

**IMMUNOPROTECTION ASSAYS AGAINST RAT DENTAL
CARIES USING *Streptococcus sobrinus* VIRULENCE-
ASSOCIATED IMMUNOMODULATORY PROTEIN AS A
TARGET ANTIGEN**

MÁRCIA DALILA BOTELHO DINIS

Dissertação de Doutoramento em Ciências Biomédicas

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Dissertação de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutora Paula Ferreira, Professor Associado do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Co-orientador – Doutora Delfina Tavares, Professora Auxiliar do Instituto de Ciências Biomédicas Abel Salazar e Investigadora do Instituto de Biologia Molecular e Celular da Universidade do Porto.

Co-orientador – Doutor António Coutinho, Director do Instituto Ciência Gulbenkian e Professor Catedrático da Faculdade de Medicina da Universidade de Lisboa.

To my parents: Ana e Augusto

To my brothers: Lita e Hugo

To my nephews: André...and others on the way

*Posso ter defeitos, viver ansioso e ficar irritado algumas vezes,
Mas não esqueço que a minha vida é a maior empresa do mundo.
E que posso evitar que ela vá à falência.
Ser feliz é reconhecer que vale a pena viver,
Apesar de todos os desafios, incompreensões e períodos de crise.
Ser feliz, é deixar de ser vítima dos problemas
E se tornar autor da sua própria história.
É atravessar desertos fora de si,
Mas ser capaz de encontrar um oásis no recôndito da sua alma.
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Ser feliz é não ter medo dos próprios sentimentos.
É falar de si mesmo.
É ter coragem de ouvir um "não".
É ter segurança para receber uma crítica, mesmo que injusta.
Pedras no caminho?
Guardo todas, um dia vou construir um castelo...*

(Fernando Pessoa)

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People say, statistically speaking, that acknowledgments have the most read chapter of any thesis. I believed that any person likes to meet the human character behind the scientific work. At this moment I think it is easier to write science than express the feelings in my heart. I apologize to those who can understand, but I am going to write this part in my mother language.

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“ O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis ” (Fernando Pessoa).

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Dinis M, Tavares D, Fonseca AJMM, Faria R, Ribeiro A, Silvério Cabrita AM and Ferreira P. (2004) Therapeutic vaccine against *Streptococcus sobrinus*-induced caries. *J Dent Res* 83: 354-8.

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No cumprimento do Decreto-Lei mencionado supra, o autor desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação e discussão dos resultados e na sua redacção.

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ABSTRACT

Dental caries is a chronic infectious disease widespread in industrialized countries and becoming also prevalent in the world's poorest developing countries. In spite of the intensive investigation dedicated to the development of a vaccine against this disease, none is so far available. *Streptococcus mutans* and *Streptococcus sobrinus* are known as the primary pathogens responsible for dental caries in humans.

Previous studies developed by Arala-Chaves and collaborators showed that some pathogens secrete proteins that were designated as virulence-associated immunomodulatory proteins (VIP) taking into account their immunobiological effects. These proteins interact with host immune system in a way that ultimately causes the suppression of specific immune response achieved through an early production of IL10 and/or by inducing a lymphocyte polyclonal activation. Moreover, they facilitate host microbial colonization therefore acting as a virulence factor for the producing microbe. Furthermore, it was shown that the immunoneutralization of the VIP effects through the immunization with these proteins confers host protection to the infection caused by the producing microbe. The bacterium *S. sobrinus* also produces a VIP, which was identified as enolase. Based on N-terminal protein sequencing, the respective gene was cloned and the recombinant protein (rEnolase) was obtained purified.

In the present thesis we first showed that rEnolase facilitates *S. sobrinus* oral colonization in Wistar rats that had as a consequence an increase in dental caries lesions in these animals. Therefore, we performed several immunoprotective assays in order to assess whether immune neutralization of rEnolase effects on the host could result in protection against rat dental caries.

The obtained results showed that therapeutic immunization with VIP confers protection against *S. sobrinus* induced-dental caries in a rat model.

Next we also showed the protective effect of oral therapeutic immunization with rEnolase against *S. sobrinus* induced-dental caries in Wistar rats. Moreover, since enolase is a ubiquitous protein, the putative cross-reactivity with human enolase of the antibodies raised by bacterial enolase immunization was also investigated. The therapeutic vaccination with rEnolase significantly reduced the cariogenic lesions induced by *S. sobrinus* colonization. The immunization protocol used induced high levels of salivary IgA and IgG antibodies specific for rEnolase. This result is directly correlated with a reduction in the number of bacteria recovered from the oral cavity of the immunized rats as well as with the reduction on the extent of dental caries

observed in these animals. Moreover, the rEnolase vaccination did not caused any detected harmful physiological effect on rats.

Since the homology between *S. sobrinus* and *S. mutans* enolase is very high (approximately 98%), we hypothesized that antibodies produced against one of these proteins also recognize the other. Therefore, to address this question a protocol for *S. mutans* infection in the rat model was established. Using this model, we investigated whether therapeutic immunization with rEnolase could protect the animals against *S. mutans* induced-dental caries. A reduction on dental lesions on rEnolase-immunized animals infected with *S. mutans* was observed comparatively with sham-immunized animals infected with the bacterium. These results strongly suggested that rEnolase is a good candidate for a vaccine against dental caries.

A preventive approach was also performed here. However, due to the so-called, “window of infectivity” preventive protocols are more difficult to achieve than the therapeutic ones. In fact, in rat caries models there is a discrete period of time, between days 19 and 22 after birth, to implant the mutans streptococci in the oral cavity of laboratory rats. The results, although preliminary, clearly showed a reduction on dental caries lesions in rEnolase-immunized rats as compared with sham-immunized controls.

We furthermore investigated whether maternal vaccination with rEnolase could protect their offspring from dental caries by passively transferred immunity. The results showed the induction of immune protection against dental caries by maternal rEnolase vaccination in wistar rats born from these mothers. In our studies, immunity conferred by breast milk was also evaluated. The obtained results showed that rats born from rEnolase-immunized dams were more protected from dental caries, even when nursed by sham-immunized control dams, than rats born from sham-immunized dams and breastfeed from rEnolase-immunized dams. These data indicates that transplacental transfer is likely to be the major mechanism of acquisition of maternal antibodies.

There is some controversy about the development of a dental caries vaccine and the immunization protocols to be assayed in children, since we are dealing with a non lethal disease. Thus, it is necessary to carefully evaluate the risks involved for the child, and the safety of these vaccines. One possible solution could encompass maternal immunization and our results clearly showed that offspring from rEnolase immunized-mothers present a significant reduction on dental caries lesions.

Overall, the results presented in this thesis indicate that rEnolase could be a promising and safe candidate for testing in vaccination trials against dental caries in human.

SUMÁRIO

A cárie dentária é uma doença infecciosa crónica prevalente em países industrializados e que começa a ser também um problema em países subdesenvolvidos. Apesar da intensa investigação dedicada ao desenvolvimento de uma vacina contra esta doença não existe ainda nenhuma disponível comercialmente. *Streptococcus mutans* e *Streptococcus sobrinus* são conhecidos como os principais agentes patogénicos responsáveis pela cárie dentária.

Arala-Chaves e colaboradores, em estudos anteriores mostraram que alguns agentes patogénicos produzem proteínas que foram designadas por proteínas imuno-moduladoras associadas à virulência do microrganismo (VIP) com base nos seus efeitos imunobiológicos. Estas proteínas interagem com o sistema imune do hospedeiro de modo a induzirem a supressão da resposta imune específica conseguida através da produção de IL-10 e/ou pela indução de uma activação linfocitária policlonal. Assim, estas proteínas facilitam a colonização do microrganismo no hospedeiro, funcionando por isso como um factor de virulência para o micróbio que as produz. Foi ainda demonstrado que imunização com estas proteínas resulta na protecção do hospedeiro contra as infecções pelos micróbios que as produzem.

S. sobrinus também produz uma VIP, que foi identificada como sendo uma enolase. Com base na sequenciação N-terminal da proteína, o respectivo gene foi clonado e a proteína recombinante (rEnolase) foi purificada.

Na presente dissertação começou-se por mostrar que a rEnolase favorece a colonização de *S. sobrinus* na cavidade oral de ratos Wistar. De seguida, vários ensaios de imuno-protecção foram desenvolvidos com intuito de investigar se a imuno-neutralização dos efeitos de rEnolase, através da imunização com esta proteína, no hospedeiro resultava em protecção contra a cárie dentária induzida em ratos.

Os resultados obtidos demonstraram que a imunização terapêutica com VIP ou com a rEnolase induz protecção contra a cárie dentária induzida por *S. sobrinus* em ratos Wistar. De facto, a vacinação com a VIP ou com a rEnolase reduziu significativamente as lesões cariogénicas induzidas pela colonização com *S. sobrinus*. Esta protecção foi devida à presença de anticorpos IgA e IgG na saliva, específicos para a VIP ou para a rEnolase nos animais vacinados. Atendendo a que a enolase é uma proteína ubíqua, foi ainda estudado a possível reactividade cruzada entre os anticorpos produzidos contra a enolase bacteriana e a enolase humana. Os resultados obtidos mostraram que não há reactividade cruzada. A avaliação histopatológica efectuada nos animais imunizados com a rEnolase mostrou ainda a inocuidade desta vacina.

Devido existir uma elevada homologia (~ 98%) entre a enolase de *S. sobrinus* e *S. mutans*, fomos verificar se a vacinação terapêutica com a rEnolase conferia protecção contra a cárie dentária induzida por *S. mutans* em ratos Wistar. Uma redução das lesões cariogénicas foi observada nos animais vacinados comparativamente aos animais controlos. Estes resultados sugerem fortemente que o antígeno rEnolase é um bom candidato para uma vacina contra a cárie dentária.

Foi também estabelecido um protocolo preventivo. No entanto, devido à existência da chamada “janela de infectividade”, que consiste no período durante o qual é possível colonizar a cavidade oral do rato, os protocolos preventivos são difíceis de obter comparativamente aos terapêuticos. Nos modelos de rato existe um período discreto de tempo, entre os dias 19 e 23 após nascimento, onde é possível colonizar com *mutans streptococci* a cavidade oral de ratos de laboratório. Os resultados, embora preliminares, demonstraram claramente uma redução das lesões cariogénicas nos ratos imunizados com rEnolase quando comparados com os animais controlos.

Adicionalmente foi ainda investigado se a vacinação das mães com rEnolase protegia a descendência da cárie dentária. Os resultados mostraram que as mães vacinadas com a rEnolase antes da gravidez protegiam a descendência da cárie dentária induzida em ratos Wistar com o *S. sobrinus*. Foi ainda avaliada a imunidade conferida pelo leite materno. Os resultados obtidos mostraram que os ratos provenientes de mães imunizadas com rEnolase estavam mais protegidos contra a carie dentária, mesmo quando amamentados por mães controlos, comparativamente a ratos provenientes de mães controlos e amamentados por mães imunizadas com rEnolase. Estes resultados indicam que a transferência de imunidade via placenta é o mecanismo mais provável para explicar a protecção observada.

Existe alguma controvérsia em relação ao desenvolvimento de uma vacina contra a cárie dentária e protocolos de imunização experimentais em crianças, dado que não se trata de uma doença mortal. Como tal, é necessário avaliar cuidadosamente os riscos envolvidos para a criança e a segurança dessas vacinas. Uma possível solução poderia passar pela imunização das mães. Os resultados por nós obtidos mostram que a vacinação das mães com rEnolase antes da gravidez é capaz de proteger a descendência contra a cárie dentária.

Por tudo isto, a rEnolase poderá ser um seguro e promissor candidato contra a cárie dentária e ser testado em ensaios clínicos em humanos.

RÉSUMÉ

La carie dentaire é une maladie infectieuse, chronique qui domine les pays industrialisés et qui commence également à être un problème des pays sous-développés. Nonobstant, l'intense investigation dédiée au développement d'un vaccin contre cette maladie, il n'en existe aucun commercialisé. *Streptococcus mutans* et *Streptococcus sobrinus* sont connus comme être les principaux agents pathogéniques responsables de la carie dentaire.

Arala-Chaves et collaborateurs, lors d'un recherche antérieur révèlent que quelques agents pathogéniques produisent des protéines dénommées protéines immuno-modulateur qui sont associées à une virulence du micro-organisme (VIP) sur le fondement de l'effet immunobiologique. Ces protéines interfèrent avec le système immunitaire de l'hôte de manière à introduire la suppression de la réponse spécifique réussie par la production de IL-10 et/ ou par l'induction d'une activation poly-clonale des lymphocytes. Ainsi, ces protéines facilitent la colonisation du micro-organisme chez l'hôte, et sont finalement des facteurs de virulence pour les microbes qui les produisent. Il a été, aussi, démontré que l'immunisation par ces protéines conduisent à la protection de l'hôte contre les infections du micro-organisme qui les produisent. *S. sobrinus* produit, également, une VIP qui a été identifiée comme enolase. Suite à la séquence du N-Terminal de la protéine, le respectif gène a été cloné et la protéine recombinant (rEnolase) a été purifiée.

Cette dissertation commence, ainsi, par démontrer que la rEnolase favorise la colonisation du *S. Sobrinus* dans la cavité orale des rats Wistar. Plusieurs essais d'immuno-protection ont été développés pour vérifier si l'immuno-neutralisation des effets de l'rEnolase, qui est dû à l'immunisation avec cette protéine chez l'hôte, conduit à une protection de la carie dentaire introduit dans les rats.

Les résultats obtenus ont démontré que l'immunisation thérapeutique avec la VIP ou la rEnolase conduisent à une protection de la carie dentaire introduite par *S. sobrinus* dans les rats Wistar. En effet, la vaccination avec la VIP ou avec l'rEnolase réduit de manière significative les lésions provoquées par la carie induite après la colonisation pour *S. sobrinus* dans les rats Wistar. Cette protection est due à la présence d'anticorps IgA et IgG dans la salive, spécifique de la VIP ou de la rEnolase chez les animaux vaccinés. Une fois que, l'enolase est une protéine doué d'ubiquité, une investigation a été également faite sur une possible réaction croisée entre les anticorps produit contre l'enolase bactérienne et l'enolase humaine. Les résultats obtenus ont

démontré l'absence de réaction croisée. Une recherche histo-pathologique faite à des animaux immunisés avec l'rEnolase a démontrée encore l'innocuité de ce vaccin.

Dû à l'existence d'une haute homologie (~98%) entre l'énolase de *S. sobrinus* et *S. mutans*, nous avons investigué si la vaccination thérapeutique avec l'rEnolase conférait une protection contre la carie dentaire introduite par *S. mutans* dans des rats Wistar. Nous avons constaté une réduction des lésions provoquées par la carie chez les animaux objets de vaccination en comparaison à l'animal témoin. Ces résultats sont révélateurs par le fait que l'antigène rEnolase est un bon candidat pour la vaccination contre la carie dentaire.

Il a été également établi un protocole préventif. Toutefois, et en raison de l'existence de ce que l'on appelle « la fenêtre de l'infection » qui consiste dans la période où c'est possible de procéder à colonisation de la cavité orale du rat, les protocoles préventifs sont plus difficiles à obtenir si l'on fait la comparaison avec les thérapeutiques. Dans les protocoles avec le rat, il est possible durant un court laps de temps, entre le dix-neuvième et vingt-troisième jour qui suit la naissance, de procéder à la colonisation de la cavité orale des rats de laboratoire à l'aide de mutants streptococci. Les résultats préliminaires obtenus permettent de conclure qu'il existe une réduction des lésions de la carie chez les rats immunisés avec la rEnolase en comparaison avec les animaux témoins.

Une autre investigation a été faite pour savoir si la vaccination des mères avec la rEnolase permettait de protéger la descendance contre la carie dentaire. Les résultats obtenus, démontrent que les mères vaccinées avec la rEnolase avant la grossesse protégeaient la descendance de la carie dentaire introduite chez les rats Wistar avec le *S. sobrinus*. Il a été aussi investigué l'immunité conférée par le lait maternel. Les résultats obtenus ont permis de démontrer que les rats qui naissent de mères immunisées par la rEnolase sont davantage protégés contre la carie dentaire, même si allaités par des mères témoins, en comparaison avec des rats qui naissent de mères témoins et allaités par des mères immunisées par la rEnolase. Ces résultats indiquent que le transfert de l'immunisation par le placenta est le mécanisme le plus probable qui explique la protection qui a été observée pendant l'investigation.

Il existe, toutefois, une controverse en relation au développement d'un vaccin contre la carie dentaire et les protocoles d'immunisation expérimentés avec des enfants, dû au fait que la carie dentaire n'est pas une maladie mortelle. Ainsi, il est nécessaire que l'on procède à une évaluation des risques qui peuvent exister pour les enfants, ainsi que sur l'efficacité de ces vaccins. Une solution possible serait de procéder à l'immunisation des mères. Les résultats obtenus démontrent que la vaccination des mères avec la rEnolase, avant la grossesse, est capable de protéger la descendance de la carie dentaire.

Ainsi, la rEnolase pourra être un sérieux candidat contre la carie dentaire et être testé sur des humains, dans les investigations cliniques.

ORGANIZATION OF THE THESIS

The present thesis represents part of the work performed at Immunology laboratory Mario Arala-Chaves, at Department of Immuno-Physiology and Pharmacology, ICBAS, and at Immunobiology Unit, IMBC, University of Porto (Porto), to complete my Ph.D. dissertation. This thesis is organized into seven chapters. The first chapter reviews and introduces the main concepts and current understanding of the dental caries, mucosal immunity and vaccines development against this disease. The second chapter addresses the main goals of this works. In the third chapter, evidence of the role of recombinant enolase from *S. sobrinus* as a virulence factor in *S. sobrinus*-induced dental caries. The fourth chapter and subsections presents the several approaches in order to develop a vaccine against dental caries using immunomodulatory proteins from *S. sobrinus*. The fifth chapter presents the preliminary results obtained in order to study the mucosal immunity associated with dental caries protection. Finally, general conclusions are presented in chapter sixth, unifying the published data and suggesting avenues for future research.

ABBREVIATIONS

AP – Alkaline Phosphatase
APC – Antigen presenting cell
ATCC - American Type Culture Collection
BALT – Bronchus associated lymphoid tissue
BSA – Bovine serum albumin
BSS – Balance Salt Solution
CEP-Ss – Extracellular Crude of *Streptococcus sobrinus*
CFU – Colony Forming Units
DC – Dendritic cells
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
dNTP – Deoxyribonucleotide triphosphate
EDTA – Ethylenediaminetetra-acetate
ELISA - Enzymatic–Linked Immunosorbent Assay
GALT – Gut associated lymphoid tissue
GBP – Glucan Binding Protein
GTF – Glucosyltransferase
i.n. – intranasal
IFN γ - Interferon γ
Ig – Immunoglobulin
IgA - Immunoglobulin A
IgG - Immunoglobulin G
IL – Interleukin
IP – Propidium iodide
I.S. – Immune System
MALT – Mucosal associated lymphoid tissue
MS - Mutans streptococci
NALT – Nasal associated lymphoid tissue
OPD - o-Phenylenediamine
PAMP's – Pathogen-associated molecular patterns
PBS - Phosphate-buffered saline
PC – Plasma cells
PCR – Polymerase Chain Reaction
PE – Peroxidase

PP – Peyer's Patches

PRRs – Pathogen recognition receptor

s.c – subcutaneous

SDS – Sodium dodecyl sulphate

SIgA – Secretory Immunoglobulin A

TAE - Tris-acetate/ EDTA

TBS - Tris-buffered saline

TBST - Tris-buffered saline-tween

TE – Tris-EDTA

TGF- β – Transforming growth factor

TLRs – Toll-like Receptors

Tris - Tris(hidroxymethyl)aminomethane

UV – Ultraviolet

VIP - Virulence Immunomodulatory proteins

CHAPTER I

GENERAL INTRODUCTION

I.1. Dental Caries

I.1.1. General

Dental caries is one of the most prevalent infectious diseases in humans associated with major medical complications (Brooks and Burnie 1994, Hamada *et al.* 1986) and its prevention would greatly benefit children and adolescents, as well as adults, both on a short and a long-term basis. The World Health Organization (WHO) recently published an global overview of oral health, describing the programme's approach to promote further improvement in oral health during the 21st century (Petersen 2003, Petersen 2005). This disease is a multifactorial, ubiquitous and plaque related chronic infection of the enamel caused by specific streptococci, being the *Streptococcus sobrinus* and the *Streptococcus mutans* the primary etiological agents (Hamada *et al.* 1986, Loesche 1986).

Despite the great improvements in the oral health status of populations across the world, the problem persists in both developed and developing countries, particularly among underprivileged groups. Although its prevalence decreased during the last few decades, dental caries continue affecting 60-90% of school children and the vast majority of adults. Even though, several studies show that after the decrease, a plateau was achieved and in some cases an increment of dental caries incidence may be observed. In Asian and Latin American countries dental caries is the most prevalent oral disease. Apparently, in African countries the disease appears to be less severe, due to low dental caries prevalence, but there are some evidences that this fact is changing because sugar consumption increases, inadequate exposure to fluorides, and lack of effective general oral care or preventive oral health systems (Petersen 2003, Petersen 2005).

This infectious disease is currently associated with cardiovascular diseases (Ylostalo *et al.* 2006), infective endocarditis (Nomura *et al.* 2006), diabetes (Hintao *et al.* 2007, Soell *et al.* 2007), obesity (Touger-Decker and Mobley 2007) and periodontal diseases (Takashi *et al.* 2005).

It must be stressed that dental caries is not eradicated; in fact a majority of children have manifest caries when they reach adulthood. This underlines the need for exploring new strategies for caries prevention and therapy.

The WHO, in 1979, at the World Health Assembly adopted a priority resolution, calling oral health for all by the year 2000. Subsequently, in 1981 the WHO adopted as the first global indicator of oral health status an average of not more than 3 DMFT (Decayed, Missing, Filled, Teeth) at the age of 12 years by the year 2000. Therefore,

the global goal average would be no more than 3 DMF teeth at 12 years of age (Report 1982). Even though, the actual caries percentage described in the WHO reports (Petersen 2003, Petersen 2005) is 27% for preschoolers, 42% in school-age children, and 91% in dentate adults having caries experience.

In Portugal tree studies concerning the oral health status have been carried out, one in 1990-1991 (Almeida *et al.* 1990a, Almeida *et al.* 1990b, Almeida *et al.* 1991), other in 1997 (Almeida and Emílio 1997), and most recently in 2003 (Almeida *et al.* 2003). This last report, indicates that the goal of the WHO for the year 2000 (DMFT under 3) has been achieved. However, the multivariate analysis suggests that such programme activities apparently had some limited impact on the prevalence of caries at the population level. Further implementation of school based oral health promotion and application of population-directed preventive strategies are needed in Portugal (Almeida *et al.* 2003). In 2005, the Portuguese Health Ministry promoted a survey on the Portuguese oral health according to the WHO criteria, including dental caries, enamel lesions, oral hygiene status and Community Periodontal Index. The results were presented in the Report of National Promotion of Oral Health from Portuguese Health General Board (*Direcção Geral de Saúde*) in 2007. In this report it was stated an increase in oral health surveillance and in caries lesions treatment between children of 3 to 16 years-old due to an high number of professional dentistry doctors, that is correlated with a decrease in disease incidence (Ferreira Cádima 2007).

I.1.2 Infectious disease

As reported above, dental caries is a transmissible infectious disease in which mutans streptococci (MS) play a major role. The MS, mainly *Streptococcus mutans* and *Streptococcus sobrinus*, are the principal etiological agents of dental caries (Loesche 1986).

Studies using phenotyping and/or genotyping methods strongly suggest that the MS strains are highly conserved within not only mothers and their children but also in the ethnic groups, suggesting vertical transmission of this organism within human populations. The mother is the major primary source of infection for children (Caufield 1997, Caufield *et al.* 1993, Caufield *et al.* 1988, Emanuelsson *et al.* 1998), and saliva is the principal vehicle by which MS transfer may occur (Napimoga 2005).

Dental caries is a chronic disease that progress slowly in most people, this is a multifactorial disease (Figure I.1) that starts with microbiological shifts within the complex biofilm (dental plaque) and is affected by salivary flow and composition,

exposure to fluoride, consumption of dietary sugars and by preventive behaviours (oral hygiene) (Selwitz *et al.* 2007).

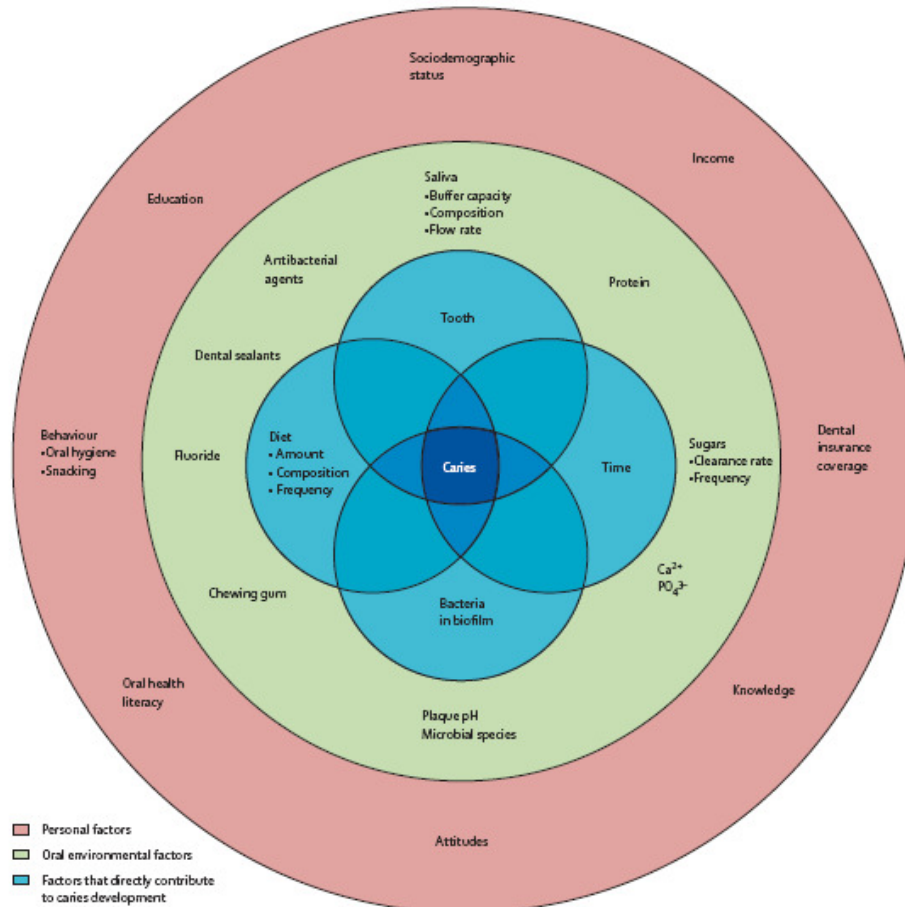


Figure I.1. Illustration of the factors involved in caries development (Selwitz *et al.* 2007).

Therefore, dental caries is a dynamic process, evolving enamel and dentine mineral destruction caused by specific group of streptococcal microorganisms, that occurs in three phases: 1) initial interaction with tooth surface mediated by adhesins; 2) accumulation of the bacteria in a biofilm in the presence of sucrose and glucans synthesised by the bacteria glucosyltransferase; and 3) lactic acid production (Anusavice 2005). The metabolism of various saccharides (including glucose and fructose) by the accumulated bacterial biofilm results in the production and secretion of considerable amounts of the metabolic end-product lactic acid, which can cause de mineralization of the tooth structure when present in sufficient amounts in close proximity to the tooth surface. This is thought to be the third event in the formation of dental plaque, and it eventually results in a carious lesion (that is, in dental caries).

I.1.3. History

Although the dental caries incidence increased after Second World War, the disease has affected humans since ancient time, infecting preferentially adults. In nowadays children are the target (Hanada 2000).

The microbiological study on human dental plaque dates back to 1924 when Clark first observed oral Streptococci (Clarke 1924). The infectious and transmissible nature of dental caries was brought in focus by the studies by Keyes on gnotobiotic rodents in decade of sixties (Keyes 1958). The gram positive bacterium isolated by Clarke was denominated by *Streptococcus mutans*, due to its streptococcus mutant form. Even though, only 35 years later Landmark established that MS were the etiological agents of dental caries and discussed the disease transmissibility (Loesche 1986).

I.1.4. Mutans streptococci

Mutans streptococci (MS) are the main etiological agents of dental caries in humans and are classified in seven species: *S. criceti*, *S. mutans*, *S. rattus*, *S. sobrinus*, *S. macacae*, *S. downei* e *S. ferus*; that can be divided in 8 serotypes (Table I.1).

Table I.1. Serotypes of several *Streptococcus* species

Species	Serotypes	G + C%	Host
<i>S. mutans</i>	c, e, f	36-38%	Primates
<i>S. sobrinus</i>	d, g, h	44-46%	Primates
<i>S. ferus</i>	c	43-45%	Wild rats
<i>S. macaque</i>	c	35-36%	Macaques
<i>S. cricetus</i>	a	42-44%	Hamsters
<i>S. rattus</i>	b	42-44%	Rats

Of these, *S. sobrinus* and *S. mutans* are the most frequently isolated from the human oral cavity (Loesche 1986). Several studies have shown that the prevalence of *S. sobrinus* is more closely related with high caries activity than *S. mutans* (Igarashi *et al.* 2001) and *S. sobrinus* also has a higher acidogenic capacity than the *S. mutans* (De

Soet *et al.* 1991). Moreover, recent research indicates the coexistence of *S. sobrinus* and *S. mutans* is an important factor for dental caries development (Nagashima *et al.* 2007, Seki *et al.* 2006).

I.1.5 Pathogenesis

The first designation of dental caries was: dental caries is a localized, progressive decay of teeth initiated by the demineralization of the outer surface of the tooth due to organic acids produced locally by bacteria that ferment deposits of dietary carbohydrates. With progression loss of the tooth mineral and secondary destruction of the tooth protein by continued bacterial action, cavities form which, if untreated, extend and destroy most of tooth, often leading to serious infection of the surrounding tissues. The development of caries requires critical relationships between tooth surface, oral microbiota and dietary carbohydrate (Scherp 1971).

The MS are not particularly good colonizers of the tooth surface, so their emergence as dominant plaque species needs to be explained. The tooth surface is unique anatomical tissue among body surfaces, crown enamel and root cementum are nonshedding hard surfaces, which selectively adsorbs various acid glycoproteins (mucins) from saliva, forming what is known as the acquired enamel pellicle (AEP). Once teeth erupt into the oral cavity, they are immediately coated with salivary glycoproteins, which act as substrate for subsequent colonization by microorganisms. The growth and co-aggregation of microbes on the surface of teeth result in the development of an adherent biofilm, otherwise known as dental plaque (Ruby and Barbeau 2003).

The MS attach to tooth surface through ionic and electrostatic interaction between the bacteria and saliva constituents, both negatively charged, using a cationic bridge of calcium ions.

There are several rodents' models in order to study the MS colonization (Jespersgaard *et al.* 1999, Tanzer 1979, 1995, Zhang *et al.* 2002). Relationship between dietary sucrose, plaque formation and dental decay can be associated with the presence and absence of various glucosyltransferases (GTF) and their resultant glucans products. The saccharose ingestion promotes the colonies adhesion to dental surface, due to production of glucans products resulting from MS GTF metabolism or the sucrose energy metabolism that results in lactic acid production. The acid production decreases biofilm pH leading to tooth demineralization and dental decay (Loesche 1986).

Generally, as schematic represented in figure I.2, the first microorganism arriving to oral cavity form a tiny pellicle that increases very rapidly due to bacteria growth and glucose extracellular polymers, glucans (Idone *et al.* 2003, Sato *et al.* 2004) produced by bacteria GTF using dietary sugars. These glucans promote the MS and other bacteria aggregation, through the interaction of glucans binding proteins (GBP) present in microorganism's surfaces and oral cavity glucans. The GTF have a domain to bind GBP that enables the glucans binding and promote MS accumulation in dental biofilm (Smith 2002a, Smith *et al.* 2001a, Smith *et al.* 2003, Taubman *et al.* 2001, Taubman *et al.* 1999). Once these microorganisms are present in teeth surface they can metabolize diverse carbohydrates, there as principal energetic source, and produce piruvate that will be transformed in lactic acid, formic acid, ethanol and acetate, there will be an environment acidification that promotes dental decay and inhibition of other bacteria that compete with MS. Since there are able to maintain there neutral intracellular pH, the dental plaque will be rapidly colonized with MS (Ajdic *et al.* 2002, Idone *et al.* 2003, Mitchell 2003).

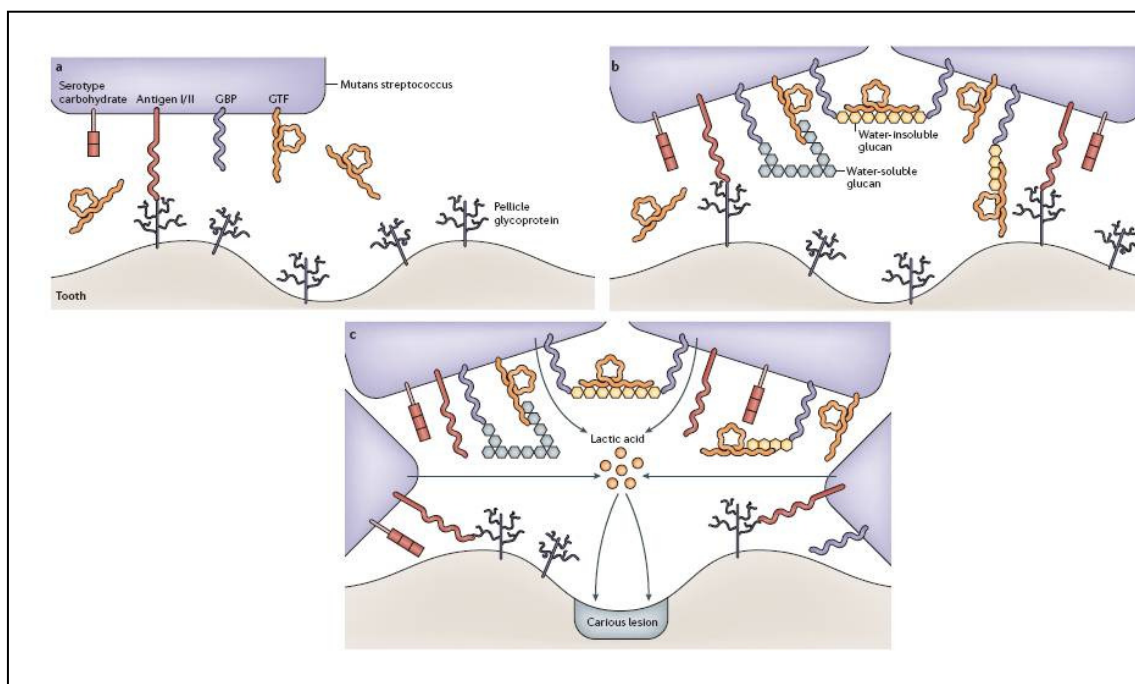


Figure I.2. The molecular pathogenesis of dental caries associated with mutans streptococci. **A)** Initial attachment of mutans streptococci to tooth surface. **B)** Accumulation of mutans streptococci on tooth surfaces in the presence of sucrose. **C)** Acid production by mutans streptococci. The metabolism of various saccharides by accumulated bacterial biofilm results in the production and secretion of considerable amounts of the metabolic end-product lactic acid, causing tooth demineralization (Taubman and Nash 2006a).

I.1.6. Window of Infectivity and caries transmission

Several investigators support that children acquire mutans streptococci (MS) during a very specific period of time, after the first tooth eruption: “no teeth, no mutans streptococci” (Caufield *et al.* 1993). It seems likely that majority of children acquire MS around 2 years-old, being 75% colonized between 19 and 23 months after birth, This is what has been described as “window of infectivity”, as shown in Figure I.3. The mothers are the vehicle of transmission, since they harbour the bacteria in there oral cavity and because the intense contact with their infants, mothers were the logical infant bacteria source (Caufield 1997, Caufield *et al.* 1993, Li and Caufield 1995). In fact, there seems to be a very strait correlation between the mothers MS salivary levels and the offspring cariogenic activity, they will present similar levels to their mothers (Caufield 1997).

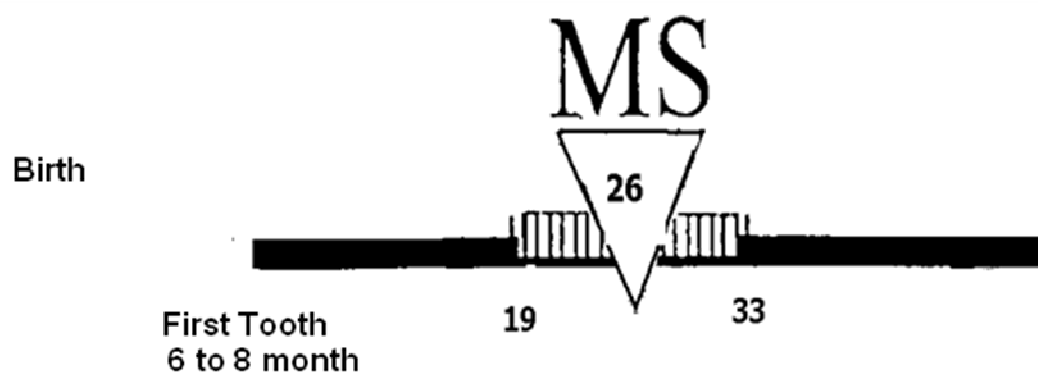


Figure I.3. Mutans streptococci “window of infectivity”, adapted by Caufield and collaborators in 1997 (Caufield 1997).

In order to address the question of which events dictate this specific period of time, “window of infectivity”, they found a very strong correlation between the total surface area of primary teeth present in infant’s mouth and the cumulative probability of infection curve (Figure I.4). This correlation, not provide causation, provided a reasonable explanation of the biological events that dictate the infant window of infectivity, i.e., the more teeth more likelihood for initial acquisition (Caufield 1997).

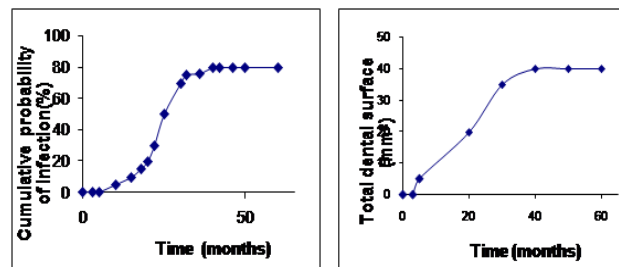


Figure I.4. Curves of cumulative probability of infection. **(A)** Cumulative probability of infection in infant's oral cavity after birth, period of time of 60 months. **(B)** Total dental surface of infants after birth, period of time of 60 months (Caufield 1997).

The presence of first and second primary molar teeth is the key event in initial acquisition, because their presence constitutes not only large surface areas but also introduces fissure surfaces, essential role in MS colonization. The recently emerged molars provide a finite period whereby the MS can colonize the oral cavity without having to compete with existing plaque microflora. Once a stable plaque or biofilm covers the infant's tooth surfaces it becomes less likely that MS can be established. This model reconciles the observation that the window closes shortly after 20 primary teeth emerge and become colonized by other plaque microorganism.

Evidence for a window, that reinforces the above explanation, can be found in experiences with laboratory animals, in which the MS are artificially introduced (Caufield 1997). The scientists using laboratory animals concluded that MS could be introduced in the animal's oral cavity during a discrete period of time, between days 18 and 22 (Figure I.5). After this period stable establishment of MS in the oral cavity of the rats was less probable.

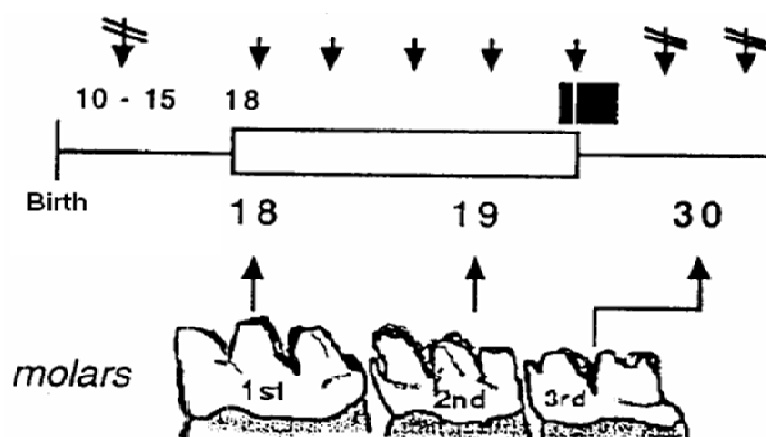


Figure I.5. "Window of infectivity" observed in laboratory rats (Caufield 1997).

I.1.6.1. Fidelity of transmission

The concept that mother are the offspring first focus of oral microorganisms was suggested by the work developed by Keyes and collaborators with laboratory animal in sixties (Keyes 1958). Since mothers have an intense contact with their children, it seems likely for them to transfer their oral bacteria to the offspring (Berkowitz and Jordan 1975).

With phenotyping studies there was high variability between different laboratories and the results were not reproducible. After the development of new molecular and genetic techniques, plasmidic DNA was epidemiologic marker, it was possible to determine the mothers and offspring strains similarity. With new genotyping techniques and longitudinal studies, observation of children microflora development, the initial acquisition period can be determined, and the answers to these problems was the chromosomal DNA fingerprinting assay (Caufield 1997).

The direction of transmission, from mother to child, could be deduced by showing that mothers harboured a particular genotype of MS prior to infant's initial acquisition. They observed in same studies that infants newly acquired specific MS strains genotypically similar their mothers (Li and Caufield 1995). It was remarkably that fidelity of transmission between mothers and their female infants approached 90% and was significantly better than the fidelity of transmission between their male infants (53%). Another remarkable observation was that African-American mothers transmitted MS to their infants with greater fidelity than did white mothers (Li and Caufield 1995). In other studies among breast feeding mothers and their infants they observed that breast fed infants acquires MS with significantly greater fidelity than non-breast fed infants (Caufield 1997, Li and Caufield 1995, Li *et al.* 1997). These observations can lead to same questions, breast fed children likely enjoy more close interaction with their mothers, fostering the transfer of oral bacteria. But mother's milk also contains immunoglobulin's, which may also play a role in directing which indigenous bacteria can colonize and play a role in guiding the infant in preferentially selecting that indigenous biota coming from the mother, while excluding those from the father and other "nonself" sources (Caufield 1997, Li *et al.* 1997).

Strains differ in virulence and a particular individual may acquire a virulent strain or a nonvirulent strain or both. Certain ethnic groups may have a virulent or a nonvirulent strain within their mating cohort; this may be the reason why caries varies so widely among different ethnic groups. Many attribute this variation to dietary or cultural

differences (Caufield 1997). Indeed, they are likely to be powerful influences, but it is known that sugar consumption, for example varies widely among individuals and does not always correlate to disease (Caufield *et al.* 1988). The presence of low counts of MS in saliva or plaque correlates poorly to caries risk (Caufield 1997). This correlation is not necessarily cause and effect, i.e., high levels of MS indicate the presence of existing caries, but does not mean that high levels of MS predispose an individual to caries. Suppose that MS is relatively noncariogenic or nonvirulent; this individual may not be at risk compared to one harbouring a more virulent strain.

Diagnostic and intervention strategies may include determining which infants are most at risk, based on a series of diagnostic tools that could include DNA based test to reveal the particular strains or combinations of strains of cariogenic bacteria, determine children with major risk to become colonized. Moreover, the possible genetic predisposition must be taken into account.

Therapeutic measures presently used, such oral hygiene instructions, periodic fluoride administration, restorative treatment, have little scientific backing or rationale in terms of treating an infectious diseases. For several years the development of a vaccine against dental caries has been subject of intense investigation.

I.2. Virulence factors

The development of bacterial diseases has been linked to a “molecular fight”, in which host tries to eliminate the bacteria and the bacteria try to survive in the host. Most of the bacteria do not cause disease, but some cause serious human infections.

Many species of streptococci (*S. sobrinus* and *S. mutans*; *S. agalactiae*; *S. pyogenes*; *S. pneumoniae*) are members of commensal microflora, bacteria are present on the mucosal surfaces of humans and animals, but normally cause no damage. The problem arises, when under specific conditions, they cause disease.

To cause disease, the bacteria pathogen must be able to adhere to a tissue or surface and compete with normal microflora already present on that surface. To survive in the host environment, bacteria must acquire nutrients and at same time avoid host immune system. The mechanisms used by bacteria for survive in the host are related with bacterial factors, designated by virulence factors (Mitchell 2003).

As previously described in this chapter, *S. sobrinus* and *S. mutans* are the causative agents of human dental caries (De Soet *et al.* 1991, Hirose *et al.* 1993, Loesche 1986). These bacteria express several virulence factors as part of the microbial biofilm that constitutes dental plaque. I will focus, however, in four of these virulent factors that have been used as a target antigen in several immunoprotection assays (Taubman and Nash 2006a). They are: the surface fibrillar adhesins known as AgI/II (synonyms: antigen B, P1, SpaP, PAc, SpaA, PAg), the glucosyltransferases (GTF), the glucan-binding proteins and the virulence-associated immunomodulatory proteins.

As described before, the molecular pathogenesis of mutans streptococcal-associated dental caries occur in three phases. The first phase involves the initial attachment of the microorganism to the dental pellicle (Staat *et al.* 1980) that is mediated by an adhesin from mutans streptococci known as antigen I/II (also known as P1 and PAc) (Hajishengallis *et al.* 1992, Lee *et al.* 1988, Russell and Lehner 1978). The second phase, which is known as accumulation, depends on the presence of sucrose, as well as glucosyltransferases (GTFs) (Guo *et al.* 2004, Nanbu *et al.* 2000) and glucan-binding proteins (GBPs) (Lynch *et al.* 2007) from mutans streptococci. After cleaving sucrose into its component saccharides (glucose and fructose), mutans streptococci GTFs synthesize glucans that have different solubility in water. In the third phase, the multivalent glucans that have been produced interact with GBPs and with the glucan-binding domain of GTFs, both of which are present at the surface of mutans streptococci.

S. sobrinus secretes a virulence-associated immunomodulatory protein (VIP) (Ferreira *et al.* 1997) that displays several biological activities similar to what has been described with other VIP (Arala-Chaves *et al.* 1988, Ferreira *et al.* 1988, Lima *et al.* 1992, Soares *et al.* 1990, Tavares *et al.* 2000, Tavares *et al.* 1993). These VIP are inhibitory for host specific immune responses by triggering polyclonal non-specific lymphocyte activation (Arala-Chaves *et al.* 1988, Ferreira *et al.* 1988, Lima *et al.* 1992, Tavares *et al.* 1993) and/or inducing an early production of IL-10 in the host (Ferreira *et al.* 1997, Tavares *et al.* 2000, Vilanova *et al.* 1999). They act as virulence factors because their production is directly correlated with pathogenicity of the secreting microorganism and host treatment with the VIP before colonization increases the specific colonization (Lima *et al.* 1992, Santarém *et al.* 1987, Tavares *et al.* 1993). Thus, the production of these VIP seems to be a mechanism of immune evasion amongst several pathogens. As previously suggested and reported for several microorganisms (Balaban *et al.* 1998, Lima *et al.* 1992, Minoprio *et al.* 1986, Soares *et al.* 1990, Tavares *et al.* 1993), the isolation and the characterization of molecules involved in microbial pathogenicity may be useful in the identification of targets for

vaccination. Our laboratory, demonstrated for systemic candidiasis in mice, that VIP can be used as a vaccination target inducing specific protection against the microorganism (Tavares *et al.* 1995). The same approach has been successfully applied with *S. sobrinus*-induced dental caries (Dinis *et al.* 2004) and will be presented in this thesis.

Based on N-terminal protein sequencing, enolase was identified as a immunomodulatory protein from *S. sobrinus* (Ferreira *et al.* 1997, Veiga-Malta *et al.* 2004). In a previous work we cloned and sequenced the enolase gene of *S. sobrinus* and purified the recombinant protein (Veiga-Malta *et al.* 2004).

Enolase is one of the important glycolytic enzymes, found generally in the cytoplasm, that catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate, an important metabolic intermediate. However, several others functions have already been ascribed to enolase (Aaronson *et al.* 1995, Al-Giery and Brewer 1992, Breitenbach *et al.* 1997, Pancholi and Fischetti 1998, Pancholi and Fischetti 2001, Williams *et al.* 1985, Wistow *et al.* 1991). Surface expression of enolase was shown to be important in the pathogenesis of *Streptococcus pyogenes* (Fontan *et al.* 2000, Pancholi and Fischetti 1998) and has also been proven its potential virulence in *S. pneumoniae* due the ability to bind plasmin and plasminogen in order to invade tissues (Bergmann *et al.* 2001).

The bacteriostatic effect of fluoride in the dental plaque is claimed to be due to the inhibition of the enolase by fluoride (Hamilton 1977, Huther *et al.* 1990).

I.3. Mucosal Immunity

I.3.1. Mucosal-associated lymphoid tissue

The vast majority of infections involve the mucosae with regard to initial microbial colonization and/or entry into the body. The mucosal surface area is approximately 400m² in a human adult and is mostly covered by a vulnerable monolayered epithelium. Although intensive research, the development of vaccines against mucosal pathogens has not yet to successful. The hypothesis is that if vaccines could be administrated simply in mucosal surfaces, immunization practice would become safer, more accepted and more suitable for mass use (Levine 2003). Mucosal vaccination should most effectively induce antibody protection at mucosal surfaces mediated principally by Immunoglobulin A (IgA) in co-operation with innate non-specific factors,

denominated the “first line” of antimicrobial protection (Brandtzaeg 2003, Neutra and Kozlowski 2006).

The secretory antibody is locally provided by the mucosae and associated exocrine glands which harbor most of the body’s activated B cells, terminally differentiated to Ig-producing plasmablasts and plasma cells (PC). Secretory IgA (SIgA) antibodies are remarkably stable molecules, mainly consisting of PC-derived IgA dimers with one (or more) ‘joining’ (J) chain(s) and an epithelial portion called bound secretory component (SC) which is disulfide linked to one of the IgA subunits. Most mucosal PC, 70–90%, do normally produce dimers and some trimers of IgA (collectively called polymeric IgA, plgA) which, together with J chain-containing pentameric IgM, are exported by secretory epithelial cells to provide SIgA and secretory IgM (SIgM) antibodies (Brandtzaeg 2003, Brandtzaeg and Johansen 2005).

It has been described that, in the first postnatal period, only traces of SIgA and SIgM occur in human external secretions, whereas some IgG is often present mainly as a result of ‘leakage’ from the mucosal lamina propria, because of placental transfer, which contains readily detectable maternal IgG as early as at 34 weeks of gestation (Brandtzaeg *et al.* 1991). Therefore, an adequate mucosal barrier function of neonates depends on a supply of SIgA antibodies from breast milk (Corthesy 2007, Hanson 1998, Hanson 2007). ‘Gut closure’ normally occurs in humans mainly before birth, but an adequate mucosal barrier may not be established until after 2 years of age; the different variables involved in this process are poorly defined but secretory immunity is most likely a major component (Brandtzaeg *et al.* 1991). They hypothesize that postnatal colonization of commensal bacteria is important both for establishing and regulating an appropriate epithelial barrier function, the effect of which could be both directly on the epithelium via microbial pattern recognition receptors (PRRs) through binding to Toll like receptors (TLRs) and by driving the development of the mucosal immune system in response to those antigens (Niers *et al.* 2007, Yamanaka *et al.* 2003).

The various secretory effector sites receive their activated B cells from inductive mucosa-associated lymphoid tissue (MALT) organized lymphoid structures that sample antigens directly from the epithelial surface (Figure 1.6). Although gut-associated lymphoid tissue (GALT) – including Peyer’s patches in the distal ileum, the appendix and numerous isolated lymphoid follicles – constitutes the major part of MALT, induction of mucosal immune responses can take place also in the palatine tonsils and other lymphoepithelial structures of Waldeyer’s pharyngeal ring, including nasopharynx-associated lymphoid tissue (NALT) such as the adenoids in humans

(Brandtzaeg 2003, Brandtzaeg *et al.* 1999a, Brandtzaeg *et al.* 1999b, Brandtzaeg *et al.* 1999c, Brandtzaeg and Pabst 2004) and probably also bronchus-associated lymphoid tissue (BALT).

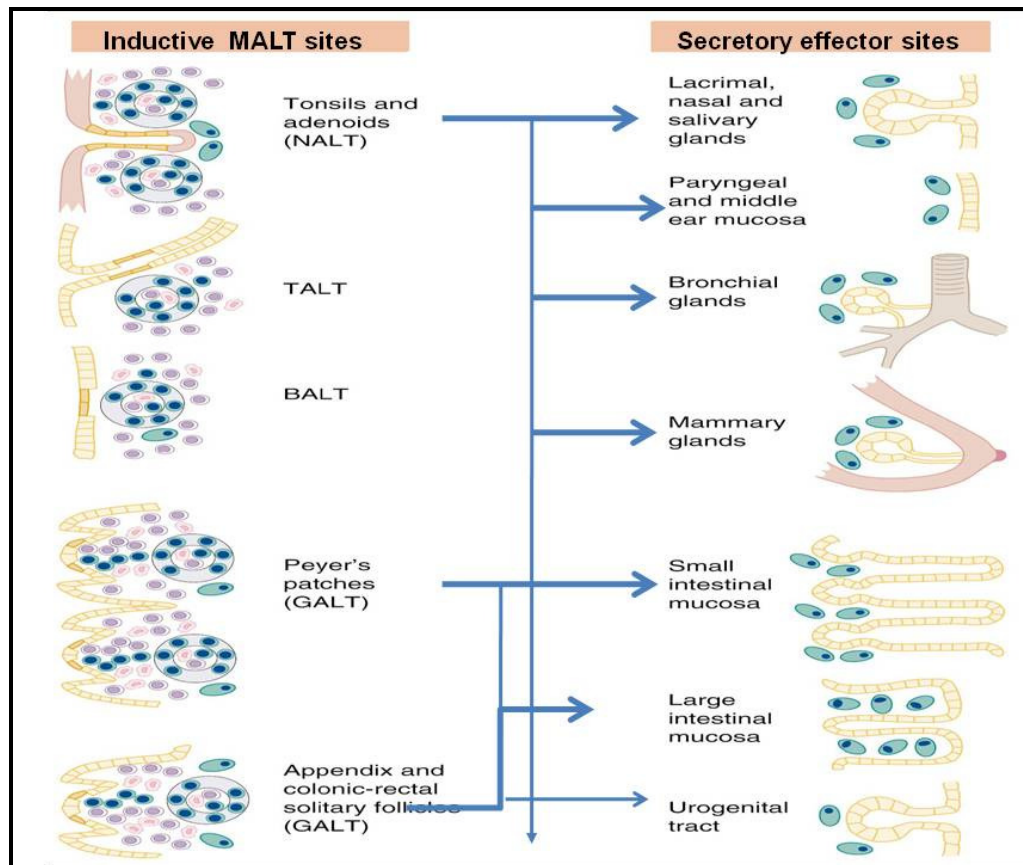


Figure I.6. Regionalization of the mucosal immune system. Model for homing of primed B cells from inductive lymphoid tissue sites with their activated follicles (germinal centers) to secretory effector sites (Brandtzaeg *et al.* 1999c).

The intestinal IgA system is the best understood contributor to mucosal immunity since the gut mucosa contains at least 80% of the body's PC, and some 90% of these activated B cells produce pIgA (Brandtzaeg 2003). Their antigen-mediated induction depends on complex stimulatory mechanisms in the organized GALT structures, while the lamina propria and epithelial compartment principally constitute effector sites receiving the primed mucosal memory/effector B and T cells. However, although such homing appears to be antigen-independent, topically available antigen will contribute to the local retention, proliferation and differentiation of effector B cells (Brandtzaeg *et al.* 1999a, Brandtzaeg *et al.* 1999b, Brandtzaeg and Pabst 2004).

The domes of GALT are covered by a characteristic follicle-associated epithelium (FAE), which contains variable numbers of antigen-sampling 'microfold' or 'membrane' (M) cells depending on the species and the degree of stimulatory activity (Brandtzaeg and Pabst 2004). GALT structures resemble lymph nodes with B-cell follicles, intervening T-cell zones, and a variety of professional antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs). Therefore, microbial stimuli must come directly from the epithelial surfaces, mainly via the M cells, but also aided by DCs which may penetrate the surface epithelium with their processes (Milling *et al.* 2005). Induction of intestinal immunity hence takes place primarily in the GALT structures and to a variable extent probably also in mesenteric lymph nodes (MLNs) (Brandtzaeg and Pabst 2004).

Naive B and T cells arrive in MALT via high endothelial venules like in other secondary lymphoid tissue. Antigens are presented to the naive T cells by APCs after intracellular processing to immunogenic peptides. CD4⁺ helper T cells activated in GALT are known to release cytokines such as transforming growth factor (TGF- β) and interleukin (IL)-10, which drive the class switch and differentiation of mucosal B cells to predominantly IgA-committed plasmablasts with J-chain expression, although their detailed regulation still remains unclear (Brandtzaeg and Johansen 2005, Fagarasan and Honjo 2003). Both naive and primed B cells migrate rapidly from GALT via draining lymphatics to MLNs where they may be further stimulated and next reach thoracic duct lymph and peripheral blood to become seeded by preferential homing mechanisms into distant mucosal effector sites, particularly the intestinal lamina propria, where they finally differentiate to PC. This terminal differentiation is modulated by 'second signals' from local antigen-sampling DCs, lamina propria CD4⁺ T cells, and available cytokines (Brandtzaeg *et al.* 1999c, Brandtzaeg and Johansen 2005).

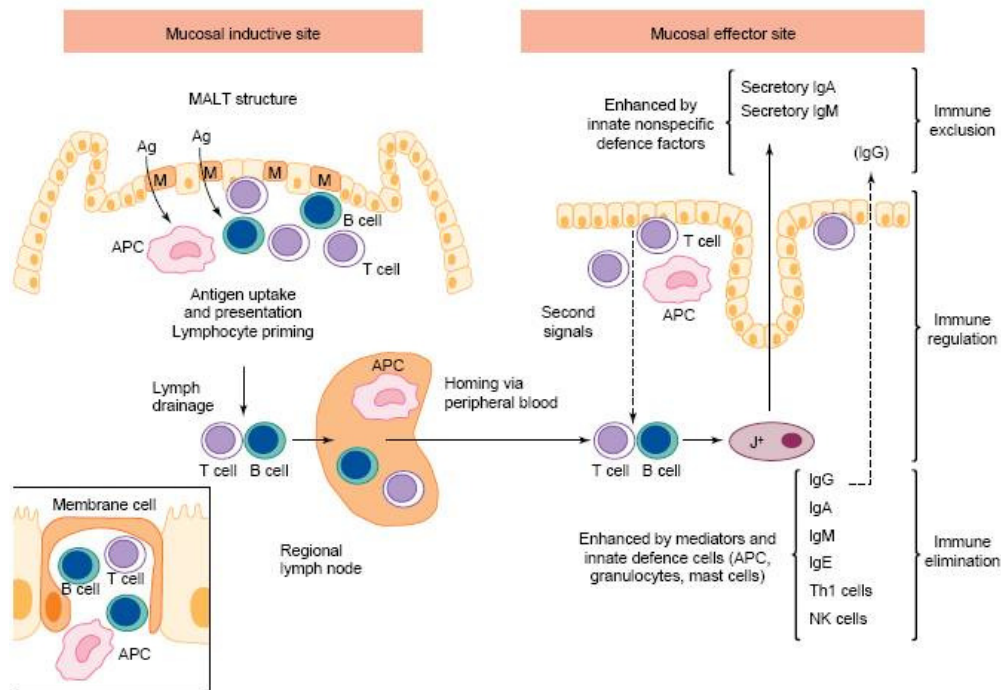


Figure I.7. Antigen stimulation in mucosa-associated lymphoid tissue (MALT) provides “first signals” to B cells, which migrate (“home”) to secretory effector sites via lymph and peripheral blood. “Second signals” (dashed heavy arrow) induce local proliferation and terminal differentiation of the extravasated B cells (Brandtzaeg *et al.* 1999a).

Accumulating evidence shows that a remarkable regionalization exists in the mucosal immune system, especially a dichotomy between the gut and the upper aero digestive tract with regard to differentiation and homing properties of B cells (Brandtzaeg 2003, Brandtzaeg and Johansen 2005, Holmgren and Czerkinsky 2005). The regional disparity is apparently explained by topical differences in the microbial antigenic repertoire as well as specific adhesion molecules and chemokines involved in local leukocyte extravasation (Johansen *et al.* 2005).

In general, it appears that primed immune cells preferentially home to effector sites corresponding to the inductive sites where they initially were triggered by antigens. Such compartmentalization (Figure I.7) combined with integration in the mucosal immune system has to be taken into account in the development of local vaccines and in their application strategy (Johansen *et al.* 2005). In addition to the variable cellular communication that exists among different mucosal regions, the mucosal and systemic lymphoid cell systems are not completely segregated, particularly not in the upper airways, where several homing molecules are shared between the two systems (Johansen *et al.* 2005).

A regionalized impact on mucosal immune regulation is also reflected by the highly distinct subclass distribution of IgA-producing PCs. In the mucosal immune system,

relatively more IgA2 is generally produced than in peripheral lymphoid tissue, reaching dominance over the IgA1 subclass in the large bowel mucosa (Brandtzaeg *et al.* 1999c, Brandtzaeg and Johansen 2005). Such an increase of the IgA2 isotype in secretions compared with serum can be important for the stability of secretory antibodies because SIgA2, in contrast to SIgA1, is resistant to several proteases synthesized by a variety of potentially pathogenic bacterial species. However, production of IgA1 is dominating both in the nasal (93%) and bronchial (75%) mucosa (Brandtzaeg and Johansen 2005).

MLNs and cervical lymph nodes apparently share immune-inductive properties with the respective regional MALT structures, that is, GALT and NALT. These lymph nodes receive antigens in MALT and mucosa-derived afferent lymph as well as via antigen-transporting DCs continuously migrating through draining lymph from the mucosal surfaces.

Nevertheless, similar mediators can probably induce homing capacity for the upper aero digestive tract when T and B cells are stimulated by antigens in Waldeyer's ring and/or cervical lymph nodes (Johansen *et al.* 2005). Antigens reaching MALT-associated lymph nodes may hence elicit or amplify mucosal immunity in the same region. Importantly, the human nasal mucosa is extremely rich in various DC types, both within and beneath the epithelium (Brandtzaeg and Pabst 2004). To be highly successful, an intranasally applied vaccine should therefore aim to target both mucosal DCs in the nasal cavity and FAE (including M cells) of MALT structures in Waldeyer's ring.

The oral mucosa has also been under intensive investigation due the fact that mouth is a opening door for several microorganisms. Adaptive immunity is antigen specific and serves to enhance the protective mechanisms of non specific innate immunity (Murphy *et al.* 2008). The microorganisms must be blocked and their systemic invasion needs to be prevented. The understanding of oral mucosal defence will be important for better comprehension of oral diseases such as dental caries or periodontitis.

Recent advances in immunology have disclosure the enormous complexity of the immune regulatory system. The oral mucosal diffuse lymphoid tissue and dental pulp are equipped to mount adaptive immune response to dental caries (Cutler and Jotwani 2006, Cutler and Teng 2007). This includes at least antigen presenting cells (APC), lymphocytes, mast cells and their cytokines and chemokines (Hahn *et al.* 2000, Hahn and Liewehr 2007a, b). There still many gaps in the current understanding of caries antigens and oral mucosa and dental pulp immune responses. The quantitative analyses of DC, cytokines and chemokines profiles related to oral microbe's infection

may elucidate the prevalence and development of dental caries and enabling future tools to prevent disease.

I.3.2. Salivary immunity

The saliva and salivary glands function has a significant impact on oral health, including dental caries. Therefore we are going to discuss above some of their chemical and immunological properties and their impact in oral disease prevention.

I.3.2.1. Saliva

Saliva is a complex secretion; 93% by volume is secreted by the major salivary glands and the remaining 7% by the minor glands. These glands are located in every region of the mouth except for the gums and the anterior part of the hard palate. Saliva is sterile when it leaves the salivary glands but ceases to be so as soon as it mixes with the crevicular fluid, remains of food, microorganisms, and desquamated oral mucous cells (Tenovuo 1997).

The salivary glands are made up of acinar and ductal cells. The acinar cells of the parotid gland produce a largely serous secretion. While this gland synthesizes most of the alpha-amylase, it produces less calcium than the submandibular gland. The mucins are mainly produced by the submandibular and sublingual glands and proline and histatin-rich proteins by the parotid and submandibular glands. The minor salivary glands are essentially mucous. Daily secretion rates range between 500 and 700 ml and the average volume in the mouth is 1.1 ml. 99% of saliva is water and the other 1% is composed of organic and inorganic molecules.

A number of physiological circumstances reduce salivary secretion. They include age, the number of teeth in the mouth, male/female, body weight and the time of day. Physiologically, the greatest salivary secretion takes place during tooth eruption and is linked to hyperstimulation of the peripheral receptors in the oral mucus.

Saliva plays important role in protecting against caries due the ability to dilute and eliminating sugars and other substances, buffer capacity, balancing demineralization/demineralization and antimicrobial action (Table I.2) (Dodds *et al.* 2005).

Table I.2. Saliva components and functions (Mandel 1993).

<u>Functions</u>	<u>Components</u>
Lubrication	Mucin, proline-rich glycoprotein, water
Antimicrobial action	Lysozyme, lactoferrin, lactoperoxidases, mucins, cystins, histatins, immunoglobulins, proline-rich glycoprotein, IgA
Maintaining mucosa integrity	Mucins, electrolytes, water
Cleansing	Water
Buffer capacity and demineralization	Bicarbonate, phosphate, calcium, staterin, proline-rich anionic proteins, fluoride
Preparing food for swallowing	Water, mucins
Digestion	Amylase, lipase, ribonucleases, proteases, water
Taste	Water, gustin
Phonation	Water, mucins

One of the most important functions of saliva is to remove microorganisms and dietary components from the mouth. The sugars in the saliva easily spread to the bacterial plaque. A few minutes after sugar ingestion, the plaque is already supersaturated with greater concentrations than are found in the saliva; there is a correlation between pH changes in the plaque and sugar clearance from the saliva (Axelson 2000).

Although saliva plays a role in reducing the acids in the plaque, it also contains specific buffer mechanisms such as bicarbonate, phosphate and some protein systems which not only have a buffer effect but also provide ideal conditions for automatically eliminating certain bacterial components that require a very low pH to survive.

Caries lesions are characterized by a sub-surface demineralization of the enamel that is covered by a fairly well mineralized layer, unlike chemical erosion, where the outer surface of the enamel is demineralized but there is no sub-surface lesion. The caries process begins when bacteria ferment carbohydrates, resulting in the production of organic acids that lower the pH of the saliva and the plaque. In the dynamic balance of the caries process, super saturation of the saliva provides a barrier to demineralisation and tips the balance towards remineralization. The presence of fluoride assists this balance.

The saliva is able to perform its function of maintaining the oral microbiota balance because it contains certain proteins. The most important proteins involved in oral ecosystem maintenance are proline-rich proteins, lysozyme, lactoferrin, peroxidases, agglutinins and histidine, as well as secretory immunoglobulin A and immunoglobulins G and M (Liébana *et al.* 2002).

I.3.2.2. Salivary Immunoglobulins

I.3.2.2.1. Salivary IgA

As stated before, SIgA antibody in saliva appears as a consequence of induction of immune responses in MALT (Brandtzaeg 2007b).

The swallowed bacteria can be taken by specialized epithelial M cells overlying Peyer's patches in the intestine; these cells then pass the bacterial components to DC, macrophages and B cells. These cells present antigen to CD4+ helper T cells which, when activated, liberate cytokines which cause mucosal B cells to differentiate, primarily to IgA-committed cells, that migrate through the lymphatic circulation to the mesenteric lymph nodes, then reach the peripheral circulation through the thoracic duct. These primed mucosal memory/effector B cells home to mucosal tissues via mechanisms which include adhesion molecules, chemokines and topically available antigen (Brandtzaeg and Johansen 2005, Johansen *et al.* 2005).

The IgA dimers bind the poly-immunoglobulin receptor (pIgR) on the glandular epithelium, are endocytosed, and finally secreted into the glandular lumen as SIgA, with pIgR covalently attached. Once in the local mucosal tissue final differentiation of IgA dimer-secreting plasma cells take place. Due the significant regionalization of the mucosal immune system, inductive sites in Waldeyer's ring, which contain MHC class II molecules-expressing follicle-associated epithelium (Johansen *et al.* 2005), are likely to be important for priming effector B cells to oral antigens and subsequent antibody-secreting plasma cells in the salivary glands. The salivary SIgA antibody levels may also be amplified by transport of oral antigens via dendritic cells to cervical lymph nodes, which also are thought to have mucosal immune inductive properties (Brandtzaeg 2007b).

The effective dental caries vaccine approaches depend on understanding mucosal immune system. Preferably, immune intervention would take place during initial mutans streptococcal colonization events. The child's mucosal immune system needs to be sufficiently mature by the end of the 1st year of life for a vaccine to induce effective

response. The knowledge of the immunological responsiveness to the secretory immune system primary comes from analysis of mucosal responses to infections with indigenous organisms.

The oral immune system environment undergoes rapid, early development; secretory IgA antibody in saliva and other secretions is essentially absent at birth but IgM and IgA-committed cells are present in saliva at this time (Thrane *et al.* 1987, Thrane PS 1987). After birth, exposure to bacterial, viral and food antigens causes rapid expansion of IgA plasma cells in mucosal lamina propria (MacDonald and Spencer 1990).

Immunomodulatory factors in breast milk apparently enhance this expansion since neonates receiving breast milk have significantly higher concentrations of salivary IgA than those receiving other nutrition means (Hayes *et al.* 1999, Newburg and Walker 2007). Salivary SIgA concentrations continue to increase at higher rate in breast *versus* bottle fed infants during the first 6 months of life (Fitzsimmons *et al.* 1994).

The mature SIgA, dimeric IgA, with bound secretory component, is the principal salivary immunoglobulin secreted by 1 month of age, at this stage, many infants saliva (Childers *et al.* 2003, Fitzsimmons *et al.* 1994, Smith *et al.* 1989) are dominated by the IgA isotype. At 6 months of age, most children exhibit a more adult-like distribution of salivary IgA1 and IgA2 subclass, both which contain antibody specificities to oral commensal microbiota (Smith and Taubman 1992).

Commensal flora represents a significant antigenic stimulus to the developing mucosal immune system. As a result, mucosal IgA antibody to pioneer gut and oral (*Streptococcus mitis*, *S. salivarius*) (Smith and Taubman 1992) microbiota can be detected within weeks of initial exposure. The importance of these responses to antigens present in the developing commensal flora is not fully understood, due to the difficulties in quantitatively assessing mucosal immune responses in young children, in addition to the increasing concentration of IgA, antibody reactivity is measured in whole saliva than the pure glandular secretions. The increasing diversity of commensal flora and dietary components, eruption of primary dentition, variations in salivary flow rates have considerable impact on salivary antibody concentrations.

The characteristics of response patterns to initially colonizing streptococci have been followed through the first's years of life in several studies (Cole *et al.* 1999, Nogueira *et al.* 2005, Smith and Taubman 1992). They suggested that predominant salivary IgA antibody response in young children to the four tested streptococcal species (*S. mitis*, *S. salivarius*, *S. sanguis* and *S. mutans*) was directed to cross-reactive antigens. This may reflect innate-like polyclonal responses observed to commensal gut flora, which are characterized by limited repertoire, cross-reactive SIgA antibody of low affinity

(Bouvet and Fischetti 1999). The low-level responses are adaptive, high affinity responses to specific antigens, which may result from initial bacterial challenge or pathogen exposure but not efficient enough to eliminate the bacteria.

The mucosal immune maturation has developed to the stage at which detectable salivary IgA immune response to mucosally applied dental caries vaccines could be expected by the end of the 1st year of life at least to protein antigens (Taubman and Nash 2006a).

Natural exposure to mutans streptococci results in mucosal immune responses to these microorganisms, the response is often observed in the 2nd year of life, when MS begin to accumulate on primary tooth surfaces (Taubman and Nash 2006a). Analyses by western blot and ELISA reveal that the major responses appear to be directed to streptococcal components which are considered to be important in bacterial colonization and accumulation (Smith *et al.* 1998). Similar results were observed in experimental models, as a consequent experimental infection of rats with mutans streptococci.

Permanent colonization with MS leading to accumulation is not always required for induction of detectable levels of salivary IgA antibody to species-specific antigens. Antibody reactivity with MS antigens can be observed independent of the ability to detect ongoing infections in some children (Nogueira *et al.* 2005, Smith *et al.* 1998). In cases of many bacterial challenges throughout the body, the threshold of immunological response is lower than that of persistent infection; therefore it is not surprising to observe antibody to *S. mutans* antigens in absence of its colonization, since most children are being exposed to these organisms by their primary caregivers from birth. Children respond at different rates following infection, a condition which may be partly as result of the infection extent, dose antigen, age at time of infection, maturation of immune system (Li and Caufield 1995, Saarela *et al.* 1993). Children become colonized with one or more of these strains, a process which is influenced by several aspects, as been discussed above, i.e., salivary protein characteristic, epithelial receptors and pre-existing microbiota. The rate, specificity and/ or extent of the mucosal immune response to previous encounters with the organism may also contribute to the success or failure of permanent colonization. There are evidences suggesting that vigorous immune responses to antigens are important for the colonization inhibition and may delay *S. mutans* infection under certain circumstances (Nogueira *et al.* 2005).

The evidence from salivary IgA responses to commensal and pathogenic oral microorganisms indicates that the mucosal immune system is well developed by the period of time during which children became infected with mutans streptococci.

However, the question remains in the antibodies specificity, although there are some salivary immunoglobulins it seems no sufficient to prevent colonization. The possibility exists that such responses could be protective if induced prior to critical colonization events, or other bacterial antigens must be studied, against whom perhaps there is not detectable response, but if we were able to increase the antibody levels against them we would obstruct MS colonization.

In cooperation with a variety of innate mucosal defence mechanisms (Müller *et al.* 2005), the function of secretory antibodies is very important. Due to the remarkable stability, SIgA can retain its antibody activity for prolonged periods in hostile environments such as the gut lumen and oral cavity (Ma *et al.* 1998). As discussed earlier, the antibody function of SIgA is most likely enhanced significantly by the high level of cross-reacting activity as also detected in human secretions (Quan *et al.* 1997). These 'natural' antibodies are apparently designed for urgent protection before an adaptive specific immune response is elicited; they are therefore reminiscent of innate immunity (Bouvet and Dighiero 1998, Bouvet and Fischetti 1999).

Although SIgA is the chief effector immune response, SIgM also contributes, particularly in the newborn period and in IgA deficiency (Brandtzaeg *et al.* 1991). In addition, there may be a significant contribution by serum-derived or locally produced IgG antibodies.

I.3.2.2.2. Salivary IgG

There have been some studies showing that induction of immunity by different mucosal routes, salivary injection (McGhee *et al.* 1975) or bacteria feeding, was sufficient to elicit a protective salivary secretory IgA response in rats (Michalek *et al.* 1976) without the induction of detectable specific serum IgG.

Limited investigations in non-human primates indicated a significant correlation between the concentration of serum and gingival crevicular fluid IgG specific for bacterial antigens and the reduction in dental caries after parenteral immunization with mutans streptococcal protein antigen (Lehner *et al.* 1981).

The gingival crevicular fluid has been collected and used in several biochemical assays in order to relate its composition and oral health status (Takashi *et al.* 2005). There are several diseases associated to oral cavity, besides dental caries, such as periodontal disease, characterized by chronic inflammatory lesions and destruction of supportive periodontal tissue.

The crevicular fluid and saliva contain substances associated with the host response, which could reflect periodontal disease and other oral diseases status. Therefore, determination of biochemical substances in oral fluid may be appropriated for early detection of oral diseases. Saliva is secreted from salivary glands; consequently, basic components may reflect a general condition rather than oral disease status (Llena-Puy 2006, Ozmeric 2004). The role of locally or serum derived IgG antibodies in the protection of dental caries is still controversial, until recently the major role was attributed to SIgA but nowadays striking results are given increasing importance to IgG induction and its function in protective immune response against this infectious disease (Taubman and Nash 2006a).

I.4. Vaccines

I.4.1. General

The vaccines constitute the most efficient advance in fighting infectious diseases. The first vaccine was discovered by Edward Jenner in 1776. This English doctor inoculated children with purulent material from infected cows (cowpox) with vaccinia virus to prevent infection with smallpox virus (Jenner 1798, Le Fanu 1951). One hundred years later, Pasteur, immunized children against rabies, using attenuated virus obtained from infected rabbits spinal medulla fluid. Even though the technique has been accepted during the next century, the vaccine development was very slow (Murphy *et al.* 2008). Although vaccine preparations bear no resemblance to the crude scab homogenates of 200 years ago, his concept of vaccination is still the used in several vaccines. A better understanding of the immune system has been gained over the last 50 years and this deeper knowledge of innate and acquired immunity is being used for the optimal targeting of vaccines.

A vaccine must stimulate the immune response in order to protect the immunized individuals against natural occurring infections. Therefore, the development of vaccines must elicit protective immune responses, humoral or cellular, against infectious pathogenic microorganisms.

Vaccination is a cost-effective weapon for disease prevention. Indeed, vaccination contributed for variola eradication, one of the most contagious and mortal human disease and control of other diseases such as rubeola, diphtheria, poliomyelitis, tetanus. Part of these success resulted from recognition of extracellular bacterial toxins

by Roux, Yersin, Behring and Kitasato that permitted the development of toxoids (inactivated toxins).

Even though it's still no efficient vaccines against several infectious diseases', there is no single licensed vaccine against fungi, protozoa and helminthes. Molecular technology has given a greater insight into the aetiology of disease, the immune system function and on mode of action of several pathogens. This knowledge has been used to develop new vaccines with antigens which elicit protective immune responses (humoral and /or cell mediated) in the host.

Nowadays, purification of microbial elements, genetic engineering and improved knowledge of immune protection allowed direct creation of attenuated mutans, expression of vaccine proteins in live vectors, purification and even synthesis of microbial antigens and induction of a variety of immune responses trough manipulation of DNA, RNA, proteins and polysaccharides (Plotkin 2005b).

Some vaccines, traditionally given by the parental route are now given by the natural route of antigen entrance, i.e., orally, (polio), intranasal (live influenza), aerosol (measles and Rubella), and transcutaneous with patches or microneedles (Hepatitis B, anthrax) (Plotkin 2005b). Since the parental route implicated injections and required needles, not available in some developing countries, the World Health Organization (WHO) extensively discuss this subject in the Expanded Programme of Immunization (EPI), defending vaccines composed by several antigens in order to reduce the number of injection and promote the development of other routes of administration (Giudice and Campbell 2006). The alternative routes (orally, intranasal, rectal, vaginal, etc.) have two major advantages, often interrelated, the rapid onset of immunity and stimulation of local, mucosal immunity. The balance has to be found between what is feasible technological and what provide a least as good protective immunity as current, conventional vaccines (Chalmers 2006).

As new and emerging diseases appear globally, new opportunities arise for molecular and conventional technologies to be applied both to the development and delivery of novel vaccines, as well as the improvement of vaccines in current use. The development of molecular biology and genetic, has had an important impact on vaccine development, providing new opportunities for construction of inactivated antigens and for rational attenuation of organisms through direct mutation (Plotkin 2005a).

The first success of genetic engineering was the hepatitis B vaccine, manufactured in a yeast recombinant carrying the gene for the S protein (McAleer *et al.* 1984), replacing the vaccine based on purification of S particles from plasma of infected individuals (Szmuness *et al.* 1981).

Recombinants of viruses and bacteria, used as live vaccine, are termed “vector vaccines”, the key issue is to have a vector or carrier that will incorporate and express the gene for a pathogen without causing illness. Many viral and bacterial vectors have been proposed, but poxviruses, and adenovirus and Bacille Calmette-Guérin are the most accepted (Plotkin 2005b). Genomic sequencing had an exponential growth in last years, almost 300 bacterial genomes have been completed and more than 500 are currently being sequenced (Mora *et al.* 2006). Now it's possible to compare sequences of related bacteria, compare pathogens with commensal of the same species or those of related species, enabling the identification of disease-related genes (Mora *et al.* 2006). For several decades, the concept for vaccine candidates has mainly been related with secreted or extracellular proteins, associated with bacterial membranes, due the fact they are more easy accessible to antibodies. After gene sequencing, the DNA fragments were screened using computer analysis to select proteins predicted to be present on the bacterial surface or proteins with homology to bacterial factors involved in pathogenesis and virulence. This genome-based approach has been denominated as “classical” reverse vaccinology (Rappuoli 2001, 2003). The reverse genetics techniques allowed the construction of novel negative-strand segment RNA viruses by mutation of their cDNA. This technique is currently being incorporated in manufactured of influenza vaccines (Wood and Robertson 2004). Because the single genomic sequence revealed to be insufficient to represent the variability of bacterial populations, multiple sequences may be required for effective vaccine formulation (Mora *et al.* 2006). The analysis of genetic variability between pathogens and closely related non-pathogenic microorganism leads to the rapid identification of genes that are potentially responsible for virulence, when the analysis is done within different strains of the same species, is referred to “pan-genomic” reverse vaccinology (Kaushik and Sehgal 2008).

The availability of such tools has made possible to predict genes that potentially encode virulence factors, toxins and other molecules that promote pathogenesis based on the sequence similarity to known pathogenic protein already present in the data base (<http://www.ncbi.nlm.nih.gov/tools>).

Prevention of infectious disease by vaccination remains a compelling goal in an effort to improve public health in industrialized and developing societies. The rapid expansion of genome-based biotechnology provides a variety of new avenues for vaccine development, but it is nevertheless essential to continue the exploration of basic immunology. Thus, vaccination relies on immunological memory, yet our understanding of this fundamental characteristic of the adaptive immune system is incomplete.

I.4.2. Dental caries vaccines

As referred above, dental caries is still one of the most prevalent infectious diseases, affecting a large population, and costly due the high distribution and treatment cost. Therefore, intensive investigations have been taking place in order to develop a vaccine against dental caries (Petersen 2003).

Nowadays the treatment is established with preventive measures, promotion of oral hygiene (tooth brushing), professional supervision (dentistry doctors), equilibrated diet (control of sugar consumption), use of dental floss (preventing dental plaque accumulation in susceptible surfaces) and topic Fluor administration and/or supplemented water. However, the combined action of these measures is not enough to inhibit dental caries formation and usually only a small reduction can be observed (Hajishengallis and Michalek 1999).

Several efforts have been made in order to produce a safe and efficient vaccine against dental caries. In the last two decades there have been some enhancements in this area; with the improvement of molecular biology: cloning and mutans streptococci (MS) virulence factors characterization, mucosal immunity better knowledge and development of sophisticated antigen delivery and new adjuvants that induced salivary IgA (Hajishengallis and Michalek 1999).

I.4.2.1. Active immunization

In the beginning of the 70's there were some animal models studies demonstrating that protection against dental caries could be induced (McGhee *et al.* 1975, Taubman and Smith 1974). Several investigators have been developing studies in order to identify the best and efficient target for immunization against this disease (Anusavice 2005).

Several strategies have been employed to test the hypothesis that the presence of antibody prior to infection can interfere with infection and disease progression. Virtually all approaches target either *Streptococcus mutans* or *S. sobrinus* as the principal etiologic agents of the disease. Most investigators sought to prevent initial colonization rather than to remove cariogenic microorganisms from the established biofilm. Bacterial colonization or accumulation was targeted by vaccines for inactivation/blockage, rather than metabolic pathways critical to the survival of the microorganisms.

The early experiments employed immunizations with intact mutans streptococci, *S. mutans* or *S. sobrinus* (Michalek *et al.* 1976, Taubman and Smith 1974). Although some protection was observed, the results were insufficient and there were serious

side effects as severe cardiac problems due to cross reactivity of the induced antibodies or the microorganisms itself and the cardiac muscle (Hughes *et al.* 1980, Michalek and Childers 1990, Wu and Russell 1990).

Afterward several investigators began to focus on the antigens involved in adherence of *S. mutans*, since the first step in the molecular pathogenesis of the disease is the tooth surface colonization, or, more accurately, colonization of the pre-existing biofilm on the tooth surface, though adhesin-mediated mechanisms.

Abundant *in vitro* and *in vivo* evidence indicates that antibody with specificity for *S. mutans* antigen I/II (Agl/II) or *S. sobrinus* SpaA can interfere with bacterial adherence and subsequent dental caries. Antibody directed to the intact AgI/II molecule or to its salivary-binding domain blocked the adherence of *S. mutans* to saliva-coated hydroxyapatite (Koga *et al.* 2002). Furthermore, a variety of immunization approaches have shown that active immunization with intact Ag I/II, or passive immunization with monoclonal or transgenic antibody to putative salivary binding domain epitopes within this component can protect rodents, primates or humans from infection caused by *S. mutans* (Koga *et al.* 2002).

Intranasal immunization with Ag I/II, coupled with cholera toxin B sub-unit, reduced dental caries by *S. mutans* in an experimental rat model (Hajishengallis *et al.* 1998). Likewise, intranasal applied DNA vaccines encoding Ag I/II sequence were shown to be protective (Jia *et al.* 2004). Immunization with *S. sobrinus* SpaA constructs protected rats, at some extent, from caries caused by *S. sobrinus* infection (Redman *et al.* 1996). Thus, these streptococcal adhesins have been discussed as possible candidates for dental caries vaccine application; but there are other molecules with the same function as the Ag I/II and immunization protocol using the Ag I/II don't protect the host against the others.

Sucrose-dependent accumulation of MS within the dental biofilm has also been immunologically targeted in the preclinical and clinical studies (Taubman and Nash 2006a), summarize in table 3. Most effort has targeted on a group of glucosyltransferase enzymes (GTFs) which catalyze the extracellular formation of glucans from sucrose. Taking into account the role of glucan formation in the molecular pathogenesis of dental caries, the GTFs from *S. mutans* and *S. sobrinus* have been used to try to induce protective immune responses in preclinical (Smith 2002b) or clinical studies with young adults (Smith and Taubman 1987). Thus the presence of antibody to glucosyltransferase in the oral cavity prior to infection can influence the disease outcome, presumably by interference with one or more of the functional activities of the

enzyme. Glucan-binding domains of GTFs have been shown to induce immune responses interfering with enzyme function and/or with cariogenic activity of MS infection (Jespersgaard *et al.* 1999, Smith 2002b). Protection has also been observed recently with GTFs peptides associated with MHC class II-binding (Culshaw *et al.* 2007).

Glucan-binding proteins have also received attention in the search for effective antigens. Although several glucan-binding proteins (GBP) have been described, only one, *S. mutans* GBP-B, has been reported to induce protective immunity in experimental systems (Smith and Taubman 1996). The ability to induce protective immunity with subunit vaccines based on adhesins, GTFs, or GBP-B sequences has led investigators to develop constructs combining epitopes from one or more proteins. Taubman and collaborators (Taubman *et al.* 2001), have shown that the combination of peptides from catalytic and glucan-binding domains of Gtfs enhances the level of experimental enzyme and caries inhibition. Fusion proteins constructed from saliva-binding alanine-rich region of Ag I/II and the glucan-binding domain of GTFs induced immune responses that inhibited water-insoluble glucan synthesis by *S. mutans* GTFs and also inhibited sucrose-independent adhesion of *S. mutans* to saliva coated hydroxyapatite beads (Yu *et al.* 1997). DNA vaccines coding for both the adhesin and GTFs also shown to induce protective responses (Guo *et al.* 2004). Although rapid progress has been made in demonstrating the efficiency of DNA vaccines against numerous infectious diseases, autoimmune diseases, allergies and cancers in the last 15 years, the poor immunogenicity is still a major problem in DNA vaccination (Vajdy *et al.* 2004).

In conclusion, there are several types of vaccine against dental caries in development, and these differ in terms of their target antigens, in the Table I.3 there is a summary of the different approaches. Importantly, each of these vaccines candidates is known to be involved in the molecular pathogenesis of dental caries. But the results obtained so far are insufficient. Only some protective immunity has been induced with partial inhibition of *mutans* streptococci colonization. Moreover, vaccination with some of these antigens only works for *S. mutans* and not for *S. sobrinus*, the other dental caries etiological agent, or *vice versa*, and the ideal vaccine should be efficient for both MS. There are some recent evidences concerning the degree of genomic diversity of pathogenic microbial species (Mora *et al.* 2006). Indeed, a universal vaccine is only obtainable using a combination of antigens chosen from different strains, or an antigen that is maintained in the *mutans* streptococci species.

Antigen	Function	Mode(s) of Immunization	Studies outcome	Mechanism of interference
Single antigen				
Antigen I/II (surface adhesin)	Bacterial-surface adhesin; receptor for attachment of dental pellicle	Subcutaneous injection near the major salivary glands, or intranasally administration	Preclinical: reduction in dental caries	Antibody blockade of adhesin mediated binding to dental pellicle, and aggregation of mutans streptococci for clearance in the salivary milieu
		Passive antibody administration	Clinical: interference in recolonization of human teeth with indigenous MS	
Intact GTF	Synthesis of glucans; required for accumulation of MS	Subcutaneous injection near the major salivary glands, or intra-rectal administration	Preclinical: reduction in experimentally induced dental caries	Antibody specific for any GTF activity domain can inactivate GTFs and interfere with synthesis of glucans, inhibiting accumulation of MS
		Oral or buccal mucosal instillation	Clinical: induction of parotid salivary IgA specific for GTF, and delayed reaccumulation of indigenous MS	
GBP	Receptor for glucans; involved in accumulation of MS	Subcutaneous injection near the major salivary glands, or passive administration of antibody	Preclinical: reduction in experimentally induced dental caries	Blockade of GBP at the surface of mutans streptococci, and interference with aggregation and accumulation of MS in dental biofilms
Combination antigen				
Conjugate of <i>S. sobrinus</i> GTF and water soluble glucans synthesized by GTF	Sucrose-dependent accumulation, and glucan-mediated aggregation	Subcutaneous injection near the major salivary glands, or intranasally instillation	Preclinical: reduction in experimentally induced dental caries	Inactivation of the enzyme mediated accumulation of MS that involves glucans and GTFs
Fusion protein of GTF and antigen I/II	Synthesis of glucans, and attachment of MS	Passive administration of antibody, or intranasally instillation	Preclinical: inhibition of <i>S. mutans</i> adherence in vitro and suppression of oral <i>S. mutans</i> colonization in vivo	Secretory IgA-mediated inactivation of GTF and antigen I/II, presumably leading to reduced attachment and accumulation of MS
Mixture of GTF and antigen I/II	Synthesis of glucans, and attachment of MS	Oral or intranasally instillation	Clinical: increase in secretory IgA1 and secretory IgA2 in nasal secretions and/or saliva	Secretory IgA-mediated inactivation of GTF and antigen I/II, presumably leading to reduced attachment and accumulation of MS
Diepitopic MAP containing GTF catalytic-domain derived peptide and glucan binding-domain derived peptide	Cleavage of sucrose, and binding of glucans	Subcutaneous injection near the major salivary glands	Preclinical: reduction in experimentally induced dental caries	Antibody specific for any GTF activity domain mediates inactivation of GTF, interferes with glucan synthesis and inhibits accumulation of MS
Diepitopic MAP of GTF catalytic domain-derived peptide and GbpB SYI peptide	Cleavage of sucrose, and binding of glucans	Subcutaneous injection near the major salivary glands	Preclinical: reduction in experimentally induced dental caries	Blockade of GbpB-mediated accumulation, and reduction of MS in dental biofilms

Table I.3. Dental caries vaccines that comprises mutans streptococcal antigens (Taubman and Nash 2006a).

I.4.2.2. Our vaccination approach

Although, several immunization protocols are in study, animal models and human clinical trials, the results are not sufficient and an ideal vaccine against dental caries is still inexistent.

As reported above, we have showed that extracellular proteins produced by diverse microorganisms interfere with the host immune response and facilitate colonization (Ferreira *et al.* 1997, Ferreira *et al.* 1988, Santarém *et al.* 1987, Soares *et al.* 1990, Tavares *et al.* 1993, Vilanova *et al.* 1999). Therefore these proteins were generally designated as Virulence-associated immunomodulatory proteins (VIP) (Tavares *et al.*

2000). Moreover, it was also shown that immunization with a particular VIP was able to protect the host against a subsequent infectious challenge with the VIP-producing microbe. The suitability of these proteins as immunoprotective antigens was demonstrated in animal models of systemic candidiasis (Tavares *et al.* 1995, Vilanova *et al.* 2004). Furthermore, we have also used the same approach on dental caries diseases (Dinis *et al.* 2004, Dinis *et al.* 2009) and the obtained results will be presented in the Results section of this thesis.

I.4.2.3. Passive Immunization

As an alternative to strategies that require active immune responses in the host, antibody to critical *S. mutans* virulence factors can be passively introduced into the oral cavity for protective effect (Koga *et al.* 2002).

The first study, in which monkeys were infused intravenously with simian IgG antibody directed to *S. mutans* cell surface antigens, indicated that non-host IgG antibody entering the oral cavity via the gingival crevicular fluid, could reduce dental caries in rhesus monkeys (Lehner *et al.* 1978). A second pioneering study showed that mucosally delivered antibody could also impart protection (Michalek and McGhee 1977). In this study, they immunized rat dams with *S. mutans* streptococcal cells, resulting in mucosal secretion of antibody to *S. mutans* in their milk. Offspring of these dams, after sucking on antibody-rich milk, showed a significant degree of protection from dental caries caused by subsequent infection with *S. mutans*. This experiment established the concept of passive protection for dental caries.

Human application of the passive immunization approach was highlighted in several small clinical trials with monoclonal antibodies directed against an epitope of *S. mutans* antigen I/II. In those experiments, the adult volunteer teeth were treated with antibacterial drug (chlorhexidine) in order to remove the dental microflora and afterwards they were topically treated with mouse monoclonal IgG (Ma and Lehner 1990) or transgenic SIgA/G (Ma and Hein 1996) antibody to an adhesin epitope, prepared in tobacco plants.

A novel method of relevant monoclonal antibody specificities delivery in the oral cavity has been reported by Krüger and collaborators (Krüger *et al.* 2002, Krüger *et al.* 2005). In this approach, vectors encoding a single-chain Fv (scFv) fragment of antibody with specificity for Ag I/II adhesin epitope were expressed in *Lactobacillus zeae*, the scFv were displayed on the bacteria surface. When de-salivated rats were orally swabbed

with these transformed lactobacilli throughout the course of *S. mutans* infection, significant reductions in infection and dental caries were observed.

Hamada and co-workers (Hamada *et al.* 1991) prepared chicken egg yolk IgY antibody to the cell-associated GTFs or GTFs from culture supernatants of *S. mutans*. *In vitro*, the more purified IgY antibody to GTF inhibited glucan formation and could interfere with sucrose-dependent adherence of *S. mutans*. *In vivo*, reduction in plaque formation and caries development occurred in the rats fed purified IgY antibody to GTF at concentrations up to 1% of the diet.

More recently, immune milk containing antibody to a fusion protein consisting of alanine-rich salivary binding region of Ag I/II and the glucan binding domain of GTF was used as mouth rise (Shimazaki *et al.* 2001).

Antibody to *S. mutans* glucan-binding protein B (GBP-B) was also shown to provide passive protection against experimental dental caries (Smith *et al.* 2001b). In this study, IgY antibody to GBP-B or pre-immune IgY was added cariogenic diet and given to experimental or control groups. Periodic measurements of the *S. mutans* infection extent indicated that IgY antibody to GBP-B had significant effect on accumulation of these cariogenic streptococci. Interestingly, the levels of caries protection after 23 days of IgY antibody feeding was similar to the experiments where rats were actively immunized with GbpB followed by a similar *S. mutans* infection period (Smith *et al.* 1997b). It seems that although passive administration induced similar results than active immunization, they needed a prolonged antibody administration. We believe that this is not a feasible protocol, and the question remains, if the infection period was prolonged for how long the antibodies will persist.

In order to address the problem of passive immunization, we studied the passive transfer of antibodies from mother to their offspring and the impact of those antibodies in their dental caries protection. The obtained results will be presented in this thesis.

I.4.3. Methods of antigen delivery and Immune Enhancement

Although the basic principle of immune protection from dental caries caused by mutans streptococci has been investigated, refinement to the effective application of this approach to human's remains. Besides the best target antigen for vaccination needs to be established, and another important issue is the route of delivery. Traditionally vaccines were administrated by parental route, nowadays are given by natural route; either orally or intranasally.

Early studies with mucosal applied vaccines used the oral or intragastrically route for antigen delivery (Michalek *et al.* 2001). Induction of immune responses by oral route (Smith 2002a) requires antigen passage through the gut prior to uptake in the gut-associated lymphoid tissue (GALT).

The antigen intranasal administration (i.n) of antigen, which targets the nasal-associated lymphoid tissue (NALT), has been used to induce experimental immunity to many bacterial antigens, including those associated with mutans streptococci colonization and accumulation (Jespersgaard *et al.* 1999, Li *et al.* 2003, Russell *et al.* 1996, Smith *et al.* 2001a, Wu *et al.* 1997). Protective immunity after infection with cariogenic mutans streptococci could be induced in rats by intranasal route with many *S. mutans* antigens or functional domains associated with these components. There were some studies which protection was accomplished by this route, using salivary domain of *S. mutans* Ag I/II, the glucan-binding domain of *S. mutans* Gtfs, the *S. mutans* GbpB or GbpB-derived peptides (Koga *et al.* 2002, Smith 2002b). More recently the intranasal route was also applied in human immunization with several *S. mutans* antigens (Childers *et al.* 2006). The results indicated that intranasal immunization primes the immune system for a localized secondary response to *S. mutans* antigens. Moreover, additional routes as such antigen exposure to tonsils (Childers *et al.* 2002), minor glands (Smith and Taubman 1990) or rectal tissue (Smith *et al.* 2003) has also been assayed.

Vaccination has been stated to be the most cost-effective way to control infectious diseases. However, with vaccines administrated to healthy individuals, only very few or preferably no side effects can be tolerated.

In the last decades the whole-cell vaccines have been replaced by purified subunits or synthetically derived peptides that can be produced with high purity, in high quantities by recombinant technology (Olive *et al.* 2001).

Intact microorganisms contain a series of highly conserved structures, known as pathogen-associated molecular patterns (PAMP's) (Medzhitov and Janeway 2000). These molecules act as ligands for as instances, Toll-like receptors (TLR) on antigen-presenting cells (APC), and this receptor-ligand interaction is important in the early phases of establishing adaptive immunity (Aderem and Ulevitch 2000).

Mucosal application of soluble antigen generally is insufficient to sustain immune response, because there is a negative impact on antigen targeting in the uptake by APC for processing. A quest for new potent adjuvants has emerge for such purpose and the number of new substances with documented adjuvant activity and the literature describing their use have expanded in the last 20 years (Lindblad 2007).

The toxicity and adjuvant activity must be balanced to obtain maximum immune stimulation with minimal adverse effects (Gupta *et al.* 1993). The actual acceptance level for adverse reactions depends on whether the adjuvant is intended for human or veterinary vaccines. The majority of adjuvants induce some effects at the injection site, the most frequent being an inflammatory response. When used in preventive medicine the vaccine is administered to healthy persons and in many cases to children, the adverse reactions to the adjuvant are not acceptable. Thus several enhancement strategies have been employed in order to improve the process.

One of them was to take advantage of the immunostimulating ability of enterotoxins, they inactivated them, such as B subunit of CT (CTB); or the closely related serogroups I and II of *E. coli* heat labile enterotoxins (LT). It has showed that CTB could enhance the immune response and/or protective potential of intragastrically or intranasally applied mutans streptococcal antigens (Russell *et al.* 1996, Russell and Wu 1991, Smith *et al.* 2001a, Taubman *et al.* 2000b).

Antigen or functional domains, where mixed with detoxified adjuvant or chemically conjugated or genetically fused with adjuvants subunits, dramatically increased salivary immune responses resulting in protective immunity (Smith 2002b, Taubman *et al.* 2000a).

Recent investigations in mucosal adjuvants field, broth new adjuvants for this discussion. The aluminium adjuvants (aluminium hydroxide and aluminium phosphate gel adjuvants) (Lindblad 2004) have been accepted for use in humans, but there are limitations for the content aluminium allowed, the limit is dependent on the aluminium intake established for each country. These adjuvants have been used more than half century and are generally regarded as safe when used according to current immunization protocols (Clements and Griffiths 2002). Since we tried to develop a vaccine against dental caries that could be used in humans in future, in our vaccination protocols we decided to use alum as adjuvant.

The effect of aluminium adjuvants at the cellular and cytokine level has just partly elucidated and further understanding still necessary.

There are some evidences that aluminium adjuvants are able to generate MHC class I restricted cytotoxic T-cells in mice (Dillon *et al.* 1992) and usually don't induce delayed-type hypersensitivity (DTH) response in rodent models (Bomford 1980). Their overall adjuvant effect was shown in the late 1970s to involve stimulation of T-cells. In mice the aluminium hydroxide attracted eosinophils to the injection site in the absence of an antigenic stimulus (Walls 1977), this reaction apparently required the presence of T-cells. The proliferating CD4⁺ T-cells from mice immunized with antigen and aluminium adjuvant were of the Th2 subpopulation Taking into account the isotypic profile of

antibody response for T-cell stimulation and for cytokine profiles induced, there will be a limit for certain adjuvant utilization. The basic question here is whether aluminium adjuvants, being Th2 stimulators, will promote such parameters of the immune response, as are essential for protective immunity against a given disease. For example, the Th2 biased immune response is not likely to protect against diseases for which Th1 immunity and MHC class I restricted CTL's are essential for protection, such as with e.g. tuberculosis (Lindblad *et al.* 1997). In our work we need to induce mucosal immune response protective against dental caries, which is related with the induction of IgA. Since Th2 response may mediate the isotype switching for IgA (Hummelshoj *et al.* 2006) , this type of adjuvants may improve the required immune response.

There have been some work using Caprylic/capric glyceride (pure) in mucosal administration (Hrafnkelsdottir *et al.* 2005), leading to good immune responses and less side effects for the immunized host. We also intent in a future to test these adjuvants. One important aspect is what antigens will be combined with adjuvant, both may contribute to the net safety profile and the possible interactions between adjuvant and antigens must be considered. The age of the person to receive the adjuvanted vaccine formulations should be considered, infants may react differently from adults and this may have impact on the dosage recommendations.

Another important aspect in mucosal vaccines is the delivery systems. Targeting vaccine to mucosal immune tissue or delivery in vehicles which prolong or promote exposure of antigen to inductive tissues has also been used in preclinical studies. Intact adhesins, chimeric proteins (Hajishengallis *et al.* 1996), or Gtfs glucan binding domains (Russell *et al.* 2004) were expressed in *Salmonella*, since these bacteria specifically bind to the epithelial cells which overlie mucosal lymphoid tissue. Such constructs, delivered in attenuated *Salmonella* expression vectors, induced protective immune responses when administrated intragastrically or intranasally (Hajishengallis *et al.* 1996, Redman *et al.* 1996).

Incorporation of antigen in or on various types of microparticles or microspheres made of poly(lactide-co-glycolide) or liposomes has also been employed in an attempt to increase uptake via antigen particularization (Yoshinori *et al.* 2006). These approaches have been shown to be useful in the delivery of dental caries vaccine antigens for protective responses in pre-clinical (Smith 2002b, 2003) or salivary antibody formation on clinical studies (Childers *et al.* 1997, Childers *et al.* 1999).

Under controlled conditions of dental caries infection, experimental vaccine approaches have shown that infection and disease can be controlled. Refinements in antigen formulation, delivery vehicles, enhancing agents and routes of administration are needed; and must be coupled with approaches that are timed to intercept most vulnerable periods of infection, primary and permanent dentition, was well provide additional tools to the health care practitioner to maintain oral health.

Therefore, developing a vaccine against dental caries is feasible, and there is a moral obligation to implement this because of the devastation caused by this disease.

In a vaccine development there is a risk when providing medical intervention in a healthy individual to protect him from a future disease. The resolution of such concerns can only be achieved by clinical trials that are appropriately designed and carried out. The clinical immunological science provide information for the public decision on the utility of a vaccine against dental caries, and the dental health of millions of children awaits for these clinical trials.

CHAPTER II

OBJECTIVES

II. Objectives

The overall aim of this study was to develop and evaluate a safe cost effective vaccine against dental caries. The work was divided in several experimental studies, as described above, and they will be presented in the next chapters.

III. *Streptococcus sobrinus* enolase plays a role in the oral colonization by this bacterium.

IV. Development of a vaccine against *S. sobrinus* and *Streptococcus mutans*-induced dental caries using native or recombinant VIP secreted by *S. sobrinus*.

IV.A. Therapeutic vaccine against *Streptococcus sobrinus*-induced caries.

IV.B. Oral therapeutic vaccination with *Streptococcus sobrinus* recombinant enolase confers protection against rats dental caries.

IV.C. Therapeutic vaccine against *Streptococcus mutans*-induced caries

IV.D. Preventive vaccination with *Streptococcus sobrinus* recombinant enolase confers protection against rats dental caries.

IV.E. Maternal immunization of rats with *Streptococcus sobrinus* recombinant enolase confers protection to offspring against dental caries.

V. A preliminary study on the characterization of the mucosal immune response after *Streptococcus sobrinus* recombinant enolase mucosal immunization in mice.

CHAPTER III

ENOLASE FROM *Streptococcus sobrinus* VIRULENCE FACTOR

III. *Streptococcus sobrinus* ENOLASE PLAYS A ROLE IN THE ORAL COLONIZATION BY THIS BACTERIUM

Abstract

In this study, we investigated the role of *S. sobrinus* recombinant enolase in the colonization of the bacterium in oral cavity and in caries lesions incidence using a rat model. Wistar rats were orally treated with *S. sobrinus* recombinant enolase (enolase-treated) in a suppressive dose or with PBS (control), seven days later orally infected with *S. sobrinus* and fed with a cariogenic diet. The evaluation of the oral colonization showed that enolase-treated rats had higher *S. sobrinus* colonization than control animals. This increase of *S. sobrinus* oral colonization was correlated with the augment of cariogenic lesions. The levels of salivary IgA antibody anti-enolase or *S. sobrinus* sonicates were lower in the enolase-treated than in control rats indicating that enolase treatments suppressed specific antibody response. In conclusion, we demonstrated that *S. sobrinus* enolase plays a role in the pathogenicity of these bacteria which could be explained by the immunosuppressive properties of this protein.

III.1. Introduction

Dental caries is one of the most prevalent infectious diseases in humans and its treatment is probably one of the most expensive in the world (Taubman and Nash 2006a) being *Streptococcus sobrinus* one of the primary pathogens responsible for dental caries in humans (De Soet *et al.* 1991). We have previously reported that this bacterium produces virulence-associated immunomodulatory proteins (VIP) (Ferreira *et al.* 1997) and a therapeutic vaccine using VIP as a target antigen showed to reduce rats cariogenic lesions in rat (Dinis *et al.* 2004). Enolase from *S. sobrinus* has been described as an immunosuppressive protein and the respective gene has been cloned (Veiga-Malta *et al.* 2004). In the present study, we investigated the role of the *S. sobrinus* recombinant enolase in the survival of *S. sobrinus* in the oral cavity and in the dental caries lesions in a rat model.

III.2. Material and Methods

III.2.1. Animal models

Male and female Wistar rats, purchased from Jackson Laboratories (Maine, USA) and bred at the animal facilities of the ICBAS (Porto, Portugal), were used at 7 days after birth, and were weaned at approximately 20 days of age. Procedures involving animals were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

III.2.2. Bacteria

Streptococcus sobrinus strain 6715 was a kind gift of Dr. B. Klausen from the Department of Oral Diagnosis and Microbiology, Royal Dental College, University of Copenhagen, Denmark. The strain was stored at -70°C in Brain Heart Infusion broth medium (Difco, Detroit, MI) with 25% (v/v) of glycerol. Bacteria inoculums were prepared as previously described (Dinis *et al.* 2004).

III.2.3. Preparation of *S. sobrinus* recombinant enolase

The isolation and purification of recombinant enolase has been described in detail (Veiga-Malta I *et al.* 2004). The protein concentration was determined by the method of Lowry (Lowry *et al.* 1951) and its purity was evaluated by SDS-PAGE. The recombinant enolase was depleted of lipopolysaccharide (LPS) using a polymyxin B column (Pierce, Rockford, IL), and all recombinant protein preparations were LPS-free, as assessed by the limulus amoebocyte lysate kit (E-Toxate; Sigma).

III.2.4. Enolase treatment protocol

Groups of 7 days-old Wistar rats were orally treated with 50 µg of recombinant enolase (suppressive dose) plus alum as a adjuvant (enolase treated) or with PBS plus alum (control). One week later the treatment was repeated with 80 µg of recombinant enolase or with PBS. After seven days, rats were orally infected for five consecutive days (from 21 to 25 days of age) with 1×10^9 colony forming units (CFU) of *S. sobrinus* and were fed with cariogenic solid diet.

III.2.5. Bacterial recoveries

The mutans streptococcal flora was assessed as previously described (Dinis *et al.* 2004).

III.2.6. Caries assessment

The mandibles were hemisectioned and the extent and depth of carious lesions in all rat molar teeth (caries score) were microscopically evaluated by a modified Keyes's method (Keyes 1958). Caries scores were determined on smooth and on occlusal dental surfaces. The analyst was blind to group assignments.

III.2.7. Antibody analyses

Salivary IgA and IgG antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Dinis *et al.* 2004). Briefly, polystyrenemicrotiter plates (Nunc, Roskilde, Denmark) were coated with 5 µg/mL of recombinant enolase, or *S. sobrinus* sonicates. After washing with Tween-saline (TS) the plates were blocked with PBS containing 1% of bovine serum albumin (Sigma, St

Louis, Mo). Antibody activity was measured by incubation with 1:2 to 1:16 dilutions of saliva. After washing, the bound antibodies were revealed by the addition of peroxidase-labelled goat antibodies anti-rat IgG (Southern Biotechnology Associates, Birmingham, USA) or peroxidase-labelled mouse anti-rat IgA (Biosource, Belgium). The reaction was stopped with 10% SDS and the colorimetric change was measured with a Biotek Chromoscan at 450 nm. Data are reported as ELISA units (EU), which were calculated relative to levels in reference from non-infected rats or non-treated rats. Dilutions of 1:4 (A_{450} of 0.1) were considered 1 EU for both reference salivary IgA and IgG specific for recombinant enolase or *S. sobrinus* sonicates.

III.2.8. Statistical analysis.

The level of significance of the results in all groups of animals was determined by t-Test. Differences were considered significant at $p < 0.05$.

III.3. Results

III.3.1. Treatment with a suppressive dose of recombinant enolase increased *S. sobrinus* oral colonization

The Wistar rats orally infected with 10^9 *S. sobrinus* cells and previously treated with suppressive doses of recombinant enolase or with PBS plus alum as adjuvant were monitored on days 30, 60 and 120 after infection to evaluate the *S. sobrinus* CFU in the tooth.

As shown in Figure III.1, an increase in oral *S. sobrinus* CFU on enolase treated rats was observed compared with control group, throughout the study. Therefore, the recombinant enolase treatment increases *S. sobrinus* oral colonization.

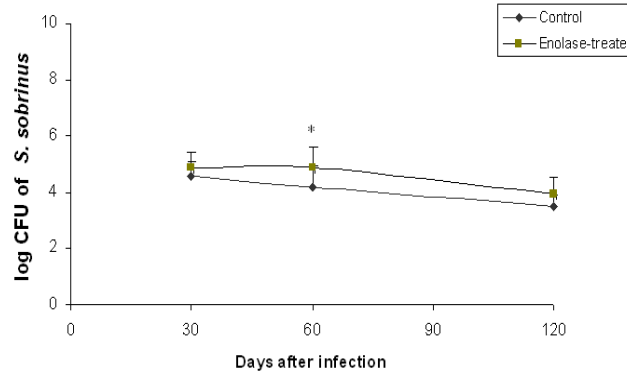


Figure III.1. Numbers of *S. sobrinus* CFU (log10) recovered from oral cavities of control and enolase treated Wistar rats at the indicated days after infection. Results are the means logCFU + SD of 10 to 12 rats per group and are one of two representative independent experiments. Comparison among groups indicated a significant difference between enolase treated and control animals (* $p < 0.05$).

III.3.2. Increase on dental caries by *S. sobrinus* recombinant enolase treatment

The increase of *S. sobrinus* oral colonization observed in the enolase treated rats in comparison with control animals is correlated with the increase on caries lesions. Actually as shown in Fig. III.2A there was a fold increase of 1.7 in the enamel sulcal caries score on enolase treated rats comparatively with control animals. This effect on teeth lesions was also evident in the enamel interproximal caries score (Fig. III.2B) where a 2.1 fold increase was observed in the interproximal caries lesion on the enolase treated group.

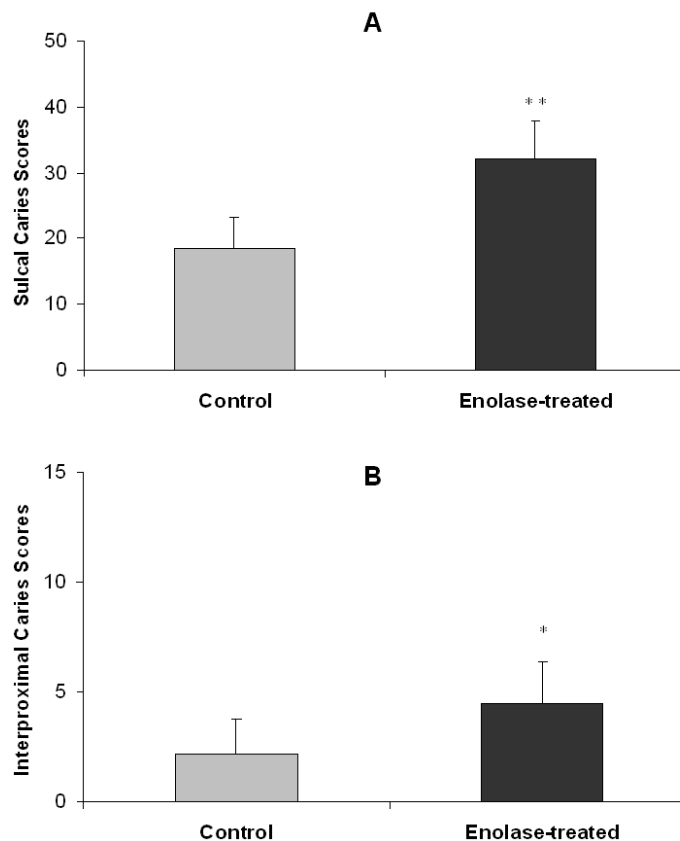


Figure III.2. Caries lesions on sulcal (A) and interproximal (B) enamel of molar surfaces in control and enolase treated rats. Values are the mean caries scores + SD of 10 to 12 Wistar rats and are one of two representative independent experiments. Comparison among groups indicated a significant difference between enolase treated and control animals (* $p < 0.05$, ** $p < 0.01$).

III.3.3. Levels of salivary IgA antibody specific for enolase or *S. sobrinus* sonicates in enolase treated rats were lower than in control animals.

Since it has been described that *S. sobrinus* enolase induced a suppression of specific immune responses of the host (Dinis *et al.* 2004) we compared the titres of IgA and IgG antibodies, specific to enolase or to *S. sobrinus* sonicates, in the enolase-treated and control rats. As shown in Fig. III.3A, the levels of salivary IgA antibodies specific for *S. sobrinus* recombinant enolase were lower ($p < 0.07$) in the enolase-treated than in control rats.

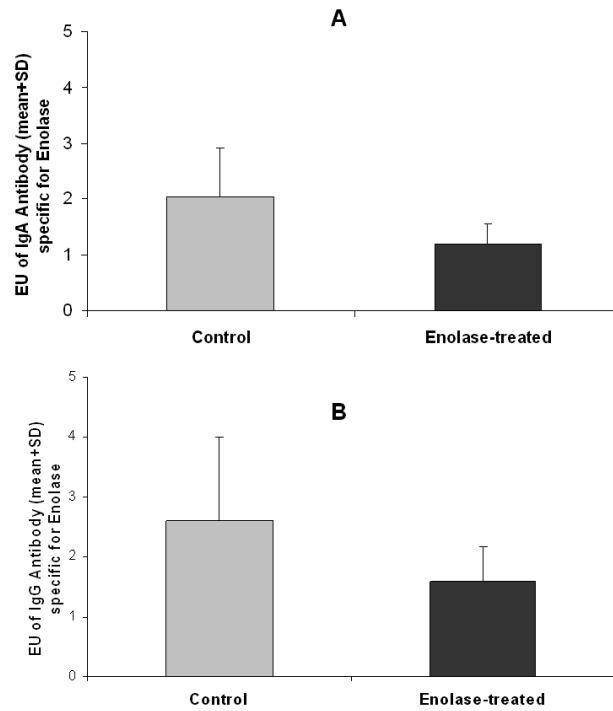


Figure III.3. Comparison between the salivary levels, expressed as ELISA units (EU) of IgA (A) or IgG (B) antibodies against enolase in control and enolase treated. The results are the mean of EU+SD of 10 to 12 Wistar rats and are one of two representative independent experiments.

Moreover, the salivary levels of IgA antibodies anti-*S. sobrinus* sonicates (Fig. III.4A) were also significantly lower in the enolase treated than in control rats. These results indicate that enolase treatment suppresses specific antibody production, suggesting that this protein is important for the colonization of *S. sobrinus* on rat oral cavity and for disease development.

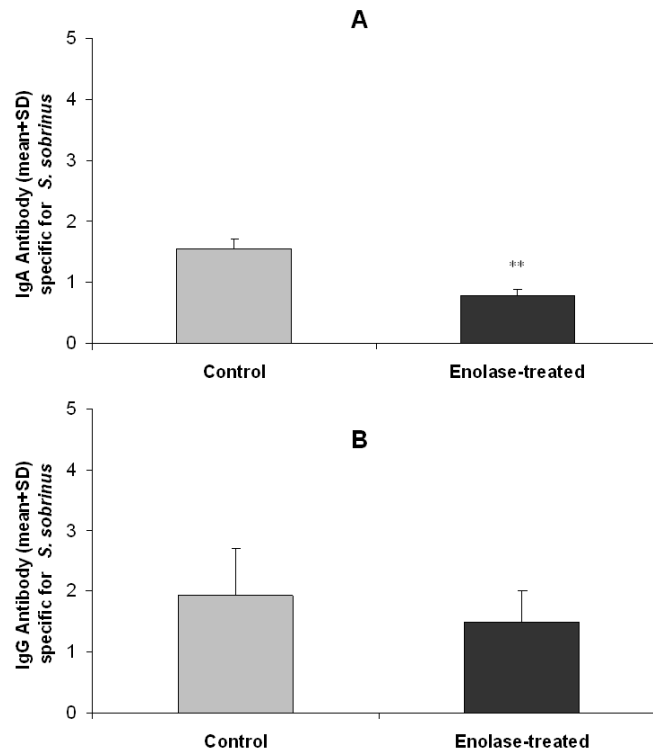


Figure III.4. Comparison between the salivary levels, expressed as ELISA units (EU) of IgA (A) or IgG (B) antibodies against *S. sobrinus* sonicates in control and enolase treated. The results are the mean of EU+SD of 10 to 12 Wistar rats and are one of two representative independent experiments. Comparison among groups indicated a significant difference between enolase treated and control animals (** $p < 0.01$).

III.4. Conclusions

In the present study we show that treatment with a suppressive dose of *S. sobrinus* recombinant enolase before bacterial infection induces an increase in oral *S. sobrinus* colonization that is correlated with an increase in caries lesions. These results could be explained by the immunosuppressive properties of this protein.

CHAPTER IV

DEVELOPMENT OF A VACCINE AGAINST *Streptococcus sobrinus* AND *S. mutans* INDUCED DENTAL CARIES USING NATIVE OR RECOMBINANT VIP SECRETED BY *S. sobrinus*

CHAPTER IV.A

A THERAPEUTIC VACCINE AGAINST *Streptococcus sobrinus*-INDUCED CARIES

IV.A. THERAPEUTIC VACCINE AGAINST *Streptococcus sobrinus*-INDUCED CARIES

Abstract

Streptococcus sobrinus produces a virulence-associated immunomodulatory protein (VIP) which suppresses the host specific immune response and induces the early production of IL-10. In this study we evaluated the effects of the therapeutic-immunization with this VIP on the incidence of caries in *S. sobrinus*-infected rats. Groups of Wistar rats were orally infected with *S. sobrinus* and fed with sucrose-sweetened drinking water *ad libitum*. Five days later rats were immunized intranasally with active- or heat-inactivated-VIP plus alum as adjuvant or PBS plus adjuvant (sham-immunized). After three weeks, all rats were re-immunized as above. Evaluation of dental caries showed that VIP-immunized animals had significantly fewer enamel sulcal and proximal caries lesions than the sham-immunized ($p < 0.001$). The protective effects following therapeutic-VIP-immunization were attributed to the induced salivary immunoglobulin A specific to the VIP. These results offer a promising and safe strategy for the development of a vaccine against dental caries.

IV.A.1. Introduction

Dental caries is one of the most prevalent infectious diseases in humans and a vaccine against this disease has been under intensive investigation. Treatment of dental caries is probably one of the most expensive in the world due to the wide distribution of this illness (Hanada 2000). Children and adolescents could benefit greatly in a short and long-term basis with the prevention of the major medical complications of caries, as well as adults. A group of related oral bacteria known as mutans streptococci are implicated as primary etiological agents of human caries (Coykendall and Gustafson 1986). Within this group, *Streptococcus sobrinus* and *Streptococcus mutans* are the species most commonly isolated from humans (De Soet *et al.* 1991, Loesche 1986). Research efforts towards developing an effective and safe caries vaccine have been directed to the functional characterization of virulence factors from the bacteria (Newbrun 1983, Walden and Wilensky 1982). As reported (Ferreira *et al.* 1997), *S. sobrinus* secretes a virulence-associated immunomodulatory protein (VIP) that like VIP secreted by other microorganisms inhibits the hosts specific responses by triggering polyclonal non-specific lymphocyte activation (Arala-Chaves *et al.* 1988, Ferreira *et al.* 1988, Lima *et al.* 1992, Tavares *et al.* 1993) and inducing the early production of IL-10 in the host (Ferreira *et al.* 1997, Tavares *et al.* 2000, Vilanova *et al.* 1999). Moreover, the VIP act as virulence factors because their production is correlated with the pathogenicity of the secreting microorganism and host treatment with the VIP before colonization increases the specific colonization (Lima *et al.* 1992, Santarém *et al.* 1987, Tavares *et al.* 1993). Thus, the production of these VIP seems to be a mechanism of immune evasion amongst pathogens.

Isolation and characterization of these molecules, which play a key role in the survival and interaction of the microorganism with the host and his immune defences, are fundamental for the development of rational strategies for vaccination and infection therapy. Indeed, as we have demonstrated for systemic candidiasis in mice, VIP can be used as a vaccination target inducing specific protection against the microorganism (Tavares *et al.* 1995).

The rat caries model has been extensively used for delineating immune protection against this disease. In these models there is a discrete period of time, between days 18 and 22, to implant the mutans streptococci in the oral cavity of laboratory rats denominated “window of infectivity” (Caufield 1997). After day 22, stable establishment of the bacteria in rats was less probable (Caufield 1997). On the other hand, induction of an effective salivary IgA response requires at least two mucosal immunizations. For these reasons, it is obvious that caries immunity is difficult to establish prior to infection

in rats. The present study reports a VIP secreted by *S. sobrinus* as a novel target for vaccination against caries induced by this bacteria. The vaccination reported here is a therapeutic-vaccination because it occurs after the implant of the bacteria in the oral cavity of the rats.

IV.A.2. Materials and Methods

IV.A.2.1. Animal models

Male and female Wistar rats, bred at Faculty of Medicine (Coimbra, Portugal) and kept at the animal facilities of the Instituto Ciências Biomédicas Abel Salazar (Porto, Portugal) during the experiments, were used at 16-days old in the caries experiments. These rats were weaned at approximately 20 days.

The animal experiments were performed according to European Economic Community animal experimentation guidelines Directive of 24 November 1986 (86/609/EEC) and approved by Veterinary Ethical Committee of Instituto Ciências Biomédicas Abel Salazar.

IV.A.2.2. Bacteria

S. sobrinus, strain 6715 obtained from ATTC (“American Type culture Collection”), was stored at -70°C in Brain Heart Infusion broth medium (Difco, Detroit, MI) with 25% (v/v) of glycerol.

IV.A.2.3. Preparation of the VIP

The isolation and purification of VIP has been described in detail (Ferreira *et al.* 1997). All VIP preparations were lipopolysaccharide (LPS)-free, as assessed by the limulus amoebocyte lysate kit (E-Toxate; Sigma, St. Louis) as described (Tavares *et al.* 2000). The protein content was determined by the methods of Lowry (Lowry *et al.* 1951).

IV.A.2.4. Protocol for caries experiments

IV.A.2.4.1. Antigens and adjuvants

The VIP isolated as described above was used as active-VIP in a submitogenic dose that was unable to induced immunosuppression in host (10 µg for the first and 50µg for the second immunization/rat) or heat-inactivated-VIP (10 µg for the first and 50µg for the second immunization/rat) for the immunoprotection assays. Alum (Alhydrogel® “85”, Brenntag Biosector, Denmark), kind gift of Erik Lindblad (Brenntag Biosector, Frederikssund, Denmark), was used as adjuvant.

IV.A.2.4.2. Immunizations

Three experimental groups of Wistar rats were done with 10 to 12 animals per group and treated as following: at the 16^o day after birth all the rats were submitted to a cariogenic diet of 8% of sucrose in the drinking water. At the 18^o day and for 4 consecutive days all the animals were infected orally with 10⁹ cells of *S. sobrinus*. Five days later the animals were immunized by intranasal route (i. n.) with active- or heat-inactivated-VIP plus alum as adjuvant or with buffer (PBS) incorporated in alum (sham-immunized). Each dose volume did not exceed 20 µl. The immunizations were repeated 3 weeks later. At the end of the experiment when the animals were 120 days old, rats were sacrificed. The serum and saliva were collected for antibodies quantification and the teeth for caries evaluation.

IV.A.2.5. Antibody analyses

Serum immunoglobulin G (IgG) and salivary immunoglobulin A (IgA) antibodies were tested by ELISA. Polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 µg of VIP per ml, at 4°C overnight. Coated plates were washed in Tween-saline (TS) (0.9% NaCl containing 0.05% Tween 20). The plates were blocked 1 h with 200 µl per well of a solution containing 10 µg of bovine serum albumin (Sigma) per ml in PBS, at room temperature. Dilutions of rat serum or saliva samples were added (50 µl/ well) in duplicate, and the plates were incubated for 2 h at 37° C. After washing with TS, the bound antibodies were revealed by the addition of 50 µl/well of peroxidase-labelled goat antibodies anti-rat IgG (Southern Biotechnology Associates, Birmingham, USA) or peroxidase-labelled mouse anti-rat IgA (Biosource, Nivelles, Belgium) and incubated

for 3 h at 37°C. After washing, the plates were incubated with orthophenylenediamine dihydrochloride (Sigma) and H₂O₂, 100 µl per well for 30 min at room temperature. The reaction was stopped with 10% SDS (50 µl/well) and the colorimetric change was measured with a Biotek Chromoscan at 450 nm. The values of Absorbance were calculated after subtraction of the background values (no serum or saliva added). Data are reported as ELISA units (EU), which were calculated using saliva or serum obtained from non-immunized and non-infected rats as a reference. Dilutions of 1:2 (A₄₅₀ of 0.1) were considered 1 EU for reference salivary IgA specific to VIP. Dilutions of 1:10 (A₄₅₀ of approximately 0.2) were considered 1 EU for reference serum IgG specific to VIP.

IV.A.2.6. Caries assessment

The extent of enamel carious lesions in the first, second and third molar teeth of all rats (caries score) were microscopically evaluated by a modified Keyes method as previously described (Keyes 1958).

IV.A.2.7. Bacterial recoveries

The mutans streptococcal flora was assessed as previously described (Taubman *et al.* 2000b). Briefly, *S. sobrinus* infection levels were assessed after systematic swabbing of teeth, sonication, and plating of appropriate dilutions on Todd-Hewitt agar [Difco] with Streptococcus Selective Supplement (0.001 mg of colistin sulphate and 0.5 µg of oxalinic acid per ml) (Oxoid, Hampshire, England). The plates were incubated at 37°C, in aerobiose, for 48 h and *S. sobrinus* CFU were then enumerated microscopically.

IV.A.2.8. Statistical analysis

The level of significance of the results in all groups of rats was determined by one-way ANOVA, calculated with Microsoft Excel 2000 software.

IV.A.3. Results

IV.A.3.1. Increased resistance to *S. sobrinus*–induced dental caries in rats by intranasal therapeutic vaccination with a VIP secreted by the bacteria

The experiments were performed to investigate the protective effect of therapeutic immunization with an active- or heat-inactivated-VIP in *S. sobrinus*-induced dental caries in Wistar rats.

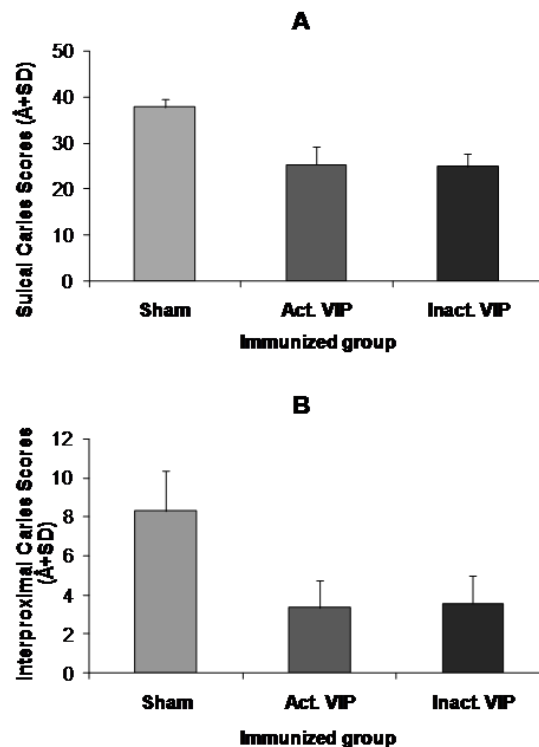


Figure IV.A.1. Evaluation of dental caries scores on proximal (A) and sulcal (B) molar surfaces involving enamel lesions in Wistar rats sham-immunized, active- or heat-inactivated-VIP immunized. Data show $\bar{A} \pm SD$ of 10 to 12 rats per group. Statistical difference in mean score among the three groups was assessed by ANOVA. Multiple comparisons between groups indicated a significantly different mean score of active- or inactive-VIP immunized in comparison with sham immunized ($p < 0.001$). No differences were detected between active- and heat-inactivated -VIP immunized groups. Figure shows results of one of three representative independent experiments.

The differences in the enamel sulcal caries score between the sham-immunized group and the other two immunized groups were statistically significant ($p < 0.001$) with a reduction of 34% in caries lesions in both immunized groups of rats (Fig. IV.A.1).

The protective effect of VIP immunization on caries lesions was more marked in the enamel proximal caries score. Indeed, the differences in the proximal caries score between the sham-immunized group and the two VIP-immunized groups were statistically relevant ($p < 0.001$) with a reduction in the caries lesions of 60% in both VIP-immunized group (Fig. IV.A.1). No statistical differences were found between active- and heat-inactivated-VIP-immunized groups ($p = 1.000$). Therefore, immunization with either active- or heat-inactivated-VIP, conferred protection against the *S. sobrinus*-induced dental caries in wistar rats.

The evaluation of *S. sobrinus* colonization in the oral cavity of the rats showed that VIP-immunized groups exhibited a significant reduction in *S. sobrinus* levels while sham-immunized group maintained high levels of the bacteria throughout the study (Table1).

Days after Immunization	Mean response (log CFU $\pm$ log SD) ^b		
	Sham- Immunized	Active-VIP Immunized	Heat-Inactivated - VIP Immunized
30	7.62 $\pm$ 0.66	6.76 $\pm$ 0.65	6.62 $\pm$ 0.54
60	6.60 $\pm$ 0.56	<u>5.43 $\pm$ 0.58</u>	5.61 $\pm$ 0.68
90	5.89 $\pm$ 0.74	<u>4.23 $\pm$ 0.60</u>	<u>4.27 $\pm$ 0.61</u>

Table IV.1. Reduction of *S. sobrinus* oral colonization in active- or heat-inactivated-VIP immunized rats ^a.

^a Numbers of *S. sobrinus* CFU recovered from the oral cavity of the different groups of Wistar rats orally infected with *S. sobrinus* four days before immunization (post-infection immunization).

^b Results are $\bar{X} \pm SD$ of 10 to 12 Wistar rats per group. The significant higher differences between Sham-Immunized and active- or heat-inactivated- VIP immunized are underlined ($p < 0.001$). Statistical difference in mean score among the three groups was assessed by ANOVA.

IV.A.3.2. Effects of immunization on salivary IgA antibodies

To quantify the antibody response after i. n. immunization with active- or heat-inactivated-VIP, the titres of serum and salivary antibodies specific for VIP were evaluated by ELISA in the VIP-immunized and in the sham-immunized groups of animals.

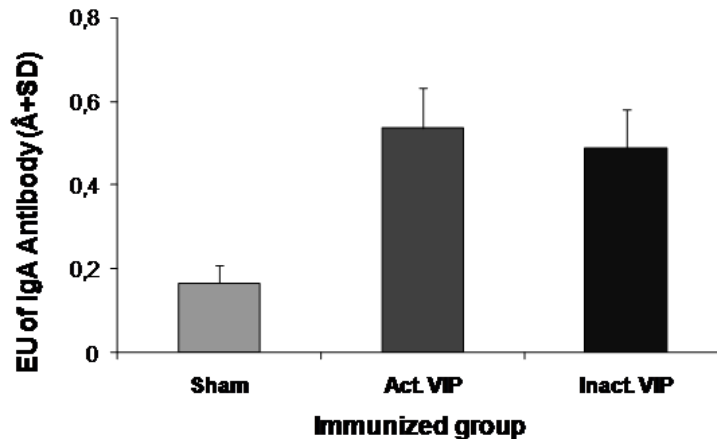


Figure IV.2. Comparison between the salivary levels, expressed as ELISA Units (EU), of IgA specific for VIP in sham-immunized, active- or heat-inactivated-VIP immunized rats. Results are $\bar{X} \pm SD$ of 10 to 12 Wistar rats. Statistical difference in mean score among the three groups was assessed by ANOVA. Multiple comparisons between groups indicated a significantly different mean score of active- or inactive-VIP immunized in comparison with sham immunized ($p < 0.001$). No differences were detected between active- and heat- inactivated -VIP immunized groups. Figure shows results of one of three representative independent experiments.

The salivary IgA antibodies specific to VIP in experimental animals were significantly different between sham-immunized and both VIP-immunized groups of rats ($p < 0.001$, Fig. IV.2). The VIP-immunized groups of rats showed similar high levels of specific IgA antibodies ($p = 0.3$), which means that either active- or heat-inactivated-VIP immunization induced a localized mucosal immunity. In contrast, no differences in serum levels of IgG antibodies specific for VIP were observed between the sham-immunized group and both VIP-immunized groups of animals (data not shown), which indicated that the intranasal immunization did not induce a systemic immunity. Therefore, the protection observed against dental caries induced by VIP-immunization correlates with the production of salivary IgA antibodies specific to VIP.

IV.A.4. Discussion

This study demonstrated the induction of a salivary IgA antibody response against a VIP secreted by *S. sobrinus* after intranasal (i.n) immunization with either an active or heat-inactivated VIP in rats which was associated with protection against *S. sobrinus* induced dental caries. Therefore, in order to create a model as similar as possible to the human, Wistar rats, an out-bred strain, were used in the experiments. Due to the mutans streptococci window of infectivity (Caufield 1997), the immunizations occurred after oral bacteria inoculation, i.e., a therapeutic immunization with either active- or heat-inactivated-VIP was done. The i.n. route was selected because the nasal region has been shown to contain organized nasal-associated lymphoid tissue (NALT) with inductive properties (Michalek *et al.* 1994, Wu *et al.* 1997), which after antigen exposure result in the induction of significant levels of salivary IgA antibodies (Smith *et al.* 1999, Wu *et al.* 1997, Wu *et al.* 1997). This route also has been used for human immunization with attenuated influenza (Michalek *et al.* 1994). All the immunizations were given with the protein or PBS incorporate with alum since it is an adjuvant approved to be used in humans (O'Hagan *et al.* 2001). The results present herein show that efficient therapeutic-VIP-immunization against *S. sobrinus*-induced caries was observed in rats. The active-VIP was used in a (submitogenic) dose that was unable to induce immunosuppression in the host (unpublished results). However, as this kind of molecules is very dependent of the reactivity of the immune system of the host (Arala-Chaves 1992), a heat-inactivated-VIP was also used in the immunoprotection assays. The results showed that rats immunized i.n. either with submitogenic- or heat-inactivated-VIP had a significant reduction in caries lesions comparatively to the sham-immunized rats. A significant reduction in the enamel sulcal and proximal caries score was observed in both immunized groups of rats, when compared to the sham-immunized rats ($p < 0,001$). We also found that protection induced by active- or heat-inactivated-VIP immunization was not significantly different ($p = 1,00$). The similar results obtained with active- or heat- inactivated-VIP could be explained by the fact that protective antigenic determinants did not depend on protein folding, i.e, are accessible in native- and in the heat-inactivated-VIP and this will be a safe molecule for the immunization strategy.

Moreover, active- or heat-inactivated-VIP immunized animals showed significant inhibition of *S. sobrinus* colonization in contrast to sham-immunized animals, which maintained high levels of the bacteria throughout the study.

The finding that the levels of salivary IgA specific for the VIP were significantly higher in both VIP-immunized groups in comparison with the sham-immunized group

($p < 0,001$) indicated that the VIP induced a mucosal immunity. There were no significant differences between the two VIP-immunized groups.

The presence of a slight level of antibodies against VIP in the saliva of sham-immunized rats at the end of experiment indicate that the bacterial infection can induce an immune response against the VIP that is secreted during the bacterial growth, but that is not enough to reduce the colonization of the bacteria. This could be explained by the described mitogenic effect of these proteins that induce a polyclonal response in the host but a very dim specific response against them (Arala-Chaves *et al.* 1988, Minoprio *et al.* 1991). With the purpose of circumventing the immunobiological dose-dependent effects of the VIP (Arala-Chaves *et al.* 1979) we used a submitogenic dose of the VIP.

The protection obtained through the VIP-immunization supports the involvement of the VIP secreted by *S. sobrinus* in the pathogenesis of the bacteria, which has also been described for other VIP secreted by *Candida albicans* (Tavares *et al.* 2000).

The i.n. VIP-immunization did not induce a systemic immunity since we could not observe differences in serum levels of IgG specific for VIP between VIP-immunized groups and sham-immunized groups. This observation could be explained by the fact that we are using alum as adjuvant. However, the role of serum antibodies against mutans streptococci and the degree of protection against caries is still controversial (Hajishengallis and Michalek 1999). There are several references to the use of structural antigens of mutans streptococci as a target for preventive vaccination against the bacteria (Hajishengallis and Michalek 1999), however, absolute protection has not yet been demonstrated (Hajishengallis and Michalek 1999). The strategy that we present in this report is based on the use of a target molecule that is important for the survival of the producing microorganism. This approach has also been successfully assayed by others (Blander and Horwitz 1991, Horwitz *et al.* 1995, Reina-San-Martin *et al.* 2000, Strindeli *et al.* 2002).

Taking into account the results obtained in this study, it seems reasonable to hypothesize that the same approaches of vaccination could be applied for mucosal therapeutic vaccination against human dental caries.

CHAPTER IV.B

PROTECTION AGAINST DENTAL CARIES BY *Streptococcus sobrinus* ENOLASE THERAPEUTIC VACCINATION

IV.B. ORAL THERAPEUTIC VACCINATION WITH *Streptococcus sobrinus* RECOMBINANT ENOLASE CONFERS PROTECTION AGAINST RATS DENTAL CARIES

Abstract

Dental caries is among the more prevalent human chronic infections for which an effective human vaccine has not yet been achieved. Enolase from *Streptococcus sobrinus* has been identified as an immunomodulatory protein. In this study, we used recombinant *S. sobrinus* enolase (rEnolase) as a target antigen and assessed its therapeutic effect in a rat model of dental caries. Wistar rats fed with a cariogenic solid diet on the day 18 after birth were orally infected with *S. sobrinus* on day 19 after birth and for 5 consecutive days thereafter. Five days after infection and, again, 3 weeks later, rEnolase plus alum adjuvant was delivered into the oral cavity of the rats. A sham-immunized group of rats was contemporarily treated with adjuvant alone. In the rEnolase-immunized rats, increased levels of salivary IgA and IgG antibodies specific for this recombinant protein were detected. A significant decrease in sulcal, proximal enamel, and dentin caries scores was observed in these animals, compared with sham-immunized controls. No detectable histopathological alterations were observed in all immunized animals. Furthermore, the antibodies produced against bacterial enolase did not react with human enolase. Overall, these results indicate that rEnolase could be a promising and safe candidate for testing in trials of vaccines against dental caries in humans.

IV.B.1. Introduction

Dental caries is one of the most prevalent infectious diseases in human (Bratthall 1991, Smith 2003) and has been associated with major clinical complications such as infective endocarditis (Brooks and Burnie 1994, Jaspers and Little 1984, Knox and Hunter 1991). The World Oral Health Report 2003 (Petersen 2003), published by WHO, indicates that dental caries is a major health problem in most industrialized countries affecting children, adolescents, and adults. Approximately 5% of the total health-care expenditure is spent in oral health care in the United States stressing the need for the development of a vaccine against dental caries (Taubman and Nash 2006a). *Streptococcus sobrinus* and *Streptococcus mutans* are known as primary pathogens responsible for dental caries. One characteristic that make mutans streptococci particularly efficient at causing dental caries is the rapid production of large amounts of lactic acid (Yamashita *et al.* 1993). Research efforts towards developing an effective and safe vaccine against dental caries have been directed to the characterization of bacterial virulence factors (Koga *et al.* 2002, Newbrun 1983, Taubman and Nash 2006a). Vaccination against this disease promotes the induction of mucosal immunity to the level of secretory IgA that has been directly correlated with a reduction in the development of the disease.

A therapeutic vaccine that uses as a target antigen the virulence-associated immunomodulatory proteins from *S. sobrinus*, was shown to reduce carious lesions in rats (Dinis *et al.* 2004). Enolase has been described as one of the immunomodulatory proteins of *S. sobrinus* (Veiga-Malta *et al.* 2004). Because, in vaccination strategies, the use of recombinant protein offers several advantages, the bacterial enolase gene has been cloned and expressed, and the recombinant protein has been purified (Veiga-Malta *et al.* 2004).

In the present study, we investigated whether oral therapeutic immunization with *S. sobrinus* recombinant enolase (rEnolase) reduces dental caries induced by this bacterium in rats, and we also tested the safety of this vaccine through histopathological evaluations. Enolase is a ubiquitous protein, and several autoimmune diseases have been associated with the production of antibodies against this protein (Fontan *et al.* 2000, Pancholi and Fischetti 1998, Pancholi and Fischetti 2001, Saulot *et al.* 2002, Witkowska *et al.* 2005). Therefore, we also investigated whether antibodies raised against bacterial enolase during systemic immunization with rEnolase recognize human enolase.

IV.B.2. Materials and methods

IV.B.2.1. Animal models

Male and female Wistar rats, purchased from Charles River Laboratoires (Barcelona, Spain), and bred at the animal facilities of the ICBAS (Porto, Portugal) were weaned at approximately 20 days of age.

Procedures involving animals were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

IV.B.2.2. Bacteria

Streptococcus sobrinus strain 6715 was provided by Dr. B. Klausen of the Department of Oral Diagnosis and Microbiology, Royal Dental College, University of Copenhagen (Copenhagen, Denmark). The strain was stored at -70°C in brain-heart Infusion broth (Difco) with 25% (v/v) of glycerol. For preparation of inocula, *S. sobrinus* cultures were diluted 10-fold in Todd-Hewitt broth (Difco), incubated overnight, and then incubated for another 8 hours to reach midexponential growth. The bacteria were then washed 3 times and suspended in cold, endotoxin-free PBS (Sigma). With the use of a standard curve plotted from colony forming units (cfu) as a function of the absorbance at 600 nm, the cultures were diluted in PBS to obtain the desired number of colony-forming units per milliliter. *S. sobrinus* colonies were identified by typical morphology on Todd Hewitt agar, by microscopic examination with Gram stain, and findings were confirmed by using the API 20 Strep System (bioMerieux, Marcy L'Etoile, France).

IV.B.2.3. Preparation of *S. sobrinus* recombinant enolase

The isolation and purification of rEnolase has been described in detail elsewhere (Veiga-Malta *et al.* 2004). In brief, *S. sobrinus* enolase was expressed in *Escherichia coli* DH5 α as a fusion protein containing 26 NH₂ extra amino acid residues including 6 histidyl. The recombinant protein was purified by affinity chromatography performed using a His-trap column (Amersham Biosciences) under native conditions. The protein concentration was determined by the method of Lowry (Lowry *et al.* 1951), and its purity was evaluated by SDS-PAGE. The rEnolase was depleted of lipopolysaccharide (LPS) by using a polymixin B column (Pierce), and all recombinant protein preparations

used were LPS-free, as assessed by the Limulus amoebocyte lysate kit (E-Toxate; Sigma).

IV.B.2.4. Saliva and serum samples

For collection of saliva and serum samples, rats were anesthetized using ketamine and xylazine, which were administrated at doses of 87 and 13 mg/kg of body weight, respectively, and they were given buprenorphine at 0.075 mg/kg, as an analgesic. Secretion of saliva was stimulated by intraperitoneal injection of 0.05 mg of pilocarpine per 100g of body weight. After centrifugation, the clarified saliva was stored at -20°C . Blood samples were collected from the tail vein during administration of anesthesia and serum samples obtained from coagulated and centrifuged blood were stored at -20°C .

IV.B.2.5. Caries induction protocol

In rat caries models, there is a discrete period, between days 19 and 22 after birth, during which mutans streptococci may be implanted in the oral cavity of laboratory rats; this period is known as the “window of infectivity” (Caufield 1997). Therefore, oral inoculation of *S. sobrinus* was started at day 19 after birth and was given for 5 consecutive days. At day 18 after birth, Wistar rats that were being weaned were fed cariogenic diet #11725 plus 62,7% of saccharose (Scientific Animal Food & Engineering); the rats were then orally infected for 5 consecutive days (from days 19 to 23 after birth) with $10\ \mu\text{l}$ containing 10^9 cfu of *S. sobrinus*. One group of rats was fed a cariogenic diet but was not infected or immunized. The oral swab specimens were obtained from individual rats to investigate bacterial colonization before and after infection. At the end of the experiment (on day 120, this length of time is necessary to observe caries lesions in control animals), the animals were euthanized, saliva and serum collected, and the mandibles removed for caries assessment. The body weight of rats was measured before and at the end of the experiments.

IV.B.2.6. Immunizations schedules

rEnolase or recombinant human enolase (rhEnolase) (provided by Franco Novelli, Laboratorio di Immunologia dei Tumori, Centro Ricerche Medicina Sperimentale, Ospedale San Giovanni Battista, Università di Torino, Turin, Italy) was used as a target

antigen and was given in the following doses: 100 µg for the first immunization and 500 µg for the second immunization for both types of immunization.

IV.B.2.6.1. Oral immunization

For vaccination purposes, 5 days after the last administration of *S. sobrinus*, 10 µl of rEnolase in a 1:1 PBS/alum (aluminium hydroxide gel; Brenntag) (provided by Erik Lindblad) suspension was delivered directly into the oral cavity of the rats. Sham-immunized animals received 10 µL of a 1:1 PBS/alum suspension. Three weeks later, the rats were immunized again.

IV.B.2.6.2. Subcutaneous immunization

For cross-reaction assays, rats 30 days of age twice received subcutaneous immunization with 100 µL of rEnolase or rhEnolase in a 1:1 PBS/alum suspension, with a 3-weeks interval between immunizations. The sham-immunized animals received 100 µL of a 1:1 PBS/alum suspension.

IV.B.2.7. Antibody analyses

Serum IgG and salivary IgA and IgG antibody levels were determined by ELISA, described elsewhere (Dinis *et al.* 2004). In brief, polystyrene microtiter plates (Nunc) were coated with rEnolase, rhEnolase or goat anti-rat IgA (Bethyl, Laboratories) given at dose of 5 µg/mL. After washing with Tween-saline, the plates were blocked with PBS containing 1% of bovine serum albumin (Sigma). Antibody activity was then measured by incubation with serum sample dilution of 1:30 to 1:2,430 or with saliva samples dilutions of 1:2 to 1:16. After washing, the bound antibodies were revealed by the addition of peroxidase-labelled goat antibodies anti-rat IgG (Southern Biotechnology Associates) or peroxidase-labelled mouse anti-rat IgA (Biosource) and they were incubated for 3 h at 37° C. After washing, the plates were incubated with orthophenylenediamine dihydrochloride (Sigma) and H₂O₂ for 30 min at room temperature. The reaction was stopped with 10% SDS and the colorimetric change was measured with a Biotek Chromoscan at 450 nm. Data are reported as ELISA units (EU) which were calculated relative to levels in reference serum samples obtained from non-immunized and non-infected rats as a reference. Dilutions of 1:30 (A₄₅₀ of 0.2) were considered to be 1 EU for reference serum IgG specific for recombinant

enolase. Dilutions of 1:4 (A_{450} of 0.1) were considered to denote 1 EU for both reference salivary IgA and reference of IgG specific for recombinant enolase.

IV.B.2.8. Caries assessment

The maxillary and mandibular soft tissue was removed. The maxilla and mandibula were put in a 1% silver nitrate aqueous solution overnight, and each sequence of first, second and third molar was hemisectioned using Keyes method (Keyes 1958) and was dried for 48 hours in a incubator by use of dry heat sterilizer at 50°C. Again, with use of the Keyes method, sulcal, proximal and dentin carious lesions in all rat molar teeth were microscopically evaluated. The analyst was blind to group assignments.

IV.B.2.9. Bacterial recoveries

The presence of *S. sobrinus* was assessed after systematic swabbing of teeth with a sterile brush for 10 s per side. The brush was placed into 500 µL of sterile PBS. After sonication and plating of appropriate dilutions on Todd-Hewitt agar with 0.001 mg/mL of colistin sulphate and 0.5 µg/mL of oxalinic acid (Streptococcus Selective Supplement, Oxoid), the plates were incubated at 37°C in aerobiose for 48 h, and colony-forming units were then enumerated. The typical morphology of *S. sobrinus* colonies grown in Todd-Hewitt agar were assessed by microscopy after Gram staining, and the species identification was confirmed using the API 20 Strep System (bioMérieux).

IV.B.2.10. Histopathology

Histopathologic evaluations were performed for all rats that were orally or systemically immunized with rEnolase. Tissues samples obtained from the lung, kidney, heart, liver, spleen, thymus, pancreas and lymph nodes of sacrificed rats were fixed in 10% buffered formaldehyde and dehydrated in graded alcohol. The fixed tissues were embedded in paraffin and sectioned at a thickness of approximately 6 microns. For histological examinations, the sections were mounted onto regular glass slides and stained with hematoxylin and eosin (HE).

IV.B.2.11. Statistical analysis

The level of significance of the results in all groups of rats was determined using Mann-Whitney test. Differences were considered significant at $P < .05$.

IV.B.3. Results

IV.B.3.1. Association between oral therapeutic vaccination with recombinant enolase and an increase in resistance to *S. sobrinus* colonization

The microbial load on the tooth surfaces of rEnolase-immunized and sham-immunized rats was evaluated to determine the effect of rEnolase immunization on *S. sobrinus* colonization. Wistar rats orally infected with 10^9 cfu of *S. sobrinus* cells and orally immunized with recombinant enolase or with PBS plus alum as adjuvant were monitored on days 0, 30, 60 and 90 after immunization, to evaluate the number of colony-forming units of *S. sobrinus* on the tooth.

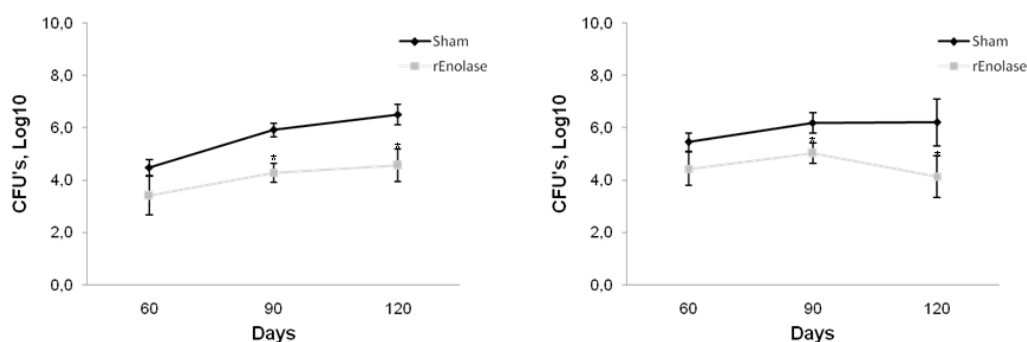


Figure IV.B.1. Reduction in oral colonization by *Streptococcus sobrinus* in *S. sobrinus* recombinant enolase (rEnolase)-immunized, in 2 independent experiments (A and B). Shown are the numbers of *S. sobrinus* colony-forming units (cfu) recovered from the oral cavity of sham-immunized rats (sham) or rEnolase-immunized rats at 60, 90 and 120 days after oral challenged with *S. sobrinus*. The rats were orally immunized with PBS (sham) or with rEnolase 5 days after the infection. In this figure and in figures 2 and 3, the results are means ($\pm$ SD) of 10-12 Wistar rats and the data correspond to results from 2 of 3 independent experiments. The significance of the results between sham- and rEnolase-immunized groups is shown (* $P < .05$; ** $P < .01$).

As shown in figure IV.B.1, throughout the study, all sham-immunized rats maintained higher levels of bacteria on tooth surfaces than did enolase-immunized rats. The reduction of *S. sobrinus* oral colonization among rEnolase-immunized animals is in accordance with the diminution of the number of caries lesions observed (see below). In the group of animals that was fed with cariogenic diet only, no *S. sobrinus* colonization was detected throughout the study (data not shown).

IV.B.3.2. Association of oral immunization with *S. sobrinus* rEnolase with increase levels of salivary IgA and IgG against this protein

The ability of oral immunization with rEnolase to induce specific antibodies in saliva and serum of immunized rats was evaluated by ELISA. At 30 and 90 days after immunization, an increase in the levels of salivary anti-rEnolase IgG antibody was observed in rats immunized with this protein, compared with sham-immunized animals (table IV.B.1). Moreover, the percentage of rEnolase-specific IgG or rEnolase-specific IgA among total IgG or total IgA in saliva of sham and rEnolase immunized rats was quantified. As shown in table 1, no significant differences in the levels of total salivary IgG was observed between rEnolase- and sham-immunized animals throughout the study. However, the proportions of rEnolase-specific IgG were elevated in the saliva of rEnolase-immunized animals as compared to that of sham-immunized controls animals.

Table IV.B.1. Total IgG antibodies or anti-*Streptococcus sobrinus* recombinant enolase (rEnolase) IgG antibodies detected in the saliva of sham- or rEnolase-immunized Wistar rats on the indicated days after immunization in 2 independent experiments.

Experiment, days after immunization	Total IgG antibody, salivary EU		Anti-rEnolase IgG antibody, salivary EU (%) ^a	
	Sham immunized	rEnolase Immunized	Sham immunized	rEnolase Immunized
A				
30	14,00 ± 1,63	15,00 ± 0,88	2,79 ± 0,47 (19,9%)	5,36 ± 1,02 ^b (35,7%)
90	17,88 ± 1,63	18,64 ± 0,80	4,48 ± 1,32 (25,1%)	7,21 ± 1,59 ^b (38,7%)
B				
30	16,90 ± 0,49	17,15 ± 4,03	2,30 ± 0,45 (13,1%)	6,31 ± 2,20 ^b (36,8%)
90	22,00 ± 2,05	24,80 ± 1,49	3,88 ± 0,36 (17,6%)	7,75 ± 1,89 ^b (31,6%)

Note: Data are means values (± SD) for 10-12 Wistar rats. Results shown are from 2 experiments (A and B) and are representative of results from 3 independent experiments. EU, ELISA Units.

^a. The percentage of salivary IgG antibodies specific to rEnolase was determined as the ratio of anti-rEnolase IgG antibodies to total IgG antibodies, expressed as EUx100.

^b. P<.05, for comparison of sham-immunized group and the rEnolase-immunized group, as determined by the use of the Mann-Whitney test.

Furthermore, as shown in table IV.B.2, no significant differences in the total levels of total salivary IgA antibodies were observed between rEnolase- and sham-immunized animals, throughout the study. In contrast, proportions of rEnolase specific IgA were elevated in the saliva of rEnolase-immunized animals, compared with sham-immunized control animals. These results point out that oral immunization with rEnolase induced a local mucosal immunity.

The serum levels of IgG antibodies specific to rEnolase showed no differences between rEnolase- and sham-immunized animals, indicating that oral immunization with rEnolase plus alum did not induce a specific systemic immune response.

Table IV.B.2. Total IgA antibodies or anti- *Streptococcus sobrinus* recombinant enolase (rEnolase), IgA antibodies detected the saliva of sham- or rEnolase-immunized Wistar rats on the indicated days after immunization in 2 independent experiments.

Experiment, days after immunization	Total IgA antibody, salivary EU		Anti-rEnolase IgA antibody, salivary EU (%) ^a	
	Sham immunized	rEnolase Immunized	Sham immunized	rEnolase Immunized
A				
30	15,77 ± 4,99	17,47 ± 4,99	2,97 ± 0,93 (18,8%) ^b	7,06 ± 2,95 ^b (40,4%)
90	33,40 ± 5,20	34,70 ± 4,10	2,07 ± 0,56 (6,2%)	15,49 ± 3,02 ^b (44,6%)
B				
30	16,00 ± 0,50	20,10 ± 3,95	2,43 ± 1,12 (15,2%)	8,31 ± 2,55 ^b (41,3%)
90	36,00 ± 2,32	35,08 ± 4,17	2,65 ± 0,45 (7,4%)	14,45 ± 3,18 ^b (41,2%)

Note: Data are means values (± SD) for 10-12 Wistar rats. Results shown are from 2 experiments (A and B) and are representative of results from 3 independent experiments. EU, ELISA Units.

^a. The percentage of salivary IgA antibodies specific to rEnolase was determined as the ratio of anti-rEnolase IgA antibodies to total IgA antibodies, expressed as EUx100.

^b. P<.05, for comparison of sham-immunized group and the rEnolase-immunized group, as determined by the use of the Mann-Whitney test.

IV.B.3.3. Protection against dental caries by oral therapeutic immunization with rEnolase

To determine whether protection against dental caries could be achieved by therapeutic oral immunization with rEnolase, groups of Wistar rats were immunized with the bacterial recombinant protein after *S. sobrinus* infection, and dental caries were evaluated on sulcal and interproximal enamel surfaces and on dentin of molar teeth.

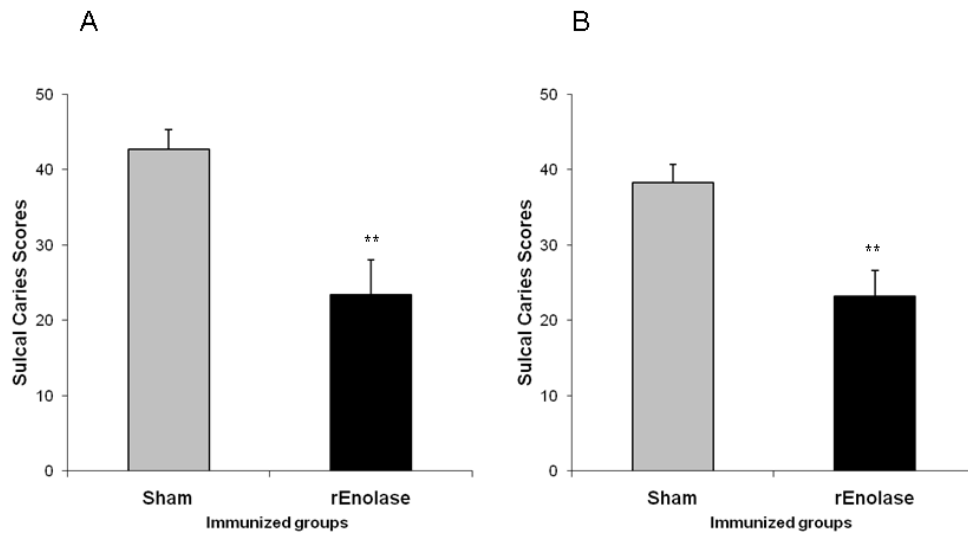


Figure IV.B.2. Evaluation of dental caries lesions on sulcal enamel surfaces of the molar teeth of sham-immunized or *S. sobrinus* recombinant enolase (rEnolase)-immunized rats in 2 independent experiments (A and B). Wistar rats were orally immunized with PBS (sham) or with *S. sobrinus* recombinant enolase (rEnolase) 5 days after being oral infected with *S. sobrinus*.

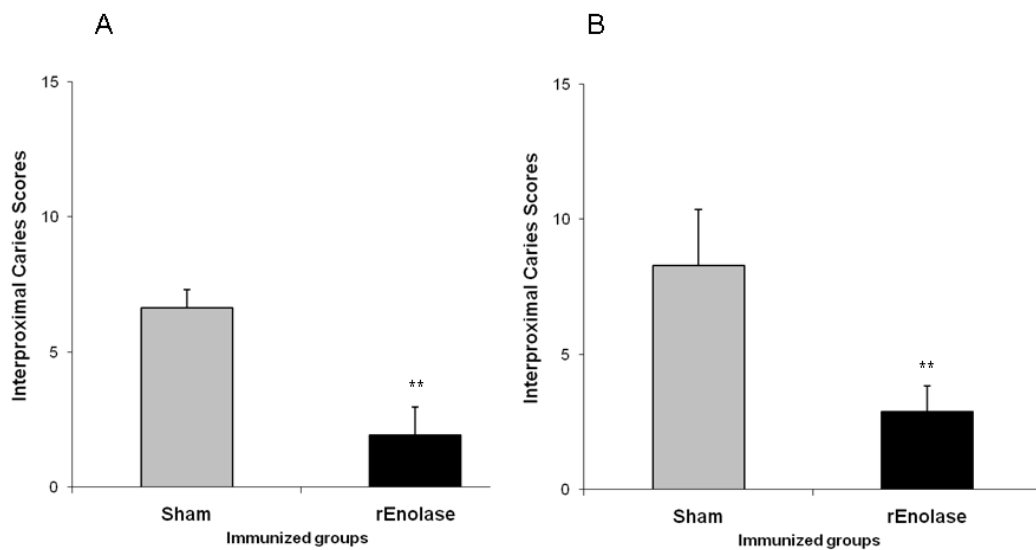


Figure IV.B.3. Evaluation of dental caries lesions on the interproximal enamel surfaces of molar teeth of sham-immunized or *S. sobrinus* recombinant enolase (rEnolase)-immunized rats in 2 independent experiments (A and B). Wistar rats were orally immunized with PBS (sham) or with rEnolase 5 days after oral infection with *S. sobrinus*. The assessment of the carious lesions was done when the rats were 120-days old.

As shown in figure IV.B.2, a significant decrease in the sulcal enamel caries score was observed in the rEnolase-immunized group of rats, compared with the sham-immunized group. These rats showed a 45% reduction in sulcal enamel carious lesions. This protective effect of rEnolase immunization on carious lesions was more evident in the proximal enamel caries score (figure IV.B.3). Indeed, the differences between the rEnolase-immunized group and the sham-immunized group were statistically significant, with a 71% reduction in the number of proximal caries lesions observed in the rEnolase-immunized group. In addition, rEnolase-immunized rats showed a 46% reduction in the number of dentin lesions, compared to the sham-immunized group (data not shown). In the group of animals that were fed a cariogenic diet only, no carious lesions was observed (data not shown).

IV.B.3.4. Absence of pathologic lesions in rats orally immunized with rEnolase

The safety of rEnolase oral vaccine was investigated through a histologic evaluation of several organs obtained from all rEnolase-immunized and sham-immunized rats. External examination of rats that the status of nutrition and development was normal, without any signal of disease with no significant differences in the weight gains noted between the 2 groups (rEnolase-immunized and sham-immunized animals). The skin and cutaneous adnexal and natural orifices showed no morphologic alterations. Macroscopic observation of superficial and deep lymph nodes demonstrated no difference between the 2 groups of rats. The same negative result was obtained for the abdominal cavity, after macroscopic and histologic observation of the spleen, pancreas, digestive tube, liver, kidneys, ureters, urinary bladder and genital apparatus, as well as in the thoracic cavity, where the thymus, lungs and heart were analyzed.

IV.B.3.5. Lack of recognition of human enolase by antibodies specific to *S. sobrinus* enolase induced by subcutaneous immunization

BLAST search of sequences deposited in computer databases (National Center for Biotechnology Information) revealed a 46-49% or 47-49% homology between *S. sobrinus* enolase and human enolase or rat enolase, respectively; it also uncovered 96% homology between rat enolase and human enolase. Therefore, to induce a systemic high level of antibodies against bacterial or human enolase, Wistar rats were subcutaneously immunized with rEnolase and with rhEnolase, and the antibodies

produced were tested in cross-reaction assays. Systemic immunization induced a high level of IgG antibodies specific to rEnolase or to rhEnolase in rEnolase- or rhEnolase-immunized animals, respectively, compared with that noted in the sham-immunized group of animals (figure IV.B.4).

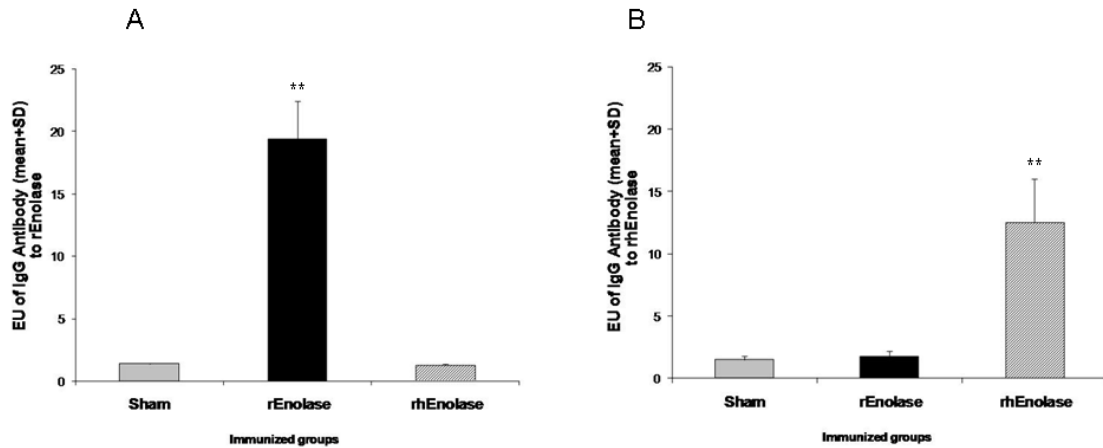


Figure IV.B.4. Evaluation of serum IgG antibody levels (expressed as ELISA Units (EU)), specific for *Streptococcus sobrinus* recombinant Enolase (rEnolase) (A) or human recombinant enolase (rhEnolase) (B) in Wistar rats subcutaneously immunized with PBS (sham), rEnolase or rhEnolase. The results shown are the mean value ($\pm$ SD) for 8 Wistar rats, and they correspond to results from 1 of 2 independent experiments.

No reactivity to rhEnolase was observed in the antibodies produced in response to rEnolase immunization, and anti-rhEnolase IgG antibodies also did not react with rEnolase (figure IV.B.4). These results indicate that antibodies specific to *S. sobrinus* enolase did not recognize human enolase, and vice versa.

Taking into account that enolase has a highly preserved structure during evolution and that the presence of circulating anti-human enolase autoantibodies was associated with several diseases in humans (Pancholi V 2001, Vicent Saulot *et al.* 2002, Witkowska *et al.* 2005), we also investigated whether systemically produced anti-rEnolase antibodies in rats would induce any disease in these animals. No morphologic changes and no signs of acute or chronic inflammation were observed in the lungs of all systemically immunized rats. Moreover, observation of their livers of these rats by optic microscopy revealed a normal architecture. No signs of degenerative alterations or inflammatory changes previously associated with systemic autoimmune disorders induced by autoantibodies have been found (data not shown). Histology examination of the

kidneys also showed no morphologic alterations. The spleen of some animals in the rEnolase-immunized group showed a cellular infiltration that conferred a more dense appearance to the organ than that observed in the sham-immunized group, which is in accordance with the activation state normally observed after a systemic immunization. Examination of these tissues demonstrated an absence of detectable histologic damage due to anti-rEnolase antibodies (data not shown).

IV.B.4. Discussion

In the present study, we demonstrated that therapeutic vaccination with rEnolase reduced the cariogenic lesions induced by this bacterium in a rodent model, and that this vaccine was well tolerated by the rats. A significant reduction in the enamel sulcal and proximal caries scores was observed for rEnolase-immunized rats. In accordance with the reduction in the number of cariogenic lesions observed, a decrease in *S. sobrinus* oral colonization was observed in rEnolase-immunized rats. The immunization induced a high level of salivary IgA antibody response specific for rEnolase, and this result is directly correlated with the reduction of the number of bacteria recovered from the immunized rats, as well as with the reduction in the extent of dental caries observed in these animals. The levels of this immunoglobulin class have been associated with protection in other dental caries vaccination studies (Russell *et al.* 2004). Moreover, we have also observed, in rats immunized with rEnolase, an induction of salivary IgG antibodies could also be associated with the protection observed. In rEnolase-immunized and sham-immunized rats, no differences were observed in serum levels of IgG antibodies specific for this recombinant protein; this finding indicates that oral rEnolase-immunization did not induce a systemic immunity Michalek *et al.* (Michalek *et al.* 1976) were the first to show that induction of immunity by an oral route in bacterial-feeding rats was sufficient to elicit a protective salivary secretory IgA response without the induction of detectable specific serum IgG. Therefore, the high level of IgG antibodies observed in the saliva of the enolase immunized animals raises the possibility that those antibodies are not derived from the serum and suggests that they arise mainly from a local production. Although IgG in saliva has been suggested to be the consequence of diffusion from the serum, there are data suggesting that it can also be produced locally as salivary antibodies (Nurkka *et al.* 2004). Induction of IgG associated with local immunity may complement IgA as an immune barrier/defence against invasion by mucosal pathogens. Thus, local

production of IgG antibodies should be considered when analyzing the protection induced by mucosal immunization.

The observed protection against *S. sobrinus*-induced dental caries by salivary anti-rEnolase antibodies could be explained by the immunoneutralization of the effects of enolase in the host. Actually, it has been reported that rEnolase (Veiga-Malta *et al.* 2004) interferes with the host immune response and facilitates host colonization by this bacterium. Therefore, the antibodies specific for rEnolase would prevent these effects and therefore protect the host against *S. sobrinus* colonization. Moreover, because this protein could be expressed on bacteria surface, other plausible, although unproven explanation could be that the anti-enolase antibodies could lead to opsonization, thus promoting the phagocytosis and destruction of the bacterium.

Examination of multiple tissues obtained from the rEnolase-immunized rats showed an absence of detectable long-term histopathologic damage. Thus, therapeutic vaccination with rEnolase seems to have no harmful physiologic effect on rats.

Enolase, a highly expressed key enzyme of glycolysis and gluconeogenesis, is present in all eukaryotic and in most prokaryotic cells (Devlin 2002), and the presence of this enzyme on the surface of streptococci has been described as a virulence factor (Bergmann *et al.* 2001, Pancholi and Fischetti 1998). The presence of circulating autoantibodies against enolase in humans has been related with various pathologic findings (Pancholi and Fischetti 2001, Saulot *et al.* 2002, Witkowska *et al.* 2005); therefore, it was important to investigate whether antibodies raised against *S. sobrinus* enolase in our immunization procedure cross-react with human enolase. To exclude any undesirable/pathogenic effect induced by anti-rEnolase antibodies, serum with high levels of antibodies directed against bacterial enolase were induced by systemic immunization in rats. Several tissues obtained from the animals systemically immunized with bacterial enolase were histologically evaluated, and no pathologic alterations were observed in any of the tissues analyzed. Moreover, the antibodies specific to bacterial enolase did not recognized human enolase, and the antibodies produced against human enolase demonstrated no cross-reaction with *S. sobrinus* enolase. This result is different from the one obtained with group A streptococci (Fontan *et al.* 2000). A likely explanation could be that exposed human enolase epitopes are similar to those to group A streptococci but not to *S. sobrinus* enolase.

This therapeutic vaccination with rEnolase seems to have no harmful physiologic effect on rats, and it may be a useful target antigen for human vaccination against dental caries. Because the homology between *S. sobrinus* and *S. mutans* enolase is very high (~ 98%) (Veiga-Malta *et al.* 2004), we anticipate that the anti-enolase antibodies produced against one of the *Streptococcus* species should recognize the other

species, and vice-versa, thereby reinforcing interest in rEnolase as a candidate vaccine antigen against dental caries induced by these related lactic acid-producing streptococci. Further studies will be done to ascertain this point.

CHAPTER IV.C

THERAPEUTIC VACCINE AGAINST *Streptococcus mutans*-INDUCED CARIES

IV.C. THERAPEUTIC VACCINE AGAINST *Streptococcus mutans*-INDUCED CARIES

Abstract

Dental caries is one of the most prevalent human chronic infections for which an effective human vaccine is not available. The mutans group of oral streptococci consists of several species, but among them, *Streptococcus sobrinus* and *Streptococcus mutans* are the most frequently isolated from human dental plaque and closely associated with human dental caries. We have described that enolase from *S. sobrinus* is an immunomodulatory protein that plays a role in the bacterial pathogenesis. Moreover, the recombinant protein (rEnolase) was purified and we showed that rEnolase vaccination confer protection against *S. sobrinus* -induced dental caries in Wistar rat. Since the homology between the enolase from *S. sobrinus* and the enolase from *S. mutans* is very high, we hypothesized that the vaccination with the enolase from *S. sobrinus* also confers protection against the *S. mutans* infection. Therefore, in this study we evaluated the effect of a therapeutic vaccination with rEnolase of *S. sobrinus* origin on *S. mutans*-induced dental caries in Wistar rats. For that purpose, Wistar rats were fed with a cariogenic solid diet on the 18th day after birth and were orally infected with *S. mutans* from the 19th day for 5 consecutive days. Five days after infection and again 3 weeks later, the rats were orally immunized with rEnolase incorporated in alum as adjuvant. A sham-immunized group of rats was contemporarily treated with adjuvant alone. Increased levels of salivary IgA and IgG antibodies specific for this recombinant protein were observed in rEnolase-immunized rats. The evaluation of *S. mutans* oral colonization showed a decrease in rats vaccinated with rEnolase comparatively with sham-immunized rats, throughout the study. This decrease on *S. mutans* colonization resulted in a reduction on sulcal dental caries in rEnolase-immunized rats compared with sham-immunized animals. These results indicate that rEnolase of *S. sobrinus* origin vaccination also confers protection against *S. mutans* -induced dental caries in rats similarly to what has been observed with *S. sobrinus* infection.

IV.C.1.Introduction

Streptococcus mutans has been implicated as the primary causative organisms of dental caries, the most common oral disease in humans (Russell 1994). Colonization of the tooth surfaces by *S. mutans* is thought to be an important step for the initiation of dental caries (Russell *et al.* 2004). *Streptococcus sobrinus* is the other cariogenic microorganism causing dental caries in humans (Russell 1994).

The genome sequence of *S. mutans* (serotype c) was recently established (<http://www.genome.ou.edu/smutans.html>), and various cell-surface antigens of mutans streptococci and their recombinant fragments have been studied as possible candidates for dental vaccines (Childers *et al.* 2006, Koga *et al.* 2002, Russell *et al.* 2004). However, in spite of intensive investigation dedicated to the development of a vaccine against this disease, none has so far available proved effective and, consequently, available. An enolase from *S. sobrinus* was identified as an immunomodulatory protein, the respective gene was cloned and the recombinant protein (rEnolase) was purified (Veiga-Malta *et al.* 2004). Furthermore, we have showed that immunization with this recombinant protein results in protection against rat *S. sobrinus*-induced dental caries (Dinis *et al.* 2009). Since the homology between *S. sobrinus* and *S. mutans* enolase is very high (nucleotides sequences 98% identical), we hypothesized that rEnolase from *S. sobrinus* could also be a target antigen for vaccination against *S. mutans*-induced dental caries. Therefore, in this study, we evaluated whether vaccination with rEnolase of *S. sobrinus* origin could protect against *S. mutans*-induced dental caries in Wistar rats. Our results showed a reduction in sulcal caries scores in rEnolase-immunized rats, compared with sham-immunized animals.

Moreover, since there is currently considerable interest in oral route in an effort to design vaccines that can be applied for immunization in a cheap way, in this study, we also investigated whether rEnolase immunization delivered by oral route induce specific serum and salivary antibody production.

IV.C.2. Materials and methods

IV.C.2.1. Animal models

Male and female Wistar rats, purchased from Charles River Laboratories (Barcelona, Spain), and bred at the animal facilities of the ICBAS (Porto, Portugal) were weaned at approximately 20 days of age.

Procedures involving animals were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

IV.C.2.2. Bacteria

The *Streptococcus mutans* (serotype c) strain was purchase from American Type Culture Collection (ATCC, nº 700611, University Blvd., Manassas, VA). The strain was stored at -70°C in Brain Heart Infusion broth medium (Difco, Detroit, MI) with 25% (v/v) of glycerol. For inocula preparation, overnight cultures of *S. mutans* were diluted ten-fold in Todd-Hewitt broth (Difco) and incubated for another 8 hours to reach mid-exponential growth. The bacteria were then washed three times and suspended in cold endotoxin-free phosphate-buffered saline (PBS, Sigma, St. Louis, MO). Using a standard curve plotted from colony forming units (CFU) as a function of the absorbance at 600 nm, the cultures were diluted in PBS to give the desired number of CFU/ml. *S. mutans* colonies were identified by typical morphology on Todd Hewitt agar, under microscope with Gram stain and confirmed by the API 20 Strep system (bioMerieux, Marcy L'Etoile, France).

IV.C.2.3. Preparation of *S. sobrinus* recombinant enolase

The isolation and purification of rEnolase has been described in detail (Veiga-Malta *et al.* 2004). Briefly, *S. sobrinus* enolase was expressed in *Escherichia coli* DH5 α as a fusion protein containing 26 NH2 extra amino acid residues including six histidine. The recombinant protein was purified by affinity chromatography using a His-trap column (Amersham Biosciences, Uppsala, Sweden) under native conditions. The protein concentration was determined by the method of Lowry (Lowry *et al.* 1951) and its purity was evaluated by SDS-PAGE. The rEnolase was depleted of lipopolysaccharide (LPS) using a polymixin B column (Pierce, Rockford, IL), and all recombinant protein

preparations used were LPS-free, as assessed by the limulus amoebocyte lysate kit (E-Toxate; Sigma).

IV.C.2.4. Saliva and serum samples

For saliva and serum collection, rats were anesthetized by using ketamine and xylazine at 87 and 13 mg/kg of body weight, respectively, and buprenorphine at 0.075 mg/kg as an analgesic. Saliva secretion was stimulated by intraperitoneal injection of 0.05 mg of pilocarpine/100g of body weight. After centrifugation, the clarified saliva was stored at -20°C. Blood was collected from the tail vein during anesthesia and sera from coagulated and centrifuged blood were stored at -20°C.

IV.C.2.5. Mucosal therapeutic immunization

For vaccination purposes 10 µl of rEnolase in a 1:1 PBS/alum (Aluminum hydroxide Gel; Brenntag, Frederikssund, Denmark, kind gift of Erik Lindblad) suspension were delivered directly into the oral cavity of the rats, 5 days after the last *S. mutans* administration. Sham-immunized animals received 10 µl of 1:1 PBS/alum suspension. Three weeks later the rats were re-immunized.

IV.C.2.6. Caries induction protocol

In rat caries models there is a discrete period of time, between days 19 and 22 after birth, to implant the mutans streptococci in the oral cavity denominated “window of infectivity” (Caufield 1997). T. We follow the infection protocol that we have established for *S. sobrinus* bacteria (Dinis *et al.* 2004). At day 18th after birth, weaning Wistar rats were fed with cariogenic diet #11725 plus 62,7% of saccharose (Scientific Animal Food & Engineering, SAFE, Villemoisson sur Orge, France) and then orally infected for five consecutive days (from day 19 to 23th of age) with 10 µl containing 1×10^{11} CFU of *S. mutans*. The oral swabs were taken from individual rats to investigate the bacterial colonization before and after infection. At the end of the experiment (on day 120, since this lapse of time is needed to observe caries lesions in control animals), all the animals were sacrificed, the saliva and serum collected, and the mandibles removed for caries assessment.

IV.C.2.7. Antibody analyses

Serum IgG and salivary IgA and IgG antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Dinis *et al.* 2004). Briefly, polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 µg/mL of rEnolase. After washing with Tween-saline (TS) the plates were blocked with PBS containing 1% of bovine serum albumin (Sigma, St Louis, Mo). Antibody activity was then measured by incubation with a 1:30 to 1:2,430 dilutions of sera or with 1:2 to 1:16 dilutions of saliva. After washing, the bound antibodies were revealed by the addition of peroxidase-labelled goat antibodies anti-rat IgG (Southern Biotechnology Associates, Birmingham, USA) or peroxidase-labelled mouse anti-rat IgA (Biosource, Belgium) and incubated for 3 h at 37° C. After washing, the plates were incubated with orthophenylenediamine dihydrochloride (Sigma) and H₂O₂ for 30 min at room temperature. The reaction was stopped with 10% SDS and the colorimetric change was measured with a Biotek Chromoscan at 450 nm. Data are reported as ELISA units (EU) which were calculated relative to levels in reference sera from non-immunized and non-infected rats as a reference. Dilutions of 1:30 (A_{450} of 0.2) were considered 1 EU for reference serum IgG specific for recombinant enolase. Dilutions of 1:4 (A_{450} of 0.1) were considered 1 EU for both reference salivary IgA and IgG specific for recombinant enolase.

IV.C.2.8. Caries assessment

For caries assessment the maxilar and mandible soft tissue was removed. Maxilla and mandible were put overnight in a 1% silver nitrate aqueous solution and each sequence of first, second and third molar has been hemisectioned using Keyes method (Keyes 1958) and dried during 48 hours in a incubator by dry heat sterilizer at 50 degrees Celsius. Still using Keys method sulcal and proximal carious lesions in all rat molar teeth were microscopically evaluated. The analyst was blind to group assignments.

IV.C.2.9. Bacterial recoveries

The presence of *S. mutans* was assessed after systematic swabbing of teeth with a sterile brush for 10 sec per side. The brush was placed into 500 µl of sterile PBS. After sonication and plating of appropriate dilutions on Todd-Hewitt agar with 0.001 mg/mL of colistin sulphate and 0.5 µg/mL of oxalinic acid (Streptococcus Selective

Supplement, Oxoid, Hampshire, England) the plates were incubated at 37°C in aerobiose for 48 h, and colony-forming units (CFU) were then enumerated. The typical morphology of *S. mutans* colonies grown in Todd-Hewitt was assessed by microscopy following Gram staining and the species identification was confirmed by the API 20 Strep system (bioMérieux, Marcy L'Etoile, France).

IV.C.2.10. Statistical analysis

The level of significance of the results in all groups of rats was determined by Mann-Whitney test. Differences were considered significant at $P < 0,05$.

IV.C.3. Results

IV.C.3.1. *S. sobrinus* rEnolase purification

The isolation and purification of *S. sobrinus* recombinant enolase (rEnolase) has been pursued as described in detail (Veiga-Malta *et al.* 2004). Briefly, the *S. sobrinus* enolase was expressed in a heterologous system as a fusion protein containing 26 extra amino acid residues at the N-terminus, including 6 consecutive histidines and was purified by affinity chromatography. We obtain the protein sequence and search of sequences deposited in computer databases of the National Center for Biotechnology Information revealed a high similarity of these sequences with bacterial enolase. There is high homology between the protein sequence from *S. sobrinus* and *S. mutans* (about 90% homology).

IV.C.3.2. Oral therapeutic vaccination with *S. sobrinus* rEnolase increased resistance to *S. mutans* colonization

The experiments were performed to investigate the protective effect of therapeutic immunization with *S. sobrinus* rEnolase on *S. mutans* oral colonization.

As shown in Figure IV.C.1, rEnolase-immunized rats exhibited a significant reduction in *S. mutans* oral colonization comparatively with sham-immunized rats, throughout the study.

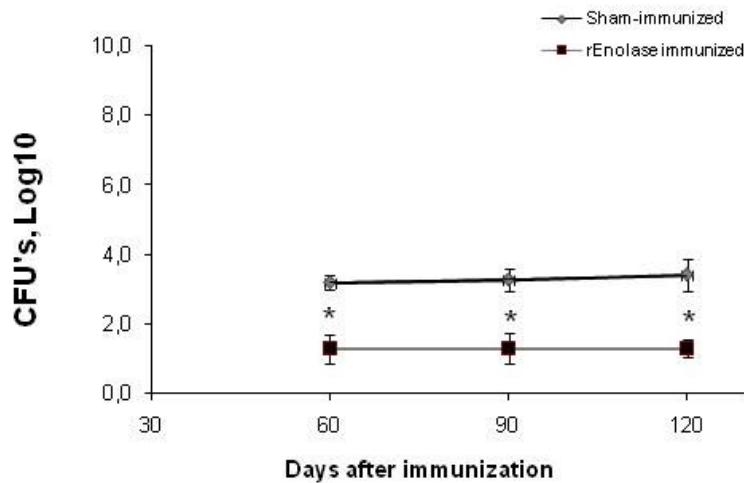


Figure IV.C.1. Evaluation of *Streptococcus mutans* oral colonization in Sham-immunized and in *S. sobrinus* rEnolase-immunized rats. Shown are the numbers of *S. mutans* colony-forming units (CFU) recovered from the oral cavity of the different groups of Wistar rats at 60, 90 and 120 days after oral challenge with *S. mutans*. The rats were orally immunized with PBS (Sham) or rEnolase, 5 days after the infection. In this figure and in figures 2 and 3, the results are presented as the mean value ($\pm$ SD) for 10 to 12 Wistar rats per group. The significance of the results between the sham- and rEnolase-immunized groups is shown (* $P < 0,05$).

IV.C.3.3. Oral immunization with rEnolase induced increased levels of salivary IgA and IgG against this protein

To evaluate our immunization protocol the specific antibody production after oral immunization with rEnolase was evaluated by ELISA in rEnolase-immunized and sham-immunized animals.

An increase in the levels of salivary anti-rEnolase IgA antibody was observed in rEnolase-immunized rats comparatively with sham-immunized animals (Figure IV.C.2). Moreover, the levels of salivary anti-rEnolase IgG antibody anti-rEnolase were also increased in rEnolase-immunized animals when compared with sham-immunized animals (Figure IV.C.2). Thus, a 4 folder increases on salivary anti-rEnolase IgA antibody and 5 folder increases on salivary anti-rEnolase IgG antibody was observed in rEnolase immunized rats.

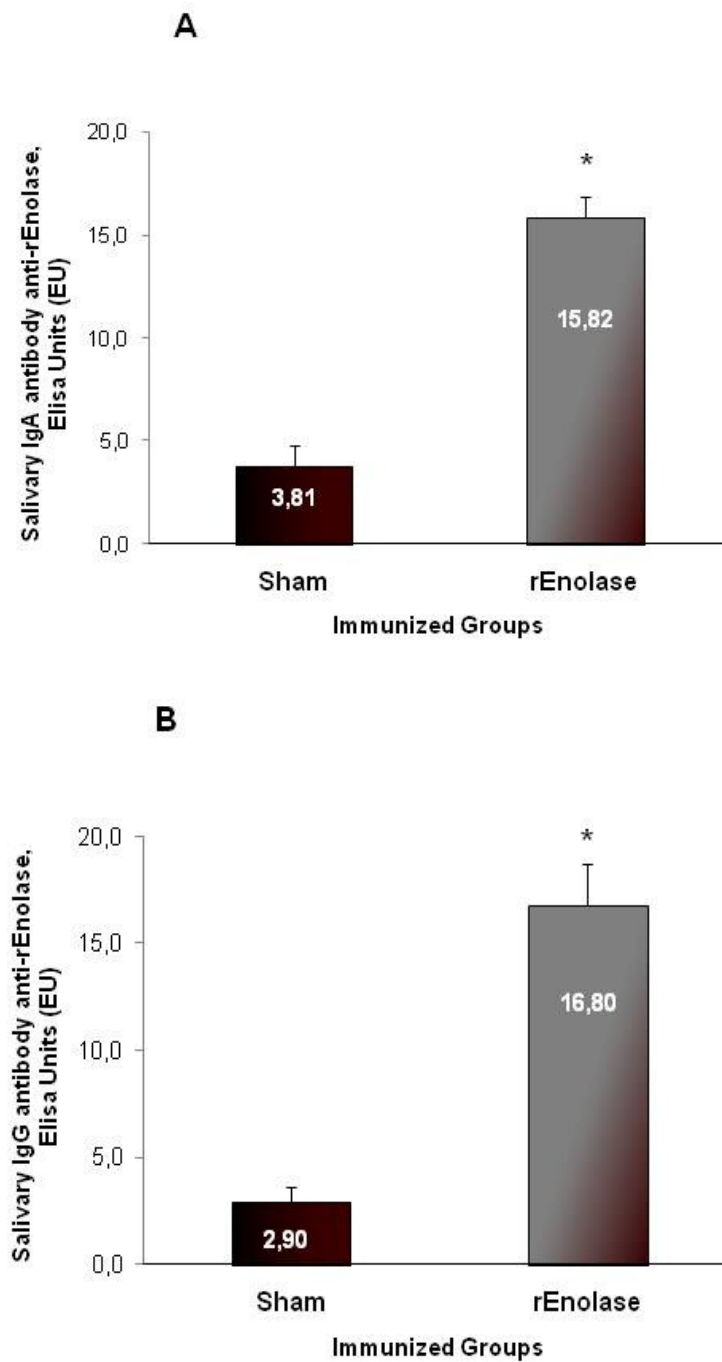


Figure IV.C. 2. Levels of salivary anti-rEnolase IgA (**A**) and IgG (**B**) antibodies, expressed as ELISA Units (EU), in rEnolase-immunized and Sham-immunized rats. The significance of the results between the Sham- and rEnolase-immunized groups is shown (* $P < 0,001$).

These results indicate that oral rEnolase immunization induced a localized mucosal immunity. In contrast, no differences in serum levels of anti-rEnolase IgG antibody were observed between the sham-immunized group and rEnolase-immunized group of

animals (data not shown), which indicated that the oral immunization did not induce a specific systemic immune response

These results indicate that the protection observed against *S. mutans*-induced dental caries by rEnolase vaccination is correlated with the production of salivary IgG and IgA antibodies specific for the recombinant protein.

IV.C.3.4. Protection against *S. mutans* induced-dental caries by oral therapeutic immunization with rEnolase of *S. sobrinus* origin

At the end of the experiment (on day 120) the caries lesions in both groups of rats were quantified.

As shown in Figure IV.C.3, a significant reduction in the sulcal enamel caries score was observed in the rEnolase immunized group of rats, compared with the sham-immunized group. These rats showed a 54% reduction in sulcal enamel carious lesions.

Unfortunately it was not possible to detect any proximal caries lesion in the sham-immunized group. This means that our caries induction protocols failed to induce this type of lesions. Since we have found this kind of lesions when the animals were infected with other cariogenic bacteria, *S. sobrinus*, the *S. mutans* seems less cariogenic in our laboratory animal model.

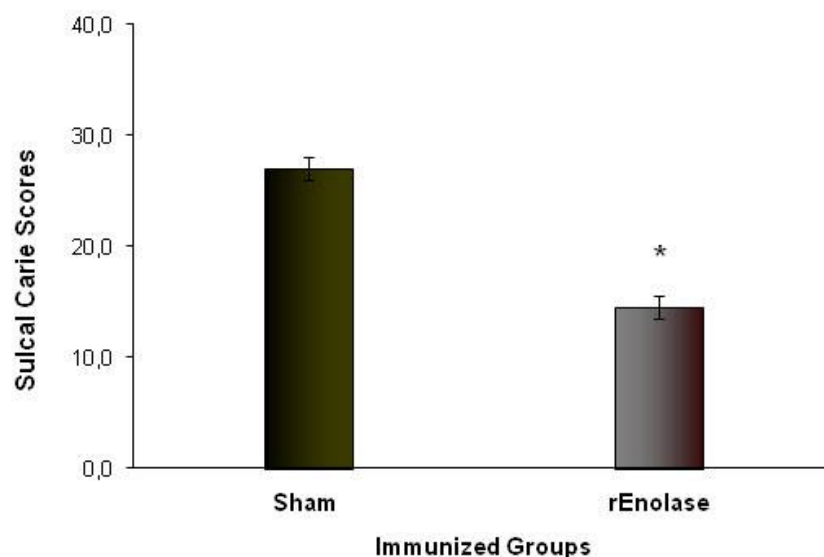


Figure IV.C.3. Evaluation of dental caries lesions on the sulcal enamel surface of the molar teeth of Sham-immunized or rEnolase-immunized rats. The significance of the results between the Sham- and rEnolase-immunized groups is shown (* $P < 0,01$).

IV.C.4. Discussion

S. mutans and *S. sobrinus* have been implicated as the primary causative agents of dental caries in humans (Loesche 1986). Various antigens of mutans streptococci and recombinant fragments have been used in several immunizations protocols (Childers *et al.* 2006, Jespersgaard *et al.* 1999, Lehner *et al.* 1981, Russell *et al.* 2004, Smith and Taubman 1996). However, the results obtained so far are unsatisfactory and some of these antigens are specific for *S. mutans* or *S. sobrinus* conferring protection only against one of these cariogenic agents. Moreover, although several mutans streptococci infection protocols have been established most of them do not resemble a normal human oral cavity. Indeed, most of them use specific pathogen free (SPF) Sprague Dawley rats (Ooshima *et al.* 1981, Smith *et al.* 1997b, Tanzer 1979, 1995), germ free Fisher rats (Michalek *et al.* 1975), gnotobiotic animals (Fitzgerald 1968) or in recent studies, with a massive antibiotics treatment previous to infection (Zhang *et al.* 2002). In all these animal models the oral microbiotic flora is manipulated in order to facilitate the MS colonization. However, the oral cavity in humans presents other bacteria and a dynamic ecosystem is maintained that mutans streptococci (MS) must overcome in order to succeed and induced dental caries. Taking into account all these concerns, we used Wistar rats as our animal model (Dinis *et al.* 2004). These rats harbor an endogenous oral flora similar to the humans and we were able to implant the bacteria throughout the “window of infectivity” (Caufield 1997) and induce dental carious lesions, observed at the end of experiment, when the rats are 120 days-old. Therefore, our infection model resembled the MS infection that occurs in humans.

The prevalence of *S. mutans* and *S. sobrinus* has been under intensive study and the more effective vaccine for dental caries would be the one that use a target antigen present in both MS.

In previous studies, we established a vaccination protocol against *S. sobrinus*-induced dental caries, using a recombinant enolase from *S. sobrinus* as target antigen. Since the homology between *S. sobrinus* and *S. mutans* enolase is very high (nucleotides sequences 98% identical), we hypothesized that the recombinant enolase from *S. sobrinus* could also be a good target for vaccination against *S. mutans*-induced dental caries. Our results indicate that rEnolase from *S. sobrinus* origin is also a good target for vaccination against *S. mutans* infection. In this way, a single protocol would enable the control and reduction of both primary causative agents of dental caries in humans.

As stated above, in this work we also assayed a protocol for *S. mutans* colonization in the oral cavity of Wistar rats, which was similar to the ones performed with *S. sobrinus* (Dinis *et al.* 2004, Dinis *et al.* 2009). However, it was only possible to maintained

moderate levels of *S. mutans* in the oral cavity of control rats, throughout the study. The number of CFU collected from oral cavity of sham-immunized control rats was lower than the ones observed with *S. sobrinus* colonization (Dinis *et al.* 2009). This data is correlated with the lower dental caries induction observed with *S. mutans* infection than with *S. sobrinus* infection. This highlight that *S. sobrinus* is more cariogenic than *S. mutans* in experimental model which has also been described by others authors (Ooshima *et al.* 1981). Further work still required for improve our *S. mutans* infection protocol.

In spite of that, a significant difference in the level of *S. mutans* oral colonization was observed in rEnolase-immunized animals, compared with sham-immunized control animals. This difference resulted in a significant decrease in sulcal enamel caries score observed in these rEnolase vaccinated animals.

Intranasal and oral administrations have been described as convenient delivery routes and have been demonstrated to be effective in inducing salivary IgA responses in anti-carries vaccination (Dinis *et al.* 2004, Taubman and Nash 2006b). In this study, we assayed oral route for immunize the rats with rEnolase. The obtained results showed that it is possible to induce a protective immune response by oral delivery of the vaccine. Indeed, the rEnolase-immunized rats showed high levels of salivary anti-rEnolase IgA antibody that were associated with the few caries lesions observed in these animals compared with sham-immunized control animals. These results suggest that inhibition of dental caries was due to the induction of salivary IgA responses. This finding is consistent with our previous work (Dinis *et al.* 2004, Dinis *et al.* 2009) and has also been described by others research groups (Zhang *et al.* 2002).

Moreover, an increase in the salivary anti-rEnolase IgG antibodies production was also detected in the animals immunized with rEnolase. Induction of IgG-associated with local immunity may complement IgA as an immune barrier/defense against mucosal pathogens and their invasion. Thus, local production of IgG antibodies should be considered when analyzing the protection induced by mucosal immunization.

In conclusion, our results indicate that vaccination with rEnolase from *S. sobrinus* induces protective immune response against dental caries caused by both mutans streptococci that are described as the responsible for the disease in humans.

CHAPTER IV.D

PREVENTIVE VACCINATION PROTOCOL

CHAPTER IV.E

MATERNAL IMMUNIZATION OF RATS WITH *Streptococcus sobrinus* RECOMBINANT ENOLASE CONFERS PROTECTION TO OFFSPRING AGAINST DENTAL CARIES

CHAPTER V

PRELIMINARY STUDY ON THE CHARACTERIZATION OF THE MUCOSAL IMMUNE RESPONSE AFTER *S. sobrinus* RECOMBINANT ENOLASE IMMUNIZATION IN MICE

V. A PRELIMINARY STUDY ON THE CHARACTERIZATION OF THE MUCOSAL IMMUNE RESPONSE AFTER *Streptococcus sobrinus* RECOMBINANT ENOLASE MUCOSAL IMMUNIZATION IN MICE

Abstract

Streptococcus sobrinus, one of the ethiological agents of dental caries, localizes in the oral cavity, where the nasopharyngeal lymphoid tissue (NALT) is believed to play an important role in host defence. As NALT and intestinal Peyer's patches (PP) are described mucosal inductive sites, we attempted in this study to characterize the NALT, cervical lymph nodes (CLN) and PP lymphoid populations after oral or intranasal immunization with recombinant enolase (rEnolase) in C57BL/6 mice. To address this issue, after isolation of the NALT, CLN and PP from sham-immunized or intranasal or oral route rEnolase-immunized mice, the cells were analyzed by flow cytometry (FACS). The obtained results indicate that, after intranasal immunization with rEnolase, germinal centers may have developed in NALT, whereas no evidence suggest that such structures formed in the NALT from oral immunized mice. Moreover, a decreased in CD4+ T cell population was observed in intranasal rEnolase-immunized mice compared with sham-immunized mice. Afterwards, we investigate the role of CLN in the mucosal immune response network after rEnolase mucosal immunization. In these organs, the animals immunized with rEnolase by intranasal route showed a significant increase in both CD4 and CD8 T cells, although most evident on the former. Furthermore, a small increase on peanut agglutinin (PNA+), B220+ B cells was also observed in these mice indicating germinal centers formation in the CLN upon rEnolase immunization by intranasal route. In oral or intranasal rEnolase-immunized animals an increase in the levels of IgA and IgG antibodies specific for rEnolase were observed in salivary glands homogenates.

These preliminary data provide evidence that NALT and CLN are involved in induction of protective immunity after mucosal vaccination with rEnolase.

V.1. Introduction

Dental caries is one of the most prevalent infectious diseases in humans, associated with major medical complications such as endocarditis. Its wide incidence, affecting children, adolescents as well as adults (Hanada 2000), highlights the impact that prevention of this oral disease by means of vaccination could have in human public health. Moreover, the treatment of dental caries is probably one of the most expensive in the world due to the wide distribution of this illness. This disease is a multifactorial, ubiquitous, plaque related chronic infection of the enamel or dentine caused by specific streptococci denominated “mutans streptococci” (Coykendall and Gustafson 1986, Hamada *et al.* 1986). These includes *Streptococcus sobrinus* and *Streptococcus mutans* as the primary etiological agents of human dental caries (Caufield 1997, De Soet *et al.* 1991).

It has been described that several pathogenic microbes produce virulence-associated immunomodulatory proteins (VIP) important for their survival in the host (Ferreira *et al.* 1997, Ferreira *et al.* 1988, Tavares *et al.* 1993, Vilanova *et al.* 1999). These VIP interfere with the host's immune system leading to a polyclonal activation of lymphocytes (Ferreira *et al.* 1988{Arala-Chaves, 1988 #4, Lima *et al.* 1992, Tavares *et al.* 2003, Tavares *et al.* 1993) and a suppression of the immune response against the producing microorganism through an early production of IL-10 (Ferreira *et al.* 1997, Tavares *et al.* 2000, Vilanova *et al.* 1999). Furthermore, specific immunization against VIP results in protection of the host against the producing microorganism (Dinis *et al.* 2004, Tavares *et al.* 1995, Vilanova *et al.* 1999). Recently, it has also been reported that specific immunization against *S. sobrinus* VIP results in protection against rat dental caries (Dinis *et al.* 2004). The VIP from *S. sobrinus* was identified as enolase (Veiga-Malta *et al.* 2004). The corresponding gene was cloned and the recombinant enolase (rEnolase) purified.

In previous studies we addressed the development of vaccination protocols using recombinant enolase from *S. sobrinus* as target antigen. Since the induction of an effective mucosal immunity is imperative for a good dental caries vaccine, we established protocols where the antigen was delivered in inductive mucosal sites such as the oral and nasal cavities (Dinis *et al.* 2009).

The mucosal immune system is of major importance in resisting infection in the oral cavity (Wu *et al.* 1996). The humoral and cellular mediated mucosal immune responses involve both a site of induction (where the antigen enters the body, is processed and an immune response initiated) and an effector site (where the immune response is mounted). Increasing evidence suggests the existence of a regional

compartmentalisation of the mucosal immune system and that a particular inductive site may be important in generating effector responses at a limited number of sites. This may have important implications in the development of efficient mucosal vaccines against oral infections and also in explaining the pattern of immunological disease affecting the oral mucosa (O'Hagan *et al.* 2001). In the mucosa, numerous and various kinds of immune-competent cells (T cells, B cells, dendritic cells, macrophages, etc) form a unique network and contribute to the induction of specific and protective immunity against foreign antigens (Iijima *et al.* 2001). This mucosal response is characterized by the predominance of local dimeric IgA production, secretory IgA (sIgA) that is responsible for the immune exclusion of bacteria and viruses (Sosa and Roux 2004). In our previous studies, mucosal immunization with rEnolase induced high levels of salivary IgA and IgG antibodies specific for the recombinant protein, in the rEnolase immunized rats. These were correlated with decreased *S. sobrinus* oral colonization and carious lesions reduction (Dinis *et al.* 2009).

S. sobrinus infection localizes in the oral cavity, where the nasopharyngeal lymphoid tissue (NALT) is thought to play an important role for host defence. In humans NALT is also known as Waldeyer's ring and it consists of the adenoids or nasopharyngeal tonsils, the bilateral pharyngeal lymphoid bands, the bilateral tubal tonsils, the bilateral palatine and lingual tonsils (Sosa and Roux 2004).

The rat has been extensively used as a model for delineating immune protection against dental caries disease. In this model a tissue equivalent to the Waldeyer's ring has been described in the nasopharyngeal duct, however, rats don't have tonsils (Sosa and Roux 2004).

The caries vaccine strategies developed so far are based in strategies of mucosal immunization that induce high levels of salivary antibodies that can persist and inhibit bacteria colonization (Russell *et al.* 2004). It has been shown that intranasal immunization is more effective than oral or vaginal immunization at inducing generalized mucosal and systemic immune responses, and it requires smaller amounts of immunogen (Kiyono and Fukuyama 2004).

Taking into account these evidences and our previously results, in this work we tried to characterize the cell population on NALT, Peyer's Patches (PP) and cervical lymph nodes (CLN) after oral and intranasal vaccination with rEnolase in C57BL/6 mice.

V.2. Materials and methods

V.2.1. Mice

Male C57BL/6 and BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Barcelona, Spain), and animals were kept at the animal facilities of the Institute Abel Salazar (ICBAS, UP) during the time of the experiments. All procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

V.2.2. Preparation of *S. sobrinus* recombinant enolase

The isolation and purification of rEnolase has been described in detail (Veiga-Malta *et al.* 2004). Briefly, *S. sobrinus* enolase was expressed in *Escherichia coli* DH5 α as a fusion protein containing 26 NH₂ extra amino acid residues including 6 histidyl. The recombinant protein was purified by affinity chromatography using a His-trap column (Amersham Biosciences, Uppsala, Sweden) under native conditions. The protein concentration was determined by the method of Lowry (Lowry *et al.* 1951) and its purity was evaluated by SDS-PAGE. The rEnolase was depleted of lipopolysaccharide (LPS) using a polymixin B column (Pierce, Rockford, IL), and all recombinant protein preparations used were LPS-free, as assessed by the limulus amoebocyte lysate kit (E-Toxate; Sigma).

V.2.3. Salivary glands and serum samples

For salivary glands and serum collection, mice were anesthetized by using ketamine and xylazine at 87 and 13 mg/kg of body weight, respectively, and buprenorphine at 0.075 mg/kg as an analgesic. Blood was collected from the tail vein during anaesthesia and sera from coagulated and centrifuged blood were stored at –20 °C. The salivary glands were removed by dissection immediately after the mice were killed and placed in a 1.5-ml conical tube containing RPMI 1640 medium supplemented with glutamine, penicillin, streptomycin, and FBS. The salivary gland was then placed inside a sterile Petri dish. The tissue was minced with scissors and then with a razor blade, transferred to conical tube containing 0.75 ml of RPMI 1640 that was supplemented additionally with collagenase (Sigma-Aldrich) at 1.5 mg/ml, and mixed end-over-end for 30 min at 37 °C using a Shaker rotisserie (Thermolyne Labquake). The contents were then

filtered through a Falcon cell strainer (70- μ m nylon; BD Biosciences), and the filtrate was saved for posterior antibodies quantification by ELISA.

V.2.4 Cell suspensions preparation

V.2.4.1. Collection of NALT cells suspension

NALT was harvested from each mouse and immediately placed in ice-cold RPMI medium (Sigma-Aldrich) and processed as previously described (Asanuma *et al.* 1997, Asanuma *et al.* 1995, Donata Medaglini 2006, Medaglini D 2006, Park *et al.* 2004). Briefly, the mice were killed by cervical dislocation and decapitated, and the lower jaw and tongues were removed. After rinsing with ice-cold sterile phosphate-buffered saline (PBS) to remove blood, the heads were immobilized with pins on a wax dissection slab to reveal the upper palate. Using a zoom stereo microscope (total magnification: 63; Model SZ6045; Olympus Melville, NY, USA), palates were excised using n^o. 11 scalpel blade (Lance Blades, Sheffield, UK). After the incisions, palates were gripped behind the incisor teeth with fine forceps and gently pulled toward the molar teeth while using the scalpel to gently release tissue between the palates, jawbones, and nasal septum. Palates were placed immediately into a 24-mm Petri dish containing 3.0 ml of ice-cold RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine at pH 7.4, (Sigma Chemical Co., St Louis, MO, USA) and the NALT (visible under low angle illumination) was teased gently into the medium to release cells. NALT cell suspensions from individual animals were pooled and cells were washed twice with RPMI-1640 medium by centrifugation (500 g for 10 min at 4°C) and resuspended in RPMI-1640 medium and cells were stained for flow cytometric analysis.

V.2.4.2 Peyer's patches (PP), spleen and CLN isolation

The entire small intestines were removed and the number of PPs was determinate by macroscopic visualization. Cell suspensions from PPs were prepared by pressing the PP's for each mouse with a 5 ml syringe piston. The CLN were removed and gently passed through a steel mesh. The mononuclear cells were further isolated by incubation with 100U/ml collagenase D (Roche, Mannheim, Germany) for 30 minutes at 37°C in RPMI media. Spleen cells were obtained by gently teasing the organ in cold RPMI-1640 (Sigma), suspension.

The cells suspensions were centrifuged at 500g for 10 minutes at room temperature and cells were submitted to flow cytometry analyses on a FACSScan (Becton-Dickinson, San Jose, CA), and analyzed using the Cell Quest software (Becton-Dickinson).

V.2.5. Mucosal Immunizations schedules

For mucosal immunization purposes 10 µl of rEnolase in a 1:1 PBS/alum (Aluminium hydroxide Gel; Brenntag, Frederikssund, Denmark, kind gift of Erik Lindblad) suspension were delivered directly into the intranasal or oral cavity of the mice. Sham-immunized animals received 10 µl of 1:1 PBS/alum suspension. The doses and routes are presented in Table V.1.

Table V.1. Schematic presentation of the rEnolase immunization protocols, respective doses and delivery routes used.

	Group	1 st Dose	Interval of time	2 nd Dose	Interval of time	Sacrifice
Exp.	Sham	PBS/alum intranasal or orally	3 weeks	PBS/alum intranasal or orally	10 or 20 days	+
	rEnolase	10 µg rEnolase intranasal or orally	3 weeks	20 µg rEnolase intranasal or orally	10 or 20 days	+

V.2.6. Flow cytometric analysis

For cytometric analysis cells suspensions from NALT, CLN, spleen, PP, prepared as above described, were resuspended in BSS supplemented with 10 ml of sodium azide and 1% BSA. The following monoclonal antibodies were used on previously titrated dilutions for immunofluorescence cytometric analysis in a FACScan (Becton Dickinson) with the CellQuest software (Becton Dickinson): hamster anti-mouse CD3 FITC conjugate (Southern Biotechnology Associates); IgM FITC conjugated (Southern Biotechnology Associates, Birmingham, ALA, USA); rat anti-mouse CD4 FITC conjugate (PharMingen); rat anti-mouse CD8 phycoerythrin (PE) conjugate (PharMingen); rat anti-mouse B220 phycoerythrin (PE) conjugate (PharMingen, San Diego, CA); hamster anti-mouse CD80 FITC conjugate (PharMingen); rat anti-mouse

CD86 FITC conjugate (PharMingen); CD69 biotin conjugate (PharMingen, San Diego, CA, USA); hamster anti-mouse CD69 PE conjugate (PharMingen); rat anti-mouse CD25 Biotin conjugated (BD Bioscience Pharmigen, San Diego, CA, USA); rat anti-mouse major histocompatibility complex (MHC) Class II biotin-conjugate (Southern Biotechnology Associates) revealed with Cy-Chrome-conjugated streptavidin (PharMingen); rat anti-mouse Mac1 (CD11b) FITC conjugated (BD Bioscience Pharmigen, San Diego, CA, USA). Peanut-agglutinin (PNA) FITC-conjugate (Sigma) was used for germinal center B cells detection. Dead cells were excluded by propidium iodide incorporation.

V.2.7. Antibody analyses

Serum IgG and salivary IgA and IgG antibody levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Dinis *et al.* 2004). Briefly, polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 µg/mL of rEnolase. After washing with Tween-saline (TS) the plates were blocked with PBS containing 1% of bovine serum albumin (Sigma, St Louis, Mo). Antibody activity was then measured by incubation with a 1:30 to 1:2,430 dilutions of sera or with 1:2 to 1:16 dilutions of saliva. After washing, the bound antibodies were revealed by the addition of peroxidase-labelled goat antibodies anti-mouse IgG (Southern Biotechnology Associates, Birmingham, USA) or peroxidase-labelled goat anti-mouse IgA (Biosource, Belgium) and incubated for 3 h at 37° C. After washing, the plates were incubated with orthophenylenediamine dihydrochloride (Sigma) and H₂O₂ for 30 min at room temperature. The reaction was stopped with 10% SDS and the colorimetric change was measured with a Biotek Chromoscan at 450 nm. Data are reported as ELISA units (EU) which were calculated relative to levels in reference sera from non-immunized and non-infected rats as a reference. Dilutions of 1:30 (A_{450} of 0.2) were considered 1 EU for reference serum IgG specific for rEnolase. Dilutions of 1:4 (A_{450} of 0.1) were considered 1 EU for both reference salivary IgA and IgG specific for rEnolase.

V.2.8. Histopathology

Tissues from the NALT, superficial and posterior cervical lymph nodes of sacrificed mice were fixed in 10% buffered formaldehyde and dehydrated in graded alcohol. The fixed tissues were then embedded in paraffin and sectioned at approximately 6

microns thickness. For histological examinations, the sections were mounted onto regular glass slides and stained with haematoxylin and eosin (H and E).

V.2.9. Statistical analysis

The level of significance of the results in all groups of mice was determined by Mann-Whitney test. Differences were considered significant at $P < 0,05$.

V.3. Results

V.3.1 Isolation of NALT lymphocytes

The NALT aggregates were clearly visible on the palate, near the entrance of nasopharyngeal duct. Lymphocytes from isolated NALT were obtained as described above. The number of lymphocytes recovered from isolated NALT of non-treated C57BL/6 mice was about $6,0 (\pm 0.7) \times 10^5$ cells for a single mouse and were about $1,0 (\pm 0.8) \times 10^6$ lymphocytes for non-treated BALB/c mice. With our experimental protocol the NALT cells suspension contained greater portion of B cells than T cells (Table V.2). There were observed tiny differences in lymphocytes percentages, dependent of the mice strain used. As shown in Table V.2, the obtained results were relatively similar to those described by other investigators.

Histological analysis from the NALT and lymph nodes tissues were also performed to confirm the existence of lymphoid tissue in our cells preparations (data not shown).

Table V.2. Surface phenotype of freshly isolated lymphocytes from nasal associated lymphoid tissue (NALT), stained with fluorescently T (CD3+) and B (B220+) lymphocytes cell marker expression with a FACScan fluorescence analyzer.

NALT					
Methods	Mice Strain	CD3	CD4	CD8	B220
(remove nose, collagenase, Percoll) (Asanuma, Inaba et al. 1995)	BALB/c		18-23%	7-10%	20-38%
(remove palato, collagenase, Percoll) (Asanuma, Hodson Thompson et al. 1997)	BALB/c	53,5%			45,1%
J Immunol, 2002 (Zuercher AW2002)	BALB/c	20-50%			47-78%
<u>Our Method:</u>	C57BL/6	26,5% ± 1,2			45,5% ± 2,5
<u>Our Method:</u>	BALB/c	18% ± 1,4			60,1% ± 1,8

V.3.2. Mucosal immune system characterization after rEnolase immunization

Our vaccination protocols against *S. sobrinus*-induced dental caries has been performed in rat (Dinis *et al.* 2004, Dinis *et al.* 2009). However, due to the limited monoclonal antibodies available for multicolor immunofluorescence staining in rats we used mice in order to characterize the effect of rEnolase immunization in the mucosal immune system. For that purpose, C57BL/6 mice were intranasal or oral immunized with two doses of rEnolase, 10 µg and 20 µg incorporated in alum as adjuvant, respectively, three weeks apart of each other. Control animals (sham) were equally treated with PBS plus alum. All animals were sacrificed 10 days after second immunization and serum several organs, NALT, CLN, salivary glands, spleen and PP were collected. Cell suspensions of these organs were analyzed in flow cytometry in order to characterize the cells population after rEnolase vaccination.

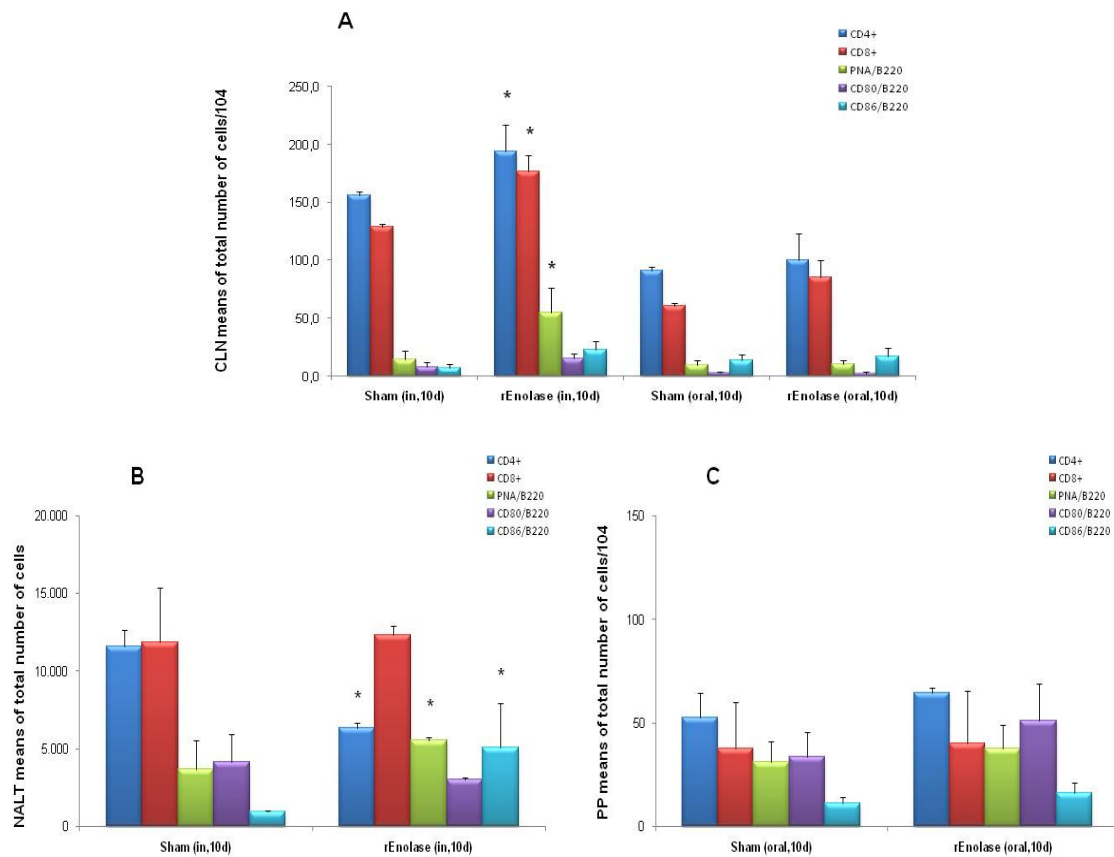


Figure V.1. Numbers of CD4+ and CD8+ T cells, PNA+, B220+, CD80+ and CD86+ B cells in the cervical lymph nodes (CLN) **(A)**, nasal-associated lymphoid tissues (NALT) **(B)**, Peyer's Patches (PP) **(C)** of C57BL/6 mice, evaluated by flow cytometric analysis. Mice were, intranasal (in) or oral (oral) immunized with recombinant enolase (rEnolase) plus alum as adjuvant, twice with 3 weeks apart, and sacrificed 10 days after second immunization. Control animals (sham) were treated with PBS plus alum. In this and in following figures, the results shown are the mean value + 1SD of 8 mice and correspond to one representative experiment out of two independent experiments.

No differences were observed in the spleen after oral or intranasal immunization with rEnolase in C57BL/6 mice (data not shown). We next evaluated the roles of NALT, CLN and PP the mucosal immune response after rEnolase mucosal vaccination. As shown in Figure V.1A, in the mice intranasal immunized with rEnolase a significant increase on T cells numbers, more evident for CD4+ T cells than for CD8+ cells, was observed in CLN. Moreover, an increase in PNA+, B220+ B cells population was also observed in these mice, indicating the induction of germinal centers in CLN upon intranasal rEnolase immunization. In contrast, no differences were observed in CLN from oral rEnolase-immunized mice compared with control animals (Fig. 1A).

The nature of the interactions between antigen and nasal mucosa (NALT) seems important in some dental caries vaccine protocols therefore, we analyzed the NALT cells suspension after intranasal treatment with rEnolase.

In NALT, a significant decrease in the CD4⁺ T cells population was observed in intranasal rEnolase-immunized mice when compared with sham immunized mice while no differences were observed in the CD8⁺ T cell population between the two groups of mice (Fig. V.1B). Moreover, in intranasal rEnolase-immunized animals, an increase in PNA-binding and CD86-expressing B cells were observed (Figure V.1B), indicating the induction of germinal centers in NALT.

The gut associated lymphoid tissue (GALT) is important in induction of mucosal immune response against antigens administrated by oral route. Therefore, in the mice oral immunized with rEnolase the PP were analyzed. As shown in Figure V.1C, no significant differences were however observed between the two groups of mice analyzed.

Taking into account the humoral immune response observed in our previous studies upon rat vaccination with rEnolase (Dinis *et al.* 2009), we also evaluated in this study the local and systemic antibody production after rEnolase-immunization. The levels of antibodies specific for rEnolase were quantified in serum and in the supernatants of NALT, PP, and salivary glands by ELISA.

As shown in Figure V.2, in salivary glands, the levels of IgA and IgG antibodies specific for rEnolase were increased in the oral or intranasal rEnolase-immunized mice (Figure V.2). In the serum, no significant differences were observed on the level of IgG antibodies specific for enolase between the different groups of mice (data not shown).

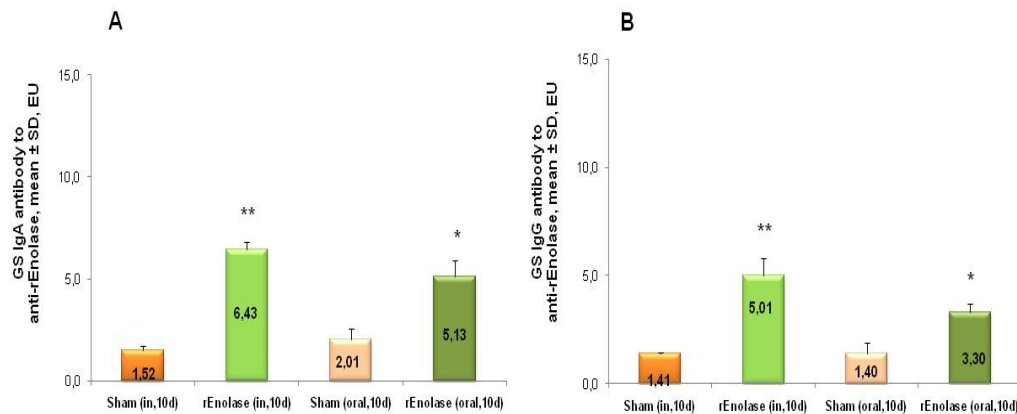


Figure V.2. Evaluation of IgA (**A**) and IgG (**B**) antibody levels, expressed as ELISA Units (EU), specific for *S. sobrinus* recombinant Enolase in the salivary glands homogenates. Mice were, intranasal (in) or oral (oral) immunized with recombinant enolase (rEnolase) plus alum, as adjuvant, twice with 3 weeks apart, and sacrificed 10 days after second immunization. Control animals (sham) were treated with PBS, plus alum.

Taking into account the obtained results with intranasal immunization, we also evaluated the mucosal immune response, 20 days after the second immunization.

In spleen, an increase on CD4⁺ T cells numbers was observed in rEnolase-immunized mice compared with sham-immunized animals (Fig. V.3A). In CLN, the increase on T cell population size observed in rEnolase mice 10 days after the second immunization was maintained 20 days after the second immunization. Again, this increased was more marked on CD4⁺ than on CD8⁺ T cell population. We also observed an increase on PNA⁺/B220⁺ cells numbers, but more pronounced than the one obtained 10 days after the second immunization (Fig. V.3B).

In NALT, the decrease in CD4⁺ and CD8⁺ T cells, observed 10 days after the second immunization in rEnolase-immunized mice was also maintained 20 days after the second immunization (Fig. V.3C). A significant increase in the expression of PNA-binding, B220⁺ and CD80⁺ B cells was observed in rEnolase-immunized mice (Fig. V.3C).

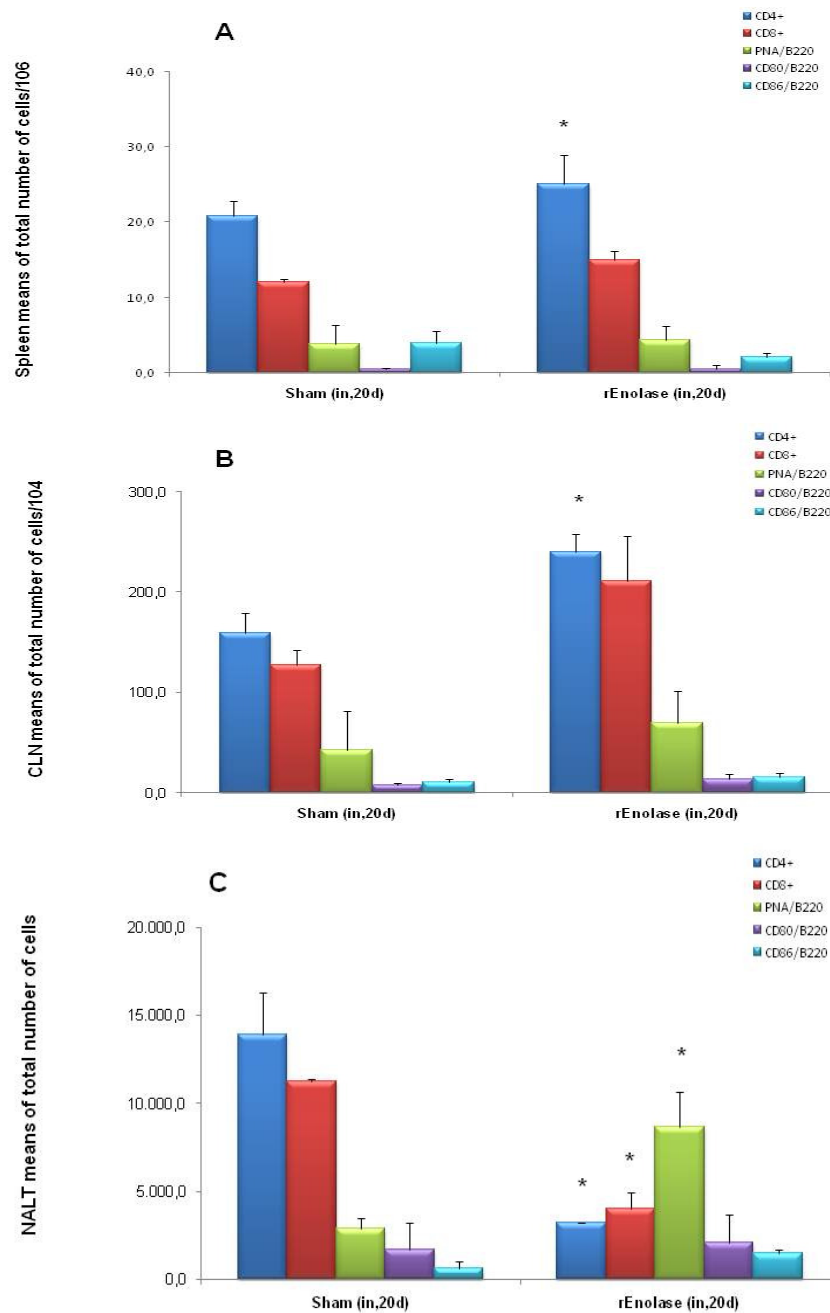


Figure V.3. Numbers of CD4+ and CD8+ T cells, PNA+, B220+, CD80+ and CD86+ B cells in the spleen **(A)**, cervical lymph nodes (CLN) **(B)** and NALT **(C)**, of C57BL/6 mice, evaluated by flow cytometric analysis. Mice were, intranasal (in) immunized with recombinant enolase (rEnolase) plus alum as adjuvant, twice with 3 weeks apart, and sacrificed 20 days after second immunization. Control animals (sham) were treated with PBS plus alum

Moreover, in rEnolase-immunized mice the levels of IgA and IgG antibodies specific for rEnolase were increased (Figure V.4).

We also evaluated the antibody production against rEnolase in serum but no significant differences were detected (data not shown).

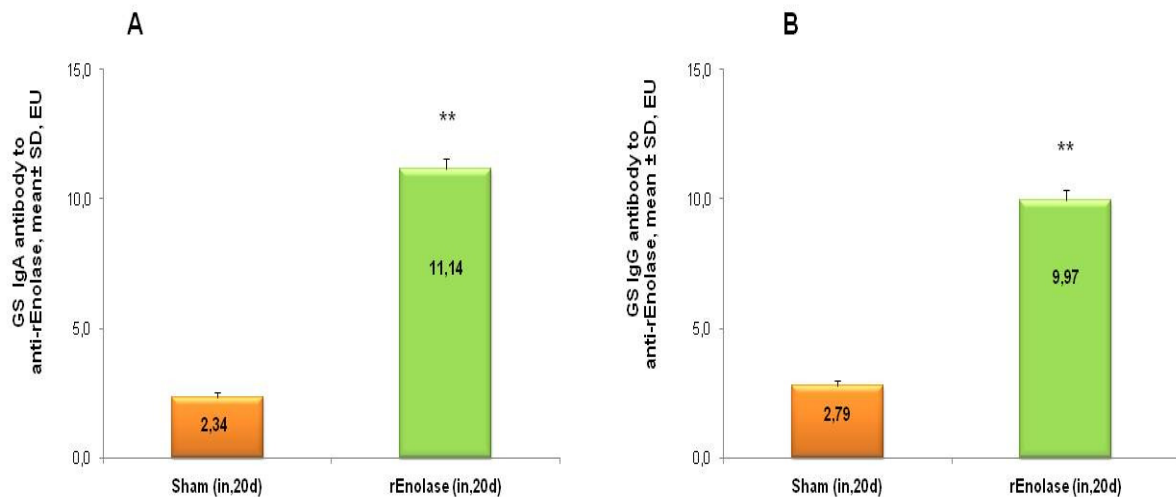


Figure V.4. Evaluation of IgA (**A**) and IgG (**B**) antibody levels, expressed as ELISA Units (EU), specific for *S. sobrinus* recombinant Enolase in salivary glands homogenates. Animals were, intranasal immunized with recombinant enolase (rEnolase) plus alum as adjuvant, twice with 3 weeks apart, and sacrificed 20 days after second immunization. Control animals (sham) were treated with PBS plus alum.

V.4. Discussion

The paired lymphoid cell aggregates of the rodent upper respiratory tract, the NALT, have been recognized as the only well organized mucosal-associated lymphoid tissue in the upper respiratory tract of rodents and is believed to be the equivalent of the Waldeyer's ring of humans (Kiyono and Fukuyama 2004). NALT is located at the floor of the nasal cavity just underneath the ciliated respiratory epithelium and consists of a reticular network filled with various types of lymphoid and non-lymphoid cells (Kiyono and Fukuyama 2004). Several evidences suggest that NALT has an important role in the induction of mucosal immune responses after nasal immunization (Rodriguez-Monroy *et al.* 2007).

In the present experiments, we have developed a method to isolate the NALT from mice. The histological observation of isolated NALT showed the entire structure, which

is composed by the accumulation of lymphoid cells, partially covered with mucosal epithelial membrane. Since there is mucosal epithelium covering NALT, the isolated NALT fraction may include a trace amount of intraepithelial lymphocytes.

NALT and the nasal mucosa drain directly to the superficial and posterior cervical lymph nodes (CLN). Although an inhaled particulate antigen impacting on the mucus layer of the nasal mucosa can be rapidly cleared by ciliary motion, it could also be selectively delivered to the organized NALT structures, thereby triggering an immune responses (Wu *et al.* 1997).

Thus, new vaccination strategies based on nasal application have been designed to prevent diseases caused by infectious agents with a mucosal portal of entry. However, the introduction of soluble antigens in the nasal mucosa predominantly leads to immunological nonresponsiveness (Unger *et al.* 2003). Therefore, there are extremely complex mechanisms operating at the level of the nasal immune system to regulate highly specialized processes, such as immune reactivity and mucosal tolerance. Despite its central role in mucosal immunity, little is known about the nasal immune system. The mucosal immune system has developed separately from the general systemic immune system. As a major consequence, only immune responses initiated at mucosal inductive sites can lead to effective immunity in mucosal tissues. Previous studies have also shown the uniqueness of immune cells from mucosal tissues with respect to those from secondary lymphoid organs (Rharbaoui *et al.* 2005).

The stimulation of NALT will depend on the composition, dose and frequency of the antigen. Some authors believed that small and soluble antigens will penetrate to the nasal epithelium to interact with lymphocytes, dendritic cells and macrophages and will be drained by superior cervical lymph nodes (SCLN) which in turn drain to posterior cervical lymph nodes (PCLN). While particulate antigens can be taken up specifically by M cells above NALT and drained preferentially to the PCLN (Brandtzaeg and Johansen 2005, Kuper *et al.* 1992). Immunochemical characterization of rat NALT has shown that B and T cells are distributed in distinct areas with high CD4:CD8 T cell ratio and predominance of B over T cells (Asanuma *et al.* 1997, Asanuma *et al.* 1995). With our mouse NALT isolation protocol we obtained similar results, greater portion of conventional mature B cells (45,5%) than T cells (26,5%). There were observed tiny differences in lymphocytes percentages, dependent of the mice strain used

Effective nasal immunization requires an appropriate antigen formulation; intranasal administration of pure protein antigens alone may not efficiently induce mucosal and systemic immune responses and may instead elicit anergy and systemic tolerance. Different adjuvants and delivery systems have therefore been proposed for the development of effective nasal vaccines (Kiyono and Fukuyama 2004). In our

vaccination protocols the rEnolase was incorporated in alum, as adjuvant, and was administrated by two routes: intranasal and oral. The obtained results showed that in the animals immunized with rEnolase by intranasal route, 10 and 20 days after immunization, a significant increase in the numbers of CD4⁺ and CD8⁺ T cells in the CLN cell suspensions were observed. Moreover, in CLN and NALT cells suspension, an increased number of PNA⁺ B cells, indicative of germinal centers formation, were observed in rEnolase-immunized mice, 10 days after the second immunization.

The gut associated lymphoid tissue (GALT) is important for oral delivery of the antigen (Kiyono and Fukuyama 2004), therefore we have evaluated the PP after oral vaccination with rEnolase. The obtained results showed that in rEnolase-immunized animals an increase in the numbers of CD4⁺ and CD8⁺ T cells were observed 10 days after the second dose immunization.

Accordingly with the mucosal immunity developed after intranasal and oral immunization with rEnolase, we next evaluated the antibody production in salivary glands. A high increase in levels of IgA and IgG antibodies specific for rEnolase in salivary glands homogenates was observed in oral or intranasal rEnolase-immunized mice, 10 and 20 days after second immunization.

With this study we provide evidence indicative of germinal centers formation in NALT and CLN after intranasal immunization with rEnolase and the expansion of IgA⁺ B cells in regional mucosal immune system. These findings strongly indicate that NALT is an inductive site for humoral mucosal immune responses.

Nonetheless, an improvement of our experimental protocol and additional experiments are still necessary to better elucidate the ability of rEnolase-immunization to induce mucosal immunity and to prevent the development of dental caries.

CHAPTER VI

CONCLUDING REMARKS

VI. CONCLUDING REMARKS

In the begging of my “immunologic adventure”, as undergraduate student, I thought of immune system as a big and complex puzzle, where cells and molecules work as pieces with specific locations, functions, and they will fit together building an amazing and complex system. I thought, and still do, that immune system is the best machinery ever developed, due it function, composition and regulation. Those were the main reasons that, although my biochemistry education, led me in the immunology field. During my “immunological education” I realize that the system was much more dynamic than a puzzle, because along with immune system pieces we also have the pathogen particles interacting. Throughout this thesis project I began to image the system as a football game with tow complex teams, in one side the immune system, on the other the pathogens, playing in the most important championship, survival fight. To understand the game and its rules, we need to understand the pathogen intervenient and techniques, what are the defence’s players, the striker and goal keeper. On the other side of the field we have the immune system team, with B cells, T cells (CD4, CD8), NK, Dendritic cells (DC), macrophages, cytokines, chemokines and so one. Now image the game between them, one fighting to survive and the others trying to eliminate the pathogens. The problem is that with so many intervenient players, and unknown rules, how can we understand the game? Who is the coach of each team? How can we help the immune system team to achieve success and block the pathogen attack? Most of the time when we began to understand the pathogen and the interaction with host immune system, the microorganism tends to change the combat approach and the game starts all over again.

In order to understand infectious diseases we need to study the microorganism involved, learn about the disease pathogenesis and investigate cellular and molecular immune responses that are taking place.

Dental caries is still one of the most prevalent infectious diseases in humans and there is a long way to go before declares victory over this disease. Major questions remain unanswered: Can infection with dental caries pathogens, *Streptococcus sobrinus* and *S. mutans*, be intercepted or modified immunologically? Can immune strategies handle effectively with chronic mutans streptococci infections? What is the most efficient, safe, protective and low-cost vaccine? What will be the route of administration and the better mucosal adjuvants? Are these vaccines safe? What comes out in oral cavity after immunization against dental caries?

Dental caries is a transmittable, infectious disease, caused by specific microorganisms. This paradigm would require caries prevention by vaccination, but there is evidence that caries is not a classical infectious disease. Rather it results from an ecological shift in the tooth-surface biofilm, leading to a mineral imbalance between plaque fluid and tooth and hence net loss of tooth mineral. Therefore, caries belongs to common 'complex' or 'multifactorial' diseases, such as cancer, cardiovascular diseases, diabetes, in which many genetic, environmental and behavioural risk factors interact.

The use of the concept 'an infectious disease' immediately signals that a disease is caused by a particular microorganism or agent, which has 'infected' an individual. As defined by John Last (2001), who uses infectious disease synonymously with communicable disease: 'an illness due to a specific infectious agent or its toxic products that arises through transmission of that agent or its products from an infected person, animal or reservoir to a susceptible host...' For about half century caries was defined as an 'infectious and transmittable' disease on the following premises.

Not all types of microorganisms in the oral cavity are equally able to ferment carbohydrates, so it has been logical to look for major caries pathogens. While research in the first half of the previous century focused on the role of *Lactobacillus*, the latter half of that century focused on the role of mutans streptococci (Loesche 1986).

The concept of dental caries being 'infectious and transmittable' grew out of the elegant rodent studies performed in the 1950s (Keyes 1958). Further proof emerged when certain streptococci isolated from caries lesions in hamsters, unlike other types of streptococci, caused rampant decay in previously caries-inactive animals (Tanzer 1995). These bacteria, later identified as *Streptococcus mutans* (SM), gave rise to the concept of caries being due to a specific infection with mutans streptococci, a concept that has gained wide support within the field of caries microbiology over the past four decades (Loesche 1986, Tanzer 1995). Mutans streptococci belong to the resident microflora and are ubiquitous in populations worldwide.

The outgrowth of mutans streptococci could be explained by a disturbance of the homeostasis in the dental biofilm. If the homeostasis of the oral microflora is lost, then an opportunistic infection can occur, i.e., these infections are derived from microorganisms that are endogenous to the host (Marsh and Martin 1992) and an ecological plaque hypothesis seems more attractive. The resident flora in the oral cavity will inevitably form biofilms on teeth, e.g. a population or community of bacteria living in organized structures at an interface between a solid and liquid.

So, dental caries is one of the most common infectious diseases. Of the oral bacteria, mutans streptococci, such as *Streptococcus sobrinus* and *S. mutans*, are considered to

be the primary agents responsible for human dental caries (Loesche 1986). There have been numerous studies on the field of mutans streptococci vaccine immunology (Taubman and Nash 2006a). To control dental caries, experimental vaccines have been produced using various cell-surface antigens of these organisms (Culshaw *et al.* 2007, Ma *et al.* 1990, Smith 2003, Smith *et al.* 1997a, Smith *et al.* 2001a, Smith and Taubman 1987, 1990, 1996, Smith *et al.* 2005, Smith *et al.* 1997b, Taubman *et al.* 2001, Taubman *et al.* 2000b). Progress in recombinant DNA technology (Guo *et al.* 2004, Xu *et al.* 2006, Xu *et al.* 2007) and peptide synthesis has been applied to the development of recombinant and synthetic peptide vaccines to control this disease. Protective effects against dental caries have been shown in experimental animals, such as mice, rats and monkeys, which have been subcutaneously, orally, or intranasally immunized with these antigens (Koga *et al.* 2002, Smith 2002a, Taubman and Nash 2006a). Some studies have examined the efficacy of dental caries vaccines in humans (Taubman and Nash 2006a). In clinical assays with passive administration of antibody anti- Ag I/II, there was interference in recolonization of human teeth with indigenous mutans streptococci (Hamada *et al.* 1991, Ma *et al.* 1990, Ma *et al.* 1987). The authors argue that the protection was mediated by the neutralization of the binding of the cariogenic bacteria to dental pellicle and aggregation of mutans streptococci for clearance in the salivary milieu. However, the mutans streptococci have other adhesins that could compensate the effect mediated by antigen I/II.

Intact glucosyltransferase (GTF), which is involved in synthesis of glucans, has also been used in clinical assays. GTF oral or buccal mucosal instillations were used in clinical assays, there was induction of parotid salivary IgA specific for GTF, and delayed reaccumulation of indigenous mutans streptococci (Smith and Taubman 1987, 1990).

A combination of antigen mixtures has also been tested. Some investigators used mixture of GTF and antigen I/II in clinical assays, with oral or intranasal instillation, and an increase in secretory IgA1 and secretory IgA2 in nasal secretions and/or saliva was observed (Childers *et al.* 2003, Childers *et al.* 2006, Childers *et al.* 2002, Childers *et al.* 1997, Childers *et al.* 1999).

We need to take in consideration that these humans' results reported only the humoral response upon antigen or antibody passive administration; there is not data about their effect in MS colonization. The results that correlate the humoral response and dental caries lesions are only described in experimental models.

It has been described that several pathogenic microbes produce and secrete virulence-associated immunomodulatory proteins (VIP) important for their survival in the host (Lima *et al.* 1992, Santarém *et al.* 1987, Tavares *et al.* 1993). As previously reported (Ferreira *et al.* 1997), *S. sobrinus* secretes a virulence-associated immunomodulatory protein (VIP). The isolation and biochemical and immunological characterization of these molecules could be useful for vaccination studies against the infection caused by the producing microbe. Indeed, it has been demonstrated for systemic candidiasis that neutralization of the VIP effects through the immunization with this protein confers protection against the infection by the fungus in mice (Tavares *et al.* 1995).

Since, rodent model has been extensively used for delineating immune protection studies; our first step was to establish an experimental model that resembled the human dental caries infection. Several mutants streptococci infection protocols have been established. However most of them do not resemble a normal oral cavity infection. Indeed, most use specific pathogen free Sprague Dawley rats (1981, Smith *et al.* 1997b, Tanzer 1979, 1995), germ free Fisher rats (Michalek *et al.* 1975), gnotobiotic animals (Fitzgerald 1968, McGhee *et al.* 1975), or administration of massive amounts of antibiotics before infection with cariogenic bacteria (Zhang *et al.* 2002). It is obvious that in all these animal models the oral microflora was manipulated in order to facilitate the MS colonization. And this is not what occurs in the oral cavity of humans, where other bacteria are present and a dynamic system is maintained. In humans, there is an ecosystem that MS must overcome to succeed and induce dental caries lesions. Taking into account all these concerns, we choose Wistar rats as our animal model (Dinis *et al.* 2004). They are outbred rats and harbor an endogenous oral flora similar to the humans. We have administrated the cariogenic bacteria taking into account the “window of infectivity” (Caufield 1997), that correspond to a discrete period of time, between days 18 and 22 after birth which it is possible to colonize the oral cavity of laboratory rats. After our animal model established we investigated whether therapeutic immunization with VIP could protect the animals against *S. sobrinus*-induced dental caries. The obtained results showed that VIP immunization confers protection against *S. sobrinus*-induced dental caries (Dinis *et al.* 2004).

Concomitantly, enolase has been described as one of the immunomodulatory proteins of *S. sobrinus* (Veiga-Malta *et al.* 2004).

In view of the fact that, in vaccination strategies, the use of recombinant protein offers several advantages, the bacterial enolase gene has been cloned and expressed, and the recombinant protein, rEnolase, was purified (Veiga-Malta I *et al.* 2004). With the

recombinant protein we first confirm that the treatment with an immunosuppressive dose of rEnolase before the infection facilitates *S. sobrinus* oral colonization (Dinis *et al.* 2007). Indeed, increases numbers of CFU was recovered from the oral cavity of the Wistar rats treated with the rEnolase comparatively with control rats. Moreover the treatment with rEnolase showed an increase in rat caries lesions, indicating that enolase is involved in the pathogenesis of *S. sobrinus* infection.

Since rEnolase facilitates *S. sobrinus* oral colonization, similarly to what has been described with other VIP (Santarém *et al.* 1987, Tavares *et al.* 1993, Vilanova *et al.* 1999), we next investigated whether the neutralization of rEnolase effects through immunization could confer protection against *S. sobrinus*-induced dental caries in Wistar rats. Therefore, a oral therapeutic immunization with rEnolase, in a dose unable to induce immunosuppression, was assayed (Dinis *et al.* 2009). The obtained results showed a reduction on dental caries lesions in rats vaccinated with the recombinant protein, compared with control rats. In accordance with this reduction, a decrease in *S. sobrinus* oral colonization was observed in rEnolase-vaccinated rats throughout the study. The evaluation of the antibodies levels specific for rEnolase showed that the immunization induced a high level of salivary IgA antibody response specific for rEnolase that is directly correlated with the reduction in the number of bacteria recovered from the immunized rats, as well as with the reduction in the extent of dental caries observed in these animals. Specific immune defense against cariogenic bacteria is provided mainly by salivary secretory IgA (SIgA), the most abundant immunoglobulin in saliva. SIgA is immunoglobulin class has been associated with protection in several dental caries vaccination studies (Russell *et al.* 2004). Immunization at various inductive sites of the common mucosal immune system (CMIS) such the gut, nasal cavity, bronchus, rectum or vagina can generate SIgA not only in the region of induction, but also at other, remote mucosal surfaces. Indeed when we used the intranasal and oral routes for immunization with rEnolase there was an effective induction of salivary IgA responses (Dinis *et al.* 2009). The significant lowest incidence of dental caries and of *S. sobrinus* oral colonization, after rEnolase immunization, was also associated with an increase in the levels of gingival crevicular IgG antibody specific for rEnolase. The role of locally or serum derived IgG antibodies in the protection of dental caries is still controversial. Until recently, the major role was attributed to SIgA but nowadays reports referred the importance of an IgG induction for protective immune response against dental caries (Taubman and Nash 2006a) or other oral diseases (Takashi *et al.* 2005). Our results reinforced the important role of both salivary IgA and IgG antibodies in dental caries protection. Although antibodies

have a fundamental role in defence against this infectious disease, cellular immune response also needs to be considered. It has been described that many commensal bacteria in human saliva are coated with SIgA (Brandtzaeg *et al.* 1968, Brandtzaeg and Johansen 2005). In the gut mucosal immune sites such coating allows M-cells mediated bacteria uptake, which improves bacteria elimination by subepithelial macrophages and also the uptake by DC (Macpherson *et al.* 2005). Since enolase has been described as a surface protein, we could hypothesize that the antibodies specific to rEnolase coated the bacteria and promoted their phagocytosis; and/or enabled T cells activation and differentiation in effector and memory cells leading to bacteria growth control and disease inhibition.

The safety of rEnolase as a target antigen was also evaluated through histopathological analysis in all vaccinated rats. The examination of multiple tissues showed an absence of detectable long-term histopathologic damage. Since enolase is an ubiquitous protein, and several autoimmune diseases have been associated with the production of antibodies against this protein (Fontan *et al.* 2000, Pancholi and Fischetti 2001, Saulot *et al.* 2002, Witkowska *et al.* 2005) we also investigated whether antibodies raised against bacterial enolase recognize human enolase. Indeed, we demonstrated that the antibodies raised against bacterial enolase do not recognize human enolase.

With this work we concluded that therapeutic vaccination with rEnolase does not have any detected harmful physiologic effect on Wistar rats. Therefore, it may be a useful target antigen for human vaccination against *S. sobrinus*-induced dental caries.

However, one of the main problems with dental caries vaccines still persists. We have showed protection only against one of the human cariogenic bacteria, *S. sobrinus* and an ideal dental caries vaccine must protect the host against the two cariogenic agents, *S. mutans* or *S. sobrinus*. The homology between the enolase from *S. sobrinus* and from *S. mutans* enolase is very high (nucleotide sequences 98% identical), therefore, we hypothesized that rEnolase could also be a target antigen for vaccination against *S. mutans*-induced dental caries. Therefore, a therapeutic vaccination with rEnolase was performed in Wistar rats infected with *S. mutans*. The obtained results showed a reduction on dental caries lesions on rEnolase-immunized rats.

This result indicates that rEnolase is a good candidate for a vaccine against dental caries induced by either of the cariogenic agents. However, further work is still required to improve our *S. mutans* infection protocol since the oral colonization with this bacterium was much lower than the one observed with *S. sobrinus*. This is in

accordance with the studies performed by Ooshima and collaborators (Ooshima *et al.* 1981) showing that *S. sobrinus* is more cariogenic than *S. mutans* in animal models.

Taking into account the importance of a preventive vaccination procedure, the next step was to establish a preventive vaccine protocol using the rEnolase as a target antigen. It is important to state that due to the “window of infectivity” preventive protocols are more difficult to achieve than the therapeutic ones. The obtained results, although preliminary, clearly showed a reduction on dental caries lesion in rats immunized with rEnolase as compared with controls rats.

In our preventive vaccination protocol we assayed the intranasal route for immunization of the rats and a protective immune response against *S. sobrinus*-induced dental caries was observed. Increased levels of salivary IgA and IgG antibodies specific for rEnolase were observed in vaccinated rats. This could be explained by the fact that after NALT vaccine uptake a mucosal immunity was generated resulting in dental caries protection of rats. We have also used a combination of intranasal and subcutaneous route for vaccine delivery that showed the best results. The introduction of the subcutaneous route, besides the intranasal route in the vaccination protocol, promoted mucosal and systemic immunity.

Some improvements in our preventive protocol are required in order to obtain a greater dental caries inhibition. We must remember that one of the major problems of vaccine administrated to newborn and to infants is the effect of mother protective immunity that inhibits the vaccine effect in infant child. This is the reason for the several boosts that is necessary. We could hypothesize that most probably the mother's milk immunoglobulins inhibit the induction of an early immune response in their offspring. In order to overcome this mother's effect we could propose boost with rEnolase after the inhibitory effect of the mother have passed. Other possibility may be the vaccination of the mother and only vaccinate children when they are older.

Therefore, we investigated whether maternal vaccination with rEnolase before pregnancy could protect the offspring from dental caries by passively transferred immunity.

The results showed that rats born from rEnolase-immunized dams were more protected from dental caries, even when nursed by sham-immunized controls dams, than rats born from sham-immunized dams indicating that transplacental transfer is likely to be the major mechanism of maternal antibodies acquisition. Moreover, our results also suggest the influence of maternal immunity on antibody response in their progeny. Indeed, the evaluation of salivary IgA and IgG antibodies specific for rEnolase

in rats born from rEnolase vaccinated dams, 120 days after birth, showed an increase in the levels of these immunoglobulins.

It is generally accepted that maternal antibodies provide passive protection to offspring and shape the developing B cell repertoire via idiotypic interactions with neonatal antibodies (Malanchère *et al.* 1997). The functions of maternal antibodies and which isotypes are transferred to the offspring have been under intensive investigation. In mammals, these are exclusively IgA and IgG antibodies (Lemke *et al.* 2004). The maternal IgG is actively transported prenatally across the placenta by the neonatal Fc-receptor (FcRn) and /or can be transferred after birth through colostrum and milk, which also contain high concentrations of IgA (Malanchère *et al.* 1997). The maternal antibodies of IgG isotype crossing the placenta will supply the immunologically immature developing fetus and neonate with immune defense (Fink *et al.* 2008). In mice but not in human, maternal IgG transfer continues after birth by uptake from milk through the intestinal neonatal Fc receptor (FcRN), until FcRn is down-regulated on gut epithelial cells approximately 16 days after birth. Despite the fact that some transcytosis of IgG through epithelial cell layers have been demonstrated in humans, the maternal antibodies uptake occurs predominantly through the placenta (Fink *et al.* 2008). It is well established that maternal antibodies can prevent bacterial and viral infections that cannot be cleared by the immature immune system (Van de Perre 2003).

More recently, maternal antibodies and their impact in the generation of humoral responses in offspring have been investigated (Glezen 2003). Those results showed that maternal antibodies seem to be required to prime offspring optimally and increase the antibodies response following subsequent infection. Long-lasting modulating effects of maternal antibodies on specific immune responses of the progeny up to adulthood have been shown for some antigens, but the mechanisms behind remains unclear (Fink *et al.* 2008, Glezen 2003).

Our results are in accordance with this study; indeed rEnolase specific maternal antibodies stimulate humoral immune response in the progeny that consequently confer protection of the offspring against *S. sobrinus*-induced dental caries.

All these results will be useful for the development of novel vaccination strategy for protection against *S. sobrinus* and/or *S. mutans* oral colonization in early life, through the maternal immunization.

Mucosal epithelia comprise an extensive vulnerable barrier which is reinforced by numerous innate defence mechanisms cooperating intimately with adaptive immunity. Local generation of secretory IgA (SIgA) constitutes the largest humoral immune

system of the body. However, there is considerably disparity with regarding the migration of memory/effector cells from mucosal inductive sites to secretory effector sites and systemic immune organs. Although immunological memory is generated after mucosal priming, this may be masked by a self-limiting response protecting the inductive lymphoid tissue in the gut.

Despite its central role in mucosal immunity, little is known about the nasal immune system. The mucosal immune system has developed separately from the general systemic immune system. As a major consequence, only immune responses initiated at mucosal inductive sites can lead to effective immunity in mucosal tissues. Previous studies have also shown the uniqueness of immune cells from mucosal tissues with respect to those from secondary lymphoid organs (Rharbaoui *et al.* 2005).

We also tried to investigate the cellular and molecular mechanisms underlying oral mucosal immunity upon immunization with rEnolase. As previously described, our model for dental caries infection was Wistar rat. So, the protection assays were performed in that model, the humoral response was quantified and the carious lesions were evaluated. However, at the time there were fewer markers for anti-rat surface molecules, so we simulated the immunoprotection assays in mice. We mimicked our vaccination protocol in mice model; therefore C57BL/6 mice were immunized with rEnolase plus alum. The main conclusion from these results was the generation of germinal centers in NALT after intranasal immunization with rEnolase and the expansion of IgA⁺ B cells in regional mucosal immune system. These findings provide direct evidence that NALT is an inductive site for humoral mucosal immune responses. In our preliminary results, B cells and T cells from regional lymph nodes (cervical lymph nodes) seemed important, leading to generation of germinal centers in the induction site (NALT). These observations could be correlated with increased levels of salivary IgA and IgG in the rEnolase immunized animals. Indeed, germinal centers are of vital importance for T cell- dependent generation of conventional memory B cells, affinity maturation of BCR and Ig-isotype switching (Brandtzaeg and Johansen 2005).

Innate immunity is activated upon the initial invasion of microbes. In the oral mucosa, the innate immunity system consists in epithelial barriers, circulating cells, proteins that block bacterial invasion and eliminate the invading microbes (Hahn and Liewehr 2007a). Due to the unique anatomic location of caries bacteria, classic phagocytic killing probably does not occur until the pulp is directly in contact with caries front. Before actual caries exposure, the dental pulp beneath shallow caries is capable of mounting innate immune response that may slow down caries invasion (Hahn and Liewehr 2007a). It has been described the components of innate response of the dentine/pulp

complex; the dentinal fluid, which composition is not fully determined, but is considered to be serum derived fluid containing serum proteins and immunoglobulins (Knutsson *et al.* 1994). The investigators raised the hypothesis of protective functions of antibodies in dentinal fluid to antigen specific or nonspecific. For example, natural antibodies against *S. mutans* in serum (Challacombe *et al.* 1984) may diffuse extravascularly into dentinal fluid and react with bacterial antigens in carious lesions (Love 2002). The participation of odontoblasts in the innate response to caries has also been investigated, they express cytokine, chemokines, chemokines receptors that are involved in recruitment of DC (Hahn and Liewehr 2007a). Odontoblasts cultured from normal pulp also constitutively express TLR (TLR1, TLR6 and TLR9) (Durand *et al.* 2006). In the dental pulp there are also innate immune cells, including immature dendritic cells, natural killer cells; and T cells, cytokines and chemokines (Hahn and Liewehr 2007a). Both T cells and DC have been considered to be important in immunosurveillance as part of innate immune response to caries. Possible interactions between innate immune cells, cytokines, odontoblasts may occur in the pulps beneath shallow caries and elicit adaptive immune response (Hahn and Liewehr 2007a, b). Recently, the role of oral mucosal dendritic cells in immune responses and tolerance, at the oral mucosa has been investigated (Cutler and Jotwani 2006, Cutler and Teng 2007). The highest numbers were found in keratinized mucosa (soft palate, ventral tongue, lip), whereas lower levels were found in hard palate and gingiva (Cutler and Jotwani 2006). In some in vitro studies they tried to isolate mouse and human dendritic cells and investigated their interaction with oral microbes implicated in chronic periodontitis (Cutler and Teng 2007). The dendritic cells can polarize effector responses toward Th1 or Th2, depending on the type of antigen used as stimulant. For example, in *P. gingivalis* infection, the interaction of dendritic cells and bacterial lipopolysaccharide induces the secretion of IL-10 (Jotwani *et al.* 2003). Contrarily, the potential role of DC in aggressive periodontitis, appears to be active/mature cells and induce a Th1 response (Kikuchi *et al.* 2004). After capturing foreign antigens, DC migrate to immune inductive sites and initiate the adaptive immune response, but can also re-stimulate a local response in peripheral tissues (Murphy *et al.* 2008). In DC oral mucosa, very little is known about these inductive and effector sites. Murine studies suggested that buccal mucosa DC capture antigen and migrate to cervical mandibular lymph nodes, where antigen is presented (Eriksson *et al.* 1996). The oral mucosal tissues and dental pulp are equipped to mount an innate response to invading caries bacteria; but the unique location of these bacteria seems to prevent their elimination by oral mucosal immune system.

In our work we tried to quantify several cytokines (TGF- β , IL-10, INF- γ) by ELISA, which could be involved in IgA production in different organs (NALT, salivary glands). Although we were not able to detect appreciable amounts of these cytokines, which can be explained by the fact that collagenase treatment used to better isolate cells from these tissues can depredated these molecules, it has been described that TGF- β , IL-2, IL-5 and IL-10 appears to be a crucial IgA switch factor and may also be important for the expansion and terminal differentiation (Brandtzaeg and Johansen 2005). Indeed, a central role of IL-10 in human B cell differentiation was supported by the fact that this cytokine could release the block of IgA-committed B cells from IgA-deficient patients (Brière 1994). Also naïve human B cells activated through CD40 can be pursue toward IgA production by TGF- β and IL-10 combination (Defrance *et al.* 1992).

In future work, we will pursue the cytokine profile characterization by correlating the levels of cytokines mRNA using reverse transcriptase PCR (RT-PCR) with protein expression in ELISA assays. Another important approach will be to determine the cell types population involved in TGF- β and IL-10 expression after rEnolase treatment by flow cytometry, using different doses and during different time points.

The CD4⁺ T cells from CLN seemed important in the immune response to rEnolase intranasal administration, further work is necessary to characterize which subsets are involved: CD4⁺ Foxp3⁺ CD25⁺ T cells, Th2 cells or Th3. In parallel, we observed that rEnolase administration had an impact in the NALT B cells; these cells seemed to be affected after rEnolase immunization. Another hypothesis is that these cells could be involved in the IL-10 production and that could lead to B cell decrease in NALT suspensions. With the purpose to identify the IL-10 producing cells, the cytokine staining would be important.

After rEnolase plus alum intranasal immunization we observed the induction of germinal centers in the inductive site (NALT) and an increase of salivary glands IgA and IgG antibodies specific for rEnolase. The results are in accordance with the postulated mucosal immune system compartmentalization, that it is, in the NALT the antigens must be captured by B cells, APC and interact with T cells that will migrate to regional lymph nodes (CLN). The cells will proliferate and since they were mucosal administrated, there will be "homing" to the inductive site, as discussed by other investigators (Brandtzaeg and Johansen 2005). In parallel, the expression of PNA was increased, correlated with germinal centers induction, it would be important, in the future, to perform the IgA⁺ PNA⁺ cells staining. The salivary levels of IgA and IgG antibodies anti-rEnolase were increased after immunization with the recombinant protein. Although we could not detected significant levels of IgA and IgG antibodies in

the NALT suspensions, we believed that this lack of antibodies may result from experimental problems as previously described.

Moreover, it will also be important to identify the specific role of different subsets and maturation state of APC in the regulation and differentiation of effector or regulatory T cells. The role of DC in oral mucosal immune system have been highlighted (Cutler and Jotwani 2006). Importantly, the human nasal mucosa is extremely rich in various DC types, both within and beneath epithelium (Jahnsen *et al.* 2004), and subepithelial band of putative APC is seen below the surface epithelium as well as FAE (Follicle-associated epithelium) in human gut (Brandtzaeg and Johansen 2005).

The mucosal B cell system responds to infection with local production of IgA and IgM, and the level of this response appears to determine whether clinical symptoms will occur or not (Brandtzaeg 2007b, Brandtzaeg and Johansen 2005). There still remaining some questions concerning the regulation of this antibody system, and the interaction of mucosal and systemic immune system. Evidence is accumulating to stress out that innate protective mechanisms are much more crucial and complex than previously believed (Sansonetti 2004). The cooperation between innate and adaptive immunity needs further exploration to better understand how the homeostasis of mucosal membrane is normally maintained in order to commensal flora persist and pathogen microorganism are eliminated. The IgA induction and production at mucosal effector sites has been discussed, (Brandtzaeg 2007a), cytokines play an important role in stimulating germline transcription of several isotypes.

Although the obtained results are very promising, more studies are required. We need to pursue more studies in the cellular mucosal immunity, in order to clarify the role of the different cells population. For example, studies addressing the role of dendritic cells in the oral mucosal interface, subsets of regulatory cells described in the NALT and other mucosal tissues. These are the first steps in order to better understand the dental caries in the context of mucosa immunity, but further work is required.

Therefore, a more thorough understanding of the mucosal immune system and its regulation will be helpful for the treatment and prevention of dental caries as well as other infectious diseases related to the mucosal immune system.

In conclusion, with this thesis we proved that enolase from *S. sobrinus* is a virulence factor and its immune-neutralization prevents or treats dental caries. With our therapeutic and preventive immunization protocols we demonstrated that rEnolase vaccine was able to reduce caries lesions caused by *S. sobrinus* or *S. mutans* and could be used as a target antigen for people that already harbouring the bacteria, or prevent people from getting it. Moreover, our results showed that rEnolase is a safe antigen candidate for dental caries vaccination.

Since children would be the best target population, but vaccination clinical trials are inadvisable in young children, the vaccination of the mother will be a good approach as our results indicate.

At this moment I'm feeling exactly as when I'm puzzling with my nephew André, a small piece fits in the mucosal immune puzzle but others are still missing.

CHAPTER VII

REFERENCES

VII. REFERENCES

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