

Gingipains, which can be both cell-bound and secreted, are the main endopeptidases produced by *P. gingivalis* and contribute to the virulence properties of *P. gingivalis* since they degrade a large variety of host proteins.[4,5] These proteases appear to be essential for *P. gingivalis* by providing iron, peptides and amino acids from environmental proteins.[21,22] They also contribute to processing/maturation of various cell-surface proteins of *P. gingivalis*.[23] Gingipains can degrade various extracellular matrix proteins,[4] as well as activate pro-matrix metalloproteinases,[24] the kallikrein/kinin pathway and the blood coagulation system.[4] Gingipains also contribute to the evasion of host defense mechanisms by modulating cytokine functions[25,26] and by degrading immunoglobulins, complement proteins[27] and cell membrane proteins such as interleukin-6 receptor[28] or CD14.[29] Interestingly, it was reported that gingipain inhibitors reduce the pathogenicity of *P. gingivalis*.[9] In this study, we showed that cranberry NDM efficiently inhibits both Arg- and Lys-gingipains activities of *P. gingivalis*. Other polyphenolic compounds, including the green tea catechins, have been previously reported to also strongly inhibit the gingipain activities of *P. gingivalis*.[20] Based on the critical roles previously suggested for gingipains in the pathogenesis of periodontitis, our results suggest that cranberry compounds found in the NDM fraction may contribute to reduce periodontal tissue destruction.

Iron plays an important role in the growth and virulence of *P. gingivalis*.[22] The ability of *P. gingivalis* to multiply under iron-limiting conditions has been correlated with its pathogenicity in an animal model.[30] One major source of iron for periodontopathogens is human transferrin, which is present in high amounts in the gingival crevicular fluid. Cleavage of human transferrin by gingipains promotes growth and formation of hydroxyl radicals which may contribute to tissue destruction during periodontitis.[31] In our study,

NDM exhibited inhibitory effect on transferrin degradation by *P. gingivalis*. This suggests that NDM may contribute to reduce the growth and virulence of *P. gingivalis in vivo*.

T. denticola chymotrypsin-like activity has been shown to degrade transferrin, fibrinogen, gelatin, immunoglobulins, α1-antitrypsin and various basement membrane proteins.[32,33] This activity may thus play a major role in the invasion and destruction of basement membrane by *T. denticola*[33] as well as in epithelial cell layer penetration by this organism.[34] The chymotrypsin-like activity of *T. denticola* was strongly inhibited by NDM, suggesting that NDM may limit the pathogenicity of *T. denticola*.

Collagen-degradating enzymes produced by periodontopathogens may be involved in the gingival connective tissue destruction.[35] Since type I collagen is the predominant protein of periodontal tissues, representing about 60% of this tissue volume, degradation of this major constituent of the gingival matrix would lead to tissue destruction. *P. gingivalis* can degrade type I collagen and the collagenolytic activity of this bacteria has been attributed to various proteases.[36-38] A pathological role of *P. gingivalis* DPP IV in the progression of periodontitis was recently suggested.[39] Indeed, DPP IV was reported to act in conjunction with collagenase. NDM, by inhibiting both the collagenolytic and DPP IV activities of *P. gingivalis*, can reduce the type I collagen disruption and the subsequent connective tissue fragilization in periodontitis patients. Others compounds, such as tetracycline, and doxycycline, which reduce the severity and progression of periodontitis in animal models and humans, also inhibit the collagenase activity of *P. gingivalis*.[11] In addition, proteases from *T. denticola* and *P. gingivalis* can activate host collagenases and plasminogen, and destroy host protease inhibitors which would enhance host enzyme-mediated tissue destruction.[6,40] NDM from cranberry, by inhibiting these proteolytic activities, may also contribute to reduce the impact of host enzyme-mediated destructive process occurring in periodontitis.

To prevent periodontal disease progression, mechanical procedures are used to remove the dental biofilm. Although these procedures are effective in managing the

majority of periodontitis patients, there are situations in which conventional therapy does not always achieve the desired clinical outcome. Control of disease in individuals with significantly increased risk for periodontitis (smokers, diabetics or individuals possessing genetic predisposition) or who do not respond to conventional therapy may require adjunctive treatments, such as use of antimicrobials or host modulators. Indeed, systemic administration of subantimicrobial doses of doxycycline, which downregulates matrix metalloproteinase activity, is indicated as an adjunctive treatment for periodontitis and confers clinical benefits to patients with periodontitis.[41] In regard to developing additional alternatives to conventional therapy for individuals with a substantially increased risk for periodontitis, local application of cranberry NDM may provide a new practical approach based on attenuation of periodontopathogen virulence.

Our result showed that NDM efficiently inhibits proteases of periodontopathogens, which are major factors for their pathogenicity and required for their growth and survival. These results suggest that NDM may contribute to reduce the proliferation of periodontopathogens in periodontal pockets and the proteinase-mediated destructive process occurring in periodontitis. NDM exhibits interesting properties for periodontal health and thus appears promising for the development of new therapeutic approaches for adjunctive treatment of periodontitis.

9.7. Acknowledgments

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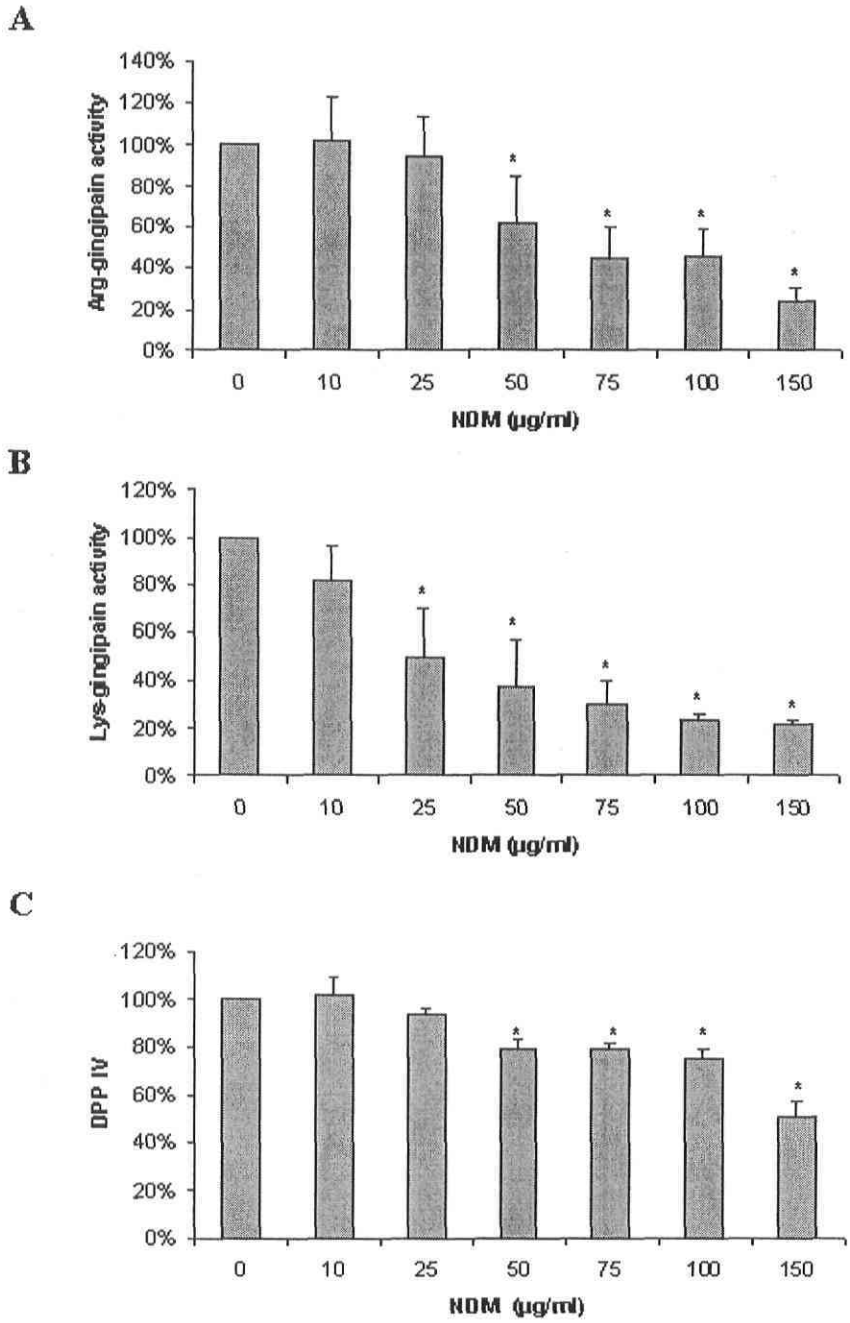

Figure 9.1. Effect of the cranberry NDM fraction on Arg-gingipain (A), Lys-gingipain (B) and DPP IV (C) activities of *P. gingivalis*. The degradation obtained in the absence of NDM was given a value of 100%. * $P < 0.05$ between NDM of various concentrations and control without NDM.

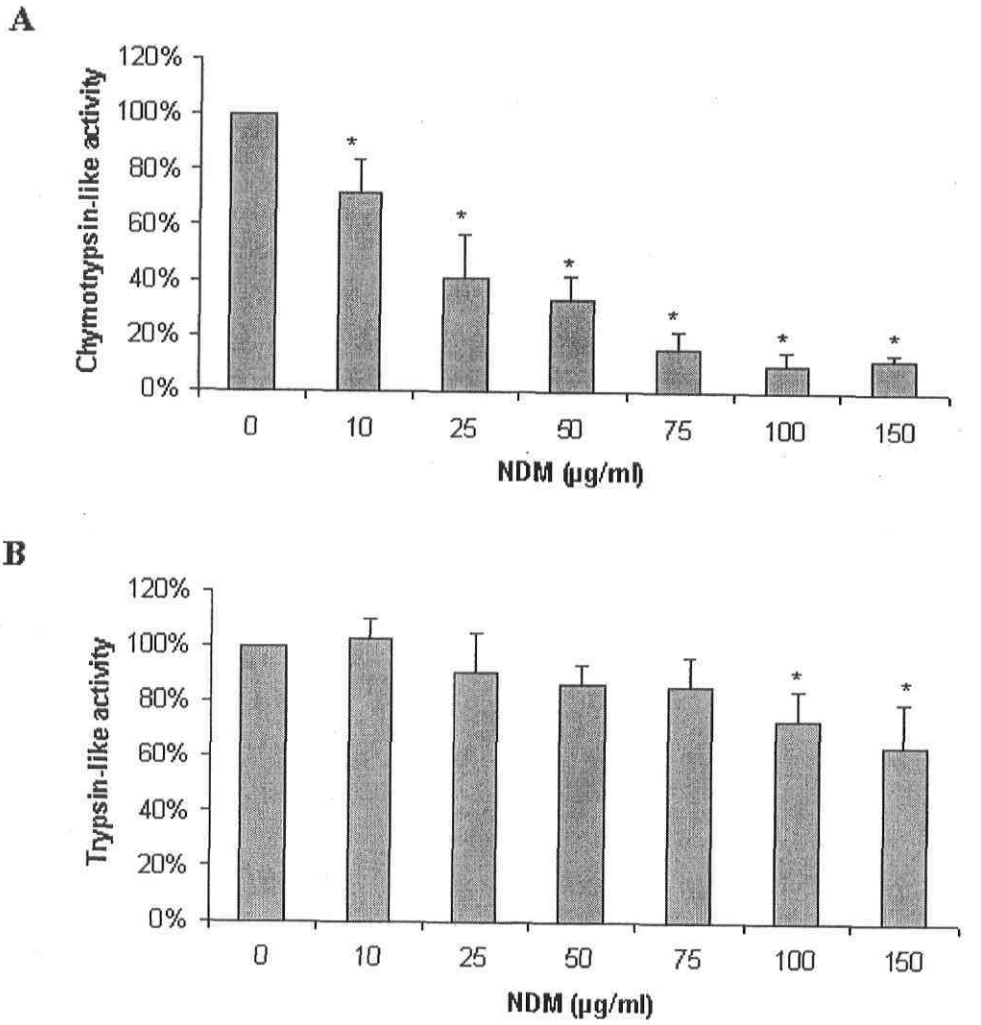

Figure 9.2. Effect of the cranberry NDM fraction on chymotrypsin-like activity of *T. denticola* (A) and trypsin-like activity of *T. forsythia* (B). The degradation obtained in the absence of NDM was given a value of 100%. * $P < 0.05$ between NDM of various concentrations and control without NDM.

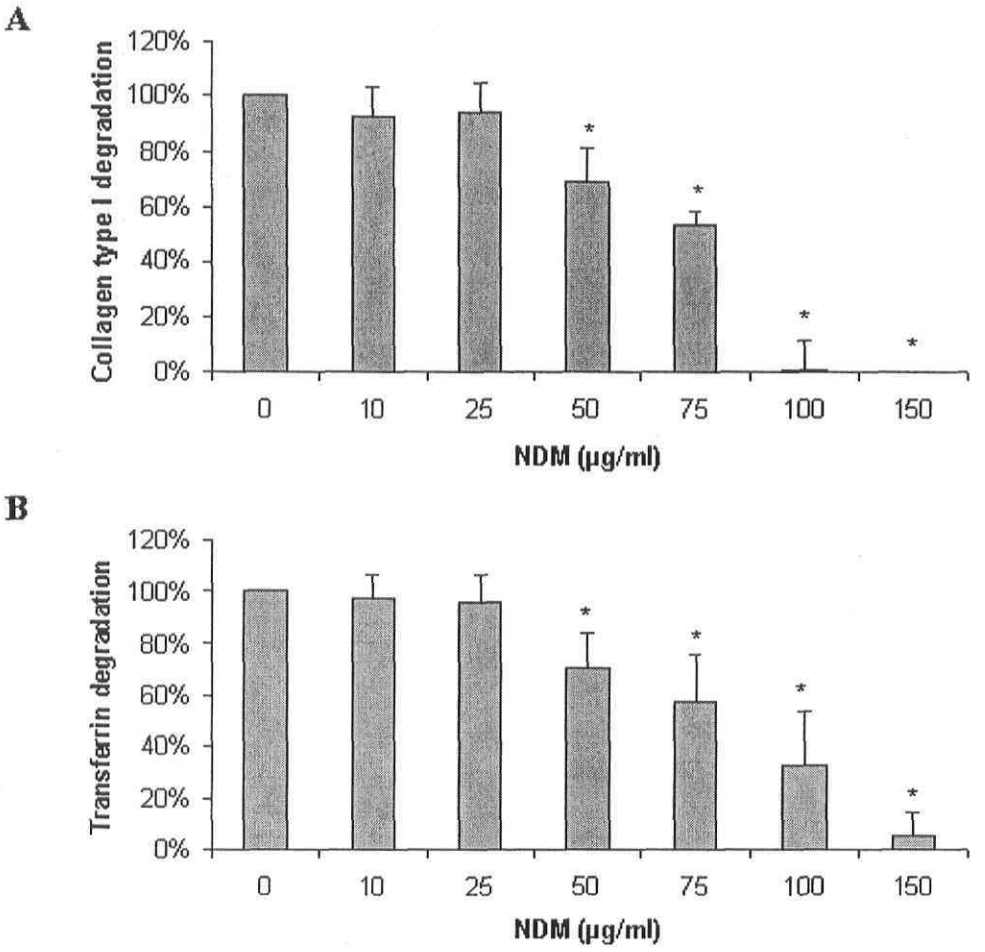

Figure 9.3. Effect of the cranberry NDM fraction on degradation of fluorescein-labeled type I collagen (A) and fluorescein-labeled transferrin (B) by *P. gingivalis*. The degradation obtained in the absence of NDM was given a value of 100%. * $P < 0.05$ between NDM of various concentrations and control without NDM.

CHAPITRE 10

Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of *Porphyromonas gingivalis*

10.1. Résumé

Porphyromonas gingivalis est un agent étiologique majeur des parodontites chroniques, une maladie destructive affectant les tissus de soutien de la dent. Des études récentes ont rapporté que les molécules de haut poids moléculaires de la canneberge pouvaient prévenir l'adhésion de pathogènes humains aux tissus. Le but de cette étude est de caractériser l'effet d'une fraction de canneberge non dialysable obtenue à partir du jus concentré sur la croissance, la formation du biofilm et les propriétés d'adhérence de *P. gingivalis*. L'effet de la fraction NDM de canneberge sur la formation du biofilm a été étudié en utilisant des essais en microplaque et de la microscopie électronique à balayage. L'effet du NDM sur les propriétés d'attachement de *P. gingivalis* a été évalué par des essais en microplaque dans lesquelles des protéines humaines étaient immobilisées au fond des puits. Les résultats ont montré que la fraction NDM de canneberge est un inhibiteur puissant de la formation du biofilm de *P. gingivalis*. Par contre, cette fraction n'a pas eu d'effet sur la croissance et la viabilité des bactéries. La fraction NDM de canneberge a inhibé également l'attachement de *P. gingivalis* aux surfaces recouvertes de collagène de type I, de fibrinogène ou de sérum humain. Ces données suggèrent que les constituants de la canneberge pourraient avoir un effet bénéfique pour la prévention et le traitement des parodontites en réduisant la capacité de *P. gingivalis* à coloniser les sites parodontaux.

10.2. Abstract

Background: *Porphyromonas gingivalis* is a major etiologic agent of periodontitis, a destructive disease affecting the tooth-supporting tissues. Recent reports have indicated that high molecular weight molecules from cranberry juice concentrate can prevent the attachment of human pathogens to host tissues.

Objectives: The aim of this study was to investigate the effect of non-dialysable material (NDM) prepared from cranberry juice concentrate on growth, biofilm formation, and adherence properties of *P. gingivalis*.

Methods: The effect of cranberry NDM on biofilm formation was studied using a polystyrene microplate assay and by scanning electron microscopy. The effect of cranberry NDM on the attachment properties of *P. gingivalis* was evaluated by a microplate assay in which mammalian proteins were immobilized into wells.

Results: Our results indicated that cranberry NDM is a potent inhibitor of biofilm formation by *P. gingivalis*. However, it has no effect on growth and viability of bacteria. Cranberry NDM also prevented significantly the attachment of *P. gingivalis* to surfaces coated with either type I collagen, fibrinogen or human serum.

Conclusion: Our data suggest that cranberry constituents may have a beneficial effect for the prevention and treatment of periodontitis by reducing the capacity of *P. gingivalis* to colonize periodontal sites.

10.3. Introduction

Periodontitis is an inflammatory disorder leading to the destruction of tooth-supporting tissues, including the periodontal ligament and the alveolar bone, and is caused by a specific group of Gram-negative anaerobic bacteria.[1] The continuous challenge to the host immune system by periodontopathogens and their products induces a number of host-mediated destructive processes.[2] Much evidence points to *Porphyromonas gingivalis* as the key pathogen in chronic periodontitis.[1] The ability of this bacterium to colonize subgingival sites is a critical step in the initiation of periodontal diseases. *P. gingivalis* is well known to

express a number of adhesins, associated with either the outer membrane or fimbriae, that promote its adhesion to tooth surfaces, gingival epithelial cells, basement membrane components, erythrocytes and oral bacteria.[3]

The cranberry is a native North American fruit for which a number of studies have reported benefit properties for human health. Cranberries are particularly rich in various polyphenolic compounds, including flavonoids, phenolic acids and complex phenolic polymers.[4] It has been reported that high molecular mass proanthocyanidins (condensed tannins) from cranberry juice inhibit the adherence of uro-pathogenic fimbriated *Escherichia coli* and thus protect against urinary tract infections.[5] Furthermore, a high molecular weight cranberry fraction was also reported to inhibit the sialic acid-specific adhesion of *Helicobacter pylori* to human gastric mucosa, a critical step for gastric ulcer development.[6] In the area of dental research, it has been reported that a non-dialysable material prepared from cranberry juice concentrate inhibits the coaggregation of many oral bacteria[7] and prevents mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) biofilm formation.[8-10] To our knowledge, no study has investigated the effect of cranberry constituents on adherence and growth of periodontopathogenic bacteria. In this study, we hypothesized that high molecular weight cranberry constituents may have a beneficial effect in the prevention of periodontitis by reducing growth and adherence of *P. gingivalis*.

10.4. Materials and methods

10.4.1. Cranberry fraction

Juice concentrate from the American cranberry *Vaccinium macrocarpon* was kindly provided by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA). The juice was exhaustively dialyzed (5 days) in 14,000 MW cut-off dialysis bags at 4°C against distilled water and then lyophilized. The non-dialysable material (NDM) was dissolved in distilled water prior to use. Chemical analyses of cranberry NDM were realized by Robin Roderick

(Ocean Spray Cranberries, Inc.) and revealed that this fraction is devoided of sugars and acids and contains 0.35% of anthocyanins (0.055% of cyanidin-3-galactoside, 0.003% of cyanidin-3-glucoside, 0.069% of cyanidin-3-arabinoside, 0.116% of peonidin-3-galactoside, 0.016% of peonidin-3-glucoside and 0.086 % of peonidin-3-arabinoside) and 65.1% of proanthocyanidins. This latter class of complex phenolic polymers was enriched 125 fold in comparison with the original juice concentrate (data not shown).

10.4.2. Bacteria and culture conditions

P. gingivalis ATCC 33277 was used throughout the study. Bacteria were routinely grown in Todd Hewitt Broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with hemin (10 μg/mL) and vitamin K (1 μg/mL) (THB-HK) and incubated for 24 h in an anaerobic chamber ($N_2/H_2/CO_2$:75/10/15) at 37°C.

10.4.3. Growth

The effect of cranberry NDM on growth of *P. gingivalis* was determined by using a plate diffusion assay. The cranberry NDM powder was sterilized by a 48 h treatment in 70% ethanol. The cranberry NDM was then diluted in sterile 50 mM phosphate-buffered saline (PBS, pH 7.2) to obtain final concentrations ranging from 0 to 500 μg/mL. Fifty-microliter samples of the dilutions were placed in glass penicylinders (8 by 8 mm; Bellco Glass Inc., Vineland, N.J.) on 3% blood (sheep)-supplemented THB-HK agar plates that had been inoculated by spreading 100 μL of an overnight culture of *P. gingivalis*. After 7 days of incubation at 37°C under anaerobiosis, growth inhibition on the agar surface was evaluated visually by comparing with the control (PBS alone).

10.4.4. Biofilm formation, desorption, and viability

A 24 h culture of *P. gingivalis* in THB-HK was diluted in fresh broth medium to obtain an optical density at 655 nm (OD_{655}) of 0.07. Samples (100 μL) were added to the

wells of a 96-well tissue culture plate (Sarstedt, Newton, NC) containing 100 µL of serial dilutions (0 to 500 µg/mL) of sterile cranberry NDM in THB-HK. Control wells with no NDM were also inoculated. After incubation for 48 h at 37°C under anaerobic conditions, spent media and free-floating bacteria were then removed by aspiration using a 26G needle and the wells were washed three times with distilled water. The *P. gingivalis* biofilms were stained with 0.4% crystal violet (100 µL) for 15 min. The wells were washed four times with distilled water to remove unbound crystal violet dye and dried for 2 h at 37°C. After adding 100 µL of 95% (v/v) ethanol to each well, the plate was shaken for 10 min to release the stain from the biofilms and the absorbance at 550 nm (A_{550}) was recorded. A preliminary assay revealed that NDM did not affect the reading of the absorbance values at 550 nm. Assays were run in triplicate and the means ± standard deviations of two independent experiments were calculated.

The effect of cranberry NDM on biofilm formation by *P. gingivalis* was also investigated by scanning electron microscopy. *P. gingivalis* was inoculated as above (2 mL/well) in 35 mm dishes in the absence and presence of cranberry NDM at 125 µg/mL. After 48 h of incubation, spent media and free-floating bacteria were removed. The biofilms on each plate were fixed overnight in fixation buffer (4% [wt/vol] paraformaldehyde and 2,5% [vol/vol] glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7,3). Samples were dehydrated through a graded series of ethanol (50, 70, 95 and 100%), critical point dried, gold sputtered and examined by a JEOL JSM-35CF scanning electron microscope operated at 15 kV.

The capacity of cranberry NDM to promote desorption of a *P. gingivalis* biofilm was investigated. Briefly, a 48 h biofilm of *P. gingivalis* was prepared as above and treated for 2 h with the cranberry NDM at final concentrations ranging from 0 to 250 µg/mL. Following these treatments, the biofilms were stained with crystal violet.

The effect of cranberry NDM on *P. gingivalis* viability was investigated using the tetrazolium sodium 3'-{1- [(phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-

nitro) benzene sulfonic acid hydrate (XTT; Sigma-Aldrich Canada Ltd., Oakville, Ontario) reduction assay. Briefly, XTT was dissolved in PBS at 1 mg/mL and menadione was prepared in acetone at 1 mM. The XTT/menadione reagent was prepared fresh and contained 12.5 parts XTT/1 part menadione. A 48 h biofilm of *P. gingivalis* was prepared as above and treated with cranberry NDM (0 to 250 μg/mL) for 4 h (anaerobiosis, 37°C) prior to adding 25 μL XTT/menadione. After 1 h at 37°C, the absorbance at 490 nm (A_{490}) was read using a microplate reader.

10.4.5. Adherence to protein-coated surfaces

Wells of a flat-bottomed microtitre plate (MaxiSorp; Nalge Nunc International, Rochester, NY) were filled with 100 μL of either rat tail type I collagen (BD Sciences, Bedford, MA; 1 mg/mL in 0.01% [v/v] acetic acid), human fibrinogen (ICN Biomedicals, Aurora, OH; 1 mg/mL in 50 mM carbonate buffer pH 9), or human serum (20% [v/v] in 50 mM carbonate buffer pH 9), and the plate was incubated overnight at room temperature. The protein solution was then removed by aspiration using a 26G needle and 0.05% glutaraldehyde (100 μL) was subsequently added. After 45 min at room temperature, the glutaraldehyde was removed and the wells were washed twice with distilled water. Bacteria from a 24 h culture were harvested by centrifugation (10 000 x g for 15 min) and suspended in PBS to a concentration of 10^{10} bacteria/mL, as determined with a Petroff-Hausser counting chamber. Equal volumes of bacteria and cranberry NDM at 250, 125, 50, 12.5, 2.5, or 0.5 μg/mL were mixed. After 10 min, 100 μL of the mixtures was added to the protein-coated wells. The plate was incubated at 37°C for 1.5 h with gentle shaking every 20 min. After the attachment period, unbound bacteria were removed by aspiration using a 26G needle and the wells were washed three times with PBS containing 0.01% Tween 20 to minimize non specific hydrophobic interactions. Adherent bacteria to the wells were fixed with methanol for 15 min, extensively washed with distilled water and then stained with 0.4% crystal violet (100 μL) for 15 min. Wells were rinsed with distilled water and dried at 37°C for 2 h. After adding 100 μL 95% (v/v) ethanol to each well, the plate was shaken to release the stain. The A_{550} was recorded using a microplate reader. Assays were run in

triplicate and the means ± standard deviations of two independent experiments were calculated.

10.4.6. Cell surface hydrophobicity

The relative cell surface hydrophobicity of *P. gingivalis* cells treated with the cranberry NDM fraction (0-125 μg/mL) was determined by measuring their absorbing property to n-hexadecane using the method of Rosenberg *et al.*[11]

10.4.7. Statistical analyses

Differences between means were analyzed for statistical significance using Student's *t*-test. Differences were considered significant at the 0.05 level (*p* value).

10.5. Results

The cranberry NDM was added at various concentrations in THB-HK to determine its inhibitory effect on biofilm formation by *P. gingivalis.* The formation of biofilm was dose-dependently inhibited by the cranberry fraction (Fig. 10.1A). A significant inhibition ($p < 0.05$) was observed when cranberry NDM was used at a concentration of 62.5 μg/mL and higher. Scanning electron microscopy confirmed the inhibitory effect of the cranberry fraction on *P. gingivalis* biofilm formation. In the control well, *P. gingivalis* formed a uniform three-dimensional structure (Fig. 10.1B). However, when cranberry NDM was added at 125 μg/mL, very few *P. gingivalis* cells were observed attached on the plate (Fig. 10.1C).

The plate diffusion assay revealed that the cranberry NDM had no effect on the growth of *P. gingivalis* even at a concentration of 250 μg/mL. In addition, the XTT reduction assay indicated that at the same high concentration, a 4 h treatment with the

cranberry fraction did not affect the viability of a 48 h biofilm of *P. gingivalis*. Lastly, such a treatment did not cause any desorption of the *P. gingivalis* biofilm.

Using a microplate assay in which proteins were immobilized onto the bottom of wells, *P. gingivalis* was found to attach to a similar extent to type I collagen, fibrinogen and human serum (Fig. 10.2). The cranberry NDM caused a significant inhibition of *P. gingivalis* attachment to all three proteins when added at 50 μg/mL. We investigated whether the cranberry NDM inhibits biofilm and adherence of *P. gingivalis* through a modification of the cell surface hydrophobicity. Untreated cells showed a % hydrophobicity of 21. Incubating cells for 1 h in the presence of cranberry NDM, up to a concentration of 125 μg/mL, did not modify the cell surface hydrophobicity of *P. gingivalis*.

10.6. Discussion

Colonization and subsequent biofilm formation by *P. gingivalis* in subgingival sites is the initial step in the pathogenesis of periodontitis. Thereafter, *P. gingivalis* together with other Gram negative bacterial species stimulate the host defense system leading to an overproduction of a large variety of inflammatory mediators, including interleukin-1β, tumor necrosis factor-α, and prostaglandin E_2.[2] We previously showed that a high molecular weight fraction prepared from cranberry juice concentrate could inhibit lipopolysaccharide-induced pro-inflammatory cytokine and chemokine production by human macrophages.[12] In this study, we investigated the inhibitory effects of this cranberry fraction on growth, biofilm formation and adherence properties of *P. gingivalis*.

The growth and viability of *P. gingivalis* was found to be unaffected by cranberry NDM for which the chemical analysis indicated that it is enriched in complex phenolic polymers. This supports the study of Ahuja *et al.*[13] who reported that cranberry juice concentrate had no antibacterial activity on *E. coli*.

Biofilms, which are defined as structured microbial communities attached to surfaces, play an important role in most bacterial infections of the human body. In the oral cavity, biofilms allow bacteria to evade immune defenses and to better resist to mechanical removal and chemotherapeutic agents. Yamanaka *et al.*[10] and Steinberg *et al.*[8] previously reported the capacity of cranberry NDM to prevent biofilm formation by oral streptococci, including *S. mutans* and *S. sobrinus*. In this study, we showed that cranberry NDM could also prevent the formation of *P. gingivalis* biofilm at a concentration of 62.5 µg/mL and higher. However, the cranberry fraction did not show any capacity to desorb a pre-formed biofilm of *P. gingivalis*. Although they used a much higher concentration of cranberry NDM (2 mg/mL), Steinberg *et al.*[9] reported a desorption effect on *S. sobrinus* biofilm. *P. gingivalis* possesses multiple structures and components for binding to host cells and proteins. Using a microplate assay in which type I collagen, fibrinogen or serum proteins were immobilized onto the bottom of wells, we demonstrated that cranberry NDM could inhibit the attachment of *P. gingivalis*. Such an inhibition was also observed with another Gram negative periodontopathogen, *Fusobacterium nucleatum* subsp. *nucleatum* (data not shown). The mechanism involved in inhibition of biofilm formation and adhesion of *P. gingivalis* does not appear not to involve a modification of the cell surface hydrophobicity, as previously reported for the inhibition of oral streptococci biofilm.[10] Additional studies will investigate whether cranberry constituents may interfere with specific receptor-ligand involved in *P. gingivalis* attachment. Indeed, proanthocyanidins with unique A-type linkages have been isolated from cranberry fruit and were found to attach to specific fimbrial adhesins on *Escherichia coli*.[5]

To prevent periodontal disease progression, mechanical procedures are used to remove the dental biofilm. Although these procedures are effective in managing the majority of periodontitis patients, there are situations in which conventional therapy does not always achieve the desired clinical outcome. Control of disease in individuals with significantly increased risk for periodontitis (smokers, diabetics or individuals possessing genetic predisposition) or who do not respond to conventional therapy may require adjunctive treatments, such as use of antimicrobials or host modulators. The inhibition of adhesion is an attractive target for the development of new therapies in the prevention of

bacterial infections, particularly infections of mucosal surfaces. Our results brought clear evidence that cranberry compounds can inhibit biofilm formation and adherence of *P. gingivalis*. The fact that cranberry NDM acts by preventing bacterial adhesion rather than by inhibiting growth may represent an advantage since bacteria cannot develop resistance. Considering that cranberry NDM was previously reported to inhibit proteinases of *P. gingivalis*[14], this cranberry fraction may offer new perspectives for the prevention/treatment of *P. gingivalis*-associated periodontitis.

10.7. Acknowledgments

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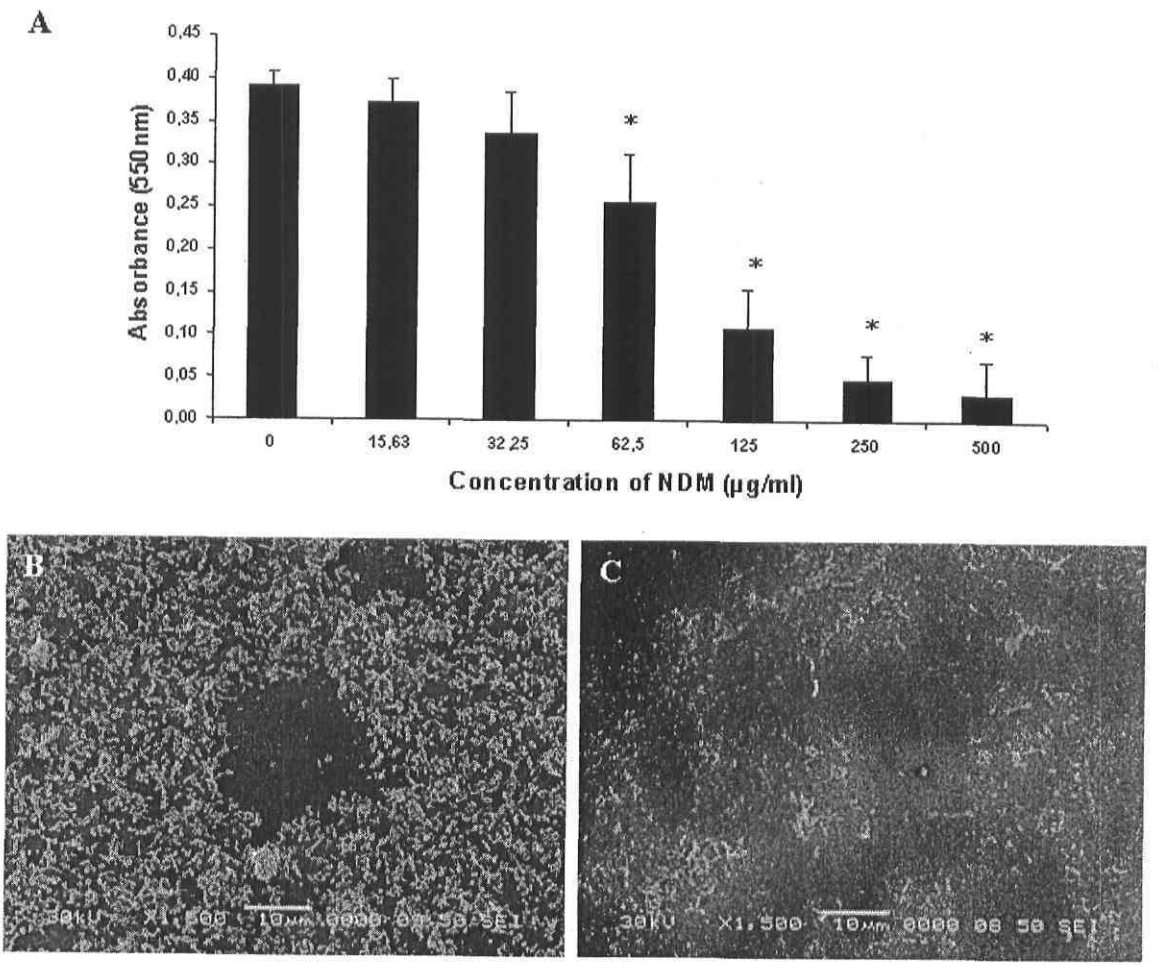

Figure 10.1. Effect of cranberry NDM on biofilm formation by *P. gingivalis*. Polystyrene microplate assay, * significantly different from control at $p < 0.05$ (panel A). Scanning electron photomicrographs of *P. gingivalis* biofilm formed in the absence (panel B) and the presence (panel C) of cranberry NDM at 125 µg/mL, magnification of 1 500 X.

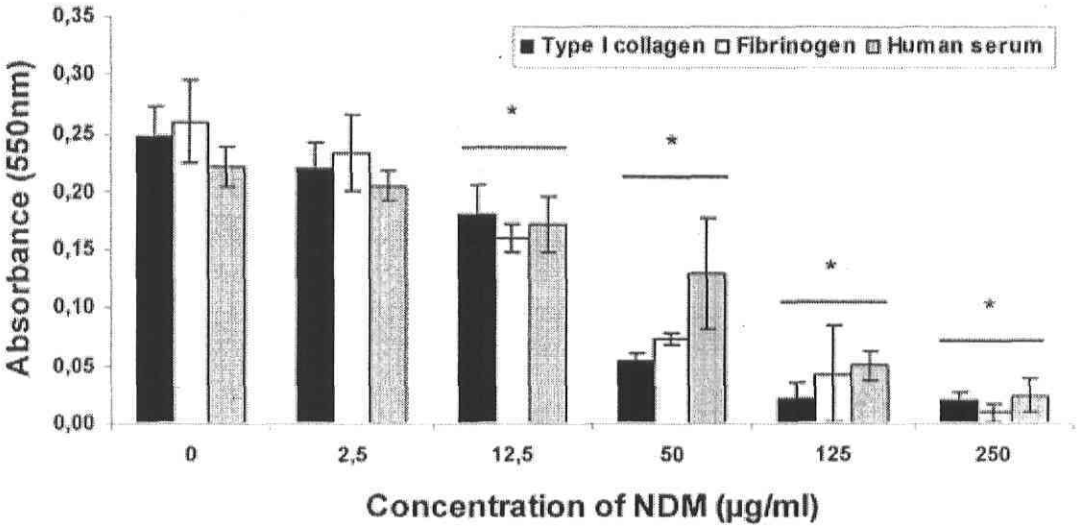

Figure 10.2. Effect of cranberry NDM on attachment of *P. gingivalis* to polystyrene surfaces coated with type I collagen, fibrinogen, and human serum. * Significantly different from control at $p < 0.05$.

CHAPITRE 11

DISCUSSION ET CONCLUSION GÉNÉRALE

Il est maintenant établi que l'initiation et la progression des parodontites dépend fortement de la virulence des bactéries parodontopathogènes impliquées et du niveau de la réponse immuno-inflammatoire de l'hôte (Kornman *et al.*, 1997; Reynolds and Meikle, 1997; Okada and Murakami, 1998; O'Brien-Simpson *et al.*, 2004; Holt and Ebersole, 2005). Les parodontites sont reconnues comme des infections polymicrobiennes et de nombreuses études désignent les espèces bactériennes du complexe rouge (*P. gingivalis*, *T. denticola* et *T. forsythia*) comme fortement impliquées dans la pathogénicité de ces maladies (Socransky *et al.*, 1998; Holt and Ebersole, 2005; Ximenez-Fyvie *et al.*, 2006). La réponse de l'hôte à ces bactéries résulte de trois types d'interactions principales : i) les interactions entre les bactéries parodontopathogènes, ii) les interactions entres les différents types de cellules de l'hôte et iii) les interactions entres les cellules de l'hôte et les bactéries parodontopathogènes. Afin de prendre en considération ces divers types d'interactions, cette étude a utilisé des modèles constitués de différents types de cellules de l'hôte, ainsi que des stimulations avec des combinaisons de bactéries parodontopathogènes. De plus, la contribution de facteurs locaux à la réponse de l'hôte, tels que la concentration d'hémoglobine, a été prise en considération. Par ailleurs, cette étude a mis en évidence la capacité d'une fraction de canneberge enrichie en proanthocyanidines à inhiber divers mécanismes pathogéniques contribuant à la parodontite.

Dans un premier temps, un modèle de co-culture de cellules épithéliales et de macrophages, deux lignées cellulaires majeures intervenant dans la réponse immuno-inflammatoire de l'hôte, a été développé. Ce modèle a été infecté par différentes souches de *P. gingivalis*. Les résultats de ces expériences ont permis de mettre en évidence que *P. gingivalis* possède la capacité d'induire la production de cytokines (IL-1β et IL-6) et de chimiokines (IL-8 et RANTES) pro-inflammatoire par les cellules de l'hôte mais également de dégrader massivement ces médiateurs inflammatoires résultant ainsi en une modulation de la réponse de l'hôte. L'induction et la dégradation des cytokines dépendent fortement de la dose bactérienne utilisée. À une forte concentration bactérienne, *P. gingivalis* dégrade massivement les cytokines suggérant qu'il pourrait ainsi contourner la réponse de l'hôte, créant une rupture des gradients de chimiokines et limitant les réactions inflammatoires locales à proximité de la plaque bactérienne (Figure 11.1). À une faible concentration bactérienne, *P. gingivalis* induit la production de médiateurs pro-inflammatoires suggérant que cet organisme pourrait favoriser la réponse immunodestructive de l'hôte dans les tissus parodontaux (Figure 11.1). Ce modèle de compartimentation de la réponse inflammatoire est supporté par d'autres résultats de notre étude. En effet, l'utilisation d'un modèle *ex-vivo* de sang complet a mis en évidence que *P. gingivalis* induit la production d'une forte quantité de médiateurs inflammatoires dans une population mixte de leucocytes. De plus, des infections mixtes du modèle de co-culture de cellules épithéliales et de macrophages par des combinaisons de bactéries du complexe rouge ont mis en évidence que *P. gingivalis*, *T. denticola* et *T. forsythia* agissent de concert pour induire la sécrétion de médiateurs pro-inflammatoires et de MMP-9 par les cellules de l'hôte. Ces résultats appuient l'hypothèse que *P. gingivalis* ainsi que les autres espèces bactériennes du complexe rouge pourraient favoriser la réponse immunodestructrice de l'hôte. Par ailleurs, nous avons rapporté la forte capacité de *T. denticola* à dégrader la chimiokine RANTES. Récemment, Miyamoto et *al.* (2006) ont mis en évidence la dégradation de l'IL-1β, de l'IL-6 et du TNF-α par la dentilisine de *T. denticola*. *T. denticola*, en association avec *P. gingivalis*, pourrait ainsi contribuer à la dégradation des cytokines à proximité de la plaque sous-gingivale. Ce phénomène pourrait notamment réduire l'afflux des leucocytes phagocytaires vers la plaque parodontopathogénique et favoriser ainsi la prolifération des bactéries parodontopathogènes. Les bactéries du complexe rouge pourraient ainsi

augmenter leur proportion relative dans la plaque sous-gingivale tout en générant une réaction inflammatoire intense dans les tissus parodontaux profonds.

Au cours de notre étude, nous avons caractérisé la production de médiateurs inflammatoires et de MMP-9 induite par des infections mixtes avec les espèces bactériennes du complexe rouge dans un modèle de co-culture de cellules épithéliales et de macrophages. Les résultats obtenus n'ont pas mis en évidence d'effet synergique sur la réponse inflammatoire lors d'infections polymicrobiennes. Pourtant, plusieurs études avaient rapporté des effets synergiques de virulence dans des modèles animaux entre *P. gingivalis* et *T. denticola* (Kesavalu *et al.*, 1998; Kimizuka *et al.*, 2003) ainsi qu'entre *P. gingivalis* et *T. forsythia* (Takemoto *et al.*, 1997; Yoneda *et al.*, 2001). Une production plus importante d'IL-1β, d'IL-6 et de TNF-α a même été observée lors d'infections avec *P. gingivalis* et *T. denticola* comparée à des infections avec *P. gingivalis* seul dans un modèle de pneumonie chez la souris (Kimizuka *et al.*, 2003). Les relations écologiques étroites mises en évidence entre les bactéries du complexe rouge, notamment les relations nutritionnelles (Grenier, 1992c; Nilius *et al.*, 1993; Yoneda *et al.*, 2005), et d'adhésion (Grenier, 1992a; Yao *et al.*, 1996; Hashimoto *et al.*, 2003; Ikegami *et al.*, 2004) ou la formation d'un biofilm synergique entre *P. gingivalis* et *T. denticola* (Kuramitsu *et al.*, 2005; Yamada *et al.*, 2005) pourraient expliquer les effets obtenus dans des modèles animaux. En effet, ces relations suggèrent que les espèces du complexe rouge seraient capables de coloniser l'hôte plus facilement lorsqu'elles sont inoculées ensemble. Cette affirmation est supportée par une étude récente démontrant que les cellules entières et les vésicules membranaires de *P. gingivalis* augmentent la capacité de *T. forsythia* à adhérer aux cellules épithéliales et à les envahir (Inagaki *et al.*, 2006). De par l'ensemble de ces interactions, les bactéries du complexe rouge se retrouveraient en plus grand nombre, provoquant une réponse inflammatoire plus importante que si elles étaient inoculées individuellement. Nos résultats suggèrent que les effets synergiques de virulence observés antérieurement entre *P. gingivalis*, *T. denticola* et *T. forsythia* ne seraient pas liés à une induction synergique de médiateurs inflammatoires. La destruction accrue des tissus parodontaux observée dans les sites contenant les trois espèces du complexe rouge (Socransky *et al.*, 1998; Kasuga *et al.*, 2000; Lopez *et al.*, 2004; Ximenez-Fyvie *et al.*,

2006) pourrait être expliquée par les relations écologiques favorisant la croissance et la survie à long terme de ces bactéries qui exprimeraient ainsi plus largement leur potentiel pathogénique.

La première partie de cette étude a caractérisé le rôle des bactéries du complexe rouge, notamment de *P. gingivalis*, dans la stimulation de la sécrétion de divers médiateurs inflammatoires par les cellules de l'hôte. Bien que les bactéries parodontopathogènes semblent déterminantes dans l'induction de la réponse immunodestructive de l'hôte, certains facteurs de l'hôte modulent de manière importante cette réponse. En effet, cette étude a mis en évidence que l'hémoglobine agissait de manière synergique avec le LPS des bactéries parodontopathogènes pour augmenter la sécrétion de cytokines pro-inflammatoires par les macrophages. Ainsi, un taux élevé d'hémoglobine dans le parodonte favoriserait la progression des parodontites. *P. gingivalis* pourrait contribuer à augmenter le taux d'hémoglobine dans le parodonte. En effet, les gingipaïnes de *P. gingivalis* sont connues pour augmenter la perméabilité vasculaire en activant le système kallikréine-kinine et perturber la coagulation sanguine en dégradant le fibrinogène, la fibrine et le facteur X (Imamura, 2003), favorisant ainsi le saignement des sites de parodontites. Par ailleurs, la culture de *P. gingivalis* en présence de sang augmente fortement la virulence de cette bactérie dans un modèle d'abcès murin (Kesavalu *et al.*, 2003). Récemment, il a été démontré que le taux d'hémine modifiait la structure du lipide A des lipopolysacharides de *P. gingivalis*, un phénomène qui pourrait avoir un effet significatif sur la réponse de l'hôte à cette bactérie (Al-Qutub *et al.*, 2006). Des changements même mineurs de la structure du lipide A peuvent en effet produire des effets importants sur la réponse de l'hôte (Dixon and Darveau, 2005). Ainsi le saignement des poches parodontales qui est fréquent lors des parodontites module à la fois la réponse inflammatoire de l'hôte et la virulence de *P. gingivalis*. Des facteurs comme le stress, l'usage du tabac ou des facteurs génétiques sont connus pour favoriser la progression des parodontites (Van Dyke and Sheilesh, 2005), les taux d'hémoglobine et d'hémine pourraient également représenter des facteurs influençant fortement le déclenchement et la progression des parodontites.

Diverses cibles thérapeutiques potentielles ont été suggérées pour le développement de nouveaux traitements pour les parodontites. La fraction de canneberge NDM qui est enrichie en polyphénols, notamment en proanthocyanidines, a montré une forte capacité à inhiber divers mécanismes pathogéniques des parodontites (Figure 11.2). Cette fraction a l'intérêt d'exercer des effets inhibiteurs contre les deux facteurs considérés déterminants pour l'initiation et la progression des parodontites : les bactéries parodontopathogènes et la réponse de l'hôte. Cette fraction a l'avantage de ne pas posséder de propriétés antibiotiques, agissant en inhibant la formation du biofilm et l'attachement aux surfaces de *P. gingivalis* ainsi que les activités protéolytiques des bactéries du complexe rouge. La fraction NDM serait ainsi peu propice au développement de mécanismes de résistance chez les bactéries parodontopathogènes contrairement aux antibiotiques utilisés dans certains cas pour traiter les parodontites. Les effets observés sur les bactéries parodontopathogènes pourraient être liés aux proanthocyanidines de haut poids moléculaire. En effet, la capacité du jus de canneberge à inhiber l'adhérence d'*E. coli* aux cellules épithéliales du tractus urinaire a été attribuée aux proanthocyanidines (Foo *et al.*, 2000; Howell *et al.*, 2005). Des résultats préliminaires récents présentés à des congrès suggèrent que les proanthocyanidines seraient les polyphénols de la canneberge les plus efficaces pour inhiber la formation d'un biofilm de *P. gingivalis* et les activités protéolytiques de *P. gingivalis* et *T. denticola* (Gafner *et al.*, 2006; Yamanaka *et al.*, 2006). On ne peut cependant pas exclure que d'autres polyphénols interviennent dans les effets observés, la complexité de la fraction NDM étant probablement impliquée dans la diversité des effets observés. La fraction NDM dérivée de la canneberge inhibe également la production de nombreux médiateurs inflammatoires (cytokines, chimiokines et prostaglandines) et de MMPs (MMP-3 et MMP-9) impliqués dans les destructions tissulaires et osseuses observées au cours des parodontites. Cette activité pléitropique suggère une grande efficacité potentielle pour limiter les processus immunodestructeurs. Les médiateurs inflammatoires et les MMPs exerçant également des fonctions biologiques essentielles à l'homéostasie des tissus parodontaux, il est important de ne pas réprimer l'expression de ces médiateurs d'une manière excessive lors d'un traitement complémentaire. En effet, il a été rapporté que l'usage d'un inhibiteur à large spectre de l'activité des MMPs entraînait une augmentation de la résorption osseuse induite par ligature chez le rat (Bjornsson *et al.*, 2004). Par ailleurs, bien que la cytotoxicité du

NDM soit à priori faible, cette fraction pouvant d'ailleurs protéger les fibroblastes et les macrophages de la cytotoxicité induite par des composantes membranaires de bactéries parodontopathogènes (appendice 1), des études utilisant des modèles animaux pourraient être requises pour valider son usage en vue d'une application clinique. Bien que des effets prometteurs ont été obtenus *in vitro* avec la fraction NDM, la dose thérapeutique, le mode d'application locale, la durée d'usage et l'efficacité clinique de cette fraction restent à déterminer pour envisager une application comme traitement complémentaire des parodontites. D'autres études ont également rapporté des effets bénéfiques des polyphénols de la canneberge sur les bactéries responsables de la carie dentaire (appendice 1) suggérant ainsi que les constituants de la canneberge possèdent un fort potentiel d'application thérapeutique pour la prévention et le traitement des maladies buccodentaires.

En résumé, cette étude a permis de caractériser la capacité de *P. gingivalis* à moduler la production de médiateurs inflammatoires dans des modèles pluricellulaires (modèle de co-culture de cellules épithéliales et de macrophages et modèle *ex vivo* de sang complet). L'utilisation de ces modèles offre l'avantage de prendre en considération les interactions entre les différents types de cellules de l'hôte qui surviennent *in vivo* et ainsi d'avoir une vision plus complète et précise des interactions hôte/*P. gingivalis*. Cette étude contribue à une meilleure compréhension de la capacité de *P. gingivalis* à moduler la réponse immunodestructrice de l'hôte par la diversité des types cellulaires utilisés et des médiateurs inflammatoires investigués. De plus, les stimulations polymicrobiennes du modèle de co-culture avec les bactéries du complexe rouge ont permis de mettre en évidence la capacité de *T. denticola* et *T. forsythia* à agir de concert avec *P. gingivalis* pour favoriser cette réponse immunodestructrice. Cette étude a démontré également que l'augmentation de la concentration d'hémoglobine dans les tissus parodontaux pourrait être un facteur critique impliqué dans la réponse inflammatoire excessive de l'hôte par son action synergique avec le LPS des bactéries parodontopathogènes. Par ailleurs, cette étude a mis en évidence diverses propriétés intéressantes de la fraction de canneberge NDM. Ainsi, l'inhibition des MMPs et les puissants effets anti-inflammatoires des polyphénols de la canneberge en font des candidats intéressants pour le développement de nouveaux traitements pour certains types de cancer et diverses maladies inflammatoires comme la

polyarthrite rhumatoïde. Les nombreux effets bénéfiques de cette fraction de canneberge sur les mécanismes pathogéniques de la carie dentaire et de la parodontite (annexe 1) mettent en évidence un fort potentiel d'application dans le domaine de la santé buccale. Par exemple, la fraction NDM pourrait être incorporée dans un bain de bouche ou une pâte dentifrice pour la prévention de la carie dentaire et des parodontites. Cette fraction pourrait aussi être incorporée dans un gel ou des bandelettes résorbables pour une application locale dans la poche parodontale comme traitement complémentaire des parodontites. Néanmoins, des études cliniques sont nécessaires pour valider l'efficacité de ces approches et les effets bénéfiques potentiels de la fraction NDM observés *in vitro*.

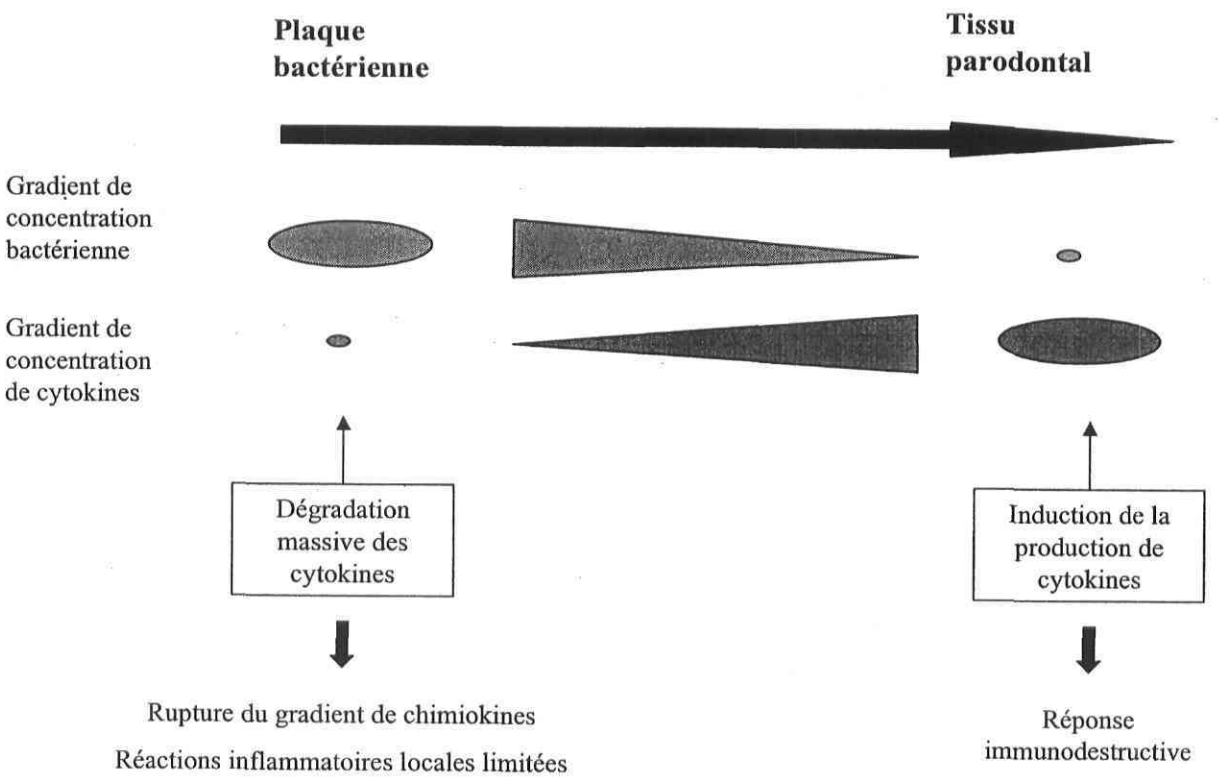

Figure 11.1. Modèle de la modulation des quantités de cytokines par *P. gingivalis*.

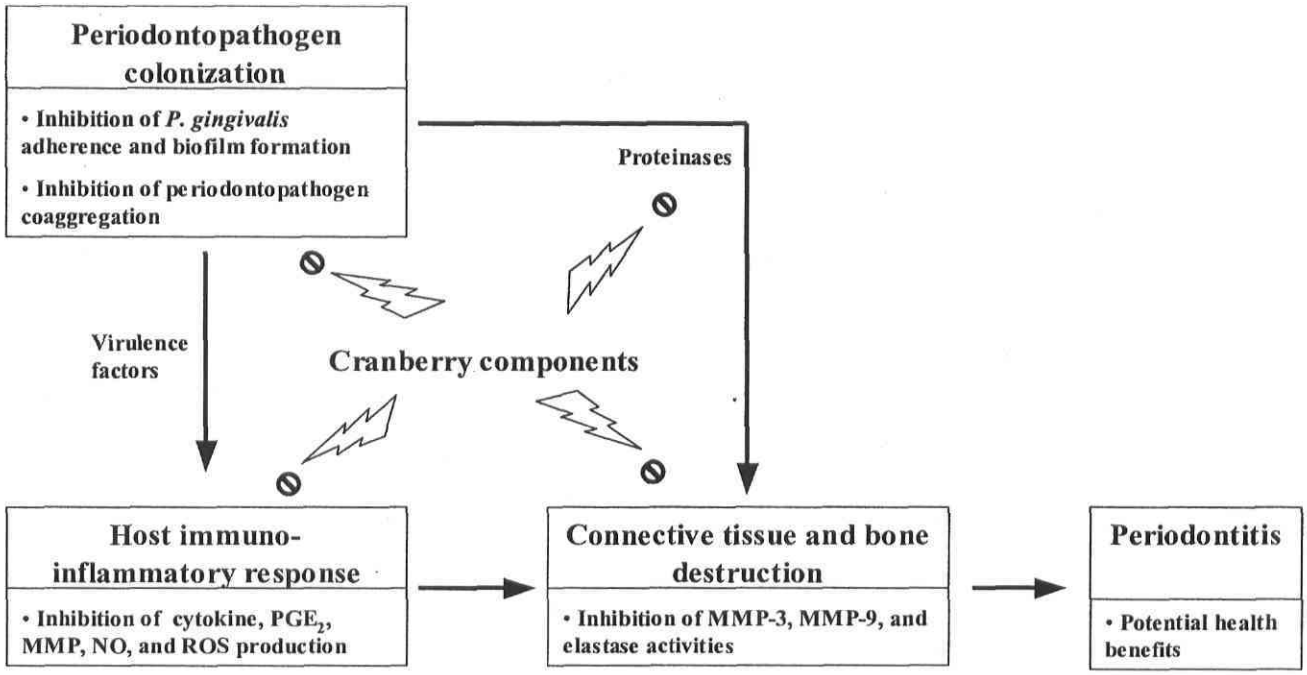

Figure 11.2. Effets bénéfiques potentiels des polyphénols de la canneberge sur les mécanismes pathogéniques contribuant à l'initiation et au développement des parodontites. (Figure tirée de la revue de littérature présentée en annexe 1).

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APPENDICE 1

Potential Oral Health Benefits of Cranberry

ABSTRACT

Dental researchers have been paying increasing attention to cranberry extracts in recent years. The potential benefits of cranberry juice in reducing oral diseases, including dental caries and periodontitis, are discussed in this review. Cranberry juice has an inhibitory effect on the attachment and biofilm formation of oral streptococci involved in dental caries. A non-dialysable fraction enriched in high-molecular-weight polyphenols prepared from cranberry juice concentrate has very promising properties with respect to cariogenic and periodontopathogenic bacteria as well as the host inflammatory response and enzymes that degrade the extracellular matrix. Cranberry components are potential anti-caries agents given that they inhibit acid production by *Streptococcus mutans*, affect glucan-binding proteins, and reduce both bacterial hydrophobicity and polysaccharide production by cariogenic streptococci. Concerning periodontitis, cranberry components inhibit host inflammatory responses, the production and activity of enzymes that cause the destruction of the extracellular matrix, the production of biofilms, the adherence of *Porphyromonas gingivalis*, and the proteolytic activities and the coaggregation of periodontopathogens. The effects enumerated above suggest that cranberry components have a promising potential as bioactive molecules for the prevention and/or treatment of oral diseases.

INTRODUCTION

The cranberry *Vaccinium macrocarpon* is one of three native North American fruits, the others being concord grapes and blueberries, that grow in the wild from the Carolinas to Canada. The fruit of the cranberry is widely consumed in various food products, including fresh and dried fruit, sauces, and juices, as well as in powder form in capsules and tablets. Cranberry extracts are a uniquely rich source of bioactive polyphenolic compounds, notably flavonoids, and possess biological properties that may provide human health benefits. Ripe fruit were used by Native Americans to treat bladder and kidney ailments. Therapeutic applications of cranberries documented during the 17th century include the relief of blood disorders, stomach ailments, liver problems, vomiting, appetite loss, scurvy, and cancer (Siciliano, 1996). Recently, cranberry extracts have been receiving increasing attention in various areas of health research, including infectious and noninfectious diseases.

Cranberry juice has been a focus of interest for their beneficial effects in preventing urinary tract infections (Howell, 2002; Raz *et al.*, 2004). In the past decade, *in vitro* and clinical studies have focused on certain microbial infections and specific cranberry components. High molecular mass proanthocyanidins (condensed tannins) from cranberry juice appear to inhibit the adhesion of uro-pathogenic fimbriated *Escherichia coli* to uroepithelial cells in the urinary tract (Howell *et al.*, 2005). A high molecular weight cranberry fraction, apparently different from tannins, has also been reported to inhibit the adherence of P-fimbriated uropathogenic *E. coli* as well as sialic acid-specific adherence of *Helicobacter pylori* to human gastric mucosa, a critical step in gastric ulcer development (Burger *et al.*, 2000; Burger *et al.*, 2002; Ofek *et al.*, 1991; Shmuely *et al.*, 2004; Zafriri *et al.*, 1989). These *in vitro* observations have been supported by clinical studies showing that regular consumption of cranberry juice can suppress urinary tract infections in women (Stothers, 2002) and *H. pylori* infections in endemically affected populations (Zhang *et al.*, 2005). Cranberry extracts also have an inhibitory effect on influenza virus adhesion and infectivity (Weiss *et al.*, 2005) and exert a fungistatic effect on dermatophytic and other

fungi, although they have no effect on the oral pathogenic fungi *Candida albicans* (Swartz and Medrek, 1968).

In addition to the effects on infectious agents, cranberry extracts inhibit the proliferation of human oral, colon, prostate, and breast cancer cell lines (Seeram *et al.*, 2004; Sun and Hai Liu, 2005). In addition, cranberry extracts have potential beneficial effects on cardiovascular diseases (Chu and Liu, 2005; Reed, 2002; Ruel *et al.*, 2005; Ruel *et al.*, 2006). Dental researchers have been paying increasing attention to the very promising effects of cranberry extracts on cariogenic and periodontopathogenic bacteria, as well as on host inflammatory responses in recent years. This article reviews the potential beneficial effects of cranberry components on oral health.

CHEMICAL COMPOSITION AND BIOACTIVE COMPOUNDS OF CRANBERRY

Early studies established that cranberry juice inhibits the adhesion of *E. coli* and that the urine of mice fed cranberry juice possesses anti-adhesion activity (Schmidt and Sobota, 1988; Sobota, 1984). Different components may be responsible for the anti-adhesion activity. Zafriri *et al.* (1989), using 12-14 kDa molecular weight cut-off dialysis membranes, first reported that most of the anti-adhesion activity in cranberry juice cocktail is in the form of high molecular weight, non-dialysable materials (NDM). Other studies showed that oligomeric proanthocyanidins (PAC) inhibit the adhesion of P-fimbriated *E. coli* (Foo *et al.*, 2000; Howell *et al.*, 2005). Cranberry PAC is unique because the oligomeric molecules are of the A type, whereas the PAC of most other fruits are of the B type, which is devoid of anti-adhesion activity. The cranberry NDM was prepared from an Ocean Spray juice concentrate (50% Brix) following extensive dialysis. NDM from other commercial juices (e.g., grape, guava, and pineapple) do not possess anti-adhesion activities (Ofek *et al.*, 1991). Although the precise nature of NDM is not known, there is no doubt that it is a molecular species distinct from PAC, as exemplified by differences in solubility in various solvents, in molecular weight, and in mass spectroscopic and NMR spectra

(Table 1). NDM and PAC also have different oxygen:hydrogen:carbon ratios. NDM has a ratio (%) of 2:41.4:56.6 (Ofek *et al.*, 1996), whereas the PAC monomer has a ratio 4.2:62.5:33.3 as deduced from the active structure described by Foo *et al.* (2000).

Further analysis performed by Ocean Spray Inc. revealed that the NDM preparation is devoid of detectable sugars and acids and contains only 0.35% anthocyanin compounds (Bodet *et al.*, 2006a). We found that it is also devoid of nitrogen (Ofek *et al.*, 1996). NMR and MALDI-TOF analyses of NDM have, thus far, not provided an interpretable spectrum. Whereas PAC can be eluted from columns with organic solvents, NDM usually remains bound to Sephadex columns. The bound material can be eluted with distilled water. After dialysis (12,000 MW cut-off), a fraction called PF-1 is obtained that exhibits strong anti-adhesion activity. The NMR spectrum of PF-1 is identical to that of NDM, with very broad unresolved peaks at 6-7.5 mhZ, unlike most proanthocyanidins, which have sharp peaks typical of phenolic compounds (Vvedenskaya *et al.*, 2004). MALDI-TOF analyses have not improved the resolution of the structure of NDM or PF-1, while mild acid hydrolysis of NDM does not yield any low molecular weight compounds (Ofek *et al.* unpublished observations), unlike PAC (Foo *et al.*, 2000). A more recent analysis of NDM revealed that it contains 65% PAC-like material as determined by the colorimetric method of Cunningham *et al.* (2002), which is 125-times higher than in concentrated juice (Bodet *et al.*, 2006a), suggesting that the procedure for obtaining NDM selectively retains these compounds. It is reasonable to assume that at least two-thirds of NDM is composed of PAC in a unique molecular form that is distinct from oligomeric PAC (e.g., tannins) and that exhibits potent anti-adhesion activity. Further studies aimed at resolving the high molecular weight components of NDM are required to resolve such a complex structure. Irrespective of its structure, the rather simple procedure used to obtain NDM from cranberry juice concentrate in an edible form makes NDM very useful as a supplement to various products to promote oral health.

CRANBERRY NDM AND DENTAL CARIES

Microscopic observations of *in situ* dental plaque samples have revealed that there is intimate contact between the vast majority of bacteria in biofilms. In some areas of the oral cavity, biofilms may be composed of several hundred layers of bacteria. Dental plaque is formed when saliva bathes tooth surfaces, forming conditioned surfaces or pellicles (Abbott and Hayes, 1984; Leach, 1970). The pellicle serves as a substratum for the adhesion of the so-called first colonizers. Streptococci and actinomycetes are the predominant early colonizers of tooth surfaces (Nyvad and Kilian, 1990). They apparently attach, via adhesin-receptor interactions (Gibbons *et al.*, 1991) or hydrophobic interactions (Weiss *et al.*, 1982), to macromolecules selectively adsorbed to the enamel. The attached bacteria proliferate, giving rise to microcolonies, the most prominent of which are composed of mutans streptococci, including *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus criceti*, and *Streptococcus rattus*, all of which are frequently associated with carious lesions. Aggregation and accretion of late colonizing bacteria to the temporary bacterial population attached to the enamel occurs mainly via cell-to-cell interactions, a phenomenon known as coaggregation. The late colonizers include a wide range of bacterial species that are considered periodontal pathogens, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Treponema denticola*, all of which interact with each other and with the early colonizers on the tooth surface to form a stable, mature dental biofilm.

There is convincing evidence that the formation of plaque on tooth surfaces is a prerequisite for dental caries. Dental caries is the direct result of the activity of bacteria inhabiting dental biofilms, especially when the biofilms are allowed to reach a critical mass due to poor oral hygiene. It is broadly accepted that regular oral hygiene reduces total bacterial mass and significantly diminishes the severity and frequency of dental caries. Recently, much research has been directed at identifying nontoxic edible compounds that affect the formation of dental biofilms by inhibiting either the adhesion of mutans streptococci to tooth surfaces or the coaggregation of oral bacteria. The high molecular

weight components of cranberry described above appear to inhibit the adhesion of and biofilm formation by mutans streptococci as well as the coaggregation of a considerable number of oral streptococcal species. Moreover, salivary mutans streptococcal counts are reduced significantly in volunteers using mouthwash supplemented with the high molecular weight components from cranberry juice. In the following section, we will summarize these studies and discuss the potential of using functional foods to prevent dental caries.

Inhibition of mutans streptococci adhesion and biofilm formation on hydroxyapatite

Since enamel surfaces are composed of hydroxyapatite, saliva-treated hydroxyapatite beads are often used to study the adhesion and biofilm formation by early colonizers, especially mutans streptococci. Yamanaka *et al.* (2004) tested the effect of 25% cranberry juice on the adhesion of a battery of oral streptococci to saliva-coated hydroxyapatite surfaces. They reported a significant inhibitory effect ranging from 80-95% on biofilm formation by *S. sobrinus* 6715, *S. mutans* JC2, *S. criceti* E49, *Streptococcus sanguinis* ATCC 10556, *Streptococcus oralis* ATCC 10557, and *Streptococcus mitis* ATCC 9811. Weiss *et al.* (2004) studied the effect of cranberry NDM on the adhesion of *S. sobrinus* 6715 to saliva-coated hydroxyapatite in the presence of sucrose. They reported that NDM has a significant inhibitory effect on biofilm formation by *S. sobrinus*, which is more prominent in the presence of sucrose. The effect is dose-dependent, with a 90% inhibition observed at 130 μg NDM/ml. Using the cariogenic strain, *S. mutans* UA 159, Duarte *et al.* (2006) found that other cranberry fractions (125 μg flavonols/ml and 500 μg proanthocyanidins/ml) also significantly affect the development of biofilms on saliva-coated hydroxyapatite surfaces via a sucrose-dependent mechanism. On the other hand, anthocyanins, another cranberry juice component, have a limited effect on bacterial adhesion. The reduction in biofilm mass and bacterial adhesion reported in these studies was not due to an antibacterial effect since, at the concentrations used, there was no significant effect on bacterial viability. This is particularly interesting since the objective of anti-adhesion therapies to fight dental caries is to inhibit the biofilm without affecting the viability of the bacteria so as to minimize the emergence of resistant strains. The anti-

biofilm phenomenon is unique to cranberry, making it a potential candidate for shifting bacterial populations without affecting their viability.

There is extensive documentation showing that the most potent mechanism regulating biofilm formation and adhesion by mutans streptococci in the oral cavity is sucrose dependent (Liljemark and Bloomquist, 1996; Steinberg, 2000). This mechanism involves the synthesis of extracellular polysaccharides by the extracellular enzymes glycosyltransferase (GTF) and fructosyltransferase (FTF). Glucans (glucose polymers) and fructans (fructose polymers) are formed rapidly from sucrose by immobilized and non-immobilized GTF and FTF (Hannig *et al.*, 2005; Steinberg *et al.*, 1996). Both Steinberg *et al.* (2004) and Duarte *et al.* (2006) have proposed that the inhibition of this sucrose-dependent adhesion mechanism by cranberry is dependent on the inactivation of GTF and FTF. Indeed, it has been shown that cranberry extracts significantly inhibit the activities of these enzymes. It is interesting to note that the inhibition depends on whether these cell-free enzymes are immobilized on a surface or are in a soluble form. NDM has less of an effect on these enzymes when they are immobilized on hydroxyapatite (Steinberg *et al.*, 2004). This differential inhibitory effect may be due to the poor diffusion of NDM into the deep layers of the polysaccharide film that is formed by the immobilized enzymes. Since the structural configuration of the enzymes may change upon immobilization, it cannot be ruled out that conformational changes may also render the enzymes less susceptible to NDM.

Glucans formed by cell-free GTF immobilized on tooth surfaces act as bacterial binding sites. The adhesion of streptococci to glucans via glucan-binding proteins (GBP) on the membranes of streptococci is one of the mechanisms by which bacteria attach to the biofilm (Banas and Vickerman, 2003). Koo *et al.* (2006) examined the ability of 25% cranberry juice to inhibit the adhesion of *S. mutans* to saliva- or glucan-coated hydroxyapatite surfaces and found that pre-coating surfaces with cranberry juice results in a statistically significant reduction in adhesion. Cranberry blocks bacterial adherence to

glucan-binding sites in the salivary pellicle coating the tooth enamel by 40-85% and reduces the mass of the biofilm.

While the production of polysaccharides and GBP-binding proteins is impaired by cranberry juice, oral bacteria have other mechanisms, such as hydrophobic interactions, that enable them to attach to the tooth surface and that may play an important role, especially in the initial adhesion of bacteria (Weiss *et al.*, 1982). Indeed, Yamanaka *et al.* (2004) found that cranberry juice significantly reduces the hydrophobicity of oral streptococci. This effect may augment the inhibitory effect of cranberry juice on biofilm formation.

Preventing the formation of biofilms on oral surfaces is the ultimate goal of dental surfaces caries therapy. This may be achieved by blocking bacterial adhesion with NDM. However, as biofilms are constantly being formed on the tooth surface, the disruption of existing biofilms is also of great clinical importance. Steinberg *et al.* (2005) tested the ability of NDM to induce *S. sobrinus* detachment from biofilms composed of conditioned films formed from immobilized cell-free GTF or FTF in the presence or absence of sucrose. In the presence of NDM, they observed extensive desorption of bacteria from GTF biofilms in the absence of *in situ* formation of glucans (no sucrose was added). The desorption effect was less pronounced in biofilms formed in the presence of sucrose. A similar trend was observed for FTF biofilms. NDM reduces bacterial adhesion to biofilms, lowers the mass of biofilms, and decreases polysaccharide production. This results in deformed biofilms that are thinner and less dense than biofilms not exposed to NDM (Steinberg *et al.*, 2005).

Once a dental biofilm is formed, its effect on dental is mainly due to the acidogenicity of the bacteria residing in the biofilm. The rapid production of organic acids by cariogenic bacteria such as mutans streptococci is the main cause of tooth enamel demineralization and cavity formation. Duarte *et al.* (2006) observed that cranberry PAC inhibits acid production by *S. mutans*. While the pH remains below the critical value of ~ 5.5 at which extensive demineralization occurs, the inhibition of acid production may still contribute to the anti-caries effect of cranberry.

As summarized in Figure 1, it appears that cranberry may affect supra-gingival biofilm formation and influence the tooth decay process either by affecting glucan/fructan formation or by affecting the GBP. In addition, it may lower the hydrophobicity of the bacteria, further reducing their capacity to adhere. The ability of cranberry to decrease acid production is yet another mean by which it may act as an anti-caries agent.

Inhibition of intergeneric bacterial coaggregation

The stability of dental plaque relies on bacterial adhesion to an acquired pellicle and on interspecies adhesion or coaggregation. Coaggregation interactions are probably the most important factor allowing bacteria to withstand both the mechanical forces and the salivary flow that tend to dislodge dental plaque. Coaggregation can be assessed *in vitro* by mixing bacterial suspensions from two different genera. A positive coaggregating pair results in the formation of large aggregates that settle to the bottom of a test tube within seconds after mixing, leaving a clear supernatant (Kolenbrander *et al.*, 1993). This test has been used for the rapid screening and identification of suitable coaggregation partners and for the isolation of mutants lacking adhesins and/or receptors (Weiss *et al.*, 1987a; Weiss *et al.*, 1987b). Weiss *et al.*, (1998) reported that a high molecular weight cranberry fraction reversed the coaggregation of 49 of 84 coaggregating bacterial pairs tested. It acted preferentially on pairs in which one or both members were Gram-negative anaerobes frequently involved in periodontal diseases. This anti-coaggregating cranberry component thus has the potential to alter the subgingival microbiota and to enable the conservative control of gingival and periodontal diseases.

Weiss *et al.* (2004) assessed the effect of a mouthwash supplemented with NDM on oral hygiene. Following six weeks of daily usage of cranberry-containing mouthwash by an experimental group ($n = 29$), we found that the salivary mutans streptococci count and the total bacterial count were reduced significantly (ANOVA, $P < 0.01$) compared with the control group ($n = 30$), who used a placebo mouthwash (Weiss *et al.*, 2004). No change in

the amount of dental plaque or gingival indices was observed. *In vitro*, cranberry inhibits the adhesion of *S. sobrinus* to saliva-coated hydroxyapatite. Findings to date suggest that the reduction of mutans streptococci counts *in vivo* results from the anti-adhesion activity of cranberry.

CRANBERRY NDM AND PERIODONTAL DISEASES

Periodontal diseases are multifactorial infections that are caused by a specific group of Gram-negative anaerobic bacteria and that lead to the destruction of tooth supporting tissues, including the alveolar bone and the periodontal ligament. Two major factors are involved in the pathogenesis of periodontitis. The first is the microbial factor, notably the accumulation of periodontopathogenic bacteria in subgingival plaque, which damage periodontal tissue via the molecules they produce, including proteolytic enzymes (Eley and Cox, 2003; O'Brien-Simpson *et al.*, 2004). The second is the host response to periodontopathogens, notably the over-production of inflammatory mediators (pro-inflammatory cytokines, matrix metalloproteinases and prostanoids), which are critical for the initiation and progression of periodontitis (Birkedal-Hansen, 1993; Offenbacher *et al.*, 1993; Okada and Murakami, 1998). Cranberry fractions have been reported to have beneficial inhibitory effects on these two factors.

Effects on periodontopathogens

Bacterial colonization

The colonization of subgingival sites by periodontopathogens is a critical step in the initiation of periodontal disease. The capacity of periodontopathogens to form biofilms and to express adhesins, which allow them to adhere to host cells, tooth surfaces, basement membrane components, and oral bacteria, play a major role in periodontitis (Rosan and Lamont, 2000). Much evidence points to *P. gingivalis* as the key pathogen in chronic

periodontitis (Haffajee and Socransky, 1994). The cranberry NDM fraction, which is composed of high molecular weight polyphenols, is a potent inhibitor of biofilm formation by *P. gingivalis*, but does not affect the growth or viability of the bacteria (Labrecque *et al.*, 2006). This cranberry fraction also inhibits the attachment of *P. gingivalis* to various proteins such as type I collagen, fibrinogen, and human serum (Labrecque *et al.*, 2006). In addition, as discussed above, cranberry NDM inhibits the coaggregation of many oral bacteria, acting notably on bacterial pairs in which one or both members are Gram-negative anaerobes frequently involved in periodontal diseases (Weiss *et al.*, 1998; Weiss *et al.*, 2002). These findings suggest that cranberry may reduce the capacity of periodontopathogens to colonize subgingival sites. In addition, the fact that cranberry NDM acts by preventing bacterial adhesion rather than by inhibiting growth may be an advantage in that it reduces the development of resistant bacteria.

Proteolytic enzymes

Members of the red complex, which include *P. gingivalis*, *T. forsythia*, and *T. denticola*, are suspected periodontopathogens that are closely linked to clinical measures of periodontitis, particularly pocket depth and bleeding on probing (Kasuga *et al.*, 2000; Socransky *et al.*, 1998). The strong proteolytic activities of bacteria of the red complex are an important factor that contributes to periodontal tissue destruction through a variety of mechanisms, including direct tissue degradation and host inflammatory response modulation (Eley and Cox, 2003; Grenier, 1995; Grenier, 1996; Holt and Ebersole, 2005; Imamura, 2003). The cranberry NDM fraction affects periodontopathogen proteinases by dose-dependently inhibiting the gingipain (both Arg- and Lys-gingipain) and dipeptidyl peptidase IV activities of *P. gingivalis*, the trypsin-like activity of *T. forsythia*, and the chymotrypsin-like activity of *T. denticola* (Bodet *et al.*, 2006d). It also blocks the ability of *P. gingivalis* to degrade native proteins, including type I collagen and transferrin (Bodet *et al.*, 2006d). This suggests that NDM has the potential to reduce the multiplication of *P. gingivalis*, *T. forsythia*, and *T. denticola* in periodontal pockets, since their growth relies on the availability of amino acids and peptides. In addition, NDM may reduce the tissue destruction mediated by proteinases produced by these bacterial species. Yamanaka *et al.*

(2006) provided additional proof of the inhibitory effect on periodontopathogen proteinases by showing that cranberry polyphenol fractions have an inhibitory effect on *P. gingivalis* gingipain and *T. denticola* chymotrypsin-like activities. Of the three fractions tested (anthocyanin, proanthocyanidin, and flavonol), the proanthocyanidin fraction was the most effective whereas the anthocyanin fraction was the least. It has been suggested that inhibitors of periodontopathogen proteinases may reduce bacterial pathogenicity and therefore are considered potentially new therapeutics agents for periodontal diseases (Grenier *et al.*, 2002; Kadowaki *et al.*, 2004; Song *et al.*, 2003). Thus, cranberry components exhibit promising properties against periodontopathogens proteinases.

Effects on host responses

Anti-inflammatory properties of cranberry

The continuous, high production of cytokines, including interleukin-1β (IL-1β), IL-6, IL-8, and tumor necrosis factor alpha (TNF-α), by host cells triggered by periodontopathogens is thought to be responsible for the periodontitis-associated destruction of tooth-supporting tissues (Okada and Murakami, 1998). It has been reported that cranberry contains molecules with anti-inflammatory properties. Cranberry polyphenols reduce TNF-α-induced up-regulation of various inflammatory mediators in human microvascular endothelial cells (Youdim *et al.*, 2002). Bodet *et al.*, (2006a) recently reported that the high molecular weight cranberry fraction inhibits the production of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and chemokines (IL-8 and RANTES) by macrophages stimulated by lipopolysaccharides (LPS) from *E. coli* and major periodontopathogens, including *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum* subsp. *nucleatum*, *P. gingivalis*, *T. denticola*, and *T. forsythia*. Protoanthocyanidins may be responsible for this effect since they were 125-times more concentrated in the high molecular weight fraction than in whole cranberry juice, which exhibited no anti-inflammatory properties (Bodet *et al.*, 2006a). The cranberry NDM

fraction also inhibits soluble RANKL-induced macrophage differentiation into osteoclasts, suggesting that cranberry components such as NDM may reduce alveolar bone resorption (unpublished data). Furthermore, NDM may diminish the release of free radicals associated with periodontal tissue destruction. Indeed, NDM decreases nitric oxide and reactive oxygen species production and inducible nitric oxide synthase expression by LPS-stimulated macrophages (Chandad *et al.*, submitted). The LPS-induced IL-6, IL-8, and prostaglandin E_2 (PGE_2) responses of gingival fibroblasts are inhibited by cranberry NDM (Bodet *et al.*, 2006b), which may act by inhibiting gingival fibroblast intracellular signaling proteins, leading to a downregulation of activating protein-1 (AP-1), a major transcriptional factor of pro-inflammatory genes. Cranberry can also reduce the expression of cyclo-oxygenase 2, an enzyme involved in PGE_2 production. The above findings indicate that cranberry may limit the inflammatory reactions of both gingival fibroblasts and macrophages elicited by periodontopathogens. Active compounds that are endowed with a capacity to modulate host inflammatory responses are receiving considerable attention because they may give rise to new therapeutic agents and approaches for treating periodontal diseases (Paquette and Williams, 2000). Cranberry constituents appear thus to have a high potential for the development of a new anti-inflammatory therapeutic approach.

Cranberry NDM may also protect host cells from the toxicity of periodontopathogen components. It significantly enhances the viability of gingival fibroblasts cultivated for five days in the presence of *A. actinomycetemcomitans* LPS (Bodet *et al.*, 2006b) and protects macrophages from the cell toxicity induced by cell wall components of *T. denticola* and *Peptostreptococcus micros* (unpublished data).

Inhibition of host tissue-degrading enzymes

Much evidence points to host-derived matrix metalloproteinases (MMPs) as key destructive enzymes in periodontal disease (Kinane, 2000). Excessive MMP activity is a hallmark of human periodontal disease leading to gingival collagen loss, periodontal ligament degradation, and alveolar bone resorption. The high molecular weight cranberry

fraction inhibits LPS-induced MMP-3 and MMP-9 production by both gingival fibroblasts and macrophages (Bodet *et al.*, 2006c), and cranberry components appear to affect the phosphorylation and expression of various intracellular fibroblast proteins implicated in MMP production. The NDM fraction may act by reducing AP-1 activity, which regulates MMP gene expression. In addition, enzymes, including MMP-3, MMP-9 and elastase, involved in extracellular matrix destructions are also efficiently inhibited by low concentrations of cranberry NDM. Interestingly, sub-antimicrobial doses of doxycycline, which downregulates MMP activity, is indicated as an adjunctive treatment for periodontitis and confers clinical benefits on patients with periodontitis (Preshaw *et al.*, 2004). Cranberry NDM, by inhibiting both the production and activity of MMPs, may be a novel potential therapeutic option for treating periodontitis.

Potential for periodontal disease treatment

To prevent the initiation and progression of periodontal disease, oral hygiene measures, mechanical debridement, surgery, and antimicrobial pharmaceutical agents are used to reduce the subgingival biofilm. While these procedures are effective in managing the majority of patients with periodontitis, conventional therapy does not always achieve the desired clinical outcome. The chronic nature of periodontitis implies that clinicians should continuously monitor their patients and use preventive treatment approaches. The management or prevention of periodontal disease in high risk individuals (smokers, diabetics, individuals with a genetic predisposition) requires either strict bacterial control or combinations of bacterial control and adjunctive treatments, such as host modulators. Various approaches are envisaged to develop new adjunctive treatments for periodontitis, including modulating MMPs, prostanoids, cytokines, and NOS activity and inhibiting periodontopathogen proteinases (Kadowaki *et al.*, 2004; Kornman, 1999; Salvi and Lang, 2005; Song *et al.*, 2003). Cranberry components have interesting inhibitory properties against many pathogenic mechanisms involved in the initiation and development of periodontal diseases (Figure 2). Interestingly, combination therapy involving a subantimicrobial doxycycline dose with non-steroidal anti-inflammatory drugs synergistically suppresses enzymes involved in extracellular matrix destruction in the

gingiva of chronic periodontitis patients (Lee *et al.*, 2004). This suggests that treatments targeting multiple pathogenic mechanisms involved in periodontal diseases may be a successful therapeutic approach. The development of strategies as adjuncts to conventional therapy for individuals with a substantially increased risk for periodontitis may potentially involve local applications of NDM to modulate the host response, inhibit enzymes involved in extracellular matrix destruction, and attenuate periodontopathogen virulence and proliferation. Cranberry components thus appear to be promising candidates for the development of novel adjunctive treatments for periodontal diseases.

CONCLUSION

The cranberry NDM fraction disrupts the pathogenic mechanisms of dental caries and periodontitis. While components isolated from cranberry juice may help fight these diseases, the high dextrose and fructose content of commercially available cranberry juice makes it unsuitable for oral hygiene use. On the other hand, recent research suggests that cranberry components possess promising potential for use as supplements for improving oral health. For example, the cranberry NDM fraction might be incorporated into mouth rinses or toothpastes to prevent oral diseases, including dental caries and periodontitis. NDM may also be added to gels or strips for local application in periodontal pockets to treat periodontitis. To validate the efficacy of these approaches and the beneficial effects of the high molecular weight components of cranberry juice observed *in vitro*, clinical studies are required.

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Table 1. Properties of cranberry NDM and PAC

Property	Cranberry Fraction NDM[a]	PAC[b]
Concentration in cranberry juice cocktail	1.5 mg/ml	2.9 mg/ml
Concentration required to inhibit the adhesion of		
P-fimbriated *E. coli*	23.5 μg/ml	60 μg/ml
mutans streptococci	130 μg/ml	500 μg/ml
P. gingivalis biofilm	62.5 μg/ml	NT
Oral bacteria coaggregation	200-500 μg/ml	NT
Concentration required to inhibit the enzymatic activity of		
S. mutans GTF or FTF	1000 μg/ml	NT
Periodontopathogen proteases	10-100 μg/ml	NT
Concentration required to inhibit acid production by *S. mutans*	NT	500 μg/ml
Taste	Not astringent	Astringent
Solubility (2 mg/ml) in[b]		
H_2O	Soluble	Soluble
80% (v/v) Me_2CO	Not soluble	Soluble
DMSO	Not soluble	Not soluble
1% HOAc (in MeOH)	Not soluble	Soluble
Dialysis (12,000 MW cut-off)	Not dialyzable	Dialyzable
NMR spectrum or MALDI-TOF	No visible protons	Sharp peak

NT: not tested , GTF and FTF: Glucosyltransferase and Fructosyltransferase

[a], Data from Zafriri *et al.* (1989), Ofek *et al.* (1991; 1996), Weiss *et al.* (1998), Bodet *et al.* (2006a), and Labrecque *et al.* (2006), unless otherwise stated

[b], Data from Howell *et al.* (2005), Foo *et al.* (2000) and Duarte *et al.* (2006) and Dixon *et al.* (2005), unless otherwise stated

[c], Used as eluants from fractogel columns (Ofek *et al.*, unpublished observations)

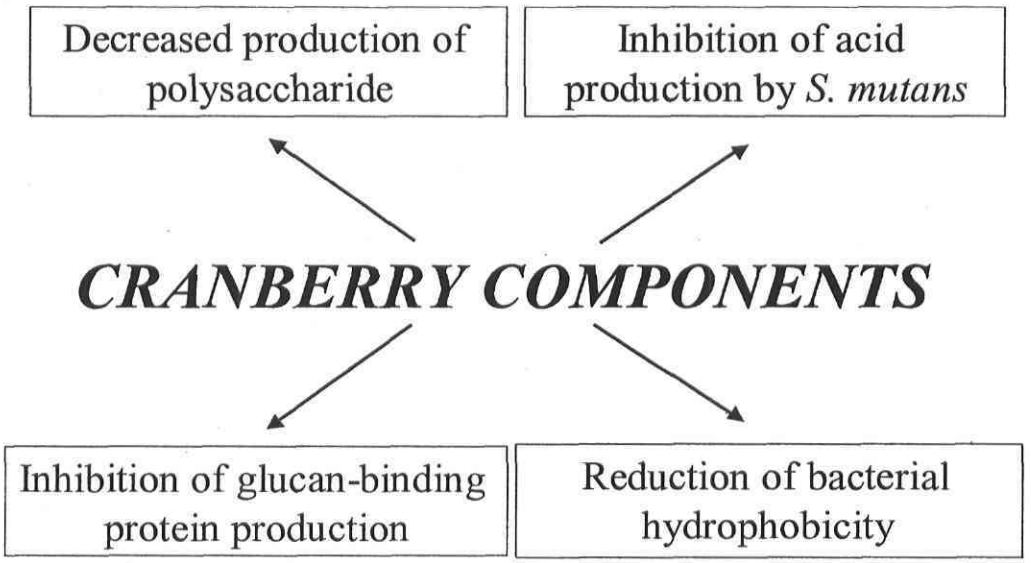

Figure 1. Properties indicating that cranberry components have an anti-caries effect

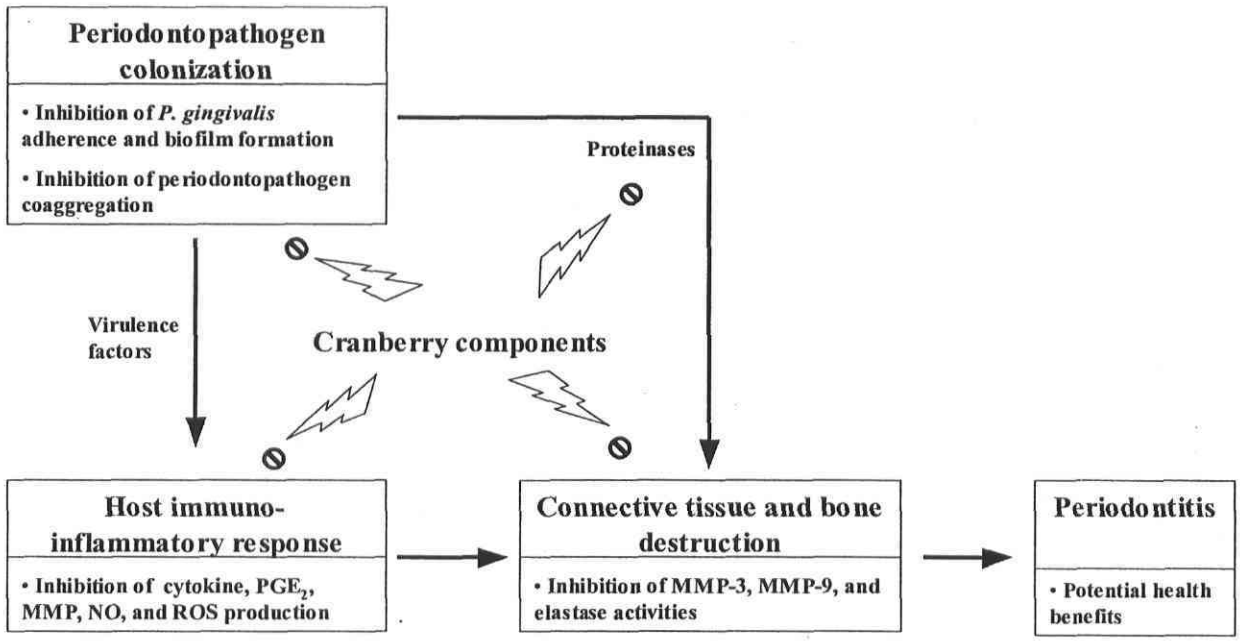

Figure 2. Potential beneficial effects of cranberry components in disrupting the pathogenic mechanisms involved in the initiation and development of periodontitis