The vaccination approach to control infections leading to dental caries

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Abstract
Dental caries is a transmissible infectious disease, in which mutans streptococci (MS) play the role of main pathogens. This oral disease represents a public health problem worldwide, and despite the advances in dental health with the use of fluoride abroad, treatment of caries manifestations and their outcomes are still highly costly to public and private healthcare systems. Lack of treatment of dental caries ultimately may have serious systemic consequences. Mutans streptococci have a panel of virulence factors important for their establishment in the complex microbial community of the dental biofilm and in the induction of caries. In this review we discuss the advances in our understanding of the molecular mechanisms which underlie MS transmission, tooth colonization and virulence. Infection and disease take place in a milieu exposed to components of the mucosal and systemic immune systems of the host. Thus, inducing host responses which target aspects of mutans streptococcal colonization and disease may provide additional measures to modify dental caries. This review also describes current strategies for anti-caries vaccination efforts with regard to important bacterial targets, routes, adjuvants and delivery systems for active and passive immunization.

Key Words:
dental caries, vaccination, mutans streptococci

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Introduction

Dental caries as a transmissible infectious disease

Dental caries is an infectious disease that occurs because of an imbalance in the homeostasis between the host and oral flora. This imbalance is created by the emergence of cariogenic microorganisms in the complex community known as dental plaque (dental biofilm). Cariogenic organisms are those able to cause tooth demineralization because of their capacity to accumulate, produce and tolerate extremely low pHs in the dental plaque, that account for demineralization of dental tissues. All host and environmental factors that favor transmission of cariogenic organisms, creating ecological advantages for their establishment in the dental biofilm may be defined as risk factors for caries development. For example, frequency and intensity of contact with already infected subjects are initial factors in the transmission of these organisms. Several additional host and environmental factors influence this transmission and caries development. These include conditions that interfere with salivary innate and adaptative immune defenses (e.g. general immune deficiencies associated with malnutrition, inherited or medication disorders, or other factors that affect salivary flow and saliva composition). Specific substrates mainly derived from diet (e.g. sucrose) can favor bacterial accumulation in the dental biofilm. Other host factors are also associated with a higher susceptibility of teeth to caries development even in the absence of a highly cariogenic microbiota. These include developmental defects in enamel formation in primary and permanent teeth[1]. The composition of the dental biofilm community also may be an important obstacle to establishment of pathogenic organism, because of antagonisms and cooperative interactions that occur among inter-genera and species during the dynamic process of microbial competition for establishment in oral niches. Thus, dental caries may be defined as a complex multifatorial disease in that a broad group of biological, socio-economic and cultural factors interact directly or indirectly in the establishment and growth of cariogenic microorganisms within the diverse microbial community of the dental biofilm.

Mutans streptococci and dental caries.

There is solid evidence that the principal organisms associated with the onset and progression of dental caries are the mutans streptococci (MS). The associations of these organisms with caries in clinical and epidemiological studies has been extensively studied and significant advances have been made in understanding the mechanisms by which these "cariogenic" streptococci bring about disease.

Mutans streptococci were initially isolated from caries lesions of British children by Clarke[2], who referred them as "mutans" streptococci because of their atypical rod-like shape resembling "mutant" streptococci. However, it was not until the 1960´s that mutans streptococci were demonstrated to be transmissible infectious agents involved in the establishment of caries disease[3]. Since then, many epidemiological studies have investigated the degree of mutans streptococcal infection in populations worldwide and the relationships between MS infection and development of dental caries lesions (for reviews see[4-10] Mutans streptococci account for 7 distinct species isolated from animals and humans: *Streptococcus cricetus*, *Streptococcus ferus*, *Streptococcus macacae*, *Streptococcus rattus*, *Streptococcus downei*, *Streptococcus mutans* and *Streptococcus sobrinus*[11]. *S. mutans* and *S. sobrinus* are exclusively isolated from humans and *S. mutans* is the most prevalent species[11]. Other bacterial aciduric species, such as lactobacilli and veillonella[12-13], and yeast[8,14], may co-infect developing carious lesions. There is little evidence however that these later organisms are implicated in the initiation of caries. Their presence may result from a favorable acidic environment and the retentive topography of caries lesions already initiated by mutans streptococci[12,14].

Mutans streptococci have been identified in all populations studied worldwide[4,7,10], but differences were observed regarding frequency and intensity of infection and associations with caries indices. Study comparisons have been made difficult because of variations in study design, age and number of subjects analyzed, socio-economic background of the studied populations, cultural traits related to self care habits and diet, genetic background and exposure to fluoride[8]. Apart from these limitations, epidemiological and clinical studies have provided a number of key notions that strengthen the need to control mutans streptococcal infection in order to limit dental caries development and progression. These studies can also provide clues about the best strategies to achieve this goal.

The earlier children are colonized by MS, the less the likelihood to control infection and caries development. Clinical studies performed with young children have identified crucial aspects of MS colonization and pathogenesis. These studies influenced the development of methods to control infection by MS. Pioneer studies analyzing initial colonization of MS and its association to caries were performed in Scandinavia. Alaluusua and Renkonen[15] first reported that children with detectable levels of MS at 2 years of age presented a caries incidence about 11-fold higher than children not colonized by the same age. Later, Köhler et al.[16]observed that children colonized by *S. mutans* before 2 years of age, during primary tooth eruption, when their commensal microbiota were still being establishmed, showed a mean caries score (mean dfs=5.0) about 6-fold higher than the dfs score of children colonized in later ages (from 0.9 to 2.5). These early colonized children also had a number of caries lesions about 10-fold higher than the index of children not colonized at 4 years of age (mean dfs=0.3)[16]. This group later described that an intense

program to reduce oral levels of MS (including daily application of 1% chlorhexidine gel for 2 weeks) in initially highly MS colonized mothers significantly delayed the colonization of their first born babies who were followed from one to month to seven years of age. This was in contrast to babies from a control group of mothers (received only routine treatment and dental prophylaxis), who were colonized significantly earlier and presented a significant higher caries incidence [17]. The preventive measures applied to mothers were performed until children reached 3 years of age. The benefits of this treatment (low levels of infection and low caries incidence), however, continued to be observed until the end of the study, when children were 7-years-old[17-18]. Understanding the effects of this treatment on the maturing biofilm underlies the explanation for these observations.

Studies of the initial colonization by mutans streptococci indicate that these bacteria require non-shedding tooth surfaces to become established in the oral cavity[19-20]. More sensitive methods using DNA specific probes indicate that the retentive surfaces of the tongue dorsum may function as a reservoir for posterior tooth colonization[21]. The molecular mechanisms by which MS colonize and accumulate in the biofilm are not fully understood but the process involves an initial adherence phase in which surface proteins (adhesins) specifically interact with salivary components adsorbed to the surfaces of teeth. This is followed by an accumulation phase in which MS increase in number as a result of the production of an extracellular matrix of glucans synthesized from sucrose by glucosyltransferase enzymes secreted by these bacteria. MS are not considered good initial colonizers of tooth surfaces, probably because other non pathogenic streptococci, e.g. *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus sanguinis*, present adhesins of higher affinity to the salivary components adsorbed to teeth[22-23]. Also, these commensal species produce IgA1-proteases, which are not produced by MS species. These proteases may confer additional ecological advantages for these pioneer streptococcal establishment in the biofilm community[24]. MS establish on dental surfaces much later than other oral streptococci[25-26]. In an American population, MS were detected in only 7% of children who hadonly incisors erupted (mean age of 9.2 months). In contrast, all were colonized by *Streptococcus mitis* biovar 1, and 50% by *Streptococcus sanguis*[25]. These latter two species are primary colonizers of tooth surfaces and out-compete MS during the initial establishment of the dental plaque microbiota[22,26]. These findings suggest that MS does not easily colonize and establish in the dental biofilm, and that specific factors may be required to favor MS colonization in relation to other commensal species.

The natural history of MS colonization was analyzed in a prospective study of 48 children from birth to 5 years of age whose mothers were heavily colonized by MS[27]. In that study it was observed that all children who acquired MS were colonized between the ages of 19 to 30 months, and that children who did not present detectable levels of this bacteria during this defined period were not colonized until the end of the study, when children were 5-years-old. This period from 19 to 31 months of age was referred to as the window of MS infectivity. The reasons that account for this window of infectivity are not fully understood, but may be associated with the eruption of molars which provide virgin and retentive occlusal surfaces for colonization. Later studies performed in American children have confirmed the notion of a window of infectivity[28]. On the other hand, initial acquisition of *S. mutans* may occur earlier than the period of 19-30 months when children are under high challenge resulting from an excessive intake of sucrose and frequent exposure to heavily infected subjects[17,21,29-32]. Other host and environmental factors may influence the time of MS acquisition[10,32], one of which may be the status of immunological maturation[33-34].

Apart from variations in the period for the opening of the "window of infectivity", the finding that this window is closed by 30 months of age supports the notion first conveyed by Köhler and Andreén[17], that preventive strategies that delay the transmission of this bacteria until after the non pathogenic commensal biofilm matures on erupted tooth surfaces (e.g., 30 months of age) may effectively prevent MS infection throughout childhood. Based on this concept, it is suggested that preventive measures to control MS colonization when permanent teeth erupt, may also reduce risks for caries development in later ages[35-6]; however, further studies are needed to investigate this issue. This may be applicable even to those populations highly exposed to sucrose and characterized by a very early MS infection[29], where it was found that MS achieve relatively stable levels after 2 years of age[29]. Several efforts to identify sources of MS infection have been performed using phenotypic typing and DNA fingerprinting methods to track *S. mutans* initial colonizers. In these studies it was determined that mothers are the main source of MS infection[37-40], although other individuals from the family may also transmit this bacteria[39-40] Few studies have, however, analyzed populations where children has less contact with family members, as for example children attending nursery schools[41-42] These studies are necessary since a significant part of the population use these institutions for early child care in several developed and developing countries. There is data suggesting that horizontal transmission of MS may occur between children attending nurseries[42], or between children and caregivers[21]. Once MS establish in the oral biofilm, they are difficult to modify, unless drastic modifications occur in sucrose consumption and oral hygiene care. Strategies available to control *S. mutans* levels in the oral cavity in public health programs are limited to attempts to change habits of sucrose consumption and periodic tooth cleaning through mechanical

and/or chemical control of biofilm[17,43] These approaches, however, are significantly challenged by behavioral, educational and socio-economic problems. Furthermore, while fluoride was responsible for much of the decrease in the caries lesion indexes in the last decades, this treatment was not sufficient to control the disease; thus, dental caries remains a major public health problem. The experience of Brazilian children in the city of Piracicaba, SP, is instructive in this regard. Piracicaba has fluoride in the drinking water at optimal levels (around 0.7 ppm) since 1971. In addition, beginning at 2 years of age, children attending public nursery schools (Escolas Municipais de Ensino Infantil – EMEIs) have their teeth brushed with fluoridated dentifrices by health agents[29] However, despite this high exposure to fluoride, about 35% of children between one to three years of age present with caries lesions[44-45]; nearly three fourths of 12-18-month-old children have shown detectable levels of *S. mutans*, and 20% of this age group showed high oral levels of *S. mutans* (>100 cfu of mutans streptococci), a level of infection much higher than that observed in communities from the U.S.A, Japan and Sweden[27,28,40,45-47]. In these latter communities, infection with ultimately cariogenic *S. mutans* usually occurs during the ages of 18-36 months at which time the oral ecology and the level of mucosal immune development are likely to be significantly different from the earlier and generally more heavily infected Brazilian children. This abnormal infection rate has severe consequences. For example, we have shown that some children in this Brazilian population already have dental decay at 12 to 18 months of age[44]. A subsequent prospective study revealed that young children carrying heavy infection levels of *S. mutans* showed a high level of risk for dental caries, which was determined as a 9-fold increase in risk for caries development when compared to children with lower levels of MS[29].

The control of MS infection levels in young children and adults proved to be extremely difficult[30-31,48-50]. Application of chlorhexidine to reduce MS currently is the main alternative choice because of its substantivity and selective effect on MS[49,51-53]. However, intense treatment (frequent applications with gels of high concentration) are necessary for effective suppression of bacteria[51,52]. In addition, recolonization occurs within three to six months even after intense suppression of MS by chlorhexidine treatment in adults[49] and in young children[48]. Thus a more effective strategy may include use of chlorhexidine for initial suppression of MS infection, coupled with additional measures which interfere with the ecological competitiveness of MS species.

Because the detection of MS is very frequently associated with occurrence of caries lesions, the use of culture methods to detect and quantify MS levels were applied in several populations in order to identify high risk subjects from adults and children, who could be targets for application of more intense health care for caries prevention (for review see van Houte, 1993[7]). However, it appears that quantitation of oral levels of MS is more efficient for detection of subjects that will not develop dental caries rather for identification of high caries risk[7] This may be explained by the complex multifactorial nature of caries disease. Among young Brazilian children from public nursery schools, it was found that children heavily colonized by MS developed a high mean of 4.6 new caries lesions per year[29] However, even among the heavily MS infected children, there were some children that did not develop caries during the follow-up period, despite socio-economic, dietary and fluoride exposure conditions similar to those of high caries active children[29,45]. These children experienced reduction in levels of MS after a one-year follow-up period. High fluctuations in MS initial infection levels were described in other populations[28], and much still is to be learned about the host factors that may influence susceptibility to the establishment of a pathogenic dental biofilm. Some socio-economic and cultural factors have already been shown to relate to caries experience in Brazilian[54] and other populations[1,41], but a complete understanding of host and bacterial factors involved in infection and disease remain. For example, children who are breast-feed for longer periods have lower caries prevalence than children who are weaned early in life[44] Colostrum is known to provide a stimulus for accelerating mucosal membrane closure in the gastrointestinal tract and to provide a stimulus for maturation of the mucosal immune system[55]. Children who are not breast fed are deprived of milk-borne passive immunity to a variety of bacterial antigens which may account for higher levels of infections[55]. There is evidence of higher susceptibility to respiratory diseases, for example, in children with transitory episodes of depressed immune response associated with reduced salivary levels of SIgA[55]. However, the effect of low levels of SIgA in caries development is unclear[56-58], probably because of the diversity in IgA specificity to virulence-relevant antigens[59-61]. In this regard, the immune response to MS during their initial establishment in the oral cavity needs more study, especially in those populations showing heavy MS colonization. Moreover, the high genetic diversity already described within the MS species indicates that some bacterial clones may be more virulent than others within a population. Large variations in the expression of different virulence factors have been described among clinical isolates of MS[62-64].

Molecular Pathogenesis of disease

Mutans streptococci produce several proteins important to the pathogenesis of dental caries. These proteins are involved in the ability to colonize teeth and to impose their ecological advantage over other commensal organisms when the dental biofilm is under the environmental stress. For initial colonization, MS are able to adhere to saliva-coated tooth

surfaces by specific interactions with salivary components and with surface proteins of other bacterial species. Secondly, MS are able to increase in proportion within the biofilm community through the synthesis and interaction with an extracellular matrix of water-insoluble glucans. Sufficient accumulation of mutans streptococci in the plaque allow them to decrease biofilm pH to less than 5.0 by virtue of the synthesis of lactic acid from fermentative metabolism of dietary sugars and from intracellular and extracellular reservoirs of saccharides. An additional virulence trait of MS is their ability to tolerate extreme stress conditions resulted from external environmental factors and from the own bacterial products, e.g., high osmotic pressure, relatively long periods of starvation and very low pHs. Extended exposure of the teeth to biofilm pH's of less than 5.5 leads to tooth demineralization, and thus carious lesions. Understanding the molecular mechanisms of pathogenesis of these SM species has allowed investigators to identify targets for controlling *S. mutans* infection and virulence.

Important surface proteins are involved in initial adherence of *S. mutans* to saliva-coated teeth and are called adhesins. Oral streptococcal species express several adhesins that specifically bind proteins and glycoproteins from saliva, from epithelium, and also from dietary and microbial components[65]. The affinities and specificities of these interactions help to determine the distribution of microbiota on oral muccosa and saliva-coated tooth surfaces. The group of surface adhesins expressed by *S. mutans* are variously termed antigen I/II (SpaP, Pac or P1) and in *S. sobrinus*, SpaA. This family of surface proteins was found to bind salivary glycoproteins and other microorganisms[66], and seem to participate in virulence. Polypeptides from this family of protein have also been observed in other commensal species as *Streptococcus gordonii*[65]. *S. mutans* also presents a cell wall-associated protein (WapA) that was suggested to bind saliva-coated smooth surfaces and to participate in sucrose-dependent adherence, but its role in cariogenesis is not fully understood[67-68].

Glucosyltransferases (Gtfs) have a crucial role in the accumulation process of *S. mutans* in the biofilm, since they catalyze the synthesis of an extracellular matrix of glucans from sucrose. *S. mutans* express three Gtfs isotypes, GtfB, GtfC and GtfD that produce glucans with distinct degrees of water-solubility, depending the proportion and type of glycosidic linkages. The isotypes GtfB and GtfC synthesize a(1-3) rich water-insoluble glucans, while GtfD synthesizes water-soluble glucans rich in a(1-6) glycosidic linkages[69-71]. The extracellular synthesis of glucan has proven to be essential for the ability of this bacteria to adhere to smooth surfaces in the presence of sucrose *in vitro* and to induce dental caries in animal models, as shown by the fact that knockout mutants of each *gtfB*, *gtfC* and *gtfD* gene were compromised in their ability to cause caries[72-74]. *S. sobrinus* secretes 4 distinct Gtfs. GtfI, is encoded by *gtfI* and synthesizes water insoluble glucans. Two other isotypes encoded by *gtfT* and *gtfU* that are involved in the synthesis of glucan with both kinds of glycosidic linkages a(1-3) and a(1-6)[75-76]. The Gtf-Si isotype synthesizes a(1-6) water-soluble glucans. Although other commensal streptococcal species also produce their own Gtfs, generally they only weakly adhere to smooth surfaces in the presence of sucrose. This may be related to the variation in the chemical structure of glucan produced. For example, growth of *S. sanguis* in the presence of *S. mutans* Gtfs and sucrose increases the ability of *S. sanguis* to adhere to smooth surfaces[77]. Although Gtfs from several oral streptococcal species show high sequence homologies, the number of isotypes produced per species seems to be variable[78]. Moreover, the mechanisms that control expression of the Gtf isozymes are distinct[78-79].

Gtfs have two functional domains, a catalytic domain located in the N-terminal third of the molecule, and a glucan-binding domain located in the carboxy-terminal third. The glucan-binding domain contain amino acid repeats that resemble ligand-binding domains of various Gram-positive bacteria, and that were found to be also necessary for the catalytic activity[78]. The number of repeats in this domain affects the structure of glucans, and thus may affect its adherence properties[78].

The molecular interaction of *S. mutans* cells with the extracellular glucan matrix is not fully understood, but a heterogeneous group of proteins have been shown to bind glucans, and for that property were named Glucan-binding proteins (Gbps). *S. mutans* expresses at least 4 distinct Gbps, that are named GpbA, GbpB, GbpC and GbpD, based in the order in that they were firstly described[78]. Apart from their affinity for glucan, genetic and biochemical studies on each protein have indicated that the biological functions of Gbps are variable[78]. GbpA has homology with the glucan-binding domain of the *S. mutans* GtfB and GtfC. However, deletion of the gene encoding GbpA modified the morphology of the biofilm *in vitro*, and caused an increase in virulence[80-81] GbpB shows no homology with other glucan-binding proteins, but has high homology to peptidoglycan hydrolases from other gram-positive organisms[63] Mutants of GbpB proved to be very unstable, suggesting that this protein has an essential role in *S. mutans* biology, probably because it plays a role in the maintenance of the cell wall integrity[82-84] Production of GbpB appears to influence the ability of *S. mutans* genotypes to grow in a biofilm phase, and is over-expressed under osmotic stress[85].

The thirth Gbp, GbpC, is also expressed under osmotic stress conditions, but differently from GbpA and GbpB which can be presented in cell-associated or secreted forms, GbpC is covalently linked to the cell wall through a LPxTG anchor motif targeted by a sortase transpeptidase[86]. GbpC is important to *S. mutans* aggregation in the presence of glucan

and dextran and adherence of *S. mutans* to saliva-coated teeth surfaces under stress conditions, which suggests its participation on *S. mutans* cariogenicity[87] GbpD has recently been identified through the screening of putative proteins of the *S. mutans* genome[88] that share homology with the glucan-binding domain of Gtfs and GbpA[78]. However, its role in the cariogenicity of *S. mutans* remains to be determined. *S. sobrinus* also has been shown to secrete several glucan-binding proteins[89]. One has been sequenced and defined as a dextransucrase inhibitor[78].

S. mutans can prevail in the cariogenic biofilm not only because of its ability to synthesize and interact with glucans from sucrose, but also because they present several mechanisms to tolerate extreme and rapid pH drops (to pHs lower than 4.0), a condition that is poorly tolerated by other commensal streptococci as *Streptococccus sanguinis*, *Streptococcus gordonii* and *Streptococcus oralis*[12]. The frequent drops in biofilm pH due to exposure to sugars and subsequent synthesis of acids from glycolysis promotes the increase in the proportion of *S. mutans*, lactobacilli, and other acid-tolerant organisms, within a microbial community[12]. Commensal streptococcal species have mechanisms to raise environmental pH by the production of ammonia from deaminase activities[90]. However only *S. mutans* can produce acids from the fermentation of sugars at pH as low as 4.4 and grow at pHs of about 4.8[91]. *S. mutans* is the gram-positive species with the ability to metabolize the highest number of carbohydrates when compared to the gram-positive species sequenced so far, and presents at least five sugar ABC transport systems, including the multiple sugar metabolism system (MSM)[88]. The genome of UA159 contains the highest number of phosphoenol pyruvate systems (PTS) among Gram positive organisms, accounting for a large number of sugar transport systems encoded in their genome[88]. Moreover, *S. mutans* can sustain acid production even during periods of fasting, because of the ability to store intracellular glycogen-like polyssacharides, a trait that has been considered to be important in virulence in animal models[92]. Mutans streptococci also express surface attached dextransucrases and fructanases that break extracellular polysaccharides for further uptake and metabolism[66].

S. mutans have several mechanisms to maintain an acid tolerant phenotype (ATP). It was shown that acid shock promotes up-regulation of 64 proteins within 30 minutes of the pH change to avoid damage to intracellular proteins and nucleic acids[93]. One of these mechanisms includes a transmembrane proton-pumping F_1F_0-ATPase system, which occurs in other oral streptococci. This remains a fertile field for investigation (for review see Quivey et al., 2001 and Burne, 1997)[91,94].

Finally, to survive and grow under all the stress conditions to which the cariogenic biofilm is exposed, MS has to sense environmental changes and rapidly modify its physiology in order to persist and grow in its ecological niche. It has become clear that expression of several virulence factors, including adhesins, Gtfs, Gbps and proteins for acid-shock resistance are all environmentally regulated[85,95-100]. Recently, an inter-cell quorum-sensing communication system that involved secretion of pheromone-like peptides was characterized in *S. mutans*, and appears to be important to biofilm growth[97,100]. Another two pheromone-like peptides were identified in the *S. mutans* genome, and may participate in an intra-species communication system[88]. A further inter-species communication system seems to be also presented in its genome[88]. Blocking these communication systems could repress expression of a group of virulence factors[101-102]. The completed sequencing of the *S. mutans* genome, and recent progress in the sequencing of the *S. sobrinus* genome as well as of other commensal species of the dental biofilm[103] coupled with new technologies for analysis of gene expression and regulation has opened a broad field to better understand the physiology of MS, their interaction to commensal organisms and expression of virulence. These advances will be very important to elucidate a variety of molecular mechanisms involved in the pathogenicity of these organisms within the complex dental biofilm community.

Pathways to Protective Immunity

The oral cavity is protected by a variety of innate and adaptive host mechanisms which enter via the major and minor salivary glands, gingival crevice, or perfuse through the oral epithelium. The principal adaptive immune component in saliva is secretory IgA antibody which is synthesized as a dimer by interstitial plasma cells and secreted into the lumen through the salivary gland epithelium. An additional glycopeptide, referred to as secretory component or poly-immunoglobulin receptor, is covalently added to the IgA dimer during transit through the salivary epithelium, imparting increased resistance to proteolysis. IgG-rich gingival crevicular fluid contributes additional antibody to the oral milieu to deal with infections. Modest amounts of IgG antibody may also be contributed by the minor salivary glands. Given that dental caries is the result of infection, a significant effort has been mounted to engage one or more of these immune systems to intercept primary colonization and growth of the acidogenic microflora that have been associated with this disease. Passive application of preformed antibody has also been employed to inhibit the re-colonization of preexistent cariogenic organisms.

Several strategies have been commonly pursued during the course of development of potential dental caries vaccines. Firstly, virtually all approaches targeted either *Streptococcus mutans* or *S. sobrinus* as the principal etiologic agents of the disease. Secondly, most investigators sought to prevent initial colonization rather than to remove cariogenic microorganisms from an established biofilm. Thirdly,

investigators sought to use antibody to block one or more aspects of bacterial colonization or accumulation was sought, rather than inactivate metabolic pathways critical to the survival of the microorganism. The following sections will describe the development of these active and passive immune approaches in preclinical studies and humans clinical trials.

Development of mucosal immunity: The development of effective dental caries vaccine approaches have paralleled the emergence our understanding of mucosal immune ontogeny. Although some immunological issues important for the application of a childhood dental caries vaccine are incompletely resolved, sufficient data are available to formulate reasonable strategies. For example, the natural history of mutans streptococcal infection described above indicates that a child's mucosal immune system needs to be sufficiently mature by the end of the first year of life in order for a vaccine to induce an effective response. Since children of this age do not receive mucosal immunizations, knowledge of the immunological responsiveness of the secretory immune system only comes from analysis of mucosal responses to infections with indigenous organisms. These studies have shown that the oral immune environment undergoes rapid, early development. Although secretory IgA antibody in saliva and other secretions is essentially absent at birth[104], exposure to bacterial, viral and food antigens causes a rapid expansion of IgA plasma cells in mucosal lamina propria[105] Mature SIgA, i.e., dimeric IgA with bound secretory component, is the principal salivary immunoglobulin secreted by one month of age[106]. Salivary IgA antibody to pioneer oral (*Streptococcus mitis, Streptococcus oralis* and *S. salivarius)*[59,60,107] microbiota appears within weeks of initial exposure. Salivary IgA antibody to tetanus toxoid and poliovirus also appears following pediatric immunization with these injected and oral vaccines[59]. Salivary antibody to oral commensal microbiota can be detected in both IgA subclasses by nine months of age[60,107]. Also, although salivary IgA concentrations are significantly below adult levels at this age, salivary immunoglobulin is > 95% SIgA. Thus, the evidence suggests that significant maturation of the mucosal immune response has occurred by the end of the first year of life.

The manner in which the child's immune system handles initial infection with mutans streptococci can also be instructive. Natural exposure to mutans streptococci results in a mucosal immune response, which can often be observed in the second and third year of life. Western blot and ELISA analyses reveal that the major responses appear to be directed primarily to MS streptococcal components which are considered to be important in colonization and accumulation, such as antigen I/II, glucosyltransferase, and glucan binding protein(s)[28]. Interestingly, in some children, antibody to mutans streptococcal antigens can also be detected independently of the ability to detect ongoing infection in the second year of life. As is the case with 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 the absence of its colonization. Longitudinal studies suggest that antibody reactive with mutans streptococci results from contact with mutans streptococci, rather than from cross-reaction with earlier colonizing oral streptococci, since well-developed salivary IgA antibody to pioneer oral streptococci can be demonstrated prior to the detection of antibody reactive with mutans streptococci[108]. However, significant individuality seems to occur in the rate, specificity, and intensity of response after mutans streptococcal infection. Even siblings may differ in the amount or specificity of salivary IgA antibody, despite being presumably challenged with the same maternal bacterial clonaltypes. Regardless of these differences, the evidence does point to the ability of the child to recognize infection with mutans streptococci by the formation of salivary IgA antibody.

Bacterial Targets for Caries Vaccines:

Targets associated with bacterial colonization and accumulation: Following early demonstrations that immunization with intact mutans streptococci could induce protective immune responses in experimental models for dental caries[109-110], investigators sought to refine vaccine targets to individual components. The first step in the molecular pathogenesis of the disease is the colonization of the tooth surface, or, more accurately, colonization of the pre-existing biofilm on the tooth surface, by cariogenic streptococci through adhesin-mediated mechanisms.

Abundant *in vitro* and *in vivo* evidence indicates that antibody with specificity for *S. mutans* AgI/II or *S. sobrinus* SpaA can interfere with bacterial adherence and subsequent dental caries. Antibody directed to the intact antigen I/II molecule or to its salivary-binding domain blocked adherence of *S. mutans* to saliva-coated hydroxyapatite[111]. Furthermore, a variety of immunization approaches have shown that active immunization with intact antigen I/II[112-113], or passive immunization with monoclonal[114] or transgenic antibody[115] to putative salivary binding domain epitopes within this component can protect rodents, primates or humans from dental caries caused by *S. mutans*. Immunization of mice with synthetic peptides (residues 301-319) from the alanine-rich region of antigen I/II suppressed tooth colonization with *S. mutans*[116]. Intranasal immunization with antigen I/II, coupled to cholera toxin B subunit, suppressed colonization of mouse teeth by *S. mutans*[116]. Fusion proteins containing this adhesin also were shown to inhibit sucrose-independent adhesion of *S. mutans* to saliva-coated hydroxyapatite beads[84]. Immunization with *S. sobrinus* SpaA constructs

protected rats from caries caused by *S. sobrinus* infection[117]. Thus, these streptococcal adhesins appear to be prime candidates for dental caries vaccine applications.

Targets associated with bacterial accumulation:

Sucrose-dependent accumulation of mutans streptococci within the dental biofilm have also been immunologically targeted in preclinical and clinical studies. Most effort has centered on a group of glucosyltransferase enzymes which catalyze the extracellular formation of alpha 1,3 and 1,6-linked glucan from sucrose. These polysaccharides provide multiple binding sites for the accumulation of mutans streptococci in the plaque as well as reservoirs of carbohydrate for cell metabolism. Given the central role of glucan formation in the molecular pathogenesis of dental caries, it is not surprising that Gtfs from *S. mutans* or *S. sobrinus* Gtf could induce protective immune responses in preclinical[110,118] or clinical studies in young adults[119-120]. Futhermore, passive administration of antibody to Gtf in the diet also protected rats from experimental dental caries[121]. Thus, the presence of antibody to glucosyltransferase in the oral cavity prior to infection can significantly influence the disease outcome, presumably by interference with one or more of the functional activities of the enzyme.

Immune responses to Gtf can also be directed to functional domains. A variety of biochemical, genetic and sequence alignment techniques have identified several residues within the N terminal region of Gtfs that appear to be associated with its catalytic activity[122-125]. Other studies have shown that the repeating sequences in the C terminal third of Gtf are associated with primer dependent glucan binding[126-129]. This information has directed the design of Gtf subunit vaccines. For example, immunization with synthetic peptide constructs corresponded to sequence containing at least five different catalytically active residues or glucan binding domains have been shown to induce immune responses interfering with enzyme function and/or with the cariogenic activity of mutans streptococcal infection[130-134].

Glucan binding proteins have also received some attention in the search for effective antigens. Although several glucan binding proteins (Gbp) have been described only one, *S. mutans* GbpB[135], has been reported to induce protective immunity in experimental systems[136]. Salivas of young children often contain IgA antibody to GbpB, indicating that initial infection with *S. mutans* can lead to natural induction of immunity to this protein[28]. The *gbpB* gene, cloned and sequenced by Mattos-Graner and co-workers in 2001[63], indicates an expressed GbpB protein of 431 residues. Bioinformatic analysis of the GbpB sequence has revealed several sequences with potential MHC class II-binding activity. A synthetic peptide based on one of these sequences in the N terminal third of the molecule has been shown to induce protective immunity in a rat model for dental caries[137].

The ability to induce protective immunity with subunit vaccines based on adhesin, Gtf or GbpB sequences has led investigators to develop constructs combining epitopes from one or more proteins. Taubman and coworkers[134] have shown that the combination of peptides from the catalytic and glucan-binding domains of Gtf enhances the level of experimental enzyme and caries inhibition. Fusion proteins constructed from the saliva-binding alanine-rich region of Ag I/II and the glucan binding domain of Gtf induce immune responses that inhibited water-insoluble glucan synthesis by *S. mutans* Gtf and also inhibited sucrose independent adhesion of *S. mutans* to saliva-coated hydroxyapatite beads[84]. DNA vaccines coding for one or more of the virulence associated proteins also have been shown to induce protective responses[138]. Incorporation of a Gtf catalytic domain peptide within a construct containing a GbpB sequence with MHC class II binding characteristics greatly enhanced antibody formation to the Gtf peptide. Thus the potential exists to broaden the protective potential of the subunit vaccine approach.

Enhancements to Active Immunization Approaches:

Routes: Although the basic principle of immune protection from dental caries caused by mutans streptococci has been established in preclinical studies, refinements to the effective application of this approach to humans remain. One of the issues deals with the route of delivery. Early studies with mucosally applied caries vaccines used the oral or intragastric route for antigen delivery. Although significant effects on mutans streptococcal infection and disease were observed in animal[109,118] and human studies[119-120], induction of immune responses by the oral route requires antigen passage through the gut prior to uptake in the gut associated lymphoid tissue (GALT). Since transit through the acidic stomach environment reduces the effective antigen stimulus, and the journey to GALT dilutes antigen concentration, induction of protective immunity in mucosal sites that are in closer anatomical relationship to the oral cavity has been pursued. Intranasal installation (IN) of antigen, which targets the nasal-associated lymphoid tissue (NALT), has been used to induce immunity to many bacterial antigens, including those associated with mutans streptococcal colonization and accumulation. Protective immunity after infection with cariogenic mutans streptococci could be induced in rats by the IN route with many *S. mutans* antigens or functional domains associated with these components. Protection could be demonstrated with *S. mutans* Ag I/II[113], the salivary binding region (SBR) of Ag I/II[139], a 19 mer sequence within the SBR[116], the glucan binding domain of *S. mutans* Gtf-B[140], *S. mutans* GbpB[132] or GbpB-derived peptides[137], and fimbrial preparations from *S. mutans*[141], using antigen alone or in combination with mucosal adjuvants. Additional routes have

included exposure of antigen to tonsils or to minor salivary glands. Each has resulted in aspects of protection in animals[142] and humans[120].

Adjuvants: Since mucosal application of soluble antigen is insufficient to sustain an immune response, several strategies have been employed. One of these has been to take advantage of the immunostimulating ability of enterotoxins such as cholera toxin (CT) or the closely related serogroups I and II of *E. coli* heat labile enterotoxins (LT). CT could be shown to markedly enhance the protective potential of intragastrically or intranasally applied mutans streptococcal antigens[143-144]. Adjuvant features of CT or LT for use in preclinical studies of dental caries vaccines were preserved by removing the A1 subunit toxin domain from the CT complex[145], mutating residues in the A subunit domain,[146]or using the B subunit[113]. Antigen or functional domains therein, when mixed with detoxified adjuvant or chemically conjugated or genetically fused with adjuvant subunits, dramatically increased salivary immune responses resulting in protective immunity[116,147-149].

Delivery systems: Targeting vaccine antigens to mucosal immune tissue or delivery in vehicles which prolong or promote exposure of antigen to inductive tissue have also been used successfully in preclinical studies. For example, intact adhesins[117], chimeric SBR-CTA2/B proteins[150-152], or Gtf glucan binding domains[153] 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 administered intragastrically or intranasally. 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. These approaches have been shown to be useful in the delivery of dental caries vaccine antigens for protective responses in preclinical studies[154] or salivary antibody formation in humans[155-157].

Passive Immune Approaches

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 (for a review, see Koga et al.[158], 2002). Pioneering studies in the 1970's by groups in the US and Great Britain provided a theoretical basis for this approach. Michalek and McGhee[159] showed caries-protective effects in rat pups that suckled on IgA-rich milk from mothers who had previously been immunized with mutans streptococci. Lehner and coworkers[160] showed that intravenous infusion of rhesus monkeys with serum IgG antibody from monkeys that had been injected with mutans streptococcal cells protected the passively transferred animals from subsequent dental caries.

Refinements in this approach occurred with respect to antibody specificity, antibody source, and method of introduction into the oral cavity. Antibody to intact streptococci[161-162], antigen I/II[114,163-166], Gtf[121] or GbpB[167] have each been reported to have a measure of success in decreasing colonization or recolonization of mutans streptococci in experimental models or in small clinical trials in humans. Thus, although no direct comparisons have been performed, the bacterial components that induce protective immunity by active immunization, also are useful for passive immune applications.

The methods of practical delivery of antibody into the oral cavity include incorporating antibody into the diet or drinking water, or exposing to the teeth to antibody reagents via a variety of dental rinsing mechanisms. Earlier applications with mouse monoclonal antibody[114,163-164] have given way to the use of IgY antibody from chicken egg yolks in which this IgG-like antibody is concentrated[121,161-162,167]. Bovine milk antibody sources have also demonstrated a protective effect. For example, antibody to intact mutans streptococci in bovine milk, incorporated into the diet of gnotobiotic rats[168] or used as a mouth rinse in humans[169] interfered with the progress of infection or disease. More recently, immune milk containing antibody to a fusion protein containing the alanine-rich salivary binding region of antigen I/II and the glucan binding domain of Gtf was also used as a mouth rinse[166]. Following cetylpyridinium treatment of the teeth of adults, and mouth rinsing with immune milk, significant reductions in recolonizing mutans streptococci were observed.

Building on the long term protection of humans from dental caries achieved after chlorhexidine treatment followed by topical application with monoclonal to *S. mutans* Ag I/II adhesin epitopes[170], Ma and coworkers[171] prepared the immune reagent by transgenic synthesis of secretory IgA/G hybrid monoclonal antibody of similar specificity in tobacco plants. Application of this hybrid transgenic antibody to humans under a similar three week protocol also resulted in protection from oral colonization with mutans streptococci for at least four months[115]. The basis for these long term protective effects after a relatively short topical antibody treatment remain unclear but might be explained ecologically by the nature of the oral biofilm that recolonizes the teeth in the presence of antibody after chlorhexidine treatment.

Sumary: Clearly there is strong evidence that *Streptococcus mutans* and *S. sobrinus* are closely associated with dental caries. Fluoride treatment used abroad has successfully limited caries progression, but was not sufficient to control this infectious disease, even when used together with professional tooth cleaning and dietary counseling in populations highly exposed to these cariogenic microbiota. As we increase our knowledge about the molecular

mechanisms involved in establishment and emergence of MS in the dental biofilm, and the influence of the biofilm on biochemical pathways leading to caries formation, new methods to intervene with the establishment of a cariogenic flora should develop [172]. Active or passive immunization strategies, which target key elements in the molecular pathogenesis of MS hold promise. Integrating these approaches into broad-based public health programs may yet forestall dental caries disease experienced by many of the world's children, among whom those of high caries risk might derive the greatest benefit.

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